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Notch-regulated Ankyrin Repeat Protein Is Overexpressed in Different Subtypes of Breast Cancer

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Abstract

Introduction: Breast cancer (BC) is a highly heterogeneous disease that has been classified into several subtypes at the molecular level, each with a distinct outcome. Recently, Notch-regulated ankyrin-repeat protein (NRARP), a gene expressed followed by Notch signal activation, has attracted interest due to its aberrant expression in different types of cancer. Accordingly, this study evaluated the expression levels of NRARP in different subtypes of BC.

Methods: The MCF-7, SKBR3, MDA-MB-468, and MDA-MB-231 human BC cell lines, which represent the luminal, human epidermal growth factor receptor 2 (HER2) overexpression, basal A, and basal B subtypes, respectively, as well as MCF-10A as a normal breast epithelial cell for comparison, were selected and grown in the appropriate medium. The relative expression of NRARP in BC cell lines was then determined using the quantitative real-time polymerase chain reaction (qRT-PCR).

Results: The results of the qRT-PCR demonstrated that the expression level of NRARP in all BC cell lines was significantly higher than that of the normal breast epithelial cells ($P<0.05$). However, the significance was more noteworthy in luminal, HER2-overexpressing, and basal A (7.72, 5.81, and 4.6 folds, respectively).

Conclusion: NRARP is a potential target gene for further interventional studies due to its abnormal expression in different subtypes of BC.

Keywords: Breast cancer, Notch signaling, NRARP

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Introduction

Breast cancer (BC) is the most common invasive cancer among women and the leading cause of malignancy-related mortality in this population.[1] BC is a highly heterogeneous disease at both molecular and clinical levels.[2] During the last 20 years, using high throughput technologies and gene expression profiling, five intrinsic molecular subtypes of BC have been defined, including luminal A, luminal B, human epidermal growth factor receptor 2 (HER2) over-expressing, basal A, and basal B.[3,4] The tumors of luminal subtypes express estrogen receptors (ER), progesterone receptors (PR), and high levels of genes related to luminal; however, luminal B tumors are more aggressive than luminal A and express genes associated with proliferation at higher levels.[5,6] HER2 is highly expressed in HER2 over-expressing BC tumors, which are typically high grade upon diagnosis and considerably more aggressive than luminal subtype tumors. They do, however, respond to anti-HER2 targeted therapy.[7,8] Basal-like breast tumor cells have a poor prognosis and generally lack the expression of ER, PR, and HER2. Hence, chemotherapy is the only treatment option that is currently available to increase the patient's chances of survival.[9,10]

The Notch signaling pathway is one of the evolutionary conserved molecular pathways from urchins to human beings.[11] This pathway has an important role in growth, cell differentiation, and morphogenesis during embryonic life and later.[12] It consists of the Notch receptor, which is a single-pass transmembrane protein, a Notch ligand, and a DNA binding sequence with the transcription factor CSL.[13] Although it has been observed that the abnormal activation of the Notch pathway is linked to poor prognosis in BC and other cancers such as gastric, colon, and T-cell

acute lymphoblastic leukemia, it is downregulated in patients with prostatic, skin, and liver malignancies.[14] Notch-regulated ankyrin-repeat protein (NRARP) is a downstream product of the Notch signaling pathway, which has structural similarities with Notch ligands and functions as a negative feedback regulator of the Notch pathway.[15] According to several studies, NRARP is abnormally expressed in human malignancies and may be a promising therapeutic target for patients diagnosed with cancer.[16,17] In the context of BC, *in vitro* studies have shown that NRARP is overexpressed in several rat models of BC.[18] However, the role of NRARP is still unclear in different intrinsic subtypes of BC which may have a worse prognosis. Therefore, the purpose of this study was to determine whether NRARP is abnormally expressed in different subtypes of BC.

Methods

Cell Culture

MCF-7, SKBR3, MDA-MB-468, and MDA-MB-231, representing luminal, HER-2-overexpressing, basal A, and basal B, respectively, were purchased from the National Cell Bank of Iran (NCBI). The MCF-10 cell line as a normal breast epithelial cell was also used for comparison. Dulbecco's modified eagle medium (Caisson, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 unit/mL penicillin, and 100 µg/mL streptomycin (Cegrogen, Germany) was used to culture BC cell lines. MCF-10A cells were cultured in the medium containing 10% horse serum (Gibco, USA), insulin, and Cholera toxin. Then, they were incubated at 37°C with 90% humidity and 5% CO_2 and evaluated every 24 hours for cellular density, morphology, and contamination using an inverted microscope.

Primer Design

AlleleID7 Software (Premier Biosoft) was used to design specific primers for NRARP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes. The primers were designed from the exon-exon junctions. The NCBI database BLAST feature was employed, and then the appropriate primer pair was ordered (Pishgam Biotech Company, Iran) to ensure that the primer pair did not duplicate non-specific targets. In this study, the *GAPDH* gene was applied as the housekeeping gene for normalization. Primer sequences are provided in Table 1.

RNA Extraction

Hybrid-R™ RNA extraction kit (Gene All, South Korea) was used to extract cellular RNA by following the manufacturer's protocol. Briefly, first, the cells were dissociated, and 500 µL of RiboEx™ lysis solution was added to the cells. The resulting suspension was gently pipetted several times to gain a homogeneous appearance and then was transferred to a microtube. After 5 minutes, 200 µL of chloroform was added, and the mixture was vigorously shaken for 15 seconds. Afterward, the microtube was centrifuged (12 000 × g) for 15 minutes at 4°C; thereafter, the supernatant of the three-phase product was carefully transferred to a new microtube, and an equal volume of RBI buffer was added to it. After being well-mixed, 700 µL of this solution was gently transferred to the mini-spin column. The column was centrifuged (12000 × g) for 1 minute at 25°C. Then, the pass-through was discarded, and the remaining solution was transferred to the column, and centrifugation was repeated. Next, SWI buffer was added to each column, and the mixture was centrifuged (12 000 × g) for 1 minute at 25°C again. After discarding pass-through, RNW buffer was added to the column, and the sample was centrifuged (12 000 × g) for 1 minute at 25°C. The mini-spin column was placed in a new RNAase-free microtube, and approximately 30 µL of distilled water was carefully added to the column. After 2 minutes, the sample was centrifuged (12 000 × g) for 1 minute at 25°C. Finally, the sample in each microtube contained purified RNA and was used for further experiments.

Quality Assessment of the Extracted RNA

Agarose gel electrophoresis was performed to evaluate the quality of the extracted RNA. The integrity of ribosomal bands and their strength indicated the high quality of the extraction of the desired RNA. To assess protein contamination, 2 µL of the extracted product was placed in a Nanodrop spectrophotometer (Aosheng, China), and its absorbance was read at 260 and 280 nm. The absorption ratio of 260-280 should be between 1/8 and 2.

Complementary DNA Synthesis and Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Complementary DNA (cDNA) synthesis was performed using the cDNA synthesis kit (Yekta Tajhiz, Tehran, Iran) containing Moloney murine leukemia virus (M-MLV)

Table 1. The Sequences and Properties of the Applied Primers

Gene Symbol	Sequence 5′→3′	Length	Product Size (bp)	Tm	GC%
NRARP (Sense)	TCTCCTGCGTCACTTTCTGT	20	195	58.96	50
NRARP (Anti-sense)	TCCTCAAGTGCTCCCCATTT	20		58.92	50
GAPDH (Sense)	CCTCAAGATCATCAGCAATG	20	167	54.15	45.00
GAPDH (Anti-sense)	CATCACGCCACAGTTTCC	18		56.43	55.56

Note. NRARP: Notch-regulated ankyrin-repeat protein; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

reverse transcriptase. The StepOnePlus™ thermal cycler (ABI, USA) was used to perform qRT-PCR using SYBR Green fluorescent dye. The PCR settings included initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 40 seconds, and 72°C for 50 seconds. The melting curve of PCR products for the presence of non-specific products and the expected melting temperature (Tm) were evaluated at the end of the experiment. The cycle threshold values were imported into the REST 2009 program, and the relative expression of each gene compared to MCF-10A was analyzed using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

GraphPad Prism, version 8.0.1 (GraphPad, La Jolla, CA) was used for statistical analysis. In addition, a Student's *t* test was employed to compare the statistical differences between the two groups, and $P<0.05$ was considered as the criterion of significance.

Results

The Expression Levels of NRARP in the MCF-7 Cell Line

MCF-7 is a human BC cell line that represents the luminal subtype of BC. As shown in Figure 1, the expression level of NRARP was 7.72 folds higher in MCF-7 cells compared to normal breast epithelial cells ($P<0.01$).

The Expression Levels of NRARP in the SKBR3 Cell Line

SKBR3 is a human BC cell line that demonstrates the HER2-overexpressing subtype of BC. As depicted in Figure 2, the expression level of NRARP was 5.81 folds higher in SKBR3 cells in comparison to normal breast epithelial cells ($P<0.01$).

The Expression Levels of NRARP in the MDA-MB-468 Cell Line

MDA-MB-468 is a human BC cell line that indicates the basal A subtype of BC. As illustrated in Figure 3, the expression level of NRARP was 4.6 folds higher in MDA-MB-468 cells compared to normal breast epithelial cells ($P<0.01$).

The Expression Levels of NRARP in the MDA-MB-231 Cell Line

MDA-MB-231 is a human BC cell line that represents the basal B subtype of BC. As shown (Figure 4), the expression level of NRARP was a 2.17-fold higher in MDA-MB-231 cells in comparison to normal breast epithelial cells ($P<0.05$).

Discussion

The molecular mechanisms behind human malignancies have yet to be fully characterized, particularly in the case of BC, which is a heterogeneous disease with

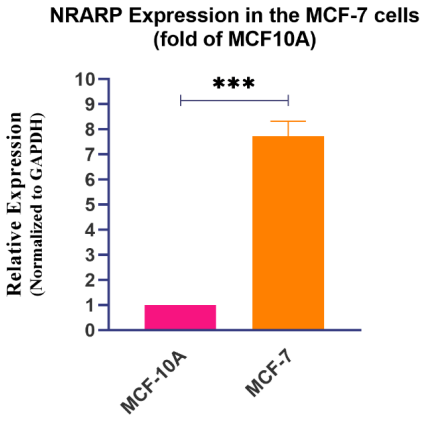

Figure 1. The mRNA Expression of NRARP in the MCF-7 Cell Line. *Note.* NRARP: Notch-regulated ankyrin-repeat protein; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; SD: Standard deviation. GAPDH was used to normalize the gene expression data, and expression values were represented as the mean ± SD of the three independent experiments (***$P\leq0.001$ versus MCF-10A)

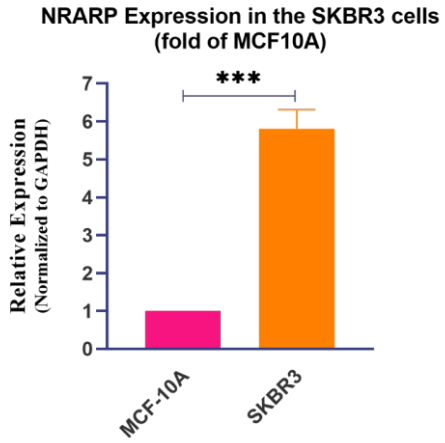

Figure 2. The mRNA Expression of NRARP in the SKBR3 Cell Line. *Note.* NRARP: Notch-regulated ankyrin-repeat protein; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; SD: Standard deviation. GAPDH was employed to normalize the gene expression data, and expression values were demonstrated as the mean ± SD of the three independent experiments (***$P\leq0.001$ versus MCF-10A)

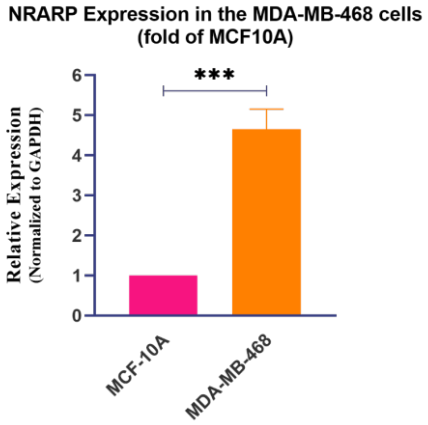

Figure 3. The mRNA Expression of NRARP in the MDA-MB-468 Cell Line. *Note.* NRARP: Notch-regulated ankyrin-repeat protein; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; SD: Standard deviation. GAPDH was used to normalize the gene expression data, and expression values were denoted as the mean ± SD of the three independent experiments (***$P\leq0.001$ versus MCF-10A)

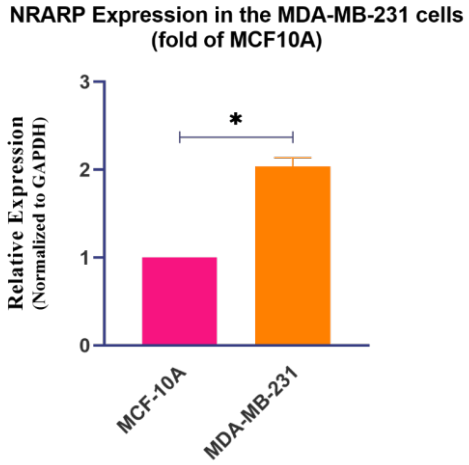

Figure 4. The mRNA Expression of NRARP in the MDA-MB-231 Cell Line. *Note*. NRARP: Notch-regulated ankyrin-repeat protein; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; SD: Standard deviation. GAPDH was applied to normalize the gene expression data, and expression values were represented as the mean ± SD of the three independent experiments (*$P \leq 0.05$ versus MCF-10A)

different molecular subtypes.[19] Hence, it is important to identify genes and molecular pathways that cause the heterogeneity of breast tumors.[20] This study attempted to further clarify the role of NRARP, a downstream target of the Notch signaling pathway, in different types of BC cell lines.

Previous studies demonstrated that although the Notch signaling pathway can play a dual role in cancer depending on the context,[21] Notch1 and Notch-4 receptors are overexpressed in the invasive subtypes of BC, and the Notch signaling pathway has the potential to be a promising treatment option in patients diagnosed with BC.[22] In addition, the expression of Notch receptors and ligands has been frequently related to breast tumor subtype, grade, and stage.[23] NRARP, a novel intracellular component of Notch signaling, inhibits the pathway through negative feedback by reducing the expression of the intracellular domain.[24] It also interacts with Wnt/β-catenin signaling, where it enhances the stability of the nuclear DNA-binding transcription factor LEF1, a downstream effector of the Wnt/β-catenin signaling pathway, to positively regulate the pathway.[25] Recently, NRARP has attracted interest due to its aberrant expression in a number of human malignancies; for example, the upregulation of NRARP has been associated with the development of papillary thyroid cancer,[17] whereas the downregulation of NRARP inhibits thyroid cancer cell proliferation and invasion.[26] The overexpression of the NRARP protein has also been associated with the development of the tumor and overall survival time in non-small lung cancer.

The results of the present study revealed that NRARP was overexpressed in different subtypes of BC. In line with these findings, Imaoka et al showed that NRARP is overexpressed in two human BC cell lines (MCF-7 and T-47D), both representing the luminal subtype, and the expression of cell cycle-related genes was significantly decreased as a result of NRARP inhibition.[18] Further, it has been reported that miR-130a-3p plays a tumor-suppressive role in BC, and its restoration in BC cell lines causes NRARP to be downregulated, resulting in a significant decrease in the proliferation, migration, and anchorage-independent growth of MCF-7 and SKBR3 BC cells.[27] No study has previously evaluated the expression of NRARP in the other subtypes of BC, and our results could be evidence of the involvement of NRARP in the aggressiveness and high metastasis rates of basal-like and HER2-overexpressing subtypes.

Conclusion

NRARP expression was significantly increased in all *in vitro* cell lines, representing BC molecular subtypes compared to normal breast epithelium. However, the increase of NRARP expression in luminal, HER2-overexpression, and basal A subtypes was more significant compared to normal cells. Therefore, NRARP could be a candidate gene for additional studies and a treatment strategy for BC patients.

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Authors' Contribution

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Supervision: Masoumeh Rajabibazl.
Validation: Masoumeh Rajabibazl, Jafar Poodineh.
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Writing – original draft: Parsa Nazarian, Masoumeh Rajabibazl, Jafar Poodineh.
Writing – review & editing: Parsa Nazarian, Masoumeh Rajabibazl, Jafar Poodineh.

Competing Interests

The authors hereby declare no conflict of interests.

Ethical Approval

This article contains no studies with human participants or animals performed by any of the authors.

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