HER-2/neu in Human Breast Cancer By Real Time Reverse Transcriptase Polymerase Chain Reaction and Immunohistochemistry

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Abstract: Breast cancer is a complex genetic disease characterized by the accumulation of multiple molecular alterations. Metastatic spreading through blood vessels is the most important factor affecting the prognosis of patients with primary carcinomas. In this regard, detection of carcinoma cells in the blood could be important to identify carcinoma patients at high risk of relapse. Thus, the development of a molecular diagnostic assay capable of detecting breast cancer-associated gene expression in the peripheral blood has the potential to vastly improve breast cancer staging and treatment. Human epidermal growth factor receptor-2 (HER2/*neu)* is an oncogene which plays an important role in the pathogenesis of breast cancer. Amplification of the *HER-2* gene plays an important role in the pathogenesis of breast cancer. Immunohistochemistry, fluorescence *in situ* hybridization, chromogenic *in situ* hybridization, and quantitative reverse transcription-PCR (RT-PCR) may be used for this purpose. Other approaches have been proposed for the assessment of HER-2 status in peripheral blood, including evaluating either circulating HER-2 extracellular domain (ECD) by ELISA or nucleated cell-associated HER-2 mRNA recently by quantitative PCR-based methods, either conventional competitive PCR and competitive reverse transcription-PCR (RT-PCR) methods or more advanced, quantitative real-time PCR methods. For this purpose, this study assessed *HER-2/neu*-mRNA, CA 15-3 and CEA in peripheral blood samples of 32 newly diagnosed breast cancer female patients presenting to the Surgery Department, Ain Shams University, and eight healthy female subjects as a control group. Our study found that the assayed serum CA15-3 and CEA did not achieve statistical significance between patients and controls and did not correlate with the circulating *HER-2/neu*. The quantified *HER-2/neu* in peripheral blood by real time PCR compared favorably with tissue IHC in breast cancer samples. HER-2 assessment by IHC has major disadvantages; it is a semi-quantitative method with considerable inter-observer variations and that IHC HER-2 score at the +2 level is an equivocal score that needs to be confirmed with another technique such as real-time PCR. Comparisons of our data with important clinical prognostic indices yielded several associations. The present study found an association between the level of circulating *HER-2/neu* gene and hormone receptor negativity, presence of circulating micro-metastases and the clinical stage. On the other hand, HER2/neu amplification was not associated with the size of the primary tumor, the status of lymph nodes, high grade tumors and tumor type. In Conclusion: Measurement of circulating HER2/neu by real time PCR in patients with breast cancer is useful for several clinical applications. These include: identification and monitoring of women with metastatic breast cancer to aid in patient management; prediction of the response to hormonal therapy; and the selection patients for trastuzumab therapy. This would improve clinical outcome and five-year survival.

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1. Introduction

Breast cancer remains an important public health problem. It is the most common malignancy in women and is the leading cause of death among them. The incidence of this disease and the resulting deaths continue to increase and is higher among women, who reside in Western, developed countries, especially among younger females ***(Espinosa et al., 2003 and Afonso, 2009).***

Breast cancer is highly curable if diagnosed at an early stage. However, a significant number of patients with early-stage breast cancer may harbor microscopic metastasis at the time of diagnosis ***(Wang et al., 2005).*** For these reasons, a large number of cellular oncogenes have been studied to determine their ability to predict prognosis, response to therapy, or both ***(Esteva and Hortobagyi, 2004***; **Bidard *et al.*, 2010**; ***mavaddat et al., 2010; and*** **Mego *et al.*, 2010**).

The activation and over expression of cellular oncogenes has been considered to play an important role in the development human cancer. One of the

important members of the oncogene family is the growth factor receptor known as human epidermal growth factor receptor-2 (HER-2), which is also referred to as HER-2/neu or cerbB-2 ***(Bieche et al., 1999).***

The HER-2/neu protooncogene encodes a transmembrane glycoprotein with tyrosine kinase activity and structural homology to the human epidermal growth factor (EGFR; ERBB1). HER-2/neu is expressed in many cell systems, triggers a network of signaling pathways, and plays an important role in normal growth and development ***(Walter et al., 2003).***

It was found that in 20%-30% of breast carcinomas, HER-2/neu status is altered, and this is manifested either as amplification of the gene or over expression of the protein product. Such alteration of this gene has been associated with poor prognosis and with resistance to conventional adjuvant chemotherapy, regardless of the nodal or hormone receptor status ***(Madrid and Lo, 2004;*** **Criscitiello *et al.*, 2010**; ***Mavaddat et al.,2010;*****.Riethdorf *et al.*, 2010 and Kallergi *et al.*, 2012.**

The most common detection methods for HER-2/neu include measurement of protein over expression by the immunohistochemical assay (IHC) and the detection of gene amplification by fluorescence in situ hybridization (FISH) techniques ***(Gupta et al., 2003) or*** PCR-based methods including conventional competitive PCR and competitive reverse transcription-PCR (RT-PCR) methods or more advanced, quantitative real-time PCR methods for RNA and DNA analysis ***(Gilbey et al., 2004).*** The latter has the advantage of being rapid and easy for screening multiple samples at the same time. Furthermore, PCR amplification and quantification are performed in the same reaction tube to eliminate manual manipulation of the PCR product after amplification. This reduces the chance of PCR product contamination and simplifies sample tracking.Moreover, rapid-cycle PCR also reduces amplification time and increases sensitivity and specificity over traditional PCR ***(Königshoff et al., 2003***; **Fabbroni and Sandri, 2010; Ahmed *et al., 2011: and Houghton et al., 2011)***.

Aim of the Work:

The aim of this study was to investigate the clinical reliability of HER-2/neu mRNA status in the blood, by real-time RT-PCR, as an early predictor of circulating micrometastases in breast cancer patients and to study the relevance of this marker to its protein expression in breast tissue by immunohistochemistry across the spectrum of breast cancer.

2. Subjects and Methods

I. Subjects:

A. Patients' Group:

This study was conducted on 32 newly diagnosed breast cancer female patients. Their ages ranged from 32 to 67 years (51.4± 8.1years). They were presented to The Surgery clinic with breast masses.The diagnos is of breast cancer was based on mammography, ultrasonography, and tissue biopsy for histopathological examination, Chest X-ray and bone scan for detection of distant metastases.

This group included 32 breast cancer patients. According to tumor size (T), five patients were T1, 16 patients were T2, 7 patients were T3 and 4 patients were T4. Regarding lymph node metastases (N): 8 cases were N0, 13 cases were N1, 10 cases were N2 and only 1 case was N3.

According to the presence of distant metastases (M), 21 patients were M0 representing, 11 cases M1. Accordingly, the patients were classified according to their clinical stage into 2 cases in stage I, 14 cases in stage II, 6 patients in stage III and 10 patients were first diagnosed in stage IV.

Another re-classification of patients was done according to the Bloom-Richardson grading system. Only 3 tumors were well differentiated, while 22 were moderately differentiated and 67were poorly differentiated.

Histologically, according to the World Health Organization classification breast cancer patients were classified into infiltrating duct carcinoma subgroup which included 28 patients and infiltrating lobular subgroup which included 4 patients.

Finally according to hormone receptors, regarding ER: 16 patients were ER positive and 16 were ER negative, whereas, regarding PR :14 patients were PR positive and 18 were PR negative.

B. Control Group:

Twenty Eight age-matched women were chosen as a control group. Their ages ranged from 32 to 64 years (48.8 ± 11.1). They were 10 healthy volunteer served as healthy control group : with no symptoms or signs suggestive of breast cancer or any other breast disease;and 18 patients served as patient control group(8 patients with fibroadenoma,4 patients with uterine carcinoma,4 patients with ovarian carcinoma, and 2 patients with cancer colon.

For histopathological studies, the healthy control group included the normal breast tissue adjacent to fibroadenomas.

A verbal consent was taken from all participants before withdrawal of the samples and their enrollment into this study

II. Sampling:

Blood Samples:

From each participant in the study, 9 mL venous blood was withdrawn by venipuncture: the first 3 mL dispensed into a plain vaccutainer left to clot,sera were separated spared for the assay of CEA and CA15.3. The following 6 mL were dispensed into two sterile K_3 EDTA vaccutainers. The first was used for CBC and the second K $_3$EDTA vaccutainer was used for RNA extraction, which was kept on ice until being processed within two hours of collection.

Tissue Biopsies:

Breast tissue samples were fixed in 10% formaldehyde, routinely processed to paraffin blocks, then 5 µm thick sections were prepared and stained with hematoxylin and eosin stain for histopathological examination.

III Methods:

Analytical Methods:

1. Serum CEA and CA15.3 Assay:

They were assayed on the IMMUNLITE[1] by chemiluminescent sequential immunometric assay using reagent products of DPC Bekton Dickenson Vaccutainer System. The solid phase is in the form of polystyrene beads coated with monoclonal murine anti-CEA or anti-CA15.3 antibodies.

Patients samples were incubated with alkaline phosphatase conjugated to either polyclonal rabbit anti-CEA or anti-CA15.3 antibodies for 30 minutes in the test units with intermittent agitation. The CEA and CA15.3 in the samples hence bind to form an antibody sandwich complex. Unbound conjugate was then removed by a centrifugal wash, after which the chemiluminescent substrate, a phosphate ester of adamantyl dioxtane, was added and the test units were incubated for further 30 minutes. The photon output monitored is directly proportional to the concentration of CEA and CA15.3 in the samples.

2. Quantification of HER2/neu mRNA by Real Time RT-PCR:

This process was performed as follows:

a) RNA extraction.

b) Reverse transcription, amplification and detection.

a)RNA extraction:

RNA was extracted from whole blood automatically using MagNA Pure Compact [2] instrument and MagNA Pure Compact Nucleic Acid Isolation Kit I.The samples were lysed by incubation by a special lysis buffer "Lysis/Binding Buffer", containing chaotrophic salts, and then the cellular proteins\were digested by proteinase K so that RNA was released. Magnetic glass particles (MGPs) were added and RNA was immobilized on the MGPs surfaces. The unbound substances were removed by several washing steps with wash buffer. Finally the purified RNA was eluted from the MGPs and stored at -70ċ until assayed**.**

b)Reverse transcription, amplification and detection and quantification of expressed gene was done :by one step RT-PCR method using LightCycler® 1.5 Instrument and LightCycler® RNA Master Hydrolysis Probes Kit, provided by Roche Diagnostics. The forward and reverse primers for each gene were chosen from different exons. The sizes of PCR products were designed to be under 400 bp to optimize the RT-PCR measurements. a single 3´ non-extendable hydrolysis probe is used to detect the accumulation of a specific target DNA sequence and is cleaved during PCR amplification.

- The Taqman probe (hydrolysis probe) carried a 5` FAM (6-carboxy-flourescein) reporter dye and a 3` TAMRA (6-carboxy-tetramethyl-rhodamine) quencher dye and contained a 3` phosphate group (p) to prevent extension during PCR. The probe is designed to hybridize specifically to the target sequence between the forward and the reverse primers. When the probe is intact, the two fluorophores are in close proximity, and the reporter dye emission is suppressed by the quencher dye. During the extension phase of the PCR, the 5` to 3` exonuclease activity of the Taq polymerase cleaves the hybridized probe and releases the reporter dye, resulting in an increase in the fluorescence signal intensity proportional to the accumulation of PCR product, as the probe gets cleaved during amplification hydrolysis probes are not available for post-PCR analysis steps.

Data Interpretation of Real-Time PCR:

Raw data were analyzed with the Light Cycler software 4.05.

- The absolute target copy numbers were determined from the standared curve automatically generated from serial dilutions of the standard TBP-RNA. For each clinical sample, the amounts of the target gene (*HER-2/neu*) and the reference gene (*TBP*) were measured in patients' samples as well as in healthy control ones, in separate PCR reactions. Finally, the relative copy number of *HER-2/neu* versus *TBP* gene was calculated. The *Her2/neu* normalized expression corresponded to a ratio: Her2/neu expression (in nanograms per microgram total RNA) to TATA-Box binding protein (TBP) expression (in nanograms per microgram total RNA). Value of 26.4 x10^{-6} is

considered the positivity threshold for Her2/neu overexpression,

Immunohistochemical Study:

Streptavidin-biotin technique was used to investigate HER-2/neu, estrogen receptors (ER) and progesterone receptors (PR) expression. Three slides from each case were deparafinized, hydrated and incubated in 3% hydrogen peroxide for 30 minutes to block the internal peroxidase activity. Antigen retrieval was done by microwave pretreatment for 10 minutes in 0.01 citrate buffer. For each case, one slide was incubated with anti-HER2 monoclonal antibody (Dako Corporation) at a dilution 1:100, the second and third slides were incubated with mouse monoclonal antibody to ER and PR respectively at a dilution 1:50 (Dako Corporation). Sections were then washed twice for 5 minutes with PBS and incubated for 10 minutes in performed avidin-biotin-peroxidase complex (Dako Cytomation). Chromogen developed was accomplished by immersion of the sections in 2,3-Diaminobenzedin tetrahydrochloride (BAB) for 5 minutes. The nuclei were counterstained with hematoxylin, dehydrated, cleared and mounted. For negative controls, the primary antibody was omitted and replaced with PBS. According to ***Peiró et al., 2007,*** HER-2/neu was scored as: Positive when at least 10% of neoplastic cells showed complete membranous staining, either strong (3+), weak to moderate (2+), weak (<10%) (1+), or faint or absent (0). ER and PR appear as nuclear staining.

C. Statistical Methods:

Data were analyzed statistically using SPSS statistical package version 15.0, 2006, Echosoft Corporation, USA. Data were expressed as percentages for qualitative values and mean ± standard deviation (SD) for quantitative data.

The association of qualitative data with different subgroups (tumor sizes, regional lymph node metastases, distant metastases, receptor positivity, histopathological stages, grades or types) was assessed by means of Chi Squared Test (X^2) or Fisher exact test when necessary. Comparison between the means of 3 or more quantitative data in the different subgroups was done after log transformation of results through one-way analysis of variance (ANOVA). Between two groups comparison study was done by student's t test after log transformation of data.A p value < 0.05 was considered significant and p < 0.01 was considered highly significant.

Correlation between quantitative data was done by Spearman Correlation Coefficient (r_s). A p value < 0.05 was considered significant and p value < 0.01 was considered highly significant.

Finally, the receiver operating characteristics (ROC) curve was done to study the performance of circulating HER-2/neu level in identifying metastatic from non metastatic breast cancer patients.

3. Results

The descriptive and comparative statistics of the present study are shown in tables (1-4) and figures (1-4)

Regarding assayed CA15-3 and CEA, there were no statistically significant difference in their levels between breast cancer patients(83.6±145 U/mL and 62±130 U/mL; respectively)and the control group (11±5 U/mL and 1.7±0.95 U/mL; respectively), (p>0.05). Both markers, CA15-3 and CEA, were not correlated with the circulating Her2/neu levels as detected in patients' samples by real time RT-PCR.

Pathologically examined biopsies for Her2/neu protein, by immunohistochemical staining, showed 11 samples (34.3%) had zero score, 7(21.9%) scored +1, another 6 samples (18.18%) scored +2 and 8 cases (25%) scored +3 (figure 1, 2). According to **Ignatiadis *et al.*,2011**,the score zero and score +1 are considered negative while +2 are considered equivocal and only +3 are considered positive cases so we had 18 negative cases,8 positive cases and 6 equivocal cases need more study by either PCR or Fish Technique.

The ability of real time PCR and tissue IHC in detection of Her2/neu in breast cancer patients was found to be highly significant when HER-2/neu was assessed by both methods (X^2=13.499, p<0.001). There were 12 of cases showing positivity by both PCR and IHC (92.3%), 5 cases PCR positive/IHC negative (26.3%), 14 cases negative PCR/negative IHC (73.7%) and 1 case negative PCR/positive IHC (7.7%).

Comparison of HER2/neu gene expression by real time PCR in different IHC scores revealed that the higher the score of tissue IHC, the higher the circulating HER2/neu mRNA levels (F=15.03, p<0.001) (fig. 3).

No significant association was recorded between either the percentage of positively expressed circulating Her2/neu or the level of Her2/neu and each of the tumor size (X^2 = 3.2, p > 0.05 and F=0.085, p>0.05, respectively); the state of regional lymph nodes ***(***(X^2 = 1.357, p > 0.05 and F=2.253, p>0.05 respectively). ***(table 1, 2)***

On the other hand, patients with distant metastases (M1) had a highly significant percent positivity of circulating Her2/neu (n =10; X^2 =9.6, p < 0.01) than non metastatic group (M0)**.** Moreover,

the mean HER2/neu expression among metastatic patients was 165 ±124 ($x10^{-6}$ ng/µg total RNA) which higher than the non metastatic patients (mean 31.9± 61.2), (t=16.76, p<0.001).

Our results demonstrated that the circulating Her2/neu mRNA increases with increasing stages of breast cancer; (X^2=9.264, p<0.05) as shown in ***(Table 1)***. More precisely there was a highly significant difference in the level of circulating Her2/neu between different clinical stages (F=5.578, p<0.01) as shown in ***(Table 2)*** Post-hoc multiple comparisons between subgroups revealed that the significance was between stages III and IV (p < 0.01) more than stages I and IV (p < 0.05) and II and IV (p < 0.05).

As regards hormone receptor positivity, there was a highly significant inverse relationship between each of estrogen and progesterone receptor positivity and the level of Her2/neu gene amplification in blood (X^2 = 21.21, p < 0.001and X^2 = 10.04, p < 0.01, respectively).) The same highly significant difference

In the levels of circulating Her2/neu mRNA was found between ER negatives and positive cases (131 $x10^{-6}$ ±109 $x10^{-6}$; versus 23.93 $x10^{-6}$ ±75.64 $x10^{-6}$ t=10.485, p<0.01). Also, the level of HER2/neu expression was 127 $x10^{-6}$ ±121$x10^{-6}$ in PR negative cases; versus 14.44$x10^{-6}$±23$x10^{-6}$ in PR positive cases (t=11.6, p <0.01).

The degree of tumor differentiation ***(tables 2)*** had no impact on Her2/neu levels as there was no significant difference observed between patients with well, moderately or poorly differentiated breast cancer (circulating levels: X^2=1.682, p >0.05 versus tissue score: X^2=0.7, p >0.05). Moreover, the tumor type, either ductal or lobular carcinoma, had no impact on our studied marker (circulating HER2: X^2=0.018, p >0.05 versus tissue score: X^2=0.167, p >0.05)**.**

Finally, the receiver operating characteristic curve (ROC curve) ***(Figure 3)*** shows validity of HER2/neu determination in the blood for the prediction of metastases in cases of breast cancer. The best cut off point is 28.3 $x10^{-6}$ at which: Sensitivity: 90.9%, Specificity: 71.4%, positive predictive value (PPV): 62.5%, negative predictive value (NPV): 71.4%, Efficiency: 78.12%, AUC (area under the curve) =0.887, p < 0.001: highly significant).

Table 1: Association between HER2/neu in peripheral blood and in breast tissue; with each of tumor size, Lymph node metastasis, distant metastasis, histopathological grade and clinical stage using Chi Squared Test

Parameter	Circulating HER2/neu n=32		X^2	Tissue HER2/neu n=26----------		X^2
	-ve No(%) 15 (42.9)	+ve No(%) 17(53.1)		-ve No(%) 18 (69.2)	ve No(%) 8(30.8)	
T1	4(26.7)	1(5.9)	3.223*	4(21.1)	0(o)	1.884*
T2	7(46.7)	9(52.9)		10(52.6)	3(37.5)	
T3	2(13.3	5(29.4)		3(15.8)	1(12.5)	
T4	2(13.3)	2(11.8)		1(5.7)	4(50)	
N0	4(26.7)	4(23.5)	1.375*	7(38.9)	1(12.5)	4.614*
N1	7(46.7)	6(35.3)		7(38.9)	3(37.5)	
N2	4(26.7)	6(35.3)		4(22.22)	3(37.5)	
N3	0(0)	1(5.9)		0(0)	1(12.5)	
M0	14(93.3)	7(41.2)	9.610***	17(94.4)	2(25)	11.792***
M1	1(6.7)	10(58.8)		1(5.7)	6(75)	
Grade I	1(6.7)	2(11.8)	1.682*	2(11.1)	1(12.5)	o.700*
Grade II	12(80)	10(58.8)		12(66.7)	6(75)	
Grade III	2(13.3)	5(29.4)		4(22.2)	1(12.5)	
Stage I	2(13.3)	0(0)	9.264**	4(22.2)	0(0)	10.067**
Stage II	8(53.3)	6(35.3)		9(50)	3(37.5)	
Stage III	4(26.7)	2(11.8)		4(22.2)	1(12.5)	
Stage IV	1(6.7)	9(52.9)		1(5.6)	4(50)	
ER -ve	1(6.7)	15()	21.21***	3(15.8)	7(87.5)	10.49***
+ve	14(93.3)	2(11.8)		15(83.3)	1(12.5)	
PR -ve	4(26.7)	14()	10.041***	14(77.8)	1(12.5)	3.802***
+ve	11(73.3)	13()		27.8()	7(38.9)	

*=non significant,**=significant,***=highly significant

Table 2: Comparative statistics of circulating Her2/neu among different tumor sizes, lymph node metastasis, distant metastasis, clinical stages, histopathological grades, clinical stages, and hormone receptors using ANOVA Test

Tumor size	HER2/neu gene expression by real-time PCR*

(n=32)	$\bar{X}$	SD	F
T1	6.39	±13.12	0.085*
T2	60.51	±87.2	
T3	114.8	±115.6	
T4	169.8	±170.4	
N0	53.5	±95.4	2.253*
N1	72.7	±108.2	
N2	77.9	±95.7	
N3	331	0	
Stage I	0	0	5.578***
Stage II	41.9	±69.6	
Stage III	29.25	±49.23	
Stage IV	172	±128	
Grade I	47.93	±46.3	0.749*
Grade II	93.29	±125.3	
Grade III	40.91	±26.25	

*=non significant,**=significant,***=highly significant

Table 3: Comparison between the levels of circulating HER2/neu among breast cancer patients regarding presence of metastasis; hormone receptors and histopathological using student's t test

Parameter	$\bar{X}$ ± SD	t
M0	31.9±61.2	**16.756*** **
M1	165±124	
ER negative	131±109	10.485***
ER positive	23.93±75.64	
PR negative	127±121	11.6***
PR positive	14.44±23	
IDC	65.77±93.42	2.888*
ILC	160.25±172.08	

*=non significant,**=significant,***=highly significant

Table 4: Post Hoc multiple comparisons between different clinical stages regarding circulating HER2/neu

Compared stages	Mean difference*	*P*
I and II	41.91	>0.05
I and III	29.25	>0.05
I and IV	172.03	<0.05
II and III	12.66	>0.05
II and IV	130.1	<0.05
III and IV	142.78	<0.01

* $X10^{-6}$ ng/µg total RNA

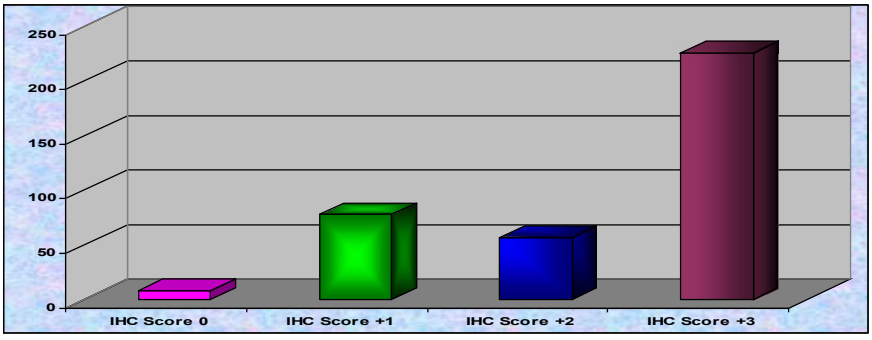

Figure (3): Mean level of HER2/neu gene expression in different IHC Scores.

[The $\bar{X}$ level of circulating HER2/neu **($X10^{-6}$ ng/µg total RNA) was in** (0) score 7.7±15.5; in (+1) was 77.4±106; in (++) was 56.6±57; in (+++) was 226±101; F =15.03; *p* <0.001]

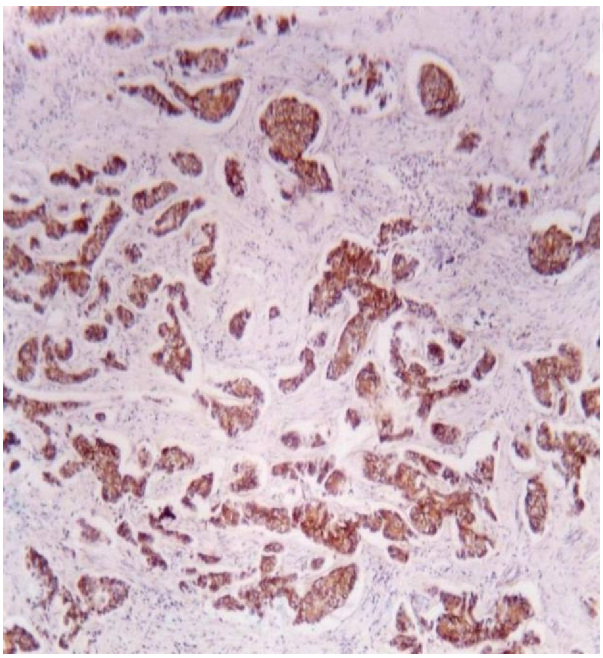

Figure 2

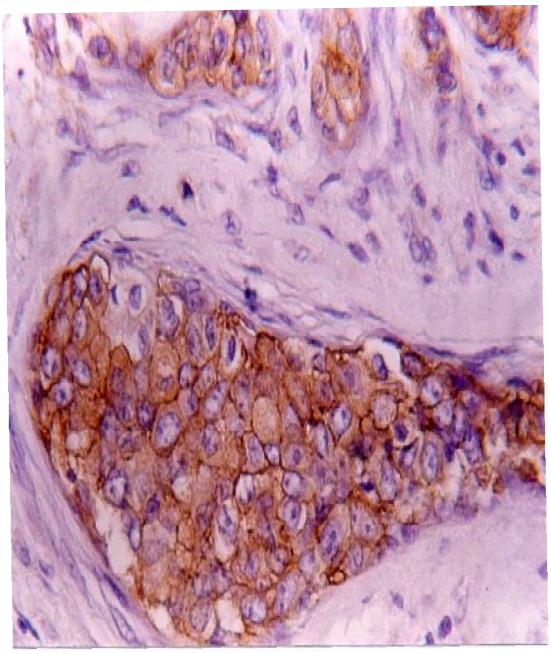

Figure 3

Figure 2: Invasive Duct Carcinoma positive membranous staining for anti Her2/neu in more than 80% of tumour cells x 40 Immunohistochemical staining

Figure3: Higher power view of the previous case showing membranous staining of ductal carcinoma cells x 200 Immunohistochemical staining

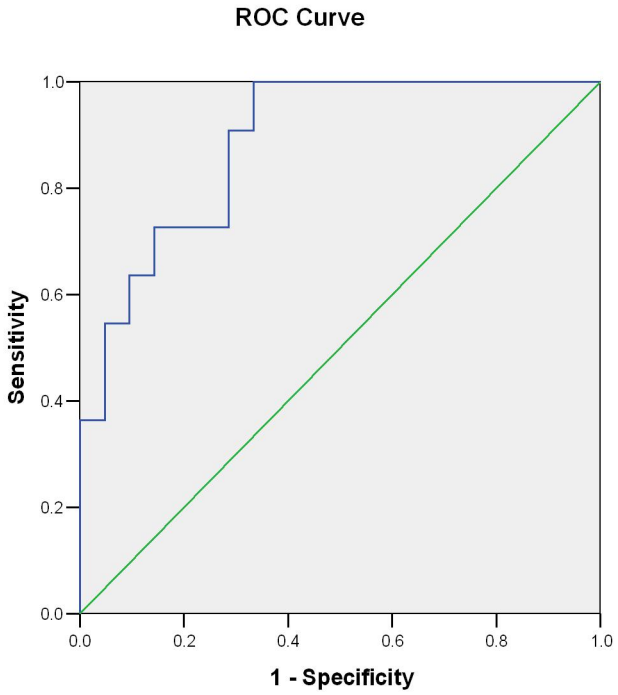

Figure 4: Receiver operating characteristics curve showing validity of HER2/neu determination in the blood for the prediction of metastases in cases of breast cancer. The best cut off point is 28.3 x10^{-6} at which: Sensitivity: 90.9%, Specificity: 71.4%, PPV: 62.5%, NPV: 71.4%, Efficiency: 78.12%, AUC (area under the curve) =0.887.

4. Discussion

Breast cancer is a complex genetic disease characterized by the accumulation of multiple molecular alterations ***(Silva et al., 2001)***. Metastatic spreading through blood vessels is the most important factor affecting the prognosis of patients with primary carcinomas. Unfortunately, patients with primary breast cancer who have undergone radical, curative surgery have a recurrence rate as high as 20–60% ***(Smith et al., 2000)***. Moreover, 30–50% of carcinoma patients who show no evidence of disease in the locoregional lymph nodes will have a recurrence at a distant site ***(De Luca et al., 2000 Gilby et al., 2004, and Autier et al., 2010)***.

In the metastatic process: tumor cells in the primary site must erode the basement membrane; penetrate a blood vessel; and spread to distant sites. In this regard, detection of carcinoma cells in the blood could be important to identify breast cancer patients at high risk of relapse ***(De Luca et al., 2000 and Rakha et al.,2007)***. Thus, the development of a molecular diagnostic assay capable of detecting breast cancer-associated gene expression in the peripheral blood has the potential to vastly improve breast cancer staging and treatment ***(Houghton et al., 2011)***. The recent identification of genes overexpressed in breast cancer, combined with advances in molecular biology, provides the opportunity to establish more sensitive, specific, and cost-effective ways of identifying metastatic disease ***(Baker et al., 2003 and Monego et al., 2007)***. Of these genes, HER2/neu was the matter of research.

In view of the previous facts, the present study addresses the feasibility of using real time RT-PCR based quantitation of circulating *Her2/neu* as a sensitive marker for detection of micrometastases in new cases of breast cancer and compares the results obtained with clinical prognostic indices and those obtained using IHC.

In the present study, the assayed serum CA15-3 and CEA tended to be higher among patients compared to healthy controls, yet, this increase did not achieve statistical significance. These findings were not surprising as they were previously revealed in 2000 by ***Cheung and coworkers and in 2007 by Carney and colleagues. The latter*** published that CA15-3 should not be used to diagnose primary breast cancer because the incidence of elevation is fairly low and that CA 15-3 is rather useful in monitoring therapy and disease progression in metastatic breast cancer patients. Moreover, ***Thriveni, et al., 2007***, generally agreed that serum CA15-3 and CEA in breast cancer are not a tool for primary diagnosis because of their low sensitivity and specificity. They added that, the increased CA15-3 values when combined with increased circulating *HER-2/neu* would predict a worse prognosis than only increased CA15-3 value.

In the present study, no amplification of HER-2/neu mRNA had been detected in blood samples at any of the studied control group weather healthy or patient control. Moreover, among the studied patients, 17 patients (53% of cases) showed positive circulating HER-2/neu gene overexpression. Our findings are in accordance to the results of ***Wasserman et al., 2000,*** who extracted RNA from peripheral blood obtained from 19 patients with breast cancer prior to surgery and from 31 normal controls. Their results found that no HER2/neu amplicon was detected in 31/31 normal controls.However,other researchers reported that the frequency of circulating Her2/neu-mRNA overexpression in breast cancer patients was 20–30% of breast cancer cases which is less than ours***(Ross et al., 2004, and Hudis, 2007).*** This discrepancy may be attributed to the fact that breast cancer in Egyptian patients is biologically more aggressive than that encountered in the West ***(Mostafa, 2005)***. Moreover ***Hüsemann, et al., 2008***, added that the late presentation of patients at an advanced stage may be an important contributing factor. Our finding support the latter suggestion, since 30% of our studied patients were in stage IV when first diagnosed.

Our results demonstrated that, by IHC 8/32 patients were positive, 18/32 was negative and 6/32 was equivocal. The 8 cases positive by IHC was also positive by PCR while from the 18 cases negative by IHC,5 cases were positive by PCR and 13 were negative,whereas from the 6 equivocal cases; 5 cases were positive by PCRand 1 was negative.Thus the frequency of positive tissue HER-2/neu by IHC is less than the circulating HER-2/neu mRNA overexpression. These results are in agreement with other studies by ***Gjerdrum et al., 2004; Tse et al., 2005*; Gown, 2008 and Alunni-Fabbroni and Sandri, 2010.** They attributed their findings to the fact that by using real-time quantitative PCR, the target can be both amplified and detected simultaneously; making the assay a potentially sensitive, specific, reliable, and cost-effective way of measuring *HER-2/neu* changes. Moreover, our results confirm those previously obtained by ----- and his co-workers, who Found that the percentage of HER2neu overexpression by IHC was less than that detected by real-time PCR. This means that real time PCR could detect HER-2/neu in patients known to be HER-2 negative in tissue samples. They explained their findings to be due to the fact that amplification of HER-2/neu is an early event and, therefore, might precede protein expression, Another possible explanation attributed that real-time polymerase chain reaction can detect and quantify very small

amounts of specific nucleic acid sequences most specifically, sensitively and reproducibly ***(Decock, 2006; and Ferro, et al., 2010***).

There was only one (7.7%) IHC-positive sample which was not identified by real-time PCR in the present study. Such results had been previously reported by ***(Königshoff et al., 2003 and Tse et al., 2005)***. The latter recorded most discrepancies between quantitative PCR and IHC, were PCR false negatives. They explained such discrepancies to be caused by transcriptional or post-translational activation. They added that there might be clonal changes that happened between the primary breast tumor and distant metastases which might affect HER-2 status, so that patients with negative tumor *HER-2/neu* status at the time of biopsy may become HER2-positive as they progress to metastatic disease ***(Lu¨ftner et al., 2004-a and Meng et al., 2004)***. Another reasonable explanation of such discrepancies was that HER-2 assessment by IHC has a major disadvantage of being at best a semi-quantitative method with considerable inter-observer variations ***(Gjerdrum et al., 2004 and Lu¨ftner et al., 2004)***. Furthermore, the many available HER2 antibodies used in IHC study show variable sensitivities and specificities that, together with differences in tumor fixation and antigen retrieval methods contribute to considerable assay imprecision. Therefore, there is widespread agreement that the assessment of HER-2/neu by IHC scores at the +2 level is an equivocal result which needs to be confirmed with another technique as FISH or PCR***(Gjerdrum et al., 2004; Criscitiello et al., 2010 and Ignatiadis et al.,2011)***

Although the present study showed that HER-2/neu could be detected in peripheral blood of patients with breast cancer, yet, the important issue was how this detection could influence the patient prognosis. Several reports demonstrated that a significant correlation of circulating HER-2/neu protein levels with disease recurrence, metastasis, or shortened survival ***(Graves [1] and Czerniecki, 2011; and Lang et al., 2011).***

In the present study, the frequency of expression of circulating Her2/neu patients with localized breast cancer (M0 or stages other than stage IV) versus patients with metastatic breast cancer (M1) was evaluated. Circulating Her2/neu was detected in 90.9% of patients with a metastatic disease and in 6.7% of patients with localized breast cancer. Our results were in agreement with those obtained by **Kallergi, 2012.** The latter suggested the assay of circulating HER2 neu in breast cancer patient for early identification of those with micro-metastasis as- by identifying metastasis earlier, patients could get benefit from neoadjuvent treatment which means starting the proper chemotherapy before surgery. This would improve clinical outcome and five-year survival. Moreover, the mean HER2/neu expression among metastatic patients was higher than the non metastatic group. These results suggest a potential value for circulating Her2/neu as a marker of tumor progression which could detect circulating micro-metastases that had not be detected radiologically when breast cancer was first diagnosed. Furthermore, recent reports supported our finding as they reported that 50% to 60% of metastatic breast cancer patients would have elevated peripheral levels ***(Valero et al., 2007 and*** **Graves and Czerniecki, 2011)**

As reported by other investigators, we have detected thatHER-2/neu positive circulating in patient with metastatic breast cancer and initially HER-2/neu negative tissue,this reflect as previously mentioned the higher sensitivity of real time PCR in identifying circulating her2 neu positive cells (**Dowsett *et al.,* 2008;Hammond *et al.,* 2010; and *skedgel et al.,2013)***.).

As reported by other investigators (**Pestrin, *et al.,* 2009**and ***skedgel et al., 2013)***, we have detected thatHER-2/neu positive circulating in patient with metastatic breast cancer and initially HER-2/neu negative tissue

When the association of HER2/neu expression and tumor size and lymph node status was studied, no significant association was found. The same results were previously obtained by **Tse *et al., 2005***, who explained that tumor size and lymph node invasion are time dependent.

The relevance of the tumor grade to the expression of HER2/neu was a matter of research by ***Denley et al., 2001 and Königshoff et al., 2003***. Results of ***Denley and colleagues,*** revealed a significant association between HER2/neu and the histological grade of the tumor. Unexpectedly in our results, association between circulating HER-2/neu-mRNA and high grade tumors was not observed. We could explain this finding to be due to the too small number of samples which showed low grade. Our results were in agreement with those of ***Michail et al., 2013*** who quantified HER2/neu in 33 clinical tissue samples of breast cancer using real-time PCR and by IHC assay. They could not find such correlation to higher grades, and explained that they had only one sample showing a low grade and that the number of patients in their study was too small to draw final conclusions about correlations. However, ***Tse and co-workers, 2001,*** reported that the grade had been shown to be an independent factor in predicting patient prognosis and correlates well with 10 year survival figures. On the other hand, some studies have shown that when estimating histological grade, there is a tendency to underscore rather than

overscore on core biopsy samples compared with excision specimens ***(Prat et al., 2013)***. Mitotic counts might be inaccurate because in tumor grading the periphery of the tumor is assessed, where mitotic figures are more frequent. In addition, the core might have an insufficient amount of tumor to allow 10 high power fields to be counted. As a result, the estimation of mitotic frequency might be inaccurate and is generally underscored in the core biopsy sample ***(Peiro et al.,2007)***.

The association of histopathological types of the tumor and circulating HER2/neu was investigated in this work. Unexpectedly, no association between HER2/neu expression and tumor type was observed. This may be due to complex inter-relations between type and grade assessment. The evaluation of tumor type is fraught with the same difficulties as grade assessment. This again stems from the possible problem of obtaining a sample that is not representative of the tumor as a whole. Another possible explanation is that the encountered studied samples were almost of infiltrating duct carcinoma type with few cases of infiltrating lobular type, so that other types, such as in situ carcinoma, could not be evaluated. However, the demonstrated non association between PCR results and tumor type in this study was supported by other reports such as that of ***Denley et al., 2001 and Königshoff et al., 2003***. The former reported that tumor type has been shown to be of less prognostic importance than the grade. They suggested that tumor grade, lymph node stage, and tumor size have all been shown to be independent prognostic factors. **Graves and Czerniecki, 2011).**

In addition to be a marker of adverse clinical outcome, HER2/neu is also regarded as a predictive marker for reduced response to certain chemotherapy and hormonal treatments. Even more importantly, positive HER-2 status predicts a response to therapy with trastuzumab (Herceptin), which is a humanized monoclonal antibody directed against the external domain of the HER-2 protein which has been shown to be effective in prolonging survival in patients with HER2 receptor-positive metastatic breast carcinoma ***(Gjerdrum et al., 2004;*** **Ejlertsen,** ***et al.,*****2010; Rack,** ***et al.,*****2010: and** ***skedgel et al.,2013)***. It had been shown that 70-80% of breast cancer patients with positive ER and PR regressed with hormone theapy ***(Duffy, 2005and Marglet et al.,2012)***. For this reason, one of the aims of this study was to investigate the relevance of circulating HER2/neu and the expression of hormone receptors (ER and PR). A significant association between the level of circulating *HER-2/neu* and receptor negativity was found in this work. This was in agreement with other reports such as that of ***Peiró and colleagues, 2007***; **Balleine and Wilcken, 2012** The latter researchers studied the impact of HER-2 status on the survival rate in patients with primary early-stage breast carcinoma and positive hormone receptor status. They found that 76.5% of patients whom had ER+ and/or PR+ tumors were predominantly HER-2 negative expression or no amplification. In contrast, 18% of patients had HER-2/neu amplification in association with lack of expression of ER and PR. Moreover, ***Peiró and co-workers*** recorded that combination of hormone receptor assessment and HER-2 status results showed highly favorable recurrence-free and overall survival rates for patients with positive hormone receptor and negative HER-2 status. Other several reports suggested that *HER-2/neu* gene amplification and protein overexpression have been associated consistently with negative assays for nuclear protein receptors for estrogen and progesterone breast cancer invasiveness and metastasis ***(Hayes and Thor, 2002, Masood et al., 2002, Zemzoum et al., 2003, Königshoff et al., 2003 and Monego et al., 2007)***. Therefore, the combination of these biologic markers has potential clinical usefulness (**Pinhel *et al.,*** 2012)

Indeed, *HER-2/neu*-overexpressing breast tumors were found to be mainly estrogen receptor negative and thus unresponsive to antiestrogens ***(Carney et al., 2003*****; Isabel Pinhel *et al.,*** 2012***; and Skedgel et al., 2013)***. Therefore, *HER-2/neu* gene status is currently considered important for clinical decisions, especially about treatment with the targeted therapy using Trastuzumab (Herceptin™) ***(Hudis, 2007)***. Because the clinical results obtained by Trastuzumab are promising, it provides a new therapeutic option for those patients who would not benefit from other chemotherapeutic agents and antiestrogens. Therefore, these results are of interest ***(Hudis, 2007)***.

Circulating HER2/neu comprises a sensitive molecular diagnostic assay for the detection of circulating breast cancer micrometastases, which may potentially replace invasive procedures for breast cancer assessment. Therefore, analysis of HER-2/neu expression should become the standard of care. The "story" of HER-2/neu testing in breast cancer will continue to unfold over the next several years.

Recommendations

- HER-2/neu should be assessed initially at the time of breast cancer diagnosis so that to identify micrometastases so as to tailor the proper chemotherapy.
- Her-2/ neu should be assayed in all breast cancer patients who have negative and equivocal results by IHC

- The blood approach has the potential advantage of allowing rapid determination of circulating HER-2/neu thus it could be used in clinical follow-up of patients postoperatively and to monitor response to chemotherapy.
- Real-time one step RT-PCR assay is an easy, rapid, sensitive, accurate and reliable method to quantify HER-2/neu expression routinely in samples exhibiting a wide range of HER-2/neu mRNA expression.

References

3. Afonso,N.(2009):Woman at high risk for breast cancer-what the primary care provider needs to know.J.AM.Board Fam MED.;22:43
4. Ahmed,S.,Iqbal.,J.,THike.A.,Lim,A.,and Tan PH(2011):HER-2/neu revisited:quality and interpretive issues.J.Clin.Pathol;64(2):120
5. **Ali, S. M.; Leitzel, K.; Chinchilli, V. M.; Engle, L.; Demers, L.; Harvey, H. A.; Carney, W.; Allard, J. W. and Lipton, A. (2002):** Relationship of serum HER-2/neu and serum CA 15-3 in patients with metastatic breast cancer. Clin Chem.; 48: 1314-1320.
6. **Astall, E.C. and Bobrow, L.G. (1998):** Assessment of the accuracy of diagnostic and prognostic information provided by core biopsy in breast carcinoma. J Pathol.; 186: ssPA6.
7. Autier,P.,Boniol,M., La Vecchia,C.,and Heanue, M., (2010): Disparities in breast cancer mortality trends between 30 European countries: retrospective trend analysis of WHO mortality database.BMJ;341:c3620
8. **Baker, M.K.; Mikhitarian, K.; Osta, W.; Callahan, K.; Hoda, R.; Brescia, F.; Kneuper-Hall, R.; Mitas, M.; Cole, D.J. and Gillanders, W.E. (2003):** Molecular Detection of Breast Cancer Cells in the Peripheral Blood of Advanced-Stage Breast Cancer Patients Using Multimarker Real-Time Reverse Transcription-Polymerase Chain Reaction and a Novel Porous Barrier Density Gradient Centrifugation Technology. Clinical Cancer Research; 9: 4865-4871.
9. Balleine RL, Wilcken NR. High-risk estrogen-receptor-positive breast cancer: identification and implications for therapy. Mol Diagn Ther. 2012;16(4):235-240
10. **Bánkfalvi, A.; Simon, R.; Brandt, B.; Bürger, H.; Vollmer, I.; Dockhorn-Dworniczak, B.; Lellé, R.J. and Boecker, W. (2000):** Comparative methodological analysis of erbB-2/HER-2 gene dosage, chromosomal copy number and protein overexpression in breast carcinoma tissues for diagnostic use. Histopathology; 37(5):411-419.
11. **Bewick, M.; Conlon, M.; Gerard, S.; Lee, H.; Parissenti, A. M.; Zhang, L.; Gluck, S. and Lafrenie, R. M. (2001):** HER-2 expression is a prognostic factor in patients with metastatic breast cancer treated with a combination of high-dose cyclophosphamide, mitoxantrone, paclitaxel and autologous blood stem cell support. Bone Marrow Transplant; 27: 847-853.
12. **Bieche, I.; Onody, P.; Laurendeau, I.; Olivi, M.; Vidaud, D.; Lidereau, R. and Vidaud, M. (1999)** Real-time reverse transcription-PCR assay for future management of ERBB2-based clinical applications. Clin Chem.; 45: 1148-1156.
13. **Buzdar, A.U. and Hortobagyi, G. (1998):** Update on endocrine therapy for breast cancer. Clin Cancer Res.; 4: 527-534.
14. **Carney, W.P.; Leitzel, K.; Ali, S.; Neumann, R. and Lipton, A. (2007):** HER-2 therapy. HER-2/neu diagnostics in breast cancer. Breast Cancer Research; 9: 207.
15. **Cheung, K.L.; Graves, C.R. and Robertson, J.F. (2000):** Tumour marker measurements in the diagnosis and monitoring of breast cancer. Cancer Treat Rev.; 26(2): 91-102.
16. ***De Luca, A.; Pignata, S.; Casamassimi, A.; D'Antonio, A.; Gridelli, C.; Rossi, A.; Cremona, F.; Parisi, V.; De Matteis, A. and Normanno, N. (2000):*** *Detection of Circulating Tumor Cells in Carcinoma Patients by a Novel Epidermal Growth Factor Receptor Reverse Transcription-PCR Assay. Clinical Cancer Research; 6: 1439-1444.*
17. **Decock, J. (2006):** How Accurate Is the Antiprimer Quenching-Based Real-Time PCR for Detection of Her2/neu in Clinical Cancer Samples? Clinical Chemistry; 52:1438-1439.
18. **Denley, H.; Pinder, S.E.; Elston, C.W.; Lee, A.H. and Ellis, I.O (2001):** Preoperative assessment of prognostic factors in breast cancer. Clin Pathol.; 54: 20-24.
19. Dowsett M, Allred C, Knox J, Quinn E, Salter J, Wale C, Cuzick J, Houghton J, Williams N, Mallon E, Bishop H, Ellis I, Larsimont D, Sasano H, Carder P, Cussac AL, Knox F, Speirs V, Forbes J, Buzdar A (2008): **Relationship between quantitative estrogen and progesterone receptor expression and human epidermal growth factor receptor 2 (HER-2) status** with **recurrence in the Arimidex, Tamoxifen, Alone or in Combination trial.** *J Clin Oncol* 2008
20. **Duffy, M.J. (2005):** Predictive Markers in Breast and Other Cancers: A Review. Clinical Chemistry; 51: 494-503.
21. Ejlertsen B, Jensen MB, Nielsen KV, Balslev E, Rasmussen BB, Willemoe GL, Hertel PB,

Knoop AS, Mouridsen HT, Brunner N: **HER2, TOP2A, and TIMP-1 and responsiveness to adjuvant anthracycline-containing chemotherapy in high-risk breast cancer patients.** *J Clin Oncol* 2010, **28:**984-990.

22. **Espinosa, A.B.; Tabernero, M.D.; Garcia, M.C.; Primo, D.; Bernal, A.G.;Cruz, J.J.; Ramos, M.; Font de Mora, J.; Alonso, A.G.; and Orfao, A. (2003):** Her-2/neu gene amplification in familial Vs sporadic breast cancer: impact on the behaviour of the disease. Am J Clin Pathol.; 120(6): 917-927.
23. **Esteva, F.J. and Hortobagyi, G.N. (2004):** Prognostic molecular markers in early breast cancer. Breast Cancer Res.; 6(3): 109-118.
24. **Esteva, F.J.; Sahin, A.A. and Cristofanilli, M. (2005):** Prognostic role of a multigene reverse transcriptase-PCR assay in patients with node-negative breast cancer not receiving adjuvant systemic therapy. Clin Cancer Res.; 11: 3315-3319.
25. Fabbroni M.A. and Sandri M. T., "Circulating tumour cells in clinical practice: methods of detection and possible characterization, (2010)" Methods, vol. 50, no. 4, pp. 289–297.
26. Ferro, P M. C. Franceschini, B. Bacigalupo et al., "Detection of circulating tumour cells in breast cancer patients using human mammaglobin RT-PCR: association with clinical prognostic factors, (2010)" Anticancer Research, vol. 30, no. 6, pp. 2377–2382.
27. **Gilbey, A.M.; Burnett, D.; Coleman, R.E. and Holen, I. (2004):** The detection of circulating breast cancer cells in blood. J. Clin Path.; 57: 675-681.
28. ***Gjerdrum, L.M.; Sorensen, B.S.; Kjeldsen, E.; Flemming Brandt Sorensen, F.B.; Nexo, E. and Hamilton-Dutoit, S. (2004):*** *Real-Time Quantitative PCR of Microdissected Paraffin-Embedded Breast Carcinoma. An Alternative Method for HER-2/neu Analysis. JMD; 6: 1.*
29. **Gown, A.M, Goldstein, LC., Barry, T.S., and Tse, C.C (2008): High concordance between immunohistochemistry and fluorescence in situ hybridization testing for HER 2 status in breast cancer requires a normalized IHC scoring system.Mod.Pathol.;21(10)127**
30. **Graves H and Czerniecki B(2011):** Circulating Tumor Cells in Breast Cancer Patients: An Evolving Role in Patient Prognosis and Disease Progression Volume 2011 (2011), Article ID 621090, 7 pages
31. **Gupta, D.; Middleton, L. P.; Whitaker, M. J. and Abrams, J. (2003)**: Comparison of fluorescence and chromogenic in situ hybridization for detection of HER-2/neu oncogene in breast cancer. Am J Clin Pathol.; 119: 381-387.
32. Hammond ME, Hayes DF, Dowsett M, Allred DC, Hagerty KL, Badve S, Fitzgibbons PL, Francis G, Goldstein NS, Hayes M, Hicks DG, Lester S, Love R, Mangu PB, and Williams RB, *(2010*): **American Society of Clinical Oncology/College Of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer.***J Clin Oncol*, **28:**2784-2795.
33. **Hayes, D. F. and Thor, A. D. (2002):** c-erbB-2 in breast cancer: Development of a clinically useful marker. Semin Oncol.; 29: 231-245.
34. **Houghton, R.; Dillon, D.; Molesh, D.; Zehentner, B.; Xu, J.; Jiang, J.; Schmidt, C.; Frudakis, A.; Repasky, E.; Filho, A.; Nolasco, M.; Badaro, R.; Zhang, X.; Roche, P.; Persing, D. and Reed, S. (2011):** Transcriptional complementarity in breast cancer: application to detection of circulating tumor cells. Mol. Diagn., 6: 79-91.
35. **Hudis, C.A. (2007):** Trastuzumab--mechanism of action and use in clinical practice. N Engl J Med.; 357(1): 39-51.
36. Hüsemann, J. B. Geigl, F. Schubert et al., (2008) "Systemic spread is an early step in breast cancer," Cancer Cell, vol. 13, no. 1, pp. 58–68, 2008. Y.
37. *KallergiG, NasiasD, Papadaki M, Mavroudis D,* **Georgoulias V, and SAgelakiS** San Antonio, TX (2012): Truncated HER2 receptor in circulating tumor cells (CTCs) of early and metastatic breast cancer patients Symposium--Dec 4-8; © 2012 American Association for Cancer Research
38. ***Königshoff, M.; Wilhelm, J.; Bohle, R.M.; Pingoud, A. and Hahn, M. (2003):*** *HER-2/neu Gene Copy Number Quantified by Real-Time PCR: Comparison of Gene Amplification, Heterozygosity, and Immunohistochemical Status in Breast Cancer Tissue. Clinical Chemistry; 49: 219-229.*
39. . Lang J. E, K. Mosalpuria, M. Cristofanilli et al., "HER2 status predicts the presence of circulating tumor cells in patients with operable breast cancer," Breast Cancer Research and Treatment, vol. 113, no. 3, pp. 501–507, 2009. Pathology Research International Volume 2011 (2011), Article ID 621090, 7 pages
40. **Liotta, L. A. and Stetler-Stevenson, W. G. (1992):** Principles of molecular cell biology of cancer: cancer metastasis DeVita V. T. Hellman S. Rosenberg S. A. eds. Cancer: Principles and

Practice of Oncology. J. B. Lippincott Co. Philadelphia; 98-115.

41. **Lu¨ftner, D.; Henschke, P.; Kafka, A.; Anagnostopoulos, I.; Wiechen, K. and Geppert, R. (2004-b):** Discordant results obtained for the different methods of HER-2/neu testing in breast cancer-a question of standardization, automation and timing. Int J Biol Markers; 19: 1-13.
42. **Madrid, M.A. and Lo, R.W. (2004):** Chromogenic in situ hybridization (CISH) a novel alternative in screening archival breast cancer tissue samples for HER-2/neu status. Breast Cancer Res.; 6(5): 593-600.
43. **Marglet, D.**N., Sreedhara,M., Chen, y., and Gloshan, M.(2012): Microinvasive breast cancer:ER,PR,and HER-2/neu status and clinical outcomes after breast conserving therapy or mastectomy. Annals of Surgical Oncology. DOI10.1245/510434-012-2640-8.
44. **Masood, S. and Bui, M. M. (2002):** Prognostic and predictive value of HER2/neu oncogene in breast cancer. Microsc Res Tech.; 59: 102-108.
45. **Mavaddat, N.**, Pharoah, P.D., Blows, F., and Antoniou, A.C.(2010):Familial relative risk for breast cancer by pathological subtype:a population based cohort study. Breast Cancer Res.;12(1):R10
46. **Meng, S.; Tripathy, D.; Shete, S.; Ashfaq, R.; Saboorian, H.; Haley, B.; Frenkel, E.; Euhus, D. and Leitch, M. (2006):** uPAR and HER-2 gene status in individual breast cancer cells from blood and tissues**.** PNAS; 103 (46): 17361-17365.
47. **Michail Ignatiadis mail, Françoise Rothé Carole Chaboteaux, Virginie Durbecq, Ghizlane Rouas,Carmen Criscitiello,Jessica Metallo, Naima Kheddoumi,Sandeep K.(2013): HER2-Positive Circulating Tumor Cells in Breast Cancer *emedicine.medscape.com/article/1689966-overview.***
48. **Monego, G.; Arena, V.; Maggiano, N.; Costarelli, L.; Crescenzi; A.; Zelano, G.; Amini, M.; Capelli, A. and A. Carbone, A (2007): Borderline HER-2 breast cancer cases: Histochemical versus real-time PCR analysis and impact of different cut-off values. Scandinavian Journal of Clinical and Laboratory Investigation; 67(4): 402-412.**
49. **Mostafa, M.Y.; Ismail, S.S.; Annous, F.A. and Hafeez, Z.M. (2005):** A prospective randomized study to evaluate doxorubicin / cyclophosphamide followed by paclitaxel versus combination chemotherapy with 5-fluorouracil / doxorubicin / cyclophosphamide as adjuvant therapy in patients with stage I and II breast cancer. Thesis.
50. **Paik, S.; Bryant, J.; Tan-Chiu, E.; Romond, E.; Hiller, W.; Park, K.; Brown, A.; Yothers, G.; Anderson, S.; Smith, R.; Wickerham, D. L. and Wolmark, N. (2002-a):** Real-world performance of HER2 testing-National Surgical Adjuvant Breast and Bowel Project experience. J. Natl. Cancer Inst.; 94: 852-854.
51. **Peiró, G.; Adrover, E.; Aranda, F.I.; Francisca M. Peiró, F.M; Maria Niveiro, M. and Sánchez-Payá, J. (2007):** Prognostic Implications of HER-2 Status in Steroid Receptor-Positive, Lymph Node-Negative Breast Carcinoma**.** Am J Clin Pathol.; 127(5): 780-786.
52. Pestrin M., Bessi S. F. Galardi et al., "Correlation of HER2 status between primary tumors and corresponding circulating tumor cells in advanced breast cancer patients," (2009) :Breast Cancer Research and Treatment, vol. 118, no. 3, pp. 523–530.
53. Prat A, Cheang MC, Martín M, et al. (2013) : Prognostic significance of progesterone receptor-positive tumor cells within immunohistochemically defined luminal a breast cancer. J Clin Oncol.; 31(2):203-209.
54. **Rakha, E.A.; El-Sayed, M.E. and Reis-Filho, J.S. (2007):** Expression profiling technology: its contribution to our understanding of breast cancer. Histopathology; 52(1): 67-81.
55. **Rhodes, A.; Jasani, B.; Anderson, E.; Dodson, A.R. and Balaton, A.J. (2002):** Evaluation of HER-2/neu immunohistochemical assay sensitivity and scoring on formalin-fixed and paraffin-processed cell lines and breast tumors: A comparative study involving results from laboratories in 21 countries. Am J Clin Pathol.; 118: 408-417.
56. **Ross, J.S.; Fletcher, J.A.; Bloom, K.J.; Linette, G.P.; Stec, J.; Symmans, W.F.; Pusztai, L. and Gabriel N. Hortobagyi, G.N. (2004):** Targeted Therapy in Breast Cancer. The HER-2/neu Gene and Protein. Molecular & Cellular Proteomics; 3: 379-398.
57. **Safi, F.; Kohler, I.; Rottinger, E. and Beger, H.E. (1991):** The value of the tumor marker CA15.3 in diagnosing and monitoring breast cancer. Cancer; 68: 574-582.
58. **Schnitt, S.J. and Jacobs, T.W. (2001):** Current status of HER2 testing: Caught between a rock and a hard place. Am J Clin Pathol.; 116: 806-810.
59. **Sharifi, S.; Peterson, M.K. and Baum, J.K. (1999):** Assessment of prognostic factors in breast core needle biopsies. Mod Pathol.; 12: 941-945.

60. ***Silva, J.M.; Dominguez, G.; Silva, J.; Garcia, J.M.; Sanchez, A.; Rodriguez, O.; Provencio, M.; España, P. and Bonilla, F. (2001):*** *Detection of Epithelial Messenger RNA in the Plasma of Breast Cancer Patients Is Associated with Poor Prognosis Tumor Characteristics. Clinical Cancer Research; 7: 2821-2825.*
61. **Skedgel**,C., Rayson, D., and Yournis,T.(2013): IS adjuvant trastuzumab a cost effective therapy for HER-2/neu positive breast cancer.Annalsof oncology.DOI:10.1093/annonc/mdt069.
62. **Smith, B. M., Slade, M. J., English, J., Graham, H., Luchtenborg, M., Sinnett, H. D., Cross, N. C., and Coombes, R. C. (2000):** Response of circulating tumor cells to systemic therapy in patients with metastatic breast cancer: comparison of quantitative polymerase chain reaction and immunocytochemical techniques. J Clin Oncol., 18: 1432-1439.
63. **Thriveni, K.; Krishnamoorthy, L. and Ramaswamy, G. (2007):** Correlation study of carcno embryonic antigen & cancer antigen 15.3 in pretreated female breast cancer patients. Indian Journal of Clinical Biochemistry; 22(1): 57-60.
64. **Tse, C.; Brault, D.; Gligorov, J.; Antoine, M.; Neumann, R.; Lotz, J-P. and Capeau, J. (2005):** Evaluation of the Quantitative Analytical Methods Real-Time PCR for HER-2 Gene Quantification and ELISA of Serum HER-2 Protein and Comparison with Fluorescence in Situ Hybridization and Immunohistochemistry for Determining HER-2 Status in Breast Cancer Patients. Clin Chem.; 51(7): 1093-1101.
65. **Valero, V.; Roche, H.; Pienkowski, T.; Canon, J.; Zhao, Y.; Carney, W.; Mackey, J.; Taupin, H.; Buyse, M. and Slamon, D. (2007):** BCIRG 007: Serum HER-2/neu levels in women with metastatic HER-2/neu-amplified breast cancer. Proc Am Soc Clin Oncol.; 12:525-533.
66. **Walter, P; Carney, W.P.; Neumann, R; Lipton, A.; Leitzel, L.; Ali, S. and Price C.P. (2003):** Potential clinical utility of serum HER-2/neu oncoprotein concentrations in patients with breast cancer. Clin Chem.; 49:1579-1598.
67. **Wang, X.; Wang, J.P.; Maughan, M. F. and Lachman, L. B. (2005):** Alphavirus replicon particles containing the gene for Her -2/neu inhibit Pathology Research International
68. **Wasserman, L.M.; Jones, V.; Easter, D. and Wallace, A. (2000):** RT-PCR detection of circulating breast cancer cells: sensitivity and specificity of HER2/neu as a marker. Cancer Deyction and Prevention; 24: (Suppl 1).
69. **Zemzoum, I.; Kates, R. E.; Ross, J. S.; Dettmar, P.; Dutta, M.; Henrichs, C.; Yurdseven, S.; Hofler, H.; Kiechle, M.; Schmitt, M. and Harbeck, N. (2003):** Invasion factors uPA/PAI-1 and HER2 status provide independent and complementary information on patient outcome in node-negative breast cancer. J. Clin. Oncol.; 21: 1022-1028.

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