RESEARCH ARTICLE

Overall Survival and Clinicopathological Characteristics of Patients with Breast Cancer in Relation to the Expression Pattern of HER-2, IL-6, TNF-α and TGF-β1

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Abstract

The present study was conducted to investigate the prognostic significance of co-expression patterna of HER-2, IL-6, TNF-a and TGF-β1 in breast cancer, by correlating the number of markers with positive expression with clinicopathological characteristics indicative of tumor progression and overall survival. One hundred thirty consecutive patients with primary breast cancer were prospectively included and evaluated. Serum concentrations of the above markers were measured by ELISA. Median split was used to subdivide patients with marker positive or negative expression. The presence of ≥3 positive markers was independently associated with extended lymph node (>3) involvement (aOR, 11.94, p=0.001) and lymphovascular invasion (aOR, 12.04, p=0.018), increasing the prognostic significance of each marker considered separately. Additional prognostic information regarding survival was also provided; as the number of positive markers increased, a gradually reduction of survival time was observed. In addition, patients with 4 positive markers had significantly shorter survival (25 vs 39 months, p=0.006) and a more than 4 fold increased risk of death (aHR, 4.35, p=0.003) compared to patients with 3 positive markers. Our findings suggest that the coexpression pattern of these four markers could be used clinically as a useful marker for tumor extension and outcome of breast cancer.

Keywords: HER-2 - IL-6 - TNF-α - TGF-β1 - breast cancer - survival

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Introduction

Breast cancer, a heterogeneous disease, is one of the most common cancers in women. Its aetiology is multifactorial, the period of development can span decades, the clinical course is highly variable and the prognosis varies depending upon the develop-mental stage of the breast tissue at diagnosis. Several clinicopathological characteristics and biological factors, such as tumor size, tumor grade, lymph node status, hormone receptors, HER-2, urokinase plasminogen activator, and plasminogen activator inhibitor 1 (Elston et al., 1999; Isaacs et al., 2001; Duffy, 2002), which may help in the initial assessment of the extent of the disease and the prediction of response or resistance to specific therapies, require tumor tissue, thus necessitating either biopsy or surgery. Although, serum tumor markers play an important role in patient management for many malignancies (Fisher and Hancock, 1997; Duffy, 2001; Rustin, 2003; Parker, 2004), their role in breast cancer is less well established (Duffy, 2006).

One of the most used serum marker, the human epidermal growth factor receptor-2 (HER-2), also known as c-erbB-2 or neu proto-oncogene is a member of the EGFR family and plays an important role in the regulation of cell growth, differentiation and survival and is involved in the regulation of normal breast growth and development (Yarden and Sliwkowski, 2001). Increased serum HER-2 is associated with rapid tumor growth (Carney et al., 2003), increased risk of earlier recurrence after surgery and shortened survival (Bewick et al., 1999; Ali et al., 2002; Fehm et al., 2004), poor response to conventional chemotherapy (Harris et al., 2001; Colomer et al., 2004) and hormonal therapy (Lipton et al., 2002) and prediction of response to trastuzumabbased treatments (Köstler et al., 2004; Esteva et al., 2005).

In addition, cytokines have received a great deal of attention by many researchers as potential diagnostic and prognostic markers in breast cancer since changes in their levels mediated by the tumour both directly and indirectly are important parameters that affect the course of disease (Nicolini et al., 2006; Chavey et al., 2007). One important cytokine in breast cancer is interleukin-6

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(IL-6), a proinflammatory cytokine involved in various other physiological and pathological processes in the body. IL-6 is active in the immune response, haematopoiesis, the acute phase response and inflammation, it can also act as an autocrine or paracrine cancer cell growth factor and contribute to recurrence and metastasis in breast cancer (Knupfer and Preiss, 2007; Heikkilä, 2008). Tumor necrosis factor-a (TNF-a), another extremely pleiotropic cytokine, is produced by a wide range of pathogenic stimuli, is also produced by tumor cells and can act as an endogenous tumor promoter (Locksley et al., 2001; Balkwill, 2002; MacEwan, 2002). TNF-a has been shown to be one of the major mediators of inflammation (Balkwill, 2002) and it is also able to affect the expression of growth factors and other cytokines, via multiple signal transduction pathways (Vilcek and Lee, 1991). Furthermore, transforming growth factor (TGF-β1), as a member of the transforming growth factor superfamily of cytokines, has diverse effects, ranging from cell growth and differentiation to immune modulation and apoptosis. It has been shown to stimulate tumor invasion by promoting angiogenesis, extracellular matrix production and through inhibition of host immune functions (Reiss and Barcellos-Hoff, 1997). Elevated plasma levels of TGF-β1 could be highly relevant to breast cancer invasion and metastasis (Perera et al. 2010).

We have previously shown, based on a smaller cohort, that elevated serum levels of HER-2, IL-6, TNF-a and TGF-β1 reflect tumor maintenance and aggressiveness in breast cancer patients, and they can be used as prognostic markers of breast cancer outcome and survival (Papadopoulou et al., 2008a; 2008b; 2010; Tripsianis et al., 2012). Despite the generally good outcome of each marker, it is well understood that multiple marker investigation rather than a single tumor marker would be of benefit towards on defining better prognostic biomarkers that would allow a more precise strategy of treatment based upon the subgrouping of patients. Therefore, in the present study, we examine the prognostic value of the coexpression pattern of these four markers (HER-2, IL-6, TNF-a and TGF-β1), by evaluating the association of the number of markers with positive expression with the clinicopathological characteristics indicative of tumor progression and the overall survival of breast cancer patients.

Materials and Methods

Study population.

One hundred thirty consecutive patients with primary breast carcinoma, admitted to the University General Hospital of Alexandroupolis were included in the present population-based study. The diagnosis of breast cancer was confirmed by histological examination, using specimens obtained from biopsy or surgical resection. Tumors were graded according to the criteria described by Bloom and Richardson (1957) and tumor stage was assigned according to the TNM classification defined by the Union International Against Cancer (1992). The expressions of estrogen receptor (ER) and progesterone receptor (PR) proteins were considered positive if 10% of the cancer cells showed immunoreactivity. Written informed consent was obtained from all women and the Regional ethical committee approved the study.

Measurement of serum HER-2, IL-6, TNF-α and TGF-β1. Peripheral blood samples were collected from each patient before operation. After centrifugation at 3000 rpm for 20 min, serum samples were frozen and stored at -70ºC until biochemical assessment. Quantitative sandwich enzyme immunoassay (ELISA) was performed for measuring concentrations of serum HER-2, IL-6, TNF-α and TGF-β1, by means of a commercially available kit (ImmunoKontact, AMS Biotechnology, U.K.).

Statistical analysis

Statistical analysis of the data was performed using the Statistical Package for the Social Sciences (SPSS), version 19.0 (IBM). The normality of HER-2, IL-6, TNF-α and TGF-β1 levels was tested with Kolmogorov-Smirnov test. All markers were expressed as the median and interquartile range (IQR, 25th to 75th percentile). In the sequence, median split was used to subdivide patients into groups with positive or negative HER-2, IL-6, TNF-α or TGF-β1 levels. The chisquare test was used to assess the association of the expression of the markers with patients' characteristics. Multivariate stepwise logistic regression analysis was constructed to explore the independent effect of clinicopathological parameters on HER-2, IL-6, TNF-α and TGF-β1 expression. Odds ratios (OR) and their 95% confidence intervals (CI) were estimated as the measure of association of markers' expression with patients' characteristics. Survival rates were calculated with the Kaplan-Meier method and the statistical difference between survival curves was determined with the log-rank test. Multivariate Cox proportional hazards regression analysis was performed to explore the independent effect of the studied markers on overall survival. All tests were two tailed and statistical significance was considered for p values<0.05.

Results

Characteristics of study population. The study population was consisted of 130 breast cancer patients with a median age of 65 years (range, 33-84 years; mean age±SD, 61.64±10.92 years). The clinicopathological characteristics of the tumors are shown in Table 1. Regarding to histology, 103 (79.2%) were ductal and 27 (20.8%) lobular carcinomas. More than 80% of cases were invasive carcinomas (106 patients, 81.5%) and the majority of the tumors had size between 2 and 5 cm (T2; 83 patients, 63.8%). Twenty two (16.9%) were well-differentiated (G1), 20 (15.4%) were moderately differentiated (G2) and 88 (67.7%) were poorly differentiated carcinomas. Half of the cases (66 patients, 50.8%) were of stage II, while in 60 patients (46.2%) lymph node metastases were detected; in 31 of them (51.7%) the number of positive lymph nodes was greater than three. ER and PR positivity was detected in 62.3% and 47.7% of the patients, respectively.

Association with clinicopathological parameters. The median levels of serum HER-2 (2.04 ng/ml), IL-6 (7.12 pg/ml), TNF-α (18.80 pg/ml) and TGF-β1 (64.10 ng/ml)

Table 1. Characteristics of Breast Cancer Patients

		No. of patients	%
Age [years; mean, (SD)]		61.64	(10.92)
Histological type	Lobular	27	20.8
	Ductal	103	79.2
Lymphovascular invasion		106	81.5
Tumor size	T1	39	30.0
	T2	83	63.8
	T3	8	6.2
Histological grade	G1	22	16.9
	G2	20	15.4
	G3	88	67.7
Clinical stage	0-I	34	26.2
	II	66	50.8
	III-IV	30	23.1
Positive lymph node status	60	46.2	
Positive lymph nodes >3	31	51.7	
HER-2 [ng/ml; median (IQR)]		2.04	(1.92-2.25)
IL-6 [pg/ml; median (IQR)]		7.12	(5.15-11.20)
TNF-a [pg/ml; median (IQR)]		18.80	(12.01-30.26)
TGF-β1 [ng/ml; median (IQR)]		64.10	(32.80-92.20)

*SD, standard deviation; IQR, interquartile range (25^{th} to 75^{th} percentile)

Table 2. Positive Expression of HER-2, IL-6, TNF-a and TGF-β1 in Patients with Breast Cancer in Relation to the Clinicopathological Characteristics**

		Positive expression			
		HER-2	IL-6	TNF-a	TGF-β1
Age	≤65	30 (46.2)	14 (21.5)	32 (49.2)	30 (46.2)
(years)	>65	35 (53.8)	51 (78.5)	33 (50.8)	35 (53.8)
Histological type					
	Lobular	10 (37.0)	12 (44.4)	14 (51.9)	14 (51.9)
	Ductal	55 (53.4)	53 (51.5)	51 (49.5)	51 (49.5)
Lymphovascular invasion					
	No	3 (12.5)	5 (20.8)	6 (25.0)	9 (37.5)
	Yes	62 (58.5)*	60 (56.6)*	59 (55.7)*	56 (52.8)
Tumor size	T1	16 (41.0)	20 (51.3)	21 (53.8)	19 (48.7)
	T2-T3	49 (53.8)	45 (49.5)	44 (48.4)	46 (50.5)
Histological grade					
	G1-G2	13 (31.0)	18 (42.9)	14 (33.3)	25 (59.5)
	G3	52 (59.1)*	47 (53.4)	51 (58.0)*	40 (45.5)
Clinical stage	I-II	38 (38.0)	45 (45.0)	44 (44.0)	45 (45.0)
	III-IV	27 (90.0)*	20 (66.7)*	21 (70.0)*	20 (66.7)*
Lymph node status					
	Negative	18 (25.7)	31 (44.3)	33 (47.1)	29 (41.4)
	Positive	47 (78.3)*	34 (56.7)	32 (53.3)	36 (60.0)*
No of positive lymph nodes					
	≤3	19 (65.5)	11 (37.9)	8 (27.6)	13 (44.8)
	>3	28 (90.3)*	23 (74.2)*	24 (77.4)*	22 (71.0)*
HER-2	Low	-	22 (33.8)	20 (30.8)	29 (44.6)
	High	-	43 (66.2)*	45 (69.2)*	36 (55.4)
IL-6	Low	22 (33.8)	-	19 (29.2)	28 (43.1)
	High	43 (66.2)*	-	46 (70.8)*	37 (56.9)
TNF-a	Low	20 (30.8)	19 (29.2)	-	31 (47.7)
	High	45 (69.2)*	46 (70.8)*	-	34 (52.3)
TGF-β1	Low	29 (44.6)	28 (43.1)	31 (47.7)	-
	High	36 (55.4)	37 (56.9)	34 (52.3)	-

*Indicates statistically significant difference; **Data are expressed as frequencies and percentages

were selected as the cut-off points to subdivide breast cancer patients into (i) patients with low levels (<median value, negative expression) and patients with high levels (≥median value, positive expression) in order to assess the relation of four markers with the clinicopathological parameters and overall survival. The presence of high HER-2, IL-6, TNF-a and TGF-β1 levels was analyzed in relation to the following parameters: patient's age, histological type, lymphovascular invasion, tumor size, histological grade, clinical stage, lymph node status and the number of positive lymph nodes (Table 2).

The following relations were obtained: *i*) High HER-2 levels were associated with lymphovascular invasion (cOR=9.86, 95%CI=2.77-35.12, p<0.001), poor differentiation (cOR=3.22, 95%CI=1.48-7.03, p=0.003), advanced stages (cOR=14.68, 95%CI=4.17-51.73, p<0.001), positive lymph node status (cOR=10.44, 95%CI=4.62-23.60, p<0.001) and the presence of >3 positive lymph nodes (cOR=4.91, 95%CI=1.19-20.23, p=0.020); *ii*) High IL-6 levels were associated with lymphovascular invasion (cOR=4.96, 95%CI=1.72-14.27, p=0.002), advanced stages (cOR=2.44, 95%CI=1.04-5.75, p=0.037) and the presence of >3 positive lymph nodes (cOR=4.71, 95%CI=1.57-14.13, p=0.005); *iii*) High TNF-a levels were associated with lymphovascular invasion (cOR=3.77, 95%CI=1.39-10.24, p=0.007), poor differentiation (cOR=2.76, 95%CI=1.28-5.95, p=0.009), advanced stages (cOR=2.97, 95%CI=1.24-7.12, p=0.012) and the presence of >3 positive lymph nodes (cOR=9.00, 95%CI=2.79-29.04, p<0.001); *iv*) High TGF-β1 levels were associated with advanced stages (cOR=2.44, 95%CI=1.04-5.75, p=0.037), positive lymph node status (cOR=2.12, 95%CI=1.05-4.28, p=0.035) and the presence of >3 positive lymph nodes (cOR=3.01, 95%CI=1.04-8.74, p=0.040). Multivariate stepwise logistic regression analysis revealed the following significant independent determinants: *i*) Lymphovascular invasion (aOR=5.76, 95%CI=1.41-23.63, p=0.015), poor differentiation (aOR=2.88, 95%CI=1.10-7.55, p=0.031) and the positive lymph node status (aOR=3.98, 95%CI=1.39-11.36, p=0.010) for high HER-2 levels; *ii*) Lymphovascular invasion (aOR=4.39, 95%CI= 1.41-13.70, p=0.011) and the presence of >3 positive lymph nodes (aOR=5.68, 95%CI= 1.32-24.38, p=0.019) for high IL-6 levels; *iii*) Lymphovascular invasion (aOR=4.46, 95%CI=1.39-14.33, p=0.012), poor differentiation (aOR=4.49, 95%CI=1.85-10.91, p<0.001) and the presence of >3 positive lymph nodes (aOR=5.10, 95%CI=1.66-15.67, p=0.004) for high TNF-a levels; (*iv*) the presence of >3 positive lymph nodes (aOR=3.03, 95%CI=1.01-9.15, p=0.049) for high TGF-β1 levels (Table 4).

In 22 (16.9%) patients all the herein studied markers were increased (≥median value); on the contrary, in 15 (11.5%) patients all markers were low (<median value). Positive expression of one, two and three markers were found in 43 (33.1%), 21 (16.2%) and 29 (22.3) patients, respectively. Statistically significant associations between the expressions of HER-2, IL-6 and TNF-a were found (Table 2); patients with high HER-2 were 4 times more likely to have high IL-6 (cOR=3.82, 95%CI=1.85-7.90, p<0.001) and 5 times more likely to have high TNF-a (cOR=5.06, 95%CI=2.40-10.66, p<0.001), while positive expression of TNF-a was more frequent among positive IL-6 patients than among negative IL-6 patients (cOR=5.86, 95%CI=2.75-12.48, p<0.001). TGF-β1 expression was independent of the three other markers. The coexpression of HER-2, IL-6, TNF-a and TGF-β1 in relation to the clinicopathological parameters was examined next (Table 3). The expression pattern of the four markers was significantly associated with lymphovascular invasion (p<0.001), clinical stage (p<0.001), lymph node

status (p<0.001) and the number of positive lymph nodes (p<0.001). In particular, the positive expression of three or four markers was more likely to be found in invasive tumors (cOR=20.54, 95%CI=2.68-157.63, p<0.001), advanced stages (cOR=8.45, 95%CI=3.26-21.89, p<0.001), patients with positive lymph status (cOR=3.07, 95%CI=1.48-6.37, p=0.002) and patients with >3 positive lymph nodes (cOR=19.93, 95%CI=5.36-74.08, p<0.001); invasive tumors (aOR=12.04, 95%CI=1.51-95.70, p=0.018) and the presence of more than three positive lymph nodes (aOR=11.94, 95%CI=2.64-54.07, p=0.001) remained significant independent determinants of the simultaneous presence of three or four positive markers (Table 4).

Association with overall survival. After a median follow up period of 31 months (range, 3-68 months), 28 (21.5%) patients have died as a consequence of disease progression. Among the entire cohort, the mean survival time was 55±2 months (95%CI=51-59 months; median survival time was not reached). The results of survival analysis based on Kaplan-Meier method are shown in Table 5. The log-rank test revealed that the positive expression of each marker was associated with statistically significant worse prognosis. In particular, shorter survival time was observed in patients with positive HER-2 (mean survival time, 44 *vs* 62 months, p<0.001), IL-6 (39 *vs* 62 months, p<0.001), TNF-a (45 *vs* 61 months, p=0.002) and TGF-β1 (46 *vs* 63 months, p<0.001). Moreover, the incidence of death was significantly higher in patients with positive HER-2 (33.8% vs. 9.2%, p=0.001), IL-6 (32.3% vs. 10.8%, p=0.003), TNF-a (30.8% vs. 12.3%, p=0.010) and TGF-β1 (38.5% vs. 4.6%, p<0.001). Patients with high TGF-β1 levels were 10 times more likely to die of cancer compared to those with low TGF-β1 levels (Hazard ratio (HR)=9.96, 95%CI=3.01-33.01, p<0.001); elevated risk of death was also observed in patients with positive HER-2 (HR=4.40, 95%CI=1.78-10.85, p=0.001), IL-6 (HR=4.35, 95%CI=1.83-10.33, p<0.001) and TNF-a (HR=3.32, 95%CI=1.45-7.58, p=0.004).

In the sequence, we defined the following groups according to the expression pattern of the four herein studied markers: group A (patients with none of the markers positive), group B (patients with one positive marker), group C (patients with two positive markers), group D (patients with three positive markers) and group E (patients with all markers positive). One, 2 and 3-year survival rates and the mean survival time gradually decrease as the number of positive markers increases (Table 5). However, the differences between groups A, B and C did not reach the statistical significance. Statistically significant worse prognosis was found in groups D and E compared to groups A, B and C; finally, patients of group E had shorter survival even when they were compared with patients of group D (25 *vs* 39 months, p=0.006) (Figure 1). During follow-up, mortality rate was 0.0%, 7.0%, 14.3%, 31.0% and 36.8% for groups A, B, C, D and E, respectively (p<0.001). Cox regression analysis revealed that patients of group D were 7.5 times (HR=7.55, 95%CI=2.04-27.97, p=0.002) and patients of group E were more than 20 times (HR=22.96, 95%CI=6.46-81.57, p<0.001) more likely to die of cancer than patients of groups A+B, respectively; a more than three-fold increased risk of death was observed in patients of group E compared to patients of group D (HR=3.04, 95%CI=1.29-7.17, p=0.011).

Investigation with multivariate Cox proportional hazards regression analysis, includ-ing all clinicopathological

Table 3. Expression Pattern of Positive (+) HER-2, IL-6, TNF-a and TGF-β1 in Breast Cancer Patients in Relation to the Clinicopathological Characteristics*

		None (+)	1 or 2 (+)	3 or 4 (+)	p value
Age	≤65	11 (16.9)	34 (52.3)	20 (30.8)	0.053
(years)	>65	4 (6.2)	30 (46.2)	31 (47.7)	
Histological type	Lobular	6 (22.2)	11 (40.7)	10 (37.0)	0.141
	Ductal	9 (8.7)	53 (51.5)	41 (39.8)	
Lymphovascular invasion					
	No	7 (29.2)	16 (66.7)	1 (4.2)	<0.001
	Yes	8 (7.5)	48 (45.3)	50 (47.2)	
Tumor size	T1	6 (15.4)	21 (53.8)	12 (30.8)	0.373
	T2-T3	9 (9.9)	43 (47.3)	39 (42.9)	
Histological grade	G1-G2	8 (19.0)	21 (50.0)	13 (31.0)	0.127
	G3	7 (8.0)	43 (48.9)	38 (43.2)	
Clinical stage	I-II	15 (15.0)	57 (57.0)	28 (28.0)	<0.001
	III-IV	-	7 (23.3)	23 (76.7)	
Lymph node status	Negative	14 (20.0)	37 (52.9)	19 (27.1)	<0.001
	Positive	1 (1.7)	27 (45.0)	32 (53.3)	
Positive lymph nodes	≤3	1 (3.4)	22 (75.9)	6 (20.7)	<0.001
	>3	-	5 (16.1)	26 (83.9)	

*Data are Expressed as Frequencies and Percentages

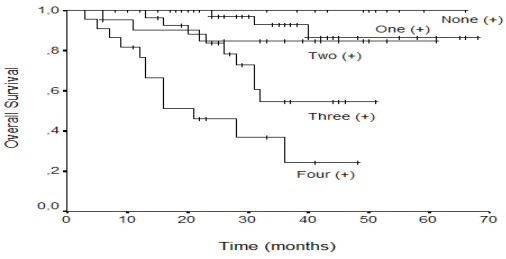

Figure 1. Overall Survival of Breast Cancer Patients According to the Number of the Following Markers: HER-2, IL-6, TNF-a and TGF-β1, with Positive (+) Expression

Table 4. Results of Multivariate Logistic Regression Analysis (Variable Selection Method: Backward Stepwise/ Likelihood Ratio) for the Association of Positive (+) Expression of HER-2, IL-6, TNF-a anf TGF-β1 with the Clinicopathological Characteristics of Breast Cancer Patients*

	HER-2 (+)	IL-6 (+)	TNF-a (+)	TGF-β1 (+)	3 or 4 (+)
Lymphovascular invasion	5.76 (1.41-23.63)	4.39 (1.41-13.70)	4.46 (1.39-14.33)	-	12.04 (1.51-95.70)
Poor (G3) histological grade	2.88 (1.10-7.55)	-	4.49 (1.85-10.91)	-	-
Positive lymph node status	3.98 (1.39-11.36)	-	-	-	-
Positive lymph nodes >3	-	5.68 (1.32-24.38)	5.10 (1.66-15.67)	3.03 (1.01-9.15)	11.94 (2.64-54.07)

*Data are Expressed as Adjusted odds Ratios (aOR) with their 95% Confidence Intervals (CI). Only Variables that Maintained Statistical Significance in Multivariate Analysis are Shown

Table 5. Survival Analysis of Breast Cancer Patients in Relation to HER-2, IL-6, TNF-a and TGF-β1 Expression

	HER-2 expression		IL-6 expression	
	Negative (-)	Positive (+)	Negative (-)	Positive (+)
1-year survival (%)	100.00	89.02±3.92	98.46±1.53	90.56±3.67
2-year survival (%)	94.25±3.22	73.53±5.71	93.43±3.19	74.04±5.85
3-year survival (%)	89.67±4.42	59.33±6.96	89.35±4.15	56.04±7.87
Survival time (years)				
Mean±SE	62±2	44±3	62±2	39±2
95%CI	58-67	38-50	58-66	34-44
Fatality (%)	6 (9.2%)	22 (33.8%)	7 (10.8%)	21 (32.3%)
Hazard ratio (95% CI)	4.40 (1.78-10.85)		4.35 (1.83-10.33)	
p value (Log Rank test)	<0.001		<0.001	
	TNF-a expression		TGF-β1 expression	
	Negative (-)	Positive (+)	Negative (-)	Positive (+)
1-year survival (%)	96.85±1.53	92.20±3.35	98.41±1.57	90.66±3.63
2-year survival (%)	93.09±3.35	74.94±5.65	96.40±2.52	71.46±5.90
3-year survival (%)	86.74±4.72	60.77±7.41	93.73±3.60	55.55±7.09
Survival time (years)				
Mean±SE	61±2	45±3	63±1	46±3
95%CI	57-66	39-51	60-66	39-52
Fatality (%)	8 (12.3%)	20 (30.8%)	3 (4.6%)	25 (38.5%)
Hazard ratio (95% CI)	3.32 (1.45-7.58)		9.96 (3.01-33.01)	
p value (Log Rank test)	0.002		<0.001	

	Expression pattern of HER-2, IL-6, TNF-a and TGF-β1				
	None (+)	One (+)	Two (+)	Three (+)	All (+)
1-year survival (%)	100.00	100.00	90.23±6.57	100.00	76.70±9.16
2-year survival (%)	100.00	97.06±2.90	84.59±8.23	83.64±7.53	46.02±11.15
3-year survival (%)	100.00	93.01±4.84	84.59±8.23	54.61±9.65	24.55±12.88
Survival time (years)					
Mean±SE	-	63±3	54±4	39±3	25±4
95%CI	-	58-68	46-61	34-45	18-33
Fatality (%)	-	3 (7.0%)	3 (14.3%)	9 (31.0%)	13 (59.1%)
Hazard ratio *	-	-	2.89	7.55	22.96
95% CI			0.58-14.31	2.04-27.97	6.46-81.57
p value (Log Rank test)					
vs None (+)	-	0.212	0.133	0.007	<0.001
vs One (+)	-	-	0.384	0.002	<0.001
vs Two (+)	-	-	-	0.097	0.002
vs Three (+)	-	-	-	-	0.006

*Reference category, patients with none (+) or one (+) marker

characteristics, ER, PR, and the expression of the four markers, revealed that the positive expression of TGF-β1 remained a statistically significant independent determinant for poor survival (adjusted Hazard ratio (aHR)=10.20, 95%CI=2.83-36.81, p<0.001); the independent effect of increased HER-2 (aHR=2.76, 95%CI=0.74-10.28, p=0.129), IL-6 (aHR=3.37, 95%CI=1.31-8.68, p=0.012) and TNF-a (aHR=1.55, 95%CI=0.43-5.59, p=0.501) was not significant. When the presence of the four markers was replaced by their coexpression (groups A+B, C, D and E), group E (aHR=40.02, 95%CI=6.89-232.50, p<0.001 compared to group A+B; aHR=8.93, 95%CI=2.24-35.51, p=0.002 com-pared to group C; aHR=4.35, 95%CI=1.63-11.60, p=0.003 compared to group D) and group D (aHR=13.00, 95%CI=2.29-73.80, p=0.004 compared to group A+B) remained independently associated with poor survival.

Discussion

HER-2 proto-oncogene is a member of the EGFR family and plays an important role in the regulation of cell growth, differentiation and survival and is involved in the regulation of normal breast growth and development (Yarden and Sliwkowski, 2001). Alterations of HER-2 have been associated with carcinogenesis and poor prognosis of breast cancer. Cytokines, which were initially discovered as secreted proteins that mediate and regulate immunity and inflammation, it is now clear that their functions extend to many other aspects of biology, including breast cancer (Thomson and Lotze, 2003; Lin and Karin, 2007). IL-6 and TNF-a are involved not only to initiation but in all stages of tumour development, including promotion, progression and metastasis (Aggarwal, 2009; Grivennikov and Karin, 2010; 2011; Grivennikov et al., 2010). TGF-β1 is involved early in mammary carcinogenesis and functions as a tumor suppressor, with cytostatic and apoptotic action (Siegel and Massagué, 2003); however, at later stages of mammary carcinogenesis, the levels of TGF-β1 increase with tumor progression and confer a poorer prognosis for human breast cancer patients (Auvinen et al., 1995; Sheen-Chen et al., 2001).

In the present study, the prognostic value of the expression pattern of these four serum markers was examined by correlating the number of markers with positive expression to the traditional surgical pathologic prognostic factors and the survival rate of breast cancer patients. We demonstrated that: (A) the positive expression of each marker was independently associated with increased likelihood of the clinicopathological characteristics indicative of tumor progression, with adjusted odds ratios ranging from almost 3 to 6; (B) the presence of at least three positive markers was also independently associated with the extension of the disease, with adjusted odds ratios of 11.94 for the presence of more than three positive lymph nodes and 12.04 for lymphovascular invasion; (C) there was a positive association between the expression of the HER-2, IL-6 and TNF-α, probably indicating that their role in the tumorigenic activity may share common molecular pathways; (D) in multivariate statistical analysis increased TGF-β1 was the only independent determinant for poor survival (aHR=10.20); (E) as the number of positive markers was increasing there was a gradually reduction of survival time and an elevation of the mortality rate and the risk of death; (F) patients with four positive markers had shorter survival even compared to patients with three positive markers (25 *vs* 39 months; aHR=4.35). Our results indicate that the combined expression of these four markers appears to be a useful independent prognostic marker for breast cancer outcome.

The present results, regarding to each of the markers separetaly, are in keeping with the preliminary results published by our group, using a smaller sample of breast cancer patients, where the quantitative expression of the serum levels of HER-2, IL-6, TNF-a and TGF-β1 was associated with the progression of breast cancer (Papadopoulou et al., 2008a; 2008b; 2010; Tripsianis et al., 2012). Since then, several other studies have also elaborated on the independent negative impact of these serum markers on the prognosis of the patients with breast cancer. Elevated serum levels of HER2 were associated with several factors related to tumor aggressiveness of breast cancer, such as tumor size, advanced stage, lymph

node involvement, poor histological differentiation and shorter disease free and/or overall survival (Azizun-Nisa et al., 2008; Samy et al., 2010; Kong et al., 2012; Ma et al. 2012; Wang et al., 2012). Sharif et al. (2010) showed a positive association of Her-2 immunohistochemical expression with tumour size and lymph node metastasis only in post-menopausal women, indicating an age-related association of Her-2 expression with the histological prognostic markers in breast carcer. Recently, Al-Hassan et al. (2012) also found elevated serum IL-6 and TNF-α levels in higher stages among newly diagnosed breast cancer patients, Al-suhail (2008) associated higher serum levels of these cytokines with higher stages and distant metastasis in Iraqi breast cancer patients, while Ravishankaran and Karunanithi (2011) associated higher serum levels of IL-6 with tumour invasion, the presence of distant metastasis and overall survival in Indian breast cancer patients. Among patients with operable breast cancer, Chod et al. (2008) found increased levels of TGF-ß1 in patients with a positive sentinel lymph node than in those with negative sentinel lymph nodes, while Dave et al. (2012), in 117 previously untreated Indian breast cancer patients, associated elevated levels of TGF-ß1 with advanced-stage and shortened overall survival. Although these studies suggest that elevated levels of the four markes may contribute to disease progression, a definite conclusion in this issue has not yet been reached (Duffy, 2006; Ali et al., 2012; Panis et al., 2013).

A first finding of our paper was that the determination of each one of the four markers could be useful for the prediction of the clinicopathological characteristics indicative of tumor progression and patients' overall survival. Moreover, among these four markers, TGF-β1 was the only independent predictor for poor overall survival. Several mechanisms have been suggested for the relation of TGF-β1 with breast cancer transformation and progression: *i*) produced TGF-β1 by tumor cells can enhance tumor growth by angiogenesis and evading immune surveillance (Ueki et al., 1992); *ii*) TGF-β1 can promote accumulation of extracellular matrix glycoproteins and cell adhesion molecules, which may enhance the metastatic potential of cancer (Massague et al., 1992); *iii*) secreted TGF-β1 may increase the cellular motility and the production of proteases, enhancing the invasive potential of fibrosarcoma (Samuel et al., 1992); *iv*) lack of TGF-β1-mediated growth inhibitory effect may be due to the absence of the TGF-β1 receptor type II, as a consequence of mutations (Derynck et al., 1987). Another major finding of this multiple marker investigation was that the presence of three or more positive markers enhanced the predictive value of the number of positive lymph nodes and lymphovascular invasion. Since the number of positive lymph nodes is a significant prognostic factors in breast cancer, any factor associated with this is likely to be associated with survival. Therefore, regardless other well-established prognostic factors, the combined analysis of the four markers gave additional prognostic information regarding patients' overall survival. Although the tendency towards reduced overall survival in patients with none, one or two positive markers did not reach the statistical significance, the presence of three positive markers significantly shortened the overall survival compared to all three previous groups of breast cancer patients. Finally, the simultaneous presence of four positive markers defined a high-risk subgroup of patients, which were independently associated with worse survival not only compared to patients with none, one or two positive markers, but also compared to patients with three positive markers, increasing the risk of death by almost 4.5 times. Our findings demonstrate that the simultaneous presence of a greater number of positive markers contributes on the progression and dissemination of breast cancer.

TNF-a and IL-6 seem to play an important role in tumor formation, invasion, and metastasis due to their ability to activate a variety of oncogenic transcription factors, such as Nuclear Factor-kappa B (NF-ϰB), protein-1 (AP-1) and Signal Transducer and Activator of Transcription (STATs 1, 3 and 5) (Bromberg et al., 1999; Moore et al., 1999; Balkwill, 2002; 2009). Cytokines not only are involved in the activation mechanisms through which they act, but they are considered to form a cytokine network, either autocrine or paracrine, acting in an amplifying cascade, to be involved in the system of invasion and metastasis through receptors expressed on cancer cells. Another major finding of the present study was the positive association between the expression of TNF-a and IL-6, which is consistent with reports demonstrating that these two cytokines are interrelated and may act in an additive manner that may affect tumor cell progression in a cooperative manner (Alvarez et al., 2002; Sharma and Anker, 2002; Ben-Baruch, 2003; Alsuhail, 2008; Kayacan et al., 2006). On the role of these two cytokines in tumor growth, overexpression of the HER-2 may play a critical role, since it also activates the above oncogenic transcription factors, without extracellular stimulation, through the major intracellular signaling cascades involved in signal transduction, including the Ras/MAPK pathway, the PI3K/Akt pathway, JAK/signal transducers and activators of transcription (STAT) pathway, which lead to cell proliferation, survival, motility and adhesion (Ross et al., 2004). The current study demonstrated a clear positive association of HER-2 with TNF-a and IL-6, which supports the suggestion that a functional interaction between their molecular pathways may promote the invasive behaviour and metastasis of breast cancer (Zhou, 2000; Badache and Hynes, 2001).

Several studies demonstrate that TGF-β1 and HER-2 cooperate at various levels; HER2 seems to provide proliferative advantage to tumor cells, increasing their survival ability during clonal selection, and TGF-β provides greater invasiveness and metastatic potential to these cells, leading to a more aggressive phenotype of breast cancer. The cooperation between TGF-β1 and HER-2 may occur through at several possible mechanisms: *i*) transcriptional modulation that targets the same downstream genes through TGF-β-induced transcription factors Smads; *ii*) activation of the Smad-independent signaling pathway; *iii*) inhibition of TGF-β-induced antiproliferative ef-fects through the up-regulation of the inhibitory Smad7; and *iv*) autocrine induction of TGF-β1

and ligands that activate RTKs. In our cohort, such a synergistic relation between elevated levels of TGF-β1 and HER-2 was not observed; this suggests a posible enhanced efficiency of conventional therapies in our breast cancer patients with HER-2 overexpression, considering the role of TGF-β1 in inducing clinical resistance to trastuzumab (Todorović-Raković, 2008; Wang, 2012).

In conclusion, the present study showed that the expression pattern of elevated HER-2, IL-6, TNF-a and TGF-β1 levels correlates significantly with classical clinicopathological parameters indicative of a more aggressive behaviour of this carcinoma and most importantly, it correlates with reduced survival rate of breast cancer patients, reinforcing the separate negative impact of each one of these four markers on cancer progression and survival. Our findings suggest that the increased number (≥3) of positive markers has a strong prognostic value for breast cancer outcome and merits to be an independent biomarker of clinical use.

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