Original Article
Running Title: Gene Expression in the Pathogenesis of Breast Cancer
Received: November 04, 2023; Accepted: April 06, 2024

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

Zahra Roshanizadeh[*,**], PhD candidate, Mohammad Reza Haghshenas[**], PhD, Amin Ramezani[**], PhD, Neda Shajari[*,**], PhD candidate, Sedigheh Tahmasebi[***], MD, Majid Akrami[****], MD, Abbas Ghaderi[*,**♦], PhD

[*]*Department of Immunology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran*
[**]*Shiraz Institute for Cancer Research, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran*
[***]*Department of General Surgery, Lymphedema Research Center, Shiraz University of Medical Sciences, Shiraz, Iran*
[****]*Breast Diseases Research Cancer, Shiraz University of Medical Sciences, Shiraz, Iran*

♦Corresponding Author
Abbas Ghaderi, PhD
Shiraz Institute for Cancer Research,
School of Medicine, Shiraz University of Medical Sciences,
Shiraz, Iran
Email: Ghaderia@sums.ac.ir

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 (TNBC) 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

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 synthesize

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).

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 µl reaction mixture consisted of 5µl SYBR® Green Master Mix, 2.1µl distilled water, 0.2µl each of forward and reverse primers, and 2.5µl 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).

Statistical analysis

and 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, BMI, and various pathological variables, including tumor size, histological grade, tumor type, TNM 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 (IHC) 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.

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 1.6fold

compared to the control group. Previously, HaukeStammet 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 to 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 OS; 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 to the >45 age group. Previous studies have shown that the age at onset of BC in Iran compared to 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.

Acknowledgment

This project forms part of Zahra Roshanizadeh's PhD thesis and received financial support from Shiraz University of Medical Sciences, Shiraz, Iran (Grant No. 23215) and the Shiraz Institute for Cancer Research (ICR-100-503). We sincerely appreciate all participating patients, the Shiraz RAZ-Laboratory, and MRI-Hospital for collaborating in this study.

Conflict of Interest

None declared.

References

1. Vázquez GP, Pozo AG, Fuertes LT, Arevalillo J, Moros AZ, Gómez MF, et al. A novel approach to triplenegative breast cancer molecular classification reveals a luminal immune-positive subgroup with good prognoses. *Sci Rep.* 2019;9(1):1538. doi: 10.1038/s41598-018-38364-y.
2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2018;68(6):394-424. doi: 10.3322/caac.21492. Erratum in: *CA Cancer J Clin.* 2020;70(4):313.
3. Anders CK, Johnson R, Litton J, Phillips M, Bleyer A. Breast cancer before age 40 years. *Semin Oncol*. 2009;36(3): 237-49. doi: 10.1053/j.seminoncol.2009.03.001.
4. Guestini F, McNamara KM, Ishida T, Sasano H. Triple negative breast cancer chemosensitivity and chemoresistance: current advances in biomarkers indentification. *Expert Opin Ther Targets.* 2016;20(6):705-20. doi: 10.1517/14728222.2016.1125469.
5. Perrot I, Michaud HA, Paoli MG, Augier S, Docquier A, Gros L, et al. Blocking antibodies targeting the Cd39/Cd73 immunosuppressive pathway unleash immune responses in combination cancer therapies. *Cell Rep*. 2019; 27(8):2411-25.e2419. doi: 10.1016/j.celrep.2019.04.091.
6. Smazynski J, Hamilton PT, Thornton S, Milne K, Wouters MC, Webb JR, et al. The immune suppressive factors Cd155 and Pd-L1 show contrasting expression patterns and immune correlates in ovarian and other cancers. *Gynecol Oncol*. 2020;158(1):167-77. doi: 10.1016/j.ygyno.2020.04.689.
7. Johnston RJ, Lee PS, Strop P, Smyth M J. Cancer immunotherapy and the Nectin family. *Annu Rev Cancer Biol.*2021;5:203-19. doi:10.1146/annurev-cancerbio-060920-084910.
8. Liu X, An T, Li D, Fan Z, Xiang cP, Li C, et al. Structure of the heterophilic interaction between the Nectin-like 4 and Nectin-like 1 molecules. *Proc Natl Acad Sci USA*. 2019;116(6): 2068-77. doi: 10.1073/pnas.1810969116.
9. Zhu Y, Paniccia A, Schulick AC, Chen W, Koenig MR, Byers JT, et al

. Identification of Cd112r as a novel checkpoint for human T cells. *J Exp Med*. 2016;213(2):167-76. doi: 10.1084/jem.20150785.

10. Salda LD, Massimini M, Romanucci M, Palmieri C, Perillo A, Grieco V, et al. Nectin-4 and P63 immunohistochemical expression in canine prostate tumourigenesis. *Vet Comp Oncol*. 2019;17(3): 298-307. doi: 10.3390/cancers14112727.
11. Xing C, Wang Z, Zhu Y, Zhang C, Liu M, Hu X, et al. Integrate analysis of the promote function of Cell division cycle-associated protein family to pancreatic adenocarcinoma. *Int J Med Sci.* 2021;18(3):672-84. doi: 10.7150/ijms.53243.
12. Ramezani A. CtNorm: Real time PCR cycle of threshold (Ct) normalization algorithm. *J Microbiol Methods.* 2021;187:106267. doi: 10.1016/j.mimet.2021.106267.
13. Zhai X, Yang Z, Liu X, Dong Z, Zhou D. Identification of Nuf2 and Fam83d as potential biomarkers in triple-negative breast cancer. *PeerJ*. 2020;8:e9975. doi: 10.7717/peerj.9975.
14. Lv S, Xu W, Zhang Y, Zhang J, Dong X. NUF2 as an anticancer therapeutic target and prognostic factor in breast cancer. *Int J Oncol.* 2020;57(6):1358-67. doi: 10.3892/ijo.2020.5141.
15. Stamm H, Klingler F, Grossjohann EM, Muschhammer J, Vettorazzi E, Heuser M, et al. Immune checkpoints Pvr and Pvrl2 Are prognostic markers in Aml and their blockade represents a new therapeutic option. *Oncogene*. 2018;37:5269-80. doi: 10.1038/s41388-018-0288-y.
16. Lanzel E, Robinson RA, Zimmerman MB, Pourian A, Hellstein JW. The use of immunohistochemistry in detection of perineural invasion in mucoepidermoid carcinoma. *Oral Surg Oral Med Oral Pathol Oral Radiol*. 2016;121(6):636-42. doi: 10.1016/j.oooo.2016.01.026.
17. Rabet M, Cabaud O, Josselin E, Finetti P, Castellano R, Farina A, et al. Nectin-4: a new prognostic biomarker for efficient therapeutic targeting of primary and metastatic triple-negative breast cancer. *Ann Oncol*. 2017;28(4):769-76. doi: 10.1093/annonc/mdw678.
18. Zeindler J, Soysal SD, Piscuoglio S, Ng C, Mechera R, Isaak A, et al. Nectin-4 expression is an independent prognostic biomarker and associated with better survival in triple-negative breast cancer.*Front Med (Lausanne)*. 2019;6:200-12. doi: 10.3389/fmed.2019.00200.
19. Bernhardt SM, Dasari P, Walsh D, Townsend AR, Price TJ, Ingman WV. Hormonal modulation of breast cancer gene expression: implications for intrinsic subtyping in premenopausal women. *Front Oncol*. 2016;6:241-52. doi: 10.3389/fonc.2016.00241.

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 (≤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

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

Gene	Sequences	TA
Nectin2	Forward5'-TGGAGGACGAGGGCAACTAC-3' Reverse 5'-CTTGGTTCTTGGGCTTGGCTAT-3'	59^0C
NUF2	Forward5'-CGGGTGAATGACTTTGAGACTGCT-3' Reverse:5'-TCCTGGTGTGCGGCGTTTA-3'	60^0C
Nectin4	Forward5'-CGTGTCCTTCCTTGCTGAG -3' Reverse: 5'- CGTAGATGCCGCTGTGCTC-3'	59^0C
β-Actin	Forward5'-GCCTTTGCCGATCCGC-3' Reverse 5'-GCCGTAGCCGTTGTCG-3'	59^0C

TA: Annealing temperature

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 involvement	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

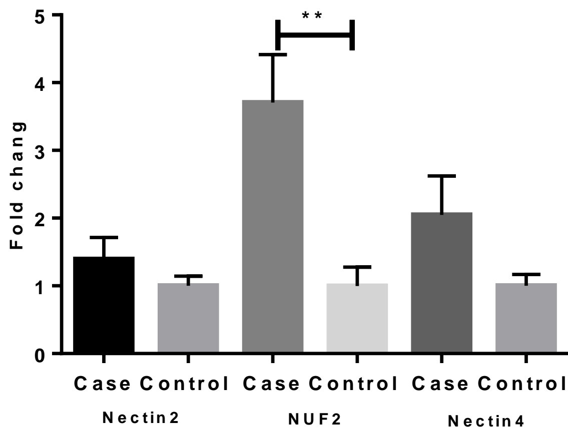

Figure 1. Comparison of *Nectin2*, *NUF2*, and Nectin4 genes expression in tumoral and adjacent breast cancer tissues. *NUF2* expression was higher in tumoral tissue than in adjacent normal tissues.
**: $P<0.005$