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EVALUATION OF RECEPTOR FOR ADVANCED GLYCATION END PRODUCT /HIGH-MOBILITY GROUP BOX 1 (RAGE/HMGB1) EXPRESSION STATUS AND ITS PROGNOSTIC VALUE IN BREAST CANCER

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ABSTRACT

Sucess in breast cancer treatment is not built only on diversity but on clinical relevance to tumor pathogenesis. So, gathering of many prognositic biomarkers involved in cancer progression could yield new treatment modalities in order to maitain good quality of life for patients .The receptor for advanced glycation end product (RAGE) and its ligand the high-mobility group box 1 (HMGB1) protein seem to play a role in many cancers , their cross-talk affects breast cancer behaviour. The aim of this study was to investigate the tissue RAGE and HMGB1 expression levels and their association with clinico-pathological features and overall survival in breast cancer patients.Tissue RAGE and HMGB1 mRNA levels were measured by real time-polymerase chain reaction (RT-PCR). Results showed tissue RAGE and HMGB1 displayed significant higher expression levels compared to benign group. RAGE and HMGB1 expression levels in breast cancer tissues were significantly associated with high tumour grade , lymph node metastasis ,stage III wih no significant relation to the molecular type of tumor nor overall survival. RAGE-HMGB1 system seems to be linked to breast cancer which may represent a prognostic biomarker of clinical and theraputic significance.

***Key words*:** Breast cancer, RAGE, HMGB1, RT-PCR .

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INTRODUCTION

worldwide and among women, breast cancer is the most frequent malignancy with high mortality rate **(Bond et al., 2018)**. Breast cancer ranks as number one among all malignant tumors in Egypt **(Stapleton et al., 2011)**. Metastasis, the causaive agent of breast cancer-related mortalities, is not fully understood **(Zheng et al., 2017)**. Therefore, identification of potential biomarkers linked to tumor proliferation and metastasis that could predict prognosis and conistitute a therapeutic target, is highly required.

Receptor for advanced glycation end product (RAGE), a member of the immunoglobulin superfamily, is a transmembrane multiligand receptor encoded by gene on chromosome 6p21.3 **(Sparvero et al., 2009).** The extracellular domain of RAGE, ligand binding part, contains one variable like (V) and two constant-like (C) type domains. The V domain poses two N-glycosylation sites. The cytoplasmic tail of RAGE is responsible for intracellular signaling transduction **(He et al., 2017).** RAGE communicates with several ligands including advanced glycation end products, HMGB1 **(Bierhaus et al., 2005).**

HMGB1 belongs to the high-mobility group (HMG) protein family that was first described by **Goodwin et al .,1973**. HMGB1 is a multifuncional protein with multiple sites of existance. Within the nucleus, as a DNA binding protein, it is concerned with regulation of replication, transcription, DNA repair, recombination and genomic stability **(Sohun and shen, 2016).**

Release of HMGB1 is mediated by passive release from necrotic cells or active release from activated immune cells **(Wittwer et al., 2013).** Once released, it carries on its extracellular functions as a damage-associated molecular pattern molecule (DAMP) by interaction with several receptors, notably RAGE **(Rai, 2018).** RAGE, beside being a fundamental partner for HMGB1-induced cell proliferation, migration, inflammation and angiogenesis, it

provides a functional platform for comunication with other HMGB1 receptors (**Kang et al., 2013**).

Growing evidences have demonstrated that RAGE orchestrates with its ligand HMGB1 to promote growth and metastasis of multiple tumors (**Dhumale et al., 2015**) This receptor-ligand pair is engaged in each of the ten hallmarks that tumor raised on, by initiating a cascade of signaling pathways controlling diverse aspects of tumor biology**.** Although, it is well recognized that HMGB1 play a paradoxical role in cancer, either as a promotor or a supressor factor (**Rai, 2018**), its role in breast cancer still confers confusion **(Sun et al., 2015, Wu et al., 2016).**

The aim of this study is to investigated the tissue expression levels of RAGE and HMGB1 and evaluate their association with the clinico-pathological features of breast cancer patients.

MATERIALS AND METHODS

This prospective case control study was carried out at Medical Biochemistry and Molecular Biology and Pathology Departments, Faculty of medicine, Menoufia University. It was performed on 68 cases of modified radical mastectomy specimens diagnosed as an invasive duct carcinoma (IDC) (malignant group), not otherwise specified and 63 cases of breast biopsy diagnosed as benign breast lesions including fibroadenoma and fibrocystic disease (control group). None of these patients were treated with radiotherapy or chemotherapy. These cases were received in Pathology Department, Faculty of medicine in the period between January 2015 and August 2016. Fresh part of the tumor mass was collected in an eppendorf tube and kept at -80°c for further RNA extraction and RT-PCR for both RAGE and HMGB1 expression level. Slices from the tumor mass were taken and immersed in formalin and was submitted to routine tissue processing ending with paraffin embedded blocks formation. Tumors were graded according to the criteria of Nottingham modification in the Bloom-Richardson system (**Elston and Ellis,**

1991). Tumor staging was performed according to Tumor Node Metastasis (TNM) staging system (**Edge et al., 2010**). According to the imunohistochemistry results of ER, PR and HER2/neu, the cases were classified into:

-Luminal subtype: positive ER and/or PR and negative HER 2/neu.

-HER 2/neu positive subtype: negative ER, negative PR and positive HER2/neu.

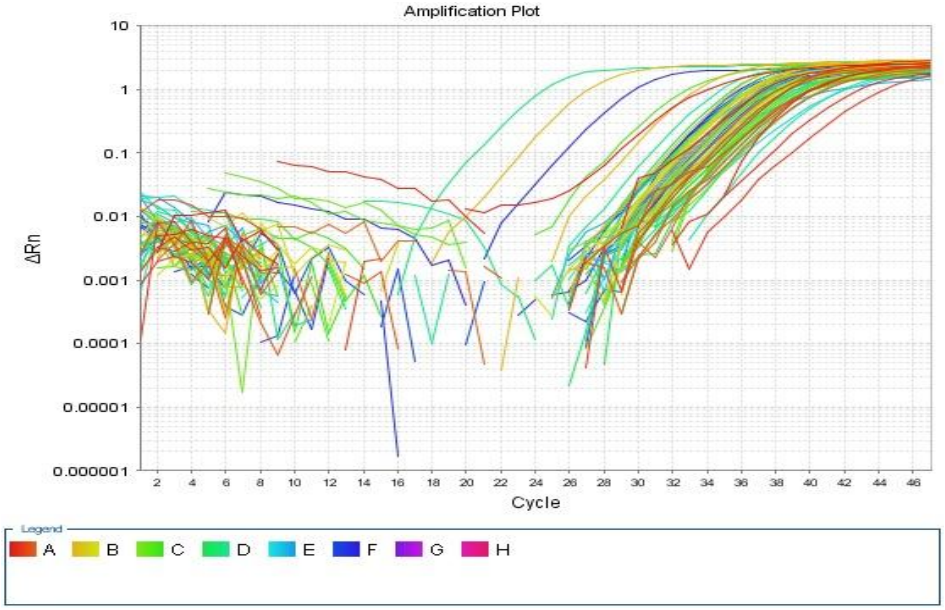
-Triple negative (TN) subtype: negative ER, negative PR and negative HER 2 neu.(**Goldhirsch et al.,2011)**. For statistical purposes, tumors with grade 1 and 2 were lumped in one group, tumors with T3 and T4 stages were lumped in one group. Also, cases with stage I and II were lumped together. The patients were followed until last follow up date August 2016. The median follow-up time was 25 months. This study was approved by ethical committee of Faculty of Medicine, Menoufia University and a written consent was obtained from all subjects before the study.

Assay of RAGE and HMGB1 mRNA expression levels:

RNA extraction from breast tissue: Total RNA was extracted from specimens using the QIAamp RNA Blood MiniKit (Qiagen, USA) according to manufacturer's specifications (**Wang et al., 2000**). The purity of RNA was determined by measuring its absorbance at 260 nm (A260). Absorbance readings should be greater than 0.15 to ensure significance. The ratio between the absorbance value at 260 and 280 nm (A260 /A280) gives an estimate of RNA purity. (A260/A280) ratio greater than 1.6 was accepted (**Dorak, 2004**).

Two-step RT-PCR: For reverse transcription step, a reverse transcriptase kit (SensiFAST cDNA synthesis kit, Bioline Reagents Ltd, United Kingdom) was used for complementary DNA (cDNA) synthesis on 2720 thermal cycler (Singapore). For cDNA synthesis, RNA (10µl) was reverse transcribed in a final volume of 20µl containing 1µl of reverse transcriptase enzyme, 4µl of 5x TransAmp buffer and 5µl of DNase/RNase free water. The samples were incubated at 25˚C for 10 min (primer annealing), and 42˚C for 15 min (reverse transcription). Reverse transcriptase was then

inactivated by heating at 85˚C for 5 min. All products were stored at -20˚C till the next step. For cDNA amplification: A relative quantification of RAGE and HMGB1 mRNA expression normalized to the endogenous reference gene β-actin was performed by real-time PCR (RT-PCR),using the 2x SensiFAST™ SYBR® Lo-ROX Kit (Bioline Reagents Ltd.), on Applied Biosystems 7500 Real-Time PCR System. RAGE primers were: 5'-AAACATCACAGCCCGGATTG-'3 (forward) and 5'-TCCGGCCTGTGTTCAGTTTCT- 3' (reverse) (**Wang et al., 2015**). HMGB1 primers were: 5'-ATATGGCAAAAGCGGACAAG-'3 (forward) and5'- GCAACATCACCAATGGACAG- 3' (reverse) (Wang et al., 2013). β-actin primers were: 5'-GGCGGCACCACCATGTACCCT-3' (forward) and 5'-AGGGGCCGGACTCGTCATACT-3' (reverse). Specificity of the primers was verified using Primer BLAST program provided by NCBI. The PCR reaction was setup with 25µl of final reacion volume consisting of 12.5µl of 2x SensiFAST™ SYBR® Lo ROX Master Mix,1 µl of each target primer (Sigma), 5.5µl of DNase/RNase free water and 5µl of cDNA. Thermal cycling conditions comprised a 10 min at 95˚C, followed by 45 cycles at 95˚C for 15 sec, and 60˚C for 1 min. For relative quantification of the results, the comparative cycle threshold (Ct) method was used. Analysis was performed using Applied Biosystems7500, software version 2.0.1. The relative expressions of RAGE and HMGB1 were calculated using the comparative Ct method (2-ΔΔCt). Each run was completed using melting curve analysis to confirm specificity of the amplification and absence of primer dimmers (figure 1 and 2).



Figure(1): Amplification plot of RAGEexpression

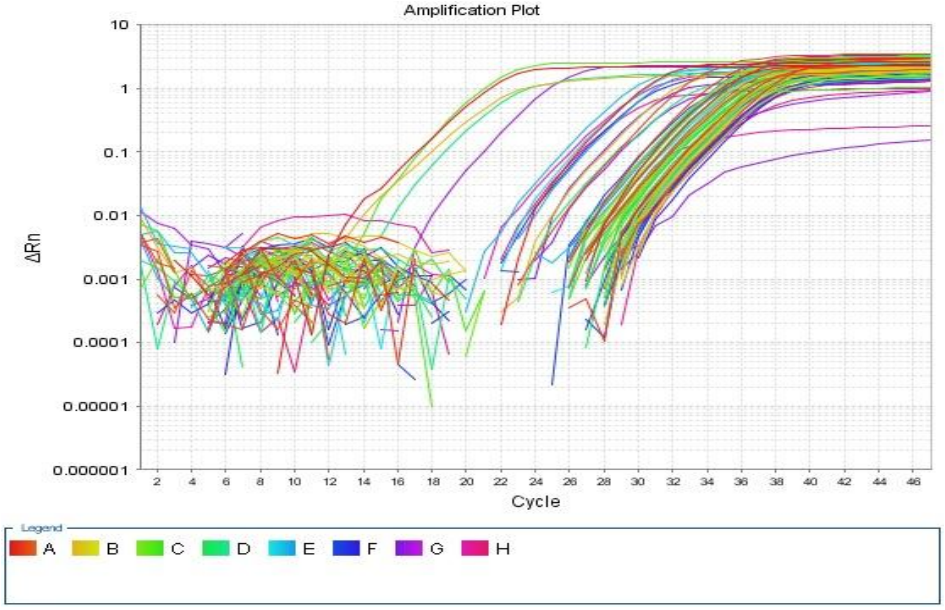


Figure (2): Amplification plot of HMGB1 expression

Statistical Analysis

Data were collected, tabulated, and statistically analyzed using a personal computer with the "statistical package for the social sciences" (SPSS) version 23. All data were expressed as median,range, number and percent. Mann-Whitney (U) and Kruskal wallis tests were used for comparisons between quantitative variables. Spearman correlation coefficient (r) were used to assess the correlation between two quantitative variants. $P \leq 0.05$ was considered significant.

RESULTS

The sixty-eight breast cancer patients were stratified according to age, tumor size, differentiation grade, lymphatic metastasis, and TNM stage and molecular type. All malignant cases were females. Their age ranged between 21 and 82 years (mean, 48.9 years). The tumor size ranged between 1 and 7.4 cm in maximal dimension. According to the differentiation grade, most of the patients were grade 2 (76.5%). In relation to T stage, 27.9%, 55.9%, 13.3% and 2.9% belonged to T1, T2, T3 and T4 stages, respectively. Regarding nodal status, 92.6% of patients showed lymph node involvement. With reference to molecular subtyping, 42.9% of patients were luminal, 30.9% were Her-2 positive and 26.5% belonged to triple negative category. Nearby carcinoma in-situ component was found in 13.2% of the breast cancer patients as shown in **(Table 1).** The mRNA expression levels of both RAGE and HMGB1 were significantly higher in breast cancer tissues compared to benign breast diseases cases ($p<0.001$) **(Table 2).**

There was significant positive linear correlation between RAGE and HMGB1 mRNA expression levels in breast cancer patients (spearman correlation coefficient r= 0.92, p<0.001) **(figure 3).** Tissue mRNA expression levels of both RAGE and HMGB1 were significantly associated with patients showing high differentiation grade (p<0.001), advanced tumor stage (T3 and T4) (p<0.001), N3 status (p<0.001), stage III (p<0.001) and with presence of in-situ component (p=0.004). Moreover, Both tissue RAGE and HMGB1 mRNA expression levels exhibited positive linear correlation with tumor size and number of positive lymph nodes (p<0.001), but no association was found between them and patient age **(Tables 3 & 4).**

Mean overall survival time was 24.7 months (mean±SD = 24.7±2.6, median 25 months). Overall survival ranges from 13 to 28 months. There was no significant relationship between overall survival and neither RAGE nor HMBG1 mRNA expression levels (p= 0.17, r=0.17 and p= 0.31, r= 0.13 respectively).

Table 1: clinico-pathological characteristics of the studied cases :

		Breast cancer patients (n=68)		Benign breast diseases cases (n=63)
		NO.	%	NO.
	Median	49		50
	Range	21 – 82		40 – 59
	Median	3.3		
	Range	1 - 7.4		
Differentiation Grade	grade 1	3	4.5	
	grade 2	52	76.5	
	grade 3	13	19.1	
T stage	T1	19	27.9	
	T2	38	55.9	
	T3	9	13.3	
	T4	2	2.9	
N stage	N0	5	7.4	
	N1	19	27.9	
	N2	28	41.2	
	N3	16	23.5	
Stage	I	4	5.9	
	II	19	27.9	
	III	45	66.2	
	Median	5		
	Range	0 – 17		
Recurrence	Negative	50	73.5	
	Positive	18	26.5	
Molecular type	Luminal	29	42.6	
	Her-2	21	30.9	
	Triple negative	18	26.5	
Presence of in-situ component	No	59	86.8	
	Yes	9	13.2	

Table 2: Comparison between control and malignant groups regarding RAGE and HMGB1 mRNA expression .

		Breast cancer patients (n=68)	Benign breast diseases cases (n=63)	Test	P.value
HMGB 1	**Median**	**8.4**	**0.8**	**U= 36**	**<0.000**
	Range	**1.35 - 89.7**	**0.04 - 2.9**		
RAGE	**Median**	**15.6**	**0.8**	**U= 15**	**<0.000**
	Range	**2.9 - 76.8**	**0.07 - 3.9**		

RAGE: receptor for advanced glycation end product , HMGB1: high-mobility group box 1 SD= standard deviation U= Mann Whitney

Table 3:The association of RAGE mRNA expression level and clinico-pathological characteristics in breast cancer cases

		RAGE in Breast cancer patients			
		Median	Range	Test	**p-value**
Age (years)				r= 0.13	0.32
Tumor Size (cm)				r= 0.66	<0.001
Differentiation Grade	grade 1& 2	13.5	2.9 - 45.6	U= 107.5	<0.001
	grade 3	20.7	15.7 - 76.8		
T stage	T1	7.5	2.9 - 16.8	K= 26.4	<0.001
	T2	15.9	4.1 - 45.6		
	T 3&4	20.7	15.7 - 76.8		
N stage	N0	5.6	4.1 – 16	K= 28.3	<0.001
	N1	7.5	4.5 - 45.6		
	N2	14.1	2.9 - 45.6		
	N3	21.9	15.8 - 76.8		
Stage	I & II	5.6	4.12 - 16.6	U= 178	<0.001
	III	16.3	2.9 - 76.8		
Number of positive Lymph Nodes				r= 0.75	<0.001
Recurrence	negative	15.7	4.6 - 76.8	U= 331	0.098
	positive	12.8	2.9 - 65.8		
Molecular type	Luminal	15.6	2.9 - 45.6	K= 0.15	0.92
	Her 2	16	4.3 - 32.5		
	Triple negative	13.9	4.9 - 76.8		
Presence of insitu component	No	14	2.9 - 45.6	U= 108.5	0.004
	Yes	19.6	12.3 - 76.8		

RAGE receptor for advanced glycation end product SD= standard deviation

r= Spearman correlation coefficient U= Mann Whitney K= Kruskal Wallis

Table 4: The association of HMBG1 mRNA expression level and

clinico-pathological chacharacteristics in breast cancer patients

		HMGB1 in Breast cancer patients			
		median	Range	Test	**p-value**
Age (years)				r= 0.08	0.46
Tumor Size (cm)				r= 0.68	<0.001
Differentiation Grade	grade 1& 2	7.4	1.35 - 85.9	U= 115.5	<0.001
	grade 3	14.5	10.7 - 89.7		
T stage	T1	4.8	1.35 - 14.5	K= 23.6	<0.001
	T2	9.7	1.4 - 85.9		
	T 3&4	14.5	10.7 - 89.7		
N stage	N0	3.8	3.8 - 5.2	K= 33.8	<0.001
	N1	3.8	1.35 - 65.6		
	N2	8.4	3.9 - 85.9		
	N3	18.9	10.7 - 89.7		
Stage	I & II	3.8	1.35 - 10.9	U= 94	<0.001
	III	12.3	3.9 - 89.7		
Number of positive Lymph Nodes				r= 0.64	<0.001
Recurrence	negative	9.4	3.2 - 85.9	U= 364.5	0.23
	Positive	6.7	1.35 - 89.7		
Molecular type	Luminal	7.4	1.4 - 65.6	K= 0.64	0.73
	Her 2	8.4	3.2 - 74.9		
	Triple negative	10.3	1.4 - 89.7		
Presence of insitu component	No	7.5	1.4 - 85.9	U= 107	0.004
	Yes	29.4	7.7 - 89.7		

HMGB1: high-mobility group box 1 SD= standard deviation r= Spearman correlation coefficient U= Mann Whitney K= Kruskal Wallis

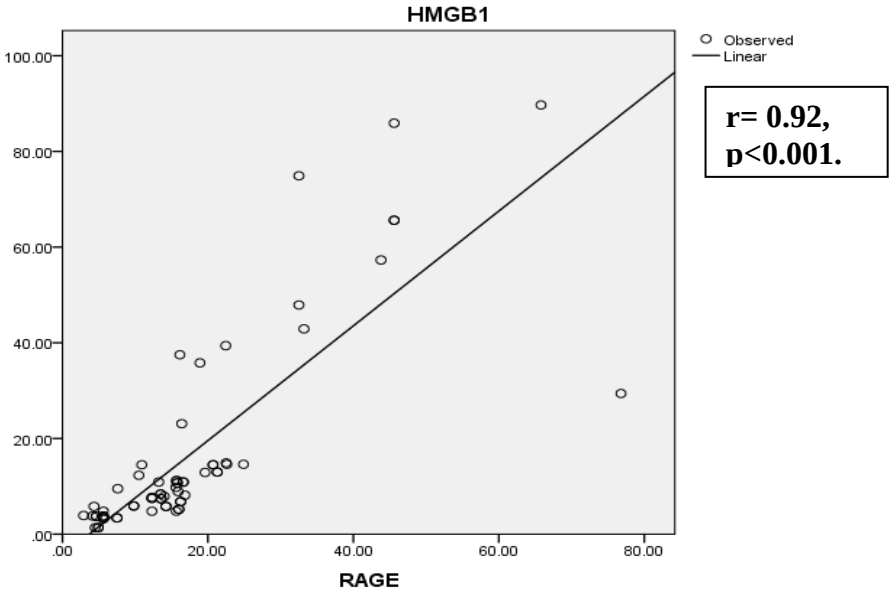


Figure 3: **Significant positive linear correlation between RAGE and HMBG1**

DISCUSSION

RAGE and its ligand HMGB1 are considered as critical mediators of cancer development and progression through activation of oncogenic signaling cascades linked to tumor cell proliferation and metastasis (**Sohun and Shen, 2016**). Based on the results of this study, RAGE-HMGB1 axis displayed higher expression levels in breast cancer tissues compared to benign group announcing for its involvement in tumour birth. Of note, there was collegial overexpression of both RAGE and HMGB1 which is proved, in this study, by the significant positive correlation between RAGE and HMGB1 levels. Previous reports augment our findings regarding HMGB1 overexpression in breast cancer (**Stoetzer et al., 2013, Sun et al., 2015, ke et al., 2017**). Within tumor cores many cells die by necrosis owing to the misery tumor microenvironment of hypoxia and nutrient shortage hence passive release of HMGB1 (**Exner et al., 2016**). Once released, it reacts with RAGE to nourish the

inflammatory tumor microenvironment through activation of nuclear factor kappa B (NF-κB) and release of proinflammatory cytokine (**Chen et al., 2014, Paek et al., 2016**). Also, extracellular HMGB1 increases mitochondrial RAGE expression and translocation, which in turn increases mitochondrial complex I activity and ATP generation encouraging tumor growth (**Kang et al., 2014**).

This study demonstrated that RAGE-HMGB1 co-expression in cancerous tissues was significantly associated with high tumour grade, advanced T stage, lymph node metastasis, stage III, and presence of in situ component. Moreover, both expression levels showed significant positive correlation with tumor size and number of positive lymph node, wih no significant relation to the molecular type. These findings make evident that RAGE-HMGB1 system is linked to aggressive breast cancer attitude representing a prognostic biomarker of remarkable clinical and therapeutic significance.

The RAGE-HMGB1 interply pushes for tumor growth and metastasis by possible mechanisms; (1) activation of mitogen-activated protein kinases, Rac1, NF-κB (**Chen et al., 2014**), extracellular signal regulated kinase 1/2 (ERK 1/2), and the protein kinase B pathway (**Angelopoulou et al., 2016)**. This in turn results in the expression of matrix metalloproteinases paving the way for tumor invasion (**Ohmori et al., 2011**), (2) induction of expression of proangiogenic growth factors and their receptors enhancing angiogenesis (**kang et al., 2013**), (3) HMGB1 enhances tumor cell motility by activating endothelial growth factor favouring invasion and metastasis **(Sparatore et al., 2005)**, (4) Escape from the host immune surveillence (**Sohun and Shen, 2016**).

Similar to our findings, several reports potentiate the link between RAGE and HMGB1 and the malignant virulence of cancer (**Tesarova et al., 2016, Dhumale et al., 2015**). **Kostova et al.,** investigated tissue samples from several cancers including 72 cases of ductal breast carcinoma and stated that beside the state of tumor differentiation, cancer prognosis can rely on HMGB1-RAGE expression and their

exact location in the cell (**Kostova et al., 2010)**. **Nankali et al., 2016** and his collegues, in their RT-PCR based study, found RAGE mRNA up-regulation in breast cancerous tissue that was significantly associated wih advanced-stage and triple-negative breast tumors, node-positive tissues and tumor size. **Sun et al., 2015,** demonstrated the close relation between HMGB1 levels and TNM stage, differentiation, and metastasis confirming its incrimination in breast cancer biological behaviour but with no association with patient age, tumor size, or HER-2/neu levels. **Chang et al., 2014** . in an immunohistochemistry based study on 60 patient with infiltrative ductal carcinoma, revealed higher HMGB1 expression in advanced stages and lymph node metastasis tissues considering it as a biomarker of unfavourable prognosis, but in contrast to us, HMGB1 showed positive correlation with HER-2. This discrepancy may be due to different study methodology and different racial background of patients.

In this study, the observed significant correlation between tumor size and RAGE-HMGB1 overexpression, was in agreement with other studies (**Sippel et al., 2008, Thompson et al., 2009**). It well established that, prognosis is profoundly dependent on tumor size (**Michaelson et al., 2003**). So this correlation indicates their participation in tumor growth and expansion.

Different studies clarified the RAGE-HMGB1 critical role in breast cancer biology by their blocking, resulting in inhibition of breast cancer cells proliferation and metastasis (**Kang et al., 2013, Radia e al., 2013 and Dhumale et al., 2015**).

Our study revealed that, there was no significant relationship between overall survival and neiher RAGE nor HMGB1 expression levels. Similary, in a meta-analysis conducted by **Wu et al**, to assess the prognostic value of HMGB1 expression in cancer, they noticed that HMGB1 overexpression was significantly associated with survivals under all studies circumstances except when the detection method of qRT-PCR was used. They explained this, at least in part, by the limited available RT-PCR based studies and their small sample size (**Wu et**

al., 2016).

Conclusion:

From this study, it could be concluded that the association of RAGE and HMGB1 overexpression with aggressive breast cancer phenotypes. Thus they may consititute prognostic biomarkers with therapeutic potential.

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الملخص العربى

تقييم مستوى التعبير الجينى لمستقبل الناتج النهائى التحللى المتقدم (RAGE)وبروتين المجموعة عالية الحركة(HMGB1)ودلالته التقدمية فى سرطان الثدى

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النجاح فى علاج سرطان الثدى غير مبنيا فقط على التنوع ولكنه يعتمد فى الحقيقة على ارتباطه الاكلينيكي بالطبيعة الباثولوجية للورم .ولذا الجمع بين العديد من الدلائل الحيوية المرتبطة بتطور الورم قد تؤدى الى انتاج نماذج جديدة للعلاج من اجل حياة افضل للمرضى ،مستقبل الناتج النهائى التحللى المتقدم (RAGE) وبروتين المجموعة عالية الحركة الرابط لهذا المستقبل (HMGB1) يبدوا انهما مرتبطان بالعديد من انواع السرطانات وهذا التداخل يؤثر على سلوك سرطان الثدى **وتهدف هذه الدراسة** الى تقييم مستوى التعبير الجينى فى الانسجة وعلاقته بالخصائص الاكلينيكية والباثولوجيا ومدة البقاء على قيد الحياة لهذين العاملين (HMGB1, RAGE) . وقد تم قياس الحامض النووى الريبوزى الرسول لكل من (HMGB1, RAGE) بواسطه التفاعل التسلسلي للبلمره الزمنيه. و قد أظهرت النتائج وجود فروق ذات دلالة إحصائية بين المجموعتين للتعبير الجينى ل (HMGB1, RAGE) وعلاوة على ذلك وجد ارتباط ذو دلاله احصائية بين مستوى التعبير الجينى لهذين العاملين (HMGB1, RAGE) فى انسجة سرطان الثدى وتقدم السرطان وانتشاره فى الغدد الليمفاوية وتم ايجاد علاقة ايجابية ذات دلالة احصائية بين مستوى التعبيرالجينى لهذين العاملين (HMGB1, RAGE)وحجم السرطان وعدد الغدد اليمفاوية الايجابية المصابة بينما لم يتبين وجود علاقة ذو دلالة احصائية بالنوع الجينى للورم ولا بمدة بقاء المريضة على الحياة ومن الممكن إعتبار هذا النظام (HMGB1, RAGE) عوامل حيوية ذات دلالة تقدمية من الناحية الاكلينيكية والعلاجية لسرطان الثدى .