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Expression profile of the *WNT3A* gene in ductal breast carcinoma

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ABSTRACT

Breast cancer is one of the leading causes of cancer-related mortality among women worldwide. The Wnt/β-catenin signaling pathway, particularly the WNT3A ligand, plays a pivotal role in mammary gland homeostasis and tumorigenesis. However, its expression profile in malignant and benign breast lesions remains unclear. This study aimed to evaluate the expression level of the *WNT3A* gene in ductal breast carcinoma compared with benign fibroadenoma tissues and to assess its potential diagnostic and prognostic value. Breast tissue samples were obtained from 31 women with ductal carcinoma and 14 women with fibroadenoma. *WNT3A* expression was analyzed using quantitative real-time polymerase chain reaction, with β-actin as the endogenous control. Statistical analyses included the ΔCt method, the Mann-Whitney U test, Pearson correlation, logistic regression, and receiver operating characteristic (ROC) analysis. *WNT3A* expression was significantly lower in ductal carcinoma tissues than in fibro-adenoma samples (fold change = 0.32; p = 0.043). ROC analysis demonstrated moderate discriminatory power (AUC = 0.695; specificity = 0.87; sensitivity = 0.46). No significant correlations were observed between *WNT3A* expression and age, tumor size, lymph node involvement, or prognostic stage. Logistic regression revealed a non-significant trend toward increased cancer risk associated with decreased *WNT3A* expression (OR = 1.442; p = 0.153). Down-regulation of WNT3A may differentiate malignant from benign breast lesions and may reflect early dysregulation of the Wnt signaling pathway. Although not associated with clinical stage or TNM classification, WNT3A shows potential as a diagnostic biomarker and warrants further investigation.

INTRODUCTION

Breast cancer is the most frequently diagnosed malignant neoplasm and one of the leading causes of cancer-related mortality among women worldwide. Among the numerous molecular mechanisms involved in the development and progression of this malignancy, particular attention has been focused on the Wnt signaling pathway. This pathway plays a pivotal role not only in embryogenesis and the maintenance of mammary gland homeostasis but also in promoting cancer cell proliferation, survival, and migration, thereby contributing to breast cancer initiation and progression. Understanding the role of this pathway in breast cancer is crucial for identifying potential therapeutic targets and developing novel treatment strategies [1,2].

The WNT signaling pathway regulates a broad range of biological processes through precise control of cellular functions. This highly conserved signaling cascade orchestrates embryogenesis, regulates cellular proliferation, differentiation, and migration, and is essential for maintaining tissue homeostasis. Dysregulation of the Wnt pathway represents one of the key mechanisms underlying the development of various cancers, including liver, lung, and breast malignancies [3,4]. Activation of WNT signaling has been detected in more than half of breast cancer patients and is associated with reduced overall survival [5].

The canonical Wnt pathway, also known as the Wnt/β-catenin pathway, is the best-characterized branch of this signaling network. Activation begins when extracellular Wnt ligands bind to membrane-bound Frizzled (FZD) receptors and co-receptors LRP5/6. This interaction activates the cytoplasmic protein Disheveled (DVL), which subsequently inhibits the "destruction complex" composed of glycogen synthase kinase 3β (GSK-3β), casein kinase 1 (CK1), and the scaffold proteins APC, Axin1, and Axin2.

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Inhibition of this complex prevents β-catenin degradation, allowing it to accumulate in the cytoplasm and translocate to the nucleus. There, β-catenin interacts with transcription factors of the TCF/LEF family to initiate transcription of target genes involved in cell cycle regulation, cell survival, and invasiveness.

Canonical pathway activation is typically driven by Wnt1-class ligands, including Wnt2, Wnt3, Wnt3a, and Wnt8a. Wnt/β-catenin signaling is essential for physiological processes but also plays a significant role in disease pathogenesis. Increasing evidence indicates that its dysregulation contributes to the development of various disorders, including solid tumors (*e.g.*, breast and colorectal cancers), hematological malignancies, and sarcomas [5,6].

Wnt3a, a member of the Wnt ligand family, plays a key role in regulating pleiotropic cellular functions, including self-renewal, proliferation, differentiation, and motility. Its role in cancer development is complex and highly context-dependent. In some experimental models, Wnt3a exhibits tumor-suppressive activity by inhibiting proliferation or promoting apoptosis, as observed in melanoma, rhabdomyosarcoma, selected B-cell acute lymphoblastic leukemia cell lines, and lung and lacrimal gland tumors. In contrast, in many other malignancies, Wnt3a promotes tumor growth by stimulating the self-renewal and proliferation of cancer stem and progenitor cells, including in leukemia, breast, prostate, colorectal, and gastric cancers [5,7].

The aim of this study was to evaluate the expression level of the *WNT3A* gene in women with ductal breast carcinoma compared with women with benign neoplastic lesions, specifically fibroadenoma, using quantitative real-time PCR. Additionally, correlations between *WNT3A* gene expression, disease stage, and patient age were analyzed to assess its potential diagnostic and prognostic value.

MATERIALS AND METHODS

Study group

The study group consisted of 31 women diagnosed with ductal breast carcinoma (CD group) and 14 women with benign fibroadenoma (FA group). All participants were hospitalized at the St. John of Dukla Center of Oncology of the Lublin Region in Lublin, Poland. The study protocol was approved by the Bioethics Committee of the Medical University of Lublin (decision No. KE-254/141/2009). All participants provided written informed consent prior to enrollment.

Patients in the CD group were clinically evaluated using the TNM tumor classification system, which assesses primary tumor size (T), regional lymph node involvement (N), and the presence of distant metastases (M) [8,9] (Table 1). None of the CD patients showed clinical or radiographic evidence of distant metastasis (M0 category). Based on TNM classification, the prognostic stage of disease progression was determined, incorporating anatomical tumor extent as well as tumor grade and malignancy assessment (Table 1). This approach ensured standardized clinical evaluation and facilitated comparison between study groups.

Tissue collection

Breast tissue samples were collected during surgery or biopsy procedures from enrolled patients. Immediately after excision, samples were placed in tubes containing RNAlater Stabilization Solution (Thermo Fisher Scientific, USA) to preserve RNA integrity.

Table 1. TNM classification of patients with ductal breast carcinoma

TNM category	Number/(%) of CD patients
T:	
T1 (Tumor ≤ 20 mm in greatest dimension)	11 (35.50)
T2 (Tumor > 20 mm but ≤ 50 mm in greatest dimension)	11 (35.50)
T3 (Tumor > 50 mm in greatest dimension)	1 (3.23)
T4 (Tumor of any size with direct extension to the chest wall and/or skin, excluding isolated dermal invasion)	6 (19.35)
N:	
N0 (No regional lymph node metastasis)	12 (38.71)
N1 (Metastasis to movable ipsilateral level I-II axillary lymph nodes)	8 (25.81)
N2 (Metastases in fixed or matted ipsilateral level I-II axillary lymph nodes or internal mammary nodes without axillary involvement)	4 (12.90)
N3 (Metastases in infraclavicular, supraclavicular, or internal mammary lymph nodes with axillary involvement)	2 (6.45)
M:	
M0 (No clinical or radiographic evidence of distant metastasis)	31 (100.00)
Prognostic stage:	
I	2 (6.45)
II	9 (29.03)
III	20 (64.52)

RNA isolation

Total RNA was isolated from breast tissue samples using the RNeasy Protect Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Tissue samples were homogenized in RLT buffer supplemented with β-mercaptoethanol using an Ultra-Turrax T-25 homogenizer (IKA-Labortechnik, Germany). The resulting lysate was loaded onto RNeasy columns, washed, and RNA was eluted with RNase-free water.

RNA concentration was measured spectrophotometrically using a Picodrop spectrophotometer (UK). RNA purity was assessed by determining the absorbance ratio at 260 and 280 nm. Samples with an A260/A280 ratio between 1.9 and 2.1 were considered acceptable. RNA integrity was evaluated by electrophoresis on a 1.5% agarose gel stained with ethidium bromide, which enabled visualization of the 28S, 18S, and 5S rRNA bands (Figure 1).

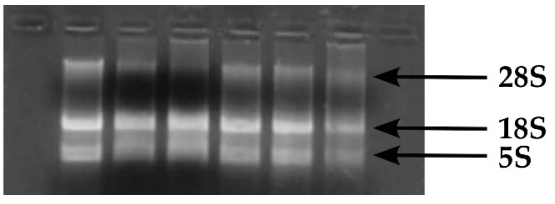


Figure 1. Representative electrophoretic profiles of total RNA showing visible 28S, 18S, and 5S rRNA bands

Reverse transcription

Reverse transcription was performed using 1 µg of total RNA and reagents from the QuantiTect Reverse Transcription Kit (Qiagen, Germany) according to the manufacturer's protocol. The reaction was carried out at 42°C for 15 minutes, followed by incubation at 95°C for 3 minutes to inactivate reverse transcriptase. The resulting complementary DNA (cDNA) was stored at -20°C until further analysis.

Real-time PCR

WNT3A gene expression was quantified using real-time polymerase chain reaction (qPCR) and the QuantiFast SYBR Green PCR Kit (Qiagen, Germany) following the manufacturer's instructions. The ACTB (β-actin) housekeeping gene was used as an endogenous control. Gene-specific QuantiTect Primer Assays (Qiagen, Germany) were applied for *WNT3A* gene (Hs_WNT3A_2_SG) and the *ACTB* gene (Hs_ACTB_2_SG). Each 25 µL reaction mixture contained 1.5 µL of cDNA, 12.5 µL of QuantiFast SYBR Green PCR Master Mix, gene-specific primers at a final concentration of 1 µM, and nuclease-free water.

Amplification was performed using a Rotor-Gene 6000 system (Qiagen, Germany). SYBR Green I fluorescence was monitored during amplification to quantify double-stranded DNA synthesis. The thermal cycling protocol consisted of an initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds and combined annealing and extension at 60°C for 30 seconds.

Melting curve analysis was performed after amplification to verify product specificity. The protocol included incubation at 70°C for 1 minute, followed by incremental heating to 94°C with fluorescence acquisition (ΔT = 0.3°C per cycle).

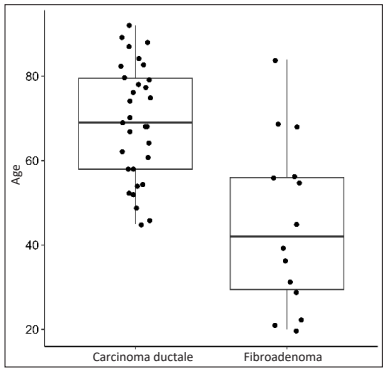
All reactions for the *WNT3A* and *ACTB* genes were performed in duplicate. The resulting data were used to quantify the initial amounts of *WNT3A* and *ACTB* mRNA, enabling normalization of *WNT3A* expression levels and assessment of the relative abundance of *WNT3A* mRNA in the CD group compared with the FA group.

Data analysis

The raw expression levels of the analyzed genes were represented as Ct values, which were calculated using the Rotor-Gene 6000 Series Software, version 1.7, coupled with the real-time PCR device used. Data analysis and visualization were performed using R 4.3.0 programming software. The mean Ct values from replicate samples were calculated, and the difference in *WNT3A* gene expression between the studied groups was analyzed using the delta Ct method [10,11]. The appropriate statistical test was chosen based on an assessment of data normality performed using the Shapiro-Wilk test (shapiro.test, a core function in R). If normality was confirmed ($p > 0.05$), a parametric two-sided Student's t-test was performed (t.test, core function in R). Otherwise ($p < 0.05$), the nonparametric two-sided Mann-Whitney test was used (wilcox.test finction in the coin 1.4-3 package). Correlation analysis was performed using the Pearson algorithm with the cor.test function in R. Logistic and linear regression models were constructed using the glm and lm functions in R, respectively. The odds ratio and its associated p-value were calculated using the or_glm function in the oddsratio 2.0.1 package in R. For the receiver operating characteristic (ROC) analysis and plotting of the ROC curve, the pROC package (version 1.18.5) in R was used. Unless stated otherwise, data visualizations were generated using the ggplot 2 3.5.0 package in R. Results with $p < 0.05$ were considered statistically significant.

RESULTS

The study population consisted of 45 female participants, including 31 women with ductal breast carcinoma (CD group) and 14 women with fibroadenoma (FA group). The mean age in the CD group was 69.06 ± 13.66 years, whereas in the FA group it was significantly lower at 45.07 ± 20.13 years (Figure 2). This difference was statistically significant, as demonstrated by a two-sided Student's t-test for independent samples ($p = 6.95 \times 10^{-4}$). Most CD patients were classified as stage III and presented with no or limited regional lymph node involvement and tumor sizes below 50 mm.



The whiskers reach the most distant point in the 1.5 interquartile range. The boxes range between the 25th and 75th percentiles. Horizontal lines inside the boxes indicate the median values

Figure 2. Distribution of age in women with ductal breast carcinoma and fibroadenoma

The raw expression levels of the *WNT3A* gene of interest and the *ACTB* endogenous control gene were represented as mean Ct values calculated from duplicate samples. To ensure the reliability of the expression data, the Ct value threshold was set to 35. One sample had a Ct value above 35 (specifically, 38.12), possibly due to low template concentration or amplification efficiency. Therefore, this sample was excluded from the analysis.

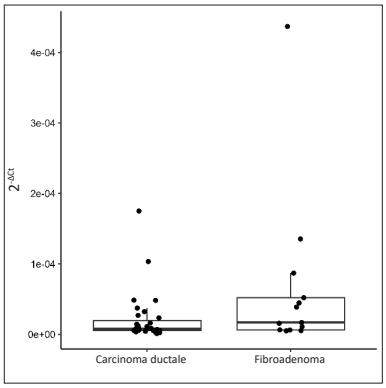
Data from the remaining 44 samples were subjected to normalization, in which the mean *WNT3A* Ct value was subtracted from the mean *ACTB* Ct value for each sample. This resulted in the calculation of delta Ct (ΔCt) values. To present the results in a linear, directly proportional form, the ΔCt values were transformed using the $2^{-\Delta Ct}$ formula. This transformed data is referenced as "expression data" throughout the text.

The mean expression of the *WNT3A* gene in the CD group was 3.125 times lower (fold change = 0.32) than in the FA group. The statistical significance of this difference was assessed using a nonparametric, two-sided Mann-Whitney test because the Shapiro-Wilk test showed that the distributions of *WNT3A* gene expression in the compared

groups did not follow a normal distribution ($p < 0.05$). The Mann-Whitney test showed a statistically significant difference ($p = 0.043$) of the observed difference in *WNT3A* expression between the CD and FA groups (Table 2). Figure 3 illustrates the distributions of *WNT3A* gene expression in the compared groups.

Table 2. Comparison of *WNT3A* gene expression between ductal breast carcinoma and fibroadenoma groups

Group	Mean	SD	Median	Variance	Fold change	*p* value
Ductal carcinoma	2.10E−05	3.53E−05	7.90E−06	1.25E−09	0.32	0.043
Fibroadenoma	6.62E−05	1.18E−04	1.68E−05	1.39E−08		



The whiskers reach the most distant point in the 1.5 interquartile range. The boxes range between the 25th and 75th percentiles. Horizontal lines inside the boxes indicate the median values

Figure 3. Distribution of *WNT3A* gene expression ($2^{-\Delta Ct}$ values) in ductal breast carcinoma and fibroadenoma groups

To analyze the differential expression of the *WNT3A* gene across the study groups further, we employed the ROC method and logistic regression. In the ROC analysis calculated the optimal threshold for grouping, along with the associated diagnostic parameters (specificity, sensitivity, accuracy, and the area under the ROC curve [ROC-AUC]). We set the cutoff value at 3.80×10^{-5}, which yielded a diagnostic accuracy of 0.75. Thus, 75% of cases were correctly classified. These results suggest that the observed difference in gene expression is moderately effective in discriminating between the compared groups. The ROC-AUC value of 0.695 supports this conclusion (Figure 4). Diagnostic specificity was quite high at 0.87, though sensitivity was lower at 0.46. Overall, better classification performance was observed in patients with CD than in patients with FA.

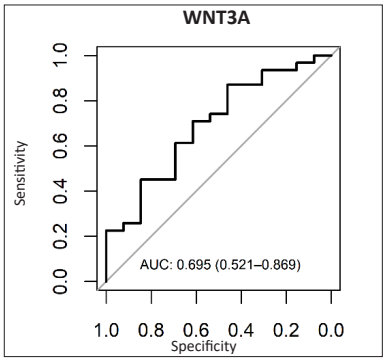


Figure 4. ROC curve generated for *WNT3A*gene expression to differentiate carcinoma ductale *vs*. fibroadenoma cases

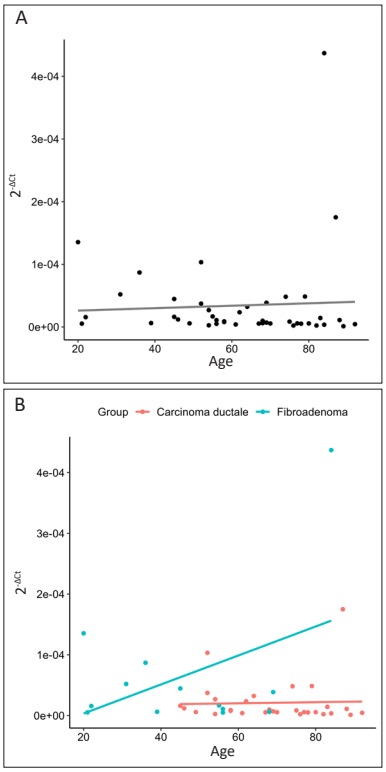
Logistic regression analysis showed that a decrease in *WNT3A*gene expression equivalent to the average expression level observed in the study population was associated with a 1.442-fold increase in the odds of developing ductal carcinoma in patients with fibroadenoma (OR = 1.442, 97.5% CI: 0.992-2.687). However, the corresponding p-value of 0.153 indicates that this association was not statistically significant at the $\alpha = 0.05$ level.

The relationship between *WNT3A* gene expression levels and age was examined across all samples and within individual groups using a two-sided Pearson correlation test and simple linear regression. No statistically significant associations were observed (Table 3, Figure 5), suggesting that *WNT3A* gene expression is likely independent of age.

Table 3. Pearson correlation and simple linear regression analyses of WNT3A expression and age

Group	R	*p* value	Regression coefficient	R^2
Ductal carcinoma	−0.0346	0.8533	8.95×10^{-8}	0.0012
Fibroadenoma	0.4129	0.1608	2.39×10^{-6}	0.1705
All cases	−0.0510	0.7421	1.95×10^{-7}	0.0026

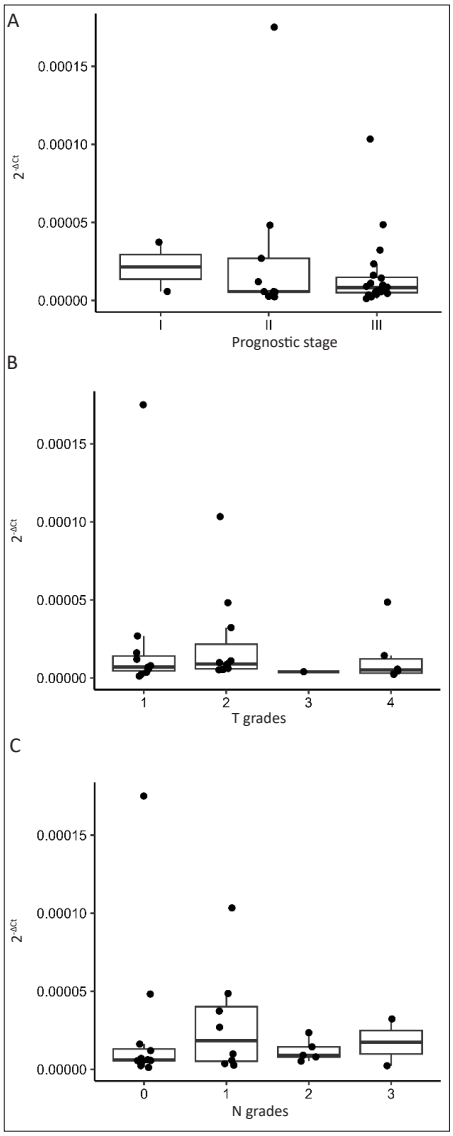
R - corelation coeficient, R^2 - determination coeficient



(A) All samples; (B) individual study groups

Figure 5. Relationship between WNT3A expression ($2^{-\Delta Ct}$ values) and age

The expression of the *WNT3A* gene was compared across prognostic stages and T and N stages of the TNM breast cancer classification system (Figure 6). The M category was excluded from the analyses because all CD patients were classified as M0. Subgroups containing at least three cases were used for analysis. For the prognostic stage classification, cases with stages II and III were compared using a nonparametric, two-sided Mann-Whitney test. No statistical difference was obtained ($p = 0.908$). For comparisons involving T and N classifications, the nonparametric



The whiskers reach the most distant point in the 1.5 interquartile range. The boxes range between the 25th and 75th percentiles. Horizontal lines inside the boxes indicate the median values

Figure 6. Distribution of *WNT3A* gene expression across prognostic stages and T and N categories of the TNM classification in ductal breast carcinoma cases

two-sided Kruskal-Wallis test was used because these analyses included more than two subgroups. No statistical differences were found between the first, second, and fourth grades of the T classification ($p = 0.437$) or between the first, second, and third grades of the N classification ($p = 0.758$).

DISCUSSION

The Wnt/β-catenin signaling pathway plays a pivotal role in regulating cellular physiology. However, constitutive activation or inactivation of this pathway can disrupt cellular homeostasis and contribute to the development of various diseases, including malignancies. There is increasing evidence highlighting the clinical significance of this pathway in cancer diagnosis, treatment, and prognosis assessment. Aberrant activation of Wnt/β-catenin pathway components promotes oncogenic transformation, supports tumor progression, and is associated with the development of many cancer types. Consequently, molecules that modulate this pathway are considered promising therapeutic targets. In breast cancer, for example, Wnt signaling regulates the tumor immune microenvironment and maintains cancer stem-like cell properties. These functions are associated with treatment resistance and the emergence of aggressive clinical phenotypes [13].

In our study, we used real-time PCR to assess *WNT3A* gene expression levels in women diagnosed with ductal breast cancer and in women with benign breast lesions (fibroadenoma). To our knowledge, this is the first study to evaluate *WNT3A* expression in breast cancer patients compared to those with fibroadenomas. The mean *WNT3A* expression level in patients with ductal breast cancer was 3.125-fold lower than in women with fibroadenoma, which was a statistically significant difference ($p < 0.043$). Further analysis comparing *WNT3A* expression across different prognostic stages and TNM classifications revealed no significant differences based on stage, tumor size (T), or lymph node involvement (N).

Other studies have demonstrated elevated WNT3A expression in breast cancer cell lines, such as MCF-7, T47D, BT474, BT20, MDA-MB-453, and MDA-MB-468, while no expression was detected in normal mammary epithelial cells (HMEC) [14]. Katoh reported WNT3A expression in MCF-7 cells, and Kaiser *et al*. confirmed the presence of this ligand in several cell lines cultured in FBS-enriched conditions, including MDA-MB468, HCC-1143, MDA-MB231, MCF-7, and T-47D. However, Huguet et al. did not detect WNT3A expression in any of the breast cancer cell lines or normal or benign mammary tissues examined (MTSV1-7, T47D, MDA231, MDA361, BT20, MDA415, MDA453, MDA157, ZR9BI1, ZR75, S-75185) [17].

The role of WNT3A in oncogenesis appears to be highly context-dependent across various cancer types. Preclinical studies have demonstrated both tumor-promoting and anti-metastatic effects. WNT3A has been reported to reduce metastasis in a murine melanoma model and inhibit multiple myeloma cell proliferation [18,19]. Conversely, WNT3A may promote mammosphere formation in estrogen receptor (ER)-positive breast cancer cell lines [20]. Elevated WNT3A expression has been noted in oral squamous cell carcinoma compared to non-malignant control tissues, in human gastric (44As3) and prostate cancer cells, as well as in advanced gliomas and glioma stem cells. In colorectal cancer tissues, Wnt3a overexpression has been associated with tumor histologic grade, clinical stage, presence of metastasis and recurrence, and patient survival outcomes [7].

These findings suggest that *WNT3A*'s role in tumorigenesis, including in breast cancer, depends heavily on the biological context and degree of pathological differentiation. The relationship between *WNT3A* expression and cancer phenotype may be further influenced by tumor molecular subtype, microenvironmental factors, and hormonal influences. It is important to consider the inherent molecular and histopathological heterogeneity of breast cancer, as this may account for discrepancies in *WNT3A* expression reported across studies.

In our CD patients, no correlation was observed between *WNT3A* expression and TNM classification or tumor stage. These results may indicate that alterations in *WNT3A*

expression occur during the early phases of neoplastic transformation and are not necessarily indicative of clinical aggressiveness. The down-regulation of *WNT3A* observed in breast cancer could reflect dysregulation of the Wnt signaling pathway, which is often disrupted during tumor progression.

In conclusion, *WNT3A* may serve as a biomarker to distinguish between benign and malignant breast lesions. Its reduced expression in malignant tumors indicates its potential diagnostic and prognostic value. Profiling *WNT3A* expression could help with biological staging and assessing progression risk.

CONCLUSIONS

This study offers new insights into how the *WNT3A* gene is expressed differently in ductal breast carcinoma and benign breast lesions (fibroadenomas) in women. However, several limitations must be considered when interpreting the findings. First, the small sample size, particularly in the fibroadenoma group, may reduce the statistical power of the analysis and the generalizability of the results to broader patient populations. Second, the investigation was restricted to mRNA expression levels without a concurrent assessment of Wnt3a protein levels. This limits comprehensive evaluation of the gene's translational activity and biological relevance.

Future research should incorporate functional analyses, such as *in vitro* and *in vivo* models with targeted modulation of *WNT3A* expression. These studies would provide a more detailed understanding of the role of *WNT3A* in tumor cell behavior, including proliferation, invasion, apoptosis, and treatment response.

Furthermore, investigating epigenetic regulation and post-transcriptional mechanisms could help clarify the pathways responsible for *WNT3A* down-regulation in ductal breast carcinoma. The overarching goal should ultimately be to determine whether *WNT3A* can serve as a differential biomarker, as well as a predictive or therapeutic target, within personalized breast cancer treatment strategies.

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