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MicroRNA-122 Is More Effective than Rapamycin in Inhibition of Epithelial-mesenchymal Transition and mTOR Signaling Pathway in Triple Negative Breast Cancer

Majdedin Ghalavand[1], Ruhollah Dorostkar[2], Hojat Borna[3], Samira Mohammadi-Yeganeh[1,4], and Seyed Mahmoud Hashemi[5,6]

[1] Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[2] Applied Virology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
[3] Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran
[4] Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[5] Medical Nanotechnology and Tissue Engineering Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[6] Department of Immunology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

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ABSTRACT

The fundamental mechanism responsible for the aggressiveness of metastatic cancers such as triple-negative breast cancer (TNBC) is the epithelial-mesenchymal transition (EMT). In cancer microenvironments, the Phosphoinositide 3-kinases (PI3K)-Akt- mammalian target of rapamycin (mTOR) signaling pathway plays a critical role in regulating the EMT mechanism. The current study focuses on the impacts of rapamycin, a newly retargeted chemotherapeutic agent against mTOR, and MicroRNA (miR)-122 on the aggressive behavior of TNBC.

The half-maximal inhibitory concentration (IC50) of rapamycin on 4T1 cells was determined using an MTT assay. Also, miR-122 was transiently transfected into 4T1 cells to study its effect on the pathway. Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted to assess the expression level of central mTOR and EMT-related cascade genes. Moreover, cell mobility and migration were evaluated using scratch and migration assays, respectively.

Both rapamycin and miR-122 significantly decreased the expression levels of *PI3K*, *AKT*, and *mTOR*, as well as *ZeB1* and *Snail* genes. However, no significant change was observed in *Twist* gene expression. Furthermore, scratch and migration assays revealed that the migration of 4T1 cells was markedly reduced, especially following miR-122 induction. Our experimental findings and gene enrichment studies indicated that miR-122 mainly operates on multiple metabolic pathways, as well as EMT and mTOR, while rapamycin has restricted targets in cancer cells.

Corresponding Authors: Samira Mohammadi-Yeganeh, PhD; Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran. Tel: (+98 21) 8866 6136, Fax: (+98 21) 8666 136-8), E-mail: s.mohammadiyeganeh@sbmu.ac.ir

Seyed Mahmoud Hashemi; PhD;
Medical Nanotechnology and Tissue Engineering Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran. Tel: (+98 21) 2243 2573), Fax: (+98 21) 2243 9970, E-mail: smmhashemi@sbmu.ac.ir

Consequently, miR-122 can be considered a potential cancer microRNA therapy option, which can be validated in the future in animal studies to demonstrate its efficacy in cancer control.

Keywords: Cancer microenvironment; MIRN122 microRNA, human; Rapamycin; Triple-negative breast cancer; Signaling pathway

INTRODUCTION

Cancer is a serious public health challenge as the world's second-largest cause of death. According to a GLOBOCAN epidemiological review of cancer prevalence in 2020, breast cancer ranked as the most widespread cancer category.[1,2]

Triple-negative breast cancer (TNBC) is the most lethal and aggressive form of breast cancer since it is resistant to current treatments.[3,4] The leading cause of cancer-related fatalities in TNBC patients is metastasis. It is a complex process involving numerous genes and signaling pathways.[5-7]

The epithelial-mesenchymal transition (EMT) process represents the initial step toward metastasis in TNBC. EMT is a biological reprogramming process that occurs when polarized epithelial cells change to a mesenchymal phenotype. It's crucial for cancer cell spread, invasion, and resistance to apoptosis.[8-10] The loss of E-cadherin in adherens junctions is the main hallmark of EMT during metastasis. To prevent transcription of E-cadherin, EMT-activating transcription factors, including *Zeb1*, *Twist*, and *Snail*, bind to E-boxes in the promoter. Consequently, they play a key role in the control algorithm of EMT, metastasis, and chemotherapy resistance.[11,12]

Emerging evidence suggests that PI3K-Akt-mTOR, autophagy, EMT, and cancer stem cells are directly engaged in metastasis and play significant roles in tumor recurrence and progression.[13-16] There are different strategies to interfere with the EMT process, including targeting the Wnt pathway with pyrvinium[17] and Evodiamine,[18] transforming growth factor-beta (TGF-β) pathway with zerumbone[19] and bufalin,[20] Akt-Snail pathway with disulfiram[21] and zidovudine,[22] Akt-HIF1α pathway with icaritin.[23] Tumor development in the breast requires constant activation of the PI3K-AKT-mTOR pathway.

The mammalian target of rapamycin (mTOR) pathway is one of the signal transduction pathways that has been investigated and analyzed the most.[24] Inhibiting the mTOR signaling pathway can also interfere with the EMT process. Rapamycin, the prototypical inhibitor of mTOR, is a macrolide antibiotic produced by the actinomycete *Streptomyces hygroscopicus* to prevent organ transplant rejection and coat coronary stents.[25,26] Based on drug repositioning investigations, scientists seek the anticancer effects of drugs such as rapamycin on tumor cells in the cancer microenvironment.

Moreover, as a subclass of noncoding RNAs, microRNAs can effectively match up with targets in signaling cascades that contribute to metastasis and cancer progression. They usually cause silencing or degradation of mRNA, leading to downregulation of gene expression.[27] Since microRNAs(miRs) have been discovered, numerous studies have demonstrated their significance as regulators of a wide range of disorders and suggested using them as therapeutic agents, particularly in cancer.[28-30]

Although standard cancer treatment procedures such as surgery, chemotherapy, and radiotherapy have shown promising efficacy, severe side effects remain as a concern. A combination of novel targeted therapy by RNA interference, immunotherapy, and drug repositioning chemotherapy is a promising method for increasing the efficacy of existing strategies. Blocking signaling pathways has proven to be an innovative and promising cancer therapy approach. miR-122 is a highly conserved microRNA in mammals that regulates many gene networks, metabolic pathways, and toxin responses.[31]

There is controversy about the tumorigenic and antiproliferative effects of miR-122 on different cancer types. In this study, we investigated the impacts of miR-122 and rapamycin on both the PI3K-Akt-mTOR signaling pathway and the EMT process.

MATERIALS AND METHODS

Chemicals and Compounds

Rapamycin was purchased from Sigma-Aldrich (USA). Temozolomide, MTT solution, and dimethyl sulfoxide were obtained from Sigma (St. Louis, USA). Penicillin-Streptomycin solution (Pen-Strep), trypan

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blue, and trypsin-EDTA were purchased from Merck (Germany). Fetal bovine serum (FBS), phosphate-buffered saline (PBS), RPMI 1640, and Dulbecco`s Modified Eagle Medium (DMEM) were provided by Gibco (USA).

Cell Line and Culture

The mouse TNBC cell line, 4T1, was purchased from Pasteur Institute of Iran (Tehran, Iran). We cultured the cells in DMEM containing 10% FBS and 1% Pen/Strep and incubated them in a humidified 5% CO_2 incubator at 37°C. The cells were trypsinized and recultured at 85% confluency.

MTT Assay

To evaluate the antiproliferative effects of rapamycin and its half-maximal inhibitory concentration (IC50), an MTT assay was carried out. 4T1 cells were seeded in 96-well plates at 5×10^3 cells/well. Following overnight incubation, increasing concentrations of rapamycin from 10–100 µM for 24, 48, and 72 h were added to wells. The relative proportion of viable cells was assessed based on the reduction of the MTT reagent to formazan. The results were read with microplate spectrophotometers by measuring the absorbance at 570 nm.

Bioinformatic Analysis and miRNA Selection

Not only do microRNAs have various targets in different cell lines and organs, but they can also play distinct actions of induction, silencing, and suppression due to their sequences and target sites in mRNAs. To analyze the predicted and validated targets of selected microRNA, TargetScan (https://www.targetscan.org/vert_71/), TargetMiner (https://www.isical.ac.in/~bioinfo_miu/targetminer20.htm), miRDB (https://mirdb.org/mirdb/index.html), and miRbase (https://www.mirbase.org) were investigated to achieve the predicted targets and miRTarBase (https://mirtarbase.cuhk.edu.cn) was studied to collect validated targets.

Enrichr (https://maayanlab.cloud/Enrichr/) was utilized to categorize and analyze the pathways involved in the interactions of miR-122.

miR-122 Transient Transfection to 4T1 Cells

In 24-well plates, 2.0×10^6 4T1 cells were cultured upon reaching 70% confluency at 37°C in 5% CO_2 to maximize the efficiency of transfection. The cells were transfected after 24 h, with pLenti-III- miR122-GFP and blank pLenti vector (Mock) using cationic polymers following the established protocol of TurboFect Transfection Reagent (Thermo Fisher Scientific Inc., USA). Following 6 to 12 h of incubation in a serum-free medium, the transfection medium was replaced with a new medium containing 10% FBS. The effectiveness of transfection was measured using a fluorescent microscope (Nikon, TE2000) and quantitative real-time polymerase chain reaction (qRT-PCR).

RNA Extraction and Synthesis of cDNA

All treatments were prepared in 5 groups, including control, pLenti (Mock), Rapamycin, miR-122, and miR-122 plus Rapamycin. The cells were gathered and washed with PBS 48 h after treatment. An RNA-hybrid kit (GeneAll, Korea) was used to extract total RNA, following the manufacturer's protocol. Then 1 µg of isolated RNA, 1 µL of 10 pM random hexamer primers, and 8.5 µL of nuclease-free distilled water were used to synthesize cDNA for gene expression evaluation. This mixture was kept for 5 min at 65°C. The solution was then supplemented with 4 µL of 5X Reverse Transcriptase (RT) buffer, 0.5 µL of RevertAid RT enzyme (200 units/µL) (Catalog No. #EP0441; Thermo Fisher Scientific), and 1 µL of dNTP mix (10 mM). For enzyme inactivation, tubes were incubated at 25°C for 10 min, 42°C for 60 min, and 70°C for 10 min. cDNA synthesis for miR-122 and the housekeeping gene (*SNORD234*) was performed according to our previously published articles.[32] The primer design was done by NCBI (http://www.ncbi.nlm.nih.gov) and Primer3 (https://primer3.ut.ee/) online software, and the sequences are provided in supplementary Table 1.

Quantitative Real-time PCR

SYBR Green Master Mix Kit (Amplicon, Denmark) was used for all quantitative real-time polymerase chain reaction (qRT-PCR) reactions. The procedures were carried out as per the manufacturer's instructions. One cycle at 95°C for 10 min was followed by 40 cycles of 15 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C for the qRT-PCR reaction. Gene and microRNA expression data were normalized to beta-2 microglobulin (*β2M*) and *SNORD234*, respectively. We used a $2^{-\Delta\Delta Ct}$ approach to determine the relative changes. The ABI StepOne System (Applied Biosciences, Foster City, CA) was used to run experiments in duplicate.

Scratch-wound Healing Assay

In 24-well plates, 6.5×10^4 transfected and untransfected 4T1 cells were seeded. When the cells had reached 90% confluency, a wound gap was made by scratching them with a sterile pipette tip, and cell debris was removed by washing them gently with PBS 1X sterile solution. The medium was replaced with a serum-free medium for 48 h. Phase-contrast images from the scratch edge were taken by inverted microscopy (Olympus, Japan). For the next 48 h, the medium was changed to one that didn't contain any serum. At time intervals of 0, 24, and 48 h, the migration of cells toward the scratched area is observed. As controls (blank), untransfected cells were evaluated, and the entire experiment was performed 3 times. The percentage of gap repair was determined using the following formula:[33]

$$\text{Wound Closure \%} = \left[\frac{\text{At=0h} - \text{At=}\Delta\text{h}}{\text{At=0h}}\right] \times 100\%$$

Transwell Cell Migration Assay

A migration experiment was conducted in 24-well transwells with a pore size of 8.0 µM (SPL, Korea). Briefly, 250 µl of 4T1 cells suspended in an FBS-free medium were transferred to the upper chambers separately (7×10^4 cells per chamber). In each example, 750 microliters of a medium containing 15% FBS as a chemoattractant were added to the bottom compartment. Non-migrated cells at the top of the transwells were manually removed using a cotton-tipped applicator after 48 h of incubation at 37°C and 5% CO2. In contrast, migrated cells on the lower surface were fixed with 70% ethanol (Sigma, Germany). An average number of migrated cells was determined by counting the number of cells in 5 randomly chosen fields after staining them with 0.05% crystal violet (Merck, Germany) and fixing them in PBS. Five separate experiments were carried out in duplicate. Cells that had not been transfected served as controls (blank).

Statistical Analysis

All information was reported as the mean±standard deviation (SD). To compare more than 2 groups, one-way analysis of variance (ANOVA) was utilized. Student's *t* test was used to compare the 2 groups using GraphPad Prism version 8.0. A *p* value of 0.05 or lower was considered to indicate statistical significance. The Shapiro-Wilk test was performed to determine if the data followed a normal distribution.

RESULTS

Cytotoxic Activity of Rapamycin

We determined the rapamycin concentration that caused 50% growth inhibition using the MTT assay. The determined IC50 value of rapamycin at 24 and 48 hours was about 39 and 17 µM for the 4T1 cell line, respectively (Figure 1). The concentration of 5 µM in 48 h was selected for further experiments.

miR-122 Practically Impacts Signaling Pathways

Studies based on target predictions of microRNAs and validated data show that miR-122 mainly acts on signaling rather than metabolic pathways. Tables 1 and 2 demonstrate the top 10 enriched KEGG pathways within which validated and predicted target mRNAs of miR-122 are enriched, respectively. All enriched pathways are statistically significant. The difference between tables of validated and predicted targets highlights the importance of the signaling pathways being highly involved in the interactions of miR-122 in different cancers.

Transient Transfection of miR-122 in the TNBC Cell Lines

Plasmids, including the pLenti-III-GFP-mmu-miR-122 overexpression vector and the empty vector (mock), were transiently transfected into 4T1 cells. Fluorescent microscopy and quantitative real-time PCR were used to evaluate miR-122 transfection efficiency (Figures 2a and 2b). The transfection was different between the 3 timeline groups, as indicated by green fluorescent protein (GFP) expression. Transfection efficiency was increased with a Turbofect DNA carrier (85%). Also, qRT- PCR approved miR-122 overexpression in the 4T1 cell line significantly ($p<0.0001$)

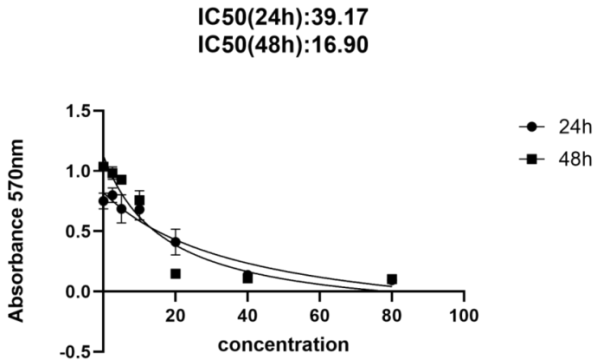

Figure 1. MTT assay shows the half-maximal inhibitory concentration (IC50) values of rapamycin in 2 time points: in 48 h, the IC50 value (16.90) was lower than 24 h of treatment (39.17).

Table 1. Top 10 validated targets of miR-122

Index	Name	*p*	Adjusted *p*	Odds Ratio	Combined Score
1	Pancreatic cancer	0.0001954	0.04429	4.93	42.14
2	Glycosaminoglycan biosynthesis	0.002870	0.08008	4.67	27.32
3	Osteoclast differentiation	0.0006350	0.04429	3.49	25.68
4	Renal cell carcinoma	0.002479	0.07684	4.13	24.80
5	AGE-RAGE signaling pathway in diabetic complications	0.001481	0.05902	3.63	23.64
6	Viral carcinogenesis	0.0004037	0.04429	2.95	23.02
7	Sphingolipid signaling pathway	0.001394	0.05902	3.37	22.16
8	Glucagon signaling pathway	0.002368	0.07684	3.37	20.36
9	Chronic myeloid leukemia	0.004280	0.09185	3.71	20.25
10	Human T-cell leukemia virus 1 infection	0.0008876	0.04953	2.71	19.06

Table 2. Top 10 predicted targets of miR-122

Index	Name	*p*	Adjusted *p*	Odds Ratio	Combined score
1	Mucin-type O-glycan biosynthesis	0.0006367	0.1242	11.43	84.10
2	Citrate cycle (tricarboxylic acid- TCA cycle)	0.004282	0.2658	10.11	55.16
3	Circadian rhythm	0.004703	0.2658	9.75	52.27
4	Riboflavin metabolism	0.08470	0.5161	12.90	31.84
5	Glycolysis / Gluconeogenesis	0.006358	0.2658	5.80	29.32
6	Epithelial cell signaling in Helicobacter pylori infection	0.007414	0.2658	5.53	27.13
7	Other types of O-glycan biosynthesis	0.01492	0.3637	6.20	26.08
8	Non-small cell lung cancer	0.008179	0.2658	5.37	25.80
9	Glycosylphosphatidylinositol-anchor biosynthesis	0.03292	0.4510	7.55	25.78
10	Hedgehog signaling pathway	0.02374	0.4510	5.15	19.25

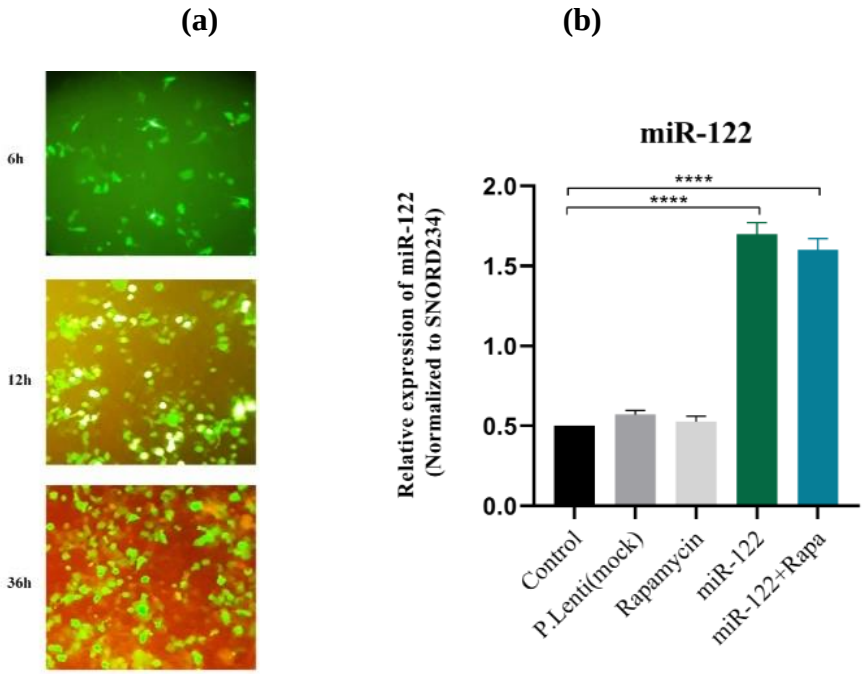

Figure 2. Establishment of transient miR-122 overexpression in 4T1 cell line (green fluorescence pictures showing that infected 4T1 cells have GFP expression). (a) GFP expression was observed by fluorescence microscopy after transfection of the *pLenti-III-GFP*-miR-122 overexpression and control vector (mock) into the 4T1 cells. Scale bar, 200µm. (b) miR-122 relative expression in the TNBC cells after transfection with TurboFect determined by quantitative real-time PCR (qRT-PCR). Data are expressed as mean±SD. Three independent experiments were done in triplicate (n=3). (*p*:*<0.05, **<0.01, *<0.001, ****<0.0001)**

Transient Transfection of miR-122 and Cotreatment with Rapamycin Downregulate PI3K-Akt-mTOR Signaling Pathway Cascade Genes in TNBC Cell Lines

mTOR Pathway Genes Suppression Induced by miR-122 Overexpression

To examined the function of miR-122 in blocking and inhibiting TNBC; PI3K-Akt-mTOR was selected due to its essential involvement in TNBC recurrence. The expression levels of *PI3K*, *Akt*, and *mTOR* genes were analyzed using quantitative real-time PCR to determine how miR-122 affected the gene expression profile of the mTOR pathway. The results of qRT-PCR demonstrated that the expression of PI3K was considerably reduced in cells treated with miR-122 compared to control groups (p<0.0001). Similarly, miR-122 treatment resulted in a marked decrease in *Akt* and *mTOR* expression as compared to control cells (p<0.0001) (Figure 3a, 3b, and 3c).

Rapamycin Decreased the Expression of PI3K-Akt-mTOR Pathway Genes

As demonstrated in Figure 3a, the rapamycin-treated group had lower *PI3K* expression levels than the control groups (p<0.05). However, after miR-122 induction, *PI3K* gene expression dropped sharply (p<0.0001). The expression level of *Akt* and *mTOR* decreased significantly in 2 experimental groups that received miR-122 and free rapamycin (p<0.0001). These data demonstrated that treatment with rapamycin and miR-122 inhibited key genes of the mTOR signaling cascade in TNBC cells. Finally, the comparison between miR-122 and miR-122 + rapamycin treatment showed that the effectiveness of miR-122 is very high (due to its involvement in many pathways, including metabolic pathways), and no significant differences were observed when combined with rapamycin. This pattern was repeated in the results of other tests as well. (Figures 3a, 3b, and 3c).

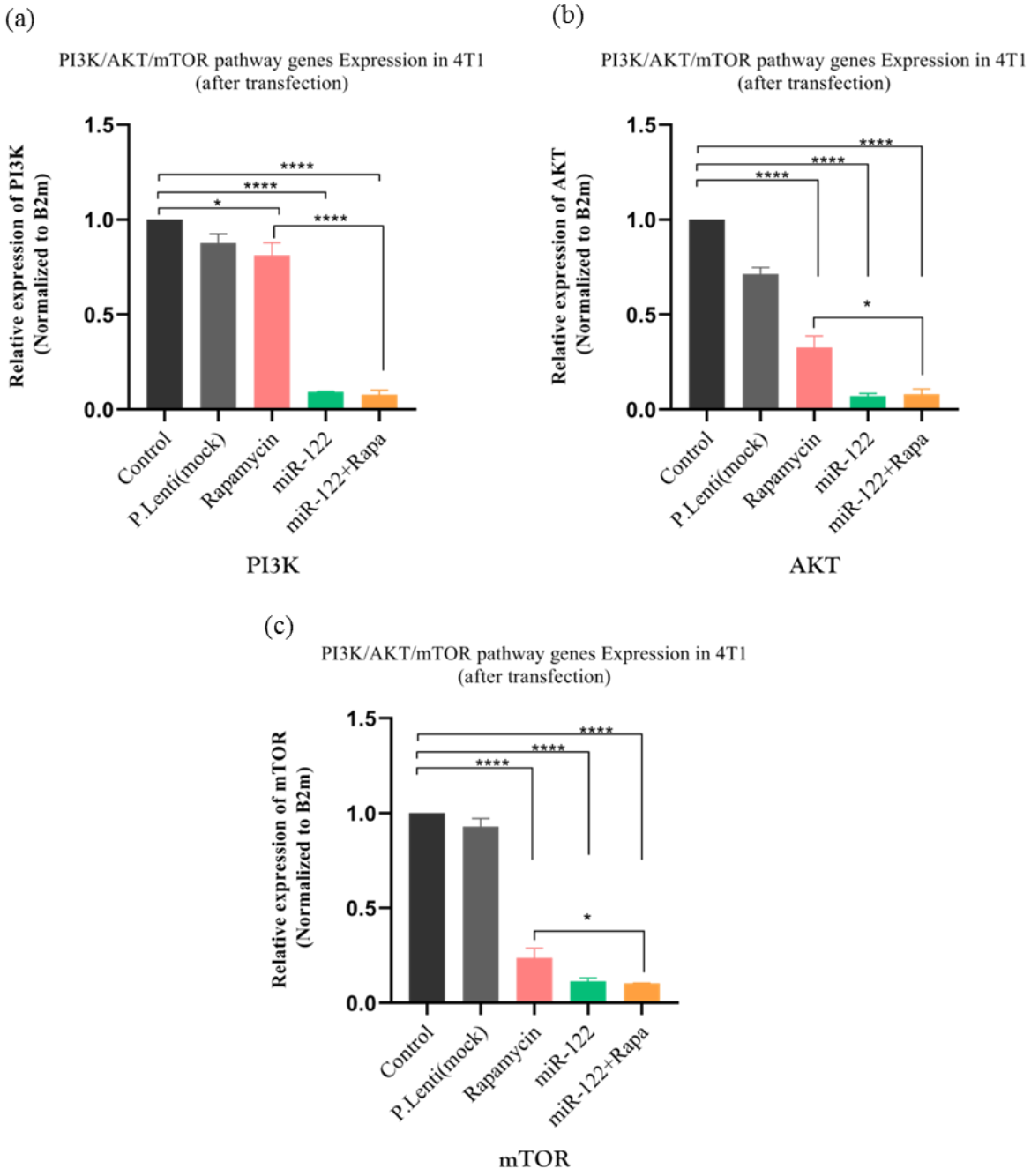

Figure 3. The relative expression of PI3K/Akt/mTOR pathway genes in 4T1 cells after treatment with miR-122 and rapamycin in 5 groups: control, pLenti (mock), rapamycin, miR-122, and miR-122 + rapamycin. (a) *PI3K*, (b) *Akt*, and (c) *mTOR* expressions were determined by qRT-PCR after treatment groups. Data are expressed as the mean±SD. Three independent experiments were done in triplicates (n=3). (*p*: * <0.05, ** <0.01, * <0.001)**

EMT Transcription Factors Expression Diminished after miR-122 Induction in 4T1 Cells

The expression level of EMT transcription factors dropped significantly in cells treated with miR-122 compared to untreated cells (p<0.0001). *Snail* gene expression was also significantly lower in miR-122-treated cells compared to control cells (p<0.0001). However, the change in the expression of the *Twist* gene was not significant in miR-122 treated cells (Figures 4a, 4b, and 4c).

Rapamycin Treatment Decreased EMT Transcription Factors' Expression

According to the qRT-PCR results, rapamycin treatment of 4T1 cells led to a considerable decrease in *Zeb1* and *Snail* transcript expression compared to the other groups (*p* value <0.0001). Likewise, in the *Twist* group, none of the gene expressions changed (Figures 4a, 4b, and 4c).

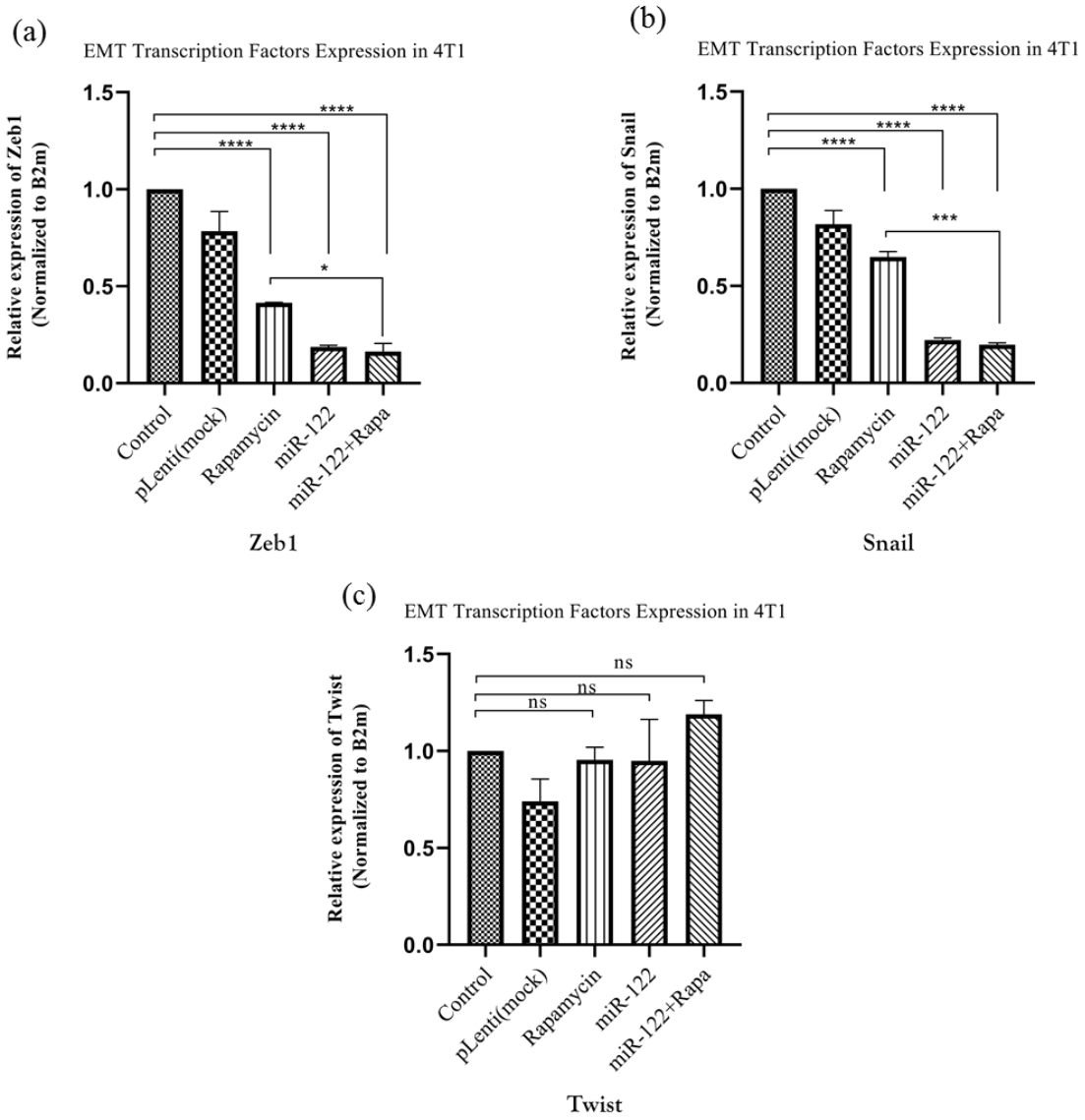

Figure 4. Expression of epithelial–mesenchymal transition (EMT)-specific transcription factors (*Zeb1*, *Snail* and *Twist*) in 4T1 cells after treatment with miR-122 and rapamycin in 5 groups; control, pLenti (mock), Rapamycin, miR-122 and miR-122 + rapamycin. (A) *Zeb1*, (B) *Snail*, and (C) *Twist* were determined by qRT-PCR after treatment groups. Data are expressed as the mean±SD. Three independent experiments were done in triplicates (n = 3). (*p* values: *<0.05, **<0.01, *<0.001)**

Delivery of miR-122 and Rapamycin Reduced Migration of 4T1 Tumor Cells

In the presence of rapamycin and miR-122, the migratory abilities of 4T1 cells were found to be diminished. Scratch assay results demonstrated that miR-122–treated 4T1 cell lines had impaired wound healing compared to rapamycin-treated and control cells. After 24 and 48 h of administration, there was no difference between the control and mockgroups (Figure 5).

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TNBC Cell Migration is Reduced by Upregulating miR-122 and Supplementing Rapamycin

According to this study looking into the effect of rapamycin and overexpression of miR-122 on the migration of 4T1 cells, miR-122 upregulation decreased the number of cells passing through the transwell chamber membrane in comparison to the mock group (p<0.001). In addition, the rapamycin-treated group displayed a significant decrease in cell migration (p<0.001) (Figure 6).

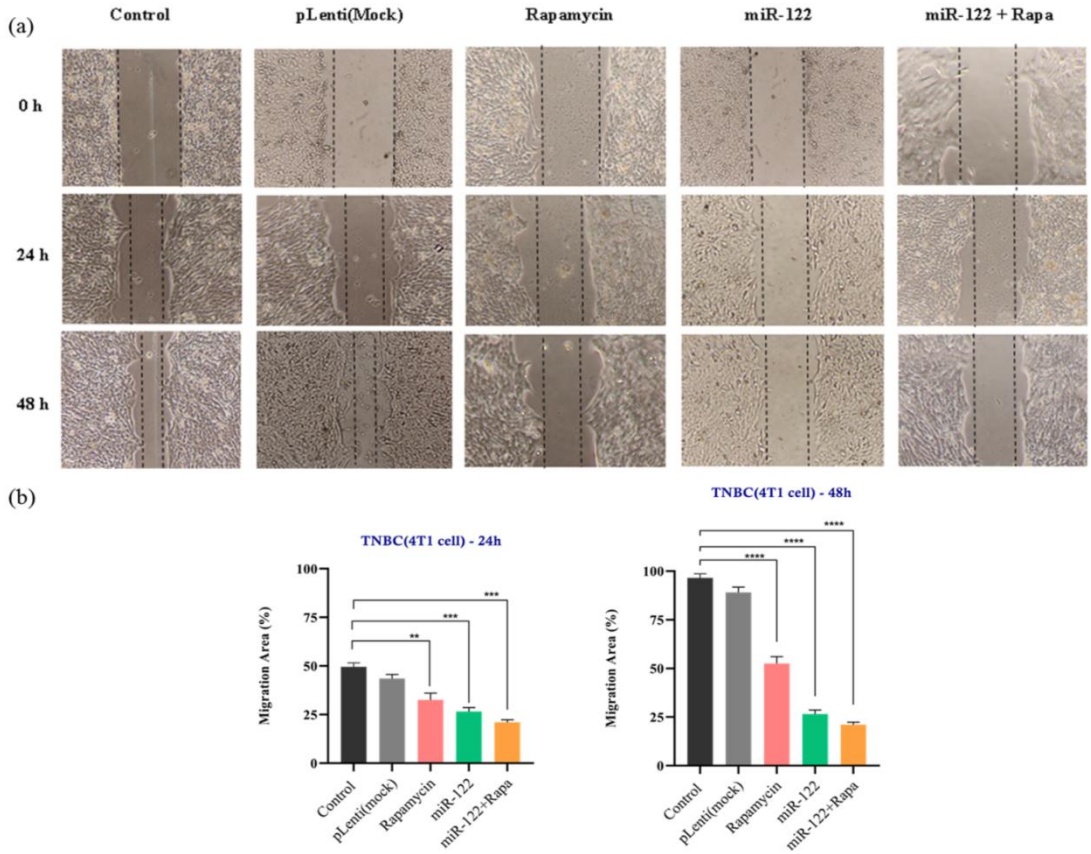

Figure 5. In vitro scratch assay. (a) 4T1 cells (magnification, ×100) were exposed to miR-122 and rapamycin in 5 groups: control, pLenti (mock), Rapamycin, miR-122 and miR-122 + rapamycin. (b) The migration area was quantified 24 and 48 h after transfection. The results are shown as mean ± SD for three independent experiments (n = 3). (*p* values: *<0.05, **<0.01, *<0.001, ****<0.0001)**

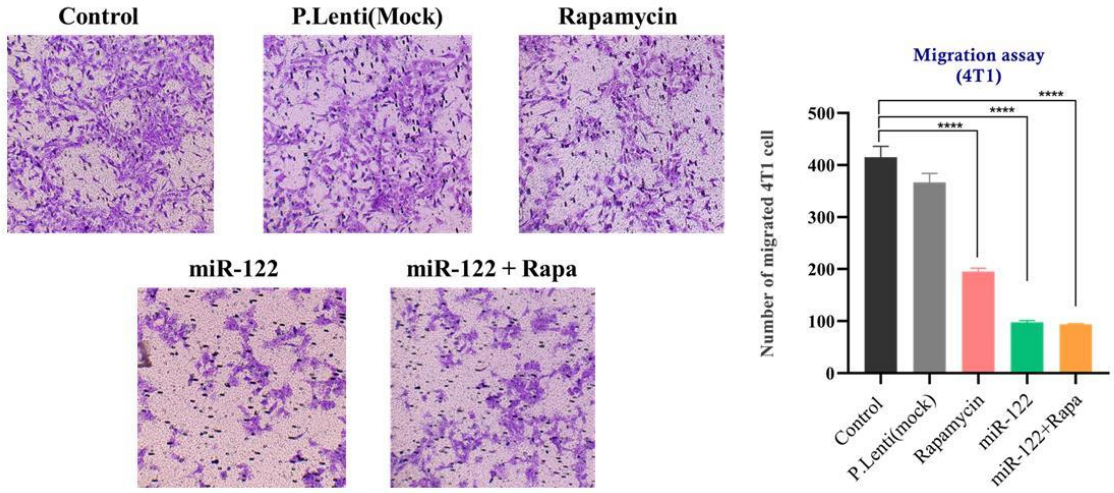

Figure 6 Transwell cell migration assay. (a) 4T1 cells (magnification, ×100) were exposed to miR-122 and rapamycin in 5 groups: control, pLenti (mock), Rapamycin, miR-122 and miR-122 + rapamycin. (b) In more than five microscopic fields, migrating cells were observed and counted. The results are shown as mean ± SD for three independent experiments (n = 3). (*p* values: *<0.05, **<0.01, *<0.001, ****<0.0001)**

DISCUSSION

This study aimed to compare the antitumor effects of miR-122 and rapamycin in triple-negative breast cancer patients (TNBC); we looked at how the PI3K-Akt-mTOR signaling pathway and the EMT process in the 4T1 cell line were affected by miR-122 and rapamycin. Molecular expression levels of important genes were examined, and their functions were verified by cellular experiments. Our study's real-time PCR, scratch, and transwell assay findings demonstrated that miR-122 performed better than rapamycin in controlling cancer.

Most strategies try to decrease or stop angiogenesis, EMT, and metastasis. Tumor migration and invasion mostly rely on the EMT process.[34,35] The hallmarks of EMT are the dissolution of intracellular adhesion, the rupture of the extracellular matrix (ECM), modifications of the cytoskeleton, an increase in cell motility and invasion, and also the general loosening of cellular structure.[36-38] As a result, the development of novel therapeutic strategies for TNBC is of great significance.[39] Considering that the EMT process is one of the important bottlenecks in metastasis, it seems necessary to select the most important factors and mediators involved in it. Therefore, three transcription factors (*Zeb1*, *Snail*, and *Twist*) were identified, based on similar articles and analysis, as one of the most important factors in the changes of this process.

In this study, we selected miR-122 and rapamycin based on literature searches and expert panels to identify possible multilateral inhibitory impacts on TNBC. They can control the PI3K/AKT/mTOR signaling pathway and EMT process. The PI3K/AKT/mTOR pathway is commonly overexpressed in TNBC.[40,41] The mTOR pathway not only plays a critical role in tumor progression but is also one of the primary activating elements of the EMT process. On the other hand, the administration of multiple therapeutic agents reduces the risk of cancer cells becoming resistant to treatment.[42-44] We have investigated variations in transcript levels of the important mTOR pathway genes (*PI3K*, *AKT*, and *mTOR*) and transcription factors associated with the EMT process (*Zeb1*, *Snail*, and *Twist*) with real-time PCR. In addition, the Transwell migration test and wound healing assay were also performed to detect cell invasiveness and migration. Downregulation of *PI3K*, *AKT*, and *mTOR*, as well as *Zeb1* and *Snail*, were significant in contrast to nonsignificant alterations of *Twist*. In addition, both rapamycin and miR-122 treatments remarkably reduced the migration of mouse mammary carcinoma 4T1 cells.

Rapamycin is a classical inhibitor of PI3K, AKT, and mTOR signaling pathways that, both directly (through the mTOR protein) and indirectly (through the P53 signaling pathway), restrains the EMT process. Interestingly, rapamycin cannot downregulate the *PI3K* as much as *AKT* and *mTOR*. Negative regulatory feedback of the S6K protein disappears following the downregulation and inhibition of *AKT* and *mTOR*, which results in a partial upregulation of PI3K compared to other pathway components. Furthermore, the downregulation of *Snail* and *Zeb1* transcription factors clearly reveals the antimigration effects of miR-122. Immunofluorescence assays and western blotting in other studies also confirm inhibition of the EMT in A549/GR tumor cells through upregulation of E-cadherin and downregulation of vimentin following miR-122 transfection.[45]

The inability of miR-122 and rapamycin to regulate *Twist*, a vital initiator of the EMT process, might be the cause of high interactions and its hub role. Different signaling pathways can regulate Twist, including TGF-β, RTK, Wnt, MAPK, and ECM/Integrin. Our results follow other studies conducted on the inhibition of TGF-β-dependent EMT with rapamycin in gallbladder cancer[46] and its protective effect against EMT through upregulation of E-cadherin as well as downregulation of *α-SMA* and *Snail*.[47,48]

Upregulation of miR-122 expression suppresses the growth and invasion of different types of malignancies. It gradually drives attention in cancer studies. Different studies have been conducted on the antiproliferative impacts of miR-122 upon different cancer types, including gastric cancer,[49,50] cervical cancer,[51] pancreatic ductal adenocarcinomas,[52] bladder cancer,[53] osteosarcomas,[54] breast,[55] ovarian,[56] and lung cancer.[45] However, some studies highlight the contribution of miR-122 towards the development and metastasis of various cancer lines, including colon cancer,[57] clear cell renal cell carcinoma,[58] and breast cancer.[59] Table 4 demonstrates the specific targets of miR-122 with which regulation took effect. Due to enrichment analysis and previous studies, it is evident that the main antiproliferative impact of miR-122 on cancer cells is through interference with different signaling pathways, as demonstrated in Figure 7. Mentioned target genes in Table 4 are thoroughly involved in cell proliferation and migration-inducing pathways such as TNF-α, IGF, EGF, VEGF, mTOR, and MAPK signaling pathways. Meanwhile, miR-122 regulates some metabolic pathways, specifically glucose metabolism, is crucial in developing resistance against starvation states in the cancer microenvironment. The regulation of pyruvate kinase and citrate synthase by miR-122 provides proper control over the energy cycle.[59,60] Metabolic pathways follows signaling regulation, and alteration in different regulatory systems, including those that control apoptosis or proliferation, affect cancer cells.

Table 4. The specific gene targets of miR-122 in cancer

Reference	Impact	Cancer Type	Target
49 and 50	-	Gastric	IYN, DUSP4
51	-	Cervices	RAD21
52	-	Pancreatic ductal adenocarcinoma	CCNG1
53	-	Bladder	VEGFC
54	-	Osteosarcoma	TP53
45	-	Lung	PRDX2
55	-	Breast	IGF1R
56	-	Ovarian	P4HA1
57	+	Colon	Glucose metabolism
58	+	Clear Cell Renal Cell Carcinoma(ccRCC)	OCLN
59	+	Breast	Glucose metabolism

This study is the first investigation of combination therapy of miR-122 and rapamycin on TNBC. The study shows high inhibitory effects of the administration of miR-122 and rapamycin on the proliferation and migration of 4T1 cells through suppression of the PI3K-Akt-mTOR signaling pathway and EMT-associated transcription factors. Administration of microRNAs would increasingly draw attention if the high off-target issue were resolved.

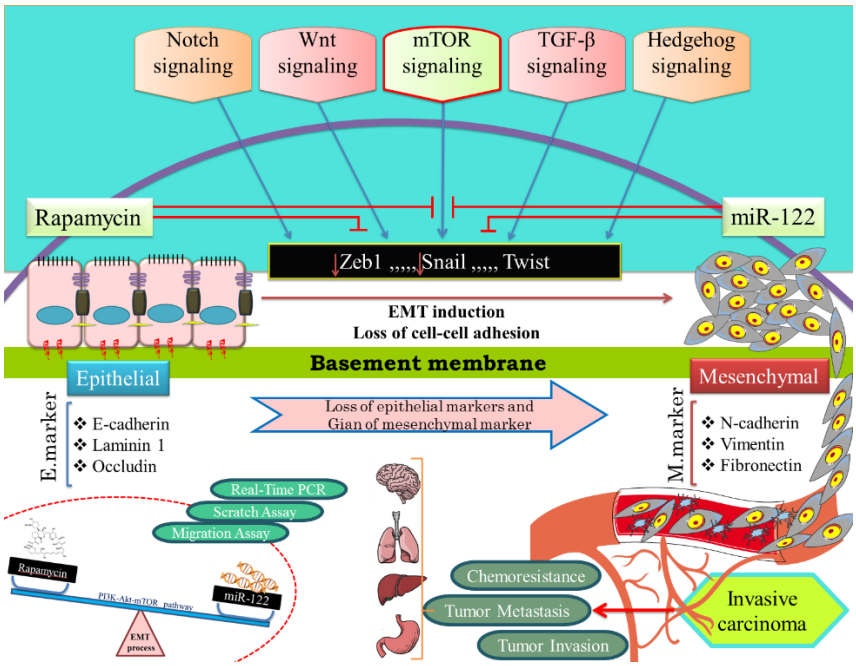

Figure 7. The schematic representation of the study design.

Different researches describe better outcomes of combination therapies of rapamycin with another inhibitor. Rapamycin and sorafenib combination therapy could be a novel and promising therapeutic method for the treatment of HCC.[61] Administration of resveratrol along with rapamycin allows blockage of rapamycin-induced Akt activation while the mTOR pathway remains restrained.[62] Another study shows that the application of rapamycin and celecoxib along with IL-15–armed oncolytic poxviruses provide potential antitumor effects against glioma.[63] However, our results demonstrate that combination therapy with miR-122 and rapamycin provides no significant difference compared with the administration of miR-122 alone. This phenomenon is because miR-122 interacts with and regulates major cellular signaling pathways, but in contrast, rapamycin only regulates the PI3K-AKT-mTOR and P53 pathways (Figure 8). miR-122 poses such a considerable amount of regulatory pressure on pathways that the alternating effects of rapamycin are faded out. It was necessary to survey the logical reasoning and determine why the inhibitory ability of miR-122 is so high. After studying the pathways and signaling pathway sites, it was found that miR-122 is not only effective in the mTOR pathway and the EMT process, but also affects the metabolic pathways and interferes with them. We suggest evaluating the effect of miR-122 and rapamycin on genes and protein expression levels involved in metabolic pathways. Future studies can also examine how miR-122 and rapamycin prevent tumor growth in an animal tumor model.

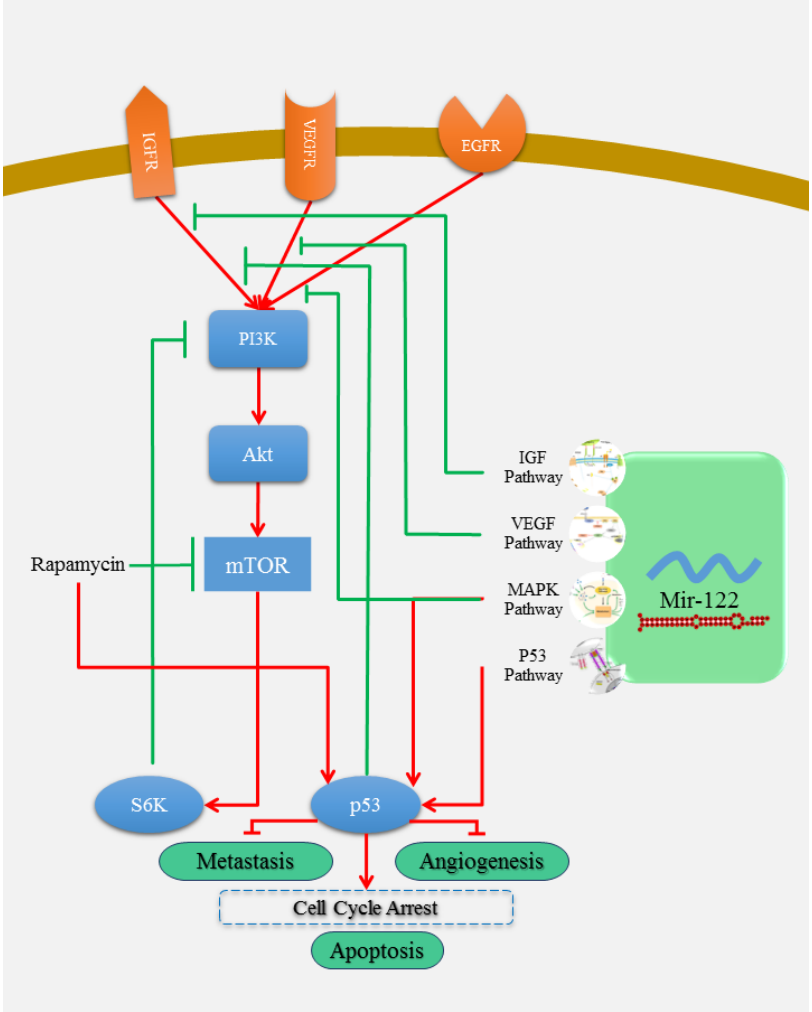

Figure 8. Enrichment analysis of the miR-122 on cancer cells is through interference with different signaling pathways

STATEMENT OF ETHICS

This study was executed under the supervision of the Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (IR.SBMU.RETECH.REC.1399.301).

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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