Up-regulation of microRNA-497 inhibits the proliferation, migration and invasion but increases the apoptosis of multiple myeloma cells through the MAPK/ERK signaling pathway by targeting Raf-1

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ABSTRACT

Multiple myeloma (MM) is a cancer that occurs in plasma cells, which fall under the category of white blood cells that are in charge of antibody production. According to previous studies, microRNA-497 (miR-497) functions as a tumor suppressor in several types of cancer, including gastric cancer and colorectal cancer. Therefore, the present study aims to investigate the effects of miR-497 on cellular function of human MM cells through the mitogen-activated protein kinase/ extracellular signal-regulated kinase (MAPK/ERK) signaling pathway by targeting Raf-1. The differentially expressed genes and miRs in MM, and the relationship between the miR and gene were verified. It was found that Raf-1 was a target gene of miR-497. The data obtained from MM tissues showed increased Raf-1 level and decreased miR-497 level. MM cells were treated with mimic, inhibitor and siRNA in order to evaluate the role of miR-497, Raf-1 and MAPK/ERK in MM. The expression pattern of miR-497, Raf-1, ERK1/2, survivin, B-cell lymphoma-2 (Bcl-2) and BCL2-Associated X (Bax) as well as the extent of ERK1/2 phosphorylation were determined. Retored miR-497 and si-Raf-1 resulted in increases in the Bax expression and cell apoptosis and decreases in the expressions of Raf-1, MEK-2, survivin, Bcl-2, along with the extent of ERK1/2 phosphorylation. In addition, the biological function evaluations of MM cells revealed that miR-497 targets Raf-1 in order to inhibit the progression of MM by blocking the MAPK/ERK signaling pathway.

1. Introduction

Multiple myeloma (MM) is a mature \(\beta\)-lymphoid cell malignancy of the bone marrow with a series of complex clinical presentations, including anemia, bone lesions, renal failure, and immune dysfunction \([1–3]\). It accounts for 1% of malignant diseases and 15% of hematological malignant tumors, as well as 20% of blood and bone marrow related cancer deaths \([1,4]\). In the early 1980s, MM inevitably resulted in a gradual decline in the life quality and even death within 2 years, while today, if properly diagnosed, there is a 50% chance of recovery with a mean survival of 5 years or even over 10 years in some cases \([5]\). The first three years after diagnosis is the optimal time for patients with MM to be cured, but MM remains one of the cancers with a high risk of mortality, especially in young patients \([6]\). The widely adopted therapies for MM are the bortezomib-based regimen and dexamethasone \([7]\). However, the efficacy is far from satisfactory. Hence, there is a need to thoroughly investigate the mechanism associated with the development of MM. A previous study has found that microRNAs (miRs) miR-15a and miR-16 regulate tumor proliferation in MM \([8]\).

miRs have been proven to have essential effects on various biological processes, including cell proliferation and apoptosis, and have been considered as new therapeutic targets for cancers and cardiovascular diseases \([9,10]\). Due to the tumor suppressor role played by miRs, they have recently been reported to have a vital involvement in MM treatment and have been regarded as important regulators of the growth and survival of MM cells \([11]\). miR-497 functions as a potential biomarker and a tumor suppressor that is involved in tumor progression, proliferation and migration in various types of cancer and its expression
has been found to be suppressed in several malignant tumors, such as human cervical cancer and non-small cell lung cancer [12–14]. miR-497 is predicted to promote apoptosis by regulating Bcl-2 expression and also to facilitate the inhibition of the invasion and migration of cancer cells [15,16]. Raf-1, a multifunctional protein, is a critical downstream target of several growth factors that encourages the proliferation and survival of various cancer cells [17]. A previous study has demonstrated that the expression of Raf-1 likely regulates the tumor metastatic potential and resistance to apoptosis [18]. Once activated, Raf-1 triggers the Raf-ERK signaling pathway, which, in turn, regulates the proliferation, migration, cell cycle entry, and apoptosis of cells [19]. Mitogen-activated protein kinases (MAPKs) are so-called evolutionarily well-conserved serine and threonine protein enzymes, which participate in signal transduction pathways linking cell surface receptors with major regulatory nuclear and intracellular targets [20]. In mammals, there are several MAPK enzymes that are responsible for cell proliferation, apoptosis, differentiation and survival [21]. Extracellular signal-regulated kinase (ERK) is embodied in the mammalian family of MAPKs [22]. In addition, the MAPK/ERK signaling pathway has been reported to be regulated by miRs [23]. These findings suggested that miR-497 could exert effects on cell proliferation, migration, invasion and apoptosis in human MM by targeting the Raf-1 gene through the MAPK/ERK signaling pathway and based on this hypothesis, we conducted the following experiments to provide further evidence on the effects of miR-497 in MM.

2. Methods

Ethics statement

All the patients involved had signed informed consent, and all experiments conducted below had been approved by the clinical trial ethics committee of Wenzhou Central Hospital, Dingli Clinical Medical School of Wenzhou Medical University.

Study subjects

A total of 152 MM patients hospitalized at the Department of Hematology of Wenzhou Central Hospital, Dingli Clinical Medical School of Wenzhou Medical University from March 2010 to September 2016 were selected for the current study, and their eligibility was determined using the MM diagnostic criteria adopted from the International Myeloma Working Group (IMWG) [24,25]. All cases had been pathologically confirmed MM, and the patients had no history of radiochemotherapy prior to the operation. Among the patients, there were 93 males and 59 females, between the ages of 24 to 85 years with a mean age of 59 years. According to the myeloma tumor node metastasis (TNM) staging criteria revised by the American Joint Committee on Cancer (AJCC) in 2010 [26], Thirty-nine cases were verified in stage II, and 113 cases were in stage III. Afterwards, MM tissue samples were collected from MM patients, while normal bone marrow tissue samples were collected from 3 healthy volunteers in order to be used as the control. All tissue samples were cut into small pieces, quickly placed into frozen tubes, stored in liquid nitrogen, and then preserved in a refrigerator at −80°C.

Immunohistochemistry

Tissue samples were rinsed 3 times with cold normal saline, embedded in optimal cutting temperature compound (OCT) and sliced into frozen sections, which were subjected to immunohistochemical staining. The sections were then incubated with rabbit polyclonal Raf-1 antibody (ab137435; Abcam, Cambridge, MA, USA) (1:1000) and were placed in a wet box at 4°C overnight. Next, the sections were incubated with biotin-labeled secondary antibody, namely, mouse anti-rabbit immunoglobulin G (IgG) antibody (ab6785; Abcam, Cambridge, MA, USA) (1:10,000) working fluid and were incubated at 37°C for 1 h, followed by the addition of diluted SP complex (horseradish peroxidase-labeled streptavidin; 1:100) (E03010; Beijing Hongyue Science and Technology Ltd., Beijing, China) and incubation at 37°C for 1 h. Thereafter, the diaminobenzidine (DAB) Kit (ab64238, Abcam, Cambridge, MA, USA) was added to the sections and color development was carried out by incubation for 10 min. The appearance of brown staining was considered a positive reaction [27]. Finally, five different fields were randomly selected from each section under an inverted microscope (XDS-800D;
Shanghai Caikon Optical Instrument Co. Ltd., Shanghai, China) to obtain images. Positive cells were identified by the presence of brown-yellow granules in the cells. The Raf-1 protein staining rate was presented as the percentage of Raf-1 positive cells in a whole field of cells. The number of positive cells over 30% was considered to be positive (+) and the number of positive cells less than 30% was considered to be negative [28].

**Hematoxylin-eosin (HE) staining**

Some of the MM tissues and normal bone marrow tissues were fixed in 3% neutral formaldehyde, made into paraffin sections, after which HE staining was performed. The sections were dewaxed routinely, hydrated with gradient alcohol, stained with hematoxylin for 2 min, rinsed with running water for 10 s, and underwent differentiation by 1% hydrochloric acid-ethanol for 10 s. After washing with distilled water for 1 min, sections were stained with eosin solution for 1 min, washed with distilled water again for 10 s, dehydrated by graded ethanol, cleared by xylene, and sealed with neutral balsam.

**Cell culture**

MM cell lines RPMI8226, U266, XG-6, XG-7, HEK293T and H929 were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. Firstly, all cells were cultured in RPMI 1640 culture medium (SP1355; Shanghai Shifeng Biological Technology Co., Ltd., Shanghai, China), 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin in a 5% CO₂ incubator (DHP-9162; Shanghai Jiechen Laboratory Instrument Co., Ltd., Shanghai, China), 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin in a 5% CO₂ incubator (DHP-9162; Shanghai Jiechen Laboratory Instrument Co., Ltd., Shanghai, China), 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin in a 5% CO₂ incubator (DHP-9162; Shanghai Jiechen Laboratory Instrument Co., Ltd., Shanghai, China), 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin in a 5% CO₂ incubator (DHP-9162; Shanghai Jiechen Laboratory Instrument Co., Ltd., Shanghai, China), 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin in a 5% CO₂ incubator (DHP-9162; Shanghai Jiechen Laboratory Instrument Co., Ltd., Shanghai, China), 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin in a 5% CO₂ incubator (DHP-9162; Shanghai Jiechen Laboratory Instrument Co., Ltd., Shanghai, China), 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin in a 5% CO₂ incubator (DHP-9162; Shanghai Jiechen Laboratory Instrument Co., Ltd., Shanghai, China), 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin in a 5% CO₂ incubator (DHP-9162; Shanghai Jiechen Laboratory Instrument Co., Ltd., Shanghai, China), 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin in a 5% CO₂ incubator (DHP-9162; Shanghai Jiechen Laboratory Instrument Co., Ltd., Shanghai, China), 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin in a 5% CO₂ incubator (DHP-9162; Shanghai Jiechen Laboratory Instrument Co., Ltd., Shanghai, China), 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin in a 5% CO₂ incubator (DHP-9162; Shanghai Jiechen Laboratory Instrument Co., Ltd., Shanghai, China), 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin in a 5% CO₂ incubator (DHP-9162; Shanghai Jiechen Laboratory Instrument Co., Ltd., Shanghai, China), 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin in a 5% CO₂ incubator (DHP-9162; Shanghai Jiechen Laboratory Instrument Co., Ltd., Shanghai, China), 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin in a 5% CO₂ incubator (DHP-9162; Shanghai Jiechen Laboratory Instrument Co., Ltd., Shanghai, China), 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin in a 5% CO₂ incubator (DHP-9162; Shanghai Jiechen Laboratory Instrument Co., Ltd., Shanghai, China), 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin in a 5% CO₂ incubator (DHP-9162; Shanghai Jiechen Laboratory Instrument Co., Ltd., Shanghai, China), 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin in a 5% CO₂ incubator (DHP-9162; Shanghai Jiechen Laboratory Instrument Co., Ltd., Shanghai, China), 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin in a 5% CO₂ incubator (DHP-9162; Shanghai Jiechen Laboratory Instrument Co., Ltd., Shanghai, China). The culture medium in U266, XG-6, XG-7 and H929 cells was changed every 1~2 days, and cell passaging was conducted when the cell confluence reached 80%~90%. Following the removal of the culture medium, the cells were rinsed with phosphate buffer saline (PBS) twice and were trypsinized in 0.25% trypsin for 2~5 min, resuspended in 5 mL of Dulbecco’s modified Eagle medium (DMEM) (190,040; Gibco, Gaithersburg, MD, USA) containing 10% FBS and sub-cultured. RPMI8226 cells were sub-cultured every two days, and, following a full mixture using a micropipette and the removal of 2/3 of the primary medium, 2/3 volume of fresh medium was added.

**Dual-luciferase reporter gene assay**

The biological prediction site microRNA.org was applied for the analysis of target genes of miR-497 and verification of whether Raf-1 was a target gene of miR-497. The full-length 3’untranslated region (UTR) of the Raf-1 gene was cloned and amplified, and polymerase chain reaction (PCR) products were cloned downstream of the pmirGLO (Promega, Beijing, China) luciferase gene. Bioinformatics sites predicted the binding sites of miR-497 and its target genes, after which site-directed mutagenesis was performed. The pRL-TK vector (TaKaRa, Dalian, Liaoning, China) expressing Renilla luciferase was used to demonstrate the difference in the transfection efficiency from that of the internal reference to adjust the difference in the cell number. The miR-497 mimic (5’-cagcagcacactgtggtttgt-3’) and corresponding negative control (NC) (5’-gtcgtcctctgtccacgacactagt -3’) were co-transfected with luciferase reporter vectors into HEK293T cells. Dual luciferase activity detection was performed according to the methods provided by Promega.

**Reverse transcription-quantitative PCR (RT-qPCR)**

Cells of the P3 generation were subjected to miR-497 quantitative detection for cell line selection. The total RNA of 5 cell lines, RPMI8226, U266, XG-6, XG-7 and H929, was extracted using a Trizol Kit (15,596–018, Invitrogen Inc., Carlsbad, CA, USA). Thereafter, the ratio of the absorbance A260/A280 and RNA concentration were determined using an ultraviolet spectrophotometer (Nanodrop 2000; Thermo, Waltham, Massachusetts, USA), and the extracted RNA was stored at −80°C for further use. RNA was reversed transcribed into cDNA according to the manufacturer’s instructions of Applied Biosystems StepOneTM and StepOnePlusTM.
Real-Time PCR Systems (4,379,704; Applied Biosystems Inc, Carlsbad, CA, USA). The RT-qPCR RNA test kit was purchased from Ambion (Austin, Texas, USA). The reaction was performed by RT-qPCR (AM1005; Invitrogen Inc., Carlsbad, CA, USA). The reaction conditions for miR-497 were as follows: pre-denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. U6 served as the internal reference for the quantitative determination of miR-497 and glyceraldehyde phosphate dehydrogenase (GAPDH) as a reference for Raf-1, methyl ethyl ketone 2 (MEK-2), ERK1/2, survivin, B-cell lymphoma-2 (Bcl-2) and BCL2-Associated X (Bax). The primers were synthesized by Shanghai Boya Biotechnology Services Co., Ltd. (Shanghai, China). The aforementioned method was also applied to detect the miR-497 level and mRNA levels of Raf-1 and MAPK/ERK pathway- and apoptosis-related genes in tissues and transfected cells. The primers of miR-497, Raf-1, MEK-2, ERK1/2, survivin, Bcl-2, Bax, U6, and GAPDH are shown in Table 1. Each experiment was carried out 3 times. The dissociation curve was used for the evaluation of the reliability of the PCR results, taking the CT value (inflexions in a kinetic PCR amplification curve), and the relative expression of target genes, which was calculated by the $2^{-\Delta\Delta Ct}$ method [29]. The formula was as follows:

$$\Delta\Delta Ct = [Ct\ (\text{target gene}) - Ct\ (\text{reference gene})]_{\text{experimental group}} - [Ct\ (\text{target gene}) - Ct\ (\text{reference gene})]_{\text{control group}}.$$ 

### Cell grouping and transfection

RPMI8226 cells in the logarithmic growth phase were categorized into the blank (without any sequence transfection), NC (transfected with sequences of miR-497 NC), miR-497 mimic (transfected with sequences of miR-497 mimic), miR-497 inhibitor (transfected with sequences of miR-497 inhibitor), si-Raf-1 (transfected with sequences of si-Raf-1), and miR-497 inhibitor + si-Raf-1 groups (transfected with sequences of miR-497 inhibitor and si-Raf-1). The cells in the logarithmic growth phase were inoculated into a 6-well plate and were transfected in accordance with the instructions provided by the manufacturer’s for Lipofectamine 2000 (11,668,019; Invitrogen Inc., Carlsbad, CA, USA) when the cell density was increased to 30 ~ 50%. The transfection sequences of cells are shown in Table 2. Frozen-dried powder samples of the miR-497 mimic, si-Raf-1, miR-497 inhibitor, miR-497 inhibitor + si-Raf-1 and NC (YDSW-D18; Invitrogen Inc., Carlsbad, CA, USA) were centrifuged and dissolved in RNase-free water. A total of 250 µL of serum-free medium Opti-MEM (31,895–070, Gibco Company, Grand Island, NY, USA) was employed to dilute Lipofectamine 2000 (5 µL), and the solution was mixed slightly and cultured at room temperature for 5 min. The above two solutions were mixed and cultured at room temperature for 20 min and were added into the cell culture well. After cell culture was carried out for 6 ~ 8 h at 37°C in a 5% CO$_2$ incubator, the medium was substituted by a complete medium. Following further incubation for 24 ~ 48 h, the RNA and protein collected were extracted for subsequent experiments.

### Table 1. Primer sequences for RT-qPCR.

| Genes    | Sequences (5’-3’)                                    |
|----------|-----------------------------------------------------|
| miR-497  | F: CAGCCCTGTCGGCACTAGC                               |
|          | R: GCCTGACCTTACGTGGC                                  |
| Raf-1    | F: CAGCCCTGTCGGCACTAGC                               |
|          | R: GCCTGACCTTACGTGGC                                  |
| MEK-2    | F: TGCTCAAACCCCAACCTTC                               |
|          | R: ACACAAACCCCGCCCAA                                  |
| ERK1/2   | F: TGTCCTGCAATACGTGGCAGATA                           |
|          | R: TCACCGAGGCGGCGGCAC                                  |
| survivin | F: CGCTGTGAATGCAGCTGGCAAC                            |
|          | R: TACGGTCTGACGCTGGCAAC                               |
| Bcl-2    | F: GTCAGTGGGGTCATGTTGGAGCC                           |
|          | R: TACGGTCTGACGCTGGCAAC                               |
| Bax      | F: GAGGATGATGGCGCAGCCGAC                             |
|          | R: GCTGGGTCAGGCGAGGAG                                  |
| U6       | F: GCTCCGGACAGCATATCATACAAAAT                        |
|          | R: CGCTAGAATTTGCTGAGC                                  |
| GAPDH    | F: GCCCTGGTGTCGGCACG                                  |
|          | R: TGGGAGGCGCCCGCC                                 |

Note: RT-qPCR: reverse transcription quantitative polymerase chain reaction; miR: microRNA; MEK-2: methyl ethyl ketone 2; ERK: extracellular regulated protein kinases; Bcl-2: B-cell lymphoma-2; Bax: BCL2-Associated X; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; F: forward; R: reverse.

### Table 2. Transfection sequences.

| Gene          | Sequence                      |
|---------------|-------------------------------|
| miR-497 mimic | CAGCCACACACUGGGGUUUUGU       |
| miR-497 inhibitor | ACAACACACUGUGGCUCGCG       |
| NC            | UUCUCCGAACGUGGAAGACGUTT      |

Note: miR-497 mimic, micro RNA-497 mimic, miR-497 inhibitors, micro RNA-497 inhibitors; NC, negative control.
Western blot analysis

The cells taken from the frozen tissue were added to an appropriate amount of protein lysate containing 60% radio-immunoprecipitation assay (RIPA) cell lysis buffer, 39% sodium dodecyl sulfate (SDS) and 1% protease inhibitor and were collected into Eppendorf (EP) tubes and allowed to stand on ice for 30 min. Thereafter, the cells were centrifuged (36,684 × g) at 4°C for 30 min, the supernatants were collected, and the protein concentrations were measured using the bicinchoninic acid (BCA) method. Following quantification according to different concentrations, the protein (20 μg) in each group was separated by polyacrylamide gel electrophoresis (PAGE), transferred onto NC membranes by the wet transfer method, and sealed in 5% bovine serum albumin (BSA) at room temperature for 1 h. Diluted primary antibodies, rabbit anti-Raf-1 antibody (1:1000; ab173539), MEK-2 antibody (1:10,000; ab32517), ERK1/2 antibody (1:1000; ab17942), survivin antibody (1:5000; ab76424), Bax antibody (1:1000; ab32503), Bcl-2 antibody (1:1000; ab32124), and GAPDH antibody (1:2500; ab9485) (all of the above were purchased from Abcam Inc., Cambridge, MA, USA) were added. The following day, the membrane was incubated with the secondary antibody (ab7312; Abcam, Cambridge, MA, USA) at 4°C for 1 h. The imaging agent was added, and the Bio-Rad gel imaging system (MG8600; Beijing Thmorgan Biotech Ltd, Beijing, China) was used for development. Quantitative analysis was performed using IPP7.0 software (Media Cybernetics, Singapore, Republic of Singapore). The gray-value ratios of Raf-1, MEK-2, p-ERK1, ERK1/2, survivin, Bcl-2, Bax to GAPDH represented the respective content. This experiment was also applicable for cell experiments.

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

The RPMI8226 cell line was transfected at predetermined concentrations and was cultured in RPMI 1640 culture medium containing 10% FBS in a 5% CO₂ incubator at 37°C for 48 h. The cells in the logarithmic growth phase in each group were selected for subsequent experiments. Cells were trypsinized at an ordinary temperature, and the cell suspension was transferred to a centrifuge tube. After being blown and sucked into a sterile micropipette, the cells were resuspended into a single-cell suspension. In addition, Trypan blue exclusion was conducted to count living cells. Cells in each group were then transferred into a 96-well plate and were plated according to the counting results, ensuring that living cells were added into the 96-well plate (5,000 cells/well), and then the plate was cultured in a 5% CO₂ incubator at 37°C. Once the solution was substituted, PBS was added to the peripheral wells in the 96-well plate to prevent evaporation. Next, the 96-well plate was placed in a 5% CO₂ incubator at 37°C for 24 h, 48 h, and 72 h. After the plate was removed, 20 μl of 5% MTT solution was added to each well, followed by incubation under dark conditions. The plate was gently shaken for the even mixture of the solution, after which the plate was cultured in a 5% CO₂ incubator at 37°C for 4 h. Later, the plate was centrifuged at 453 × g for 5 min for the removal of the supernatant. Thereafter, the plate was added with 150 μL of dimethyl sulfoxide (DMSO) solution, followed by shaking on a table concentrator at 37°C for 30 min in the dark. Next, the plate was removed, and the optical density (OD) value at the wavelength of 490 nm was detected using an enzyme analyzer (SAF-680T, Multiskan, GO, Thermo, USA). The cell growth curve was plotted with transfection time on the X-axis and the OD value on the Y-axis.

Scratch test

Confluent RPMI-8226 cells on fibronectin (10 μg/mL)-coated (Sigma-Aldrich) 6-well plates were incubated with serum-free RPMI 1640 following cell adhesion. When cells reached 90% – 100% confluency, a 10-μL pipette tip was employed to gently make vertical scratches at the bottom of the 6-well plate, with approximately 4 to 5 scratches with the same width made in each well. The cells were then rinsed with PBS 3 times with dripped cells eliminated and were placed in an incubator. At 0 h and 48 h following scratching, the distance of cell migration in the scratch area was observed under the inverted microscope, and several fields were randomly selected and photographed. IPP7.0 software (Media, Cybernetics,
Singapore) was used for the analysis of the percentage of wound healing (cell surface in wound area/wound area). Three duplicated wells were established for each group, and each experiment was repeated 3 times.

Transwell assay
The Transwell chamber was placed into the 24-well plate, and the Matrigel-diluted solution (1:8) was used to cover the upper surface of the basement membrane of the Transwell chamber, which was dried at room temperature. Cells in the blank, NC, miR-497 mimic, miR-497 inhibitor, si-Raf-1, and miR-497 inhibitor + si-Raf-1 groups were centrifuged (1000 × g) for 3 min, rinsed with PBS twice and re-suspended with serum-free RPMI 1640 culture medium. The cell density was adjusted to 1 × 10^5 cells/mL, with 200 μL of the cell suspension added to the upper chamber, and 600 μL of serum-free RPMI 1640 culture medium added to the lower chamber. After conventional culture for 24 h, the Transwell chamber was removed and the cell suspension in the lower chamber was collected. The number of cells was then counted, and each group was established with 3 duplicated wells. The measurements were repeated 3 times in order to obtain the mean value.

Flow cytometry
After transfection for 48 h, the cells were removed and rinsed with cold PBS 3 times, centrifuged, and resuspended in PBS at a concentration of 1 × 10^5 cells/mL. Next, 1 mL of −20°C precooled 75% ethanol was added to fix the cells at 4°C for 1 h. Thereafter, centrifugation was carried out to remove the cold ethanol followed by washing twice with PBS to remove the supernatant, after which 100 μL of RNase A was added and the solution was incubated in a water bath at 37°C for 30 min under dark conditions. Later, 400 μL of propidium iodide (PI) (D0820, Sigma, San Francisco, California, USA) was added at 4°C for 30 min with the avoidance of light for staining. Flow cytometry (Gallios, Beckman Coulter Life Sciences, Brea, CA, USA) was performed to record red fluorescence at the excitation wavelength of 488 nm and to measure the cell cycle entry.

Xenograft tumors in nude mice
Eighteen male Kunming nude mice (aging 3 months old and weighing [20 ± 2] g) with clean grade were selected and purchased from the Animal Experimental Center of Southern Medical University. The RPMI8226 cell line was used for the preparation of the single-cell suspension, and PBS and Matrigel were mixed at a volume ratio of 1:1. The cells were resuspended in the mixture, and the cell concentration was finally adjusted to 1 × 10^6 cells/200 μL. The 18 nude mice were then classified into the blank, NC, miR-497 mimic, miR-497 inhibitor, si-Raf-1, and miR-497 inhibitor + si-Raf-1 groups (n = 3). After the mice were anesthetized with ether, 1 × 10^6 cells/200 μL of RPMI8226 cells were subcutaneously inoculated into the right hind limb of nude mice in each group. The mice were raised in the same environment and observed every 7 d, with the length and width of the tumor recorded cautiously. The tumor volume was calculated using the formula: volume = length × width^2/2. On the 35th day, the nude mice were sacrificed, and the tumors were dissected out. Three tumors were collected and weighed in each group. The animals enrolled in this study were fed in a pathogen-free
environment ad libitum. All animal experiments were conducted in accordance with the animal care and use guidelines provided by the Ethics Committee of Wenzhou Medical University. This study also followed the applicable institutional governmental regulations concerning the ethical use of animals.

**Statistical analysis**

SPSS 18.0 statistical software (IBM Corp. Armonk, NY, USA) was used for data analysis. The measurement data were expressed using means ± standard deviation. The data of normal distribution were assessed by the D’Agostino & Pearson omnibus normality test, and the comparisons among multiple groups were conducted by one-way analysis of variance (ANOVA) using Prism 6.0 software (GraphPad Inc, La Jolla, CA, USA). Pairwise comparisons were performed using Turkey’s post hoc test. The comparisons of the data with skewed normal distribution were examined by Dunn’s multiple comparison post hoc test in Kruskal-Wallis test. A value of $p < 0.05$ was considered to be statistically significant.

**Bioinformatic analysis**

GenomicScape (http://www.genomicscape.com) was adopted for the analysis of differentially expressed genes and miRs in MM cells in comparison with the normal counterpart. All parameters were screened with $p < 0.05$ considered as the standard.

**3. Results**

**A higher positive expression rate of Raf-1 protein is found in MM tissues**

Raf-1 is one of the important signaling molecules during signal transduction related to tyrosine kinase, which is also a cross-linking point associated with multiple signaling pathways. Raf-1 regulates tumor progression by activating the down-stream signaling pathways such as the ERK signaling pathway [31]. As an oncogene, Raf-1 was rarely reported in MM, and its function in MM remains unclear. The positive expression rates of Raf-1 protein in MM tissues and normal bone marrow tissues were detected by immunohistochemistry (Figure 1(a)). Based on the results, the positive expression rate of Raf-1 protein was 36.18% in normal bone marrow tissues and 78.29% in MM tissues (Figure 1(b)). Compared with normal bone marrow tissues, there was a significant increase in positive expression rate of Raf-1 protein in MM tissues ($p<0.05$). There was a high expression of Raf-1 in MM tissues, indicating that the Raf-1 signaling pathway was activated in MM.

**Identification of MM tissues and normal bone marrow tissues**

HE staining was applied for histopathological observation of MM tissues and normal bone marrow tissues in order to determine whether the collected samples were MM or bone marrow tissues. In the MM tissues, tumors presented with an

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**Figure 1.** A higher positive expression rate of Raf-1 protein was found in MM tissues. a, Immunohistochemical staining of the positive Raf-1 protein expression in normal bone marrow tissues and MM tissues ($\times$ 200); b, positive expression rate of Raf-1 protein in normal bone marrow tissues (n = 3) and MM tissues (n = 152); *, $p < 0.05$, vs. the normal bone marrow tissues; MM, multiple myeloma; PCs, plasma cells. The values refer to measurement data, which are expressed as mean ± standard deviation. Student t test was used for data analysis. The experiment was repeated 3 times.
invasive growth and invaded surrounding skeletal muscle tissues, and the tumor cells were also found to have typical morphological characteristics of malignant plasma cells, with an irregular nuclear pattern, a clear nucleolus, rich cytoplasm, and mitotic figures. There were no necrotic zones and infiltration of fibrous connective tissue observed. However, in the normal bone marrow tissues, the vessels were small and smooth, with a slightly increased diameter of individual vessels, a round lumen without distortion, and a regularly shaped nucleus (Figure 2).

**miR-497 was poorly expressed and Raf-1/ERK signaling pathway is activated in MM tissues**

RT-qPCR and western blot analysis were performed to detect the mRNA and protein levels of RAF-1, MEK-2, ERK1/2, and apoptosis-related factors (Bax, Bcl-2 and survivin) as well as the extent of ERK1/2 phosphorylation and verify the results of immunohistochemistry. As shown in Figure 3(a–c), compared with the normal bone marrow tissues, there were decreases in the levels of miR-497 and mRNA and protein levels of Bax in MM tissues, while mRNA and protein levels of Raf-1, MEK-2, Bcl-2 and surviving were elevated, along with the extent of ERK1/2 phosphorylation (all \( p < 0.05 \)), and there was no significant difference observed in the ERK1/2 mRNA and protein levels (\( p > 0.05 \)). The correlation analysis was conducted, and found that there was a negative correlation between miR-497 level and RAF-1 mRNA level, \( R \) value = −0.93 (Figure 3D). The above results suggested that there is a poor expression of miR-497, and a high expression of Raf-1, while the ERK signaling pathway was activated in MM tissues. In addition, the Raf-1/ERK signaling pathway activation is one of the key factors associated with MM progression.

**High miR-497 level and low Raf-1 mRNA level are associated with MM progression**

The above results showed that an increase in Raf-1 expression might be correlated with the activation of the ERK signaling pathway in MM. However, the key factor regulating Raf-1 is still unknown. We also found that miR-497 level was negatively correlated with Raf-1 mRNA level. Based on the bioinformatic analysis, miR-497 was predicted to be a candidate miR that regulates Raf-1. The potential role of miR-497 and Raf-1 in MM development were further analyzed by investigating the relationship between the miR-497 level and Raf-1 mRNA level and the pathological characteristics of MM patients (Table 3). The miR-497 level and Raf-1 mRNA level were unrelated to age or gender but related to the degree of anemia and renal function impairment, ISS staging and D-S staging. These findings indicated that the increase in the degree of anemia, the degree of renal function impairment, ISS staging and D-S staging resulted in a reduction of miR-497 level and an elevation in mRNA level of Raf-1 (\( p > 0.05 \)). Hence, low miR-497 level but high Raf-1 level was correlated with higher degree of anemia and renal function impairment and ISS.

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**Figure 2.** HE staining revealed that tumor cells in MM tissues presented with typical morphological characteristics of malignant plasma cells (× 400). HE, hematoxylin and eosin; MM, multiple myeloma; PCs, plasma cells. The arrow in the figures refers to malignant plasma cells and an irregular nucleus pattern.
Figure 3. Lower miR-497 level and higher mRNA and protein levels of Raf-1, MEK-2 and apoptosis-related factors were found in MM tissues. a, miR-497 level and mRNA levels of Raf-1, MEK-2, ERK 1/2, Bcl-2, Bax, and survivin in tissues; b, Protein levels of Raf-1, MEK-2, ERK 1/2, Bcl-2, Bax, and survivin in tissues; c, protein bands of the protein levels of Raf-1, MEK-2, ERK 1/2, Bcl-2, Bax, survivin and GAPDH in tissues; d, correlation analysis of miR-497 expression and mRNA expression of RAF-1,* p < 0.05, vs. the normal bone marrow tissues; MM, multiple myeloma. The values refer to the measurement data that were expressed as mean ± standard deviation. Student t test was used for comparison between two groups (n = 152). The experiment was repeated 3 times.

Table 3. The expression of miR-497 and Raf-1 and the pathological characteristics of patients.

| Pathological characteristics | n  | Expression of miR-497 | p value | Expression of Raf-1 | p value |
|-----------------------------|----|----------------------|---------|---------------------|---------|
| Age (years)                 |    |                      |         |                     |         |
| < 50                        | 44 | 0.172 ± 0.021        | 0.974   | 2.233 ± 0.122       | 0.858   |
| ≥ 50                        | 108| 0.170 ± 0.020        | 0.999   | 2.229 ± 0.120       | 0.960   |
| Gender                      |    |                      |         |                     |         |
| Male                        | 59 | 0.171 ± 0.019        | < 0.0001| 2.227 ± 0.114       | < 0.0001|
| Female                      | 93 | 0.171 ± 0.021        | < 0.0001| 2.228 ± 0.124       | < 0.0001|
| Anemia                      |    |                      |         |                     |         |
| Mild anemia                 | 23 | 0.187 ± 0.016        | < 0.0001| 2.124 ± 0.077       | < 0.0001|
| Moderate anemia             | 30 | 0.184 ± 0.006        | < 0.0001| 2.132 ± 0.040       | < 0.0001|
| Severe anemia               | 99 | 0.163 ± 0.006        | < 0.0001| 2.279 ± 0.040       | < 0.0001|
| Renal function impairment   |    |                      |         |                     |         |
| Renal inadequacy            | 27 | 0.193 ± 0.010        | < 0.0001| 2.092 ± 0.036       | < 0.0001|
| Dropsical nephritis         | 33 | 0.182 ± 0.003        | < 0.0001| 2.151 ± 0.029       | < 0.0001|
| No impairment               | 92 | 0.160 ± 0.003        | < 0.0001| 2.294 ± 0.029       | < 0.0001|
| ISS staging                 |    |                      |         |                     |         |
| Stage I                     | 61 | 0.187 ± 0.012        | < 0.0001| 2.123 ± 0.059       | < 0.0001|
| Stage II-III                | 91 | 0.160 ± 0.014        | < 0.0001| 2.296 ± 0.079       | < 0.0001|
| D-S staging                 |    |                      |         |                     |         |
| Stage I                     | 35 | 0.191 ± 0.011        | < 0.0001| 2.097 ± 0.041       | < 0.0001|
| Stage II                    | 40 | 0.179 ± 0.005        | < 0.0001| 2.171 ± 0.029       | < 0.0001|
| Stage III                   | 77 | 0.157 ± 0.005        | < 0.0001| 2.315 ± 0.029       | < 0.0001|

Note: miR-497, microRNA-497.
staging and D-S staging, which provided further proof on the hypothesis that miR-497 was negatively correlated with Raf-1 in MM progression.

**Raf-1 is confirmed as the target gene of miR-497, and the RPMI8226 cell line is used in subsequent experiments**

To verify whether miR-497 negatively regulates Raf-1, microRNA.org, a biology prediction site was employed and the results showed that miR-497 can target Raf-1 (Figure 4(a)). To confirm that Raf-1 was a target gene of miR-497, first, recombinant luciferase reporter vectors pRaf-1-Wt and pRaf-1-Mut were constructed with Raf-1 mRNA 3′-UTR inserted into the luciferase reporter vector. Additionally, the miR-497 mimic and NC were respectively co-transfected with recombinant luciferase reporter vectors into HEK293T cells. The results of the dual luciferase reporter gene system showed that the luciferase activity of Raf-1wt-3′-UTR co-transfection was decreased by approximately 42% in the miR-497 mimic group compared with that in the NC group \((p < 0.05)\) (Figure 4(b)). However, there was no significant difference in the luciferase activity of mutant Raf-1mut-3′-UTR luciferase between the NC and miR-497 mimic groups \((p > 0.05)\). Therefore, Raf-1 was confirmed to be a target gene of miR-497. Therefore, miR-497 can target Raf-1, in turn negatively regulating its mRNA level.

RT-qPCR was used for cell line selection among the RPMI8226, U266, XG-6, XG-7 and H929 cell lines. As shown in Figure 4(c), the miR-497 level was decreased successively as RPMI8226 > H929 > U266 > XG-6 > XG-7. The highest level of miR-497 was detected in RPMI8226; thus, the RPMI8226 cell line was selected for subsequent experiments.

**miR-497 down-regulates Raf-1 to suppress the MAPK/ERK signaling pathway**

To investigate the underlying mechanism of miR-497, Raf-1 and the ERK signaling pathway in MM cells, the cells were treated with miR-497 mimic, inhibitor and siRNA targeting Raf-1 to interfere the expression of miR-497 and/or Raf-1. Following treatment, western blot analysis and RT-qPCR were performed to detect miR-497 level, mRNA and protein level of Raf-1 and the ERK signaling pathway-related genes, and the extent of ERK1/2 phosphorylation. As shown in Figure 5(a–c), there were no notable differences observed in the expression of ERK1/2 in all groups. There was no statistically significant
difference in the miR-497 level and mRNA and protein levels of Bax, Raf-1, MEK-2, Bcl-2 and survivin, as well as in the extent of ERK1/2 phosphorylation between the blank and NC groups ($p > 0.05$). Compared with the blank and NC groups, the miR-497 mimic group presented with a significantly increased miR-497 level ($p < 0.05$), while the miR-497 inhibitor group has a markedly decreased miR-497 level ($p < 0.05$), and there were no significant differences in the si-Raf-1 group and miR-497 inhibitor + si-Raf-1 group ($p > 0.05$). Compared with the blank and NC groups, the MM cell viability was decreased in the miR-497 mimic and si-Raf-1 groups ($p < 0.05$) but increased in the miR-497 inhibitor group ($p < 0.05$), while the miR-497 inhibitor + si-Raf-1 group showed no significant difference ($p > 0.05$). These findings revealed that the overexpression of miR-497 or silencing of Raf-1 could lead to the inhibition of MM cell growth.

### Overexpression of miR-497 or silencing of Raf-1 suppresses cell migration and invasion

The scratch test and Transwell assay were performed to detect cell migration and invasion to show the effect of miR-497 and Raf-1 on MM cell migration and invasion. As shown in Figure 7(a,b), there were no significant differences among the blank, NC and miR-497 inhibitor + si-Raf-1 groups (all $p > 0.05$). Compared with the blank and NC groups, the cell migration ability was decreased in the miR-497 mimic and si-Raf-1
groups ($p < 0.05$) and increased in the miR-497 inhibitor group ($p < 0.05$).

As shown in Figure 7(c–d), there were no significant differences among the blank, NC and miR-497 inhibitor + si-Raf-1 groups (all $p > 0.05$). Compared with the blank and NC groups, the cell invasion ability was decreased in the miR-497 mimic and si-Raf-1 groups.
and increased in the miR-497 inhibitor group \(p < 0.05\).

These results implied that the enforcement of miR-497 or depletion in Raf-1 expression could inhibit cell migration and invasion.

**Overexpression of miR-497 or silencing of Raf-1 promotes cell apoptosis and arrests cells in the G1 phase**

Flow cytometry was applied to detect the cell cycle distribution and cell apoptosis in MM cells with overexpressed miR-497 or silenced Raf-1 expression. PI staining indicated (Figure 8(a–b)) that there were no significant differences among the blank, NC and miR-497 inhibitor + si-Raf-1 groups (all \(p > 0.05\)). Compared with the blank and NC groups, cell growth in the miR-497 mimic and si-Raf-1 groups was mainly arrested in G1 phase, increased in G1 phase, and decreased in S and G2/M phase (all \(p < 0.05\)).

According to the result of Annexin V/PI double staining (Figure 8(c–d)), there were no significant differences among the blank, NC and miR-497 inhibitor + si-Raf-1 groups (all \(p > 0.05\)). In comparison to the blank and NC groups, the cell apoptosis rate decreased in the miR-497 inhibitor group \(p < 0.05\) and increased in the miR-497 mimic group and si-Raf-1 group \(p < 0.05\).

Taken together, miR-497 arrests cells in G1 phase, and overexpression of miR-497 or si-Raf-1 promotes cell apoptosis.

![Figure 8. miR-497 mimic or si-Raf-1 promoted the apoptosis of MM cells, as detected by flow cytometry. a and b, cell cycle distribution in different transfection groups, G0/G1 were the early stage of DNA synthetic phase and G2 phase was the later phase stage of DNA synthetic phase; c and d, cell apoptosis in different transfection groups. The experiment was repeated 3 times, and the obtained mean value \(\pm\) standard deviation was presented as experiment results. One-way analysis of variance (ANOVA) was used for data comparison. *, \(p < 0.05\), vs. the blank and NC group; NC, negative control; miR-497, microRNA-497.](image-url)
miR-497 up-regulation or Raf-1 knockdown contributes to a decrease in tumor size and growth

Finally, in vivo experiments using xenograft tumors in nude mice were conducted to investigate the effect of miR-497 and Raf-1 on MM formation ability. Based on the findings, there were no significant differences in tumor growth in the blank and NC groups ($p > 0.05$). Compared with the blank group, nude mice in the miR-497 inhibitor group had increased subcutaneous tumors and the fastest tumor growth rate ($p < 0.05$). There were decreases in subcutaneous tumor size and tumor growth rate in nude mice in the miR-497 mimic and si-Raf-1 groups ($p < 0.05$), while the miR-497 inhibitor + si-Raf-1 group showed no significant difference ($p > 0.05$) (Figure 9(a–c)). Therefore, tumor size and growth was reduced in nude mice transfected with miR-497 mimic or si-Raf-1.

The differentially expressed genes and miRs in MM were analyzed on the bioinformatics platform GenomicScape. The obtained data displayed that there was a low expression of miR-497 but a high expression of RAF-1 in MM cells (Figure 11(a,b)), which is consistent with our experiments. Meanwhile, a co-expression network of RAF-1 and MM molecular subgroups (Wnt signaling pathway: ZNF family, CK1A, FZD family, BMP6 and inflammatory cytokine STAT3) was found on the basis of bioinformatics analysis (Figure 11(c)).

4. Discussion

MM is a disease related to a hematological disorder that appears at various stages of β-cells, and it accounts for 1% of all cancers, as well as 10% of hematological malignancies [32,33]. Based on recent studies, miRs have been identified as tumor-suppressor genes and play important roles in tumor pathogenesis [34,35]. In this study, we conducted a series of experiments in order to determine the effects of miR-497 on proliferation, migration, invasion and apoptosis in human MM cells by targeting Raf-1 through the MAPK/ERK signaling pathway. Finally, our experimental results indicated that the overexpression of miR-497 resulted in the inhibition in the proliferation, migration, and invasion of MM cells and promoted apoptosis through the reduction of Raf-1 expression.

MM tissues presented with decreased levels of miR-497 and Bax and elevated levels of Raf-1, MEK-2, Bcl-2, survivin, along with an increase in the extent of ERK1/2 phosphorylation. Based on a global miR-profiling study conducted on miR expression in normal plasma cells and myeloma cells, 38 miRs were found to have been significantly down-regulated while 29 miRs were significantly up-regulated in myeloma cells in comparison with their normal counterpart [36]. A previous study has also identified miR-30, miR-106b and miR-16 as plasma cell differentiation stage specific miRNAs, which were aberrantly

Figure 9. Nude mice transfected with miR-497 mimic or si-Raf-1 showed a decreased tumor volume and weight. a, tumor growth curve of nude mice in each group examined by the experiment of xenograft tumors in nude mice; b, the tumor size in each group on the 35th day; c, comparison of the tumor weight in each group on the 35th day; the experiment was repeated 3 times, and the obtained mean value and standard deviation were presented as experimental results. One-way analysis of variance (ANOVA) was used for data comparison *, $p < 0.05$, vs. the blank and NC groups; NC, negative control; miR-497, microRNA-497.
overexpressed in MM cells [37]. However, a number of studies revealed that the miR-497 level was reduced and the expression of Raf-1 was increased in MM tissues [18,38]. The high expression of Bcl-2 in MM cell lines was found in a former study [39]. Survivin is known to be expressed in most human cancer tissues instead of normal tissues [40]. In a study conducted by Tsubaki et al., increased levels of both ERK1/2 and survivin were found in MM [41]. The down-regulation of Bax was also observed in an MM cell line in a previous study [42].

Moreover, cells transfected with the miR-497 mimic and si-Raf-1 had lower expression levels of Raf-1, Bcl-2, survivin and there was also a decrease in the extent of ERK1/2 phosphorylation, while higher expression levels of Bax were observed. Survivin has been identified as a key factor in cell division and the inhibition of apoptosis in adult cancer tissues [40]. Bcl-2, ERK, Raf-1 and survivin are anti-apoptotic proteins that play crucial roles in the regulation of cell survival and death. The inhibition of these proteins by miR-497 could be a potential therapeutic strategy for MM treatment.

Figure 10. miR-497 mediated the MAPK/ERK pathway by targeting Raf-1; miR-497 inhibited Raf-1 and then inhibited the activation of the MAPK/ERK pathway to promote the apoptosis of multiple myeloma cells. miR-497, microRNA-497; MAPK/ERK, mitogen-activated protein kinase/extracellular signal-regulated kinase.

Figure 11. miR-497 was lowly expressed but RAF-1 highly expressed in MM cells. a, RAF-1 was highly expressed in MM cells, as analyzed on the bioinformatics platform GenomicScape; b miR-497 was lowly expressed in MM cells, as analyzed on the bioinformatics platform GenomicScap; c, a co-expression network of RAF-1 and MM molecular subgroups (Wnt signaling, ZNF, CK1A, FZD, BMP6 and STAT3) on the basis of bioinformatic analysis.
genes [43–45], while Bax is a pro-apoptotic gene [46,47]. There was a positive correlation between the levels of survivin, Bcl-2 and ERK with the anti-apoptotic action, while Bax was negatively associated with the levels of survivin and Bcl-2 [48]. The inhibition of Raf-1 expression could potentially result in the suppression of Bcl-2 expression [49]. Previous studies have also found that miRs negatively regulate gene expressions [50,51]. Additionally, the results from the dual fluorescence reporter assay confirmed that Raf-1 was a target gene of miR-497, which was consistent with a previous study suggesting that Raf-1 was a key activator of the ERK signaling pathway and could be negatively regulated by miR-497 [52,53]. Another study uncovered that the overexpression of miR-497 could decrease the level of Bcl-2 in human umbilical vein endothelial cells [54]. The down-regulation of Bcl-2 and up-regulation Bax could also occur as a result of miR-142 overexpression [55]. Therefore, the results implied that the overexpression of miR-497 results in the reduction of Raf-1 expression and anti-apoptotic genes, while it promotes the expression of pro-apoptotic genes via the MAPK/ERK signaling pathway.

Finally, MM cells transfected with the miR-497 mimic and si-Raf-1 presented with decreased cell proliferation, migration and invasion and increased cell apoptosis, while the mice that had received miR-497 mimic or si-Raf-1 treatment had reduced subcutaneous tumor size and weight and tumor growth rate was also found to be inhibited. Zhang Y et al. and Han L et al. have also demonstrated that the overexpression of miR-497 resulted in decreased cell proliferation, migration and invasion [56,57]. Shen L et al. also found that the upregulation of miR-497 can lead to apoptotic enhancement [58]. miR-497 regulates the multidrug resistance of human cancer by targeting Bcl2 and inducing apoptosis and inhibiting proliferation via Bcl-2/Bax [54,59]. In addition, Raf-1 protein actively participates in cell growth and proliferation, and its inhibition is related to tumor growth arrest and cell apoptosis [60]. A study performed by Xu J et al. also showed that the up-regulation of miR-497 led to the inhibition of tumor growth [61]. The aforementioned findings suggested that the overexpression of miR-497 or Raf-1 silencing inhibits the proliferation, migration and invasion of MM cells, along with tumor growth.

In summary, our data provided further evidence on previous observations in support of the hypothesis that the overexpression of miR-497 inhibits MM cell proliferation, migration and invasion by reducing the expression of Raf-1 through the MAPK/ERK signaling pathway (Figure 10), which can be considered as promising perspective for future clinical practice. However, due to the presence of a number of factors affecting miR-497 and the MAPK/ERK signaling pathway, additional studies are required in order to investigate investigating the direct relationship between miR-497 and the MAPK/ERK signaling pathway.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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