Immunohistochemical Aspects on (HSP70) and its Correlation to Clinical Stages, Pathological Grading and Hormones Receptors (ER, PR, HER-2) in Breast Cancer (BC) Women from South of Iraq

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Abstract: Breast cancer considered the most common malignancy .Therefore this study was aimed to review the observations and information on relationship of preoperative level of (HSP70) with hormones receptors (ER,PR,HER-2) and the expression of these receptors in biopsies from patients with different stages and grades of disease by immunohistochemical method. In this study total number of (130)cases was analyzed and most patients diagnosed with stage (T111) then (T11)and (T1) with highly significant difference at ($p \leq 0.05$) and the same results was obtained from classified tumor grading with (BC)patients. The recent study evaluated level of (HSP70) in all (BC)patients ,the data showed highly significant difference and the mean level was increased in (71)case compared to control ,also we evaluated the relationship between tumor staging and grading with level of (HSP70) and data revealed to high level in (T111) patients while no signification between (T1,T3) ,the same results recorded between tumor grading and level (HSP70), hormones receptors expression (ER,PR,HER-2) and its correlation with (IL-6)level was also determined ,the(HSP70) with (ER+)compared to (ER-) with high significance (p=0.00),the same results shown with (PR+) and(HER-2) expression and (HSP70)in all (BC)patients. The role of (HSP70) in (BC) immunity ,carcinogenesis ,cancer pathogenesis ,tumor initiation ,growth and metastasis .Results also explained correlate of (HSP70)with clinical staging and grading ,these findings pointed to an important role in the progression of (BC) at least in patients with advanced disease stages. Data determined that the (ER+)(PR+) positive tumor more reactive with(HSP70) which explained the effect of this factor on hormones receptors. Moreover present study showed   little elevated in concentrate of (HSP70) with Her2+as compared with Her2- but with no significant differences,(p=.158).Immunhistochemical staining was used in this study and results showed variable degree of staining Within invasive ductal and lobular carcinoma types compared to control.

1- Introduction

Breast cancer is a complex disease resulting from abnormal growth of cells begins in the lining layer of duct or lobe of the breast. that leads to malignant tumor formation . It is the commonest malignancy in women, affecting women during their lifetime .Bc, considered the most common cancer, is now leveled as the first among all cancers diagnosed in women (Kolahdoozan, *etal*, 2010; Ferlay *et al*., 2015) **.**The HSP70 family of heat shock proteins consists of molecular chaperones approximately (70kDa) size that serve critical roles in protein homeostasis, it is the most abundant protein of eukaryotic cells and essential for cell survival under stress conditions (Daugaard *et al.,*2007).

Marginally this proteins was expressed in non transformed cells, but is greatly overexpressed in tumor cells, (Balaburski *et al.*,2013), it is aberrantly expressed in different malignancies and has a cancer specific cell protective effect, it has emerged as a promising target for anticancer therapy(Granato *et al*.,2013). The overexpression of HSP70 occurs in many different tumor types, and in general high levels of this protein are correlated with poor prognosis, increased tumor grade and drug resistance(Powers *et al* ,2010). Hsp70, which is correlated with prognosis in breast, endometrial,

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uterine cervical, and bladder carcinomas. Increased Hsp expression may also predict the response to some anticancer treatments. (Lebert ,*etal.*,2003 ;Ciocca and Calderwood, 2005; Tavassol *et al*. 2011). Hsp expression has been analyzed in relation to the histopathological characteristics of the tumor tissues as, tumor type, grade of differentiation with the expression of other molecules as estrogen receptors, and mutated p53, also with patient parameters like sex and age. In addition, levels of circulating Hsps have been correlated with patient and tumor characteristics. (Ciocca and Calderwood, 2005).in (BC) expression of HSP70 has been found associated with tumor clinical stage and histological grade with significant differences(Ciocca and Calderwood, 2005; Asfour *et al*., 2011). Several reports have showed correlated the expression of HSPs with hormonal status, in particular, Small HSP, HSP70 and HSP90 families are involved in the regulation of (ER,PR) and hence have been extensively studied in human breast cancer where they play a role in tumour cell proliferation, differentiation, invasion, metastasis, cell death and tumour immune response (Conroy and Latchman,1996 ;Ciocca and Calderwood, 2005), In breast cancer, HSP70 has been found associated with ER where it increases ER transcriptional activity and hence results in increased cell proliferation breast tumors (Sherman and Multhoff , 2007). A strong association between HSP70 expression and ER receptors was found in previous reports (JKalogeraki *et al*., 2007;Asfour *et al*.2011), the role of steroid receptors in the induction of heat shock proteins, and these agree with (Asfour,*etal*.,2011; Salda and Romanucci,2012). HSP70 plays acritical role in human carcinogenesis by enhancing cancer cell proliferation, protecting cancer cells against apoptosis and promoting cancer angiogenesis (Wadhwa,*etal*.,2006; Yang ,*etal*.,2008). Morevore The correlations between overexpression of HSP70 and the clinical features and HER-2 of patients with breast cancer were evaluated(Jin *et al*.2016).

Material and mothed

Sample collection
In this study total number of (130)cases was analyzed and (60) cases were subjected to Histopathological and Immunohistochemcal studies. The recent study evaluated level of (HSP70) in all (BC) patients and (16)control ,the data showed highly significant difference and the mean level was increased in (71)case compared to control

Immunological studies: In this study we measured (HSP70) level in the serum of the breast cancer patients by using Enzyme-Linked Immuno Sorbent Assay (ELISA) , human (HSP70) ELISA kit.

Histopathological studies: the histopathological study of tumor stage was assessed according to the criteria established by the seventh edition of the American Joint Committee on Cancer (AJCC) staging manual as TI, TII and TIII , while the tumor grade was determined according to the Scarff-Bloom-Richardson classification modified by Henson *etal*., 1991 also as GI,GII and GIII.

Immunohistochemstiry: Hormone receptors(ER,PR and HER-2) were assessed via immunohistochemistery mothed (IHC) .

Statistical analysis: The data of this study were analytic with one way anova ,t-test and chi-square by using Spss program version 22.

Results:
1- Clinical staging and histologic grading
Dependent on Tumor-node-metastasis (TNM) system was used to classified the stage of BC, in our patients out of (130) cases , staging system can be applied only to (60) cases , this study had reported (1)cases (1.7%) diagnosed as T I, (28) cases with (46.7%) are clarified as T II and (31) cases with (51.7%) are T III, there were a significant differences among different stages at(P≤0.05),(table1).

Table (1) : Tumor histologic staging in (BC) patients.

Staging	no. of patients	Percentage rate%
T I	1	1.7%
T II	28	46.7%
T III	31	51.7%
Total	60	100%

Chi-square = 27.30 df= 3   p- value = 0.00

In this study grading system can be applied only to 60 cases , and we find that 2 cases (3.33%) were diagnosed as grade I, low grade ,figure (1), 30 cases with (50 %) are grade II, medium grade , figure (2) and 20 cases with

(46.67%)recorded as grade III high grade figure (3)and(4). There was a significant differences among different grades (P ≤0.05) , table (4-10).figure

Table (2): Tumor pathological grading in (BC) patients.

Grade	no. of patients	Percentage rate %
G I	2	3.33%
G II	30	50%
G III	28	46.67%
Total	60	100%

Chi-square = 24.40   df= 2   p- value = 0.00
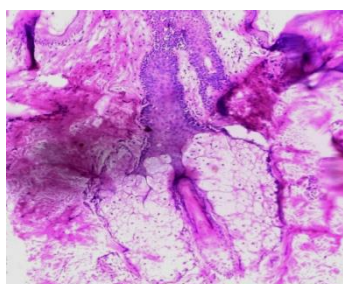
Figure(1): lesion from breast tissue (IDC) with low grade showed hyperplasia with scalp sheath and sebaceous metaplasia, Magnification x400.
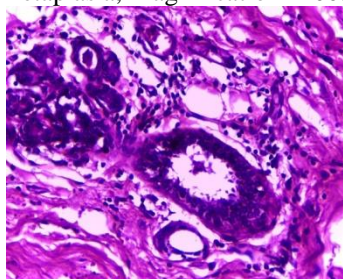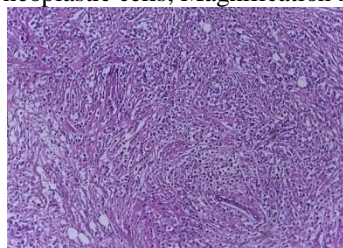

Figure (2): lesion from breast tissue (IDC) with medium grade showed Large ducts with epiheliosis but with intact basement membrane anl eosinophil secretion in the lumen number of ductules and large number of neoplastic cells, Magnification x400.

Figure(3): lesion from breast tissue (IDC) with high grade of breast carcinoma showed abnormal growth of malignant cells ,changes with architecture, dense, ovale nuclei ,vacuolated cytoplasm ,with mitotic fig ,irregular strand of collagen fibers , there was no ducts no necrosis observed ,mild collagen and hyaline matrial deposit and some empty vacuoles were noticed in matrix**, Magnification x400.**
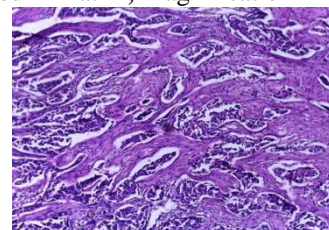

Figure (4): lesion from breast tissue (IDC) with high grade showed changes with malignant cells nuclei (dense) and hyperohromatic ,the stroma showed like trabecullae infiltrating with malignant and inflammatory cells ,most cribriform duct with hyperplasia**, Magnification x400**

2. 1 Expression of (ER) and (PR) receptors.
Results showed that(40) women was positive for (ER) receptors expression with (66.7%) rate distributed according scoring system, other (20) patients were regarded negative to expressed (ER) receptor with (33.3%) rate, significant differences(P > 0.05) between these two percentages rate table (3).

Table (3):Expression of(ER) in breast cancer patients.

Hormone receptor	no. of patient	Percentage rate%
ER +	40	66.7%
ER -	20	33.3%
Total	60	100%

X^2= 6.67   df= 1   p- value = 0.010

While (45)cases were positive for PR (75%) and (15) cases were (25%) significant (P > 0.05), Table (4)Figure(2).

Table (4): BC patients distributed by the expression of progesterone receptor.

Hormone receptor	No.	Percentage rate%
PR +	45	75%
PR -	15	25%
Total	60	100%

X^2= 15.00   df= 1   p- value = 0.00

2-2 HER-2
Result found (33)cases were positive (55%),and(27) cases were negative (45%) of patients, the percentage of cases of breast tumor were significant (P > 0.05) Table (5) Figure(3)

Table (5): BC patients distributed by the expression of human epidermal growth factor-2 receptor.

HER-2	No.	Percentage rate%
Positive status	33	55%
Negative status	27	45%
Total	60	100%

X^2= 0.60   df= 1   p- value = 0.43

3-Determination of (HSP70) concentration in (BC) patients serum
Results showed high significant difference at (P ≤ 0.05) that the concentration of(HSP70) in (BC) patient serum was (9.6641 ±1.66887ng /ml) comparative to healthy women that the (HSP70) level was(20.1589± 1.28444ng /ml) between patients group and control group, increased mean levels of HSP70, p ≤0.05), as compared with the control (9.6641ng /ml) (Table (3).

Table(6) : Level of (HSP70) in blood serum of(BC) patients compared to control values expressed as (mean ±SD)

Groups	no.of patient	(HSP70) concentration ng/ml	±SD	P value
(BC) patient	71	20.1589	1.28444	.025
control	16	9.6641	1.66887	

3-1- Correlation between (HSP70) concentration with tumor staging and grading
The relationship between (HSP70) levels and tumor staging of BC patients, showed a high significant increased with mean level of(HSP70) (16.4261± 3.25819ng/ml) in all patients at (T3) tumor stage compared with patients with stage T2 the level of (HSP70) was (4.6650± 1.14025mg /ml) and less than in T1(2.9300± .02000ng /ml) , while there were no significant difference between T2 and T1 (4.6650 and 2.9300 ng/ml, respectively p=.863) and no significant between T3 and T1 patients (16.4261and 2.9300 ng /ml ,respectively p =.182),result showed significant difference between T3 and T2(16.4261and 4.6650 ng /ml ,respectively p =.002)(Table 4).

Table (7): Serum level of HSP70 in tumor stages in BC patients

Tumor Stage	no.of patient	(HSP70) concentration ng/ml) ±SE	Sig. between groups	P value
T1	1	2.9300± .02000	T1-T2	.863
T2	28	4.6650± 1.14025	T1-T3	.182
T3	31	16.4261± 3.25819	T2-T3	.002

The relationship between(HSP70) levels and tumor grades of BC patients, showed a high significant increased with level of HSP70 in patients with G3 was (17.2366± 3.20763 ng /ml) ,patients with G2 (3.9941± .76354 g/ml) and with G1 was (1.5155± 1.39750 ng /ml), while there was no significant difference between G1 and G2 (1.5155 and 3.9941ng /ml, respectively p=.779) and no significant static .

betweenG1 and G3 patients (1.5155 ng /ml and 17.2366 ng /ml, respectively p=.080) , and also there was significant difference betweenG1 and G3 were observed(3.9941 ng/ml and17.2366 ng /ml, respectively p=.000) . However ,the results to indicated increased HSP70 levels in patients serum with advanced grades G3. (Table 5)

Table(8): Serum level of HSP70 in tumor grades of BC patients

Groups	No.	(HSP70) concentration ng/ml) mean ±SE	Sig.between groups	P Value
G1	2	1.5155± 1.39750	G1-G2	.779
G2	30	3.9941± .76354	G1-G3	.080
G3	28	17.2366± 3.20763	G2-G3	.000

3-2 Correlation between (HSP70) concentration and(ER,PR,HER2) receptors.
also result of present study showed relationship between increased mean level of (HSP70) with ER+(12.7015 ng/ml) as compared with ER-(5.8325 ng/ml) with high significant differences(p=.004),and increased in PR+(12.3227ng/ml) as compared with

PR-(5.0102 ng/ml) with significant differences(p=.018) Table(6)(7),while the result showed little elevated in concentrate of (HSP70) with Her2+(13.1479 ng/ml) as compared with Her2-(8.7178 ng/ml) but with no significant differences,(p=.158)Table(8).

Table(9): Illustrate the relationship between level of(HSP70) and (ER) receptor values expressed as(mean± SE)

Tissue receptors	no.of patient	(HSP70) concentrate ng/ml	SE	T	Df	Pvalue
ER+	40	12.7015	±2.65388	1.765	58	.004
ER-	20	5.8325	±1.38548			

Table(10): Illustrate the relationship between level of(HSP70) and (PR) receptor values expressed as(mean± SE)

Tissue receptors	no.of patient	(HSP70) concentrate ng/ml	SE	T	Df;	P value
PR+	45	12.3227	2.38270	.723	58	.018
PR-	15	5.0102	1.55285			

Table(11): Illustrate the relationship between level of(HSP70) and (HER-2) receptor values expressed as(mean± SE)

Tissue receptors	no.of patient	(HSP70) concentrate ng/ml	SE	T	Df	P value
HER2+	33	13.1479	3.25202	1.155 -	58	=.158
HER2-	27	8.7178	2.22703			

Discussion

1- (HSP70) concentration in (BC) patients serum

present study observed increase level of HSP70 with significant difference ($P \leq 0.05$) between patients group and control group, increased mean levels of HSP70 significantly higher in(BC) patients, as compared with the control and this results clarified the role of (HSP70)in association with (BC) cells, it may be preserved and maintenance the cells surface and prevent their resistance to stress and apoptotic so its level increased with tumor growth , these results in agreement with previous studies showed that (HSP70) was located on tumor cells (Hsp70) have been located on tumor cells surfaces of tumor cell lines Multhoff *et al*.,1995) , these cells have(Hsp70) on their surfaces were more resistant to apoptosis and to high doses of drugs , several studies have recognized the survival function of (Hsp70) to the ability to suppress the joint of apoptosis, since cells that contain elevated levels of this chaperone have an increased ability to tolerate different types of stress at several point of the apoptotic cascade(Didelot, C., *et al*.,(2006) .(Hsp70) actually, acts as a natural inhibitor of numerous stress kinesis at the beginning of cell death, as well as it protects cells from apoptosis by binding and modulating the activity of various pro- and anti-apoptotic proteins at transcriptional and post translational level by impairing Janus kinas (JNK) activity, in fact, Hsp70 prevents JNK-induced phosphorylation and inhibition of Bcl-2 and Bcl-xL anti-apoptotic proteins, and promotes cell survival through the maintenance of mitochondria stability Nishitai and Matsuoka,(2008).

Our results showed high level of (HSP70) associated with tumor progression with significant difference ($P \leq 0.05$) and these findings were agree with Asfour *et al*.,(2011) on (BC) women who observed high level of (HSP70) in patients more than healthy group, also agreed with study by Rohde,M. *et al*. (2005) they displayed over expression of (HSP70) in (BC) cell lines , these combined findings strongly indicate that HSP70 can act as an oncogene, although some evidence implicates(HSP70) in human cancer, the predominant form of HSP70 over expressed in human cancer is the major, cytosolic, stress induced gene HSP70, hereafter for simplicity, it referred to HSP70 in serum of patients caused promote cancer cell growth by distinct mechanisms.

Furthermore our results deal with Iraqi study made by Jassem *etal*.,(2014) on patients with bladder cancer who found high level of (HSP70) and suggested that HSP70 was urinary biomarker for diagnosis and staging of bladder cancer ,also agreed with Meral , G.,*et al*.,(2015) on patients with colorectal cancer (CRC) whose significantly higher HSP70 concentrations compared with the control group at (p=0.001). Further Moshino *et al*.,(1994) indicated that (HSP70) in (BC) patients showing that human tumor infiltrating CD4+T cells are able to react with B cells expressing hsp70. Along this line Marianna H.,*et al*.,(2015) showed that inhibition of HSP70 led to the restoration of FOXM1 (Forkhead Box M1) protein level to basal level following treatment with proteasome inhibitor thiostrepton, this protein was oncogenic transcription factor that is also a well recognized cell cycle regulator, FOXM1

expression is eliminated from quiescent or differentiated cells, but it is highly expressed in proliferating and malignant cells Laoukili J ,*etal*.,(2007),while over expression of(HSP70) led to correlates with decreased FOXM1 protein and that mean HSP70 suppresses FOXM1 during stresses also in cancer. According to the these data HSP70 considered as important biomarker in cancer this agreed with Rohde, *et al.* (2005); Suzuki,*etal*.,(2006); Asfour *et al*.,(2011), who showed , HSP70 might contribute to cancer progress by regulating multiple signaling pathways such as NFkB ,on the other hand HSP70 considered anti apoptotic and a natural inhibitor of numerous stress kinesis at the start of cell death , suppressor to FOXM1 that lead to decreased risk of cancer development agree with Nishitai and Matsuoka,(2008) ; Halasi , *et al* ,(2015).HSP70 can become an important biomarker in cancer because its over expression in serum is associated with many malignant tumors (Suzuki ,*etal*.,2006)**.**

2-Correlation of (HSP70) with tumor staging and grading

HSPs are expressed in many types of cancer and act as immune dominant antigen Suzuki, *etal*.(2006).Therefore, the ability to down regulate HSPs may be of therapeutic use for treating patients with cancer, Our data from the present study referred to high level of (HSP70)in mean concentration of all (BC) patient and the level increased in associated with tumor (T3)compared with patients at (T2) and (T1) this results may be related that the (BC) cells may produced (HSP70) or induced other inflammatory cells to secrete it, thus its associated with tumor staging and grading ,these results agreed with previous studies showed high level of (HSP70)in (BC) women with late stage and correlate to histological grade ,this protein expressed in many other types of cancers and act as immune dominant antigen Suzuki,*etal*.,(2006); Asfour *et al*.,(2011) Ciocca and Calderwood, 2005),they suggested that Hsp70, which is correlated with prognosis stages and grade in breast, endometrial, uterine cervical, and bladder carcinomas(Lebert,*etal*.,2003 ;Ciocca and Calderwood, 2005; Tavassol *et al*. 2011) . Recent data in agreement with study on oral cancer patients done by Tavassol *et al.* (2011)they observed immunoreactivity for HSP70 was positive in tumor cells in all patients positive immunoreactivity of tumor cells could be detected in 17 of 28 patients with advanced stages prognostic significance of HSP70 expression in tumor cells ,finally deal with Yu *et al.* (2013) who suggested a significance Prognostic of HSPs in urothelial carcinoma of the urinary bladder, but disagree with study by Watson, *etal*.,(2003) on patients with bladder carcinoma they showed no correlation between HSP70 expression and clinical stages and histological grades.

Our findings showed correlation between (HSP70) expression and clinical stages and grades in all(BC) patients which may caused by inhibition in immune system and effect of other factors that regulate growth and proliferation of breast cancer cells .Previous studies referred that (BC) cells might evade immune surveillance by induced cells to produce(HSP70) molecules resulting in the suppression of effectors immunity and suppress apoptosis and thus promote tumorigenesis. The present results agreed with the results of ,who suggested that (HSP70) was urinary biomarker for diagnosis and staging of bladder cancer (Garrido *et al*.,2001; Margel *et al*.,2011) .

3-Correlation of (HSP70) with (ER,PR,HER2) receptors

This study showed relationship between increased mean level of (HSP70) with ER+ positive (ER+) compared with (ER-) with high significant differences at (p=.004),and increased in PR+ as compared with(PR-)with significant differences(p=.018),while the result showed little elevated of (HSP70) level with (Her2+) compared with (Her2-) but no significant differences (p=.158),these findings regarded to the (HSP70) and its expression in high level with hormone recaptores in all (BC) patients compared to healthy Beere,(2004); Asfour,*etal*.,(2011); Salda and Romanucci,(2012). Also recent data agreed with study made by Salda and Romanucci,(2012) they observed correlation among members of small HSP, HSP70 and HSP90 families have been studied extensively in (BC) with ER,PR and HER-2.and suggested with by Jin *et al*.,(2016) they reported HSP70 is upregulated in (BC), and may be a useful poor prognostic biomarker as well as a potential therapeutic target for patients with(BC). Small HSP, HSP70 and HSP90 families are involved in the regulation of estrogen receptors (ER) and hence have been extensively studied in human (BC) where they play a role in tumor cell proliferation, differentiation, invasion, metastasis, cell death and tumor immune response (Conroy and Latchman,1996; Ciocca and Calderwood,(2005) . in particular, HSP27 and HSP70 have been shown to exert a premalignant effect in (BC) , by blocking programmed cell death and senescence, elevated expression of HSPs has also been observed in canine mammary tumors (Romanucci,*etal*.,2006; Aguruparan,*etal*.,2006; Romanucci,*etal*.,2008),this is consistent with the association of HSP70 with some of the diagnostic parameters of malignancy poor differentiation, lymph node metastasis, increased cell proliferation, block of apoptosis, and higher clinical stage Torronteguy,*etal* .,(2006),investigations into the genetic polymorphism of HSP genes indicate that homozygosis for HSP70 genes is significantly associated with increased in breast carcinoma patients Chouchane,*etal*.,(1997) ;Vargas

Roig,*etal*.,(1998). It has been established that HSP70 has various sub cellular localizations, interacts with multiple binding partners, and plays a role in carcinogenesis, also, it was elevated in immortalized cell lines and tumor cells, and additional up regulation of HSP70 expression at later stages of carcinogenesis coincides with the acquisition of invasiveness (Wadhwa,*etal*.,2006). functions contributing to continued proliferation of cancer cells, including mitochondrial-biogenesis, ATP production, anti-apoptosis, chaperoning, inactivation of tumor suppressor p53(Yang L,*etal*.,2011 ;Deocaris,*etal*.,2013).Furthermore, the presence of HSP70 in breast cancer is also significantly associated with histological grade, LN metastasis and clinical stage, the three critical prognostic factors indicative of poor outcomes and cancer recurrence in breast cancer patients. Study on human colorectal adenocarcinoma, found higher HSP70 expression correlated with poor patient survival (Dundas,*etal*.,2005; Gunaldi,*etal*.,2015).

Further multivariate survival analysis showed that HSP70 expression was one of the independent prognostic factors, along with clinical stage and Her2 status(Dundas,*etal*.,2005; Jin, *et al*.2016). Apparently, HSP70 may be a novel marker related to poor survival of breast cancer patients, most importantly, breast cancer patients with over expression of HSP70 concomitant with advanced clinical stages and lymph node metastasis had a lower survival rate than those with low HSP70 expression. Furthermore, Her2 positive was determined to be the important factor in predicting long term of breast cancer patients ,also it has been founded that expression of HSP70 was notably higher in the a liver derived tumor cell line than in a normal liver cell line (Chen X,*etal*.,2011). The expression of HSP70 was correlated with Her-2 status (Dundas,*etal*.,2005; Jin *et al*.2016)), also showed the expression of HSP70 inhibit EMT, decrease tumor progression and lose the metastasis inducing capability (Chen J,*etal*.,2014) .Our findings contribute to the better understanding of the role of heat shock proteins in breast cancer plays an important role in breast cancer progression; it might be a new attractive biomarker for prognostic evaluation and a molecular therapeutic target in patients with breast cancer. , also, the role of steroid receptors in the induction of heat shock proteins, and these agree with Asfour ,*etal*.,(2011); Salda and Romanucci,(2012). In spite of these remarkable findings, larger sample size in randomized studies is needed to assess the potential value of HSP70 as candidate biomarker for breast cancer surveillance. Further studies on a larger number of cases is needed to elucidate the biological significance of high HSPs expression which will assist in clarifying the prognostic role and the possible mechanism of action

of HSPs and its relation to steroid receptors in breast cancer.

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