

Importance of *Nectin2*, *NUF2*, and *Nectin4* Gene Expression in the Pathogenesis of Different Subtypes of Breast Cancer

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Abstract

Background: The targeted therapy using breast cancer (BC)-associated biomarkers has significantly minimized the side-effects of BC treatment. This study aims to elucidate the role of *Nectin2*, *NUF2*, and *Nectin4* gene expression in the pathogenesis of BC.

Method: In this case-control study, the expression of *Nectin2*, *Nectin4*, and *NUF2* genes was investigated through real-time polymerase chain reaction assay in 46 tumor tissues from BC patients and 46 adjacent non-tumorous tissues as a control group. Data were analyzed using SPSS-21 software, employing independent t-tests and one-way ANOVA. A *P*-value of <0.05 was considered statistically significant.

Results: The results demonstrated a significant increase in the expression of the *NUF2* gene in tumor tissues compared with adjacent normal tissues (*P* = 0.005, fold change = 3.7). No statistically significant difference was observed in the expression of *Nectin2* and *Nectin4* between the tumor and adjacent tissues. However, higher expression of *Nectin2* was noted in the early stages of the disease, particularly in subtypes with estrogen receptor-positive (ER+), progesterone receptor positive (PR+), and human epidermal growth factor receptor 2 negative (HER2-). Furthermore, the expression of *NUF2* and *Nectin4* was elevated in advanced stages and triple-negative BC subtypes. Notably, the expression of these three genes was higher in patients aged ≤ 45 years.

Conclusion: The findings suggest that the expression levels of *NUF2*, *Nectin2*, and *Nectin4* genes may influence the initiation, progression, and pathogenesis of BC subtypes.

Keywords: Triple negative breast neoplasms, Biomarker, Estrogen receptor, Progesterone receptor

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Introduction

Cancer is a growing global problem, and breast cancer (BC) is a heterogeneous disease with high incidence and mortality worldwide. BC is the most common neoplasia in women and causes the death of 450,000 people worldwide every year.¹ According to Globocan, the estimated incidence of BC was 2,088,849 new cases worldwide in 2018.² The incidence of this cancer is often at the age of 50 years and above (post-menopausal) in western women. However, the age of BC in Iran is about a decade lower than in women in developed countries, knowing that the probability of death is 39% higher in women under 40 years of age.³ Molecular subtyping of BC based on estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor (HER2) expression is known to have an essential role in therapeutic strategies. Most BC patients receive adjuvant or neoadjuvant chemotherapy, but in some cases, this leads to over-treatment. Therefore, unnecessary treatments and toxicity are avoided by identifying potential biomarkers, including proteins related to immune checkpoints and cell division-associated proteins.

Advances in microarray and high throughput sequencing have provided valuable tools to identify more reliable biomarkers for diagnosis, survival, and prognosis.⁴ Tumors can upregulate immune checkpoint ligands to inhibit the T cell function; hence, immune checkpoint inhibitors (ICISs) have revolutionized the field of immunoncology and cancer treatment.⁵ It has been shown that immune checkpoint inhibitors that target CTLA4, PD1, and PDL1 are beneficial for all types of solid tumors, especially in immunogenic cancers such as melanoma and lung cancer. This approach for most cancers with less immunogenic profile is a challenging issue. However, the activation of alternative immune checkpoints, which modulate the T cell response and participate in severe T cell exhaustion, could be caused by patients' resistance to ICISs.⁶

The Nectin family is a new immune checkpoint that includes Nectin1 to 4 or (PVRL1-4) classified as CD111, CD112, CD113, and PVRL4.⁷ Nectins are single-membrane type proteins with an external

region consisting of three immunoglobulin domains, which leads to the creation of homodimer or heterodimer complexes with hemophilic and heterophilic interactions.⁸

Nectin2 is a ligand abundantly expressed on the surface of APCs and tumor cells and binds to the co-stimulatory receptor CD226 (DNAM1) and the co-inhibitory receptor TIGIT. *Nectin2* was first identified as an adhesion factor involved in creating cell junctions, which has higher expression in several cancer cells, including acute myeloid leukemia (AML), multiple myeloma, and epithelial cell cancer.⁹ CD112R was detected as a new CD112 inhibitory receptor by Zhu et al. in 2016.⁹ CD112R is expressed in human T cells and inhibits human TCR-dependent signals, so knocking down the CD112-CD112R interaction enhances human T cell response.

The Nectin4 molecule is another member of the Nectin family; unlike other Nectins, which are often expressed in adult tissues, Nectin4 is highly expressed during embryonic development, while its expression decreases in puberty, more commonly detected in breast, bladder, lung, and pancreatic cancers.¹⁰ It has been reported that Nectin4 exclusively binds to TIGIT, inhibiting NK cell activity and compromising the innate immune response.¹⁰ Besides immune checkpoints, the proteins related to the cell cycle (e.g., NUF2) are another factor in cancer development.

NUF2 was first detected as a centromere protein called a cell division-associated protein. NUF2 is the main component of the kinetochore-associated complex (NDC80), participates in the stable spindle kinetochore-microtubule attachments, and plays a regulatory role in chromosome segregation; hence, the down-regulation of NUF2 induces a defect in kinetochore attachment and also causes the death of dividing cells.¹¹ This study evaluated the gene expression of *NUF2*, *Nectin2*, and *Nectin4* in breast tumor tissues and analyzed and compared the findings with the expression status of ER, PR, HER2, and Ki-67 molecules.

Materials and Methods

Sample gathering

In this case-control study, the research population consisted of 46 female patients with BC, averaging 48.9 ± 10.9 years of age, who visited hospitals affiliated with Shiraz University of Medical Sciences and underwent surgical procedures. Immediately after surgery and upon obtaining informed consent, 100 mg of fresh tissue was collected from both the tumor and adjacent sites. The samples were washed several times with PBS buffer and stored at -80°C .

The inclusion criteria for the study encompassed patients diagnosed with BC based on clinical and pathological findings who had not received any therapeutic interventions, such as chemotherapy or radiotherapy, prior to surgery. Exclusion criteria included patients who had undergone treatments like chemotherapy, radiotherapy, and hormone therapy before surgery, as well as those with autoimmune diseases or other malignancies in themselves or their first-degree relatives. The clinicopathological characteristics of the patients are summarized in table 1.

RNA extraction

Total RNA was extracted from the tumor and adjacent tissues using a commercial total RNA extraction kit (Parstous, Mashhad, Iran). The concentration of the extracted RNA was quantitatively assessed using a NanoDrop Spectrophotometer, followed by agarose gel electrophoresis to evaluate RNA quality.

cDNA synthesis

cDNA was synthesized from 1 μg of the extracted total RNA using a commercial kit and a thermocycler (Parstous, Mashhad, Iran). The synthesis mixture, containing random hexamer primers, was added to the RNA in an RNase-free microtube, incubated at 25°C for 10 minutes, then at 47°C for 60 minutes. Following the reaction, the microtube was incubated at 70°C for 5 minutes and immediately transferred to an ice container. The synthesized cDNA was stored at -20°C until used for real-time polymerase chain reaction (PCR).

Table 1. Clinicopathological characteristics of breast cancer patients

CPC	Factors	Number (%)
Age group (year)	≤ 45	18 (39.1)
	> 45	28 (60.9)
Tumor size (cm)	< 2	19 (41.3)
	2-5	26 (56.5)
	> 5	1 (2.2)
Histological grade	Low (I+II)	32 (69.6)
	High (III)	11 (23.9)
	ND**	3 (6.5)
Tumor type	IDC	35 (76.1)
	Others	11 (23.9)
TNM stage	Early	38 (82.6)
	Advanced	8 (17.4)
LN involved	0	23 (50)
	1-3	15 (32.6)
	4-9	5 (10.9)
	> 9	3 (6.5)
Skin involved	Involved	3 (6.5)
	Free	43 (93.5)
Lymphovascular invasion	Involved	21 (45.7)
	Free	24 (52.2)
	ND	1 (2.2)
Perineural invasion	Involved	11 (23.9)
	Free	34 (73.9)
	ND	1 (2.2)
Family history	Yes	4 (8.7)
	No	34 (73.9)
	ND	8 (17.4)
ER status	Positive	33 (71.7)
	Negative	4 (8.7)
	ND	9 (19.6)
PR status	Positive	29 (63)
	Negative	8 (17.4)
	ND	9 (19.6)
HER2 status	Positive	9 (19.6)
	Negative	27 (58.7)
	ND	10 (21.7)
Ki-67 status	Positive ($> 14\%$)	10 (21.7)
	Negative ($\leq 14\%$)	16 (34.8)
	ND	20 (43.5)

CPC: Clinical and pathological characteristics; ND: Not determined; LN: Lymph node; ER: Estrogen receptor; PR: Progesterone receptor; HER2: Human epidermal growth factor; IDC: Invasive ductal carcinoma; TNM: Tumor node metastases

Real-time PCR

Gene expression was quantified using forward and reverse primers for *NUF2*, *Nectin2*, and

Nectin4, combined with the prepared cDNAs, employing the SYBR[®] Green real-time PCR Master Mix kit (AMPLIQON, Low ROX, Denmark, Batch NO: 21J1301) and an Applied Biosystems QuantStudio 3 real-time PCR instrument (USA) in 96-well plates. The β -actin gene served as an internal control for data normalization.

The 10 μ l reaction mixture consisted of 5 μ l SYBR[®] Green Master Mix, 2.1 μ l distilled water, 0.2 μ l each of forward and reverse primers, and 2.5 μ l diluted cDNA. Amplification conditions were as follows: initial denaturation at 95°C for 15 minutes, followed by 40 cycles of 15-30 seconds at 95°C for denaturation, and 60 seconds at 60°C for extension. Primer sequences are listed in table 2.

The cycle threshold (CT) value for each gene was determined using ABI Quant Studio software, and the CT Norm algorithm adjusted the amplification efficiency to 100%. Gene expression fold changes were calculated using the $2^{(-\Delta CT)}$ method.

Ethical considerations

All participants provided informed consent for tissue sampling. The study was approved by the Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1400.430), Shiraz, Iran.

Statistical analysis

Chart creation were conducted using SPSS-

21 and GraphPad Prism 6 software. The mean expression of *NUF2*, *Nectin2*, and *Nectin4* genes between tumor and adjacent tissues was compared using an independent t-test. Differences in gene expression among various groups were assessed using one-way ANOVA. A *P*-value of <0.05 was considered statistically significant.

Results

Comparison of *Nectin2*, *Nectin4*, and *NUF2* genes expression in tumoral and adjacent normal tissues

The analysis revealed no statistically significant differences in the expression of the *Nectin2* and *Nectin4* genes between tumoral and adjacent normal tissues (*P* = 0.36 and 0.18, respectively). However, a significant difference was observed in the expression of the *NUF2* gene between the case and control groups (*P* = 0.005). The fold increase for the *Nectin2*, *NUF2*, and *Nectin4* genes in the case group was 1.6, 3.7, and 2.0 times higher than in the control group. These data are summarized in figure 1.

Association between *Nectin2*, *NUF2*, and *Nectin4* genes expression and demographic and clinicopathological characteristics

Gene expression was examined about demographic factors, family history, body mass index, and various pathological variables, including tumor size, histological grade, tumor

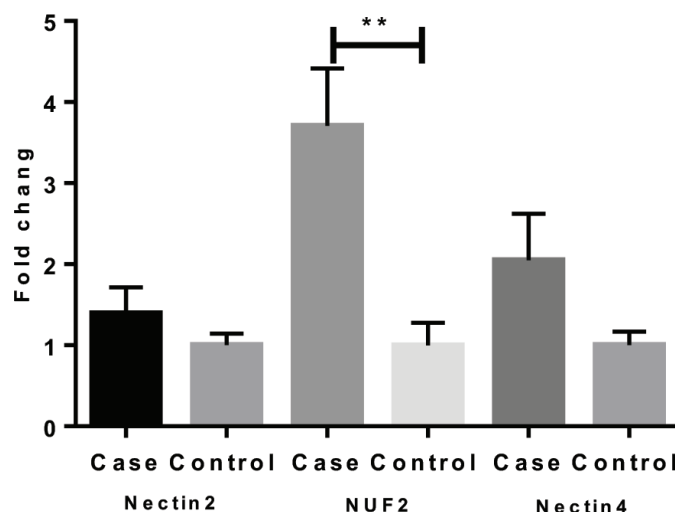


Figure 1. Comparison of *Nectin2*, *NUF2*, and *Nectin4* genes expression in tumoral and adjacent breast cancer tissues. *NUF2* expression was higher in tumoral tissue than in adjacent normal tissues.

***P* < 0.005

Table 2. Primer sequences of *Nectin2*, *Nectin4*, and *NUF2* genes

Gene	Sequences	TA
<i>Nectin2</i>	Forward 5'-TGGAGGACGAGGGCAACTAC-3' Reverse 5'-CTTGTTCTTGGGCTTGGCTAT-3'	590C
<i>NUF2</i>	Forward 5'-CGGGTGAATGACTTTGAGACTGCT-3' Reverse: 5'-TCCTGGTGTGCGGCGTTTA-3'	600C
<i>Nectin4</i>	Forward 5'-CGTGTCTTCCTTGCTGAG -3' Reverse: 5'-CGTAGATGCCGCTGTGCTC-3'	590C
β -Actin	Forward 5'-GCCTTTGCCGATCCGC-3' Reverse 5'-GCCGTAGCCGTTGTCG-3'	590C

TA: Annealing temperature

type, tumor node metastases stage, lymphovascular invasion, perineural invasion (PNI), lymph node (LN), and skin involvement. *Nectin2* expression was found to be associated with lymphovascular and PNI.

Nevertheless, no statistically significant association was observed between the expression of the *Nectin2*, *NUF2*, and *Nectin4* genes and the other variables. Furthermore, *Nectin2* expression was not significantly elevated in patients with early-stage, low-grade tumors (<2 cm), without skin involvement, PNI-, or those with a positive family history. Conversely, *NUF2* expression was higher in patients with advanced-stage, high-grade tumors (2-5 cm), LN involvement, no skin involvement, PNI-, and a positive family history. Moreover, *Nectin4* expression did not significantly differ in patients with advanced-stage, high-grade tumors, 2-5 cm in size, LN and no skin involvement, PNI-, and without a family history.

Association between Nectin2, NUF2, and Nectin4 genes expression and frequency of ER, PR, HER2, and Ki-67

The immunohistochemistry assay results extracted from patient files indicated a statistically significant association between *NUF2* expression and PR status. However, no significant associations were found between *Nectin2*, *NUF2*, and *Nectin4* expression and the ER, PR, HER2, and Ki-67 markers (Table 3). Interestingly, the expression of *NUF2* and *Nectin4* in patients with triple-negative breast cancer (TNBC) (ER-, PR-, and HER2-) was not significantly different from that in triple-positive breast cancer (TPBC) (ER+, PR+, and HER2+). Furthermore, *Nectin2*

expression in patients with ER+, PR+, and HER2- status did not significantly differ.

Comparison of Nectin2, NUF2, and Nectin4 expression in two age groups

Patients were divided into two age groups: ≤ 45 and >45 years old. There was no statistically significant difference in the expression of *NUF2*, *Nectin2*, and *Nectin4* genes between these two groups. However, *Nectin2*, *NUF2*, and *Nectin4* expression levels were insignificantly higher in patients aged ≤ 45 years.

Discussion

The results showed a significant increase in the expression of the *NUF2* gene in tumoral tissues compared with adjacent normal tissues. Although, no statistically significant difference was found between tumoral and adjacent tissues for the *Nectin2* and *Nectin4* genes. The results also showed a higher expression of *Nectin2* in the early stages of the disease and subtypes with ER+, PR+, and HER2-. Additionally, the expression of *NUF2* and *Nectin4* was higher in advanced stage and TNBC subtypes. Finally, these three genes' expression was higher in patients aged ≤ 45 years.

In silicoanalysis of human protein databases, many genes with minimum expression in normal tissues were revealed. Among them, there is a minimum expression for *NUF2* in normal tissues, and the score of *NUF2* genes was higher than 19% in BC. According to data from the human protein atlas database, GEPIA, *Nectin2*, and *Nectin4* were expressed in normal tissues, but there are differences in expression in tumor tissues.

Table 3. Association between *Nectin2*, *NUF2*, and *Nectin4* genes and the clinicopathological characteristics

Gene	Age group	Histological grade	Tumor size	Skin involvement	Lymphovascular invasion	Perineural invasion	LN invasion	TNM stage	BMI	Family history	ER	PR	HER2	Ki-67
Nectin2 (P- value)	0.55	0.44	0.79	0.29	0.04	0.01	0.47	0.84	0.23	0.32	0.29	0.3	0.12	0.3
NUF2 (P- value)	0.18	0.24	0.62	0.34	0.92	0.95	0.91	0.17	0.38	0.45	0.1	0.04	0.37	0.79
Nectin4 (P-value)	0.67	0.89	0.22	0.63	0.86	0.92	0.89	0.42	0.32	0.57	0.95	0.32	0.45	0.2

LN: Lymph node; BMI: Body mass index; ER: Estrogen receptor; PR: Progesterone receptor; HER2: Human epidermal growth factor

Thus, this study examined the expression of *Nectin2*, *NUF2*, and *Nectin4* genes between tumoral and adjacent normal tissue and their association with clinicopathological characteristics.

The data indicated a statistically significant difference between the tumor and adjacent tissues for the *NUF2* gene expression. In this regard, the expression value for the *NUF2* gene in the tumoral tissue was 3.7-fold higher than the adjacent normal tissue. Considering the role of *NUF2* in controlling the cell cycle, increasing its expression may contribute to the magnitude of tumor cell proliferation. Hence, it could be considered a biomarker for diagnosis, prognosis, and treatment of BC, a suggestion that essentially required more investigation in a larger cohort and assessing the protein expression. In addition, the study results showed that the expression of *NUF2* was higher in patients with advanced stage, high grade, tumor size 2-5cm, LN involvement, and positive family history. Therefore, based on this finding, it is assumed that overexpression of *NUF2* may play an essential role in tumor development and progression.

However, similarly, this finding needs to be reassessed by other investigations in a follow-up manner. Previous studies demonstrated that the expression levels of *NUF2* and *FAM83D* were significantly higher in TNBC tissue than in the adjacent tissues. According to the Kaplan-Meier survival curve, the expression levels of *NUF2* and *FAM83D* were associated with recurrence-free survival in TNBC samples ($P < 0.05$).¹³ The cell cycle analysis indicated that *NUF2* induced the G0/G1 cell cycle arrest by inhibiting the

expression of Cyclin B.¹⁴

In this study, the expression levels of particular immune checkpoints, including the *Nectin2* and *Nectin4* genes, were also evaluated.

In this regard, the results showed no statistically significant difference between tumoral and adjacent normal tissues. However, the expression value for the *Nectin2* gene in the case group was 1.6 fold compared with the control group. Previously, Hauke Stammet al.¹⁵ indicated that AML patients had higher *Nectin2* or TIGIT ligand expression. High expression of *Nectin2* was associated with a poor outcome in AML. Blocking *Nectin2* on AML cell lines or primary AML cells or blocking TIGIT on immune cells increased anti-leukemia effects. The difference in the type of cancer, type of assay, method used, and number of the studied populations may be the probable reasons for differences in the outcome. In the study, *Nectin2* expression was associated with lymphovascular and PNI. The prevalence of PNI was previously indicated as a risk factor for recurrence and poor survival in adenoid cystic carcinoma and high-grade mucoepidermoid carcinoma of salivary gland tumors.¹⁶ However, there was no statistically significant relationship between the expression of the *Nectin2* gene and other variables in the study. However, the findings showed a higher expression of *Nectin2* in BC patients in the early clinical stage, negative for LN involvement, and lower tumor size and grade. Interestingly, it may suggested that *Nectin2* is probably reduced during disease progression, and thereby, it can be an important therapeutic and diagnostic target in the onset of BC disease.

In concordance with other studies,^{17,18} the

results indicated the 2-fold higher expression of *Nectin4* in tumors compared with adjacent normal tissues. However, the difference was not statistically significant. Additionally, the expression of *Nectin4* was higher in advanced stage, high grade, tumor size 2-5cm, LN⁺, and PNI⁺ patients. Therefore, its increased expression may be indicated as a poor prognostic factor. Recently, it has been reported that the higher *Nectin4* expression in triple-negative and basal subtypes was associated with poor prognosis and showed a negative prognostic role for metastasis-free survival (MFS) in TNBC. The 5-year MFS rate was 61%, and high expression of *Nectin4* was associated with shorter MFS in the whole population.¹⁷ Jasmin Zeindler et al.¹⁸ studied the expression of *Nectin4* using immunohistochemical staining on 148 TNBC tissue samples. In 58% of TNBC cases, there was a high expression of *Nectin4*, and it was associated with better overall survival; hence, the expression of *Nectin4* can be considered a prognostic marker in TNBC. The significant increase of *Nectin4* in advanced-stage breast tumors makes this protein a potential target for immunotherapy.

The expression of *Nectin2*, *NUF2*, and *Nectin4* genes was insignificantly higher in the ≤45 age group compared with the >45 age group. Previous studies have shown that the age at onset of BC in Iran compared with women in developed countries is about one decade lower, and the probability of death is 39% higher in women under 40 years of age.³ The value of these markers as screening tools in younger cases of BC required further investigation. Finally, the study investigated the relationship between the expression of *Nectin2*, *NUF2*, and *Nectin4* genes and ER, PR, and HER2 expression, observing that both *NUF2* and *Nectin4* gene expression were higher in patients with TNBC than in those with TPBC.

TNBC patients are usually young people under 40 and have shorter disease-free survival and overall survival than non-TNBC patients due to the absence of specific molecular targets.¹⁹ Thus, *NUF2* and *Nectin4* might be important therapeutic targets in TNBC patients.

The most important limitation of the study

was the difficulty of performing the RNA extraction steps due to the presence of much fat in the BC tissue samples.

Conclusion

The findings suggest that *NUF2*, *Nectin2*, and *Nectin4* may play crucial roles in the initiation and progression of BC, particularly influencing the pathogenesis of the TNBC subtype and affecting patients aged ≤45 years. Further research with larger sample sizes is essential to precisely elucidate the critical roles of *NUF2*, *Nectin2*, and *Nectin4* in the molecular pathology of BC.

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Authors' Contribution

Abbas Ghaderi: Contributed to the conception and academics grants. Zahra Roshanzadeh, Mohammad Reza Haghshenas, and Amin Ramezani: Contributed to study design, lab experiments, statistical analysis, and the data interpretation. Sedigheh Tahmasebi and Majid Akrami: Contributed to data gathering and clinical confirmation of cancer. Abbas Ghaderi, Amin Ramezani and Mohammad Reza Haghshenas: Supervised the project. Zahra Roshanzadeh and Neda Shajari Drafted the proposal and the manuscript, and then it was revised by Mohammad Reza Haghshenas, Amin Ramezani and Abbas Ghaderi. All authors read and approved the final manuscript.

Conflict of Interest

None declared.

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