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RESEARCH ARTICLE

PROGNOSTIC VALUE OF THE EXPRESSION OF ENDOGENOUS HYPOXIA ASSOCIATED PROTEINS HYPOXIA INDUCIBLE FACTOR-1 ALPHA (HIF-1A) AND CARBONIC ANHYDRASE ISOFORM 9 (CAIX) EXPRESSIONS IN BREAST CARCINOMA.

Ola A. Harb[1], Ibtesam Elhasadi[2], Amal M A Adim[2], Warda M Said[2], Sharifa A. M. El.Ammari[2], Basant Sh El Shafaay[3] Fady M. Habib[4] and Loay M. Gertallah[4].

1. Department of Pathology, Faculty of Medicine, Zagazig University, Zagazig 44519, Egypt.
2. Department of Pathology, Faculty of Medicine, Benghazi University, Benghazi, Libya.
3. Department of Clinical Oncology and nuclear medicine, Faculty of Medicine, Zagazig University, Zagazig 44519, Egypt.
4. Department of General surgery, Faculty of Medicine, Zagazig University, Zagazig 44519, Egypt.

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breast carcinoma, hypoxia; HIF-1α; CAIX; immunohistochemistry; prognosis.

Abstract

Background: hypoxia has been found to be related to malignant initiation, progression, increasing the occurrence of metastasis and therapy resistance in many cancer types, which made a real need for discovering drugs that could antagonize the bad effect of hypoxia in cancer, decide which patients will have benefit from such anti-hypoxia therapy then to monitor response to therapy, especially in breast carcinoma.

It is important to detect degree of hypoxia in each cancer that could be done by evaluation of the expression of hypoxia-associated proteins in cancer biopsies e.g. hypoxia inducible factor-1 alpha (HIF-1α) and carbonic anhydrase IX (CAIX) and their detailed role in breast cancer is still uncertain and gives conflicting results.

Aim of the work: was to evaluate HIF-1α and CAIX expressions in breast carcinoma, correlating their expressions with each other, with presence of lymph node & distant metastases, with recurrence free and overall survival rates of female patients with breast cancer.

Methods: we evaluated HIF-1α & CAIX expressions in sections from 90 paraffin blocks of breast carcinoma using immunohistochemistry**.** We analyzed correlations between their levels of expressions, clinic-pathological and prognostic parameters of our patients.

Results: HIF-1α and CAIX positive expression in breast carcinoma was related to advanced stage, presence of lymph node metastases, HER2 amplified and triple negative molecular subtypes ($p<0.001$), higher tumor grade ($p= 0.001$& 0.02 respectively) and negative ER ($p= 0.005$& 0.008 respectively) & PR ($p= 0.009$& 0.027 respectively) hormonal receptors, The expression of both markers was significantly positively correlated with each other ($p<0.001$). HIF-1α and CAIX positive expression in breast carcinoma was associated with shortened recurrence free and overall survival rates ($p<0.001$).

Conclusion: HIF-1α and CAIX are markers of poor prognosis of breast carcinoma patients**.**

Corresponding Author:- Basant Sh El Shafaay.
Address:- Department of Clinical Oncology and nuclear medicine, Faculty of Medicine, Zagazig University, Zagazig 44519, Egypt.

Introduction:-

Breast carcinoma is considered the commonest cancer type and the 2nd leading cause of females' cancer related mortality **[Lakhani et al., 2012]**. Breast carcinoma lymph node and distant metastasis are the most important prognostic factor for patients [**Sugie et al. 2013**]. Because of perfusion deficits, solid tumors have heterogeneous regions of hypoxia (reduced pO2). Additionally, it has been reported that the altered tumor metabolism can also contribute to tumor hypoxia [**Wojtkowiak et al. 2015**]. Hypoxia has been found to be related to malignant initiation, progression, increasing the occurrence of metastasis and therapy resistance in many cancer types, also it has a prognostic marker of poor patients' survival rates [**Wigerup et al., 2016].**The negative consequences of tumor hypoxia on cancer of various types, made a real need for discovering drugs that could antagonize the bad effect of hypoxia in cancer [**Sun et al. 2012**]. Also, hypoxia assessment in different cancer regions which can help to decide which patients will have benefit from such anti-hypoxia therapy then to monitor response to therapy, especially in breast carcinoma. An easy method of hypoxia detection could be done by assessment of endogenous hypoxia-associated proteins expression in tumor biopsies using immunohistochemistry e.g. hypoxia inducible factor-1 alpha (HIF-1α) and carbonic anhydrase IX (CAIX) [**Bussink et al., 2003**].

HIF-1α protein is destroyed and removed within minutes in conditions of normal oxygen concentration, while it is stabilized and up regulated during hypoxia. When it is stabilized, it is translocated to the nucleus, activated and forming active transcription complex. After that it binds to hypoxia response element in promoters of different target genes that could allow increase in oxygen availability and/or increase metabolic adaptation to hypoxia [**Semenza GL. 2010**]. CAIX is a glycoprotein that is considered a major HIF-1α downstream target; its expression has been related to prognosis in some types of cancer [**Zatovicova et al. 2010**]. So it is considered an attractive endogenous marker of detection of hypoxia and evaluating its role in cancer prognosis [**Supuran, 2008**]. Relation between HIF-1α and CAIX expression in cancer cells, the underlying mechanism of actions of both markers and their roles in induction by hypoxia remain unclear. Although several studies have evaluated expression of both markers in many cancers including breast carcinoma but up till now no accurate role has been detected regarding their clinicopathological and prognostic role in breast carcinoma patients [**Wigerup et al., 2016]** and also, there is no previous studies that have studied them both in a large number of Egyptian females.

Aim of the work; was to evaluate HIF-1α and CAIX expressions in breast carcinoma, correlating their expressions with each other, with presence of lymph node & distant metastases, with recurrence free and overall survival rates of female patients with breast cancer.

Patients and methods:-

We started our prospective cohort study in July 2014 finished it in July 2017, where we included ninety female patients that are having breast carcinoma that were admitted to general surgery department oncology unit, faculty of medicine Zagazig university, Zagazig Egypt, where mastectomy were surgeons that are sharing in the study performed modified radical mastectomy with axillary clearance for all cases, then sent the biopsies to Pathology Department, Faculty of Medicine, Zagazig University where they are processed and diagnosed as breast carcinoma by routine H&E histopathological examination pathologests from Department of Pathology, Faculty of Medicine, Benghazi University, Benghazi, Libya revise the diagnosis of all slides. Pathologists from Pathology Department, Faculty of Medicine, Zagazig University Zagazig, Egypt and from Department of Pathology, Faculty of Medicine, Benghazi University, Benghazi, Libya used the American Joint Committee on Cancer staging system classification (8th edition) for cancer staging **(Giuliano et al., 2017)** and the Nottingham (Elston–Ellis) modification of the [Scarff. Bloom Richardson] grading system for cancer grading **(Elston, Ellis IO, 2002).** We identified age, tumor size, histopathological subtype, grade, stage of cancer by examination of the patient's and the slide files of the Pathology Department. ER, PR hormonal receptors& Her2 neu expressions and Ki67 labeling index were evaluated for all cases. All cases are followed up for therapy response, recurrence and survival in clinical oncology and laboratory medicine department, faculty of medicine, Zagazig University. We followed up our patients until death or until the last seen alive with the median follow-up period of 30 month with range from (15-36 month).

The technique of i**mmunohistochemical staining:-**

We used the common technique of streptavidin–biotin immunoperoxidase for **staining (Hsu et al., 1981). We cut sections of five-**μm thichness of formalin-fixed, paraffin-embedded tissues blocks prepared from surgically excised breast carcinoma tissue; we placed sections on positively charged slides, de-wax and rehydrate them. We block the

activity of endogenous peroxidase; we exposed the sections to heat for antigen retrieval in the autoclave, incubated them overnight with primary mouse monoclonal anti- HIF-1α (Calbiochem, Germany, diluted 1:300), and primary rabbit polyclonal anti-CAIX (Santa Cruz Bioscience, Santa Cruz, CA, USA, diluted 1:100) antibodies at 4°C. We used the chromogen diaminobenzidine substrate (DAB). Lastly we counterstained sections with hematoxylin. We included positive and negative controls of both markers in all cases. We considered sections from cervical squamous cell carcinoma that was positive for HIF-1α, and CAIX as positive control for both markers [**Lee et al., 2008)**]. And we have omitted the primary antibodies and replaced the by non-immune serum for the negative controls.

Evaluation of immunohistochemical expression of HIF-1α and CAIX**:-**
We considered any dark stained nuclei and positive membranous & cytoplasmic stain in >1% of the tumor cells as positive for HIF-1α and CAIX respectively [**Trastour et al., 2007**].

Results:-

Ninety females' patients were included in our study with 49 (54.4%) patients were >55years. All detailed clinicopathological criteria are included in table (1).

HIF-1α expression, correlation to clinical and histopathological findings Tables 2 &3; fig 1
HIF-1α positive expression in breast carcinoma was significantly correlated with older age of the patients, higher grade and advanced stage of the tumor. HIF-1α positive expression was significantly correlated with aggressive molecular subtypes as HER2 amplified and triple negative subtypes, presence of lymph node metastases, high KI67 index ($p<0.001$ for all of them), presence of distant metastasis ($p=0.041$), negative ER ($p= 0.005$) & PR ($p= 0.009$), but it had no significant correlation with histopathological subtype of breast cancer.

CAIX expression, correlation to clinical and histopathological findings Tables 2 &3; fig 2
The positive expression of CAIX in breast carcinoma was significantly correlated with older age of the patients, advanced stage of the tumor, aggressive molecular type, presence of lymph node metastases, high KI67 index, aggressive molecular subtypes as HER2 amplified and triple negative subtypes, ($p<0.001$ for all of them), higher grade ($p=0.02$) negative ER ($p= 0.008$) & PR ($p= 0.027$) hormonal receptors, But it had no significant correlation with histopathological subtype of breast cancer or presence of distant metastasis.

The expression of HIF-1α and CAIX in breast carcinoma was significantly positively correlated with each other ($p<0.001$).

Survival analysis: Tables 4 &5; fig 3
After the follow-up period of 30 months 27.8% of patients died; The 3-year overall survival rate was 74.4% with a mean of 32.6 ± 0.62 months (95% CI; 31.4 – 33.8 months) while the median OS was not detected.
The 3-year RFS rate was 56.1% with a mean of 30.3± 0.8 months (95% CI; 28.7- 32.2 months); however the median RFS was not detected
At the end of follow up there was 38.9% of patients [35 /90 patients] developed cancer recurrence.
In multi variant analysis LN metastasis is the most significant factor of RFS & OS rates

Progression follow-up and survival results in correlation to HIF-1α **&** CAIX **expression**
Cases with positive HIF-1α **&** CAIX **expression** had a higher rate of carcinoma recurrence ($p<0.001$).
In univariant analysis patients with positive HIF-1α **&** CAIX **expression** had poor RFS and 3 year OS rates ($p=0.007$).
We found a significant correlations between HIF-1α & CAIX positive expressions in carcinoma of the breast ($p<0.001$). Table 4; Fig 3

Table1:- The clinicopathological features of our 90 patients.

Clinicopathological feature		**No. (%)**
Age group	<55y	41 (45.6%)
	>55y	49 (54.4%)
Pathology	IDC (NST)	70 (77.8%)

	ILC	20 (22.2%)
Grade	1	20 (22.2%)
	2	40 (44.4%)
	3	30 (33.3%)
LVI	Absent	26 (28.9%)
	Present	64 (71.1%)
ER	Negative	42 (46.7%)
	Positive	48 (53.3%)
PR	Negative	42 (46.7%)
	Positive	48 (53.3%)
ER/PR	Positive/Positive	44 (48.9%)
	Positive/Negative	4 (4.4%)
	Negative/Positive	4 (4.4%)
	Negative/Negative	38 (42.2%)
HER2	Negative	54 (60.0%)
	Positive	36 (40.0%)
KI 67	Low	31 (34.4%)
	High	59 (65.6%)
Molecular	Luminal A	34 (37.8%)
	Luminal B	12 (13.3%)
	HER2 amplified	24 (26.7%)
	Triple -ve	20 (22.2%)
LN	Negative	26 (28.9%)
	Positive	64 (71.1%)
DM	Absent	69 (76.7%)
	Present	21 (23.3%)
T classification	T1	19 (21.1%)
	T2	37 (41.1%)
	T3	21 (23.3%)
	T4	13 (14.4%)
N classification	N0	26 (28.9%)
	N1	18 (20.0%)
	N2	27 (30.0%)
	N3	19 (21.1%)
Stage	Stage I	14 (15.6%)
	Stage II	30 (33.3%)
	Stage III	25 (27.8%)
	Stage IV	21 (23.3%)

Table2:- Frequency of HIF-1a and CAIX expressions in our 90 patients.

Markers		**No. (%)**
CAIX	Negative	39 (43.3%)
	Positive	51 (56.7%)
HIF-1α	Negative	32 (35.6%)
	Positive	58 (64.4%)
CAIX / HIF-1α	Positive/Positive	51 (56.7%)
	Negative/Positive	7 (7.8%)
	Negative/Negative	32 (35.6%)

Table3:- Association of clinicopathological features with HIF-1a and CAIX expressions in our 90 patients.

		CAIX		**P**	**HIF-1α**		**P**
		Positive	***Negative***		***Positive***	***Negative***	
		N=51	***N=39***		***N=58***	***N=32***	
Age group	<55y	13 (25.5%)	28 (71.8%)	**<0.001**	18 (31.0%)	23 (71.9%)	**<0.001**

	>55y	38 (74.5%)	11 (28.2%)		40 (69.0%)	9 (28.1%)	
Pathology	IDC (NST)	40 (78.4%)	30 (76.9%)	0.865	45 (77.6%)	25 (78.1%)	0.953
	ILC	11 (21.6%)	9 (23.1%)		13 (22.4%)	7 (21.9%)	
Grade	1	8 (15.7%)	12 (30.8%)	**0.02**	9 (15.5%)	11 (34.4%)	**0.001**
	2	20 (39.2%)	20 (51.3%)		22 (37.9%)	18 (56.3%)	
	3	23 (45.1%)	7 (17.9%)		27 (46.6%)	3 (9.4%)	
LVI	Absent	5 (9.8%)	21 (53.8%)	**<0.001**	5 (8.6%)	21 (65.6%)	**<0.001**
	Present	46 (90.2%)	18 (46.2%)		53 (91.4%)	11 (34.4%)	
ER	Negative	30 (58.8%)	12 (30.8%)	**0.008**	34 (58.6%)	8 (25.0%)	**0.005**
	Positive	21 (41.2%)	27 (69.2%)		24 (41.4%)	24 (75.0%)	
PR	Negative	29 (56.9%)	13 (33.3%)	**0.027**	33 (56.9%)	9 (28.1%)	**0.009**
	Positive	22 (43.1%)	26 (66.7%)		25 (43.1%)	23 (71.9%)	
ER/PR	Positive/Positive	18 (35.3%)	26 (66.7%)	**0.017**	21 (36.2%)	23 (71.9%)	**0.01**
	Positive/Negative	3 (5.9%)	1 (2.6%)		3 (5.2%)	1 (3.1%)	
	Negative/Positive	4 (7.8%)	0 (0.0%)		4 (6.9%)	0 (0.0%)	
	Negative/Negative	26 (51.0%)	12 (30.8%)		30 (51.7%)	8 (25.0%)	
HER2	Negative	21 (41.2%)	33 (84.6%)	**<0.001**	27 (46.6%)	27 (84.4%)	**<0.001**
	Positive	30 (58.8%)	6 (15.4%)		31 (53.4%)	5 (15.6%)	
KI 67	Low	8 (15.7%)	23 (59.0%)	**<0.001**	10 (17.2%)	21 (65.6%)	**<0.001**
	High	43 (84.3%)	16 (41.0%)		48 (82.8%)	11 (34.4%)	
Molecular	Luminal A	11 (21.6%)	23 (59.0%)	**<0.001**	13 (22.4%)	21 (65.6%)	**<0.001**
	Luminal B	9 (17.6%)	3 (7.7%)		10 (17.2%)	2 (6.3%)	
	HER2 amplified	21 (41.2%)	3 (7.7%)		21 (36.2%)	3 (9.4%)	
	Triple -ve	10 (19.6%)	10 (25.6%)		14 (24.1%)	6 (18.8%)	
LN	Negative	5 (9.8%)	21 (53.8%)	**<0.001**	5 (8.6%)	21 (65.6%)	**<0.001**
	Positive	46 (90.2%)	18 (46.2%)		53 (91.4%)	11 (34.4%)	
DM	Absent	36 (70.6%)	33 (84.6%)	0.113	41 (70.7%)	28 (87.5%)	0.041
	Present	15 (29.4%)	6 (15.4%)		17 (29.3%)	4 (12.5%)	
T	T1	3 (5.9%)	16 (41.0%)	**<0.001**	5 (8.6%)	14 (43.8%)	**<0.001**
	T2	18 (35.3%)	19 (48.7%)		22 (37.9%)	15 (46.9%)	
	T3	20 (39.2%)	1 (2.6%)		21 (36.2%)	0 (0.0%)	
	T4	10 (19.6%)	3 (7.7%)		10 (17.2%)	3 (9.4%)	
N	N0	5 (9.8%)	21 (53.8%)	**<0.001**	5 (8.6%)	21 (65.6%)	**<0.001**
	N1	9 (17.6%)	9 (23.1%)		11 (19.0%)	7 (21.9%)	
	N2	21 (41.2%)	6 (15.4%)		26 (44.8%)	1 (3.1%)	
	N3	16 (31.4%)	3 (7.7%)		16 (27.6%)	3 (9.4%)	
Stage	Stage I	0 (0.0%)	14 (35.9%)	**<0.001**	0 (0.0%)	14 (43.8%)	**<0.001**
	Stage II	13 (25.5%)	17 (43.6%)		16 (27.6%)	14 (43.8%)	
	Stage III	23 (45.1%)	2 (5.1%)		25 (43.1%)	0 (0.0%)	
	Stage IV	15 (29.4%)	6 (15.4%)		17 (29.3%)	4 (12.5%)	
HIF-1α	Negative	0 (0.0%)	32 (82.1%)	**<0.001**			
	Positive	51 (100.0%)	7 (17.9%)				

Table4:- Univariate analysis of overall and Recurrence-Free Survival in relation to clinicopathological parameters of our 90 patients.

Variables		**3-year overall survival Rate (%)**	**p-value**	**3-year Recurrence Free survival Rate (%)**	**p-value**
Age group	<55y	78.9%	**0.001**	69.3%	**0.002**
	>55y	54.5%		46.8%	
Pathology	IDC (NST)	67.6%	0.141	60.1%	**0.029**
	ILC	59.5%		39.7%	
Grade	1	100.0%	**< 0.001**	90.0%	**< 0.001**
	2	45.7%		56.6%	

	3	47.5%		25.3%	
LVI	Absent	78.5%	**0.032**	80.2%	**0.001**
	Present	59.4%		44.8%	
ER	Negative	54.3%	**0.004**	38.9%	**< 0.001**
	Positive	74.7%		70.9%	
PR	Negative	57.6%	**0.010**	40.7%	**< 0.001**
	Positive	74.3%		70.0%	
ER/PR	Positive/Positive	73.8%	**0.016**	68.3%	**< 0.001**
	Positive/Negative	100.0%		100.0%	
	Negative/Positive	100.0%		100.0%	
	Negative/Negative	52.2%		32.8%	
HER2	Negative	70.2%	**0.039**	61.1%	**0.027**
	Positive	60.7%		50.1%	
KI 67	Low	69.8%	**0.135**	63.5%	**0.094**
	High	64.8%		54.4%	
Molecular	Luminal A	69.6%	**< 0.001**	62.7%	**< 0.001**
	Luminal B	100.0%		100.0%	
	HER2 amplified	33.5%		20.8%	
	Triple -ve	71.6%		59.0%	
LN	Negative	78.5%	**0.032**	80.2%	**0.001**
	Positive	59.4%		44.8%	
DM	Absent	73.9%	**< 0.001**	69.9%	**< 0.001**
	Present	36.1%		0.0%	
T	T1	88.9%	**< 0.001**	83.3%	**< 0.001**
	T2	70.1%		70.5%	
	T3	45.4%		25.9%	
	T4	35.2%		0.0%	
N	N0	78.5%	**< 0.001**	80.2%	**< 0.001**
	N1	74.7%		68.8%	
	N2	69.9%		44.1%	
	N3	25.9%		23.7%	
Stage	Stage I	85.7%	**< 0.001**	85.7%	**< 0.001**
	Stage II	72.8%		76.7%	
	Stage III	68.6%		44.8%	
	Stage IV	36.1%		0.0%	
CAIX	Negative	70.6%	**0.043**	69.6%	**0.005**
	Positive	60.5%		45.5%	
HIF-1α	Negative	74.5%	**0.037**	72.5%	**0.007**
	Positive	60.1%		46.2%	
CAIX / HIF-1α	Positive/Positive	60.5%	**0.102**	45.5%	**0.016**
	Negative/Positive	40.0%		40.0%	
	Negative/Negative	74.5%		72.5%	

P value< 0.05 is significant.

Table5:- Multivariate analysis of overall and Recurrence-Free Survival in relation to clinicopathological parameters of our 90 patients.

Variables	***3 Years RFS***		***3 Years OS***	
	HR (95 % CI)	***Sig.***	***HR (95 % CI)***	***Sig***
Age >55y	1.2 (0.3-5.03)	0.770	3.7 (0.68-20.06)	0.130
Pathology	1.1 (0.42-2.8)	0.870	0.7 (0.23-1.89)	0.430
Grade	2.7 (1.17-6.2)	**0.020**	6.4 (1.98-20.76)	**< 0.001**
LVI	0.1 (0.01-1.15)	0.060	0.01 (0.001-0.33)	**0.010**
ER	2.1 (0.03-154.62)	0.740	34.6 (0.39-3081.22)	0.120
PR	0.1 (0.01-0.94)	**0.040**	0.2 (0.01-2.23)	0.180

HER2	0.3 (0.06-1.37)	0.120	0.2 (0.03-1.07)	0.060
KI67	0.1 (0.01-0.85)	**0.040**	0.1 (0.01-2.62)	0.180
Molecular	0.7 (0.14-3.81)	0.710	1.2 (0.28-5.39)	0.790
DM	2.3 (0.29-17.67)	0.430	2.0 (0.2-19.1)	0.570
T	2.1 (0.74-6.04)	0.160	1.7 (0.55-5.41)	0.350
N	10.6 (2.32-47.88)	**< 0.001**	56.3 (5.52-574.02)	**< 0.001**
Stage	0.4 (0.07-2.69)	0.360	0.1 (0.02-1.11)	0.060
CAIX	0.9 (0.17-5.01)	0.920	0.3 (0.05-2.17)	0.240
HIF1α	3.1 (0.47-20.54)	0.240	6.5 (0.65-65.63)	0.110

HR: Hazard ratio; 95%CI: 95% confidence interval, p< 0.05 is significant. OS overall survival, RFS Recurrence - free survival

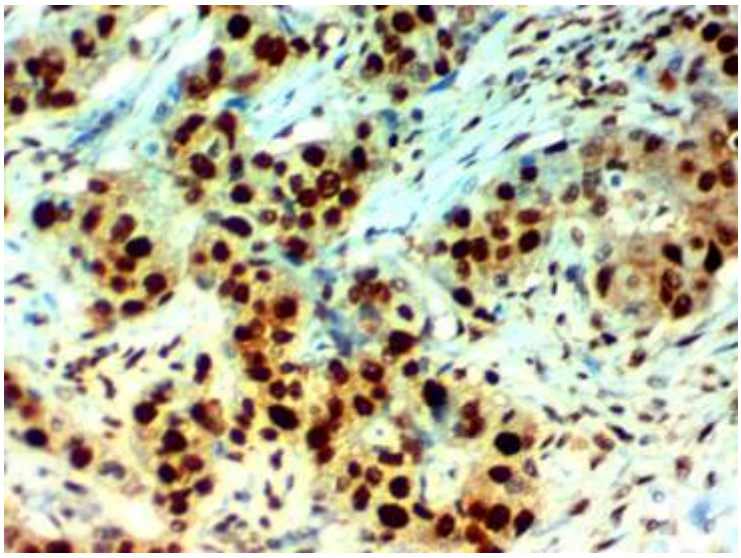


Fig 1 A

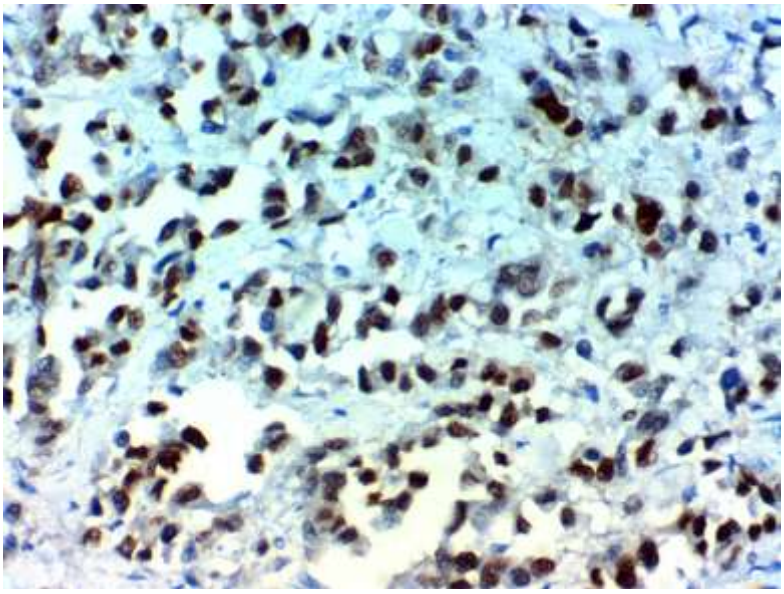


Fig 1 B

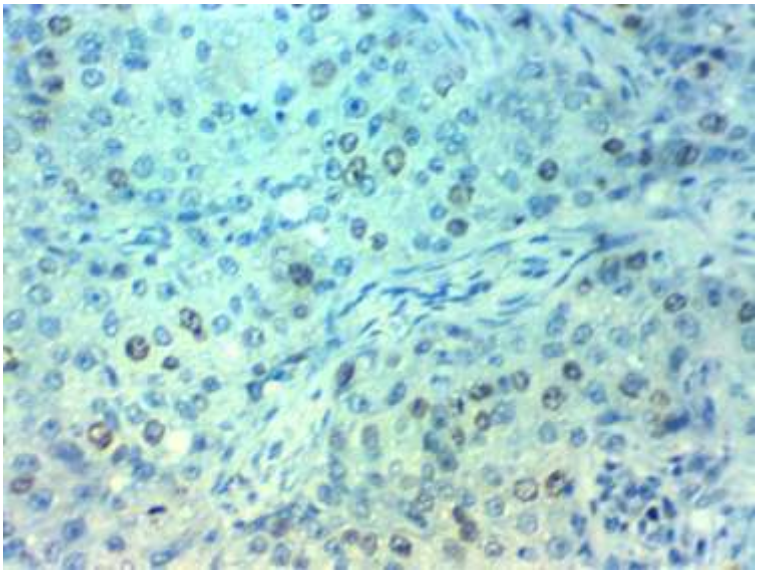


Fig 1 C

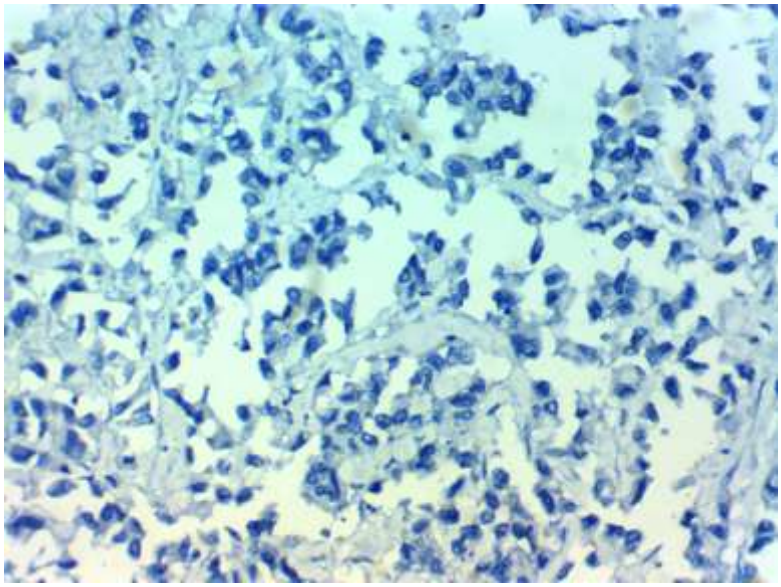


Fig 1 D

Figure1:- Immunohistochemical staining of HIF-1α in invasive carcinoma of the breast: (A) High expression in the nucleus of high grade invasive duct carcinoma of the breast (NOS) x400. (B) High expression in the nucleus of high grade invasive lobular carcinoma of the breast x400 (C) Low expression in the nucleus of high grade invasive duct carcinoma of the breast (NOS)x400(D) Low expression in the nucleus of high grade invasive lobular carcinoma of the breastx1400

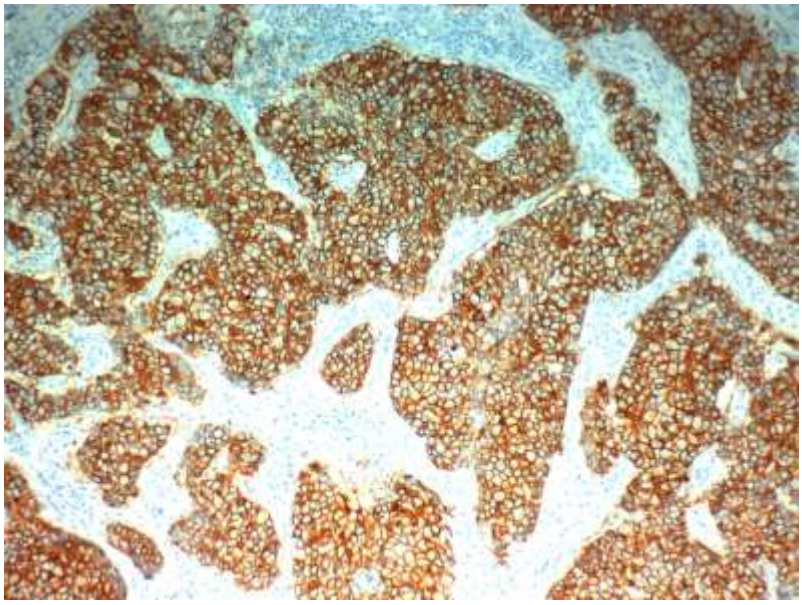


Fig 2 A

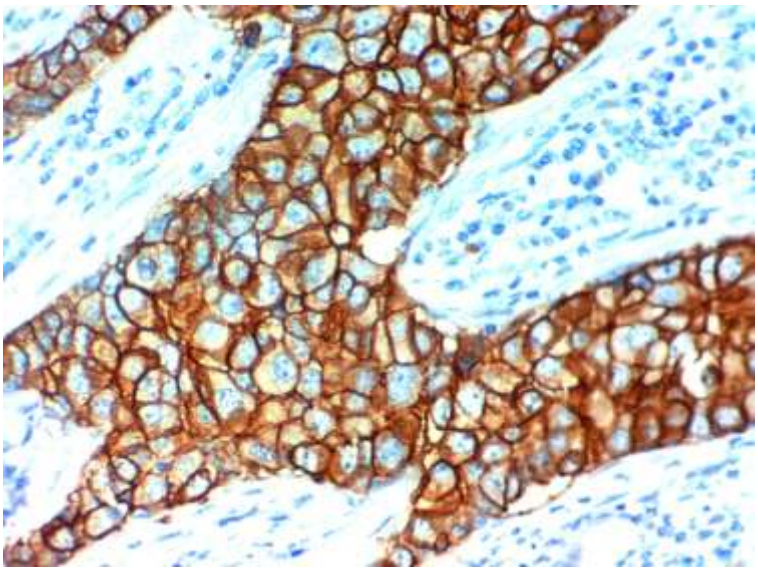


Fig 2 B

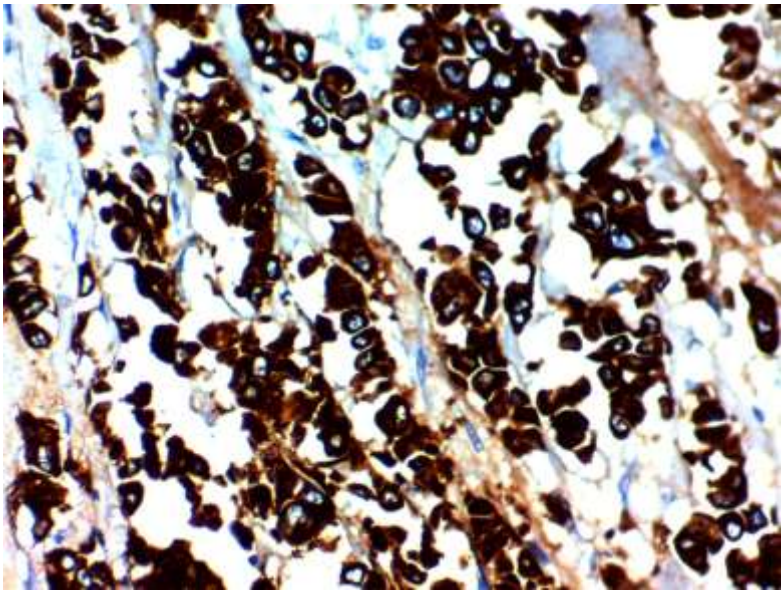


Fig 2 C

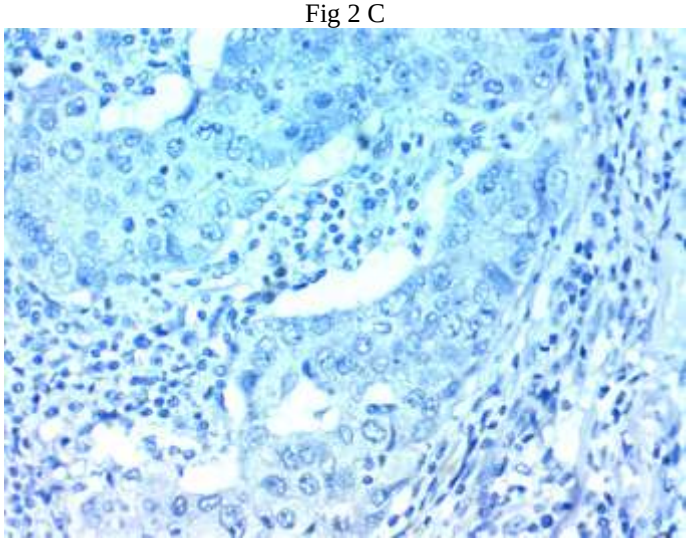


Fig 2 D

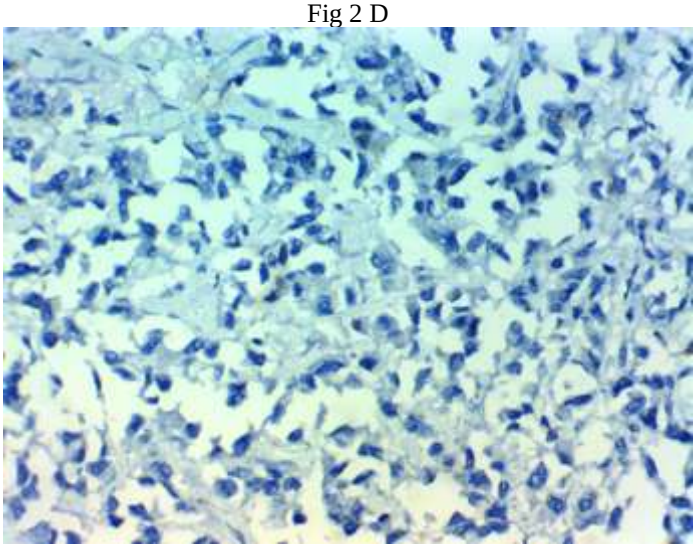


Fig 2 E

Figure2:- Immunohistochemical staining of CAIX in invasive carcinoma of the breast: (A) High expression in the cytoplasm of high grade invasive duct carcinoma of the breast (NOS) x100. (B) High expression in the cytoplasm of high grade invasive duct carcinoma of the breast (NOS) x400. (C) High expression in the cytoplasm of high grade invasive lobular carcinoma of the breast x400 (D) Low expression in the cytoplasm of high grade invasive duct carcinoma of the breast (NOS)x400(E) Low expression in the cytoplasm of high grade invasive lobular carcinoma of the breastx400

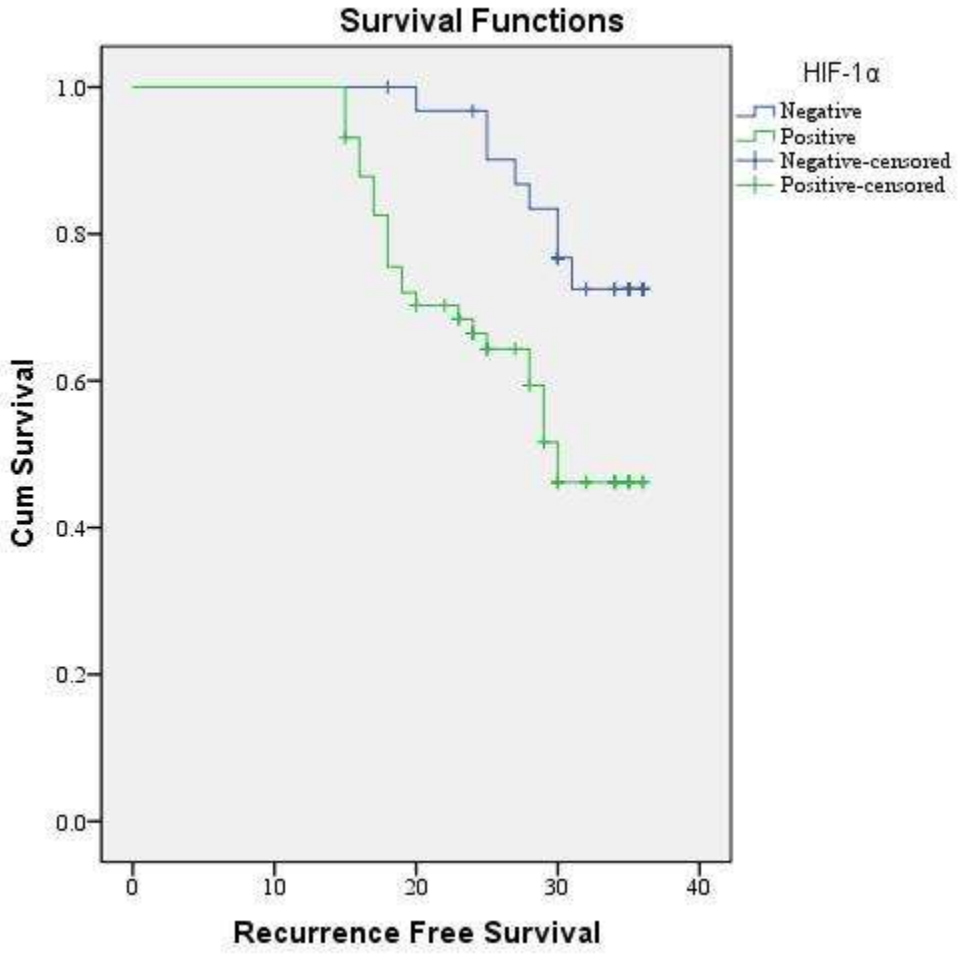


Fig 3 A

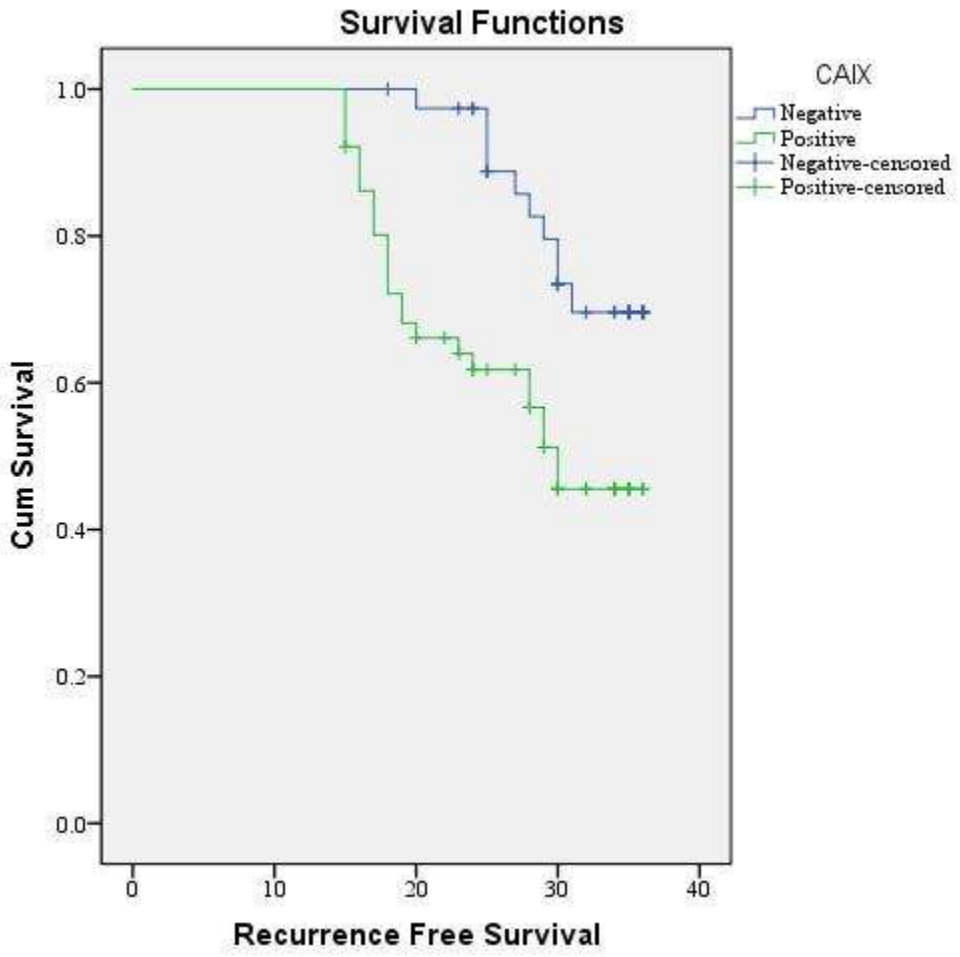

Survival Functions
CAIX
Negative
Positive
Negative-censored
Positive-censored
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
Recurrence Free Survival

Fig 3 B

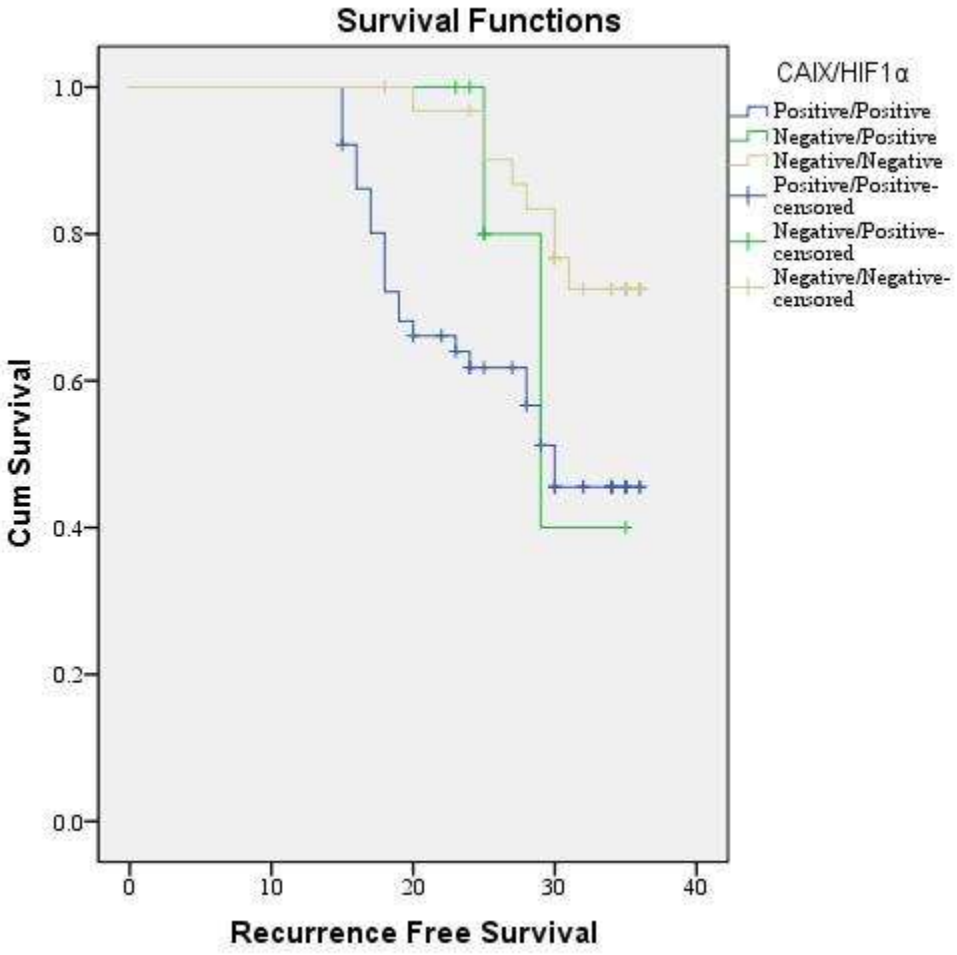

Survival Functions
CAIX/HIF1α
Positive/Positive
Negative/Positive
Negative/Negative
Positive/Positive-censored
Negative/Positive-censored
Negative/Negative-censored
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
Recurrence Free Survival

Fig 3 C

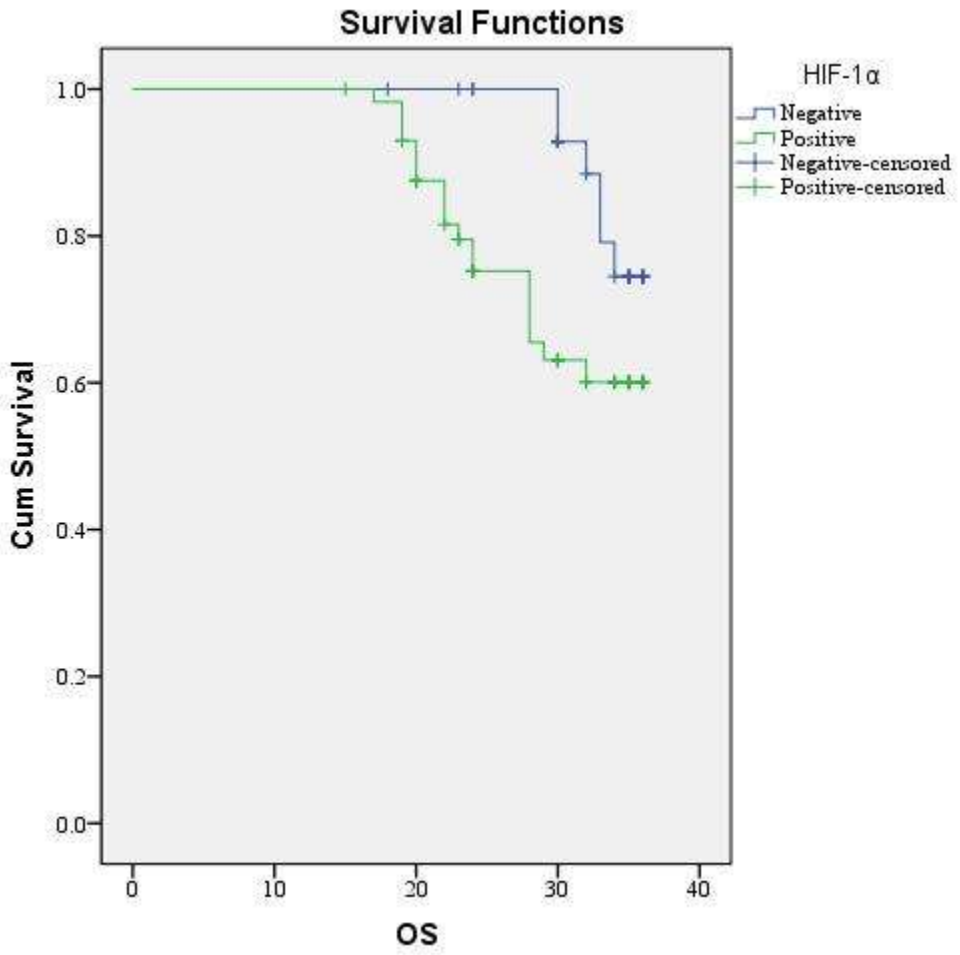
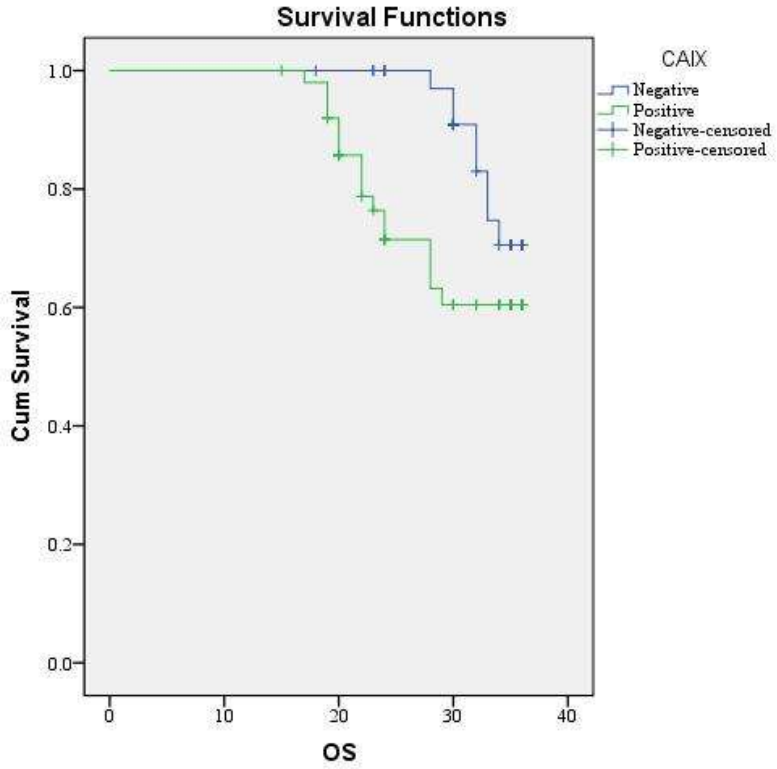

Survival Functions
HIF-1α
Negative
Positive
Negative-censored
Positive-censored
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
OS

Fig 3 D


Survival Functions
CAIX
Negative
Positive
Negative-censored
Positive-censored
Cum Survival
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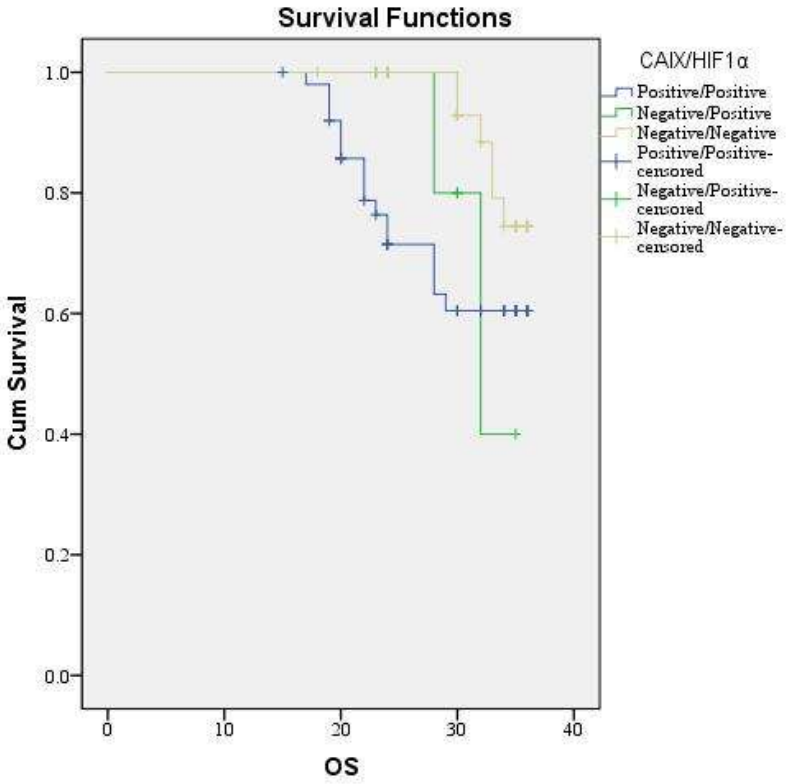
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
OS

Fig 3 E


Survival Functions
CAIX/HIF1α
Positive/Positive
Negative/Positive
Negative/Negative
Positive/Positive-censored
Negative/Positive-censored
Negative/Negative-censored
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
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10
20
30
40
OS

FIG 3 F

Figure 3:- A.The 3-year Recurrence-Free survival Rate in relation to HIF-1α Expression, B. The 3-year Recurrence-Free survival Rate in relation to CAIX Expression, C; The 3-year Recurrence-Free survival Rate in relation to CAIX/ HIF-1α Expression, D; The 3-year overall survival in relation to HIF-1α Expression, E; The 3-year overall survival in relation to CAIX Expression, F. The 3-year overall survival in relation to CAIX / HIF-1α Expression

Discussion:-

Former researchers had explored the role of HIF- 1α is involved in breast carcinogenesis [**Kronblad et al., 2006**], and detected that it could influence its growth rate and metastatic ability and subsequently could be associated with poor patient prognosis [**Liu et al., 2015**], but proved results are still conflicting and lacking accurate sharp data.

Our present results detected that when HIF-1α positively expressed in breast carcinoma that will be significantly related to worse clinic pathological findings like older age of the patients, higher grade and advanced stage of the tumor, aggressive molecular subtypes, presence of LN and distant metastasis, also we found that cases with positive HIF-1α **e**xpression had a higher rate of carcinoma recurrence, poor RFS and 3 year OS rates.

Our results were near results of former research that was done by **Nalwoga et al., 2016 that** evaluate the expression of HIF-1α in relation to markers of angiogenesis and other clinicopathological criteria in a cohort of breast cancer from Africa and they detected that positive HIF-1α expression was associated with increased tumor angiogenesis, high cancer cell proliferation rate that was evidenced by increased Ki-67 labeling index, high cancer grade that points to HIF-1α as a poor prognostic marker of breast carcinoma and a therapeutic target for breast cancer patients. We detected an association between HIF-1α expression and the presence of LN & distant metastasis in breast carcinoma; our results were near results of

Liu, et al., 2015 who had proved that proved that HIF- 1α is a regulator of cell hypoxia response and focused on HIF- 1α role in increasing breast carcinoma metastasis, as they explored that HIF-1 had several roles in metastasis, e.g. increasing malignant cells invasion, up-regulating epithelial-mesenchymal transition (EMT), and formation of metastatic niche. **Liu, et al., 2015** also discuss the values of therapeutic benefits of targeting the HIF- 1α for management of breast cancer patients that is considered a recent therapeutic approach that could be used in combination with currently used therapies.

Wigerup et al., 2016 found that positive HIF-1α protein expression is present in malignant tumors of many organs and that is associated with poor prognosis of carcinoma of cervix, endometrium and ovary. They stated that HIFs had many roles in cancer cells growth, proliferation, differentiation, angiogenesis, cancer cell metabolism, local invasion, lymph nodes and distant metastasis. Subsequently, HIFs could be responsible for to chemo- and radiotherapy resistance, so they are associated with poor prognosis of cancer patients.

In addition HIF-1α could increase the expression of PD-L1 that is an immune checkpoint protein, which could be responsible for immune suppression (Noman et al., 2014).

But **Wigerup et al., 2016** stated that the prognostic value of HIF-1α in breast carcinoma patients has conflicting results in many follow-up studies,

Zhang *et al.* (2012) showed results similar to ours that there is strong association between HIF-1α expression and presence of distant metastases in breast carcinoma, and also HIF- 1α increased the extravasation of breast carcinoma cells in the lung, that was explained by **Wong *et al.*, 2012** by the ability of HIF-1 in regulation of metastatic niche formation at distant sites before cancer cell arrival**.**

Solid malignant tumors, like carcinoma of the breast contain hypoxic areas due to presence of vascularization defects in these rapidly growing cancer cells. HIF- 1α plays an essential role in cancer cells adaptation to hypoxia by increasing transcription of many genes that could regulate angiogenesis, proliferation, invasion, and metastasis **(Semenza, 2012).**

Another mechanism by which HIF- 1α can act is by up-regulating epithelial–mesenchymal transition (EMT) process which is essential for tumor progression. EMT could be stimulated by hypoxia, by many mechanisms like HIF-1α pathways in several human malignancies. HIF- 1α induce EMT by up-regulation of EMT transcription factors e.g.

Twist, Snail, Slug and Zeb in many cancer types [**Zhang et al., 2015**], In addition, hypoxia is an angiogenesis stimulating agent via production of many HIF-1 transcription factors, moreover during the EMT process HIF- 1α stimulated angiogenesis by up-regulating VEGF transcription, and associated with microvessel growth which is an evidence of activated angiogenesis [**Wigerup et al., 2016].**

As we found that increased positive HIF- 1α expression shows strong association with poor outcome and dismal survival rates of breast carcinoma patients**, so** that hypoxia is considered a hallmark of aggressive behavior of many solid tumors and responsible for metastases and therapy resistance, so it is considered a cancer attractive therapeutic targets like the recently discovered HIF- 1α inhibitors **Liu, et al., 2015**.

Targeting hypoxic cancer cells have been explored by many approaches e.g. hypoxia-activated prodrugs, and HIF-1α specific targeting **(Semenza, 2012).**

HIF- 1α inhibitors, like digoxin and acriflavine, had potential therapeutic roles in decreasing cancer growth, invasion, metastasis and vascularization in breast cancer **(Wong *et al.*, 2012),** HIF- 1α targeting is considered as a novel therapeutic modality for management of breast cancer patients and improving their prognosis which could be used in combination with currently used therapies.

Many researchers have studied CAIX expression in a plethora of human malignancies and stated that it was associated with poor patient's outcome, but its role in breast carcinoma patients still needs further clarifications [**Thiry et al., 2006**].

Here we proved that positive CAIX expression in breast carcinoma tissues was correlated related to worse clinic pathological findings like older age of the patients, higher grade and advanced stage of the tumor, aggressive molecular subtypes, presence of LN metastasis, also we found that cases with positive CAIX expression had a higher rate of carcinoma recurrence, poor RFS and 3 year OS rates.

Glaberman et al., 2016 also found nearly the same, that CAIX expression was related to aggressive pathological phenotype, chemotherapy resistance and poor prognosis of patients with breast cancer.

We proved that the positive expression of CAIX in breast carcinoma was related to the presence of LN metastases, that was like results of **Aomatsu et al., 2014**] who proved the same results, and results of **Keun-Yong et al., 2016** who found that positive CAIX expression was strongly correlated with sentinel LN metastasis in addition to invasion of lymphatic vessels by the primary tumors, also many previous studies proved results similar to us; **Aomatsu, et al., 2014 found that** breast cancer patients with positive expression of CAIX had lower pathologic complete response (pCR) rates when treated with neoadjuvant chemo-therapy.

We proved that the positive expression of CAIX in breast carcinoma was correlated to larger tumor size, higher tumor grade, stage and aggressive molecular type similar to our results **Sch¨utze et al. who** detected upregulation of CAIX expression in breast carcinoma patients with advanced stages [**Sch¨utze et al., 2013**]. And results of, **Lou, et al., 2011, Chen et al., 2010 who f**ound a positive correlation between CAIX expression, aggressive phenotype of breast carcinoma and poor patient prognosis.

In our study we found that patients with high CAIX expression had shorter RFS and 3 year OS rates. Similar to our results **Tan et al., 2009**, observed that that positive CAIX expression was related to chemo-resistance and shorter survival rates in breast cancer patients.

Thus, these data suggest that CAIX is a predictive and a prognostic marker for breast cancer patients.

Different from our results, **Chen et al 2010** found no association between CAIX expression in breast cancer tissues and patients' nodal status tha could be explained by different number of patients, variable technique of staining and different antibody clone which gives different results.

There are multiple theories which could explain the association between positive CAIX expression and poor patients' outcome in breast cancer. That, CAIX expression is linked to cancer tissue hypoxia and acidosis and its upregulation is a step in cancer cells adaptation to survive under hypoxic conditions [**Chen et al., 2010,**], also,

CAIX is related to cancer hypoxia and stimulates cancer cell spread and invasion, which incriminated tumor hypoxia to increase cancer cells invasion and metastasis [**Shin et al., 2011**]. Tumors with upregulation of CAIX could be able to maintain their intracellular pH, but it increased acidification in extracellular space, which leads to extracellular matrix breakdown which could increase malignant cells invasive ability [**Chen et al., 2010,**, **M¨uller et al., 2011**], in addition increased hypoxia in the malignant cells leads to genome instability. Also, CAIX could influence breast cancer stem cells growth and survival under hypoxic conditions [**Lock et al., 2013**].

That association of CAIX positive expression with aggressive clinicopathological and prognostic parameters of breast cancer patients that proved by our results and results of previous studies, could support the theory that discovering selective CAIX inhibitors could be used to manage cancer patients and improving their prognosis, moreover some of such inhibitors are in the preclinical setting and still under evaluation [**Ward, et al., 2013, McDonald, et al., 2012].**

We found positive correlation between HIF-1α & CAIX expression in breast cancer tissue, that was similar to results of **Brennan et al., 2006, but** different from **Chen et al 2010, Tan et al., 2009**, who found no an association between both markers expression, which could be explained by that they have done their studies on tissue microarray that allow analyses of results based on only minute tissue samples and their tissue sections were acquired from only non-necrotic areas.

In addition, Tan, et al., 2009 explained the absence of association between both markers expression in their study by different half-lives of HIF-1α and CAIX, as HIF-1α was found to be rapidly destroyed and removed within minutes of re-oxygenation **(Jiang et al, 1996),** while, CAIX has a longer half-life of 2–3 days **[Rafajova et al, 2004),** so they stated that they could not be present together.

Sobhanifar et al., (2005 found positive correlation between HIF-1α and CAIX expression in breast carcinoma tissue, which was similar to ours but they detected CAIX expression only without HIF- 1α expression in peri-necrotic regions in cancers, which is also due to differences in half-lives of HIF-1α and CAIX [**Tan et al., (2009**].

Summary, Conclusions and future suggestions:-

HIF-1 and its downstream target HIF- 1α are considered regulators of cancer cell response to hypoxic stress and play important roles in breast carcinoma cells growth, invasion and metastasis.

Both markers, mainly HIF- 1α, is involved in the key step of the metastatic process e.g. EMT, malignant cell invasion, and metastatic niche formation.

As we demonstrated that breast carcinogenesis is stimulated by cells adaptation to hypoxia and acidosis, moreover the glycolytic, acid-resistant phenotype that has HIF- 1α and CAIX positive expression is an aggressive phenotype.

Hence it will be better that tumor management strategies should aim at antagonizing the sequence of hypoxia, glycolysis and acidosis.

Moreover, identification of the metabolic phenotype of breast carcinoma will allow discovering to novel therapeutic modalities.

Also, the aggressive triple negative molecular subtype that is difficult to treat, as they are both chemo-resistant and hormonal non-responsive, and as we detected that such subtype showed positive expression of both HIF- 1α and its downstream target CAIX, so targeting them both e.g. targeting HIF- 1α with its inhibitors, gene therapies and CAIX inhibitors could be of particular importance in managing this aggressive cancer and improving patients prognosis **(Supuran, 2008).** The combination of HIF- 1α & CAIX inhibitors with existing therapeutic modalities might be found to be useful clinically.

Clinical therapeutic trials are needed to determine if they could increase the survival of patients having breast cancer alone or in addition to currently used therapies.

Future studies are needed to discover more specific HIF- 1α & CAIX inhibitors, to study their detailed mechanism of action, and to include them in clinical therapeutic trials of breast cancer patients.

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