Down-regulation of NAMPT expression by miR-154 reduces the viability of breast cancer cells and increases their susceptibility to doxorubicin

Zahra Bolandghamtpour  
IRAN University of Medical Sciences

Mitra Nourbakhsh (✉ nourbakhsh.m@iums.ac.ir)  
https://orcid.org/0000-0002-6716-0354

Kazem Mousavizadeh  
IRAN University of Medical Sciences

Zahra Madjd  
Iran University of Medical Sciences

Seyedeh Sara Ghorbanhosseini  
Iran University of Medical Sciences

Zohreh Abdolvahabi  
Iran University of Medical Sciences

Zahra Hesari  
Iran University of Medical Sciences

Samira Ezzati  
Iran University of Medical Sciences

Research article

Keywords: Nicotinamide phosphoribosyltransferase, Breast cancer, miR-154, Duxorubicin

Posted Date: June 20th, 2019

DOI: https://doi.org/10.21203/rs.2.10551/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. 
Read Full License

Version of Record: A version of this preprint was published on November 1st, 2019. See the published version at https://doi.org/10.1186/s12885-019-6221-0.
Abstract

Background Nicotinamide phosphoribosyltransferase (NAMPT) acts as an important enzyme in the salvage pathway of nicotinamide adenine dinucleotide (NAD) biosynthesis. Deregulation of NAD could be associated with progression of many cancers including breast cancer. Here, we evaluated the effect of NAMPT inhibition via miR-154 on survival of breast cancer cells. Methods Breast cancer cell lines including MCF-7 and MDA-MB-231 were transfected with miR-154 mimic, inhibitor and their negative controls. Using real-time PCR and western blotting techniques the expression levels of NAMPT and miR-154 were determined and compared with the untreated cells. NAD levels were measured by an enzymatic method. Subsequently, colorimetric methods and flow cytometry were performed to evaluate cell viability and apoptosis. Bioinformatics analyses were performed to investigate whether NAMPT 3′-UTR is a direct target of miR-154 and the findings were confirmed by luciferase reporter assay. Results According to the obtained results, NAMPT 3′-UTR was identified as a direct target of miR-154 and the levels of this miRNA was inversely associated with NAMPT expression both at mRNA and protein levels in breast cancer cell lines. Functionally, miR-154 inhibited the NAD salvage pathway leading to a remarkable decrease in cell survival and induction of apoptosis. Co-treatment of breast cancer cells with doxorubicin and miR-154 mimic significantly reduced cell viability compared to treatment with doxorubicin alone in both cell lines. Conclusions Hence, it was concluded that the inhibition of NAD production by miR-154 might be introduced as a promising therapeutic strategy for the treatment of breast cancer either alone or in combination with other conventional chemotherapeutic agents.

Background

Breast cancer is known as one of the leading causes of cancer-related mortality in women worldwide (1, 2). In spite of substantial progresses in the treatment of breast cancer, identification of novel therapeutic targets for overcoming current obstacles is still required. In recent years, inhibition of cellular and molecular mechanisms that interfere with development of breast cancer is one of the critical diagnostic and therapeutic strategies (1-3). Nicotinamide phosphoribosyltransferase (NAMPT) is known as the main enzyme in the biosynthesis of nicotinamide adenine dinucleotide (NAD) (4, 5). NAMPT is a key enzyme in the NAD biosynthesis pathway and plays important roles in a wide range of biological processes such as metabolism and immune response. In the meantime, deregulation of NAMPT expression is related to initiation and progression of various human malignancies (6). Several studies have indicated that inhibition of NAMPT expression is associated with a remarkable increase in metabolic collapse and apoptosis in breast cancer cells both in vitro and in vivo (7). On the contrary, up-regulation of NAMPT is closely related to poor response of patients with breast cancer to chemotherapeutic drugs such as doxorubicin (8). Hence, it seems that novel therapeutic approaches based on inhibition of NAMPT could be introduced as effective therapeutic strategies for the treatment of breast cancer. Among various cellular and molecular targets involved in breast cancer pathogenesis, microRNAs are proved to act as key epigenetic regulators (9). These molecules are known as a class of short non-coding RNAs which have critical roles in the regulation of a variety of biological processes such as growth, angiogenesis,
development, and differentiation (10). Multiple lines of evidence confirmed that deregulation of miRNAs is associated with various aspects of tumorigenesis such as angiogenesis, tumor growth, metastasis and, response to therapy in breast cancer (11, 12). miR-154 is a tumor suppressor which is located at chromosome 14q32 (12). Down-regulation of miR-154 is associated with progression of many cancers such as breast cancer (12), osteosarcoma (13), hepatocellular carcinoma (14), prostate cancer (15), non-small cell lung cancer (16), thyroid cancer (17), and colorectal cancer (18). However, the function and underlying cellular and molecular pathways related to miR-154 has not been determined in breast cancer. In the current study, we accomplished a bioinformatics analysis and found that there is a binding site for miR-154 in the 3′-UTR of NAMPT mRNA and therefore we aimed to investigate whether the up-regulation of miR-154 was related with a significant decrease in NAD levels and suppression of breast cancer cells through targeting of NAMPT 3′-UTR. We assessed the regulatory effects of miR-154 on NAMPT and the subsequent cell death in breast cancer cells and cellular response to doxorubicin (DOX).

**Methods**

**Cell lines and cell culture**

Four cell lines including MCF-7, MCF-10A, MDA-MB-231 and HEK-293T were obtained from the Cell Bank of the Iranian Biological Resource Center (Tehran, Iran) and authenticated regularly. Dulbecco's Modified Eagle's Medium (DMEM, Biosera, France) was used for culturing MDA-MB-231 and MCF-7 cells. Mammary epithelial cell growth medium (MEGM; Lonza/ Clonetics, Switzerland) and DMEM/F12 (Biosera, France) were used for culturing MCF-10A and HEK-293T cells, respectively. Cells in different media were supplemented with 10% fetal bovine serum (FBS) (Invitrogen, UK), 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were incubated in a humidified atmosphere with 5% CO₂ at 37°C. For culturing of MCF-10A cells, other supplements including 10 μg/ml insulin, 20 ng/ml epithelial growth factor (EGF), 0.5 μg/ml hydrocortisone (HC) and 100 ng/ml cholera toxin (CT) (all from Sigma-Aldrich, Germany) were added to MEGM.

**Cell transfection**

**RNA isolation and real-time RT-PCR**

In order to evaluate gene expression at mRNA levels, real-time RT-PCR was employed. Total RNA was first extracted from different cells by miRCURY™ RNA isolation kit (Exiqon, USA). Then, both the quantity and quality of the extracted RNA was analyzed using Nanodrop spectrophotometer (Nanodrop, Thermo Fisher Scientific, USA). The complementary DNA (cDNA) was synthesized using 1 μg template RNA and RevertAid first strand cDNA synthesis kit (Thermo Fisher Scientific, USA). In order to synthesize miR-154 cDNA, a poly (A) tail was first linked to the 3′-end of the miRNA transcripts, employing *E. coli* Poly (A) Polymerase (PAP) (New England Biolabs, UK). A hybrid primer containing an adapter sequence and complementary sequence for the poly (A) tail was used for cDNA synthesis. The SYBR Green kit (SYBR
Premix Ex Taq II, Takara, Japan) was used for performing the qRT-PCR technique. The expression of NAMPT and miR-154 were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene and human U6 small nuclear RNA as internal controls, respectively. Each sample was analyzed in triplicate. The relative quantitation of gene expression was calculated using the $2^{\Delta\Delta CT}$ formula. Supplementary Table 1, additional le 1, lists the used primer sequences.

**Cell survival assay**

To assess the effect of miR-154 mimic and its inhibitor on cell proliferation and to show direct cytotoxic effects that lead to cell death, cell survival assay was performed using WST-1 cell survival assay kit (Roche Applied Science, Germany). A cell suspension containing total number of $5 \times 10^3$ cells/100 μl was seeded into each well of a 96-well plate. Subsequently, Plates were incubated at 37 °C with 5% CO$_2$ overnight. Then, miRNA mimic, inhibitor and their negative controls were transfected into MCF-7 and MDA-MB-231 cells. Finally, 10 μl of reconstituted WST-1 reagent was added to each well and the absorbance was measured after incubation at 37 °C for 4 h, by an ELISA plate reader (BioTek Instruments Inc., Winooski, USA) at 450/650 nm wavelength. In order to assess the effect of doxorubicin on cell viability, either untransfected cells or cells transfected with different miR-154-related oligonucleotides were treated with 0.1 μM doxorubicin and the cell viability was evaluated as described above.

**Apoptosis assay**

The effect of miR-154 on cell apoptosis was assessed using FITC Annexin V apoptosis detection kit (Roche Applied Science, Germany). A total of $3 \times 10^5$ of MCF-7 and MDA-MB-231 cells were seeded in 6-well plates and incubated for 24 h at 37 °C, with 5% CO$_2$. After 48 h, cells were harvested, washed twice with cold PBS, and subsequently stained with Annexin V and propidium iodide (PI). Finally, the Stained cells were evaluated by a flow cytometer (FACScan, BD Biosciences, USA) for apoptotic rate determination. The device was equipped with 488 nm laser for excitation, 515 nm bandpass filter for detection of FITC and 600 nm bandpass filter for PI detection. The obtained results were analyzed using CellQuest software (BD Biosciences). The Annexin V-FITC positive cells were presented as apoptotic cells.

**Western blotting**

Western blot technique was used to investigate the effect of miR-154 up-regulation on NAMPT expression levels in MCF-7, MDA-MB231, and MCF-10A cell lines. After 48 h, cells were lysed in lysis buffer (10mM Tris-Cl, pH 7.4, 0.15M NaCl, 5mM EDTA, and 1% Triton X-100) containing 100 g/mL protease inhibitors (all from Sigma-Aldrich, Germany). Ten micrograms of the total protein from each sample was separated by 10% SDS-polyacrylamide gel electrophoresis and trans-blotted onto polyvinylidene difluoride (PVDF) membrane. The membranes were blocked in blocking buffer (5% powdered non-fat milk in PBS)
containing 0.05% Tween-20. The blot was incubated with a PBEF/NAMPT Rabbit antibody at 1:1000 concentration (cell signaling technology, USA) followed by incubation with anti-Rabbit HRP-conjugated secondary antibody at a dilution of 1:5000 (Cell Signaling Technology, USA). NAMPT protein and GAPDH (control) bands were identified using chemiluminescence kit (Amersham Biosciences/GE Healthcare, Little Chalfont, U.K.).

**NAD level assay**

The concentration of intracellular NAD+/NADH was measured using NAD assay kit (Abcam, UK) according to instructions provided by the manufacturer. After lysis of the transfected cells using lysis buffer, the total protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific, USA). The proteins were removed using perchloric acid and the NAD levels were obtained by measuring the absorbance at 450 nm. The quantity of NAD was normalized against the protein content in each sample which was measured by the Bradford assay.

**Prediction of candidate miRNA targeting NAMPT 3′-UTR**

To predict the candidate miRNAs that directly bind to NAMPT mRNA, three main miRNA target prediction sites including microRNA.org (miRanda algorithm) (www.microRNA.org), MiRmap (http://mirmap.ezlab.org) and TargetScan (http://targetscan.org) were used. The predicted binding affinities and their scores were obtained and compared with the other miRNAs.

**Investigation of miR-154-target interaction**

To investigate the direct interaction of miR-154 with NAMPT 3′-UTR, the sequence of the target microRNA binding sites was cloned in the psiCHECK-2 reporter plasmid. To this purpose, the NAMPT 3′-UTR sequence was first determined using Gene Header on PubMed database. The target microRNA binding site were identified and determined by bioinformatics algorithms. The relevant region was amplified by the primers listed in Supplementary Table 1, Additional file 1 (synthesized by Macrogen Inc., South Korea). The tandem mutant of NAMPT 3′-UTR was also constructed to serve as a negative control. To create this sequence, the forward and reverse primers containing restriction sites for NotI and XhoI were used (Supplementary Table 1, Additional file 1). To ensure the presence of fused sequences and the absence of mutations in the sequence, the recombinant plasmid of NAMPT-MRE-tandem-mut-psiCHECK2 was sequenced by Macrogen Inc. The recombinant constructs were cotransfected with miR mimic, miR inhibitor or their negative controls into HEK293T cell line. The activity of renilla luciferase was measured over the activity of firefly luciferase as an internal control. Luciferase activity was done by a dual luciferase assay kit (Promega, USA).

**Statistical analysis**

Statistical analysis was done using GraphPad Prism software, version 5.01 (USA, San Diego). The statistical differences between the groups were performed by one way- ANOVA. All results were presented as mean ± standard deviation (S.D.). P values less than 0.05 were considered statistically significant.
Results

The expression levels of miR-154 and NAMPT in breast cancer cell lines

Figure 1a shows the relative expression of miR-154 in untreated MCF-7 and MDA-MB-231 cell lines compared to normal epithelial cell line (MCF-10A) that was used as control. It can be observed that miR-154 expression levels were considerably lower in MCF-7 and MDA-MB-231 (both $P<0.01$) cell lines compared with MCF-10A cells. In addition, measurement of the NAMPT mRNA expression indicated that the expression of NAMPT was significantly higher in MCF-7 ($P<0.01$) and MDA-MB-231 ($P<0.05$) cell lines compared to MCF-10A (Figure 1b). Furthermore, the blotting results revealed that the basal level of NAMPT protein in MCF-7 and MDA-MB-231 cell lines was higher than MCF-10A ($P<0.01$ and $P<0.05$, respectively) (Figure 1c, d).

Basal expression level of miR-154 and NAMPT in MCF-7 & MDA-MB-231 cell lines, basal level of NAMPT protein in MCF-7 & MDA-MB-231 cell lines. Basal expression of (a) miR-154 and (b) NAMPT in MCF-7 and MDA-MB-231 cell lines, compared with MCF-10A, each vertical bar represents the mean ± SD of triplicate determinations. *$P<0.05$; **$P<0.01$. (c) Evaluation of NAMPT basal expression at translational level by immunoblotting in MCF-7 and MDA-MB-231. (d) The basal protein expression levels of NAMPT in MCF-7 and MDA-MB-231 cells compared to MCF-10A cells. Each column represents the mean ± SD. *$P<0.05$; **$P<0.01$.

miR-154 cellular levels was up-regulated via miRNA mimic transfection

In order to understand the mechanism by which miR-154 controls NAMPT expression in breast cancer cells, transfection with miR-154 mimic and miR-154 inhibitor was conducted. The expression level of miR-154 was also measured after transfection of cells with either miR-154 mimic or its antisense oligonucleotide serving as miR-154 inhibitor. The MCF-7 cell line transfected with miR-154 mimic exhibited a significant increase in miR-154 levels ($P<0.01$), while, a decline in miR-154 expression was observed following transfection with its inhibitor ($P<0.001$) (Figure 2a). Meanwhile, the MDA-MB-231 cell line displayed a significantly enhanced cellular levels of miR-154 after transfection with miRNA-mimic ($P<0.001$). In contrast, transfection with miR-154 inhibitor was associated with a remarkable decrease in miR-154 level in MDA-MB-231 cells ($P<0.001$) (Figure 2b). Fluorescence microscopy results of the cells transfected with FAM-labeled microRNAs confirmed successful transfection (Supplementary figure 1, Additional file 1).
Relative expression of miR-154 after transfection in MCF-7 & MDA-MB-231. miR-154 cellular level after transfection of (a) MCF-7 and (b) MDA-MB-231 cells with miR-154 mimic, inhibitor or their negative controls (NC), compared to untreated cells. **P<0.01, *** P<0.001.

miR-154 and NAMPT gene expression

As described earlier, bioinformatics analysis anticipated that 3’-UTR of NAMPT is a target for miR-154. Subsequently, it was supposed that down-regulated miR-154 in cancer cells might be involved in NAMPT up-regulation. To evaluate whether miR-154 would exert an inhibitory effect on NAMPT expression, RT-PCR were performed on human breast cancer cells (HBC) transfected with mimic, inhibitor, and their corresponding NCs. At the mRNA level, NAMPT gene revealed a significantly reduced expression in MCF-7 and MDA-MB-231 cell (both P<0.001) due to miR-154 augmentation by its mimic. Quite the reverse, blocking miR-154 by its corresponding inhibitor caused a significant increase in NAMPT mRNA expression in both MCF-7 and MDA-MB-231 cell lines (P<0.001 and P<0.05, respectively) (Figure 3 a, b).

NAMPT gene expression in MCF-7 & MDA-MB-231 cell lines after treatment. Relative NAMPT mRNA expression in (a) MCF-7 and (b) MDA-MB-231 cells transfected with miR-154 mimic, inhibitor, their negative controls (NC) and mock compared to untreated cells. Each column represents the mean ± SD of at least three separate experiments. *P<0.05; ***P<0.001

Suppression of NAMPT protein expression by miR-154

The cell lines were transfected with miR-154 mimic and miR-154 inhibitor and western blotting was performed to evaluate whether miR-154 interfered with the regulation of NAMPT protein expression. The obtained results indicated that the up-regulation of miR-154 via transfection with miR-154 mimic, remarkably decreased the levels of NAMPT protein in both MCF-7 (P<0.05) and MDA-MB-231 (P<0.05) cells (Figure 4a, 4b). However, NAMPT protein expression was enhanced in both MCF-7 (P<0.01) and MDA-MB-231 (P<0.001) cell lines following transfection with miR-154 inhibitor (Figure 4a, 4b).

Suppression of NAMPT protein expression by miR-154. Representative immunoblot image showing the increased basal NAMPT protein levels in MCF-7 and MDA-MB-231 compared to MCF-10A cells. Quantitation of NAMPT protein level in (a) MCF-7 and (b) MDA-MB-231 cells transfected with miR-154 mimic, inhibitor or their negative controls (NC) compared to untreated controls. Graphs represent the mean± SD of the results of the densitometric analysis of the blotting images normalized to GAPDH as the internal control and expressed relative to control cells. *P<0.05, **P<0.01, *** P<0.001.
The effect of miR-154 on NAMPT-induced NAD depletion

Increased NAMPT level is associated with high concentration of NAD in cancer cells (5). Our results showed that NAD level was decreased in the MCF-7 cells transfected with miR-154 mimic compared to un-transfected control cells ($P<0.001$). Moreover, NAD level was increased in the cells transfected with miR-154 inhibitor ($P<0.05$) (Figure 5a). Similarly, the NAD levels in MDA-MB-231 cells transfected with miR-154 mimic exhibited a significant increase ($P<0.01$), while a considerable decrease was observed in those transfected with miR-154 inhibitor ($P<0.05$) (Figure 5b).

The effect of miR-154 on intracellular NAD levels. Transfection with miR-154 mimic induced intracellular NAD depletion and reduction in cell viability. Evaluation of relative NAD levels in (a) MCF-7 and (b) MDA-MB-231 cells transfected with miR-154 mimic, inhibitor or their negative controls (NC) compared to un-transfected control. Results are presented as mean ± SD from three separate duplicate experiments.*$P<0.05$, **$P<0.01$, ***$P<0.001$.

Over-expression of miR-154 in breast cancer cells reduced cell viability

NAMPT is elevated in a variety of human malignancy types including breast cancer. This enzyme induces proliferation and survival in cancer cells (19). In the present research, we studied the effect of miR-154 on the survival of breast cancer cells using WST-1 cell survival assay. The obtained results revealed that miR-154 mimic considerably reduced cell survival in MCF-7 ($P<0.05$) and MDA-MB-231 ($P<0.01$) cell lines compared with the untreated cells. Whereas, treating the cells with miR-154 inhibitor significantly increased cell survival in both cell lines (both $P<0.01$). The obtained results are shown in figure 6.

WST-1 cell survival assay. Survival rate of (a) MCF-7 and (b) MDA-MB-231 cells in the response to increased and decreased levels of miR-154 by its mimic and inhibitor, respectively. The obtained results are expressed as percentage to untreated control. Data are presented as mean ± SD of triplicate experiments that were repeated at least three times. * $P<0.05$, ** $P<0.01$.

miR-154 increased the susceptibility of breast cancer cells to doxorubicin

Considering the effect of miR-154 on cell viability, we treated MCF-7 and MDA-MB-231 cells with doxorubicin after transfection with miR-154 mimic, miR-154 inhibitor or their negative controls. As it is shown in figure 7, miR-154 mimic synergistically decreased viability when used in combination with
doxorubicin and the cell viability was significantly lower compared to either doxorubicin or miR-154 mimic alone. This effect was observed in both MCF-7 and MDA-MB-231 (Figure 7 a, b). On the contrary, down-regulation of cellular miR-154 by its inhibitor led to a lower response to doxorubicin treatment and the cell viability in this group (miR-154 inhibitor + doxorubicin) was similar to untreated control cells (Figure 7).

**Effects of miR-154 on susceptibility of breast cancer cells to doxorubicin.** The effect of miR-154 and DOX either alone or in combination on the viability of (a) MCF-7 and (b) MDA-MB-231 breast cancer cells. The results are presented as mean ± SD, relative to un-transfected controls. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

**Up-regulation of miR-154 promoted apoptosis in breast cancer cells**

The results of flow cytometry analysis revealed that transfection with miR-154 mimic significantly induced apoptosis in MCF-7 and MDA-MB-231 cells (both $P<0.001$). On the contrary, down-regulation of miR-154 by its inhibitor decreased cell death rate in MCF-7 and MDA-MB-231 cells (both $P<0.001$) (Figure 8).

**Cell apoptosis assay using Annexin V and propidium iodide.** Representative quadrant dot plot of the flowcytometric analysis of apoptosis in breast cancer cells. Representative quadrant dot plot of Annexin V/PI staining and average percentage of apoptotic cells in (a) MCF-7 and (b) MDA-MB-231 cells transfected with miR-154 mimic, inhibitor or their negative controls. Lower-right region of each plot is indicative of the population of apoptotic cells. The blue dots in lower right quadrant of each diagram indicate apoptotic cells. (c) and (d) The obtained results were compared to the untransfected controls and are presented as mean±SD. *** $P<0.001$.

**miR-154 regulated NAMPT by direct binding to its 3′-UTR**

As previously stated, bioinformatics analysis predicted that 3′-UTR region of NAMPT mRNA could be a potential target for miR-154. To confirm this, the luciferase reporter activity of psiCHECK2 vector containing NAMPT-related 3′-UTR in the presence of miR-154 mimic, inhibitor or their negative controls was investigated. miR-154 mimic decreased the luciferase activity by 59.5 ± 0.03% compared to untreated control cells ($P<0.01$); however, miR-154 inhibitor led to a significant increase in luciferase activity ($P<0.05$) (Figure 9). None of the controls significantly affected the luciferase activity.

**Luciferase reporter assay verifying the predicted interaction between miR-154 and 3′-UTR of NAMPT.** PsiCHECK2 vector harboring NAMPT 3′-UTR or the mutant form of miR-154 recognition element (NAMPT-
MRE-tandem-mut) were co-transfected with miR-154 mimic, inhibitor, their negative controls and mock into HEK293T. Firefly luciferase activity was normalized with respect to renilla luciferase as control. Results are shown as mean±SD of three independent experiments. * \( P<0.05 \), ** \( P<0.01 \).

**Discussion**

According to a report released by the World Health Organization (WHO), in 2018, cancer was known as the second leading cause of death worldwide and it was responsible for an estimated 9.6 million deaths (20). Among various types of cancer, breast and lung cancer have been the most prevalent cancers in the world (21). Breast cancer has been reported to be the most commonly occurring cancer in women and generally the most prevalent malignancy after lung cancer (21, 22). Over 2 million new cases of breast cancer have been reported in 2018 (21). Therefore, novel methods including strategies based on cancer-related molecular changes are essential for breast cancer management. In this study, we aimed to investigate whether miR-154 can effectively reduce the survival of breast cancer cells and increase their treatment response to doxorubicin as a widely-used chemotherapeutic agent.

Recent studies have demonstrated the abnormal expression of miRNAs in a variety of human pathological conditions including cancer (23, 24). miR-154 is one of the cancer-related miRNAs that is down-regulated and can act as a tumor suppressor in some human cancers (24).

In this research, we indicated that the expression of miR-154 was significantly reduced in breast cancer cell lines compared to normal mammary cells. Consistently, Qin et al. studied the changes of miR-154 levels in breast cancer. They showed that miR-154 was down-regulated in this malignancy and increased breast cancer cell proliferation, migration and invasion through targeting ADAM metallopeptidase domain 9 (ADAM9) as a novel direct target of miR-154 in breast cancer (25). In another study by Xu et al., E2F transcription factor 5 protein (E2F5) was recognized as a miR-154 target, and its expression was inversely correlated with miR-154 expression levels in breast cancer cell lines (26).

miR-154 has also been implicated as tumor suppressor in other cancer types and it has been offered as a suitable molecular treatment strategy in various cancers. For example, in glioma patients, down-regulation of miR-154 and a strong correlation between miR-154 levels and various clinicopathological features of the disease has been reported. Therefore, miR-154 has been suggested a suitable prognostic biomarker for patients with this disease (24). Additionally, reduced levels of miR-154 levels in colorectal cancer tissues compared with non-cancerous tissues has been reported and this low expression has been shown to be significantly correlated with aggressive clinicopathological characteristics such as large tumor size, positive lymph node metastasis, and advanced clinical stage (27). Low expression of miR-154 and its association with cell proliferation, migration and invasion has also been described in prostate cancer (28). miR-154 may also function as a tumor suppressor in non-small cell lung cancer (NSCLC) by binding to B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI-1) (29).

In the current study, a negative correlation was found between miR-154 and NAMPT expression (under- and over-expressed, respectively) in breast cancer cells, suggesting the inhibitory effect of miR-154 on
NAMPT expression. Significantly higher expression of NAMPT in breast cancer tissues compared with normal mammary gland tissue has been previously reported and has been shown to be associated with a larger tumor size, advanced clinical tumor-node-metastasis stages, increased expression of estrogen and progesterone receptors and lymph node metastasis. Meanwhile, patients with increased expression of NAMPT had a poor overall or disease-free survival (30). Various studies have reported that NAMPT up-regulation is associated with the development and progression of breast cancer, suggesting NAMPT as a potential biomarker for the early detection of breast cancer (30-36). Additionally, exogenous administration of recombinant NAMPT not only results in increased cell proliferation by activation of signaling pathways, but also increases cell survival by NAD production (37, 38).

The results obtained in our study revealed that the up-regulation of miR-154 significantly suppressed NAMPT expression both at mRNA and protein levels; indicating that this interaction leads to mRNA degradation or suppression of translation as has been suggested as mechanisms of action of miRNAs (24). The regulatory effect of miR-154 was further confirmed by reducing its cellular levels using miR-154 inhibitor which in turn led to a significant rise in NAMPT expression levels. The obtained results from luciferase reporter assay demonstrated that the aforementioned regulatory effect of miR-154 on NAMPT was the result of the direct binding of miR-154 to the NAMPT 3´-UTR, ruling out the possibility of off-targets and indirect effects. These findings are consistent with our recent study in which, miR-206 was introduced as a potential inhibitor of NAD biosynthesis in HBC cells via direct binding to NAMPT 3´-UTR (33). Consistently, NAMPT 3´-UTR has been reported in a number of studies, as the potential target for a wide variety of miRNAs in other malignancies and diseases including miR-182 in the ossification of ligamentum flavum (39), miR-300 in neonatal sepsis (40), miR-34a in obesity (41), miR-206 in pancreatic cancer (42), miR-26b in colorectal cancer (43), miR-410 in pulmonary arterial hypertension (PAH) (44) and miR-182 in HIV-1 contaminated cells (45).

The results of NAD assay, revealed that miR-154 caused the attenuation of intracellular NAD via inhibition of NAMPT salvage pathway. NAMPT is an essential enzyme in NAD biosynthesis and therefore its inhibition is a plausible approach in depleting intracellular NAD (30, 46). NAD plays an important role in the cellular processes such as genomic stability and metabolism and thus is essential for cell survival. Here we showed that inhibition of NAMPT and the subsequent decline in NAD levels leads to diminished cell viability and a prominent induction of apoptosis. We had previously reported that chemical inhibition of NAMPT by its specific inhibitor could effectively reduce NAD levels and induce apoptosis in breast cancer cells (47). In line with our findings, inhibition of NAMPT by microRNAs has also been shown to be an appropriate method in modulating NAD content and cell viability (41-43).

One major obstacle in cancer therapy is chemoresistance to the commercial cancer therapeutic drugs, contributing to cancer progression, recurrence and mortality (48). Aberrant miRNA expression is related to anti-cancer drug resistance (48). Doxorubicin (DOX), an antitumor antibiotic, is widely used for the treatment of a variety of cancers including breast cancer. The cytotoxic mechanism of DOX is dependent on apoptotic pathway and involves the inhibition of DNA or RNA synthesis following penetration into DNA. The resistance of breast cancer cells to apoptotic pathways has reduced the clinical effectiveness
of this chemotherapeutic drug (49). In the present study, we showed that miR-154 increased the cellular response to DOX and cell survival is significantly decreased when DOX treatment was combined with up-regulation of miR-154. Consistently, up-regulation of miR-760 has been shown to be able to sensitize breast cancer cells to the anti-cancer drugs (50). miR-133b has also been found to be functionally involved in the resistance of breast cancer cells to DOX and the intra-tumoral delivery of this miRNA increased the treatment response to DOX in DOX-resistant xenografts (51). miR-125b has also been introduces as another regulator of DOX-resistance in breast cancer cells via targeting hematopoietic cell-specific protein 1-associated protein X-1 (HAX-1) (46, 49). NAMPT overexpression has been shown to be associated with poor treatment response to chemotherapeutic agents including doxorubicin, etoposide, fluorouracil, paclitaxel, and phenylethyl isothiocyanate (52). Thus, over-expression of miR-154 might be an effective approach to increase the sensitivity of breast cancer cells to DOX treatment.

Conclusions

Our findings indicated that miR-154 plays an important role in the inhibition of NAMPT-mediated NAD production pathway by directly targeting NAMPT 3'-UTR. Overexpression of this miRNA reduces cell survival and promotes cell apoptosis in breast cancer cells. As a result, this molecular mechanism might be considered as a promising therapeutic strategy in management of breast cancer.

Abbreviations

NAMPT, nicotinamide phosphoribosyltransferase; NAD, nicotinamide adenine dinucleotide; miRNA, microRNA; 3'-UTR, 3'-untranslated region; DMEM, Dulbecco's modified Eagle medium; FBS, Fetal bovine serum; MEGM, mammary epithelial cell growth medium; CT, cholera toxin; EGF, epithelial growth factor; PEI, polyethylenimine; PAPA, Poly (A) Polymerase; GAPDH, Glyceraldehyde 3-Phosphate Dehydrogenase; PMSF, phenylethane sulfanyl fluoride; BCA, bicinechonic acid assay; PVDF, polyvinilidene difluoride membrane; MRE, microRNA response element; HBC, human breast cancer; PARP1, poly ADP-ribose polymerase 1; NC, negative control.

Declarations

Acknowledgement

The authors wish to acknowledge Research Council of Iran University of Medical Sciences for financial support of this research by grant number 27355.

Availability of data and materials

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

Competing interests:
The authors declare they have no competing interests.

**Authors’ contributions:**

ZBP carried out the experiments and drafted the manuscript. MN and KM designed the study and performed the analysis. ZM contributed with the critical revision of the manuscript. SSGHH contributed in performing the experiments and statistical analysis. ZA, ZH and SE contributed in performing the experiments. All authors have read and approved the final manuscript.

**References**

1. Jafari SH, Saadatpour Z, Salmaninejad A, Momeni F, Mokhtari M, Nahand JS, et al. Breast cancer diagnosis: Imaging techniques and biochemical markers. Journal of cellular physiology. 2018;233(7):5200-13.

2. Ju J, Zhu AJ, Yuan P. Progress in targeted therapy for breast cancer. Chronic diseases and translational medicine. 2018;4(3):164-75.

3. Vranic S, Palazzo J, Sanati S, Florento E, Contreras E, Xiu J, et al. Potential Novel Therapy Targets in Neuroendocrine Carcinomas of the Breast. Clinical breast cancer. 2018.

4. Grolla AA, Travelli C, Genazzani AA, Sethi JK. Extracellular nicotinamide phosphoribosyltransferase, a new cancer metabokine. British journal of pharmacology. 2016;173(14):2182-94.

5. Shackelford RE, Mayhall K, Maxwell NM, Kandil E, Coppola D. Nicotinamide phosphoribosyltransferase in malignancy: a review. Genes & cancer. 2013;4(11-12):447-56.

6. Sampath D, Zabka TS, Misner DL, O’Brien T, Dragovich PS. Inhibition of nicotinamide phosphoribosyltransferase (NAMPT) as a therapeutic strategy in cancer. Pharmacology & therapeutics. 2015;151:16-31.

7. Chini CC, Guerrico AM, Nin V, Camacho-Pereira J, Escande C, Barbosa MT, et al. Targeting of NAD metabolism in pancreatic cancer cells: potential novel therapy for pancreatic tumors. Clinical cancer research: an official journal of the American Association for Cancer Research. 2014;20(1):120-30.

8. Folgueira MA, Carraro DM, Brentani H, Patrao DF, Barbosa EM, Netto MM, et al. Gene expression profile associated with response to doxorubicin-based therapy in breast cancer. Clin Cancer Res. 2005;11(20):7434-43.

9. Noruzi S, Azizian M, Mohammadi R, Hosseini SA, Rashidi B, Mohamadi Y, et al. Micro-RNAs as critical regulators of matrix metalloproteinases in cancer. Journal of cellular biochemistry. 2018.

10. Keshavarz M, Dianat-Moghadam H, Sofiani VH, Karimzadeh M, Zargar M, Moghoofei M, et al. miRNA-based strategy for modulation of influenza A virus infection. Epigenomics. 2018;10(6):829-44.

11. Wang W, Luo YP. MicroRNAs in breast cancer: oncogene and tumor suppressors with clinical potential. Journal of Zhejiang University Science B. 2015;16(1):18-31.
12. Xu H, Fei D, Zong S, Fan Z. MicroRNA-154 inhibits growth and invasion of breast cancer cells through targeting E2F5. American journal of translational research. 2016;8(6):2620-30.
13. Zhou H, Zhang M, Yuan H, Zheng W, Meng C, Zhao D. MicroRNA-154 functions as a tumor suppressor in osteosarcoma by targeting Wnt5a. Oncology reports. 2016;35(3):1851-8.
14. Pang X, Huang K, Zhang Q, Zhang Y, Niu J. miR-154 targeting ZEB2 in hepatocellular carcinoma functions as a potential tumor suppressor. Oncology reports. 2015;34(6):3272-9.
15. Wang W, Peng B, Wang D, Ma X, Jiang D, Zhao J, et al. Human tumor microRNA signatures derived from large-scale oligonucleotide microarray datasets. International journal of cancer. 2011;129(7):1624-34.
16. Lin X, Yang Z, Zhang P, Shao G. miR-154 suppresses non-small cell lung cancer growth in vitro and in vivo. Oncology reports. 2015;33(6):3053-60.
17. Mian C, Pennelli G, Fassan M, Balistreri M, Barollo S, Cavedon E, et al. MicroRNA profiles in familial and sporadic medullary thyroid carcinoma: preliminary relationships with RET status and outcome. Thyroid : official journal of the American Thyroid Association. 2012;22(9):890-6.
18. Xin C, Zhang H, Liu Z. miR-154 suppresses colorectal cancer cell growth and motility by targeting TLR2. Molecular and cellular biochemistry. 2014;387(1-2):271-7.
19. Zhou SJ, Bi TQ, Qin CX, Yang XQ, Pang K. Expression of NAMPT is associated with breast invasive ductal carcinoma development and prognosis. Oncology letters. 2018;15(5):6648-54.
20. https://www.who.int/news-room/fact-sheets/detail/cancer.
21. https://www.wcrf.org/dietandcancer/cancer-trends/breast-cancer-statistics.
22. https://www.who.int/cancer/detection/breastcancer/en/index1.html.
23. Hosseinahi N, Aghapour M, Duijf PH, Baradaran B. Treating cancer with microRNA replacement therapy: A literature review. Journal of cellular physiology. 2018;233(8):5574-88.
24. Wang L, Wu L, Wu J. Downregulation of miR-154 in human glioma and its clinicopathological and prognostic significance. Journal of International Medical Research. 2016;44(5):994-1001.
25. Qin C, Zhao Y, Gong C, Yang Z. MicroRNA-154/ADAM9 axis inhibits the proliferation, migration and invasion of breast cancer cells. Oncology letters. 2017;14(6):6969-75.
26. Xu H, Fei D, Zong S, Fan Z. MicroRNA-154 inhibits growth and invasion of breast cancer cells through targeting E2F5. American journal of translational research. 2016;8(6):2620.
27. Kai Y, Qiang C, Xinxin P, Miaomiao Z, Kuailu L. Decreased miR-154 expression and its clinical significance in human colorectal cancer. World journal of surgical oncology. 2015;13(1):195.
28. Qiao W, Cao N, Yang L. MicroRNA-154 inhibits the growth and metastasis of gastric cancer cells by directly targeting MTDH. Oncology letters. 2017;14(3):3268-74.
29. Liu S, Yang Y, Chen L, Liu D, Dong H. MicroRNA-154 functions as a tumor suppressor in non-small cell lung cancer through directly targeting B-cell-specific Moloney murine leukemia virus insertion site 1. Oncology letters. 2018;15(6):10098-104.
30. Zhou SJ, Bi TQ, Qin CX, Yang XQ, Pang K. Expression of NAMPT is associated with breast invasive ductal carcinoma development and prognosis. Oncology letters. 2018;15(5):6648-54.

31. Hong S, Park C, Kim S, Nam Y, Yu J, Shin J, et al. NAMPT suppresses glucose deprivation-induced oxidative stress by increasing NADPH levels in breast cancer. Oncogene. 2016;35(27):3544.

32. Kim JG, Kim EO, Jeong BR, Min YJ, Park JW, Kim ES, et al. Visfatin stimulates proliferation of MCF-7 human breast cancer cells. Molecules and cells. 2010;30(4):341-5.

33. Hesari Z, Nourbakhsh M, Hosseinkhani S, Abdolvahabi Z, Alipour M, Tavakoli-Yaraki M, et al. Down-regulation of NAMPT expression by mir-206 reduces cell survival of breast cancer cells. Gene. 2018.

34. Lee Y-C, Yang Y-H, Su J-H, Chang H-L, Hou M-F, Yuan S-SF. High visfatin expression in breast cancer tissue is associated with poor survival. Cancer Epidemiology and Prevention Biomarkers. 2011.

35. Zhou T, Wang T, Garcia JG. Expression of nicotinamide phosphoribosyltransferase-influenced genes predicts recurrence-free survival in lung and breast cancers. Scientific reports. 2014;4:6107.

36. Park H-J, Kim S-R, Kim SS, Wee H-J, Bae M-K, Ryu MH, et al. Visfatin promotes cell and tumor growth by upregulating Notch1 in breast cancer. Oncotarget. 2014;5(13):5087.

37. Gholinejad Z, Kheiripour N, Nourbakhsh M, Ilbeigi D, Behroozfar K, Hesari Z, et al. Extracellular NAMPT/Visfatin induces proliferation through ERK1/2 and AKT and inhibits apoptosis in breast cancer cells. Peptides. 2017;92(Supplement C):9-15.

38. Behrouzfar K, Alaee M, Nourbakhsh M, Gholinejad Z, Golestani A. Extracellular NAMPT/visfatin causes p53 deacetylation via NAD production and SIRT1 activation in breast cancer cells. Cell biochemistry and function. 2017;35(6):327-33.

39. Zhang Q, Shen Y, Jiang Y, Zhao S, Zhou D, Xu N. Overexpression of miR-182 inhibits ossification of ligamentum flavum cells by targeting NAMPT. Experimental cell research. 2018;367(2):119-31.

40. Li Y, Ke J, Peng C, Wu F, Song Y. MicroRNA-300/NAMPT regulates inflammatory responses through activation of AMPK/mTOR signaling pathway in neonatal sepsis. Biomedicine & Pharmacotherapy. 2018;108:271-9.

41. Choi SE, Fu T, Seok S, Kim DH, Yu E, Lee KW, et al. Elevated microRNA-34a in obesity reduces NAD+ levels and SIRT1 activity by directly targeting NAMPT. Aging cell. 2013;12(6):1062-72.

42. Ju H-Q, Zhuang Z-N, Li H, Tian T, Lu Y-X, Fan X-Q, et al. Regulation of the Nampt-mediated NAD salvage pathway and its therapeutic implications in pancreatic cancer. Cancer letters. 2016;379(1):11.

43. Zhang C, Tong J, Huang G. Nicotinamide phosphoribosyl transferase (Nampt) is a target of microRNA-26b in colorectal cancer cells. PloS one. 2013;8(7):e69963.

44. Gao H, Chen J, Chen T, Zhao S, Machado RF. Microrna-410 Is Downregulated By Hypoxia And VEGF Inhibits Proliferation Of Pulmonary Artery Smooth Muscle Cells And Pulmonary Artery Endothelial Cells Via Regulation Of Nicotinamide Phosphoribosyl Transferase. B71 PULMONARY HYPERTENSION LIFE: ANIMAL MODELS AND EX VIVO STUDIES IN PULMONARY HYPERTENSION: American Thoracic Society; 2017. p. A4217-A.
45. Chen X-Y, Zhang H-S, Wu T-C, Sang W-W, Ruan Z. Down-regulation of NAMPT expression by miR-182 is involved in Tat-induced HIV-1 long terminal repeat (LTR) transactivation. The international journal of biochemistry & cell biology. 2013;45(2):292-8.

46. Sawicka-Gutaj N, Waligórska-Stachura J, Andrusiewicz M, Biczysko M, Sowiński J, Skrobisz J, et al. Nicotinamide phosphorybosiltransferase overexpression in thyroid malignancies and its correlation with tumor stage and with survivin/survivin DEx3 expression. Tumor Biology. 2015;36(10):7859-63.

47. Alaee M, Khaghani S, Behroozfar K, Hesari Z, Ghorbanhosseini SS, Nourbakhsh M. Inhibition of nicotinamide phosphoribosyltransferase induces apoptosis in estrogen receptor-positive MCF-7 breast cancer cells. Journal of breast cancer. 2017;20(1):20-6.

48. Allen KE, Weiss GJ. Resistance may not be futile: microRNA biomarkers for chemoresistance and potential therapeutics. Molecular cancer therapeutics. 2010;9(12):3126-36.

49. Hu G, Zhao X, Wang J, Lv L, Wang C, Feng L, et al. miR-125b regulates the drug-resistance of breast cancer cells to doxorubicin by targeting HAX-1. Oncology letters. 2018;15(2):1621-9.

50. Hu S, Wang C, Huang Z, Liu F, Xu C, Li X, et al. miR-760 mediates chemoresistance through inhibition of epithelial mesenchymal transition in breast cancer cells. Eur Rev Med Pharmacol Sci. 2016;20(23):5002-8.

51. Yuan Y, Yao YF, Hu SN, Gao J, Zhang L-L. MiR-133a is functionally involved in doxorubicin-resistance in breast cancer cells MCF-7 via its regulation of the expression of uncoupling protein 2. PLoS One. 2015;10(6):e0129843.

52. Sampath D, Zabka TS, Misner DL, O’Brien T, Dragovich PS. Inhibition of nicotinamide phosphoribosyltransferase (NAMPT) as a therapeutic strategy in cancer. Pharmacology & therapeutics. 2015;151:16-31.

Tables

Table 1

| microRNA            | Seq. (5′-3′)                     |
|---------------------|---------------------------------|
| microRNA Inhibitor N.C | 5′-CAGUACUUUUUGUGUAGUACAA-3′   |
| microRNA mimic N.C    | 5′-UUGUACUACAAAGAGUGUGUGACG-3′ |
| miR-154 Inhibitor    | 5′-CGAAGGCAACACGGAUAACCUA-3′   |
| miR-154 mimic        | 5′-UAGGUUAUCCUGUGUGCUCUUG-3′  |

Figures
Figure 1
Figure 4
Figure 5

Figure 6
Figure 7
Figure 8
Figure 9

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplement1.pdf
- supplement2.tif