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The role of Ghrelin Receptor Expression in the Diagnosis of breast cancer

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ABSTRACT

To determine the possible role of the assessment of Ghrelin receptor expression in breast tissues as a tool for the diagnosis of breast cancer and differentiate it from a benign breast tumor. A case-control study was done on 60 female patients with breast cancer and 60 female patients with benign breast tumors (Fibroadenoma) who were recruited from Al Imamain Al-Kadhemain Medical City and Oncology teaching Hospital, Baghdad, Iraq between May 2018 and December 2018. Immunohistochemical staining was done on the breast tissue samples obtained from patients and compared with the control group, which comprised 75 fibrocystic tissue samples obtained from age, BMI and sex-matched females. The degree of Ghrelin Receptor expression was determined immunohistochemically. The expression of Ghrelin receptors in breast malignant tumor tissues was higher than that in benign breast tumor tissues and controls, in addition to that, results obtained from all groups revealed that Ghrelin receptor intensity and its expression proportion were strongly and significantly associated with the type of tissues. The expression of the Ghrelin receptor can be considered as a highly significant immunohistochemical marker for the detection of breast tumors and for the differentiation between both types of tumors; benign and malignant.

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INTRODUCTION

Malignant breast tumor is considered globally as the most frequently diagnosed cancer and the leading cause of cancer death in women given that it accounts for about 23% of the total new cancer cases and approximately 14% of the total cancer deaths in 2008 (Jemal *et al.*, 2011; Bray *et al.*, 2018). Breast cancer considered a heterogeneous disease that showed a wide range of clinical, histological and molecular presentations. Unfortunately, other than a definitive diagnosis that performed by biopsy and histopathology, no diagnostic or screening test can be considered presently as a suitable marker for early detection of this cancer in females (Harris *et al.*, 2007).

At present, the detection of breast cancer relies mainly on mammography that appears clearly to associate with a decrease in the mortality of breast cancer (Jatoi, 1999; Gøtzsche and Jørgensen, 2013). However, mammography screening has generated controversy due to the risks of false-positive results and overdiagnosis of indolent disease (Baum, 2010;

Gøtzsche and Jørgensen, 2013; Pace and Keating, 2014; Welch *et al.*, 2016). Despite the usefulness of Mammography in the diagnosis of breast cancer, it showed a limited sensitivity for the detection of tumors in dense breast tissue (Boyd *et al.*, 2007). Therefore, there is an urgent need for biochemical and histological markers that can be used in the early diagnosis and prediction of the disease outcome in addition to the need for marker that provides a prognostic information to the clinician for treatment stratification, so, various markers either biochemical or immunohistochemical were discovered and tested widely for this purpose (Kazarian *et al.*, 2017).

Ghrelin is a peptide that consists of twenty-eight amino acids which were identified firstly as a ligand of growth hormone (GH) secretagogue receptors (GHSRs). Its name derived from "ghre," the Proto-Indo European root of the word "grow," and it was originally isolated from the stomach mucosa that regulates GH secretion. It was reported that Ghrelin had a wide variety of physiological functions that range from regulation of food intake, hormonal secretion, insulin secretion modulation, adipogenesis and gastrointestinal motility, and one of the most important orexigenic peptides currently known. The possible role of Ghrelin in tumorigenesis might be contributed to its involvement in proliferation of cells and the stimulation of milk production, together with the its stimulatory effect on Growth hormone levels via Ghrelin axis, in addition to its high levels in females (Chopin *et al.*, 2011; Delporte, 2013; Khatib *et al.*, 2014; Grönberg *et al.*, 2017).

It was reported previously that the expression of Ghrelin was associated with a positive outcome in a nonconsecutive and selected patient population of invasive breast tumors, demonstrating a 3-fold lower risk for breast cancer death in patients with tumors expressing Ghrelin compared to those lacking Ghrelin expression. Moreover, it had been documented that the increment in the risk of breast cancer was correlated with various Ghrelin gene polymorphisms. Previous literature assumed that the variations in the Ghrelin system regulation in breast tissue might be considered as a pathological influence of breast tumor development. These findings run parallel to other studies which demonstrated that Ghrelin gene-derived splice forms in breast cancer were also over expressed (Gahete *et al.*, 2010, 2011; Grönberg *et al.*, 2012).

Furthermore, it was demonstrated that breast carcinomas tissues in humans showed an expression of receptors bind specifically to natural Ghrelin and synthetic GH secretagogues (GHSs). The binding of these receptors with their legends reported to occur independently from the histological type, stage, Ki67, pre- or postmenopausal status and ER status of the tumor, but showed a correlation to the tumor's grade of differentiation. It was reported previously that well-differentiated malignant cells of breast cancer showed an elevated level of GHS binding in comparison with moderately and poorly differentiated cancerous cells.

Several types of research conducted previously on the Ghrelin system and its role in the regulation of the processes of the progression and development of breast carcinoma still not fully comprehensive and could not provide a solution to the emerging conflicts. It was reported clearly that Ghrelin participates in the inhibition of breast carcinoma proliferation in humans that may be used in the future as a promising treatment for breast cancer (Grönberg *et al.*, 2017). It was obviously demonstrated that Ghrelin's expression associates significantly with the positivity of estrogen receptor, low histologic grade, low rate of proliferation and small tumor size in human breast tumors. On the other hand, some studies observed promoting an effect of Ghrelin on proliferation rate that makes this system an attractive target for a new therapeutic approach which may be possible by using Ghrelin antagonists or compounds that mimic the effect of Ghrelin in clinical disease. The trails to develop treatments depending on the results obtained from the assuming role of Ghrelin and its receptor are currently developed. The use of Ghrelin as a good agent for catabolic states/situations is owned to its GH releasing and orexigenic effects. Synthetic Ghrelin receptor agonists and antagonists have been developed recently for its possible role in the treatment of metabolic or nutritional disorders (Costantini *et al.*, 2011; Grönberg *et al.*, 2012; Garcia *et al.*, 2013).

Previously documented that the axis of Ghrelin /GH may participate in the tumorigenesis of breast cancer even though that the precise role of this axis has not been established yet (Gahete *et al.*, 2011). So, even that Ghrelin has both proliferative and antiproliferative roles, its usefulness still uncertain as a powerful therapeutic approach for breast cancer. (Chopin *et al.*, 2012; Lin and Hsiao, 2017)

The present study aimed to determine the extent of expression of Ghrelin receptor in the breast tissues of patients with a benign and malignant tumor and compare it with the normal expression of this marker in controls to evaluate the possibility of utilizing Ghrelin receptor expression as novel immunohistological markers for differentiation and diagno-

sis of breast tumors.

MATERIALS AND METHODS

Subjects

A case-control study was done on 60 female patients with breast cancer and 60 female patients with benign breast tumors (Fibroadenoma) who were recruited from Al Imamain Al-Kadhemain Medical City and Oncology teaching Hospital, Baghdad, Iraq between May 2018 and December 2018. Ages of the malignant group ranged between 30 and 48 years (mean± SD 42.83±4.27 years) and benign group's ages were ranged between 27 and 44 years (mean± SD 41.73±5.09 years). The control group comprised of 60 fibrocystic tissue samples obtained from age, BMI and sex-matched females with mean ± SD age 41.32±4.77 years. The practical part of the study was conducted at the Department of Chemistry and Biochemistry and Department of Pathology, College of Medicine, Al-Nahrain University, Baghdad, Iraq.

In the current research, women were eligible for this study if they had a suspicious breast lesion (newly diagnosed), which was recorded by clinical breast examination and/or imaging technology. Patients were subjected to physical breast examination (PBE), mammography and approved by a histopathologist. The Exclusion criteria for this study include subjects who had a history of any acute or chronic diseases or any type of cancer and received hormonal treatment or chemotherapy.

The study was approved by the Institutional Review Board (IRB) of the College of Medicine, University of Al-Nahrain, Baghdad, Iraq. Additionally, all subjected women have signed the informed written consents of participation in accordance with the Helsinki principles

All eligible control subjects and studied patients were subjected to thorough clinical examinations with full medical history in addition to Immunohistopathological examinations for Ghrelin receptor with histopathological assessment for all breast tissue samples involved in the present study using formalin-fixed, paraffin-embedded tissue sections.

Immunohistochemistry

Immunohistochemical staining was performed using the Anti-Ghrelin Receptor/GHS-R antibody kit (Abcam, ab188986, UK) in accordance with instructions provided by the manufacturer. Sections of Five micrometers were cut from blocks fixed in formalin, embedded in paraffin and placed on charged slides. After overnight packing at 65ºC, deparaffinization process was performed by using xylene followed by a rehydration step by using ascending grades of alcohol. Tissue sections were incubated in 0.3% H_2O_2 to exhaust the activity of endogenous peroxidase for ten minutes at room temperature. Slides then washed in Tris buffer saline (TBS) for five minutes. Protein block was applied and incubated at 25°C for ten minutes to block nonspecific background staining and washed again by TBS for 5 minutes. The section was then incubated at room temperature for 15 minutes with Anti-Ghrelin Receptor/GHS-R antibody ab188986 and the detection step performed by using an HRP conjugated compact polymer system. DAB was used as the chromogen. Hematoxylin sued to counterstain the section and mounted then with DPX. Human skin, hair follicle tissue was used as a positive control. Microphotographs were taken using a digital camera with 12.1 pixels and using polyvar microscopy.

Scoring of immunohistochemical staining of Ghrelin receptor

The staining intensity of in tumor cells was examined and scored with letters A, C, and D that represent non-immunoreactive (non-IR), weak, moderate and strong staining, respectively. Each of the two cores from every tumor on the array was examined and scored separately. The entire sections from each tumor were subjected to examination with at least 200 tumor cells to designate the score of intensity score. Complete and/or partial staining with any intensity (>50% IR tumor cells) was designated as positive staining that differs from truly negative staining (< 50% IR tumor cells), background and diffuse non-specific staining. The staining of cytoplasmic that can be observed with high-power fields (40X objective) was considered a positive reaction (Grönberg *et al.*, 2017).

Statistical analysis

The results of the current study were firstly stored in a Microsoft Excel format and the numerical variables were expressed in the form of mean ± SD. All the comparisons that performed were accomplished statistically by using an independent t-test to compare two independent groups (patients and controls). Categorical variables were expressed as numbers and analyzed by cross-tabulation to assess the frequency and percentage of each variable among studied groups. The correlation was performed between Ghrelin parameters and the groups subjected to the current study were performed by Pearson correlation test (Norman, 2010) and Chi-square to test the relationships between categorical variables. All statistical analyses used in this study were carried out by using the IBM SPSS Statistics for Windows, Version 20.0 (Armonk, NY: IBM Corp).

RESULTS AND DISCUSSION

Some demographic characteristics of the studied groups were summarized in Table 1 and Figure 1. Table 1 showed non-significant differences in age and body mass index (BMI) among all studied groups.

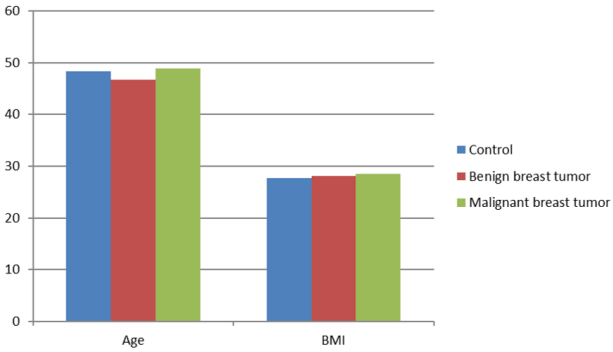

Figure 1: Age and BMI levels in all studied groups

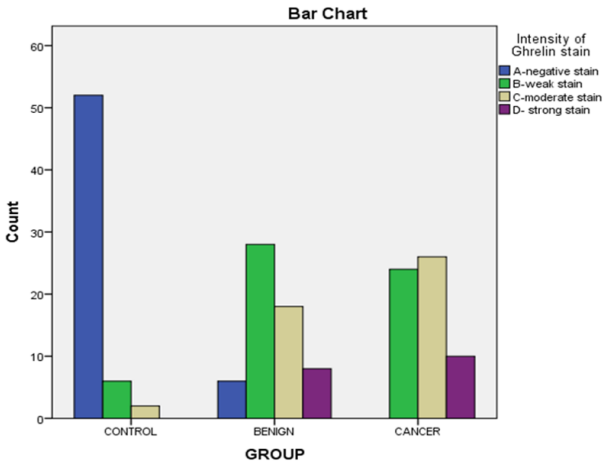

Figure 2: The intensity of the Ghrelin Receptor stain in all studies groups

Table 2 showed that the intensity of Ghrelin Receptor stain in healthy tissues showed only 2.7% with moderate intensity and 12% with weak stain intensity while the rest of the samples (85.3%) were negatively stained. Benign tissues showed noticeable different results from that of healthy tissues in which only 10% of samples were negatively stained, whereas 46.7%, 30% and 13.3% of samples were weakly, moderately and strongly stained, respectively. On the other hand, malignant samples showed more stain intensity than that of benign in which all samples showed weak, moderate and strong stain in a proportion of 40%, 43.3% and 16.7%, respectively, as postulated in Figure 2.

Controversially, the proportions of Ghrelin Receptor expression showed a different pattern in which healthy tissues showed only 3.3% of the samples

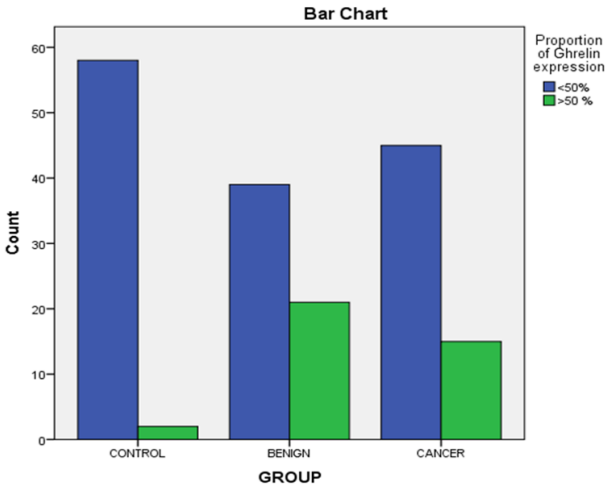

Figure 3: The proportion of Ghrelin Receptor expression in all studies groups

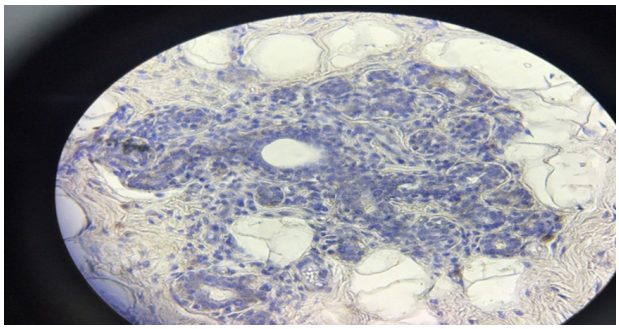

Figure 4: Negative Ghrelin Receptor expression in fibrocystic tissue (-ve control) (X40)

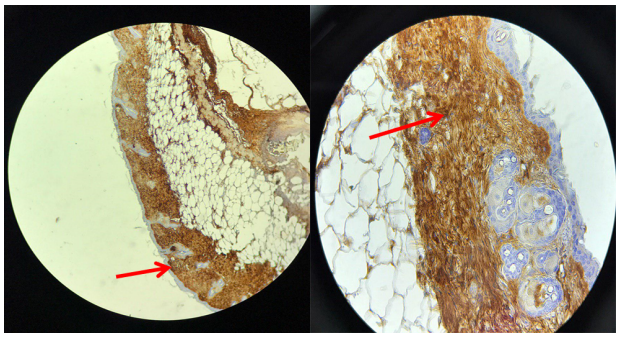

Figure 5: Positive Ghrelin Receptor expression in the hair follicle (+ve control). Notice brown cytoplasmic staining (X40 and X100)

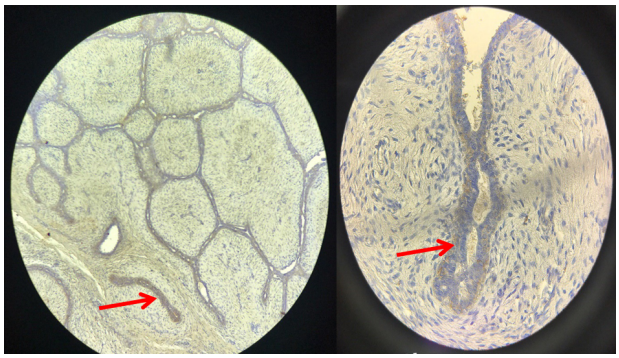

Figure 6: Positive Ghrelin Receptor expression in Benign tissue (A; X10; B; X40)

Table 1: Demographic characteristics of the patients with malignant and benign (Fibroadenoma) tumor in comparison with controls

	Control	Benign breast tumor	Malignant breast tumor
n	75	60	60
Age (year)	41.32±4.77	41.73±5.09	42.83±4.27
P-value with control		0.13	0.57
Weight (Kg)	70.28±9.06	69.82±8.93	73.37±9.87
Height (cm)	160.15±14.73	159.42±13.6	159.7±14.55
BMI (Kg/cm^3)	27.63±5.04	28.13±5.22	28.53±5.34
P-value with control		0.61	0.47

Table 2: Cross-tabulation of the intensity of Ghrelin Receptor stain in all studied groups

			The intensity of Ghrelin stain				
			A-negative stain	B-weak stain	C-moderate stain	D- strong stain	Total
Group	Control	Count	52	6	2	0	60
		% within Group	86.7%	10.0%	3.3%	0.0%	100.0%
	Benign	Count	6	28	18	8	60
		% within Group	10.0%	46.7%	30.0%	13.3%	100.0%
	Cancer	Count	0	24	26	10	60
		% within Group	0.0%	40.0%	43.3%	16.7%	100.0%
Total		Count	58	58	46	18	180
		% within Group	32.2%	32.2%	25.6%	10.0%	100.0%

Table 3: Cross-tabulation of the expression of the Ghrelin Receptor in all studied groups

			The proportion of Ghrelin expression		Total
			<50%	>50 %	
Group	control	Count	58	2	60
		% within Group	96.7%	3.3%	100.0%
	Benign	Count	39	21	60
		% within Group	65.0%	35.0%	100.0%
	Cancer	Count	45	15	60
		% within Group	75.0%	25.0%	100.0%
Total		Count	142	38	180
		% within Group	78.9%	21.1%	100.0%

Table 4: The correlations between the proportion of Ghrelin receptor expression and the intensity of a stain with the tumor type

		The proportion of Ghrelin Receptor expression	Group
The intensity of Ghrelin Receptor stain	Pearson Correlation	0.636**	0.688**
	Sig. (2-tailed)	0.000	0.000
	N	195	195
The proportion of Ghrelin Receptor expression	Pearson Correlation		0.232**
	Sig. (2-tailed)		.001
	N		195

** Correlation is significant at the 0.01 level (2-tailed)

Table 5: Chi-square and Phi results between immunohistochemical parameters among all groups

		Ghrelin Receptor Intensity	Ghrelin Receptor Proportion
Tissue type*	Phi	0.839	0.324
	P	<0.001	<0.001
Ghrelin Receptor Proportion	Phi	0.706	
	P	<0.001	

*either fibrocystic, benign or malignant tissues

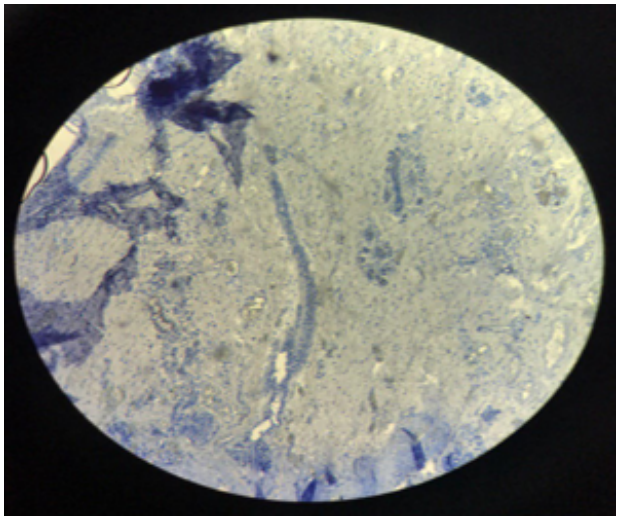

Figure 7: Negative Ghrelin Receptor expression in Benign tissue (X10)

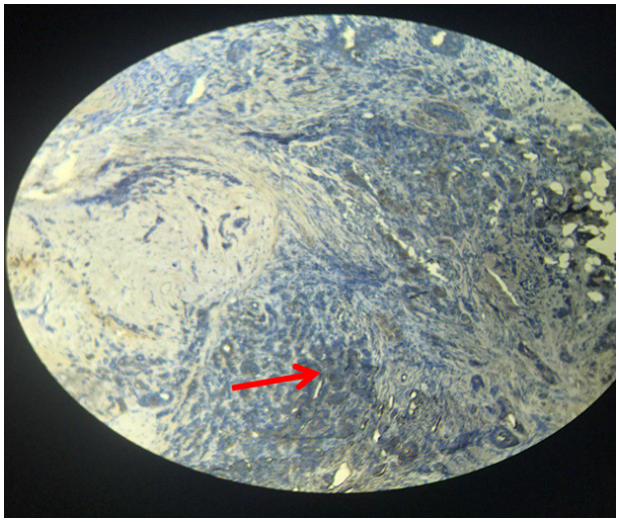

Figure 8: Positive Ghrelin Receptor expression in Malignant tissue (X10)

with proportion of more than 50% which is comparable to results obtained above for Ghrelin Receptor stain but the difference lies in the non-significant difference in the proportions of Ghrelin Receptor expression between benign and malignant tumor tissues where the benign and malignant tissues showed 35% and 25%; respectively with proportion of more than 50% and 65% and 75%; respectively with proportion of less than 50% as tabulated in Table 3 and illustrated more clearly in Figure 3. Figures 4, 5, 6, 7, 8, 9 and 10 illustrated the negative and positive expression of Ghrelin Receptor in all subjected groups.

Immunohistochemical results obtained from all groups revealed that Ghrelin Receptor intensity and its expression proportion were strongly and significantly associated with the type of tissues as demonstrated by Pearson correlation results illustrated in the Table 4 and Chi-square test results postulated in the Table 5. In addition, Ghrelin Receptor expression proportions were strongly associated with the Ghre-

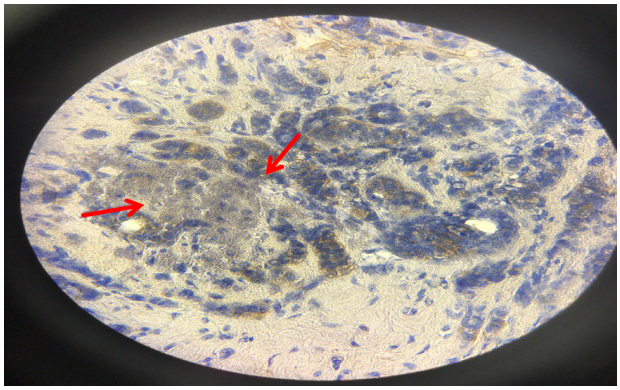

Figure 9: Positive Ghrelin Receptor expression in Malignant tissue (X40)

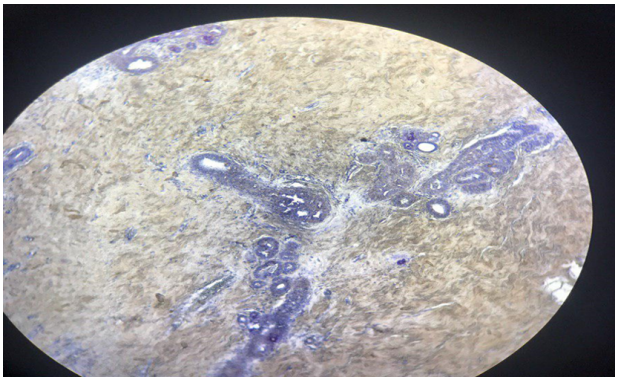

Figure 10: Negative Ghrelin Receptor expression in fibrocystic tissue (X10)

lin Receptor intensity.

Ghrelin is a hormone that possesses multiple physiologic functions which include release promotion of growth hormone in addition to its role in appetite stimulation and energy homeostasis regulation which may be considered as one of the possible causes of elevated level of Ghrelin expression in tumor tissues (Armaou *et al.*, 2009; Au *et al.*, 2017). Using Ghrelin /Ghrelin -receptor agonists as treatment becomes considered as a prospective therapy for malnutrition and disease-related cachexia. In vitro studies reported a high expression rate of Ghrelin in tumors tissue, although its exact role, including its influence on the risk of tumor progression, has not been fully understood and established (Sever *et al.*, 2016).

The present study aimed to compare the expression of the Ghrelin receptor in benign and malignant tumor tissues and also compare them with the normal pattern of expression in fibrocystic breast tissues. The groups of patients subjected to the current study were non-significantly differ from each other in BMI and age that may affect the rate of Ghrelin receptor expression as described in previous literature (Au *et al.*, 2017; Schalla and Stengel, 2018).

Previously documented that Ghrelin receptor may be expressed in normal breast tissues in either male or female, but in the breast tumor tissues, the rate and pattern of this expression become different in accordance with the type of tumor in addition to the therapy used and the response to that particular therapy (Grönberg *et al.*, 2017). These findings were consistent with results obtained in the current study in which a small number of fibrocystic tissues showed a weak stain and an expression proportion exceed 50 %, whereas malignant and benign tumor tissues showed higher stain intensity with an expression proportion exceed 50 % in a considerable number of samples.

An increase in the intensity of Ghrelin receptor stain was observed in benign tumor tissues which are in turn showed to be considerably lower than these values in malignant tissues which is clearly demonstrated in Table 2 and Figure 2 and confirmed by Pearson correlation and Chi-square results displayed in Table 4 and Table 5 that showed strong positive association between the intensity of Ghrelin receptor stain and the type of tissues in the studied groups. These results prove that the intensity of Ghrelin receptor stain can be considered as a promising marker for the diagnosis of tumors and differentiate between malignant and benign ones which is consistent with other studies which reported that Ghrelin was considered as a novel markers for breast tumors and also represent a potential therapeutic targets beside its assumed capability to distinguish between benign and malignant tumors with a specificity and sensitivity of 96% and 90%, respectively (Stefanaki *et al.*, 2012; Omoto *et al.*, 2014; Grönberg *et al.*, 2017).

On the contrary, Ghrelin receptor expression proportion showed a less obvious effect of the type of tumor on proportion values that may indicate that its reliability is less than that of the intensity of Ghrelin receptor stain in the differentiation diagnosis. Table 3 showed that the expression proportions of Ghrelin receptor in tumor (benign and malignant) tissues were considerably higher than that in normal tissues which confirmed by the strong significant correlation and highly significant association results obtained from Pearson correlation and Chi-square data, respectively that illustrated in Table 4 and Table 5 but the count of the tissues that showed an expression more than 50% demonstrate a non-significantly difference between the two tumor types investigated in the present study which indicate that the expression proportion can be used as diagnostic marker for the tumors generally but it may fail to differentiate benign from malignant breast tumors. Actually, the results pre-

sented in Figure 3 showed that the number of samples with an expression proportion of more than 50% in benign tumor tissues were slightly higher than that in malignant tumor tissues which are in agreement with previously reported data which suggest that Ghrelin receptor expression in cancer was lower than in benign tissues (Omoto *et al.*, 2014).

CONCLUSION

In conclusion, the results of the present study revealed that the expression of Ghrelin showed a valuable increase in benign tumor tissues which are in turn showed to be considerably lower than these values in malignant tissues that can be considered as highly significant immunohistochemical marker for the detection of breast tumor and for the differentiation between both types of tumors; benign and malignant.

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Author contributions

Abbas M. Ajeed: Performing laboratory measurements.

Omar F.Abdul-Rasheed: Writing of the manuscript and statistical analysis.

Alaa G. Hussein: Providing patient samples and diagnosing breast tumor patient cases.

Nazar Alwakeel: Providing patient samples and diagnosis.

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