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Original article

# Ceramide expression in relation to breast cancer molecular subtypes in Saudi women

Aliyah Almomen<sup>a,b,1,\*</sup>, Maria Arafah<sup>c,1</sup>, Monira Alwhaibi<sup>d</sup>, Norah Alsaigh<sup>a</sup>, Abdullah Alshememry<sup>b,e</sup>, Nasser B. Alsaleh<sup>f</sup>, Danah Alrabeeh<sup>g</sup>, Khalid Al Saleh<sup>h</sup>, Aws Alshamsan<sup>b,e</sup>, Musaed Alkholief<sup>b,e,\*</sup><sup>a</sup> Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, Riyadh 11495, Saudi Arabia<sup>b</sup> Nanobiotechnology Unit, College of Pharmacy, King Saud University, Riyadh 11495, Saudi Arabia<sup>c</sup> Department of Pathology, College of Medicine, King Saud University, Riyadh 11495, Saudi Arabia<sup>d</sup> Department of Clinical Pharmacy, College of Pharmacy, King Saud University, Riyadh 11495, Saudi Arabia<sup>e</sup> Department of Pharmaceutics, College of Pharmacy, King Saud University, Riyadh 11495, Saudi Arabia<sup>f</sup> Department of Pharmacology and Toxicology, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia<sup>g</sup> Department of Pharmaceutical Services, Prince Sultan Military Medical City, Riyadh 12233, Saudi Arabia<sup>h</sup> Division of Hematology-Oncology, Oncology Center, King Saud University Medical City, Riyadh 11495, Saudi Arabia

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## ABSTRACT

**Background/Introduction:** Despite advances in the diagnosis and management of breast cancer (BC), it is still associated with high mortality rates. New biomarkers are being developed for the diagnosis, treatment, and prediction of responses of BC. Ceramide (CER), a bioactive sphingolipid, has emerged recently as a useful diagnostic tool in several types of tumors. In this study, we evaluated CER expression in invasive BC and assessed its relation to the molecular subtypes of BC.

**Materials and methods:** The clinical data and histopathological slides of 50 patients with invasive ductal carcinoma were retrieved and reviewed. The cases were then stained with a mouse monoclonal anti-ceramide antibody. Pearson correlation was used to assess the correlation between CER percentage and intensity and other clinical and pathological variables.

**Results:** CER expression showed a direct relationship with estrogen and progesterone receptors Allred scores. However, it showed an inverse relation with tumor grade, HER2/neu status and Ki-67 index.

**Conclusions:** CER expression is likely to be associated with luminal BC molecular subtypes. However, more research is needed to confirm these results and to explore its relation to the different clinical outcomes, including response to treatment and prognosis.

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## 1. Introduction

Breast cancer (BC) is the most diagnosed cancer in women affecting 2.26 million women and accounting for 6.9% of cancer

\* Corresponding authors at: Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, Riyadh 11495, Saudi Arabia (A. Almomen). Department of Pharmaceutics, College of Pharmacy, King Saud University, Riyadh 11495, Saudi Arabia (M. Alkholief).

E-mail addresses: [alalmomen@ksu.edu.sa](mailto:alalmomen@ksu.edu.sa) (A. Almomen), [malkholief@ksu.edu.sa](mailto:malkholief@ksu.edu.sa) (M. Alkholief).

<sup>1</sup> Authors contributed equally to this work.

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deaths worldwide in 2020 (Howlader et al., 2018; Sung et al., 2020). Saudi Arabia is no different, where 29.7% of newly diagnosed cancer cases were BC accounting for 11.39% of women cancer-related deaths in 2018 (Alotaibi et al., 2018; Wolff et al., 2018). Although an early diagnosis of BC has been linked to a reduction in mortality and improved survival, inadequate patient awareness of BC's warning signs and screening methods, especially in developing countries, is associated with high mortality rates (Balekouzou et al., 2016; Solikhah et al., 2019; Tazhibi and Feizi, 2014). Resistance to breast cancer treatment is another hurdle making existing treatment fall short in providing adequate therapy (Ji et al., 2019). Moreover, a patient exhibiting resistance to a chemotherapeutic agent will most probably end up being resistant to other agents in a case described as multidrug resistance (MDR) phenotype of BC (Lage, 2003). The overexpression of resistance genes such as Twist, multidrug resistance gene 1 (MDR1),

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adenosine triphosphate binding cassette (ABC) transporters like P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), or the activation of programmed cell death pathways such PI3K/AKT/mTOR and RAS /MAPK/ERK signaling pathway are all linked to BC drug resistance (Lage, 2003; Wind and Holen, 2011) (Lee et al., 2015; Salaroglio et al., 2019).

For better therapy in BC, it is crucial to understand the different aspects of disease development and progression. A balance between the signaling pathways controlling proliferation and apoptosis and normal cell differentiation like estrogen receptors, epidermal growth factor receptor-2 (HER-2/neu), and Canonical Wnt/ $\beta$ -catenin signaling is crucial for the development of normal breast (Parton et al., 2001; Sever and Brugge, 2015). Alterations in signaling pathways induced by mutations in a protooncogene and/or dysregulation of tumor suppressor genes are risk factors for BC development. These genetic alterations are now used as biomarkers for the disease (Feng et al., 2018).

BC is a heterogeneous tumor with a wide range of morphological variants and molecular alterations, eventually leading to different tumor behaviors, presentations, and therapy responses. Therefore, many biological and molecular markers were studied and are now considered vital in diagnosing and managing patients, determining prognosis, and predicting response to treatment. The most commonly used biomarkers are the estrogen receptor (ER), progesterone receptor (PR), and HER-2/neu (Howlander et al., 2018). Other markers include the Ki-67 proliferation index, the urokinase plasminogen activator (uPA), and PAI-1 (Duffy et al., 2017). Although advances in technology have led to the discovery of newer biomarkers, BC's heterogeneity makes a single biomarker not very efficient in providing precise information regarding drug resistance, metastasis and recurrence risks. This heterogeneity highlights the need to explore and combine old and recent biomarkers to better design treatment strategies (Duffy et al., 2017; Weigel and Dowsett, 2010).

In recent years, bioactive sphingolipids have emerged as key molecules involved in many diseases, including cancer (Morales et al., 2007; Pettus et al., 2002; Smyth et al., 1997). Of these sphingolipids, ceramide has gained the greatest attention. Ceramide, a membrane lipid, increases the activation of protein kinases and proteases, activates apoptosis caspases, and impairs some cell organelles, which in turn activates both intrinsic and extrinsic apoptotic pathways (Dany and Ogretmen, 2015; Hait and Maiti, 2017; Lee et al., 1996; Morales et al., 2007; Moro et al., 2018). Therefore, an alteration in the ceramide signaling pathway could enhance cell survival and possibly induces tumorigenesis. The alteration of ceramide species and subsequent enzymes was evident in certain types of cancer such as breast, colon, and prostate and it was proposed to be a useful diagnostic tool in these tumors (Eto et al., 2006; Moro et al., 2018; Separovic et al., 2017).

In this study, we report the potential role and expression levels of ceramide in BC among Saudi patients and assess the presence of any correlation between the expression of ceramide and the molecular subtypes of BC.

## 2. Materials and methods

The records of the Department of Pathology at King Saud University Medical City were examined retrospectively for 50 consecutive cases of invasive ductal carcinoma (IDC) of the breast. This study was conducted under an institutional review board-approved protocol E-20-5119. The search included cases diagnosed with in-house material. The diagnosis of IDC was confirmed for each case, and the type of tumor, grade “modified Scarff-Bloom Richardson (mSBR) grade,” and lymph nodes status (when avail-

able) were reviewed. The age of the patients and follow-up information, wherever possible, were obtained from the electronic files.

The reviewed histopathological material comprised routinely processed and prepared hematoxylin-eosin-stained slides as well as routinely prepared immunohistochemical stains including estrogen receptor (ER: SP1; rabbit monoclonal primary antibody; Ventana), progesterone receptor (PR: 1E2; rabbit monoclonal primary antibody; Ventana), HER-2/neu (HER-2/neu: 4B5; rabbit monoclonal primary antibody; Ventana) and in some cases Ki-67 index (Ki-67: 30–9; rabbit monoclonal primary antibody; Ventana). Unstained coated slides were prepared from formalin-fixed paraffin-embedded tissue blocks and subsequently stained for ceramide using a mouse monoclonal anti-ceramide antibody (MID 15B4; Enzo). External and, in most cases, internal tissue controls were available and were assessed for all stains.

ER and PR immunohistochemical stains were evaluated and scored according to the American Society of Clinical Oncology/College of American Pathologists guidelines (ASCO/CAP) (Allison et al., 2020). All cases with at least 1% of positive cells are classified as receptor-positive. The Allred score (which combines the percentage of positive cells and the stain's intensity) was calculated for all ER and PR positive cases (Allison et al., 2020). The results of HER-2/neu stain were also reported per the ASCO/CAP recommendations (Wolff et al., 2018). Fluorescence in situ hybridization was performed for all HER-2/neu-equivocal cases. Due to the lack of consensus on scoring, the definition of low versus high expression, an appropriate cut-point for positivity, or which part of the tumor

**Table 1**  
Clinicopathological characteristics of invasive ductal carcinoma cases (n = 50).

Age Mean (in years) (SD)	50.1 (11.9)	
Variable	Number	Percentage (%)
<b>Side</b>		
Right	27	54.0
Left	23	46.0
<b>Grade</b>		
Grade 1	1	2.0
Grade 2	19	38.0
Grade 3	30	60.0
<b>Estrogen receptor status</b>		
Positive	28	56.0
Negative	22	44.0
<b>Progesterone receptor status</b>		
Positive	24	48.0
Negative	26	52.0
<b>HER2/neu Status</b>		
Positive	25	50.0
Negative	25	50.0
<b>Ki-67 index</b>		
Not available	28	56.0
20–39	5	10.0
40–59%	10	20.0
>60%	7	14.0
<b>Immunophenotype of breast cancer</b>		
HR-positive, HER2/neu negative	21	42.0
HR-positive, HER2/neu positive	7	14.0
HR negative, HER2/neu positive	16	32.0
Triple-negative	6	12.0
<b>Lymph nodes involvement</b>		
Cases with positive lymph nodes	31	62.0
Cases with negative lymph nodes	19	38.0
<b>Recurrence/Metastases</b>		
Unknown	8	16.0
No recurrence/metastases	27	54.0
Recurrence/metastases	15	30.0
<b>Status at last follow up</b>		
Alive without disease	32	64.0
Alive with disease	11	22.0
Died of disease	7	14.0

HR: Hormonal receptors.

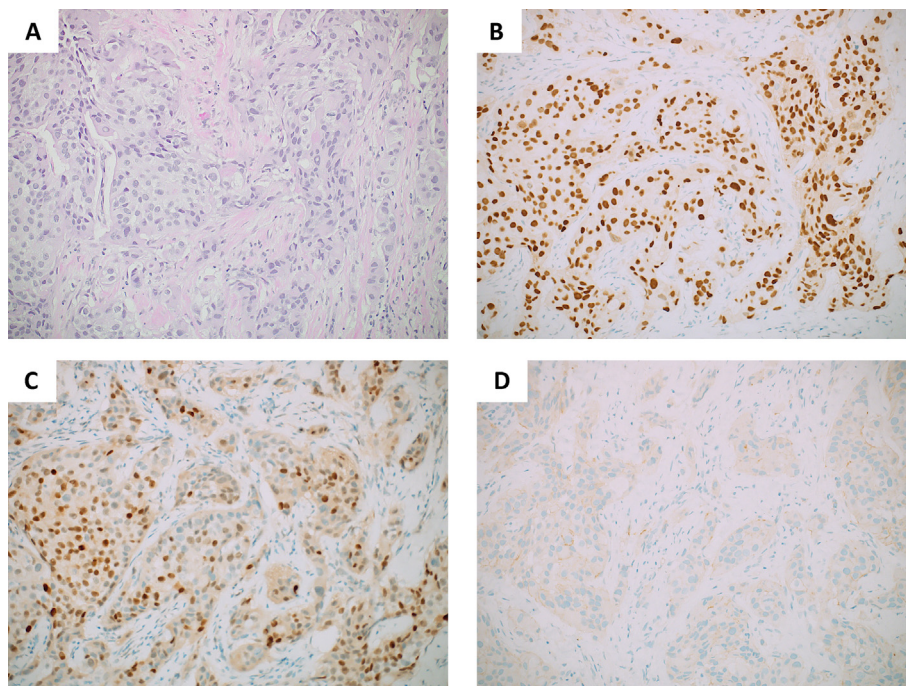
should be scored (e.g., leading-edge, hot spots, overall average), we opted to determine the percentages of Ki-67 positive nuclei subjectively by eye-balling the most proliferative area of the tumor (hot spots) (Dowsett et al., 2011). There is also a lack of data on the appropriate scoring system of ceramide immunohistochemical stain (CER). Thus, we considered any case with at least 1% positive cells as positive. The percentage of positive tumor cells and the intensity of the stain were also recorded.

Descriptive statistics were used to describe the study findings. Means and standard deviations were used to describe continuous data (i.e., age), and numbers and percentages were used to describe

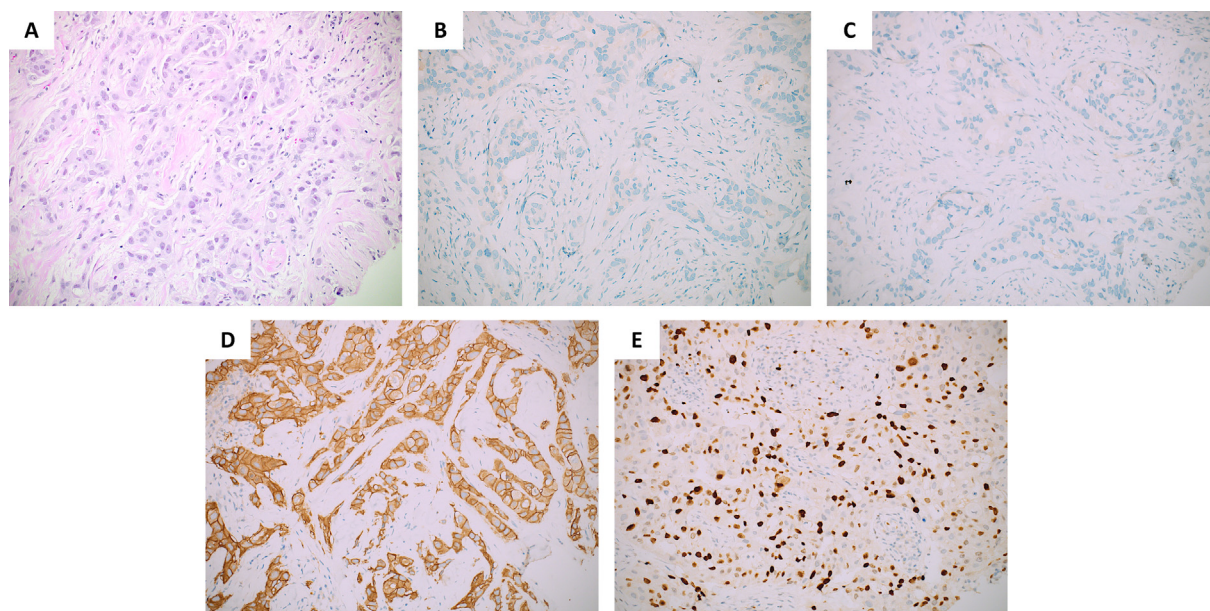
categorical variables (e.g., side, type, grade, etc.). Pearson correlation was used to assess the association between ceramide percentage and intensity and other variables. P-values less than 0.05 were considered statistically significant. All statistical analyses were carried out using SPSS software version 21.0.

### 3. Results

Fifty consecutive cases of IDC were included in this study. All patients were females (age range = 25–80 years, mean = 50.1 years).



**Fig. 1.** A photomicrograph of a hormonal-receptor positive invasive ductal carcinoma (A) (H&E-magnification  $\times 200$ ). The nuclei of tumor cells are positive to both ER (B) and PR (C) stains (ER and PR-magnifications  $\times 200$ ). HER-2/neu stain (D) is negative in this case (HER-2/neu-magnification  $\times 200$ ).



**Fig. 2.** A photomicrograph showing a high grade invasive ductal carcinoma (mSBR grade 3) (A) (H&E-magnification  $\times 200$ ). Tumor cells are negative for nuclear ER (B) and PR (C) stains (ER and PR-magnifications  $\times 200$ ). HER-2/neu stain (D) is positive in the membranes of the tumor cells (HER-2/neu-magnification  $\times 200$ ), and the Ki-67 index (E) is high, reaching up to 60% (Ki-67-magnification  $\times 200$ ).

All patients had unilateral disease (54% had right-sided breast cancer), and one patient had bilateral disease four years apart. Most patients had a diagnosis of IDC, not otherwise specified (94%), while there was a case of IDC apocrine type, a case of IDC micropapillary type, and a case of metaplastic carcinoma. Thirty cases (60%) were high-grade tumors qualifying for an mSBR grade 3. Follow up data (for 36 months) was available for 42 patients (84%). Thirty-one patients (62%) had positive lymph node metas-

tases at the time of diagnosis or during the disease. Fifteen patients (30%) developed recurrence or metastasis, and the most common sites for metastasis were the lung and liver (14%), followed by a recurrence to the chest wall (6%). Table 1 summarizes the clinicopathological characteristics of all cases.

Twenty-eight cases were luminal-type hormonal receptor-positive tumors (56%). Twenty-one cases (42%) were hormonal receptor-positive and HER-2/neu negative (Fig. 1), while 7 cases

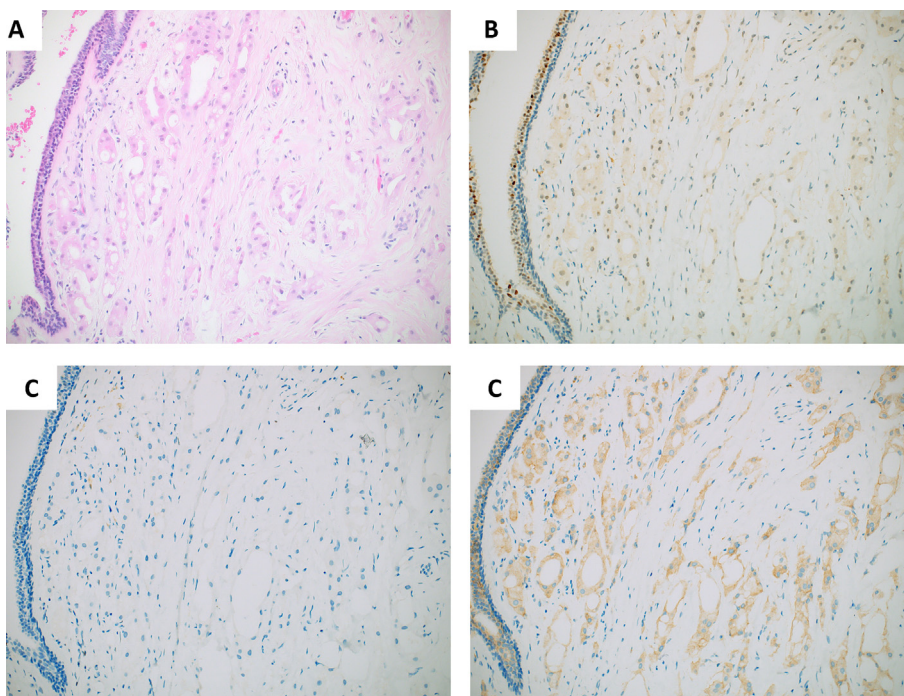


Fig. 3. This panel of images shows an example of a triple-negative invasive ductal carcinoma. (A) The invasive cells are seen adjacent to a benign duct (H&E-magnification  $\times 200$ ). The tumor cells are negative to ER (B), PR (C), and HER-2/neu (D), as proven by FISH (ER, PR, and HER-2/neu-magnifications  $\times 200$ ).

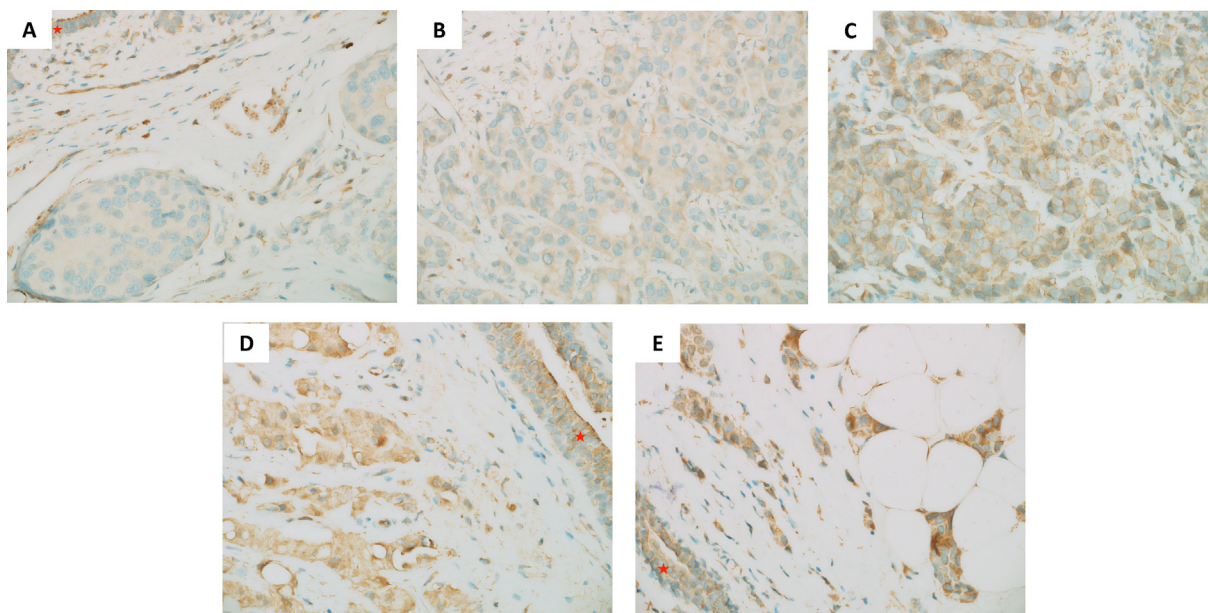


Fig. 4. A series of photomicrographs (all taken at  $\times 400$  magnification) showing the different percentages and staining intensities of ceramide in cases of invasive ductal carcinoma of the breast. (A) Ceramide stain is negative in the invasive tumor cells with positive internal control in the cytoplasm of the surrounding inflammatory cells, endothelial cells, and benign ductal epithelial cells (star). (B–C) Weak ceramide staining is seen in variable proportions of tumor cells “(B)  $<10\%$  of tumor cells and (C) around  $70\%$  of tumor cells”. (D–E) A more intense cytoplasmic staining is seen in these photomicrographs with an intact positive internal control in the benign ductal epithelial cells (stars).

**Table 2**  
Patterns of ER, PR, and CER immunohistochemical stains.

Variable	Number	Percentage %
<b>Estrogen percentage</b>		
No expression	22	44.0
1–10%	1	2.0
11–30%	2	4.0
31–60%	1	2.0
>61%	24	48.0
<b>Estrogen stain intensity</b>		
No expression	22	44.0
Faint/Weak	2	4.0
Moderate	7	14.0
Strong	19	38.0
<b>Progesterone percentage</b>		
Negative	26	52.0
1–10%	3	6.0
11–30%	2	4.0
31–60%	8	16.0
>60%	11	22.0
<b>Progesterone stain intensity</b>		
No Expression	26	52.0
Faint/Weak	0	0.0
Moderate	12	24.0
Strong	12	24.0
<b>Ceramide percentage</b>		
No expression	8	16.0
1–10%	13	26.0
11–30%	10	20.0
31–60%	11	22.0
>61%	8	16.0
<b>Ceramide stain intensity</b>		
No expression	8	16.0
Faint/Weak	33	66.0
Moderate	9	18.0
Strong	0	0.0

ER: Estrogen receptor, PR: Progesterone receptor, CER: Ceramide stain.

(14%) showed positivity to ER, PR, and HER-2/neu. Sixteen cases (32%) were HER-2/neu enriched tumors and showed negative ER and PR staining (Fig. 2). Six cases (12%) were triple-negative breast carcinomas (Fig. 3). Ki-67 index was available for 22 cases (44%), and it was recorded to be  $\geq 20\%$  in all of them. Most cases (84%) showed cytoplasmic expression of CER stain (Fig. 4). The positive stain was seen in variable percentages of tumor cells: 13 cases showed positive staining in 1–10% of tumor cells, 10 cases showed positive staining in 11–30% of tumor cells, 11 cases showed positive staining in 31–60% of tumor cells and 8 cases showed positive staining in most tumor cells (>60%). However, the stain was weak to faint in most cases (66%). Nine cases (18%) showed moderate intensity staining, and there was no strong intensity to CER in any IDC case. Table 2 shows the percentage and intensity of ER, PR, and CER immunohistochemical stains.

Statistical analysis using Pearson correlation showed no statistically significant correlation between the percentage of CER positive tumor cells or CER staining intensity and the evaluated clinicopathological variables. Table 3 shows the statistical correlation between the variables.

#### 4. Discussion

Alterations in the expression of ceramide species and subsequent enzymes were evident in certain types of cancer and have been proposed to be useful diagnostic tools (Moro et al., 2019). The accumulation of ceramide within a cancer cell often indicates that the cell is undergoing apoptosis (Huang et al., 2011). However, this is not always the case since some ceramide species are involved in anti-apoptotic mechanisms (Huang et al., 2011). Although total ceramide levels were reported to be elevated in

**Table 3**  
Correlation between ER and PR Allred scores, HER2/neu status, Ki-67 index, CER percentage and intensity, and other clinicopathological factors.

	ER status	ER Allred Score	PR status	PR Allred Score	HER2/neu Status	Ki-67 Index	CER %	CER Intensity	
<b>Age</b>	Corr.	0.008	−0.013	0.011	0.003	−0.025	0.003	0.046	0.003
	p-value	0.958	0.928	0.937	0.984	0.865	0.984	0.753	0.978
<b>Side</b>	Corr.	0.152	−0.157	0.164	−0.106	−0.040	−0.078	−0.056	0.103
	p-value	0.292	0.275	0.256	0.462	0.782	0.626	0.699	0.476
<b>Grade</b>	Corr.	0.245	−0.244	<b>0.295*</b>	<b>−0.340*</b>	−0.038	0.252	−0.243	−0.137
	p-value	0.086	0.087	<b>0.038</b>	<b>0.016</b>	0.796	0.107	0.090	0.343
<b>ER status</b>	Corr.	<b>1</b>	<b>−0.971**</b>	<b>0.852**</b>	<b>−0.818**</b>	−0.403**	−0.074	−0.088	−0.075
	p-value		<b>0.000</b>	<b>0.000</b>	<b>0.000</b>	0.004	0.642	0.542	0.603
<b>ER %</b>	Corr.	<b>0.732**</b>	<b>−0.581**</b>	<b>0.349*</b>	<b>−0.337*</b>	−0.228	−0.089	−0.034	0.059
	p-value	<b>0.000</b>	<b>0.000</b>	<b>0.013</b>	<b>0.017</b>	0.111	0.574	0.812	0.683
<b>ER Intensity</b>	Corr.	<b>0.831**</b>	<b>−0.693**</b>	<b>0.543**</b>	<b>−0.523**</b>	−0.313*	0.050	0.064	0.025
	p-value	<b>0.000</b>	<b>0.000</b>	<b>0.000</b>	<b>0.000</b>	0.027	0.752	0.656	0.861
<b>ER Allred Score</b>	Corr.	<b>−0.971**</b>	<b>1</b>	<b>−0.927**</b>	<b>0.891**</b>	0.415**	0.089	0.124	0.099
	p-value	<b>0.000</b>		<b>0.000</b>	<b>0.000</b>	0.003	0.574	0.392	0.492
<b>PR Status</b>	Corr.	<b>0.852**</b>	<b>−0.927**</b>	<b>1</b>	<b>−0.961**</b>	−0.400**	−0.085	−0.051	−0.081
	p-value	<b>0.000</b>	<b>0.000</b>		<b>0.000</b>	0.004	0.591	0.723	0.574
<b>PR %</b>	Corr.	<b>0.681**</b>	<b>−0.755**</b>	<b>0.800**</b>	−0.617**	−0.285*	−0.270	−0.097	−0.013
	p-value	<b>0.000</b>	<b>0.000</b>	<b>0.000</b>	0.000	0.045	0.084	0.503	0.931
<b>PR Intensity</b>	Corr.	<b>0.773**</b>	<b>−0.829**</b>	<b>0.908**</b>	−0.777**	−0.339*	−0.151	−0.005	−0.014
	p-value	<b>0.000</b>	<b>0.000</b>	<b>0.000</b>	0.000	0.016	0.339	0.970	0.926
<b>PR Allred Score</b>	Corr.	<b>−0.818**</b>	<b>0.891**</b>	<b>−0.961**</b>	<b>1</b>	0.401**	−0.009	0.048	0.116
	p-value	<b>0.000</b>	<b>0.000</b>	<b>0.000</b>		0.004	0.953	0.741	0.421

(continued on next page)

Table 3 (continued)

		ER status	ER Allred Score	PR status	PR Allred Score	HER2/neu Status	Ki-67 Index	CER %	CER Intensity
<b>Lymph nodes Involvement</b>	value								
	Corr.	−0.013	0.018	−0.017	−0.042	0.050	−0.246	0.130	0.031
	p-value	0.931	0.902	0.905	0.773	0.728	0.116	0.369	0.832
<b>HER2/neu Status</b>	value								
	Corr.	<b>−0.403**</b>	<b>0.415**</b>	<b>−0.400**</b>	<b>0.401**</b>	1	−0.041	−0.098	−0.219
	p-value	<b>0.004</b>	<b>0.003</b>	<b>0.004</b>	<b>0.004</b>		0.798	0.497	0.126
<b>Ki-67 index</b>	value								
	Corr.	−0.074	0.089	−0.085	−0.009	−0.041	1	−0.034	−0.041
	p-value	0.642	0.574	0.591	0.953	0.798		0.830	0.795
<b>Immunophenotype of breast cancer</b>	value								
	Corr.	0.956	0.628	0.943	0.894	0.865	0.184	0.543	0.348
	p-value	0.019	0.038	0.087	0.078	0.050	0.256	0.130	0.031
<b>Recurrence</b>	value								
	Corr.	<b>−0.336*</b>	<b>0.316*</b>	<b>−0.374*</b>	<b>0.365*</b>	0.252	0.188	−0.067	−0.096
	p-value	<b>0.022</b>	<b>0.033</b>	<b>0.011</b>	<b>0.013</b>	0.091	0.259	0.658	0.524
<b>Status at last follow-up.</b>	value								
	Corr.	<b>−0.316*</b>	<b>0.324*</b>	<b>−0.381*</b>	<b>0.359*</b>	0.350*	0.186	0.018	0.042
	p-value	<b>0.044</b>	<b>0.039</b>	<b>0.014</b>	<b>0.021</b>	0.025	0.301	0.913	0.792

CER: Ceramide; Corr.: Pearson Correlation; ER: Estrogen Receptor; PR: Progesterone Receptor; %: Percentage.

\* Correlation is significant at the 0.05 level.

\*\* Correlation is significant at the 0.01 level.

breast cancer (Supplementary table 1), studies have shown that the tumor’s response to chemotherapy and/or radiation is dependent on the accumulation of intracellular ceramides (Moro et al., 2018). More interestingly, the overexpression of ceramide was linked to the upregulation of MDR genes which could eventually lead to the resistance of some cancers to chemotherapy (Huang et al., 2011). Herein, we evaluated the pattern of ceramide expression and its relation to the clinical and pathological variables in BC cases among Saudi patients. Our results indicated an inverse association between the percentage of ceramide expression and the intensity of the stain with tumor grade, HER2/neu status and cell proliferation potency depicted by the Ki-67 expression levels. These results are in concordance with a previous study by Moro et al., which showed that an elevated total ceramide level (C<sub>16:00</sub>-C<sub>26:1</sub>) was correlated with a less aggressive phenotype of BC (Moro et al., 2018). However, this association was not statistically significant and this may be attributed to our small sample size.

It is noteworthy to mention that, to our knowledge, this study is the first study to evaluate the association between the expression of ceramide and ER and PR in BC. Our data shows a direct relation between CER expression and the Allred scores of ER and PR. Moreover, it shows a negative correlation between CER and HER2/neu status. These results suggest that CER expression may be more evident in the luminal subtype of BC (hormonal receptor positive and HER2/neu negative). In addition, our data showed no relation between CER and the clinical characteristics included in this study e.g., age, side of the tumor, the status of lymph nodes and recurrence rates.

Several limitations were encountered in this study. In addition to the small sample size, which may have contributed to the lack of statistical significance, the CER antibody utilized in this study recognizes CER16 and CER24 only and did not cover all the ceramide species and enzymes.

### 5. Conclusions

CER expression is likely associated with luminal BC molecular subtypes and shows a negative correlation with tumor grade, HER2/neu status and Ki-67 levels. There has been no statistical association between CER expression, and the other variables included in this study. However, larger-scale studies should be

conducted to further confirm these results and to explore the relation between CER expression and the different clinical outcomes, including response to treatment and prognosis.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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### Author’s Note

M Arafah is considered a co-first author and contributed equally to the first author.

### Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jsps.2021.04.022>.

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