Downregulation of NRARP exerts anti-tumor activities in the breast tumor cells depending on Wnt/ β -catenin mediated signals; the role of miR-130a-3p

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Abstract

Objectives: The Notch-regulated ankyrin repeat protein (*NRARP*) functions as a molecular link between Notch and Wnt signaling pathways. Although it has recently been identified to be overexpressed in breast cancer (BC), the molecular mechanisms regulating *NRARP* remain unknown. Since microRNAs (miRNAs) regulate gene expression post-transcriptionally, miRNA dysregulation could explain the abnormal gene expression. Here, we identified miR-130a-3p as an *NRARP* regulator and evaluated its effects on the behavior of BC cells.

Methods: Quantitative real-time PCR (qRT-PCR) was performed to assess the transcriptional levels of miR-130a-3p and *NRARP* in BC cells. Next, miR-130a-3p was transiently transfected into BC cells to assess its influence on *NRARP* expression. Owing to the positive regulatory effects of *NRARP* on the Wnt/ β -catenin signaling pathway, we also analyzed the expression levels of five Wnt/ β -catenin pathway genes in BC cells. We then assessed anti-tumor activities of miR-130a-3p in BC cells using the MTT proliferation assay, the soft agar colony formation assay for anchorage-independent growth (AIG), as well as scratch and transwell assays for cell migration.

Results: MiR-130a-3p was found to be downregulated in BC cells, whereas *NRARP* was upregulated. Overexpression of miR-130a-3p inhibited the expression of *NRARP* and some Wnt/ β -catenin signaling pathway genes, as well as exerted anti-tumor effects as evidenced by decreased cell proliferation, AIG, and migration of BC cells.

Conclusion: In conclusion, the tumor-suppressive function of miR-130a-3p in BC may be mediated by inhibiting *NRARP* and Wnt/ β -catenin signaling pathway. As a result, miR-130a-3p could be introduced as a therapeutic target for miRNA therapy in BC.

Introduction

According to the data obtained from both developed and developing countries, breast cancer (BC) is the most common cancer in women, ranking first on the list of the most frequently diagnosed malignancies in 2020 [1, 2]. The complexity of the disease has led scientists to analyze the expression of many genes in breast tumors; for example, human epidermal growth factor receptor-2 (HER2) is overexpressed in 15–20% of all breast tumors [3]. Although drugs that target HER2 could reduce the severity of disease and mortality in these patients, drug resistance and tumor recurrence remain serious issues, and novel molecular targets to improve BC treatment are welcomed [4].

The Notch and Wnt signaling pathways are essential for a variety of cellular functions such as cell proliferation, apoptosis, and differentiation [5, 6]. It has been demonstrated that abnormal activation of these pathways is associated with the development and progression of BC [4]. However, there is evidence that aberrant Wnt/ β -catenin signaling pathway is sufficient to cause mammary cell transformation, and HER2 positive breast tumors are recognized to have an overactivated Wnt/ β -catenin signaling pathway [7]. The Notch and Wnt signaling pathways interact in various ways; Notch-regulated ankyrin repeat

protein (*NRARP*) is an important molecule that regulates both Notch and Wnt/ β -catenin signaling pathways [8]. *NRARP* is expressed in response to increased Notch signals; however, it suppresses Notch signaling through feedback inhibition [9]. In the Wnt signaling pathway, *NRARP* functions as a potent inducer, increasing the expression of canonical Wnt pathway genes [10]. *NRARP* has recently been demonstrated to act as an oncogene in a number of malignancies [11]. In the context of BC, while microarray data indicate that *NRARP* is overexpressed in various rat models of BC, the molecular mechanisms regulating *NRARP* remain unknown [12].

MicroRNAs (miRNAs) are single-stranded RNA sequences of 20–22 nucleotides in length that function in messenger RNAs (mRNAs) silencing and post-transcriptional inhibition of gene expression [13]. The miRNA research has gotten a lot of attention because of the wide range of target genes and the fact that they regulate almost every biological pathway. Therefore, oncogenic or tumor suppressive miRNAs could be investigated for therapeutic purposes [14].

In the present study, we aimed at finding the putative miRNA that potentially targets *NRARP* in BC. Interestingly, miR-130a-3p was identified to be a potential miRNA involved in cell proliferation, migration, and drug resistance, as well as being dysregulated in BC cells [15]. It was then transfected into the MCF-7, a human BC cell line with estrogen receptor, progesterone receptor, and HER2 positivity, as well as SKBR3, a HER2 overexpressing BC cell line. Next, the effect of miR-130a-3p restoration on *NRARP* expression was investigated using quantitative real-time PCR (qRT-PCR). Besides this, as *NRARP* is a positive regulator of the Wnt/ β -catenin signaling pathway, we also assessed the expression of pathway's key oncogenic components. Among the genes that scrutinized, *Wnt2B*, *FZD6*, *LRP6*, *CTNNB1*, *TCF4*, and a downstream target gene, *ZEB1* were chosen for further investigation. Furthermore, reliable in vitro assays such as MTT, the formation of cell colonies on soft agar, scratch, and transwell cell migration assays were performed to investigate the mechanisms of miR-130a-3p's involvement in BC cells.

Material And Methods

Bioinformatics prediction

TargetScan, miRanda, and miRDB were used to predict putative miRNA that potentially targets *NRARP*. As shown in Fig. 2(a), with a perfect seed match model (7mer-m8), TargetScan indicated that miR-130a-3p potentially block *NRARP*. Other algorithms also predicted miR-130a-3p as a potential regulator of *NRARP*.

Cell Lines and Culture

The MCF-7 and SKBR3 human breast cancer cell lines, as well as MCF-10A as a non-cancerous breast epithelial cell line, were obtained from the National Cell Bank of Iran (NCBI, Tehran, Iran). To verify that the cells are free of mycoplasma, a PCR test was performed. In order to culture BC cell lines, complete cell culture medium containing high-glucose Dulbecco's modified eagle medium (DMEM), 10% fetal bovine serum (FBS-Gibco, USA), 100 units/ml penicillin, and 100 g/ml streptomycin was used. A medium

containing 10% horse serum (HBS-Gibco, USA) was utilized for the growth of MCF-10A cell line. The cells were incubated at 37 °C with 90% humidity and 5% CO₂. Every 24 hours, the cells were observed under an inverted microscope for cellular density, morphology, and contamination.

RNA Extraction

To assess the mRNA expression of miR-130a-3p, *NRARP*, and Wnt/ β -catenin pathway genes in BC cells or normal breast epithelial cells, the cellular RNA was isolated using hybrid-RTM RNA extraction kit (GeneAll, Korea) according to the manufacturer's protocol. Briefly, the cells were lysed with 1ml of RiboExTM lysis buffer for 5 minutes. Next, the cells were transferred to a microtube and each tube was mixed intensely with 0.2 ml of chloroform for 15 seconds and then centrifuged for 15 minutes at 12000 $\times$ g for phase separation. Afterward, the upper phase was moved to a small spin column. The column was centrifuged for 30 seconds ($\sim$ 10000 $\times$ g), and then passed-through material was removed. The spin column was washed twice using wash solutions, and then RNase-free water was used to elute the purified RNA. The cellular RNA isolated from MCF-10A was considered as control sample. NanoDrop (Aosheng, China) and denaturing agarose gel electrophoresis were used to determine the concentration and integrity of isolated RNAs, respectively. Extracted RNAs were stored at -80 °C for future experiments.

Synthesis of cDNA and qRT-PCR

In a reaction catalyzed by the RevertAid enzyme (Thermo Scientific, USA), cDNA was synthesized from isolated RNA in the presence of stem-loop primers for miR-130a-3p and SNORD47, and random hexamer primers for genes. The StepOne plusTM thermal cycler (ABI, USA) was used to perform qRT-PCR using SYBR Green fluorescent dye and primers listed in Table1. The PCR was carried out for 10 minutes at 95°C for initial denaturation. After that, 40 cycles of 95°C for 15 seconds, 60°C for 40 seconds, and 72°C for 50 seconds were performed. At the end of the experiment, the qRT-PCR amplification efficiency and cycle threshold (C_t) was analyzed using LinRegPCR program (J.M. Ruijter, Version 1.2). Expression values were normalized using Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference gene for the *NRARP* and Wnt/ β -catenin pathway genes, and SNORD47 (U47) for the miR-130a-3p, and presented as a relative expression using the $2^{-\Delta\Delta C_t}$ method.

Transient transfection

The miR-130a-3p miRNA mimics or scramble miRNA as negative control were purchased from Exiqon-Qiagen (Germany). Transfection of these sequences (5nM) into BC cell lines was carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. In brief, MCF-7 or SKBR3 cells from low passage were seeded in plates (24-well) and incubated at 37 °C until 90% confluence was achieved. The complete medium in each well was then changed with FBS-free medium. Following that, the cells were exposed to respective sequences. Cells were processed two days

after transfection for further testing. Non-transfected MCF-7 or SKBR3 cells (untreated cells) were investigated as control.

qRT-PCR after transfection

Total RNA was extracted, cDNA was synthesized, and qRT-PCR was performed on either transfected or untreated cells, as described above. For normalization of qRT-PCR data, SNORD47 and GAPDH were used as reference genes.

Proliferation assay

The proliferation of BC cells after miR-130a-3p restoration was assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. In brief, BC cells were dissociated and suspension of 3×10^3 cells for each well were transferred into a 96-well plate. The cells were transfected as described above or left untreated, and then were further grown for 24, 48, and 72 hours. Following that, the cells were incubated in the MTT reagent (0.05 g/ml in PBS) for 3 hours at 37 °C in order to develop formazan crystals. In the next step, the formazan crystals were dissolved using dimethyl sulfoxide (DMSO), and color intensity was quantified spectrophotometrically at 570 nm after 5–10 minutes shaking. Non-transfected MCF-7 or SKBR3 cells were investigated as control.

Soft agar colony formation assay

The ability of BC cells to grow anchorage-independently in response to miR-130a-3p restoration was tested using a soft agar colony formation assay, which evaluates cellular transformation *in vitro* [16]. First, the cells were exposed to respective treatments as described above or left untreated. Next, the experiment was carried out in a 6-well plate with two layers of agar; the bottom layer containing 0.8% agar and 2X cell culture medium in a 1:1 ratio, and the upper layer containing 0.5% agar and the suspension of cells (5×10^3 cells for each well) in a 1:1 ratio. After chilling and forming agar layers, plates were transferred to 37 °C temperature and 5% CO₂ incubator. After 3 weeks, each well was stained with 0.2 ml of nitroblue tetrazolium chloride solution. The plates were then incubated at 37 °C for 24 hours, and the colonies were counted.

Scratch test

Wound closure or scratch test is a type of whole-cell mass migration that is commonly used by researchers to determine the migratory potential of cancer cells [17]. For performing the scratch test, the cells were seeded at a density of 1×10^5 cells per well in a 24-well plate and grown to form a cell monolayer. The scratch was created on the cell layer using a pipette tip. Following that, the cells were

washed thoroughly twice with PBS, and a low-FBS medium (0.5 %) was added to the wells. The first image of the scratch area was captured after it was formed and viewed under an inverted microscope, and subsequent images in the same area were captured at regular time intervals for up to 48 hours. ImageJ software (v1.8.0, NIH, USA) was used to quantify snapshots, and the following formula was used to determine the migration area:

Migration Area = (the scratch area measured after the scratch has formed) – (the scratch area measured at regular time intervals)

In vitro cell migration assay

The ability of BC cells to migrate in response to attractant was appraised using an 8 µm pore transwell plate (SPL, Life Bioscience, Korea). To do so, at first, the cells were exposed to respective treatments as described above or left untreated. Next, the upper compartment of the insert was filled with 0.2 ml of FBS-free cell solution (5×10^4 cells per well), and to the well of the plate (bottom of the lower chamber), 0.7 ml DMEM supplemented with 10% FBS as a chemoattractant was added. The cells were incubated for 24 hours at 37 °C, and then the cells that migrated from the upper to lower surface of the membrane were fixed with 70% ethanol (Sigma, Germany), exposed to the 0.05% crystal violet (Merck-Darmstadt, Germany), randomly different views were chosen, and the average of the cells per views was computed. Non-transfected MCF-7 or SKBR3 cells were investigated as control.

Statistical analysis

GraphPad Prism version 8.0.1 (GraphPad, La Jolla, CA) was used for statistical analysis. For multiple comparisons, analysis of variance (ANOVA) test was employed followed by Tukey post hoc tests. The results are presented as the mean $\pm$ SD of three independent experiments, and the *P-value* of less than 0.05 was considered statistically significant.

Results

The expression of miR-130a-3p decreases in BC cell lines, whereas the expression of NRARP and Wnt/ β -catenin pathway genes increase

To investigate the involvement of miR-130a-3p, *NRARP*, and Wnt/ β -catenin pathway genes in the pathogenesis of BC, their expression was analyzed using qRT-PCR. As indicated in Fig. 1(a), the mRNA level of miR-130a-3p was lower in MCF-7 and SKBR3 cells than in MCF-10A cells (193- and 17-folds, respectively). Furthermore, *NRARP* expression was 7.7 and 5.8-folds higher in MCF-7 and SKBR3 cells, respectively, than in normal breast epithelial cells (Fig. 1(b)). Owing to the positive regulatory effects of

NRARP on Wnt/ β -catenin signaling pathway, we also analyzed the expression levels of the five Wnt/ β -catenin pathway genes (*Wnt2B*, *FZD6*, *LRP6*, *CTNNB1*, *TCF4*) and a downstream target gene, *ZEB1* in BC cells. As shown in Fig. 1(c), the expression levels of *Wnt2B*, *FZD6*, *LRP6*, and *ZEB1* were higher in MCF-7 cells compared to normal breast epithelial cells (fold changes ≥ 2 , $p \leq 0.05$). Furthermore, except for *LRP6*, the mRNA levels of Wnt pathway genes and *ZEB1* were increased in SKBR3 cells than in normal breast epithelial cells, which is shown in Fig. 1(d) (fold changes ≥ 2 , $p \leq 0.0001$).

Overexpression of miR-130a-3p is associated with NRARP downregulation in BC cell lines

To investigate how miR-130a-3p and *NRARP* are related, it was transiently transfected into BC cells, and transfection efficiency was assessed by qRT-PCR. As shown in Fig. 2(b), miR-130a-3p was upregulated in MCF-7 and SKBR3 transfected cells compared to control cells. Regarding *NRARP*, as shown in Fig. 2(c), the expression level of *NRARP* decreased significantly in the MCF-7 and SKBR3 cells after miR-130a-3p restoration compared to the MCF-10A cells. No statistically significant changes were detected between scramble-transfected cells and control cells in both cell lines ($p \geq 0.05$).

The expression of Wnt/ β -catenin pathway genes in BC cell lines after miR-130a-3p restoration

We further want to explore whether miR-130a-3p restoration and downregulation of *NRARP* has any effects on Wnt/ β -catenin signaling pathway. To address this question, the mRNA levels of some genes in the pathway were examined following transfection of miR-130a-3p into BC cells. As indicated in Fig. 2(d), all genes evaluated in the present study were downregulated in MCF-7 cells exposed to miR-130a-3p in comparison to control cells (fold change ≥ 0.5 , $p \leq 0.05$). Similar findings were observed in SKBR3 cells, as the mRNA expression of these genes decreased significantly following miR-130a-3p restoration compared to control SKBR3 cells (fold change ≥ 0.5 , $p \leq 0.001$), although *TCF4* was not (Fig. 2(e)). No statistically significant changes were detected between scramble-transfected cells and control cells in both cell lines ($p \geq 0.05$).

The proliferation and AIG of BC cell are both decreased by miR-130a-3p.

As shown in Fig. 3(a), MCF-7 cells showed a decrease in cell proliferation 72 hours after exposure to miR-130a-3p in comparison to control cells ($p \leq 0.01$). Furthermore, 48 and 72 hours after miR-130a-3p restoration in SKBR3 cells, cell proliferation decreased significantly compared to control SKBR3 cells ($p \leq 0.05$). No statistically significant changes were detected between scramble-transfected cells and control cells in both cell lines ($p \geq 0.05$).

The results of AIG indicated that the number of cell colonies decreased significantly in MCF-7 cells exposed to miR-130a-3p in comparison to the cells exposed to scramble sequences and control cells ($p \leq 0.0001$ for both, Fig. 3(b)). Furthermore, SKBR3 cells exposed to miR-130a-3p formed fewer colonies than cells exposed to scramble sequences or control cells ($p = 0.0001$ and $p = 0.0003$, respectively). No statistically significant changes were detected between scramble-transfected cells and control cells in both cell lines ($p \geq 0.05$).

Cell migration decreases in BC cell lines after miR-130a-3p restoration

The scratch test was performed by creating a line in a layer of cells and photographing it at regular intervals to follow whole-cell masses migration. As shown in Fig. 4(a-d), scratch closure decreased considerably, in both cell lines, within 24 and 48 hours after miR-130a-3p restoration compared to cells treated with scramble sequences ($p = 0.0001$ and $p = 0.0007$ in MCF-7 and SKBR3, respectively) or control cells ($p \leq 0.0001$ in both cell lines).

The single-cell migration of BC cells after ectopic expression of miR-130a-3p was more assessed by performing transwell cell migration assay. As shown in Fig. 5(a and c), MCF-7 cells exposed to miR-130a-3p exhibited a significant reduction in the migrating cells compared with the cells exposed to scramble sequences or control cells ($p = 0.0002$ and $p = 0.0001$, respectively). In SKBR3 cells, restoration of miR-130a-3p resulted in a significant reduction in single-cell migration when compared to cells exposed to scramble sequences or control cells ($p = 0.0008$, and $p = 0.0019$, respectively, Fig. 5(b and d)).

Discussion

Given the lack of a radical cure and the limitations of current therapies, human cancers should be extensively investigated at the molecular level and signaling pathways, where many potential opportunities for oligonucleotide-based therapies may emerge [18]. In this study, we evaluated the molecular mechanism regulating *NRARP*, which is a molecular link between the Notch and Wnt signaling pathways. Our results indicated that miR-130a-3p is a regulator of *NRARP* in BC, and suggested that downregulation of *NRARP* could be followed by the suppression of Wnt/ β -catenin signaling pathway, as well as decreased cell proliferation, AIG, and cell migration.

The existence of functional crosstalk between Wnt and Notch signaling has been confirmed in several studies; *NRARP* could be involved in tumorigenesis as a result of this crosstalk [19]. To date, only a few reports on *NRARP*'s function have been published. It was first shown to be a downstream target of Notch signaling, in which luciferase reporter results revealed that Notch signals increase the expression of *NRARP*. When expressed, it suppresses Notch signaling by increasing the degradation of Notch intracellular domain [20, 21]. Our findings demonstrated that *NRARP* expression was higher in BC cell

lines than in non-malignant cells, and that *NRARP* expression was dramatically reduced after exposure to miR-130a-3p. In line with our findings, Imaoka et al showed that *NRARP* was overexpressed in the two human breast cancer cell lines, namely MCF-7 and T47D, and that *NRARP* silencing inhibited cell growth and decreased the expression of cell cycle-related genes [12]. Another study in MCF-7 cells revealed that combining Emodin with 5-Fluorouracil (5-FU) causes *NRARP* to be downregulated and that suppressing *NRARP* with siRNA technology causes cellular senescence [22]. These findings give support to the hypothesis that *NRARP* plays an oncogenic role in BC. In the current study, *NRARP* was predicted as a target gene for the miR-130a-3p using several bioinformatics algorithms. Furthermore, qRT-PCR results demonstrated that exposure to miR-130a-3p can diminish *NRARP* expression.

In this study, the expression of certain genes of Wnt/ β -catenin signaling was higher in the BC cell lines than in normal cells. The Wnt/ β -catenin signaling pathway is abnormally activated in the human BC through different mechanisms [23]. Ishitani et al. [24] reported that zebrafish *Nrarp* inhibits the ubiquitination and subsequent degradation of Wnt/ β -catenin signaling transcription factor, *lef1*, resulting in the pathway activation. Also, Phng et al. [25] reported that *Nrarp* knockdown inhibits the function of Wnt3a, a Wnt/ β -catenin signaling ligand and that induction of *Nrarp* expression by Dll4/Notch promotes Wnt/Ctnnb1 signaling in the endothelial stalk cells through interactions with *Lef1*. Supporting this concept, Pinto et al. [8] have recently demonstrated that *NRARP* interacts with *LEF1* in T-cell acute lymphoblastic leukemia cells and that *NRARP* overexpression results in elevated levels of β -catenin, a main player in the pathway; so, they suggested that these findings could be extended to other malignancies in which Notch and Wnt are involved. Accordingly, it is reasonable to assume that the aberrant Wnt/ β -catenin signaling pathway in BC cells could be a result of *NRARP* upregulation. We also observed that the expression of some Wnt/ β -catenin pathway genes were significantly decreased after miR-130a-3p restoration. As illustrated in Fig. 6, It is possible that by downregulating *NRARP*, its inducing effects on the Wnt/ β -catenin signaling pathway have been reduced. Another possibility is that miR-130a-3p directly inhibited the Wnt/ β -catenin signaling pathway, as it has been suggested in our previous study in the triple-negative BC [26].

The epithelial-mesenchymal transition (EMT) is a dynamic process by which cancer cells acquire an aggressive phenotype. Zinc finger E-box-binding homeobox 1 (*ZEB1*) is the main transcription factor that regulates EMT [27, 28]. In this study, following miR-130a-3p overexpression, the expression of *ZEB1* was significantly decreased. There are three possible explanations for the *ZEB1* downregulation (Fig. 6): First, Fazio et al. [29] found that colon cancer cells that express high transcriptional levels of *ZEB1*, also express high levels of *NRARP*. Besides this, Zhu et al. [30] reported that by knocking down *NRARP*, the stemness and EMT are decreased in liver tumor cells. These findings suggest a functional link between *NRARP* and *ZEB1* as an EMT marker, and our results could be evidence that downregulation of *NRARP* may be resulted in the inhibition of *ZEB1*. Second, *ZEB1*, as a direct downstream target gene, is regulated by the Wnt/ β -catenin signaling pathway, in which β -catenin promotes the expression of *ZEB1* by binding to its promoter. Moreover, *ZEB1* expression is reduced when β -catenin is knocked down [31]. Hence, the downregulation of β -catenin could be another explanation for the significant decrease in the *ZEB1* expression after treatment with the miR-130a-3p. Third, as reported by Chi et al. [32] miR-130a-3p has

target sequences in the 3'-UTR of *ZEB1*. Thus, the decreased transcriptional levels of *ZEB1* found in the current study could be a direct impact of miR-130a-3p.

We also assessed cell proliferation, AIG, single-cell and whole-cell mass migrations of BC cells after exposure to miR-130a-3p. Our results showed that ectopic miR-130a-3p expression could reduce the AIG in BC cells. Cancer cells manifest adherent-independent cell growth, a process by which cells separate from the extracellular matrix and attach to an inappropriate matrix, resulting in colonization in distant organs [33]. The Wnt/ β -catenin is one of several molecular pathways that regulate the expression of downstream genes that are involved in the adherent-independent growth of cancer cells [34]. Therefore, the decrease in the AIG observed in this study could be a result of inhibition of Wnt/ β -catenin signaling pathway following miR-130a-3p overexpression and/or *NRARP* downregulation. In addition, ectopic miR-130a-3p expression reduced both single-cell and whole-cell mass migrations in BC cells. Because *NRARP*, *FZD6*, *LRP6*, and *ZEB1* have been linked to cell migration and invasion, the reduced cell migration seen after miR-130a-3p restoration could be attributed to miR-130a-3p directly inhibiting these genes.[35–37].

Overall, the purpose of this study was on deciphering the processes by which *NRARP* and Wnt/ β -catenin signaling pathway are regulated in breast cancer. Our results showed that miR-130a-3p is an *NRARP* regulator, and its overexpression exerts anti-tumor activities. Although qRT-PCR results indicated that overexpression of miR-130a-3p and *NRARP* downregulation could be followed by the inhibition of some Wnt/ β -catenin signaling pathway genes and *ZEB1*, the exact molecular mechanisms by which these genes are regulated were not fully elucidated in this study, emphasizing that more studies are needed.

Declarations

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Disclosure Statement:

The authors declare that there is no conflict of interest regarding this article.

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All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Masoumeh Rajabibazl, Jafar Poodineh, Samira Mohammadi-Yeganeh, and Majid Sirati-Sabet. The first draft of the manuscript was written by Jafar Poodineh and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript

References

1. Raj, U., et al. Identification of novel dysregulated key genes in Breast cancer through high throughput ChIP-Seq data analysis. *Sci Rep.* 2017;7(1):p.3229.
2. Sung, H., et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin.* 2021;71(3):p.209-249.
3. Singh, R. and Y.-Y. Mo. Role of microRNAs in breast cancer. *Cancer Biol Ther.* 2013;14(3):p.201-212.
4. Shah, D. and C. Osipo. Cancer stem cells and HER2 positive breast cancer: The story so far. *Genes & Diseases.* 2016;3(2):p.114-123.
5. South, A.P., R.J. Cho, and J.C. Aster. The double-edged sword of Notch signaling in cancer. *Semin Cell Dev Biol.* 2012;23(4):p.458-464.
6. Yang, K., et al. The evolving roles of canonical WNT signaling in stem cells and tumorigenesis: implications in targeted cancer therapies. *Lab Invest.* 2016;96(2):p.116-36.
7. Polakis, P. Wnt signaling in cancer. *Cold Spring Harb Perspect Biol.* 2012;4(5):p.a008052.
8. Pinto, I., et al. NRARP displays either pro- or anti-tumoral roles in T-cell acute lymphoblastic leukemia depending on Notch and Wnt signaling. *Oncogene.* 2020;39(5):p.975-986.
9. Lamar, E., et al. Nrarp is a novel intracellular component of the Notch signaling pathway. *Genes & development.* 2001;15(15):p.1885-1899.
10. Borggreffe, T., et al. The Notch intracellular domain integrates signals from Wnt, Hedgehog, TGF β /BMP and hypoxia pathways. *Biochim Biophys Acta.* 2016;1863(2):p.303-313.
11. Liao, Y., et al. Notch-regulated ankyrin-repeat protein is a novel tissue biomarker that predicts poor prognosis in non-small cell lung cancer. *Oncol Lett.* 2018;16(2):p.1885-1891.
12. Imaoka, T., et al. Overexpression of NOTCH-regulated ankyrin repeat protein is associated with breast cancer cell proliferation. *Anticancer Res.* 2014;34(5):p.2165-71.
13. Peng, Y., et al. The crosstalk between microRNAs and the Wnt/ β -catenin signaling pathway in cancer. *Oncotarget.* 2016;8(8).
14. Thorsen, S.B., et al. The therapeutic potential of microRNAs in cancer. *Cancer J.* 2012;18(3):p.275-84.
15. Zhang, H.D., et al. The role of miR-130a in cancer. *Breast Cancer.* 2017;24(4):p.521-527.

16. Borowicz, S., et al. The soft agar colony formation assay. *J Vis Exp*. 2014(92):p.e51998.
17. Grada, A., et al. Research Techniques Made Simple: Analysis of Collective Cell Migration Using the Wound Healing Assay. *J Invest Dermatol*. 2017;137(2):p.e11-e16.
18. Xiao, Y.-F., et al. Notch and Wnt signaling pathway in cancer: Crucial role and potential therapeutic targets (Review). *Int J Oncol*. 2016;48(2):p.437-449.
19. Hayward, P., T. Kalmar, and A. Martinez Arias. Wnt/Notch signalling and information processing during development. *Development*. 2008;135(3):p.411-424.
20. Krebs, L.T., et al. The Nrarp gene encodes an ankyrin-repeat protein that is transcriptionally regulated by the notch signaling pathway. *Dev Biol*. 2001;238(1):p.110-9.
21. Pirot, P., et al. *Biochem Biophys Res Commun*. Biochemical and Biophysical Research Communications. 2004;322(2):p.526-534.
22. Zu, C., et al. Low dose Emodin induces tumor senescence for boosting breast cancer chemotherapy via silencing NRARP. *Biochem Biophys Res Commun*. 2018;505(4):p.973-978.
23. Howe, L.R. and A.M. Brown. Wnt signaling and breast cancer. *Cancer Biol Ther*. 2004;3(1):p.36-41.
24. Ishitani, T., et al. Nrarp functions to modulate neural-crest-cell differentiation by regulating LEF1 protein stability. *Nat Cell Biol*. 2005;7(11):p.1106-1112.
25. Phng, L.-K., et al. Nrarp coordinates endothelial Notch and Wnt signaling to control vessel density in angiogenesis. *Developmental cell*. 2009;16(1):p.70-82.
26. Poodineh, J., et al. MiR-130a-3p blocks Wnt signaling cascade in the triple-negative breast cancer by targeting the key players at multiple points. *Heliyon*. 2020;6(11):p.e05434.
27. Brabletz, T. EMT and MET in metastasis: where are the cancer stem cells? *Cancer Cell*. 2012;22(6):p.699-701.
28. Preca, B.T., et al. A self-enforcing CD44s/ZEB1 feedback loop maintains EMT and stemness properties in cancer cells. *Int J Cancer*. 2015;137(11):p.2566-77.
29. Fazio, C., et al. Inflammation increases NOTCH1 activity via MMP9 and is counteracted by Eicosapentaenoic Acid-free fatty acid in colon cancer cells. *Sci Rep*. 2016;6:p.20670.
30. Zhu, P., et al. C8orf4 negatively regulates self-renewal of liver cancer stem cells via suppression of NOTCH2 signalling. *Nat Commun*. 2015;6(1):p.7122.
31. Simeone, P., et al. The multiverse nature of epithelial to mesenchymal transition. *Semin Cancer Biol*. 2019;58:p.1-10.
32. Chi, C., et al. HOXD-AS1 Exerts Oncogenic Functions and Promotes Chemoresistance in Cisplatin-Resistant Cervical Cancer Cells. *Hum Gene Ther*. 2018;29(12):p.1438-1448.
33. Paoli, P., E. Giannoni, and P. Chiarugi. Anoikis molecular pathways and its role in cancer progression. *Biochim Biophys Acta*. 2013;1833(12):p.3481-3498.
34. Malagobadan, S. and N.H. Nagoor. Evaluation of MicroRNAs Regulating Anoikis Pathways and Its Therapeutic Potential. *Biomed Res Int*. 2015;2015:p.716816.

35. Corda, G., et al. Functional and prognostic significance of the genomic amplification of frizzled 6 (FZD6) in breast cancer. *J Pathol.* 2017;241(3):p.350-361.
36. Ibrahim, S.A., et al. Syndecan-1 (CD138) modulates triple-negative breast cancer stem cell properties via regulation of LRP-6 and IL-6-mediated STAT3 signaling. *PLoS One.* 2013;8(12):p.e85737.
37. Yang, X., et al. Wnt signaling through Snail1 and Zeb1 regulates bone metastasis in lung cancer. *Am J Cancer Res.* 2015;5(2):p.748-755.

Table

Table 1 is not available with this version

Figures

Figure 1

The expression levels of miR-130a-3p, *NRARP*, and Wnt/ β -catenin pathway genes determined by qRT-PCR. (a) The expression levels of miR-130a-3p in MCF-7 and SKBR3 cell lines (normalized to SNORD47, compared to MCF-10A). (b) The mRNA expression of *NRARP* in MCF-7 and SKBR3 cells. (c-d) The mRNA expression of Wnt pathway genes in BC cell lines; (c) MCF-7 cell line; (d) SKBR3 cell line. GAPDH was used to normalize the gene expression data, and expression values were represented as the mean $\pm$ SD of the three independent experiments (* $p < 0.05$, *** $P \leq 0.001$ versus MCF-10A)

Figure 2

Transient miR-130a-3p transfection in BC cell lines. (a) a screenshot of the matched miR-130a-3p and *NRARP* sequences is presented in accordance with TargetScan, (www.targetscan.org). (b) After transfection, qRT-PCR was used to evaluate relative miR-130a-3p expression in BC cells. Restoration of miR-130a-3p affects the expression of (c) *NRARP*, (d) Wnt pathway genes in MCF-7 cell line, and (d) Wnt pathway genes in SKBR3 cell line. Expression values were represented as the mean $\pm$ SD of the three independent experiments (* $p < 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$ versus MCF-10A or control)

Figure 3

Cell proliferation and anchorage independent growth in BC cells. (a) MTT proliferation test. BC cells were either exposed to miR-130a-3p miRNA mimics or scrambled miRNA, or they were not treated. For comparison between groups, One-way ANOVA followed by Tukey's post-hoc test was used. (b) Soft agar colony formation assay. BC cells were either exposed with miR-130a-3p miRNA mimics or scrambled miRNA, or they were not treated (* $p < 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ versus control). The results are shown as mean $\pm$ SD for three independent experiments

Figure 4

***In vitro* scratch assay.** (a-b) BC cells (magnification, X 100) were exposed to miR-130a-3p miRNA mimics or scrambled miRNA or left untreated. (c-d) The migration area was quantified 24 and 48 hours after transfection. The results are shown as mean $\pm$ SD for three independent experiments. (** $P \leq 0.01$, *** $P \leq 0.001$)

Figure 5

Transwell cell migration assay. (a-b) BC cells (magnification, X 200) were exposed to miR-130a-3p miRNA mimics or scrambled miRNA or left untreated. (c-d) In more than five microscopic fields, migrating cells were observed and counted. The data in the bar charts are the mean $\pm$ SD of three separate experiments (** $P \leq 0.01$, *** $P \leq 0.001$)

Figure 6

The involvement of miR130a-3p and *NRARP* in the regulation of Wnt/ β -catenin signaling pathway