

Evaluation of the Ultrastructure and Expression of Desmoglein 2 in Breast Cancer: A Novel Biomarker

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Abstract

Background

Breast cancer is the most common malignancy among Iranian women. In recent years, the study of dysfunction in the expression of cell-cell junction genes and the related proteins in the malignant process has been at the center of attention.

Methods

In this study, 50 patients were selected who had both cancerous tissue and adjacent healthy tissue. The expression of the desmoglein 2 gene was evaluated. Healthy and cancerous tissue were compared using routine hematoxylin and eosin staining. The total protein was also compared between these two groups. The ultrastructural examination was performed.

Results

The real-time polymerase chain reaction results showed a decrease in the expression of the desmoglein 2 gene in all tumor samples compared to the healthy samples (p -value <0.0001). Besides, receiver operating characteristic curve analysis showed that the area under the curve was equal to 0.98. Transmission electron microscopy microscopic studies revealed a change in the status of desmosomal junctions. These findings were consistent with the qualitative decrease in the protein expression between the two target groups.

Conclusion

Overall, the findings showed that the association between desmoglein 2 gene expression and alterations in cellular connections leads to impaired cellular connections, which is an important risk factor for breast cancer. This result proposed the understudy gene as a new biomarker in the development of breast cancer.

Introduction

A total of 12.5% of all cancers are breast cancer in Iran, making it the 6th leading cause of death in this nation [1, 2]. In light of the reports enrolled in the National Cancer Registry of Iran (INCR), the yearly ASIR for malignant breast growth is 27.4 (Per 100,000) with a crude rate of 22.6 (per 100,000) [2, 3]. In recent years, breast carcinoma growth in Iran has had an expanding pattern in rate and mortality [4, 5].

Conventional diagnosis and treatment of breast cancer are based on prognostic estimates using the anatomical features of cancer (TNM system) and clinical findings [6]. However, studies have shown that individuals respond differently to these treatments, and some patients, after the treatment, experience recurrent problems. This indicates that molecular changes occur before any phenotypic, clinical, or pathological changes and that molecular evaluations, along with clinical and pathological findings, are of

paramount importance [7]. One of the most important of these assessments is the study of functional defects in cell junction genes and the related proteins [8]; studies show that disorders in the regulation of their components play an important role in the process of malignancy and metastasis [9].

Breast cancer is regulated in part by various adhesion molecules known as cadherin [10, 11]. These molecules are responsible for key cell functions such as programmed cell death, growth, migration, and differentiation [9]. Cadherin is also known as tumor suppressor genes that play a unique role in tissue development and differentiation. If any dysfunction occurs due to various genetic processes, epigenetics, and mutations, it can lead to tumor growth, invasion, and metastasis [12-14].

Breast tumor cells express two main subtypes of cadherin types: classical cadherin, such as E-cadherin or P-cadherin, and non-classical cadherin (desmosomes), including desmogleins and desmocollins [15]. Classical cadherins and their role in the development and progression of cancer have been widely studied, while on the other hand, the role of desmosome cadherin and their functional defects in the development of breast cancer is not frequently studied. Further studies are needed to determine the full function of desmosomal cadherin in the malignancy [10, 15, 16]. Studies have shown that desmosomes contain desmoglein (DSG1, 2,3,4) and desmocollin (DSC1, 2,3) that cross-bind these molecules together and other intracellular proteins, including plakophilin, plakoglobin, and desmoplakin, as well as calcium ion-dependent hydrophilic interactions with cytoskeletal intermediate fibers. In humans, there are four genes encoding desmoglein and three genes encoding desmocollin [17, 18]. However, the role of Desmoglein 2 (DSG2) in cancer has not been definitively determined. According to the recent research, the expression of this gene can be used as a cancer suppressor in malignancies such as gastric, prostate, melanoma, pancreas, and colon cancers. On the other hand, research shows that this gene is overexpressed in skin cancer, stem cells carcinoma, and lung cancer [19].

Desmoglein 2 (DSG2) is one of the desmosomal cadherins identified in the mammary gland. However, it is unclear how this cadherin is involved in breast cancer development and progression [12, 20].

In the present study, given the high prevalence of breast cancer in Iran, in addition to the special and ambiguous role of desmoglein 2 in the development of cancer, we aimed to compare the expression of desmoglein 2 in cancer tissue and healthy tissue of patients and analyzed the location of desmosomal cadherin.

Material & Method

Patients and Tissue Samples

After obtaining the necessary permits and obtaining the code of ethics from the Ethics Committee of the Islamic Azad University, Tehran North Branch to maintain patient confidentiality in accordance with the Helsinki Agreement, patients' information was considered confidential and used only for research purposes. The patients were individuals referred to Rasoul Akram Hospital from the beginning of June

2018 to July 2019 with a breast cancer pathology diagnosis. The written informed consent was obtained from all the patients (code of ethic: IR. IUMS.REC.1399.1210

). One hundred pairs of formalin-fixed, paraffin-embedded (FFPE) ductal carcinoma breast tumor tissues and adjacent normal tissues were collected. The patients' clinicopathological variables, including age, tumor size, histological grade, involvement/non-involvement of lymph nodes, and involvement/non-involvement of vascular node are summarized in Table I.

Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from the FFPE using TRIzol reagent (Geneall, South Korea) according to the manufacturer's instructions. RNA concentration was quantified by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). A hundred ng RNA was used to synthesize the first-strand cDNA using the BeyoRT™ II First Strand cDNA Synthesis Kit (*SMOBIO*, Taiwan) following the manufacturer's instructions.

RT-PCR Assay

RT-PCR was performed to measure the mRNA expression level of Dsg2 using the **5x Hot FIREPOL Eva Green qPCR Mix No ROX** (Solis BioDyne, Estonia) at an ABI 7300 Real Time-PCR System (Applied Biosystems, Life Technologies GmbH, Darmstadt, Germany). The following PCR reaction was used: step 1: 95°C for 15 min, 1 cycle; step 2: 95°C for 15 sec, 60°C for 45 sec, 40 cycles. The primers used in this study were the same as the previous one 21 and listed as follows: Dsg2: forward 5'-TGGACACCCAAACAGTGGCCCT-3', reverse 5'-CTCACTTTGTTGCAGCAGCACAC-3'; β -actin: forward 5'-GGCACCACACCTTCTACAATGA-3', reverse 5'-TCTCCTTAATGTCACGCACGAT-3'. All samples were run in triplicates, and samples were normalized against an endogenous internal control, β -actin. Levels of Dsg2 mRNA were quantified using the $2^{-\Delta\Delta Cq}$ method.

Data analysis

All data are presented as the means \pm standard deviation (SD). Statistical analyses (student's *t*-test and one-way analysis of variance (ANOVA)) were performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Also, in these studies, the 95% confidence level (CI) was determined. To evaluate the biomarker potential of the Desmoglein 2 gene, software GraphPad Prism 5 was used to draw a ROC diagram. A value of * was considered to indicate a statistically significant difference. All results presented in the study figures were obtained from at least three independent experiments.

H&E staining

First, the tissues were recovered with the Harris' hematoxylin arrangement for 6 h at a temperature of 60–70 °C and then washed in tap water until the water was colorless. Then, 10% acetic acid and 85% ethanol in water was utilized to distinguish the tissue two times for 2 h and 10 h, and the tissues were rinsed with tap water. Within the bluing step, the tissue was doused in a saturated lithium carbonate

arrangement for 12 h and after that washed with tap water. At long last, recoloring was performed with eosin Y ethanol arrangement for 48 h.

SDS-PAGE analysis

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was done considering the optimized convention for protein extraction from the breast tissue compatible with two-dimensional gel electrophoresis [21]. The present study applied the same approach. Breast cancer tissues were homogenized in PBS, centrifuged, and the supernatants lysed in a 2X SDS-PAGE test buffer. The resultant substance at that point bubbled and the supernatants were subjected to SDS-PAGE. The supernatants were exchanged into the Mini-V 8-10 vertical gel electrophoresis framework, and the tank was filled with electrophoresis buffer. The division of the protein was carried out at a steady voltage of 80 V for 2 h. At that point, the settling gel was exchanged to the recoloring arrangement and put on a shaker for 24 h at room temperature. The recoloring arrangement was at that point supplanted by the staining arrangement, which was changed until the protein groups became clear. The overall proteins were divided on SDS-PAGE.

Electron microscopy

The examples were minced into 1–2 mm³ pieces, hatched for 2 h in fixative, and 1 h in 1% OsO₄ in a 0.2 M phosphate cushion (pH 7.3). The tissue was then treated with 0.5% uranyl acetic acid derivation in 0.05 M sodium maleate cushion (pH 5.2) for 2 h in obscurity. The tissue was dried out and implanted in Araldite utilizing CH₃)₂CO as intermedium. At 60 °C for 48 h, functionalization was performed. Semithin areas of 0.35 µm were acquired utilizing glass cuts and stained with toluidine blue. Ultrathin areas of 50 nm were then set up with an ultramicrotome (Reichert Ultracut S, Leica, Wetzlar, Germany) utilizing precious stone blades. To upgrade contrast, the segments on copper frameworks were first treated with 3% uranyl acetic acid derivation for 5 min and afterward with lead citrate arrangement (as indicated by Reynolds 1963) for 4 min. Pictures were taken on an EM 10 (Zeiss) with a computerized camera (Olympus, Münster, Germany) utilizing the iTEM programming (Olympus). Transmission electron microscopy was utilized to describe the ultrastructure and desmosomes.

Results

Dsg2 expression in ductal carcinoma tissues

The results showed that the mRNA expression level of Dsg2 was significantly decreased in cancer tissues compared to the matched noncancerous tissues ($p < 0.01$, Figure 1A). The status of differentially expressed Dsg2 gene in the breast cancer tissues was calculated as the ratio of Dsg2 mRNA expression in tumor tissue to the matched normal tissue (T/N ratio).

Correlation between Dsg2 Expression and Clinicopathological Variables

To better understand the clinical significance of Dsg2 expression in breast cancer, we analyzed the correlation between Dsg2 expression and clinicopathological variables, including involvement/non-involvement lymph node, age, histological grade, vascular non-vascular involvement and tumor size (all $p > 0.05$, respectively Figure 1B, C, D, E, F). However, Dsg2 expression was not associated with the clinicopathological variables (Table I). ROC curve analysis was performed to evaluate the biomarker potential of desmoglein 2. The results showed that the area under curve (AUC) was equal to 0.98. This rate is at a very acceptable level, indicating the expression of this biomarker gene is suitable for diagnosing breast cancer in tissue samples. The sensitivity was 98, specificity 70, and the cutoff value 0.47 (Figure 1G).

Increased number of inflammatory nuclei and cells

In this regard, after general H&E staining, the difference between healthy tissues and tumors was observed with a light microscope. An increase in the number of nuclei was seen in tumor tissue compared to adjacent healthy tissue (Fig 2A & B).

In many patients, the ductal incidence was observed in cancer specimens, while in healthy specimens, none was observed (Fig 2D). The observation of ductal formation is consistent with lymph node involvement and metastasis in these patients.

Inflammatory cells were observed in the tumor tissue. However, no inflammatory cells were found in healthy tissue (Fig 2C). The presence of these cells indicates that the tissue is cancerous.

Comparison of breast cancer proteome with adjacent healthy tissue using SDS-PAGE technique

The study of SDS-PAGE preparations of proteins showed a difference in protein expression in the tumoral and normal tissues. The results showed differences in protein expression between these two groups. In the band range of 120 kDa, due to the molecular weight of desmoglein 2, the band difference was well observed (Figure 3).

Ultrastructure disruption and desmosome junctions

In the study of normal tissue, cell cohesion, an appropriate number of nuclei, heterochromatin state, appropriate collagen fibers, interconnected cell membranes, and milk proteins are well observed, which is consistent with the definition of a normal cell (Fig 4 A&B). In contrast, in cancer cells, an increase in the number of nuclei is observed in the euchromatin state, cell membrane rupture, cell vacuolation, and a decrease in milk proteins, which is consistent with the definition of a cancer cell (Fig 4 C&D).

Desmosome cohesion was also observed in normal cells, but desmosome disintegration was observed in tumor tissue. The results are consistent with reduced desmoglein 2 gene expression and show the importance of cellular connections in malignancy (Fig 4 E&F). To better show the change in desmosome connections in healthy and tumor cells, we focused on the image obtained.)Respectively Fig E-1 & F-1).

Nucleus in heterochromatin state 12.6 μ m. Tumor cell: C. Cell membrane rupture, increase in the number of nuclei relative to healthy tissue, cell vacuolation 12.6 μ m. D. The nucleus is in the euchromatin state 12.6 μ m. E. Desmosomes in a healthy cell (E1- Focused image). F. Desmosomes in the cancer cell (F1- Focused image).

Discussion

Breast cancer is one of the most common cancers [22]. In recent years, several studies have been performed to identify and evaluate the genetic markers involved in cancer, which has led to identifying several predisposing genes. Among the genes, those related to cell-to-cell communication are of particular importance. Studies have recently shown that desmosomal proteins play a special role in tumor progression and inhibitory functions in different cancer types [8, 23].

Desmogleins are a collection of adhesion cadherins and membrane proteins that bind to other cadherins to provide the ability to bind as desmosomes between cells. Desmosomal cadherins and classical cadherins are critical for the stabilization of tissue integrity [24]. The main function of DSG2 is to form desmosomal adhesion structures in the epithelium, myocardium, and cardiomyocytes. However, emerging reports have suggested that DSG2 also has essential tumorigenic functions, yet its specific role is unclear [25]. Kai et al. showed that DSG2 expression is increased in lung cancer, and decreased regulation could suppress tumor growth [26]. Similarly, Barber et al., Abulrob et al., Plus Kamekura et al. found that DSG2 deficiency leads to inhibition of cell proliferation and tumor growth in colon epithelial cancer [27-29]. Shuhang showed that desmoglein 2 is a biomarker that causes tumor proliferation and metastasis and is associated with a poor prognosis in the early stages of cervical cancer [30]. In 1997, Davies and colleagues studied DSG2 (although they did not study the expression of the desmoglein 2 gene) and found a negative role in breast cancer onset and motility. They stated that the cause of this phenomenon was still unknown but reported that it was undeniable that DSG2 does not have a significant effect on cancer. Besides, DSG2 can cause angiogenesis [31]. Therefore, in the present study, we investigated the expression of the DSG2 gene in breast cancer because the expression of this important desmosome binding molecule, which has a special role in the progression and metastasis of other cancers, has not been investigated. In our breast cancer studies, we found that the expression of the desmoglein 2 gene in tumor cells was decreased compared to healthy breast cells. Also, we revealed that DSG2 could be a potential biomarker. The precise and dependable estimations of the particular changes in protein biomarkers for cancer location and treatment are critical challenges [32]. In the following, for the importance of the subject, we examined this cadherin in more detail.

A prominent and unique feature of the structure of desmosomes is the existence of a dense electron midline between the plasma membranes in the intercellular space, which was observed by electron microscopy. Desmosomes are button-like spots. The plate desmosome connection is known as the sticky button structure. The distance between the two membranes is 200 angstroms, and it is located just below the strong junction, which acts as an intercellular bridge to connect two adjacent cells [33]. Rayns et al. described the regular structure of a desmosome [34]. Waschke observed that removing the DSG2 gene

with the help of an electron microscope leads to the rupture of desmosomes [35]. With the help of the study findings, Burke and colleagues showed that shortening of desmoglein 2 leads to a noticeable change in the ultrastructural level and, to a significant extent, in the desmosomes, which is directly related to pathogenicity [36]. COTRUTZ, by studying E-cadherin, showed that changes in desmosomal structures promote metastatic and aggressive behavior in ductal carcinoma. He considered these changes as prognostic markers [37]. The COTRUTZ studies were consistent with our findings. We also showed in our study that in healthy breast tissue, desmosomal junctions are cohesive and integrated, while in cancerous tissue, this type of junction is disrupted. Our findings were also consistent with the pathological descriptions of patients who presented metastatic behavior and lymph node invasion.

In order to elucidate the molecular mechanisms involved in the development and progression of breast cancer and to discover new diagnostic and therapeutic markers of the disease, in recent years, using the proteomics method, many efforts have been made to investigate the expression of proteins in breast cancer cells [38, 39]. Wulfschlegel et al. studied the expression of mitochondrial proteins and RhoGDI while studying proteomics adjacent to healthy breast cancer tissue [40]. Gam. Et al. demonstrated the protein expression profile of cancerous and adjacent normal tissues in the breast with a proteomics approach involving two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). In this regard, they concluded that given the cellular location of membrane proteins in cells, their expression in cancerous tissues could potentially be used as indicators for the diagnosis or pharmacological treatment of breast cancer [41]. The present study suggests that proteomics is an appropriate approach to identify the protein factors involved in breast cancer. Our study found that the qualitative expression of desmoglein 2 protein was reduced in tumor cells compared to adjacent healthy cells.

Conclusions

In this study, for the first time, the expression of the desmoglein 2 was examined in breast cancer. We found in our studies that the decrease of expression can alter the status of cell connections. Also, we observed a quality decrease of protein between tumor and adjacent healthy tissue. Therefore, with the obtained results, the desmoglein 2 gene can be introduced as an effective biomarker in cancer progression and malignancy in breast cancer. Further research with longer follow-ups and a larger study population is required to study the importance of the biomarker and examine OS.

Declarations

Ethics approval and consent to participate

- All experimental protocols were **approved** by Ethics Committee of Iran University of Medical Sciences (A code of ethic: IUMS.REC.1399.1210).
- All methods were carried out in **accordance** with relevant guidelines and regulations.
- **informed** consent was obtained from all patients.

Consent for publication

All authors are satisfied with the publication.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare no conflict of interest.

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Author's contributions

1. Maryam mohamadhoseini. Performing and analyzing the research topic in the laboratory, provides research funding and writing an article.
2. mona Farhadi. Consult the professor and check the test results.
3. Professor ahmad majd. Supervisor and guidance in choosing a research topic.
4. hamidreza Mirzaei. Supervisor and suggest review of cell connections in breast cancer. Oncologist.
5. nasrin shayanfar. Confirm and identify cancerous tissue from healthy tissue from the same patient. H&E staining review and approval. Pathologist.

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Tables

Table I. The correlations between clinicopathological variables and Dsg2 expression.

variables	N	P value
Age (years)		
≥45	18	0.1692
<45	32	
Tumor size		
> 2 cm	9	
2-5 cm	39	0.3102
≤5 cm	2	
Lymph node involvement		
Yes	21	0.1604
NO	29	
Tumor grade		
I-II	35	0.3876
III	15	
Vascular involvement		
Yes	10	0.3406
NO	40	

Figures

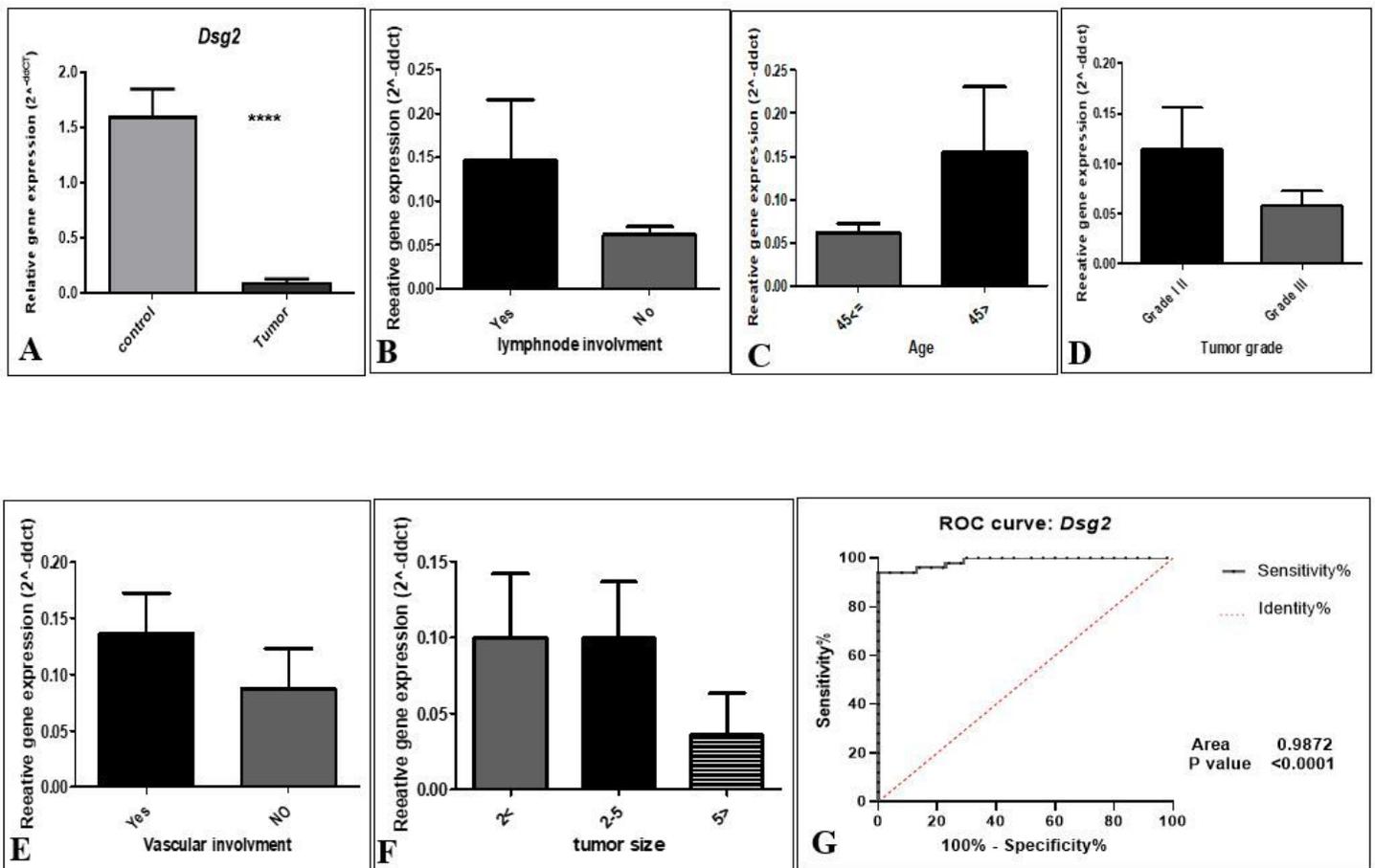


Figure 1

A: Significant decrease in the expression level of desmoglein 2 gene in tumor cells compared to adjacent healthy tissue. Figures B, C, D, E, F show the difference in the level of expression between the lymph node involvement, age, tumor grade, vascular involvement, and tumor size, respectively. Of course, this difference is not statistically significant. G: ROC curve for desmoglein2. The area under the curve shows the biomarker potential of desmoglein 2 in breast cancer tumor tissues.

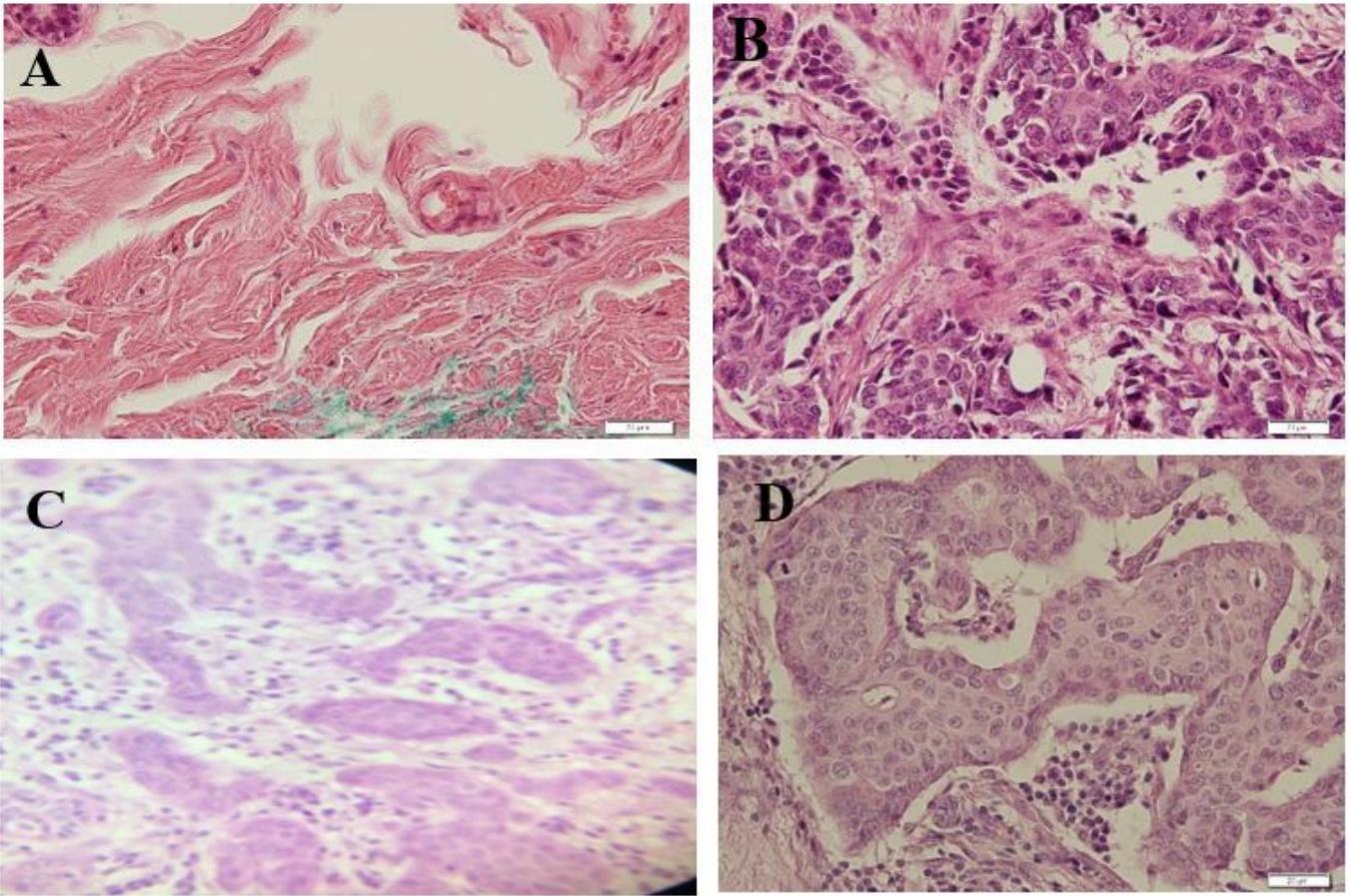


Figure 2

Increase in the number of nuclei in the cancer cell (B) compared to the same type in a healthy sample (A) 40X. Inflammatory cells in the cancer cell (C) 40X. Ductal formation in the cancer cell (D) 40X.

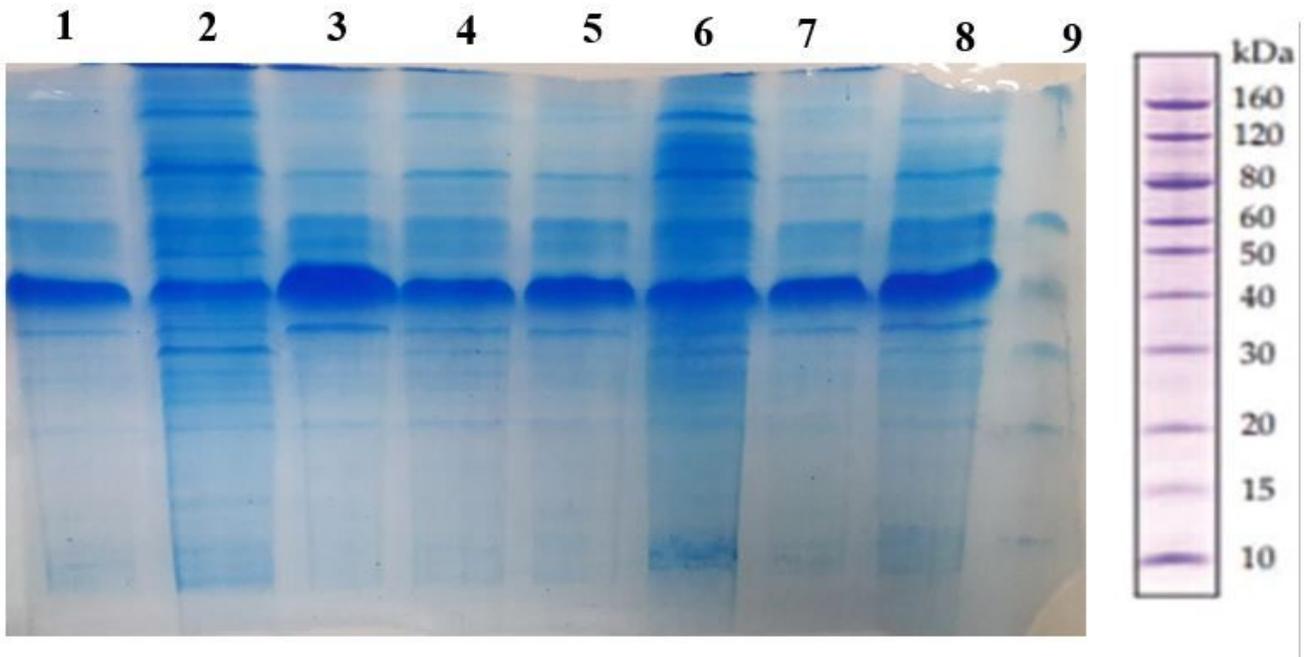


Figure 3

The SDS-PAGE protein profile of breast cancer patients and adjacent health tissues stained with Coomassie brilliant blue. Lanes 1, 3, 5, 7 are breast cancer tissues, and 2, 4, 6, 8 are healthy tissues. The result in this test also showed high expression of proteins in healthy tissues than in cancer tissues.

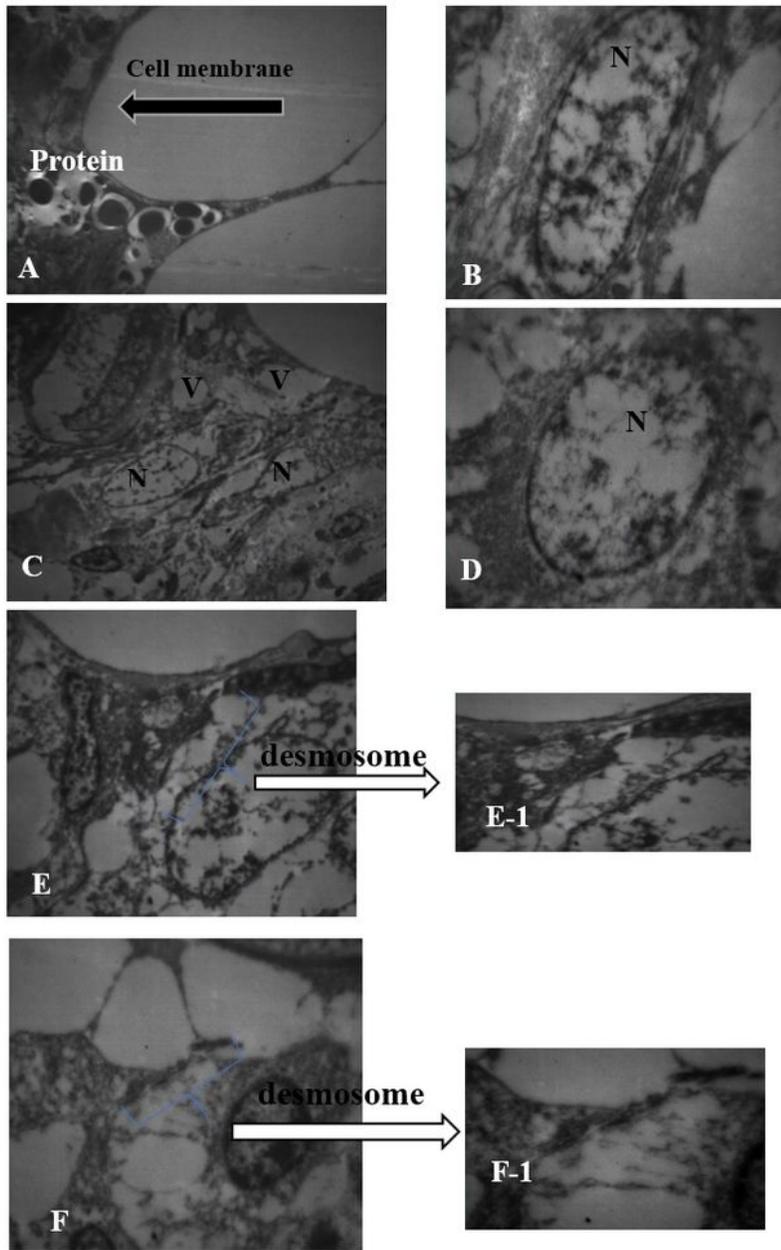


Figure 4

Healthy cell: A. the interconnection of cell membranes and milk proteins 20.1 μ m. B.