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Histopathology and Cytology

Immunohistochemical Expression of Fn14 and Cath-D as Prognostic Biological Markers and Compare with Histological Reaction in Invasive Human Breast Cancer

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Abstract **Original Research Article**

Fibroblast growth factor-inducible-14 (Fn14) is a 14-kDa type I transmembrane receptor located on chromosome 16p13. Fn14 is a member of the tumor necrosis factor receptor super family that normally expressed in healthy tissues, but its expression is increased in injured tissue where it thought to play role in tissue remodeling. Cathepsin D (Cath-D) is a soluble lysosomal aspartyl glycoprotease that can degrade the protein components of the matrix and free growth factors therein embedded, thus favoring tumor growth, invasion and angiogenesis. The aim of the present work was to investigate the expression of Fn14 and Cathepsin D as novel prognostic biomarkers in human invasive ductal carcinoma (IDC) versus benign tumors and normal breast tissues as well as their correlation with different pathological and histological parameters. Immunohistochemical technique was used to examine the expression of Fn14 and Cath-D in normal, benign as well as in IDC. Present results showed higher expression of Fn14 and Cath-D in IDC comparing to normal and benign breast tissues.

Keywords: Fn14 and Cathepsin D prognostic marker.

INTRODUCTION

Breast cancer is the most frequently diagnosed cancer and a second leading cause of cancer death in women worldwide [1]. Breast cancer represents a major scientific, clinical and societal problem. It is the most common malignancy and the second leading cause of cancer death in females following lung cancer [2] with more than 1,000,000 new cases and 370,000 deaths yearly worldwide [3]. In many developing countries, the incidence of breast cancer is now rising sharply due to changes in reproductive factors, lifestyle, and increased life expectancy [4].

Fibroblast growth factor-inducible-14 (Fn14) is the smallest member of the tumor necrosis factor (TNF) super family of receptors identified so far [5]. It is a type I trans-membrane receptor of a tumor necrosis factor likes weak inducer of apoptosis (TWEAK). Fn14 binding to its ligand (TWEAK) induces receptor trimerization, tumor necrosis factor receptor associated factors (TRAF) association with the cytoplasmic tail and activation of intracellular signaling cascades such MAPK, and NF-κB which seems to be a universal cellular response [6].

Cathepsin D (Cath-D) is a soluble lysosomal aspartyl glycoprotease [7] that can degrade the protein components of the matrix and free growth factors therein embedded, thus favoring tumor growth, invasion and angiogenesis. The aspartic protease Cath-D, a poor prognostic indicator of breast cancer, is abundantly secreted as pro-Cath-D by human breast cancer cells and self-activates at low pH in vitro, giving rise to catalytically active Cath-D [8].

MATERIAL AND METHODS

Tissue samples were obtained from patients diagnosed with breast tumors in the Department of Pathology, Medical Research Institute, Alexandria University, Egypt. Formalin-fixed and paraffin embedded tissue specimens from 60 patients diagnosed with IDC, 30 patients diagnosed with benign breast tumor and 10 were taken from normal breast tissue adjacent to the tumors were included. All the cases were asked to freely volunteer to the study and informed written consents were gathered prior to their inclusion in the study. Hematoxlin and eosin (H&E) stained slides for each patient were reviewed by two pathologists. Diagnosis of the specimens was made according to the WHO classification of the Tumors. Clinical parameters included patients' age, tumor size, lymph node metastasis (LNM).

Immunohistochemical investigation of Fn14, Cath-D

Immunohistochemical method was utilized to study the expression of Fn14 and Cath-D in 60 paraffin-embedded breast tissues. In brief, paraffin-embedded specimens were cut into 5µm thick sections. The sections were deparaffinized using 2 changes of xylen and rehydrated. The sections were submerged in an antigen retrieval (citrate buffer saline pH 6) in an oven at 95°C for 20 minutes and then left at room temperature for 20 minutes to cool. The sections were treated with 3% H_2O_2 in PBS to quench the endogenous peroxidase activity, and then incubated with serum blocking reagent for 30 minutes to block nonspecific binding. The sections were incubated with primary antibody for Fn14 and Cath-D (Biorbyt Company, London, UK) at 4ºC overnight. Sections were treated with conjugated 2nd antibody (ABC-HRP reagent) for 30 minutes, stained with diaminobenzedine (DAB) and counter stained with hematoxylin. For negative controls, antibody was replaced with PBS. Each step was followed by PBS washing. Evaluation of Fn14 and Cath-D immunohistochemical results was arbitrarily graded as negative (0), weak (+1), moderate (+2) and strong (+3).

Statistical Analysis

Data were normally distributed according to the Kolmogorov-Smirnov (K-S) normality test, and then analyzed using statistical software package SPSS 20. P values≤ 0.05 were considered statistically significant.

RESULTS

1-Hormonal status of the studied cancer cases:

a. Estrogen Receptor (ER) status:

According to the immunostaining results illustrated in table (1) and figure (1), 40% (18/45) of IDC grade II cases were ER moderate positive (2+), while 46% (6/13) of grade III were ER weak positive (1+).

Table 1: Estrogen Receptor (ER) distribution among breast cancer grades

Estrogen Receptor (ER)	Grade II		Grade III	
	No	%	No	%
Negative (-ve)	5	11	3	23
Weak positive (1+)	10	22	6	46
Moderate positive (2+)	18	40	3	23
Strong positive (3+)	12	27	1	8
Total	45	100	13	100
X^2 = 5.6 , p = 0.14 (statistically not significant)				

X^2: Chi square test

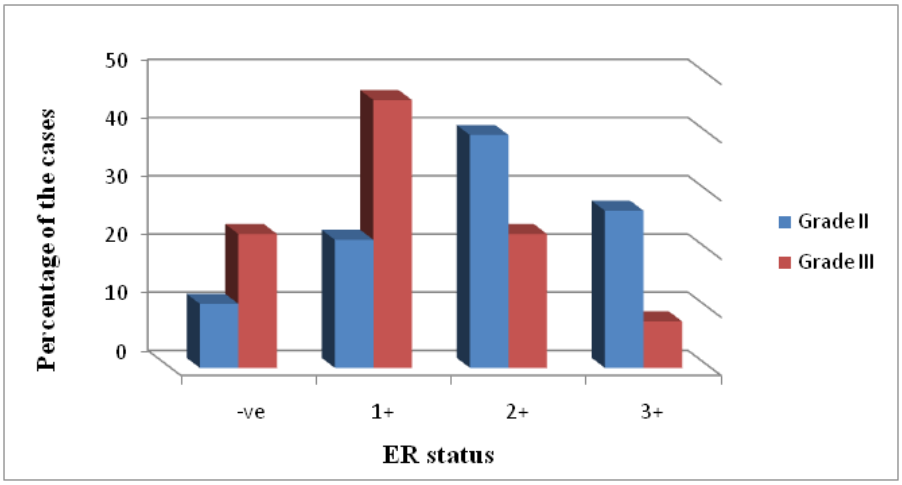

Figure 1: Distribution of estrogen receptor (ER) among breast cancer cases

b. Progesterone Receptor (PR) status:

Immunostaining results of PR showed that 46% **(**21/45) of IDC grade II were PR moderate positive (2+), while 54% (7/13) of grade III were weak positive (1+) (Table 2 and Figure 2).

Table 2: Progesterone Receptor (PR) distribution among breast cancer cases

Progesterone Receptor (PR)	Grade II		Grade III	
	No	%	No	%
Negateve (-ve)	7	16	3	23
Weak positive (1+)	8	18	7	54
Moderate positive (2+)	21	46	2	15
Strong positive (3+)	9	20	1	8
Total	45	100	13	100
X^2 = 5.04 p = 0.2 (statistically not significant)				

X^2: Chi square test

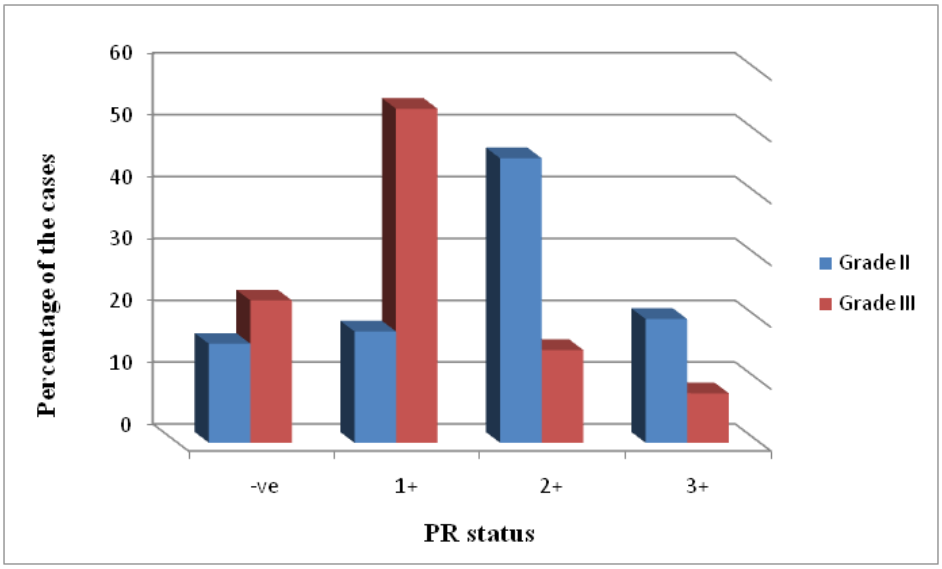

Figure 2: Progesterone receptor (ER) distribution among malignant group cases

2-Epidermal growth factor receptor-2 (HER2\neu) status:

According to the results of HER2\neu expression, 53% (24/45) of IDC grade II were weak positive (-ve), while 38% (5/13) of IDC grade III were strong positive (3+), as shown in Table (3) and Figure (3).

Table 3: HER2\neu distribution among breast cancer cases

HER2/neu status	Grade II		Grade III	
	No	%	No	%
Negateve (-ve)	8	18	2	15
Weak positive (1+)	24	53	2	15
Moderate positive (2+)	9	20	4	31
Strong positive (3+)	4	9	5	38
Total	45	100	13	100
X2 = 9.5 , p = 0.02 (statistically not significant)				

X^2: Chi square test

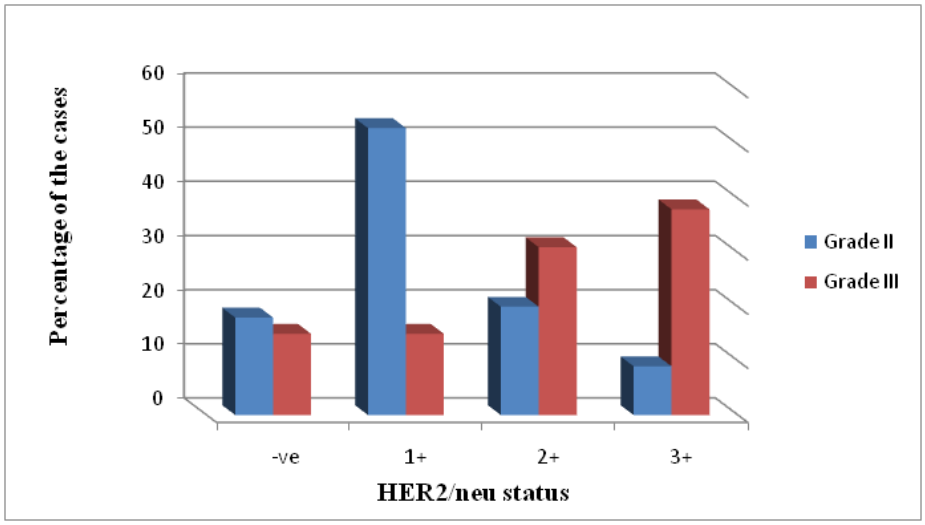

Figure 3: HER2\neu distribution among breast cancer cases

B-Histopathological results:

a. Haematoxylin and Eosin (H&E) staining.

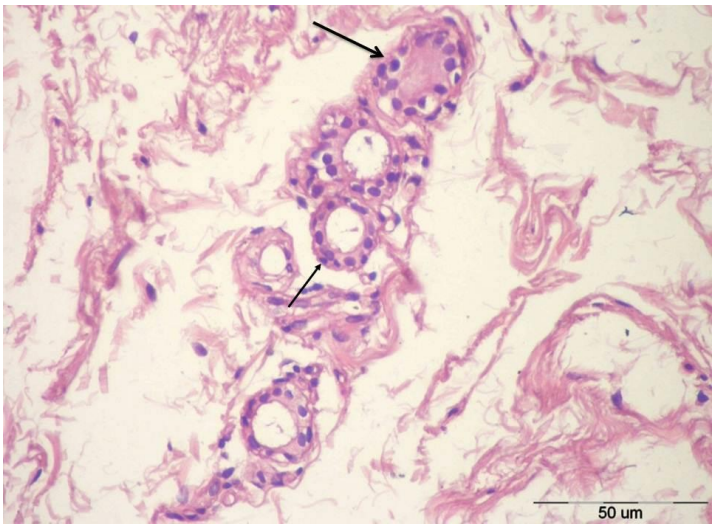

Figure 4: A view of the acini present in a normal lobule. The acini are lined by cuboidal epithelium (thick arrow) with underlying myoepithelial cells having clear cytoplasm (thin arrow) (H & E. Bar =50 µm)

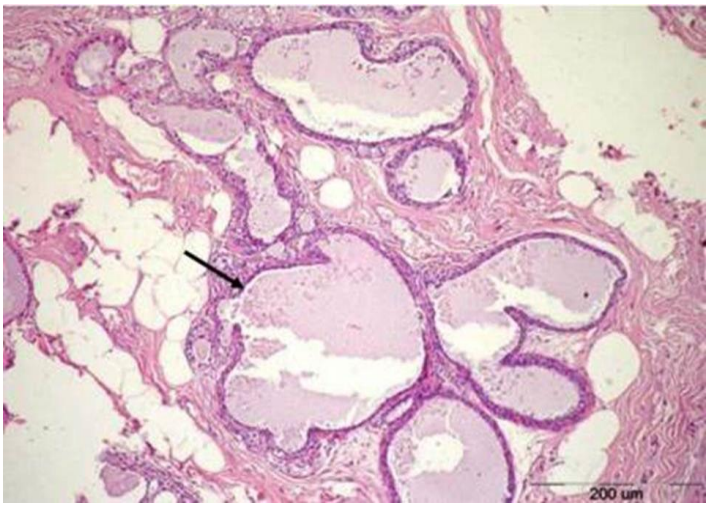

Figure 5: A section of fibrocystic disease with cyst formation (H & E. Bar = 200µm)

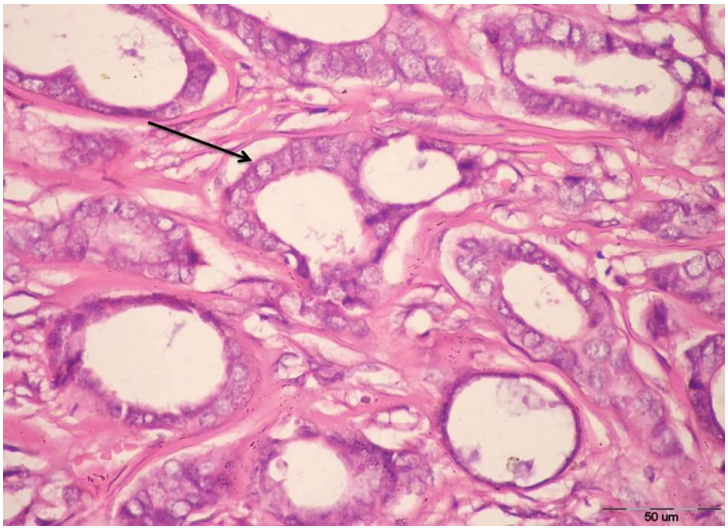

Figure 6: A section of IDC grade I showing well-defined ducts lined by cuboidal epithelial cells with vesicular nuclei (H&E. Bar =50 µm)

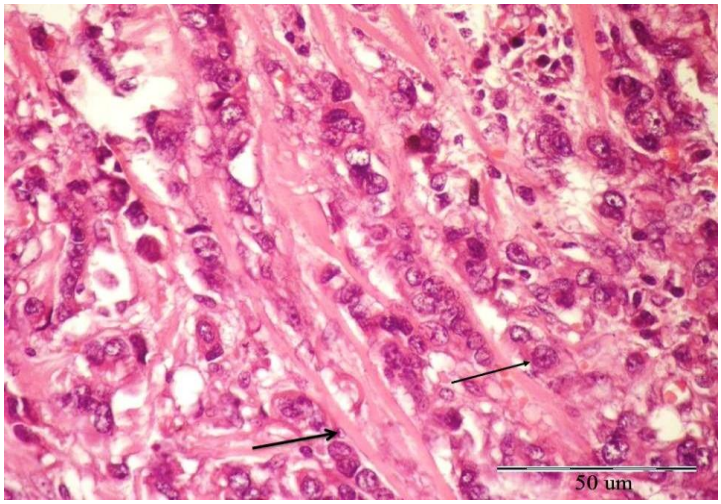

Figure 7: A section of IDC grade II showing tumor cells with abundant eosinophlic cytoplasm and pleomorphic round to ovoid vesicular nuclei (thin arraow). The cells which are arranged in cords infiltrate the desmoplastic stroma (thick arrow) (H&E. Bar =50 µm)

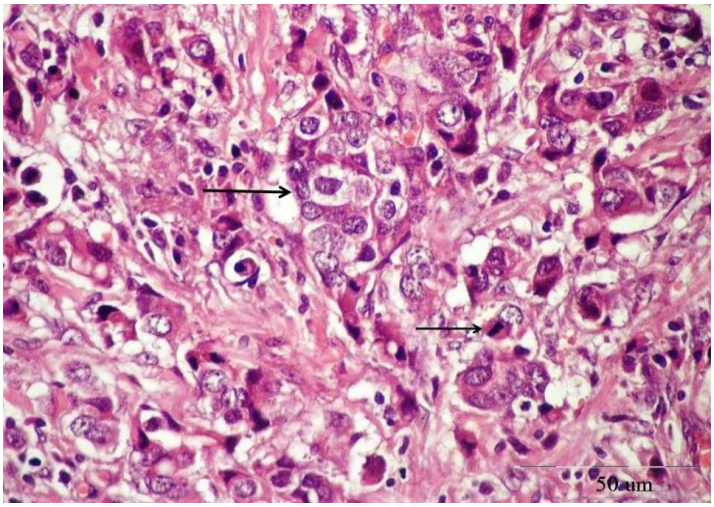

Figure 8: A section of IDC grade III showing solid nests (thick arrow) of tumor cells with large pleomorphic nuclei and some prominent nucleoli.There are numerous mitotic figures (thin arrow) (H&E. Bar=50 µm)

b. Periodic acid-Schiff (PAS) staining for basement membrane:

In the present study, the results of PAS staining showed a well-defined and continuous basement membrane (BM) surrounding the breast ducts and lobules of the control and benign tumors (fibroadenoma and fibrocystic disease), while malignant tumors exhibited fragmented or completely absent BM.

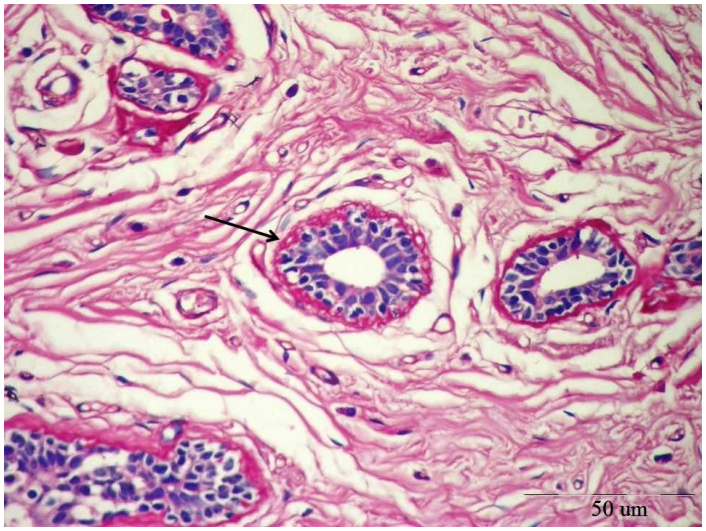

Figure 9: A PAS stained control breast tissue showing intact BMs around the acini (arrow) (Bar=200 µm)

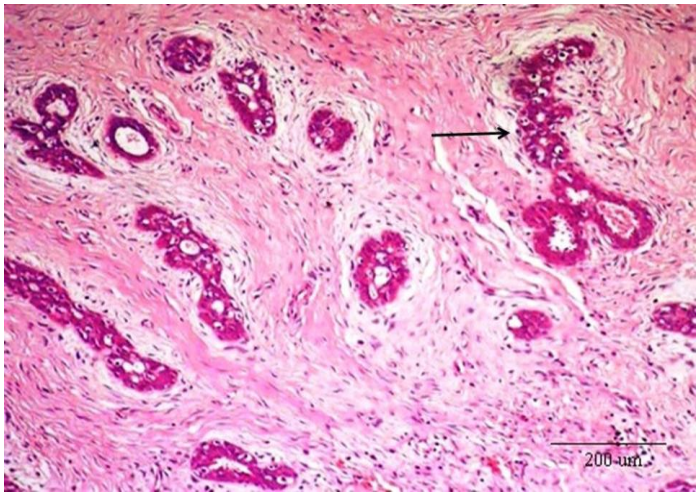

Figure 10: A PAS stained fibroadenoma section showing a continuous and well-defined BM (Bar=200 µm)

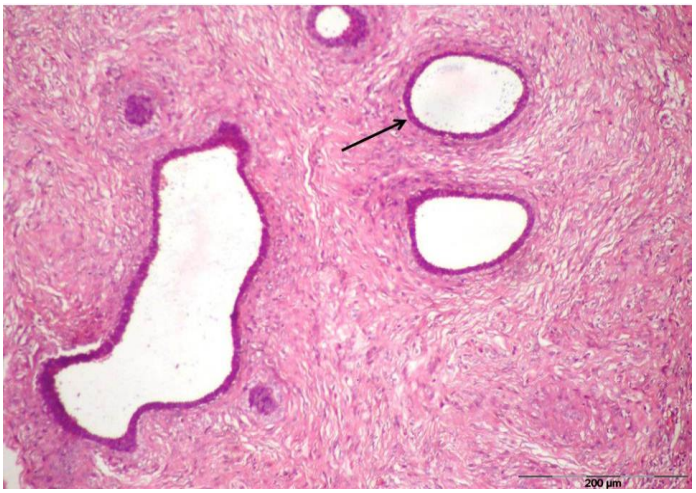

Figure 11: A PAS stained fibrocystic disease section showing a continuous and well-defined BM (Bar=200 µm)

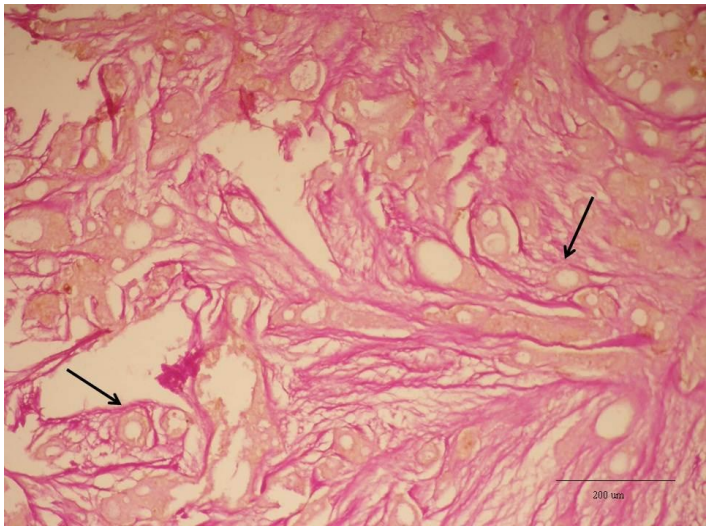

Figure 12: A PAS stained grade I IDC tissue showing partially detached BM (arrow) (Bar=200 µm)

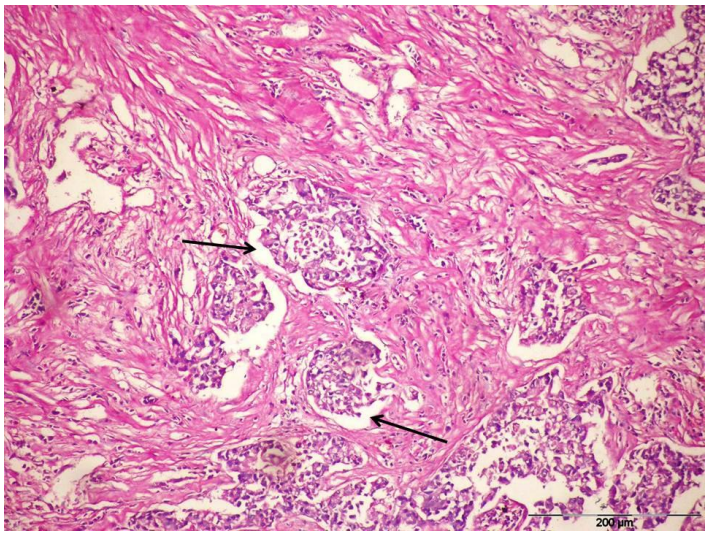

Figure 13: A PAS stained grade II IDC tissue showing degraded BM (arrow) (Bar=200 µm)

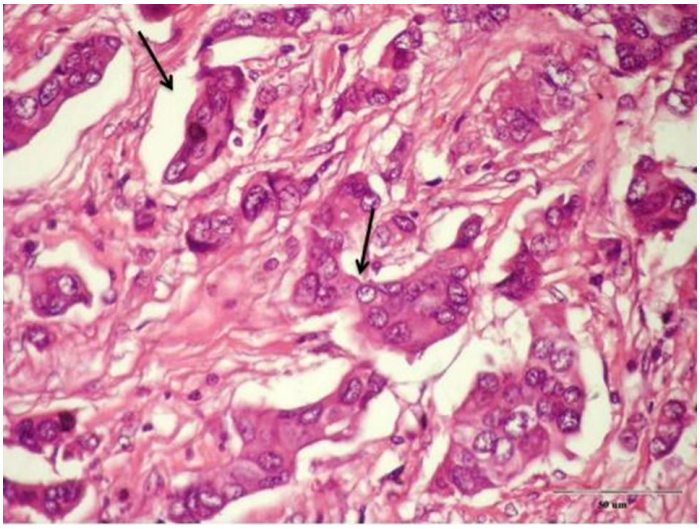

Figure 14: A PAS stained grade III IDC tissue showing completely degraded BM and malignant cells invading the surrounding microenvironment (arrow) (Bar=200 µm)

2-Immunohistochemical results:

I-Fn14:

a. Immunohistochemical reactivity of Fn14:

- Immunostaining reactivity of Fn14 was detected as granular brown stain observed in the cytoplasm and cytomembrane of the epithelial cells of the studied groups.
- Fn14 immunostaining reactivity was negative (-ve) in 70% (7/10) of control breast tissues, weak +ve (1+) in 70% (21/30) of benign group, while it was moderate +ve (2+) in 78% (35/45) of IDC grade II and strong +ve (3+) in 77% (10/13) of IDC grade III (Table 4 and Figure 15).

Table 4: Fn14 immunostaining reactivity in the different studied groups

Fn14	Control group		Benign group		Malignant group				Total	
					Grade II		Grade III			
	No	%	No	%	No	%	No	%	No	%
Negative (-ve)	7	70	6	20%	2	4%	0	0	16	16
Weak +ve (1+)	3	30	21	70%	3	7%	1	8	37	38
Moderate +ve (2+)	0	0	2	7%	35	78%	2	15	32	33
Strong +ve (3+)	0	0	1	3%	5	11%	10	77	13	13
Total	10	100	30	100%	45	100%	13	100	98	100
X^2 = 118, p = 0.000 (statistically significant)										

X^2: Chi square test

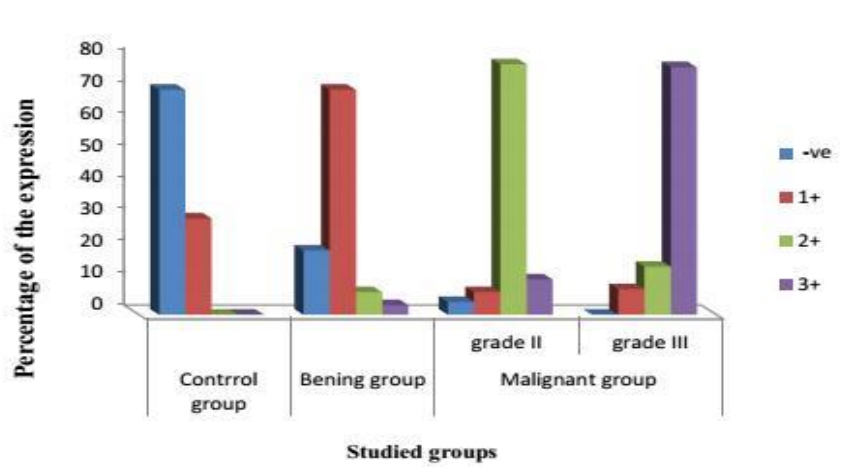

Figure 15: Immunostaining reactivity of Fn14 in the different studied groups. Note the overexpression of Fn14 in malignant group versus control and benign groups

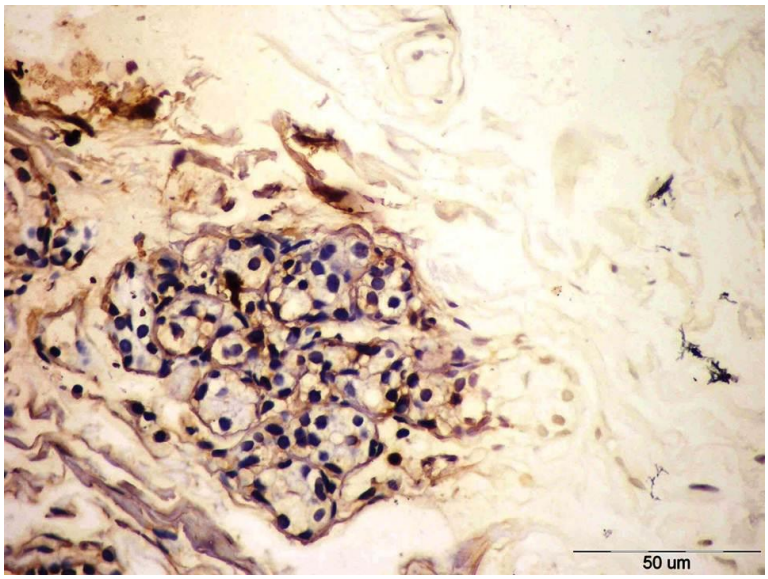

Figure 16: A control breast tissue showing negative (-ve) expression of Fn14 in the cytoplasm of the ductal epithelial cells (Bar =50 µm)

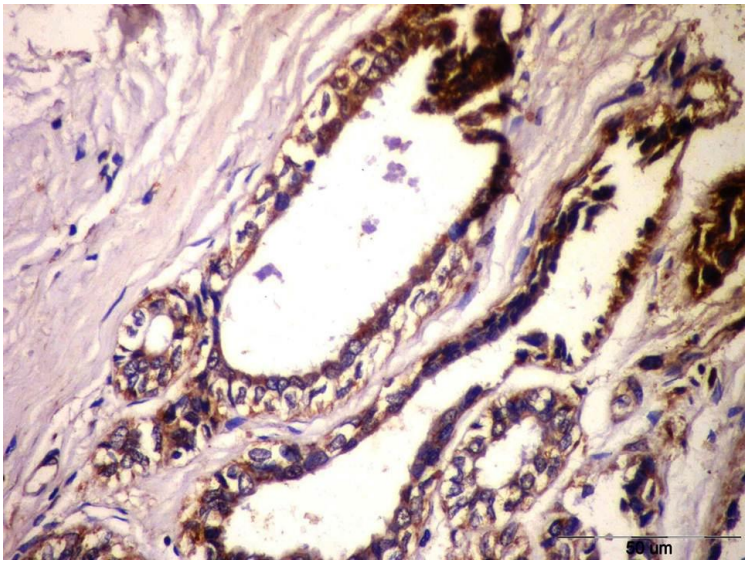

Figure 17: A benign breast tissue showing a weak (1+), granulated and membranous expression of Fn14 in the myoepithelial and ductal cells (Bar =50 µm)

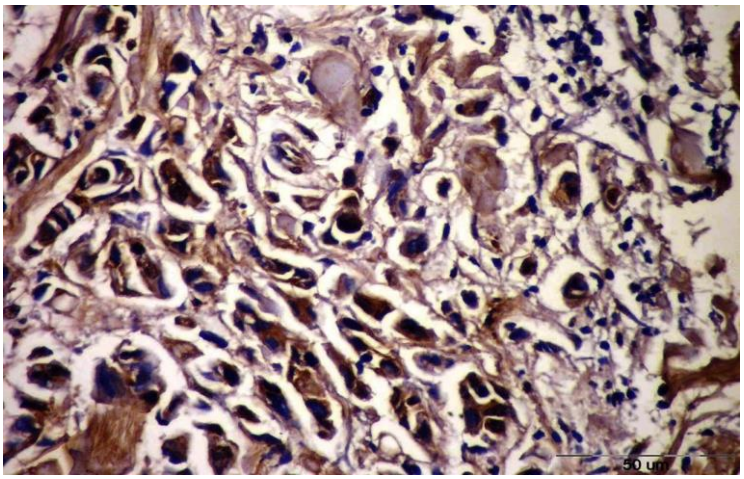

Figure 18: An IDC grade II breast tissue showing moderate (2+) expression of Fn14 in the cytoplasm of the ductal epithelial cells (Bar =50 µm)

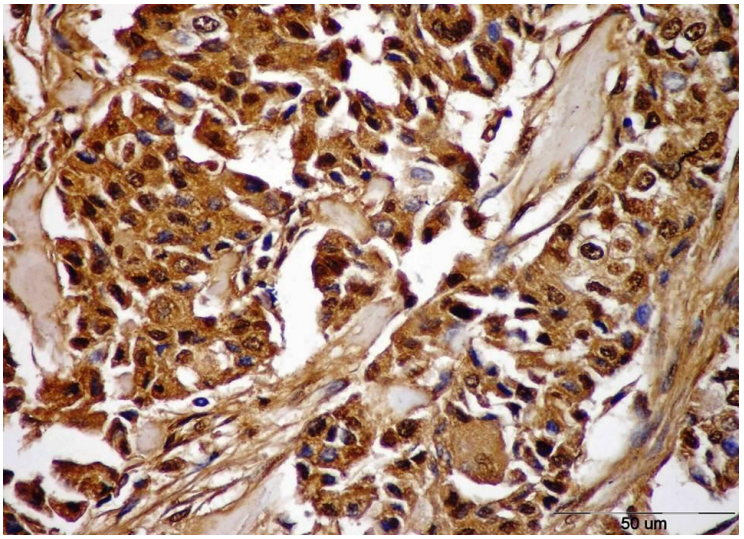

Figure 19: An IDC grade III breast tissue showing strong (3+) granulated, cytoplasmic and membranous expression of Fn14 in the ductal epithelial cells (Bar =50 µm)

II- Cath-D:

a. Immunohistochemical reactivity of Cath-D:

- Immunoreactivity of Cath-D was detected as brown course, or tiny granules detected in the cytoplasm of the ductal epithelial cells of the studied groups.
- Cath-D immunostaining reactivity was weak +ve (1+) in 70% (7/10) of control group, moderate +ve (2+) in 83% (25/30) and 53% (24/45) of benign and grade II IDC groups respectively, while it was strong +ve (3+) in 36% (16/45) and 92% (12/13) of grade II and grade III IDC respectively as illustrated in table (5) and figures (20).

Table 5: Cath-D immunostaining reactivity in the different studied groups

Cath-D	Control group		Benign group		Malignant group				Total	
					Grade II		Grade III			
	No	%	No	%	No	%	No	%	No	%
Negateve (-ve)	0	0	1	3	2	4	0	0	3	3
Weak +ve (1+)	7	70	2	7	3	7	0	0	7	7
Moderate +ve (2+)	1	10	25	83	24	53	1	8	57	58
Strong +ve (3+)	2	20	2	7	16	36	12	92	31	32
Total	10	100	30	100	45	100	13	100	98	100
$X^2 = 66.7$, $p = 0.000$ (statistically significant)										

X^2: chi-square

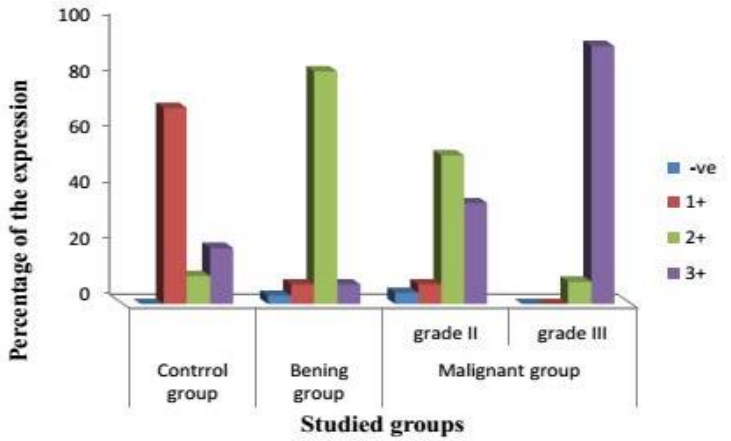

Figure 20: Cath-D immunostaining reactivity in the different studied groups

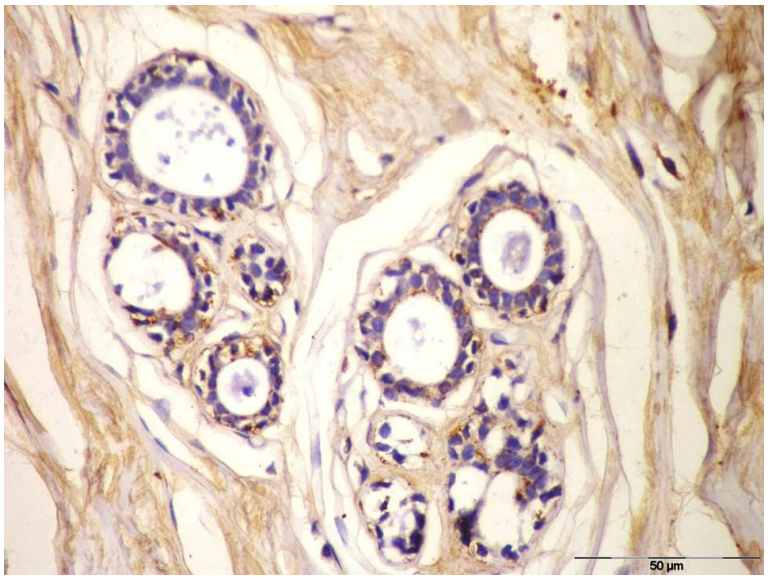

Figure 21: Immunohistochemical staining of a control breast tissue showing weak (1+) expression of Cath-D (Bar=50 µm)

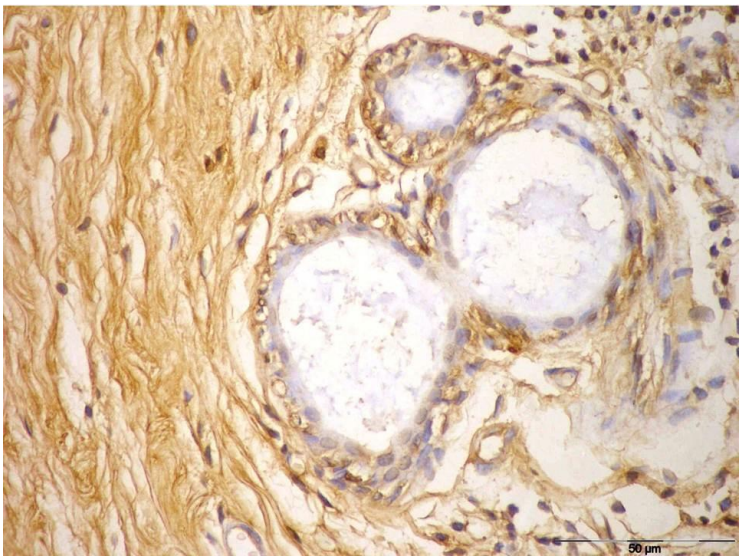

Figure 22: A benign breast tissue showing moderate (2+) expression of Cath-D enzyme in the cytoplasm of the ductal epithelial cells and surrounding extracellular matrix (Bar=50 µm)

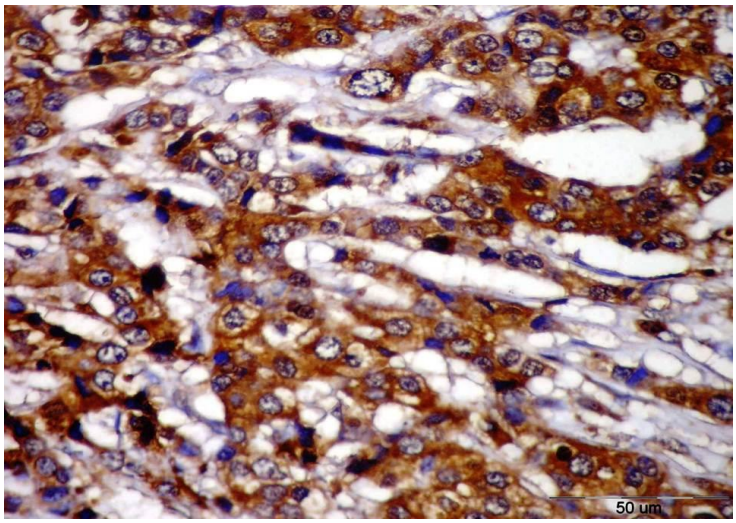

Figure 23: An IDC grade II breast tissue showing moderate (2+) expression of Cath-D in the cytoplasm of the ductal epithelial cells (Bar=50 µm)

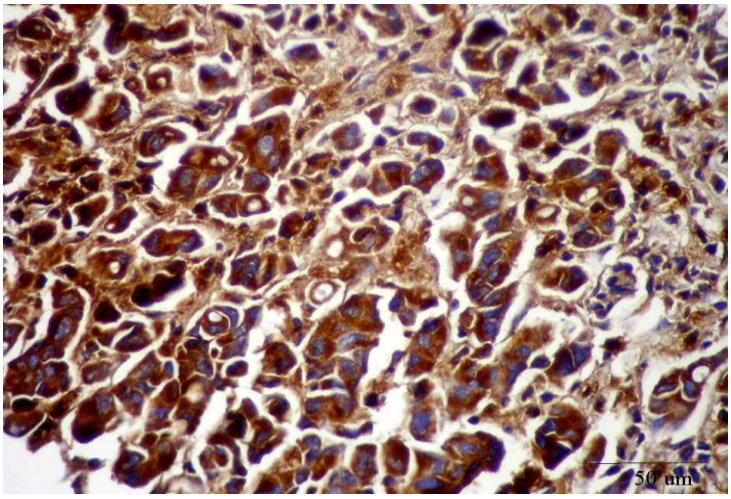

Figure 24: An IDC grade III breast tissue showing strong (3+) expression of Cath-D in the cytoplasm of the ductal epithelial cells (Bar=50 µm)

DISCUSSION

The results of the present study showed that 98% of the breast cancer cases were invasive ductal carcinoma (IDC), most of which were allocated to the age range of (>35-55) years. This results is consistent with several previous studies reported that IDC is the most common histological type of invasive breast cancer, and in the developing world it characterized by an early peak age of onset [9].

In the current study, the majority (75%) of the studied cases was grade II, followed by grade III (22%). This result is supported by previous studies claimed that most of the breast cancer cases undergoing surgical resection are at grade II and III [10].

Concerning tumor size, most of the studied breast cancer cases in the present work were allocated to the tumor size T2 (>2-5) cm and lymph node involvement was present in 73% of the studied cases. These results were in accordance with Sofi *et al*., (2012) [11].

The current results showed that most of the studied malignant cases were ER and PR positive (59%, 57%), while 38% were HER2/neu positive. These results are in agreement with several studies [12].

In the present study the results of the periodic acid-Schiff (PAS) stain showed degradation of myoepithelial cell layer with the underlying basement membrane and invasion of the malignant cells to the surrounding microenvironment in IDC group, while in normal breast tissues as well as in all benign tumors a continuous BM was found around the ducts and tubules.

During malignant transformation normal tissue architecture is disrupted by factors produced and secreted either by cancer cells or other cells associated with the tumor microenvironment [13]. When the breast tissue undergoes focal disruption of the myoepithelial cell layer and degradation of the underlying basement membrane, tumor cells invade surrounding tissues and migrate to distant organs, eventually leading to metastasis [14].

Immunohistochemical technique is an effective method for clinical determination of antibody proteins expression owing to specific targeting of tumor cells, nowadays; it is used in the investigation of a broad range of disease processes with applications in diagnosis, prognostication and therapeutic decisions [15].

The present study was undertaken to assess the immunohistochemical expression of Fn14, and Cath-D in human breast invasive carcinoma versus normal control and benign breast tumors, as well as to investigate the correlation of their immunohistochemical expression with clinicopathological parameters.

Fibroblast growth factor–inducible 14-kDa protein (Fn14) is the cell surface receptor for the cytokine TNF-like weak (TWEAK) inducer of apoptosis [16]. Fn14 receptor is a regulator of breast cancer cell invasive capacity in multiple biological contexts because of its strong overexpression in many types of solid tumors and the intrinsic tumor cell killing capacity of the TWEAK-Fn14 pathway and thus a negative prognostic indicator and potential therapeutic target for breast cancer [17].

The present study showed a statistical significant increase in the immunohistochemical expression of Fn14 in the malignant group versus normal and benign groups ($p < 0.05$). This finding is in agreement with those reported by other previous studies [17].

In the largest survey by Culp *et al*., (2010) [18] examining 1,655 tumor samples across 22 solid tumor subtypes by immunohistochemistry, Fn14 expression was detected in the majority of tumor types, including pancreatic cancer (60%), non-small cell lung cancer (55%), bone metastases (54%) and liver metastases in colorectal cancer (50%).

The fact that Fn14 expression is elevated in malignant tumors as compared with normal tissues suggests that it may be a potential tumor antigen and therefore, on the basis of expression alone, a valuable therapeutic target [19].

The results of the current study showed no statistical significant correlation between the immunohistochemical expression of Fn14 and clinical parameters such as patients' age ($r = -.03$, $p = .82$) and tumor size ($r = .246$, $P = .06$). These findings are consistent with those reported by Wang *et al*., (2013) [20].

A statistical significant correlation was noticed in the present study between Fn14's immunohistochemical expression and LNM ($r = 0.28^*$, $P = 0.03$). This is in agreement with previous studies showed that expression of Fn14 and its ligand TWEAK were both associated with metastasis and with four or more positive lymph nodes [21].

In the current study, Fn14's immunohistochemical expression has shown to be highly correlated with tumor histological grade ($r = 0.76^{**}$, $P < .001$). This finding is contrasted with Wang *et al*., (2013) [22], however the relationship between Fn14's expression and higher tumor grade that was noticed in the present study is going in accordance with Whitsett *et al*., (2014) [23] and Li *et al*., (2013) [24] who stated that Fn14 expression was significantly

correlated with more advanced grade and poorer prognosis.

The results of the current study showed that Cath-D expression was increased in breast cancer cases than in normal and benign cases. Previous study reported that normal lobular or ductal epithelia both from non-tumoral and tumoral lesions showed no Cath-D specific Staining [25].

Interestingly, the present results showed a statistical significant difference between expression of Cath-D in normal and benign cases. This finding is agreed with Brujan, *et al*., (2009) [26], who noticed that expression of Cath-D in benign breast tumors was higher than normal breast tissues, but still lesser than malignant breast tumors.

The current results showed no statistical significant correlation between the immunohistochemical expression of Cath-D and patients' age (r = .22, *P* = .09). This lack of correlation between expression of Cath-D and patients' age was also reported by several previous studies [27].

The results of the current study showed no statistical significant correlation between the immunohistochemical expression of Cath-D and tumor size (r = .04, *P* = .77). This result is consistent with Gion *et al*., (1995) [28], but contrasted with Ruibal *et al*., (2012) [29] who found that cytosolic concentration of Cath-D was associated with large tumors.

The represented data showed a statistical significant correlation between the immunohistochemical expression of Cath-D and tumor histological grade (r = 0.3*, *P* = 0.05). This result is going in accordance with Paksoy *et al*., (2011) [30], but contrasted with Carrascosa Lloret *et al*., (2002) [31].

In the present study there was a highly statistical significant correlation between the immunohistochemical expression of Cath-D and LNM (r = .35**, *P* = .006) of the studied breast cancer cases. This result is consistent with other studies stated that concentrations of Cath-D were associated with axillary lymph node involvement (119,195,198), but contrasted with others found no statistical significant relationship between Cathepsin's D level and lymph node metastasis [32].

Cath-D is involved in the pathogenesis of neurodegenerative, skin, cardiovascular and tumoral diseases [33]. In these pathologies, Cath-D is aberrantly produced and processed in malignancy and over-secreted to the cell microenvironment where it acts as tumor and stromal cells mitogen, also its hyper secretion leads to excessive degradation of the extracellular matrix, which contribute to tumor progression and metastases [34].

CONCLUSION

From the results of the present study, it could be concluded that:

- The marked immunohistochemical expression of Fn14 and Cath-D in malignant group versus control and benign groups indicates that these biomarkers might be potential tumor antigens and therefore, valuable therapeutic targets in breast cancer.
- The highly statistical correlation between the immunohistochemical expression of Fn14, and Cath-D with the high tumor grade, LNM and HER2/neu overexpression suggests that new therapeutic agents could target Fn14 and Cath-D or their downstream signaling mediators.

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