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OCT4 IMMUNOSTAINING IS ASSOCIATED WITH BREAST CANCER GRADE AND TUMOR SIZE

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Breast cancer remains one of the most prevalent tumors among females worldwide, and current prognostic methods are limited in their ability to accurately predict tumor aggressiveness. Octamer-binding transcription factor 4 (Oct4), a key regulator of pluripotency and cancer stem cell characteristics, has emerged as a promising biomarker in various cancers. This study aimed to evaluate the results of Oct4 immunostaining in benign and malignant breast tissues and its link with cancer grade and tumor size. The archival formalin-fixed breast tissue specimens, comprising 110 breast carcinoma and 20 benign tumors were examined. Oct4 expression was assessed immunohistochemically using a rabbit monoclonal anti-Oct4 antibody. The study demonstrated a significant increase in nuclear Oct4 staining in malignant breast cancer tissues compared to benign tumors. Elevated Oct4 immunostaining was positively associated with high grade and larger tumor size. However, no significant correlation was observed between Oct4 level and lymph node status. Thus, elevated Oct4 expression is associated with higher tumor grade and larger tumor size, suggesting its potential relevance in breast cancer progression.

K e y w o r d s: breast cancer, Oct4, immunohistochemistry, tumor grades and size.

Breast cancer (BC) remains a significant health issue, being the most common non-epidermal cancer and the second leading cause of cancer-related mortality among women worldwide [1]. In 2022, BC accounted for approximately 2.3 million new cases and 666,000 deaths globally, representing about 23.8% of all cancer diagnoses and 15.4% of cancer-related deaths among females [2]. Projections indicate a slight increase in disability-adjusted life-years (DALYs) from 238.6 per 100,000 in 2022 to 239.5 per 100,000 by 2050 [3]. According to the Iraqi Cancer Registry (2022) and the recently published Iraqi paper in 2025, BC is the most frequently diagnosed cancer and comprised 8,299 new cases that represented 21.2% of the total cancer cases [4, 5].

The most common systems that are used to evaluate BC progression are the Nottingham grading and the Tumor-Node-Metastasis (TNM) systems [6]. The Nottingham system evaluates how abnormal BC cells appear based on tubule formation, nuclear pleomorphism, and mitotic activity. This system ranges from grade I (well-differentiated) to grade III (poorly differentiated) [7]. The TNM system can describe the anatomical spread of the tumor by evaluating tumor size (T), lymph node involvement (N), and metastasis [8]. The TNM system is used before treatment and post-surgery to guide therapy choices and predict patient outcomes. In addition, it provides a detailed and structured assessment of cancer progression [8, 9]. However, these systems cannot be used to identify which tumors will follow an aggressive clinical course from those that will remain relatively indolent, and they cannot predict the post-treatment outcomes like relapse or long-term remission [10, 11].

The estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) have become very important prognostic biomarkers for BC, necessary for diagnosis and treatment. They are useful in classifying tumors and guiding targeted therapies, such as hormone therapy for ER/PR-positive tumors and HER2-targeted treatments [12]. However, these biomarkers have limitations. They cannot predict the patient's response to treatment or relapse. Some ER-positive tumors develop resistance to hormonal therapy, and HER2-positive cancers can relapse despite treat-

ment. Triple-negative BC has fewer treatment options and a poorer prognosis. Therefore, there is a need to find new biomarkers to better predict tumor behavior, treatment response, and patient outcomes [12].

Octamer-binding transcription factor 4 (Oct4), which belongs to the POU family of transcription factors, is crucial for maintaining pluripotency and self-renewal in embryonic stem cells [13-15]. Due to its vital role in these stem cell functions, Oct4 has gained attention as a key biomarker of cancer stem cells [16]. Furthermore, a previous study found that increased Oct4 expression has been linked to aggressive tumor behavior, suggesting its potential as a biomarker for predicting poor prognosis in various cancers [13]. In Iraq, the number of studies that have reported on Oct4's role in BC is quite limited, and no previous Oct4 study in BC tissues using IHC has been published in my city. For this reason, the objective of this research is to assess nuclear Oct4 immunostaining in benign and malignant breast tissues and to warrant further investigation into the relationship between Oct4 immunostaining and histopathological features such as BC grade and stage, using immunohistochemistry (IHC).

Materials and Methods

Patients and control samples. This retrospective study received approval from the Ethics Committee of the College of Medicine, University of Thi-Qar, and Al Hussein Teaching Hospital, Thi-Qar, Iraq (Approval No. 2021159, dated 7/12/2022). A total of 130 archival formalin-fixed, paraffin-embedded breast tissue specimens were examined, comprising 110 BC tissues and 20 benign breast tissues, such as fibroadenoma, utilized as controls, surgically excised between April 2022 and December 2023. Demographic and clinical information, including age, grade, stage, and treatment history, was taken from histopathological archives. Patients who underwent chemotherapy or hormonal treatment were excluded. Negative controls, in which no primary antibody was employed, were implemented to validate the IHC staining specificity. BC clinical data are included in Table 1.

Immunohistochemistry. IHC was performed on breast tissue samples using a rabbit monoclonal anti-Oct4 antibody (1:200 dilution; Santa Cruz Biotechnology, sc-5279). This study used the Novolink polymer detection system (RE7140K, Leica Biosystems, UK). The procedure was carried out following the method established by Alalwany et al. [17].

Four-micrometer sections from each formalin-fixed, paraffin-embedded block were cut and mounted on positively charged slides, followed by incubation at 37°C overnight. These sections undergo several pretreatment steps, including deparaffinization, rehydration, antigen retrieval, and blocking. Deparaffinization was performed using two washes with Histoclear (H5-200, National Diagnostics, UK), followed by a graded ethanol rehydration series (100%, 95%, 70%) using ethanol (20821-330, VWR, UK). Heat-induced epitope retrieval was carried out using PT link (PT200, Agilent Technologies, Denmark) at 90°C for 30 min in citrate buffer (pH 6) (RE7113, Novocastra, UK), followed by a cooling step. Endogenous peroxidase activity was subsequently inhibited with a 3% hydrogen peroxide solution in methanol for ten minutes. Proteinase K drops were then added to these sections for 5 min.

The tissue section was incubated overnight at 4°C with anti-Oct4 antibody. The following day, the slides were then washed three times in phosphate-buffered saline (PBS) for 10 min each. Subsequently, the secondary antibody was then added to these sections for thirty minutes at room temperature. After washing with PBS, drops of diaminobenzidine (DAB) solution were then added to these sections for 5 min at room temperature. Finally, the sections were counterstained with Mayer's hematoxylin (H-3401, Vector, UK) and mounted using Histofluid mounting medium (6900002, Marienfeld, Germany).

IHC quantification. The nuclear expression of Oct4 was semi-quantitatively assessed in breast tissue samples, evaluating the percentage and intensity of stained cells. Five random images were used to score the Oct4 nuclear stain in breast tissues. The percentage positive cells score was defined: 0 (no staining), 1 (1–25%), 2 (26–50%), 3 (51–75%), and 4 (76–100%). Additionally, three grades of intensity were observed on a scale from 0 to 3: 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). Summation of the percentage and intensity of the positive cells represents the final score, ranging from 0 to 7 [18].

Statistical analysis. GraphPad Prism version 8.4.2 (GraphPad Software, La Jolla, California, USA; www.graphpad.com) was used to analyze the data in this study. Data distribution normality was evaluated using the Shapiro–Wilk test. Furthermore, variables with normal distributions ($P > 0.05$) were compared for group means with the unpaired t-tests. Non-normally distributed data ($P \leq 0.05$) were analyzed with the Mann–Whitney U test. Categorical

variables were compared using the Chi-square (χ^2) test. A P value < 0.05 was regarded as statistically significant.

Results and Discussion

BC has a considerable variety, manifested in various biological characteristics, epidemiological profiles, natural histories, treatment responses, and prognostic outcomes [19]. The BC treatment approaches were mostly founded on histopathological grading and TNM staging [7, 20]. This disease has five distinct molecular subtypes, each exhibiting unique biological characteristics, natural histories, responses to personalized treatment, and prognostic implications.

Cancer stem cells (CSCs) have been a crucial focus in oncology due to their unique biological properties [21]. These cells have the capacity for self-renewal, which not only contributes to resistance against conventional therapies but also facilitates metastasis. The identification and targeted eradication of CSCs are seen as essential techniques for improving long-term treatment outcomes in cancer patients [22, 23].

One of these stem cell proteins is Oct4, which plays an important role in maintaining pluripotency and self-renewal in embryonic stem cells [1, 24]. Due to its central involvement in these stem cell functions, Oct4 has increasingly been considered a potential biomarker for identifying CSCs. Furthermore, overexpression of Oct4 has been associated with tumor aggressiveness and may be used as a prognostic biomarker for poor clinical outcomes across various malignancies [25].

This investigation aims to evaluate Oct4 expression levels in malignant and non-malignant breast tissues and to determine whether it is associated with BC clinical data, such as grades and stages. In addition, it aims to detect whether Oct4 can serve as a potential biomarker for BC prognosis and therapeutic management.

Clinical data of the study population. This study included 110 (84.62%) cases of BC and 20 cases of benign breast tissues (15.38%), and the incidence rate was statistically significant (P < 0.001). The age range of BC patients was between 22 and 73 years, whereas the range of benign tissue individuals was between 19-95 years. The age groups were categorized into three groups: <40 years (n = 12; 60%), 40–60 years (n = 5; 25%), and >60 years (n = 3; 15%).

The result found a significant difference among the age groups (P = 0.009). The BC samples were also divided into the same age categories: <40 years (n = 24; 21.82%), 40–60 years (n = 76; 69.09%), and >60 years (n = 10; 9.09%). A significant difference was observed among these age groups (P = 0.009).

Fifty-five BC cases (50%) were classified as grade 2, 47 cases (42.73%) were classified as grade 3, and only 8 cases had no available clinical data. Statistical analysis revealed a significant difference among these grade groups (P < 0.001). The majority of BC cases were classified as T2 (n = 35; 31.82%) and T3 (n = 30; 27.27%), followed by T4 (n = 12; 12.73%), with only a few cases classified as T1 (n = 3; 2.73%). The results displayed a statistically significant difference when comparing patients with T1-2 to those with T3-4 (P < 0.001). In addition, according to lymph node status, the malignant tissues were classified as N0 (n = 12; 10.91%) and N1-3 (n = 65; 59.09%). The results had a significant difference between these groups (P < 0.001) (Table 1).

The breast tissue samples' demographic and pathological characteristics showed a significant correlation between age, grade, tumor size, and lymph node status. The observed age distribution, with a significant difference across age groups in both malignant and non-malignant samples, underscores the age-associated prevalence of breast abnormalities. This is further corroborated by the prevalence of malignant cases among those aged 40-60 years, suggesting this demographic as a critical window for increased vigilance and screening. The predominant classification of malignant cases as Nottingham grades 2 and 3 further emphasizes the aggressive nature of the tumors typically encountered in this cohort, aligning with the established prognostic significance of the Nottingham grading system in breast cancer[7, 26]. This system, which evaluates tubule formation, nuclear pleomorphism, and mitotic activity, is crucial for stratifying patients into favorable and less-favorable-outcome groups and directly influencing treatment strategies [27]. Furthermore, the significant differences observed across tumor sizes (T1-T4) and lymph node statuses (N0 vs. N1-3) underscore their established roles as critical prognostic indicators, reflecting the extent of local and regional disease progression, respectively.

Oct4 staining in breast tissues using IHC. Oct4 immunostaining was assessed using IHC on benign and malignant breast tissue samples. This study found that the malignant and non-malignant breast

Table 1. The benign and malignant breast information according to histopathological data

Breast histopathological data		Number	Percentage %	*P* value
Sample size	Benign	20	15.38	<0.001
	Malignant	110	84.62	
Benign (age range)	<40	12	60	0.009
	40-65	5	25	
	>65	3	15	
Cancer (age range)	<40	24	21.82	<0.001
	40-65	76	69.09	
	>65	10	9.09	
Grade	Grade 2	55	50	<0.001
	Grade 3	47	42.73	
	N/A	8	7.27	
Stage T	T1	3	2.73	<0.001
	T2	35	31.82	
	T3	30	27.27	
	T4	14	12.73	
	N/A	28	25.45	
Stage N	N0	12	10.91	<0.001
	N1-3	65	59.09	
	N/A	33	30	
Stage M	M0	0	0	N/A
	M1	0	0	
	MX	100	100	

tissues had nuclear Oct4 staining, with staining intensities ranging from strong to weak.

The nuclei of benign breast tissues had different levels of Oct4 staining, with some areas displaying no detectable signal (Fig. 1, *A*, red arrow), while others demonstrated a more noticeable, moderate level of staining (Fig. 1, *B*, red arrow). BC had nuclear Oct4 staining with notable variation in signal strength, including strong (Fig. 1, *C*, red arrow), moderate (Fig. 1, *D*, red arrow), weak (Fig. 1, *E*, red arrow), and negative expression. The negative control did not show Oct4 staining (Fig. 1, *F*, arrow).

Quantification of Oct4 staining in breast tissue. Oct4 has a role in maintaining pluripotency and self-renewal in embryonic stem cells [14]. It has gained attention as a crucial biomarker of CSCs because of its essential participation in these stem cell capabilities [15]. There was a significant increase in nuclear Oct4 staining in BC compared to benign breast tissues ($P < 0.001$; Table 2 and Fig. 2, *A*). The result is consistent with the previous reports [1, 28-33]. Increased nuclear Oct4 staining was also linked to aggressive tumor behavior, suggesting its potential as a biomarker for predicting poor prognosis in various cancers [14]. The examination of Oct4 expression in BC provides critical insights into its involvement in tumor advancement and potential prognostic significance.

In addition, this study reported a positive association between Oct4 immunostaining and BC grade, indicating that higher Oct4 levels were more commonly observed in grade III ($P = 0.002$; Table 2 and Fig. 2, *B*). This data was consistent with previous reports [25, 28, 29, 31, 34, 35]. In addition, another study found that Oct4 can stimulate cell proliferation and differentiation [33]. However, our data were inconsistent with the previous reports that found no association between Oct 4 staining and grades or tumor size [1, 15, 33]. The sensitivity and specificity of Oct4 detection can be affected by a variety of factors, including patient ethnicity, sample quantity, antigen retrieval procedures, and the use of various antibodies. Previous research showed that Oct4 is found in tumor cells and that it is concentrated among a subpopulation of undifferentiated tumor-initiating cells that are crucial for the development of resistance to cancer therapies, the genesis of tumors, and their spread [14]. Taken together, these findings suggest that Oct4 may play a significant role in tumor formation and cell proliferation in BC.

Additionally, there was a positive association between nuclear Oct4 immunostaining and BC tumor size (T1-2 vs T3-4) ($P = 0.042$; Table 2, Fig. 2, *C*). This data was consistent with previous studies [15, 28, 34, 35], indicating its potential involvement in tumor aggressiveness. However, this data was inconsistent with other reports [1, 33]. Sample size, the use of different methods, different antibodies, or antigen retrieval methods may explain these differences. These data suggest that Oct4 may have a role in tumor proliferation and aggressiveness.

This data showed no significant association between Oct4 immunostaining and lymph node status ($P = 0.295$; Table 2 and Fig. 2, *D*), suggesting that Oct4 levels in the nucleus do not reliably predict lymphatic tumor spread. This result agreed with previous findings [28, 33, 34]. This result, however, was inconsistent with other studies [1, 15].

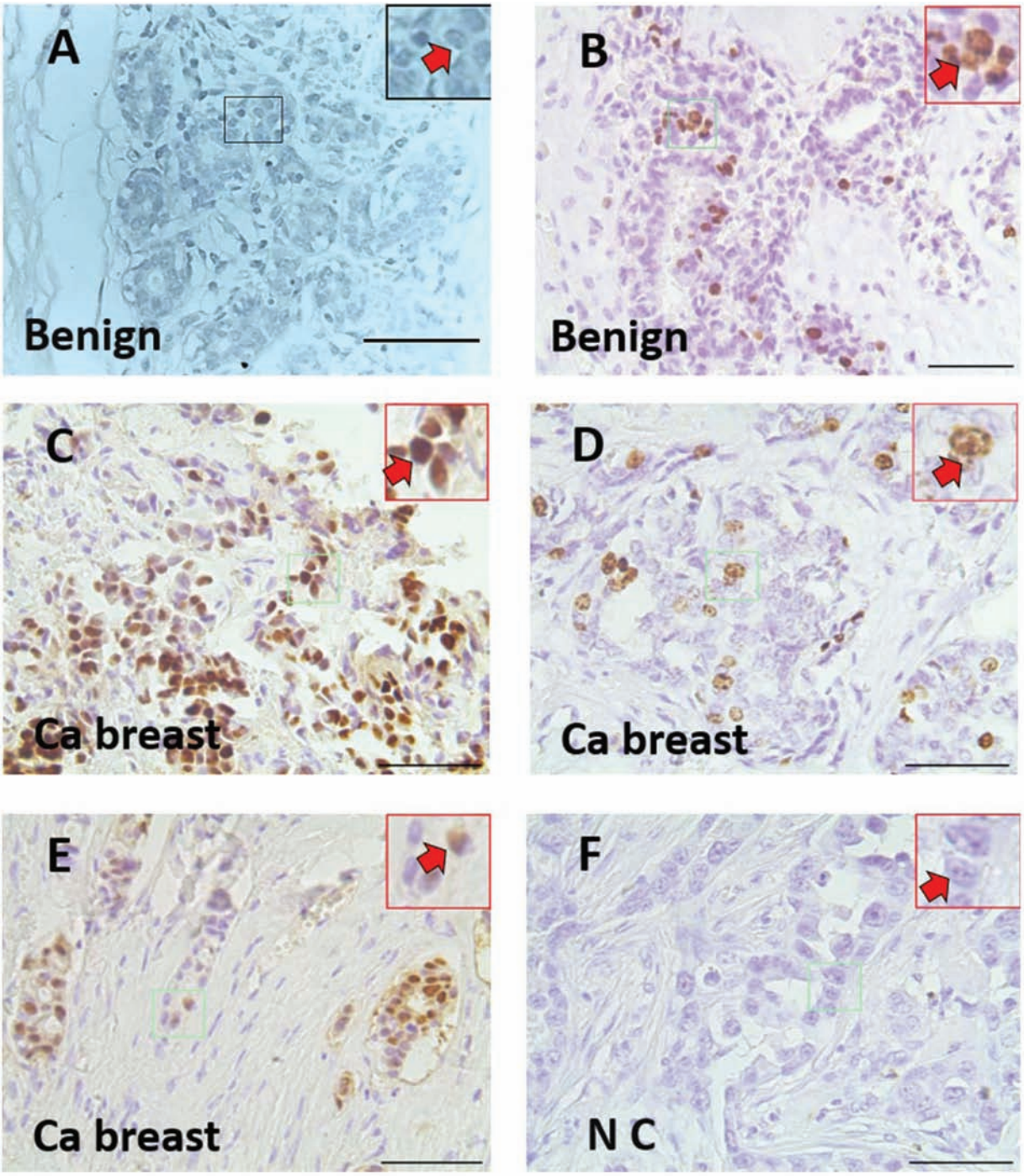

*Fig. 1. The Oct4 expression in breast tissues. (**A**) No Oct4 staining was seen in benign breast samples (arrow). (**B**) Moderate nuclear Oct4 staining was found in benign breast samples (arrow). (**C**) BC tissues showed strong nuclear Oct4 staining (arrow). (**D**) Moderate Oct4 staining was found in the nuclei of BC tissues (arrow). (**E**) Weak nuclear Oct4 staining was found in BC (arrow). (**F**) No staining was found in the negative control tissue. Scale bars = 50 μm; insets show 3x magnification*

Table 2. Oct4 Quantification in breast cancer histopathological data.

Histopathological data	Oct4 nuclear staining	Results	
		mean ± SD	*P* value
Cancer vs Benign	Increased in malignancy	3.569 ± 1.242 vs 2.278 ± 1.132	<0.001
Grade 2 vs Grade 3	Decreased in high-grade	3.963 ± 1.148 vs 3.280 ± 1.306	0.002
T1-2 vs T3-4	Increased in T3-4	3.343 ± 1.457 vs 3.949 ± 1.133	0.042
N0 vs N1-3	No sig. difference	0.7200 ± 0.7729 vs 1.311 ± 1.719	0.295

It has been found that Oct4 may enhance migration and invasion capabilities both *in vivo* and *in vitro* [25]. Despite its correlation with tumor features, Oct4 expression did not show a significant association with lymph node metastasis, suggesting that other determinants may affect metastatic capability [28, 29]. Our study had a small sample size of N0 cases, which may account for the differences observed in the literature.

It has been found that Oct4 plays a crucial role in regulating various genes or signaling pathways that affect carcinoma cell behavior. Chen et. al. demonstrated that it can induce metastasis of lung cancer cells by inducing epithelial–mesenchymal transition [36]. Additionally, Oct4 knockout was shown to reduce proliferation in hepatocellular carcinoma cell lines via EMT modulation. In cervical cancer, Oct4 overexpression has been implicated in enhancing tumorigenesis and suppressing apoptosis through regulation of the miR-125b/BAK1 axis [31]. In addition, a previous report found a link between elevated Oct4 levels and the aggressive behavior of cancer cells; thus, it may serve as a possible biomarke for poor prognosis in various cancers [33], including breast cancer. These findings suggest that Oct4 may influence several types of cancer cell biology, including breast cancer, by promoting proliferation, inhibiting apoptosis, and enhancing invasion and adhesion capabilities.

This study has several limitations including a small sample size, the absence of data on metastasis, and a relatively short follow-up period. As a result, it is necessary to conduct additional studies that involve larger patient cohorts and longer follow-up periods. Additionally, tumor-related outcomes could have been affected by variations in the adjuvant and first-line chemotherapy regimens used. Furthermore, IHC is frequently used to identify significant proteins in cells and tissues [37, 38]. However, several variables, including antibody selection, antibody concentrations, and antigen preparation methods, may affect IHC results and lead to variations in staining outcomes [39]. The absence of a standardized IHC protocol makes its application even more complex, and non-specific staining can lead to false-positive results [38]. Even with its limitations, IHC

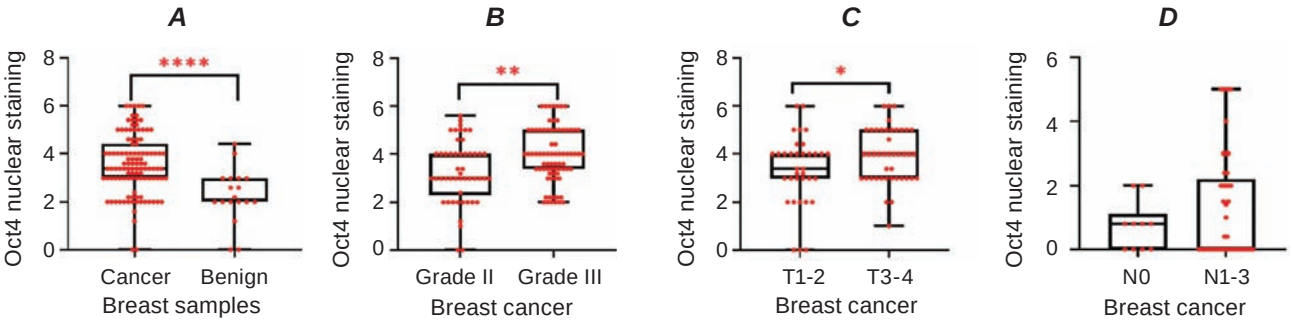

Fig. 2. Oct4 immunostaining quantification in breast samples. ***A****) High Oct4 expression was found in the nuclei of BC tissues compared to the benign samples (P < 0.001).* ***B****) Increased Oct4 expression was recorded in high-grade BC (P = 0.002). C) Oct4 immunostaining was increased significantly in T3-4 compared to T1-2 (P = 0.042). D) No significant association was seen between Oct4 and lymph node status (p0.295). Benign (n = 20), breast carcinoma (n = 110), Grade II (n = 55), Grade III (n = 47), T1-2 (n = 38), T3-4 (n = 44), N0 (n = 12), N1 (n = 65).*

remains a clinically relevant and widely accessible technique for localizing proteins.

Conclusion. This research demonstrates that Oct4 expression is elevated in BC and correlates with tumor grade and size. However, it was not linked to lymph node status, indicating Oct4's involvement in the onset and advancement of the disease. Oct4 shows promise as a BC biomarker, offering diagnostic and prognostic insights. To validate and expand upon our findings, further research utilizing additional breast cancer cell lines, larger cohorts that include more normal breast tissue samples, and complementary molecular techniques such as RNA sequencing and multivariate analyses will be essential.

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ЕКСПРЕСІЯ OCT4 АСОЦІЙОВАНА ЗІ СТУПЕНЕМ І РОЗМІРОМ ПУХЛИНИ ПРИ РАКУ МОЛОЧНОЇ ЗАЛОЗИ

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Рак молочної залози залишається однією з найпоширеніших пухлин серед жінок у всьому світі, при цьому сучасні прогностичні методи мають обмежену здатність точно передбачати агресивність пухлини. Октамер-зв'язувальний транскрипційний фактор 4 (Oct4), ключовий регулятор плюрипотентності та характеристик ракових стовбурових клітин, розглядається як перспективний біомаркер при різних типах раку. Метою цього дослідження було оцінити результати імунофарбування Oct4 у доброякісних та злоякісних тканинах молочної залози та проаналізувати його зв'язок зі ступенем злоякісності раку та розміром пухлини. Було досліджено архівні формалін-фіксовані зразки тканини молочної залози, що включали 110 випадків карциноми молочної залози та 20 зразків доброякісних утворень. Імуногістохімічний аналіз експресії Oct4 був проведений за допомогою моноклонального антитіла кролика проти Oct4. Результати дослідження показали достовірне підвищення ядерної експресії Oct4 у злоякісних тканинах молочної залози порівняно з доброякісними. Підвищений рівень імунореактивності Oct4 був позитивно асоційованим із високим ступенем злоякісності та більшим розміром пухлини. Однак суттєвої кореляції між рівнем Oct4 та станом лімфатичних вузлів не спостерігалося. Таким чином, підвищена експресія Oct4 може бути пов'язана з вищим ступенем злоякісної пухлини та більшим розміром пухлини, зо свідчить про її потенційну роль у прогресуванні раку молочної залози.

Ключові слова: рак молочної залози, Oct4, імуногістохімія, ступінь злоякісності та розмір пухлини.

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