Scientific Research Publishing

Advances in Breast Cancer Research, 2018, 7, 211-230
http://www.scirp.org/journal/abcr
ISSN Online: 2168-1597
ISSN Print: 2168-1589

Prognostic and Clinicopathological Value of the Expression of FOXC2 & YKL-40 in Carcinoma of the Breast, an Immunohistochemical Study

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How to cite this paper: Fouad, E.M., Mahmoud, A.S., Ahmed, R.Z., El Shafaay, B.Sh., Mahmoud, R., Osman, G., Amin, M.F., Gertallah, L.M. and Bessar, M.A. (2018) Prognostic and Clinicopathological Value of the Expression of FOXC2 & YKL-40 in Carcinoma of the Breast, an Immunohistochemical Study. *Advances in Breast Cancer Research*, **7**, 211-230.
https://doi.org/10.4236/abcr.2018.73013

Received: May 2, 2018
Accepted: June 3, 2018
Published: June 6, 2018

Abstract

Background: Breast cancer is considered the commonest and the most fatal female cancer worldwide. There is to an urgent need for discovering recent therapies to identify patient prognosis and improve treatment strategies. Fork-head Box C2 (FOXC2) is a transcription factor which is a key regulator of cancer stem cells (CSC) properties and epithelial mesenchymal transition (EMT) e.g. cancer initiation, metastatic capacity, and resistance to chemotherapy. FOXC2 roles in CSCs properties and EMT regulation in breast cancer needs detailed studies. YKL-40 is known as chitinase-3-like-1 belongs to a family of mammalian proteins that have an amino acid sequence which is similar to the 18-glycosyl hydrolase bacterial chitinases group. Recent studies have found aberrant YKL-40 elevated expression in cancer of various organs, so it may be used as a recent prognostic biomarker for patients with breast cancer. Former researchers have assessed the expression of FOXC2 & YKL-40 separately in cancer patients and relations to prognosis of patients; however, no studies assessed them together in breast cancer patients and the previous results were inconclusive. Accordingly, our study aimed at evaluation of immunohistochemical expressions of FOXC2 & YKL-40 in carcinoma of the breast in a trial to clarify the relation among their expressions, clinicopathological parameters and recurrence of the disease after successive therapy and patients' prognosis. **Methods:** we have evaluated expressions of FOXC2 & YKL-40 in sections from 50 paraffin blocks of carcinoma of the breast using

immunohistochemistry. We followed up our patients for 3 years for assessment of recurrence of the disease after successive therapy and survival rates. We analyzed the relationship between their combined expression clinicopathological and prognostic parameters. **Results:** high FOXC2 expression was associated with older age of the patient ($p = 0.002$), negative ER ($p = 0.009$), & PR ($p = 0.008$), positive HER2neu ($p = 0.02$), aggressive molecular subtype, higher grade of the tumor ($p = 0.03$), high incidence of distant metastasis ($p = 0.011$), high incidence of lymph node metastasis, higher KI labeling index, advanced stage, ($p < 0.001$). high YKL-40 expression was positively correlated with older age of the patient ($p = 0.002$), negative ER ($p = 0.03$), & PR ($p = 0.04$), positive HER2neu ($p = 0.02$), higher grade of the tumor ($p = 0.003$), high incidence of distant metastasis ($p = 0.04$), higher KI labeling index, aggressive molecular subtype, advanced stage, high incidence of lymph node metastasis ($p < 0.001$). We have found that patients with FOXC2 and YKL-40 overexpression have higher incidence of recurrence of the disease after therapy, poor RFS& OS rates ($p < 0.001$). **Conclusion:** Higher expression levels of FOXC2 & YKL-40 are markers of poor prognosis in breast cancer.

Keywords

Carcinoma of the Breast, FOXC2, YKL-40, Prognosis, Immunohistochemistry

1. Introduction

Breast cancer is considered the commonest and the most fatal female cancer worldwide [1]. The incidence and seriousness of such type of cancer continue to rise although there is improvement in the clinical outcome and patients' prognosis due to advances in treatments approaches recently that point to an urgent need for discovering recent therapies to identify patient prognosis and improve treatment strategies [2]. The most commonly used prognostic markers of breast cancer are TNM stage, histological grade and status of lymph node in addition to hormonal receptors and human epidermal growth factor (EGF) receptor 2 (HER2) statuses [3]. But the value of these markers not sufficient to be individualized and of high effect for most patients, that leads much to identify more clinically applicable and reliable biomarkers for identification of prognosis of such a type of cancer.

So many studies have identified activation of the epithelial mesenchymal transition (EMT) which is a trans-differentiation program which is markedly activated by malignant cells. EMT is a process in which the malignant epithelial cells have lost their epithelial characters and experience down regulation of epithelial biomarkers then acquire mesenchymal characters like increased motility, invasion and increased apoptosis resistance; EMT is induced by so many signaling pathways [4]. FOX (Fork-head box) are a family of transcription factors that play important roles in regulating expression of genes which could be involved in growth, differentiation, and proliferation of cells. There are plethora of FOX

proteins that play essential roles in carcinogenesis, particularly involved in cancer progression, so that FOX proteins might be beneficial as therapeutic targets and prognostic markers for cancer [5].

Cancer stem cells (CSCs) which are cells having stem cell criteria are a subpopulation of cancer cells that is particularly play an essential role in the aggressive behavior and chemoresistance of the cancer. Fork-head Box C2 (FOXC2) is a transcription factor which is a key regulator of cancer stem cells properties and EMT e.g. cancer initiation, metastatic capacity, and resistance to chemotherapy. FOXC2 roles in CSC properties and EMT regulation in breast cancer needs detailed studies [6].

YKL-40 that is known as chitinase-3-like-1 belongs to a family of mammalian proteins, have an amino acid sequence which is similar to the 18-glycosyl hydrolase bacterial chitinases group [7] & [8]. YKL-40 is implicated in fibroblasts and chondrocytes proliferation, macrophage differentiation, inflammation and extracellular matrix remodeling [9].

Recent studies have found aberrant YKL-40 elevated expression in cancer of various organs [10], so it may be used as a recent prognostic biomarker for cancer patients. Previous studies have evaluated the expression of FOXC2 & YKL-40 separately in cancer patients and relations to prognosis of patients; however, no studies assessed them together in breast cancer patients and the previous results were inconclusive.

Accordingly, our study aimed at evaluation of immunohistochemical expressions of FOXC2 & YKL-40 in carcinoma of the breast in a trial to clarify the relation between their expressions, clinicopathological parameters and recurrence of the disease after successive therapy and patients' prognosis.

2. Patients and Methods

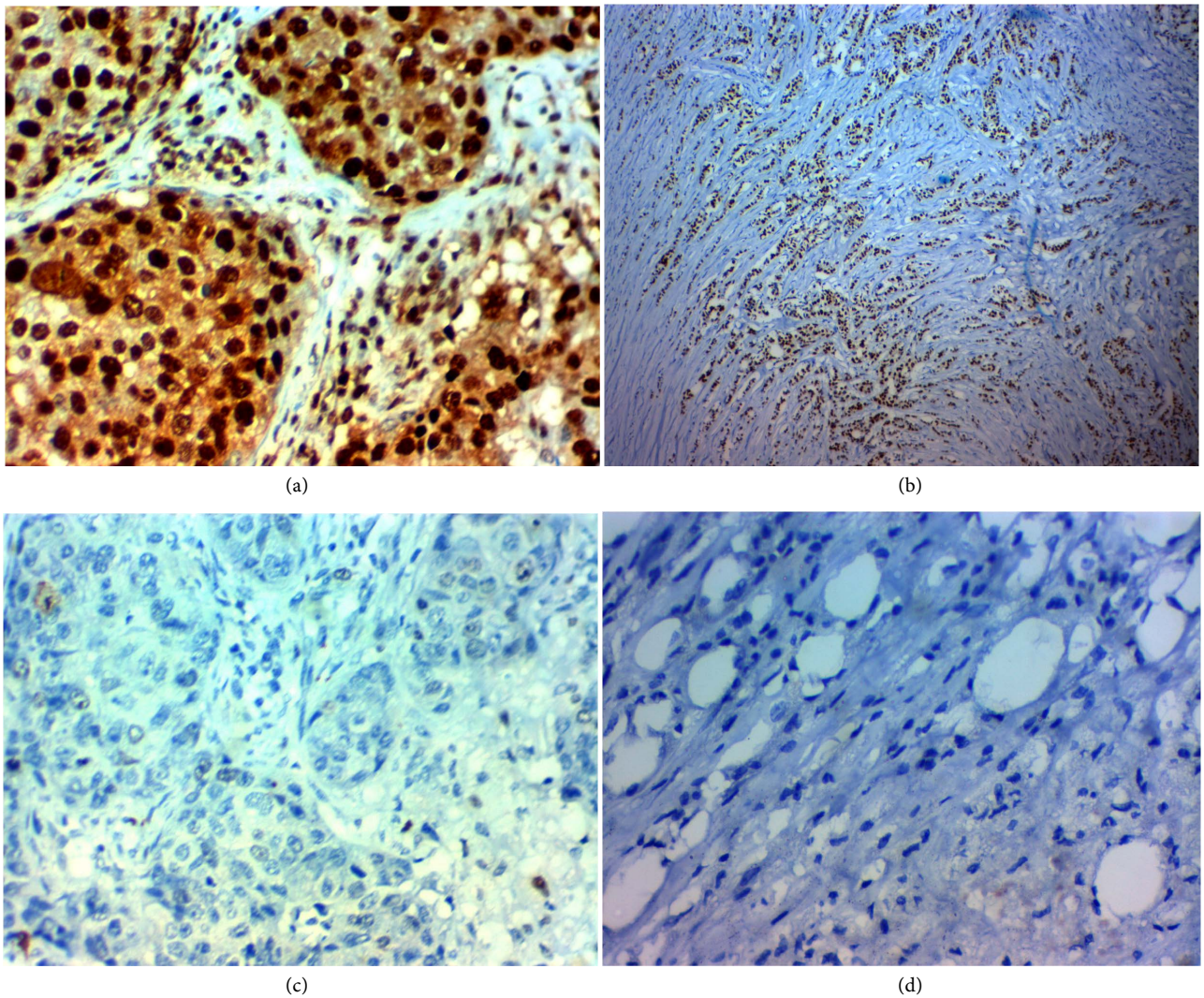
Our study includes fifty breast cancer cases which have been referred to General Surgery departments in Faculty of Medicine in Zagazig University in time from November 2014 to November 2017 as they are having a breast lump the patients sent to Department of Radiology to take true cut needle biopsy, that is sent to Department, of Pathology in both Zagazig and Beni-Suef Universities, where all samples are processed, diagnosed and evaluated as breast carcinoma. Modified radical mastectomy was done in addition to axillary clearance, then all samples are processed, diagnosed, graded and staged in Pathology Departments in both Zagazig and Beni-Suef Universities, Patients were managed, treated later on and followed up in Clinical Oncology and nuclear medicine Department, Zagazig University for further management and follow-up for 3 years. Patients were followed up till death or their most recent medical examination. The follow-up deadline was December 2017.

We prepared 50 formalin fixed paraffin embedded blocks of breast carcinoma that we have prepared from the 50 patients in Pathology Department, Faculty of Medicine, Zagazig University. We did immunohistochemistry for FOXC-2 & YKL-40 on these 50 formalin fixed paraffin embedded blocks of breast carcino-

ma. We identify the age of all cases, tumor size. graded all samples and staged them, evaluated the satus of lymph nodes and detect if there is distant metastasis (**Figures 1-5**).

2.1. Immunohistochemical Staining

We have used the avidin-biotin-peroxidase complex method for immunohistochemical staining [11], where we have incubated the sections with primary Goat polyclonal anti-Anti-FOXC2 antibody-ChIP Grade (Abcam ab5060) and primary mouse monoclonal anti-YKL-40 antibody [AT2C10] (ab86428) overnight at 4˚C and this was followed by incubation with the secondary antibody (SP-9000) and DAB (C-0010) stain. The sections were incubated with Mayer's haematoxylin as a counterstained, dehydrated, then cleaned, and mounted. The negative controls



Fgure 1. Immunohistochemical staining of FOXC2 in invasive carcinoma of the breast: (a) High nuclear expression in high grade invasive duct carcinoma of the breast (NOS) stage IV×400; (b) High nuclear expression in high grade invasive lobular carcinoma of the breast (NOS) stage III×400); (c) Low nuclear expression low grade invasive duct carcinoma of the breast (NOS) stage I×400); (d) Low nuclear expression in Low grade invasive lobular carcinoma of the breast (NOS) stage II×400.

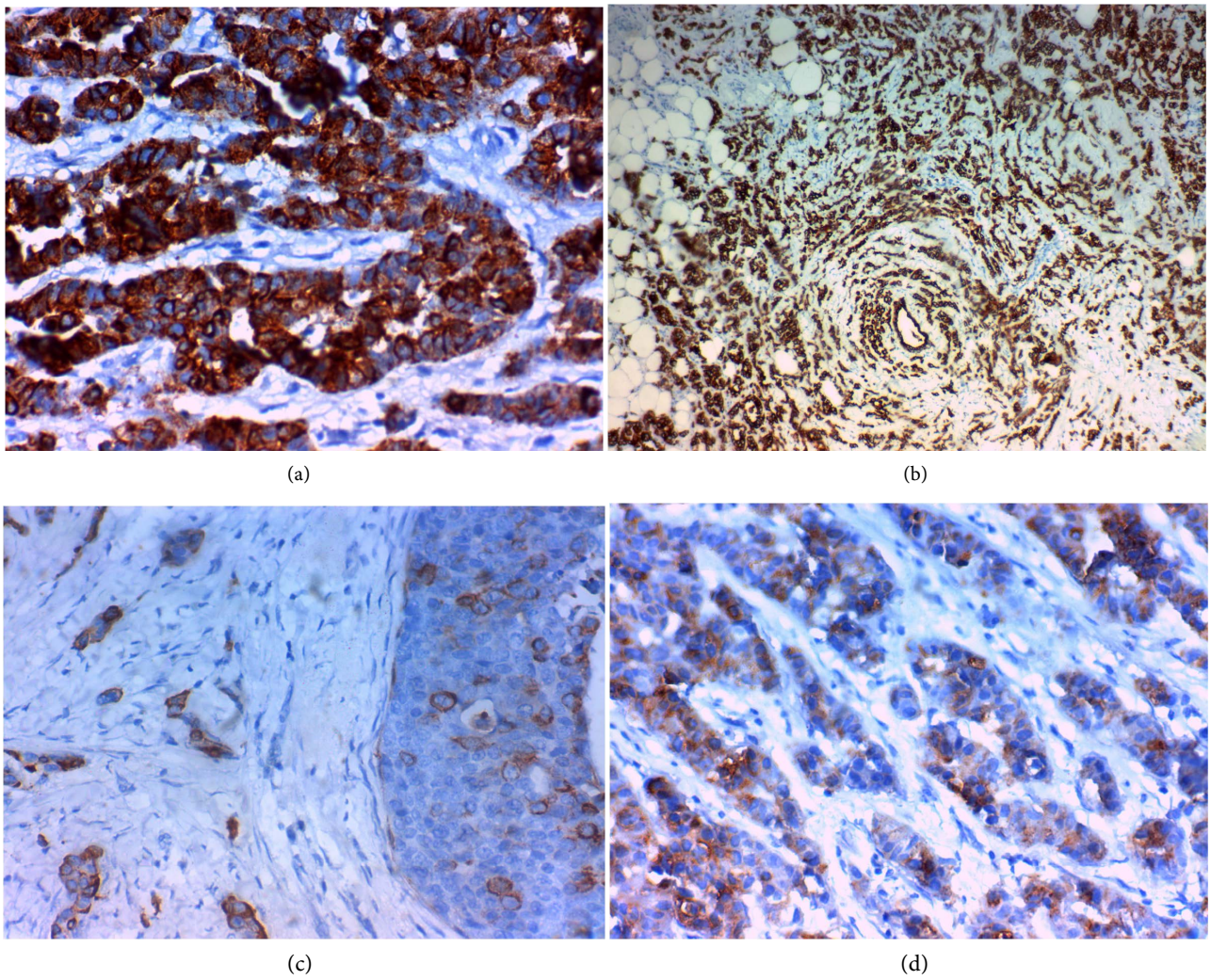


Figure 2. Immunohistochemical staining of YKL-40 in invasive carcinoma of the breast: (a) High cytoplasmic expression in high grade invasive duct carcinoma of the breast (NOS) stage IV×400; (b) High cytoplasmic expression in high grade invasive lobular carcinoma of the breast (NOS) stage III×400); (c) Low cytoplasmic expression low grade invasive duct carcinoma of the breast (NOS) stage I×400); (d) Low cytoplasmic expression in Low grade invasive duct carcinoma of the breast stage II×400.

were stained without adding the primary antibodies but replaced them with usual phosphate buffered saline (PBS).

2.2. Evaluation of Immunohistochemical Expressions of FOXC-2

We recognized FOXC-2 expression as brown staining that was localized mainly to the nucleus of cancer cells. We recognized YKL-40 expression as brown staining that was localized mainly to the cytoplasm of cancer cells.

We have evaluated FOXC2 & YKL-40 expression by using a combined scoring system that is based on summation of staining intensity and extent of the stain. Both intensity and extent of positive cells take scores from 0 to 3 respectively, as follows: 0, no staining, or staining in <10% of cancer cells; 1+, weak staining in ≥10% of the tumor cells; 2+, moderate staining in ≥10% of the tumor cells; and 3+, strong staining in ≥10% of cancer cells. We have considered 0 and 1+ scores

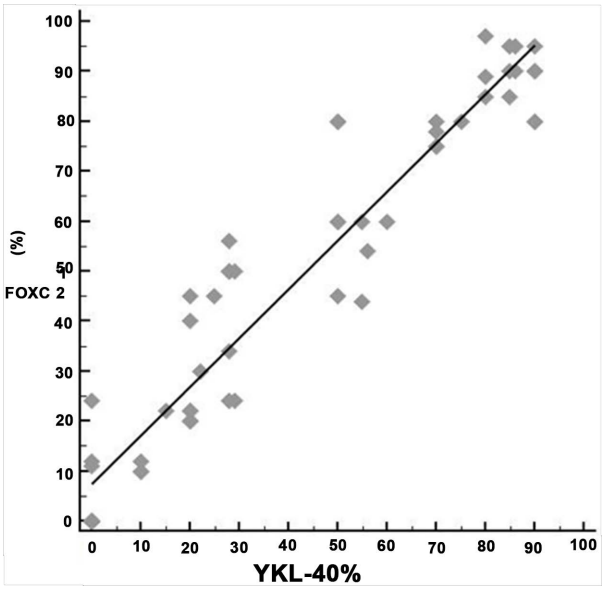
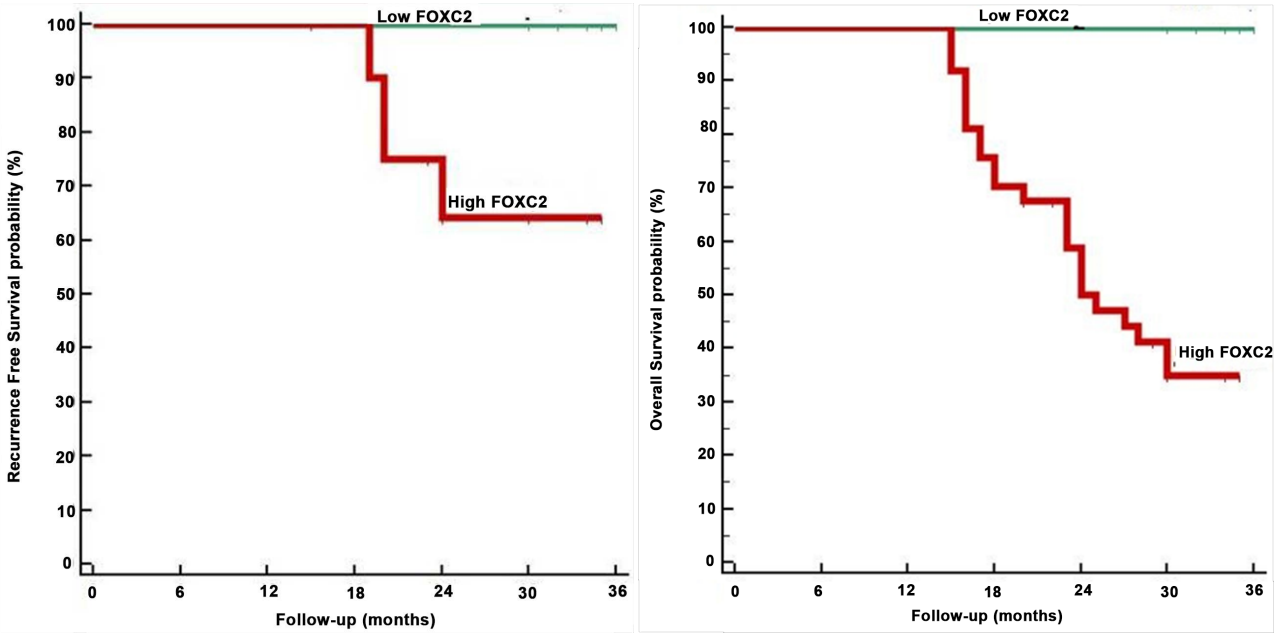


Figure 3. Scatter plot with regression line shows: (a) a significant strong direct correlation between FOXC2 and YKL41 ($r = +0.949$, $p < 0.001$).



(a) (b)

Figure 4. Kaplan-Meier plot of local recurrence free survival: (a) stratified according to FOXC2; (b) stratified according to YKL-40.

to have low FOXC2 expression, and considered scores of 2+ and 3+ to be high FOXC2 expression [12].

2.3. Statistical Analysis

We have performed all our statistics by using SPSS 22.0 for windows. We have used Mann Whitney U test for the comparison between 2 sets of non-normally

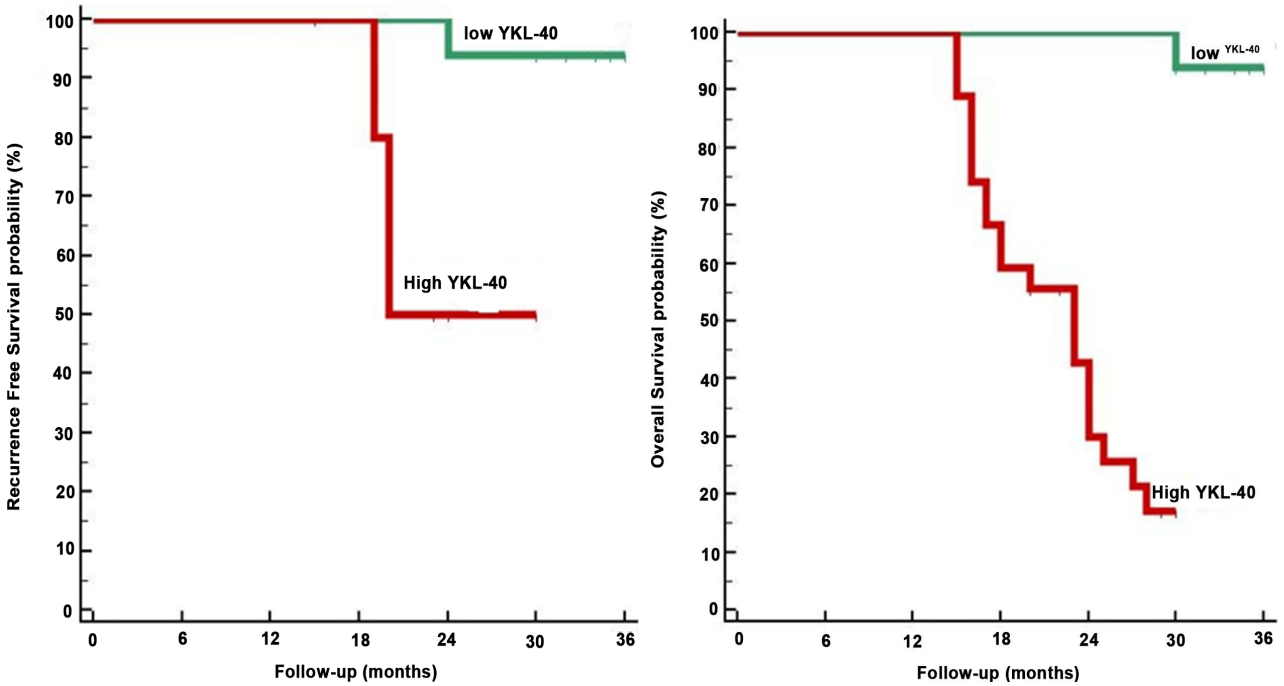


Figure 5. Kaplan-Meier plot of overall survival: (a) stratified according to FOXC2; (b) stratified according to YKL-40.

distributed parameters. And Kruskal Wallis H test to compare between more than two sets of normally distributed parameters. We have used Pearson's Chi-square test or Fisher's exact test to compare percent of categorical variables when was appropriate. Strength of relationship between FOXC2, YKL-40 & clinicopathological parameters were assessed by using appropriate coefficient e.g. Spearman's. (+) sign is an indicator for direct relationship while (−) sign is an indicator for inverse relationship. Local Recurrence Free Survival (LRFS) is the time from surgery to time of detection of local recurrence or till the most recent follow-up in which no local recurrence was detected (censored). Overall Survival (OS) is the time from cancer diagnosis up till death or the most recent follow-up data (censored). Stratification of OS and LRFS was done according to clinicopathological parameters and immunohistochemical markers. These survival rates were estimated using the Kaplan-Meier plot, and we have compared them by using two-sided exact log-rank test. A p-value < 0.05 was considered significant.

3. Results

3.1. Clinicopathological Data of Our Patients Were Detailed in Table 1

We have included 50 female patients with carcinoma breast in our study with age ranged from (35 - 75) years (Mean: 50.23 ± 11.99 years).

Thirty nine (78%) cases were diagnosed as invasive duct carcinoma no special type (IDC) and the remaining 11 (22%) cases were diagnosed as other subtypes e.g. invasive lobular carcinoma (ILC).

Table 1. Clinicopathological parameters, FOXC2 & YKL-40 expression in our 50 patients with breast carcinoma.

Characteristics	Number	Percent	Characteristics	Number	Percent
Age (years)			T		
Mean ± SD	50.23 ± 11.99		T1	8	16%
Median Range	50 (35 - 75)		T2	10	20%
≤55 years	18	36%	T3	22	44%
>55 years	32	64%	T4	10	20%
Pathological type			Lymph node		
IDC	39	78%	Negative	18	36%
Other	11	22%	Positive	32	64%
Grade			N		
Grade I	7	14%	N0	18	36%
Grade II	13	26%	N1	6	12%
Grade III	30	60%	N2	16	32%
			N3	10	20%
ER			M		
Negative	20	40%	M0	40	80%
Positive	30	60%	M1	10	20%
PR			AJCC Stage group		
Negative	22	45%	Stage I	8	16%
Positive	28	55%	Stage II	10	20%
HER2/neu			Stage III	22	44%
Negative	30	40%	Stage IV	10	20%
Positive	20	60%			
Ki-67			FOXC2		
low	20	40%	Low	20	40%
high	30	60%	High	30	60%
Molecular type			YKL-40		
Luminal A	10	20%	Low	23	46%
Luminal B	10	20%	High	27	54%
HER2 amplified	20	40%			
Triple -ve	10	20%			
			Follow-up (months)		
			Mean ± SD	28.31 ± 7.27	
			Median (Range)	30 (15 - 36)	
			Outcome		
			Disease free	30/34	84.1%
			Local recurrence (LR)	7/34	15.9%
			Died	13/50	38.3%

Categorical variables were expressed as number (percentage). Continuous variables were expressed as mean ± SD & median (range).

3.2. Correlation between FOXC2 Immunoexpression and Clinicopathological Parameters: Table 2

FOXC2 was nuclear and its high expression was detected in 30 (60%) of cases and its expression was significantly correlated positively with advanced age of the patient ($p = 0.002$), poor differentiation of the tumor ($p = 0.03$), negative ER ($p= 0.009$), & PR ($p = 0.008$), positive HER2neu ($p = 0.02$), aggressive molecular subtype, high incidence of distant metastasis ($p = 0.011$), higher KI labeling index, advanced stage, high incidence of lymph node metastasis ($p < 0.001$).

No association correlation was found between FOXC-2 expression and histopathological subtype.

3.3. YKL-40 Immunoexpression and Its Correlation Clinicopathological Features: Table 3

YKL-40 was cytoplasmic and its high expression was detected in 27 (54%) of cases and its expression was significantly correlated positively with older patients age ($p = 0.002$), poor tumor differentiation ($p = 0.003$), negative ER ($p = 0.03$), & PR ($p = 0.04$), positive HER2neu ($p = 0.02$), high incidence of distant metastasis ($p = 0.04$), aggressive molecular subtype, higher KI labeling index, advanced stage, high incidence of lymph node metastasis ($p < 0.001$).

No association was found was found between FOXC-2 expression and histopathological subtype.

Correlation between tissue protein expression of FOXC2 and YKL-40 in our cases.

We found a direct relationship between FOXC2 and YKL-40 (Spearman's $r = +0.949$), ($p < 0.001$) (Table 4).

3.4. Follow-Up Results: Table 1 & Table 5

We have found that 84.1% of our cases were disease free after 3years 15.9% of patients had local recurrence and 38.3% have died.

The 3-year recurrence free survival (RFS) and overall survival (OS) rates of our patients were 83.6% & 60.8% for all cases respectively.

We have found that patients with FOXC2 and YKL-40 overexpression have dismal out come as they have higher incidence of recurrence of the disease after therapy, poor RFS & OS rates and these results were statistically highly significant ($p < 0.001$).

4. Discussion

When we assessed the expression of FOXC-2 in 50 cases that were diagnosed with breast carcinoma with breast cancer of different grades and stages we have found that high FOXC-2 protein expression was positively associated with poor clincopathological criteria like older age of the patient ($p = 0.002$), negative ER ($p = 0.009$), & PR ($p = 0.008$), positive HER2neu ($p = 0.02$), aggressive molecular subtype, higher grade of the tumor ($p = 0.03$), high incidence of distant

Table 2. Correlations between clinicopathological parameters and FOXC2 expression in our patients.

Characteristics	All		FOXC2				p-value
	(N = 50)		Low (N = 20)		High (N = 30)		
	No.	(%)	No.	(40%)	No.	(60%)	
Age (years)							
Mean ± SD	56.23 ± 10.99		49.91	±8.72	60.16	±10.50	0.002
Median (Range)	56 (39 - 87)		45	(39 - 67)	60	(40 - 87)	
≤55 years	18	36%	14	(65.2%)	4	(21.6%)	<0.001‡
>55 years	32	64%	6	(34.8%)	26	(78.4%)	
Pathological type							
IDC	39	78%	14	(49%)	25	(51%)	0.448‡
Other	11	22%	6	(36.4%)	5	(63.6%)	
Grade							
Grade I	7	14%	5	(30.4%)	2	(8.1%)	
Grade II	13	26%	9	(39.1%)	4	(16.2%)	0.03§
Grade III	30	60%	6	(30.4%)	24	(75.7%)	
ER							
Negative	20	40%	2	(4.2%)	18	(95.8%)	0.009‡
Positive	30	60%	18	(75%)	12	(25%)	
PR							
Negative	22	45%	2	(4.2%)	20	(80%)	0.008‡
Positive	28	55%	18	(75%)	10	(20%)	
HER2/neu							
Negative	30	40%	18	(77.1%)	12	(22.9%)	0.02‡
Positive	20	60%	2	(4%)	18	(96%)	
Ki-67							
Negative	20	40%	15	(87%)	5	(13%)	<0.001‡
Positive	30	60%	5	(21.6%)	25	(78.4%)	
Molecular type							
Luminal A	25	41.7%	25	(100%)	0	(0%)	
Luminal B	10	16.7%	1	(10%)	9	(90%)	<0.001‡
HER2 amplified	15	25%	0	(0%)	15	(100%)	
Triple-ve	10	16.7%	2	(20%)	8	(80%)	
T							
T1	8	16%	8	(39.1%)	0	(0%)	
T2	10	20%	6	(26.1%)	4	(13.5%)	0.002§
T3	22	44%	5	(34.8%)	17	(37.8%0	
T4	10	20%	1	(0%)	9	(37.8%)	
N							

Continued

N0	18	36%	18	(82.6%)	0	(0%)	0.002§
N1	6	12%	2	(8.7%)	4	(13.5%)	
N2	16	32%	1	(4.3%)	15	(54.1%)	
N3	10	20%	0	(4.3%)	10	(32.4%)	
Lymph node							
Negative	18	36%	18	(82.6%)	0	(0%)	<0.001‡
Positive	32	64%	2	(17.4%)	30	(83%)	
M							
M0	40	80%	26	(55.3%)	21	(44.7%)	0.04‡
M1	10	20%	2	(15.4%)	11	(48.6%)	
AJCC Stage group							
Stage I	8	16%	9	(39.1%)	0	(0%)	<0.001§
Stage II	10	20%	10	(43.5%)	4	(10.8%)	
Stage III	22%	44%	4	(17.4%)	17	(45.6%)	
Stage IV	10%	20%	0	(0%)	16	(43.2%)	
YKL-40			18	(100%)	5	(27%)	
Low	23	46%	2	(0%)	25	(73%)	<0.001‡
High	27	54%	9	(39.1%)	0	(0%)	

Categorical variables were expressed as number (percentage), continuous variables were expressed as mean ± SD & median (range); Mann Whitney U test; ‡ Chi-square test; § Chi-square test for trend; $p < 0.05$ is significant.

metastasis ($p = 0.011$), high incidence of lymph node metastasis, higher KI labeling index, advanced stage, ($p < 0.001$) and our results were similar to results of [6] & [13] that found the same results in breast cancer and also similar to previous studies in cancers of other organs e.g. Quan *et al.* [14], & LIU B *et al.* [11], in ovarian carcinomas and Cui *et al.* [15], in colon cancer who proved the association between FOXC2 overexpression and poor prognosis in those cancer types.

Watanabe *et al.* [16], showed that the high expression of FOXC2 in primary extra-hepatic cholangiocarcinoma (EHCC) samples was associated with cancer progression and a poor prognosis that was similar to our results. Also Nishida *et al.* [17], results proved that high expression of FOXC2 is associated with cancer progression and poor prognosis of esophageal cancer. Our results could be explained by the role of FOXC-2 in EMT induction and stem cell properties in malignant cells as stated by Pietilä *et al.* [6], that identified FOXC2 as an EMT key regulator and could stimulate stem cell characters in cancer initiation, progression and resistance to chemotherapy.

We have proved that FOXC-2 over expression was related to aggressive molecular subtype particularly triple negative breast carcinomas (TNBCs) that was in line with results of Hollier *et al.* [13], who found that FOXC2 levels were up-regulated in triple negative breast carcinoma and also up-regulated in the

Table 3. Correlations between clinicopathological parameters and YKL-40 expression in our patients.

Characteristics	All		YKL-40				p-value
	(N = 50)		Low (N = 23)		High (N = 27)		
	No.	(%)	No.	(46%)	No.	(54%)	
Age (years)							
Mean ± SD	56.23 ± 10.99		49.54	±7.94	64.40	±8.39	0.002
Median (Range)	56 (39 - 87)		48	(39 - 67)	60	(55 - 87)	
≤55 years	18	36%	15	(69.7%)	3	(0%)	<0.001‡
>55 years	32	64%	8	(30.3%)	24	(100%)	
Pathological type							
IDC	39	81.7%	24	(49%)	25	(51%)	0.448‡
Other	11	18.3%	4	(36.4%)	7	(63.6%)	
Grade							
Grade I	7	14%	7	(30.3%)	0	(0%)	
Grade II	13	26%	13	(45.5%)	0	(0%)	0.003§
Grade III	30	60%	3	(24.2%)	27	(100%)	
ER							
Negative	20	40%	2	(4.2%)	18	(95.8%)	0.03‡
Positive	30	60%	27	(75%)	9	(25%)	
PR							
Negative	22	45%	3	(4.2%)	19	(95.8%)	0.04‡
Positive	28	55%	20	(75%)	8	(25%)	
HER2/neu							
Negative	30	40%	20	(87%)	3	(13%)	0.02‡
Positive	20	60%	3	(21.6%)	27	(78.4%)	
Ki-67							
Low	20	40%	20	(87%)	0	(0%)	<0.001‡
High	30	60%	3	(21.6%)	27	(78.4%)	
Molecular type							
Luminal A	25	41.7%	25	(100%)	0	(0%)	
Luminal B	10	16.7%	1	(10%)	9	(90%)	<0.001‡
HER2 amplified	15	25%	0	(0%)	15	(100%)	
Triple-ve	10	16.7%	2	(20%)	8	(80%)	
T							
T1	8	16%	8	(39.4%)	0	(0%)	
T2	10	20%	10	(33.3%)	0	(0%)	0.002§
T3	22	44%	3	(27.3%)	18	(48.1%)	
T4	10	20%	2	(0%)	18	(51.9%)	
N							

Continued

N0	18	36%	14	(57.6%)	4	(0%)	0.005§
N1	6	12%	5	(21.2%)	1	(0%)	
N2	16	32%	3	(18.2%)	13	(55.6%)	
N3	10	20%	1	(3%)	9	(44.4%)	
Lymph node							
Negative	18	36%	18	(57.6%)	0	(0%)	<0.001‡
Positive	32	64%	15	(42.4%)	27	(100%)	
M							
M0	40	80%	26	(55.3%)	21	(44.7%)	0.011‡
M1	10	20%	2	(15.4%)	11	(48.6%)	
AJCC Stage group							
Stage I	8	16%	8	(27.3%)	0	(0%)	<0.001§
Stage II	10	20%	8	(42.4%)	2	(0%)	
Stage III	22	44%	7	(30.3%)	15	(40.7%)	
Stage IV	10	20%	0	(0%)	10	(59.3%)	
FOXC2							
Low	20	40%	21.30	±18.41	77.48	±15.41	
High	30	60%	20	(0 - 56)	80	(44 - 97)	

Categorical variables were expressed as number (percentage), continuous variables were expressed as mean ± SD & median (range); Mann Whitney U test; ‡ Chi-square test; § Chi-square test for trend; $p < 0.05$ is significant.

Table 4. Correlations between clinicopathological parameters, FOXC2 & YKL-40 expression in our patients.

	FOXC2		FOXC2 (%)		YKL-40		YKL-40 (%)	
	r	p-value	r	p-value	r	p-value	r	p-value
Age (years)	+0.436	<0.001	+0.681	<0.001	+0.713	<0.001	+0.726	<0.001
Size	+0.525	<0.001	+0.709	<0.001	+0.770	<0.001	+0.747	<0.001
Grade	+0.444	<0.001	+0.675	<0.001	+0.750	<0.001	+0.728	<0.001
T	+0.534	<0.001	+0.757	<0.001	+0.860	<0.001	+0.795	<0.001
N	+0.832	<0.001	+0.842	<0.001	+0.843	<0.001	+0.833	<0.001
Stage	+0.790	<0.001	+0.893	<0.001	+0.868	<0.001	+0.862	<0.001
FOXC2	---	---	---	---	+0.713	<0.001	+0.806	<0.001
FOXC2 (%)	---	---	---	---	+0.837	<0.001	+0.949	<0.001
YKL-40	+0.713	<0.001	+0.837	<0.001	---	---	---	---
YKL-40 (%)	+0.806	<0.001	+0.949	<0.001	---	---	---	---

r correlation coefficient; $p < 0.05$ is significant.

remaining malignant cells that were retrieved from patients with carcinoma of the breast. Patients managed with conventional therapeutic modalities, that have been enriched with mesenchymal and stem cell characteristics. Hence all these

Table 5. Correlations between clinicopathological parameters, FOXC2 & YKL-40 expression, recurrence and survival rates in our patients.

Characteristics	All (N = 50)		LR free survival		p-value	Overall survival		p-value
			Median LRFS (months)	3 years LRFS		Median OS (months)	3 years OS	
All patients			NR	83.6%		NR	60.8%	
Age (years)								
≤50 years	17	(38.3%)	NR	91.3%	0.119§	NR	91.3%	<0.001§
>50 years	33	(61.7%)	NR	75%		24	41.1%	
Size (cm)								
<5 cm	18	36%	NR	91.7%	0.087§	NR	91.7%	<0.001§
≥5 cm	32	64%	NR	73.7%		24	39.4%	
Grade								
Grade I	7	(16.7%)	NR	100%	0.139§	NR	100%	<0.001§
Grade II	13	(25%)	NR	86.7%		NR	86.7%	
Grade III	30	(58.3%)	NR	72.2%		24	37.6%	
Lymph node								
Negative	32	(31.7%)	NR	100%	0.010§	NR	100%	<0.001§
Positive	18	(68.3%)	NR	70.4%		27	41.6%	
T								
T1	10	(21.7%)	NR	84.6%	0.028§	NR	84.6%	<0.001§
T2	22	(18.3%)	NR	100%		NR	100%	
T3	10	(36.7%)	NR	77.8%		NR	62%	
T4	18	(23.3%)	20	0%		17	0%	
N								
N0	6	(31.7%)	NR	100%	0.006§	NR	100%	<0.001§
N1	16	(11.7%)	NR	71.4%		NR	71.4%	
N2	8	(35%)	NR	78.6%		28	45.4%	
N3	10	(21.7%)	20	33.3%		17	20.5%	
Stage								
Stage I	8	(15%)	NR	100%	0.226§	NR	100%	<0.001§
Stage II	10	(23.3%)	NR	85.7%		NR	85.7%	
Stage III	22	(35%)	NR	75%		NR	66.7%	
Stage IV	10	(26.7%)	---	---		17.5%	0%	
FOXC2								
Low	23	(38.3%)	NR	100%	0.001§	NR	100%	<0.001§
High	37	(61.7%)	NR	64.3%		24	34.8%	
YKL-40								
Low	23	(55%)	NR	93.9%	<0.001§	NR	93.9%	<0.001§
High	27	(45%)	NR	50%		23	17.1%	

Categorical variables were expressed as number (percentage); NR denote not reached yet; § Log rank test; $p < 0.05$ is significant

results incriminate FOXC2 in chemo-radio-therapy resistance, breast cancer recurrence and progression. Another explanation for the association of FOXC-2 expression levels and aggressive behavior of breast cancer that FOXC2 was able to induce the expression of several cell cycle regulators, including cyclin-dependent kinase 1 (CDK1), suggesting a role for FOXC2 phosphorylation in the expression of cell cycle-specific genes and that FOXC2 may regulate the cell cycle in CSC-rich cell populations [18], that subsequently might increased proliferation in plethora of cancer types [19] [20] [21].

Pietilä *et al.* [6] and Hollier *et al.* [13], also has stated that increased FOXC2 expression has been shown to be limited to cancer cells which have stem cell properties, moreover it has a central role in EMT regulation, cancer cells invasion, metastasis and resistance to chemotherapeutics so it may be used as a novel promising therapeutic target as targeting FOXC2 pathway might be considered an effective novel therapeutic approach for malignant tumors that are rich in EMT/CSC properties. These explanations were confirmed by Quan Y, *et al.* [14], who explained the role of FOXC2 as an EMT-inducing transcription factor that has essential roles in cancer invasion and progression of ovarian cancer.

Moreover, Watanabe A. *et al.* [16], study showed that by high FOXC2 expression is associated with increased the expression of N-cadherin, MMP-2, and Ang-2 and decreased E-cadherin expression that is associated with increased invasion in EHCC and increases in the incidence of lymph node and distant metastasis.

Besides, FOXC2 plays an essential role in tumor angiogenesis [22]. Meanwhile, FOXC2 promoted colorectal cancer proliferation through inhibiting the activation of AKT and MAPK signaling pathways and it regulates MET expression so it promotes invasion and metastasis of CRC cells [23]. Our results highlighted the molecular mechanisms that are underlying the metastatic behavior of breast cancer cells which suggested that FOXC2 represents a potential target for therapeutic strategies in breast cancer.

Pietilä *et al.* [6], showed that FOXC2 protein levels are capable of predicting clinical outcome. As most anti-mitotic chemotherapeutic agents kill malignant differentiated cells so as to lead to shrinkage of the tumor, they are unable to kill the CSCs that is responsible for to malignant relapse, Pietilä *et al.* [6], study suggested that inhibition of PLK1-FOXC2 signaling pathway might be helpful in inhibition the CSC-rich triple negative carcinomas and showed that FOXC2 protein expression levels are capable of expecting breast cancer patients clinical outcome.

There are plethora of studies which have evaluated the role of YKL-40 expression in breast cancer patients; however, the results were not completely accurate. Results of some studies have demonstrated that YKL-40 overexpression is related to dismal outcome of breast cancer patients [24], while other studies had failed to prove such association [25] [26].

When we assessed the tissue protein expression of YKL-40 in 50 breast carcinoma patients of different grades and stages we have found that high YKL-40

protein expression was positively related to poor clincopathological criteria like older age of the patient ($p = 0.002$), negative ER ($p = 0.03$), & PR ($p = 0.04$), positive HER2neu ($p = 0.02$), higher grade of the tumor ($p = 0.003$), high incidence of distant metastasis ($p = 0.04$), higher KI labeling index, aggressive molecular subtype, advanced stage, high incidence of lymph node metastasis ($p < 0.001$). Also we have proved that YKL-40 was a significant predictor of the recurrence of breast cancer after successful therapy, shorter OS & RFS rates in this subset of patients, our results were nearly similar to results of Wan *et al.* [2], that assessed YKL-40 tissue protein expression levels in carcinoma of the breast and performed a meta-analysis about its expression iv various cancer types and he found the association between high expression of YKL-40 and poor prognosis and poor survival of patients. Additionally, Shao *et al.* [25], have identified that the YKL-40 expression levels are associated with breast cancer vascular invasion. Jefri *et al.* [27], assessed YKL-40 gene expression in lung cancer and found similar association between gene expression and patients' prognosis of lung cancer.

Moreover tissue protein expression of YKL-40 in prostate cancer was higher than that in adjacent non-malignant tissues, and migration and invasion of cells with high YKL-40 expression was significantly higher than that of cells with low YKL-40 expression [28] [29].

All these studies have pointed to that high YKL-40 expression could be a useful prognostic biomarker for cancer patients.

Roslind *et al.* [26] and Kim *et al.* [24] studies, found different results from us that high YKL-40 protein expression was significantly associated with well differentiated breast cancer and positive ER and PR expression which are considered standard prognostic factors that traditionally point to a good patients prognosis. Kim *et al.* [24], found a negative association between YKL-40 expression and DFS, that was similar to our results but could not be confirmed in Roslind *et al.*, 2008 study that found no association between high YKL-40 expression and survival advantage and they have not explained the biological mechanism of such results, they also found no association between expression of YKL-40 and HER2 in tumor tissue in their study all these results were different from ours.

These discrepancies could be explained by different antibodies used in the study, variable number of patients, different follow up period and variable detection methods of YKL-40 expression.

Qin *et al.* [30], in glioblastoma found results that are in line with our results.

Previous studies have provided many explanations of the role of YKL-40 in cancer progression, as it has a role in malignant initiation and proliferation of cancer cells [31], it promotes angiogenesis in malignancies by increasing levels of expression of the vascular endothelial growth factor (VEGF) & interacting with syndecan-1 in the endothelial cells, it could stimulate distant spread of the cancer by production of MMP-9 and other pro-invasive molecules [32] [33]. Hence, YKL-40 could to be used as a novel prognostic factor for expecting out come of carcinoma patients, more over it could be a promising targeted therapy [34]. Jefri *et al.* [27] & Hao *et al.* [28], proved the role of YKL-40 in EMT induc-

tion that has an important role in cancer progression, invasion, and metastasis that leads to dismal prognosis.

Hao *et al.*, 2017 detailed roles of YKL-40 expression in induction of EMT that it increase the expression of mesenchymal markers e.g. N-cadherin and Vimentin and EMT inducers e.g. such as Snail and Twist, while it decreases the expression of epithelial markers like E-cadherin

Moreover, YKL-40 plays a vital role in regulation of phosphatidylinositol 3 kinase (PI3K)/AKT/mTOR cascade, which is connected with tumor survival, invasion, and metastasis and is a central feature of EMT [28].

In our study we have evaluated the combined tissue protein expression of FOXC2 & YKL-40 in breast cancer tissue of different grades and stages and we have found that there is positive association between both markers (Spearman's r= +0.949), ($p < 0.001$) and both markers related to poor clinicopathological, prognostic criteria and were significant predictors of breast cancer relapse in this subset of patients as both markers are related to EMT and tumor progression our study is the first study that used both of them together. Both markers can be used as therapeutic targets for breast cancer especially aggressive molecular subtypes as TNBC.

In summary &, FOXC2 & YKL-40 are found to be important proteins that affect the invasion, metastasis and progression of breast cancer several mechanisms e.g. by regulating EMT, and elevated expression of both proteins is markedly associated with a shorter RFS and reduced cancer specific survival rates in patients with breast carcinoma, hence they might be considered as promising predictive markers for expecting the prognosis of breast carcinoma patients.

5. Conclusion

The expression levels of FOXC2 & YKL-40 were positively correlated with the migration and invasion of breast cancer cells and worse patient prognosis, so combination of both markers may be a potential therapeutic target for tumor cell invasion and metastasis.

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