RESEARCH ARTICLE

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Immunohistochemical Evaluation of Perlecan (Heparan Sulphate Proteoglycan 2) Expression in Invasive Female Breast Carcinoma

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Abstract

Background: Breast cancer (BC) is the commonest type of female cancer worldwide and a leading cause of cancer-related deaths. Perlecan expression increases in aggressive breast cancers, pointing to a possible significance of this novel target therapy. Consequently, the current research investigates the immunohistochemical expression of Perlecan by tumor cells in female breast cancer and the correlation of such expression with the pathologic parameters of the tumors. Methods: A retrospective cross-sectional investigation was carried out. Seventy-four formalin-fixed, paraffin-embedded breast carcinoma tissue samples from patients undergoing modified radical mastectomy (MRM) or conservative breast surgery (CBS) were collected from the pathology department at Kasr El Aini Hospital. The paraffin blocks were sectioned and immunostained with Perlecan, and their expression was investigated in tumor cells and evaluated according to the H-score and classified into low and high expression. The expression was statistically correlated with the clinicopathological parameters of the cases. **Results:** Perlecan expression was low in 41 cases (55.4%) and high in 33 cases (44.6%). It showed a statistically insignificant correlation with all studied parameters, but increased Perlecan expression was directly associated with poor tumor prognostic factors including higher tumor grade, advanced T stage, N3 stage, advanced anatomic stage, high Ki-67 index, positive lymphovascular invasion and perineural invasion, luminal B molecular subtype, and HER2 over-expression. **Conclusion:** Perlecan expression measured by immunohistochemical staining is associated with aggressive characteristics in breast cancer, suggesting that Perlecan may help in cancer progression and can be investigated as a possible target therapy.

Keywords: Perlecan- Heparan- breast cancer- immunohistochemistry- tumor cells.

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Introduction

Breast cancer (BC) is the most common type of cancer diagnosed in females, with over 1.8 million cases diagnosed annually worldwide. It is also the leading cause of cancer related death in women (15%) (Bray et al., 2018).). In Egypt, BC represents 38% of cancers among females. Advanced disease and high mortality rates remain common in Egypt (Abd El Naby et al., 2019).

Histologic classification of breast cancers according to the WHO is based on the growth pattern and cytologic features. There are over 20 histologic types of invasive breast carcinoma. The most common is invasive ductal carcinoma of no special type (IDC-NST), accounting for up to 80% of all invasive breast cancers, followed by invasive lobular carcinomas (ILC); around 10%. The remainder represents less common histologic types, such as mucinous, cribriform, papillary, tubular, apocrine, micropapillary and metaplastic carcinomas (Tsang and Tse, 2020).

The main prognostic factors of breast cancer are age, histological type, grade and stage, as well as expression of estrogen receptor (ER) and progesterone receptors (PR), human epidermal growth factor (HER2) over-expression, ki-67 cellular proliferation index, gene expression signature and molecular subtype (Resende et al., 2019).

The extracellular matrix (ECM) component has an eminent role in the development and the homeostasis of the normal breast. It is also an important structural component of the tumor microenvironment (TMA). It is composed of a network of biochemically distinct components, including proteins (collagen), proteoglycans, glycoproteins and polysaccharides (Nallanthighal et al., 2019).

The extracellular matrix shows changes in organization and amount during breast cancer development. These changes contribute to tumor progression and therapy resistance. Additionally, such ECM deregulation impacts

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the tumor associated stromal cells, including endothelial, immune, and other cells which may come in favor of tumor development (Giussani et al., 2015).

Cancer metastasis is a complex process, regulated spatially and temporally by intrinsic and extrinsic factors. EMA is one of the important extrinsic factors. Heparan sulfate proteoglycans (HSPGs) are constituents of the extracellular matrix. Through their heparan sulfate chains and protein core, they modulate multiple events occuring during the metastatic cascade (Elgundi et al., 2020).

Perlecan is a basement membrane HSPG2. It normally supports the cerebral vasculature by responding to dynamic changes in the cerebral blood flow. It is also a critical proteoglycan found normally in the ECM of cartilage (Trout et al., 2020).

Perlecan is a key contender in promoting carcinogenesis, as it functions as a source of pro-angiogenic factors in the extracellular matrix that provoke neovascularization and tumor development. Studies have shown that Perlecan promotes tumor growth, migration, invasion and chemoresistance, by regulating heparin-binding growth factors such as VEGF-A, FGF-2 and Hedgehog (Hh) in melanoma, prostate carcinoma and oral squamous cell carcinoma, as well as colonic and breast carcinomas (Cruz et al., 2020).

In breast carcinoma, concerning the relation between Perlecan expression and tumor pathological parameters, a weak correlation between Perlecan expression and high tumor grade was observed (Jansson et al., 2020). It was also shown that triple negative breast cancer (TNBC) patients with high HSPG2 expression have a significant poorer survival rate compared to those with low HPSG2 expression, pointing to the possible clinical significance of such novel therapeutic target (Khanna et al., 2019). This work aimed at detection of immunohistochemical (IHC) expression of Perlecan in the tumor cells of female breast carcinoma cases and correlation of such expression with the clinicopathological parameters of the tumors.

Materials and Methods

Cases selection and Material collection:

A retrospective cross-sectional study was performed. Seventy-four formalin-fixed, paraffin-embedded full face tumor tissue sections were collected from modified radical mastectomy and conservative breast surgery specimens of breast cancer patients. These cases were collected from the pathology department at Kasr El Aini Hospital during the period from November 2017 to May 2020. To preserve the patients' privacy, the names of the cases were replaced by an ID number. Only this number was used afterward on the glass slides and in the datasheet.

Exclusion criteria included: Missing data such as the age of the patient or the IHC report; patients performing lumpectomy or simple mastectomy without and axillary sampling; pure ductal carcinoma in situ (DCIS) cases; Patients who received neoadjuvant chemo- or hormonal therapy; cases with equivocal Her2 status, with no available DISH (Dual In situ Hybridization) results.

The data collected from the pathology requests and reports for each case included patient's age, tumor size, lymph node status, as well as the results of ER, PR, HER2 and Ki67.

Histopathololgic examination:

The paraffin blocks of the tumor sections were serially sectioned at 4 μm thickness and stained with hematoxylin & eosin (H&E) stains for routine histopathological examination. The tumors were histologically typed according to the latest available World Health Organization recommendations (5th edition) (Tan et al., 2020). Tumors were divided into three grades according to the extent of pleomorphism, percentage of tubular formation, and Mitotic activity (Zhu et al., 2018). For further statistical evaluation, Grade 1 and 2 cases were lumped as low grade while Grade 3 cases were considered as high grade (Wang et al., 2020). Presence of in situ component, perineural & lymphovascular invasion (LVI) were also detected.

Staging and molecular subtypes

Staging of the cases was performed according to the AJCC TNM staging system (Hortobagyi et al., 2017). During statistical evaluation, stages (T stage and anatomic stage) were divided into early; stages I & II and advanced; stages III & IV (Zhou et al., 2019).

Regarding the molecular subtypes, the tumors were classified into Luminal A (ER-positive, PR-positive, HER2-negative, and low Ki-67 index), Luminal B-HER2 negative (ER-positive, HER2-negative and either low PR or high ki-67 index), Luminal B-HER2 positive (ER-positive, HER2-positive, any ki-67 index, and any PR), Triple negative (ER, PR, and HER2 negative with any Ki-67 index & HER2neu enriched (HER2-positive and ER and PR negative with any Ki-67 index). The used cut-off of Ki-67 to designated the tumor as Luminal A or B was 20% (the threshold voted for by most of the panel in the St. Gallen 2013. Additionally, luminal cases having high histologic grades were considered as luminal B according to St. Gallen International Expert Consensus 2017 recommendations.

Immunohistochemistry Evaluation

For immunostaining, additional section was cut on charged slides. Antigen retrieval was performed with citrate buffer pH 7.4 in an automated water bath (DAKO PT link). Sections were stained for anti-Perlecan antibody (rabbit polyclonal, IgG) manufactured by Abbexa Ltd, Cambridge, UK, with a dilution of 1:50. Immunohistochemistry staining was performed in a Dako autostainer link 48. Diaminobenzidine (DAB) was used as chromogen and Hematoxylin as counterstain. Sections of normal skin were used as a positive control according to the manufacturer's recommendation and as a negative control, a tumor tissue section was processed in the same setting, without adding a primary antibody.

Perlecan immunostaining interpretation

Perlecan positive results showed brownish staining in the cytoplasm and membrane of tumor cells. Due to the heterogeneity of Perlecan expression levels, we used the H-scoring system which is obtained by the formula: (3 x percentage of strongly staining cells + 2 x percentage

of moderately staining cells + 1 x percentage of weakly staining cells + 0 x percentage of negatively staining cells) giving a range of 0 to 300, then we defined "high expression" if the score was above the mean value and "low expression" if the score was equal to or below the mean value (Rangel et al., 2018).

Statistical analysis

Microsoft Excel 2010 was used for data entry and the statistical package for social science (SPSS version 25) was used for data analysis. Frequencies were used to summarize the qualitative data. A comparison of proportions was performed using the chi-square test. A P value of ≤ 0.05 was used to consider statistical significance.

Slides Screening And Imaging

The slides were screened using a Leica DM500 microscope. Microscopic photos were captured using a digital camera attached to the microscope.

Results

The age of our cases ranged between 31 and 85 years old with mean age of about 57 years. Concerning the histologic types, IDC NST was the commonest (55%), Figure 1. As for the histologic grade, high grade cases took the upper hand (63.5%). Regarding the T stage, early disease was the more common (74.3%). Concerning the N stage, 29.7% of the cases were N2. LVI and perineural invasion were detected in 48.6% and 24.3% of the cases respectively. ER was positive in 48.6% of the cases, PR was positive in 37.8% of the cases, Her 2 neu was over-expressed in 14.9% of the cases and half of the cases (50%) showed high ki-67 proliferation index.

The pathological data of the cases are summarized in Table 1. Perlecan immunohistochemical expression was low in 41 cases (55.4%) and high in 33 cases (44.6%), Figure 2. Regarding the relation between the histologic types and Perlecan expression, IDC showed 41.5% cases with high expression. ILC showed 50% cases with high expression. Mixed IDC and ILC showed 57.1% cases with high expression. All 5 cases of mucoid carcinoma (100%) showed low expression (Figure 3). The single tubular carcinoma case (100%) showed low expression. All metaplastic carcinoma cases (4) showed high expression (100%). So, the highest expression of Perlecan was found in the more aggressive metaplastic carcinoma and the lowest expression was found in the more indolent subtypes (mucoid and tubular).

As for the relation between the histologic grade and Perlecan expression, high grade cases showed higher Perlecan expression than low grade cases (55.6% of high grade cases showed high expression versus 38.3% of low grade cases showed high expression). Concerning the relation between the T stage and Perlecan expression, 57 % of the advanced staged tumors showed high Perlecan expression versus 40% for the early staged tumors. Regarding the relation between the N- stage and Perlecan expression, 50% of node-negative cases showed high Perlecan expression while 42.9% of node-positive cases showed high Perlecan expression. N1 tumors showed 8 cases (38.1%) with high expression. N2 tumors showed 7 cases (31.8%) with high expression. N3 tumors showed 9 cases (64.3%) with high expression, so, N3 tumors showed the highest Perlecan expression. As for the anatomical staging, 45.9% of early disease cases showed high expression while 56.8% of advanced disease cases showed low expression.

Regarding the relation between the presence of LVI

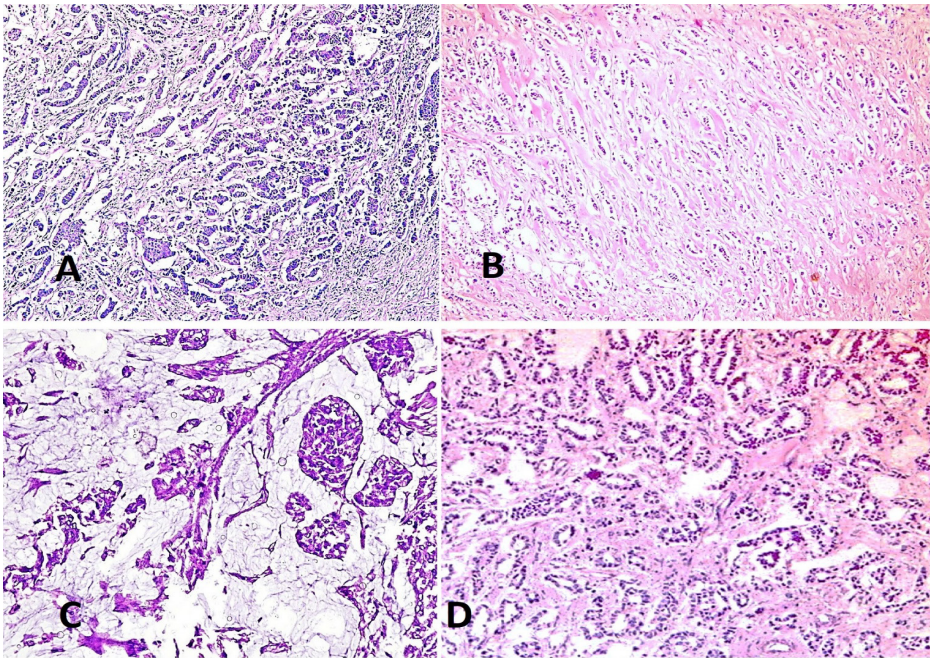

Figure 1. Various Histological Breast Cancer Subtypes Stained by H&E: (A) Invasive duct carcinoma of no special type (X40 original magnification), (B) Invasive lobular carcinoma (X40 original magnification), (C) Mucoid carcinoma (X100 original magnification), and (D) Tubular carcinoma (X40 original magnification).

Table 1. The Pathological Data of the Collected Cases

Parameter		Number (%)
Histological type	IDC-NST	41 (55.4%)
	ILC	16 (21.6%)
	Mixed Duct And Lobular	7 (9.5%)
	Mucinous Carcinoma	5 (6.8%)
	Metaplastic Carcinoma	4 (5.4%)
	Tubular carcinoma	1 (1.3%)
Histological grade	Low	27 (36.5%)
	High	47 (63.5%)
T stage	Early (T1and T2)	55 (74.3%)
	Advanced (T3 and T4)	19 (25.7%)
Lymph node Metastasis	Negative	17 (23%)
	N1	21 (28.4%)
	N2	22 (29.7%)
	N3	14 (18.9%)
Anatomic Stage	Early [I and II)	37 (50%)
	Advanced [III and IV)	37 (50%)
ER	Positive	36 (48.6%)
	Negative	38 (51.4%)
PR	Positive	28 (37.8%)
	Negative	46 (62.2%)
HER2	Positive	11 (14.9%)
	Negative	63 (85.1%)
Ki-67 index		
	Low	37 (50%)
	High	37 (50%)
BC Subtypes	Luminal A Like	27 (36.5%)
	Luminal B Like	16 (21.6)
	HER2 positive	10 (13.5%)
	Triple negative	21 (28.4%)
LVI	Positive	36 (48.6%)
	Negative	38 (51.4%)
Perineural invasion	Low	56 (75.7%)
	High	18 (24.3%)
Perlecan expression	Low	41 (55.4%)
	High	33 (44.6%)

IDC-NST, Invasive Duct carcinoma of no special type; ILC, Invasive lobular carcinoma.

and Perlecan expression, the LVI-positive cases included 50% with high Perlecan expression. The LVI negative cases included 39.5% with high Perlecan expression. So, LVI-positive cases showed higher expression than LVI-negative cases. By studying the relationship between the ER status and Perlecan expression, our study showed that higher expression was found in ER-positive cases. The collected PR negative cases showed slightly higher expression than the PR positive ones. Concerning the relation between the HER2 neu status and Perlecan expression, it was found in that Her2 overexpressing cases showed slightly higher expression of Perlecan. By studying the relation between the Ki-67 proliferation index and Perlecan expression, it was found that higher expression was found in cases with a high Ki-67 proliferation index. The relation between the molecular subtypes and Perlecan expression was studied. Luminal A, Luminal B, Her2 enriched and Triple negative cases showed subtype showed 44.4%, 56.3.7%, 50% and 33.3% Perlecan highly expressed cases respectively. So, the highest Perlecan expression was detected in Luminal B cases. The pathologic characteristics of the studied cases stratified by Perlecan expression are summarized in Table 2.

Discussion

The tumor microenvironment (TME) is rich in matrix components, cytokines and growth factors that respond to changing conditions to alter the properties of the tumor bed. Proteoglycans affect multiple cellular and molecular processes during tumors progression. Perlecan /HSPG2, a large, multi-domain heparan sulfate proteoglycan, was found to be a participant in abnormal signaling that enhances tumor progression, invasion, and metastasis, through its ability to affect tumor growth, angiogenesis, vessels integrity, endothelial proliferation, tumor cell adhesion, and motility (Cruz et al., 2020). In this study, we examined Perlecan immunohistochemical expression in breast cancer cells which was found to be low in 41 cases (55.4%) and high in 33 cases (44.6%). This was consistent with Rangel et al., (2018), yet slightly different from what Kazanskaya et al., (2018) found, where cases with high expression took the upper hand (55%). Regarding the tumor grade in this study, high grade tumors showed higher rate of expression than low grade tumors. This

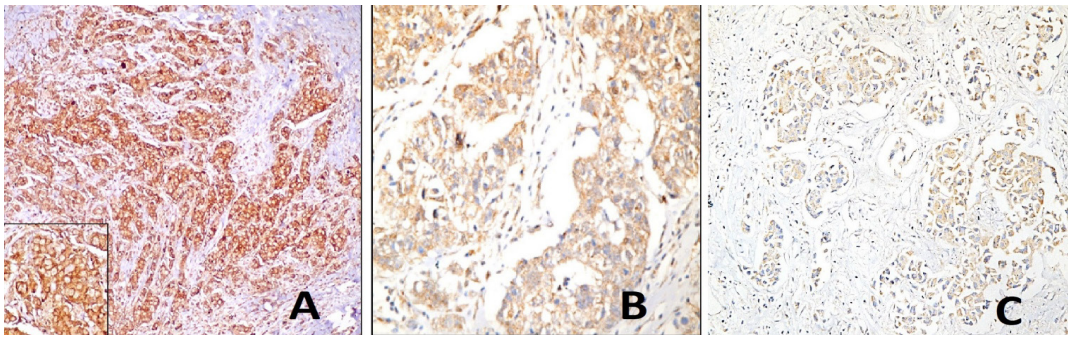

Figure 2. Different Instensities of Perlecan Expression by Immunohistochemistry: (A) strong (X100 original magnifications), (B) moderate (X200 original magnification), and (C) weak (X100 original magnification).

Table 2. The Pathologic Characteristics Correlated with Perlecan Protein Expression

Parameter		Perlecan		P value
		High (% within parameter)	Low (% within parameter)	
Histological Type	IDC-NST	17 (41.5%)	24 (58.5%)	0.06
	ILC	8 (50%)	8 (50%)	
	Mixed (IDC & ILC)	4 (57.10%)	3 (42.90%)	
	Mucoid	-	5 (100%)	
	Tubular	-	1 (100%)	
	Metaplastic	4 (100%)	-	
Histological grade	Low	18 (38.3%)	29 (61.7%)	0.151
	High	15 (55.6%)	12 (44.4%)	
T Stage	Early (T1+T2)	22 (40%)	33 (60%)	0.176
	Advanced (T3+T4)	11 (57.9%)	8 (42.1%)	
Lymph node metastasis	N0	8 (50%)	8 (50%)	0.596
	N1	8 (38.1%)	13 (61.8%)	
	N2	7 (31.8%)	15 (68.2%)	
	N3	9 (64.3%)	5 (35.7%)	
Anatomic Stage	Early (I and II]	17 (45.9%)	20 (54.1%)	0.55
	Advanced (III and IV]	16 (43.2%)	21 (56.8%)	
ER	Positive	19 (52.8%)	17 (47.2%)	1.9
	Negative	14 (36.8%)	24 (63.2%)	
PR	Positive	12 (42.9%)	16 (57.1%)	0.815
	Negative	21 (45.7%)	25 (54.3%)	
HER2	Positive	5 (45.5%)	6 (54.5%)	0.95
	Negative	28 (44.4%)	35 (55.6%)	
Ki-67 index	Low	15 (40.5%)	22 (59.5%)	0.483
	High	18 (48.6%)	19 (51.4%)	
BC Subtypes	Luminal A Like	12 (44.4%)	15 (55.6%)	2.076
	Luminal B Like	9 (56.3%)	7 (43.7%)	
	HER2 positive	5 (50%)	5 (50%)	
	Triple negative	7 (33.3%)	14 (66.7%)	
LVI	Positive	18 (50%)	18 (50%)	0.363
	Negative	15 (39.5%)	23 (60.5%)	
Perineural invasion	Low	22 (39.3%)	34 (60.7%)	0.105
	High	11 (61.1%)	7 (38.9%)	

agreed with Jansson et al., (2020) and kalscheuer et al., (2019) who stated that high-grade tumors had a stronger expression of Perlecan

As for the T stage, advanced stage tumor showed higher rate of Perlecan expression than early stage tumors. This result was consistent with what was stated in Kalscheuer et al., (2019) that there is significant increase in Perlecan expression with tumor stage. However, this may oppose what was reported in Jansson et al., (2020) which stated that Perlecan expression did not differ with T-stage.

In our study, on dividing the cases into lymph node negative and positive, it was found that 50% of node-negative cases showed high Perlecan expression while 42.9% of node-positive cases showed high Perlecan expression. N3 tumors showed the highest Perlecan expression. However, Jansson et al., (2020) reported that Perlecan expression did not differ in various node statuses. As for the anatomical staging, 54.1% of early disease cases showed low expression while 56.8% of advanced disease cases showed low expression. However, this relation was not studied before in other studies.

In this study, LVI-positive cases showed higher Perlecan expression than LVI-negative cases, compatible with our reported higher Perlecan expression in cases with positive lymph node metastasis. This was consistent with Fejza et al., (2021), who found increased Perlecan

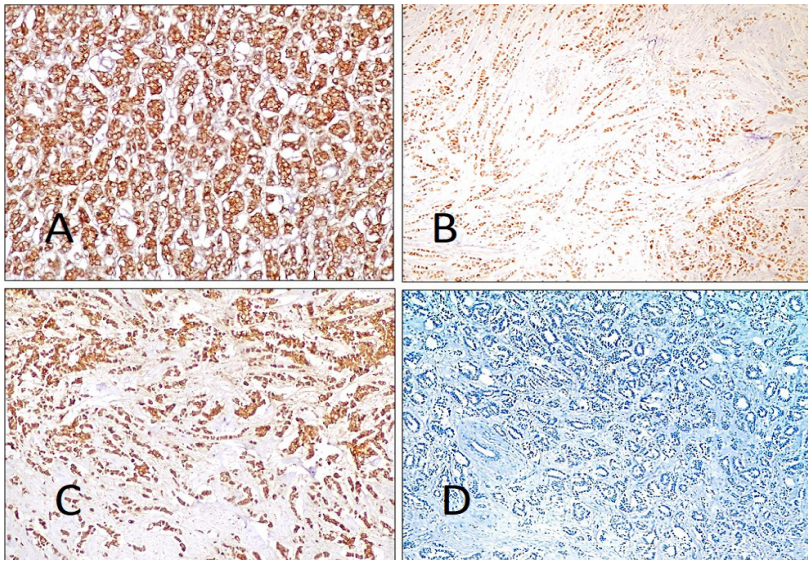

Figure 3. Perlecan Immunohistochemical Expression in Different Histologic Types of Breast Carcinoma: (A) A case of IDC-NST showing strong Perlecan expression (X100 original magnification), (B) A case of ILC showing strong Perlecan expression (X40 original magnification), (C) A case of mixed IDC & ILC showing strong Perlecan expression (X100 original magnification) and (D) A case of tubular carcinoma, low grade showing negative Perlecan expression (X100 original magnification).

expression in LVI positive cases.

Regarding the ER status, our study showed that higher expression was found in ER-positive cases. The collected PR positive cases showed slightly higher expression than the PR negative ones However, Jansson et al., (2020) stated that Perlecan expression did not differ between neither between ER negative and positive cases nor the PR negative and positive cases.

Concerning the HER2 neu expression, it was found in our study that Her2 overexpression cases showed slightly higher expression of Perlecan. However, to our knowledge, this relation was not discussed before in other studies. As for the Ki-67 proliferation index, higher expression was found in cases with a high Ki-67 proliferation index keeping with our detected higher Perlecan expression in high grade cases. However, this was opposite to the study of Kazanskaya et al., (2018) which showed higher Perlecan expression in low Ki-67 index tumors, suggesting that Perlecan expression in tumor cells is not associated with their proliferative activity. Concerning the molecular subtypes, our study showed that the highest Perlecan expression was detected in Luminal B cases. However, Jansson et al., (2020) showed that there was no difference in Perlecan expression as regarding the molecular subtypes of the tumor.

In conclusion, although Perlecan immunohistochemical expression did not reach statistical significance with any of the studied parameters, we observed higher expression in cases with poor prognostic factors, including high grade, more advanced stage with nodal metastasis and LVI, as well as cases with Her2 neu overexpression and higher Ki-67 proliferation index. A limitation of our study is the lack of detection of Perlecan high expression impact on the patients' prognosis and survival. Further studies addressing such point is required to resolve this controversial issue in the literature.

Author Contribution Statement

Dr. Samar Ibrahim Amer Ismail: Study design, Literature search, immunostaining, slides examination, data analysis and statistical analysis. Dr. Ahmed Mahmoud Abdel Aziz: Cases collection and data acquisition. Dr. Passant Essam Eldin Shibel: Literature search, slides examination and manuscript preparation.

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