Evaluation of predictive and prognostic significance of serum TGF-β1 levels in breast cancer according to HER-2 codon 655 polymorphism

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The present study was conducted to clarify the predictive and prognostic significance of serum TGF-β1 in breast cancer in relation to Ile655Val single nucleotide polymorphism (SNP) of human epidermal growth factor receptor-2 (HER-2).

In a case-control study, 56 consecutive patients with primary breast cancer were prospectively included and evaluated. The control group consisted of 45 healthy women. Serum concentrations of TGF-β1 were measured by quantitative sandwich enzyme immunoassay (ELISA). HER-2 SNP was genotyped using PCR-RFLP method.

Serum levels of TGF-β1 were significantly increased in breast cancer patients compared to healthy controls ($p<0.001$). For the evaluation of the diagnostic significance of serum TGF-β1 the area under the receiver operating characteristic (ROC) curve (AUC) was 0.804, while the optimal cut-off point of 30.86 ng/ml was determined to classify breast cancer patients, which yielded sensitivity of 77%, specificity of 78% and accuracy of 77%. Significantly elevated serum TGF-β1 levels were associated with advanced stages ($p=0.023$), positive lymph nodes ($p=0.019$) and postmenopausal status ($p=0.031$). A marginal trend towards higher TGF-β1 levels was found among patients with Val-containing genotypes compared to homozygous Ile-Ile ($p=0.094$). In multivariate analysis lymph node metastases ($p=0.009$) remained the only significant independent determinant of high TGF-β1 levels. With regard to prognostic significance for advanced stages (AUC, 0.704) and lymph node metastasis (AUC, 0.683), when the optimal cut-off value was set at 65.15 pg/ml, the sensitivity was 86% and 67%, the specificity was 60% and 62% and accuracy was 66% and 64%, respectively. Survival was shorter in patients with increased serum TGF-β1 (36 months vs 46 months, $p=0.022$). Multivariate analysis demonstrated a marginal prognostic significance of serum TGF-β1 for survival ($p=0.072$). The combination of high TGF-β1 and Val-Val genotype predicts a worse prognosis than high serum TGF-β1 alone.

Our findings suggest that serum TGF-β1 is involved in tumor malignancy and lymph node metastasis and could be used clinically as a useful tumor marker for evaluation, the extension and the outcome of the disease. They also provide clinical evidence for a significant association between HER-2 Ile655Val SNP and serum TGF-β1, resulting to more aggressive phenotype of the tumor and poor prognosis.

Keywords: serum TGF-β1, HER-2, polymorphism, breast cancer, survival

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. One of the most widely studied roles of TGF-β1 is its function as a tumor suppressor, by blocking cell cycle progression through the transcriptional upregulation of the cyclin-depended kinase inhibitors p15 and p21 [1]. However, TGF-β1 may have both inhibitory and stimulatory effects. Excess production and/or activation of TGF-β1 in tumors may facilitate cell motility and survival. Moreover, it has been shown to stimulate tumor invasion by promoting angiogen-

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esis, extracellular matrix production and through inhibition of host immune functions [2].

Elevated plasma levels of TGF-β1 have been reported in patients with prostate, gastric, colorectal and breast cancer as compared to healthy controls and is thought to be relevant to tumor transformation and progression [3–6]. Although the possibility of using TGF-β1 levels measured in serum as a tumor marker has been suggested, the available data about its role in breast cancer are conflicting.

The epidermal growth factor receptor-2, HER-2 (also known as c-erbB-2/neu), is an 185 kDa transmembrane glycoprotein receptor, with tyrosine kinase activity and is a member of the EGFR family [7]. It 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 [8]. However, HER-2 overexpression has been found in a variety of malignant diseases, including invasive breast carcinomas, in which amplification, protein overexpression, or both, of this receptor have been found in 20-35% of the tumors [9]. HER-2, is an established prognostic factor in breast cancer, which determines the trastuzumab therapy [10]. Muraoka et al. suggested that TGF-β1 can cooperate with HER-2, acting directly on epithelial cells overexpressing the HER-2 receptor to induce aggressive behavior [11].

The gene encoding HER-2 receptor, is a natural target for investigation regarding polymorphisms that might indicate resistance or susceptibility to breast cancer development. One common variant, a single nucleotide polymorphism (SNP) in the transmembrane coding region of the HER-2 gene at codon 655 (Ile655Val), encoding either isoleucine (Ile: ATC) or valine (Val: GTC), has been reported in different cancer types [12]. Changing the existing isoleucine (Ile: ATC) to valine (Val: GTC) at codon 655, suggests an increased dimerization, autophosphorylation of HER-2 and tyrosine kinase activity, which may cause the transformation of cells [13]. Xie et al. [14] first reported that this SNP has been associated with an increased risk of breast cancer development [odds ratio, 1.4]. However, several following studies have cast doubt on this association, which remains controversial [15–20]. One reason for these contradictory results might be the substantial differences in genetic polymorphism in the HER-2 codon 655 between ethnic groups [21]. In a recent study we have showed an allelic imbalance of Ile655Val SNP between Greek Christian and Greek Muslim populations of Thrace and its possible implication on the more aggressive phenotype in breast cancer patients [22]. The aim of our study was to investigate the diagnostic significance of serum levels of TGF-β1 for breast cancer and to examine their association with the clinicopathologic parameters of breast cancer patients and the HER-2 Ile655Val polymorphism. The clinical value of TGF-β1 in predicting advanced stages of the disease and lymph node involvement, as well as its prognostic relevance, were also evaluated.

Table 1. Characteristics of breast cancer patients and healthy controls

	Patients	Controls
Age		
≤ 45 years	11 (19.6)	12 (26.7)
46-65 years	20 (35.7)	21 (46.6)
>65 years	25 (44.6)	12 (26.7)
Religious/ethnic group		
Greek Christians	43 (76.8)	32 (71.1)
Greek Muslims	13 (23.2)	13 (28.9)
Menopausal status		
Premenopausal	16 (28.6)	16 (35.6)
Postmenopausal	40 (71.4)	29 (64.4)
Body mass index (BMI)		
≤ 25 Kg/m^2	21 (37.5)	22 (48.9)
>25 Kg/m^2	35 (62.5)	23 (51.1)
Histological type		
Lobular	13 (23.2)	
Ductal	43 (76.8)	
Lymphovascular invasion		
No	9 (16.1)	
Yes	47 (83.9)	
Tumor size		
T1	17 (30.4)	
T2	34 (60.7)	
T3	5 (8.9)	
Histological grade		
G1	9 (16.1)	
G2	11 (19.6)	
G3	36 (64.3)	
Clinical stage		
0-I	15 (26.8)	
II	27 (48.2)	
III-IV	14 (25.0)	
Lymph node status		
Negative	26 (46.4)	
Positive	30 (53.6)	
No of positive lymph nodes		
≤ 3 lymph nodes	14 (46.7)	
>3 lymph nodes	16 (53.3)	
Estrogen receptors		
Negative	19 (33.9)	
Positive	37 (66.1)	
Progesterone receptors		
Negative	31 (55.4)	
Positive	25 (44.6)	
HER-2 protein		
Negative	25 (44.6)	
Positive	31 (55.4)	

Materials and methods

Study population. From February 2003 to December 2005, 56 consecutive patients with primary breast carcinoma, admitted to the University General Hospital of Alexandroupolis were included in the present population-based, case-control study. Patients' age ranged from 33 to 80 years, with a median age of 64 years (mean age ± SD, 61.34 ± 11.64 years). Menopausal status was recorded and body mass index (BMI), expressed as weight/height^2 (kg/m^2), was used as a standard for the assessment of obesity. Overweight was defined as a BMI>25.0. Patients were also divided into two

different self-reported religious groups: Greek Christians (43 patients) and Greek Muslims (13 patients). Forty five healthy females with no evidence of neoplastic disease, who visited our hospital for routine health checkup, were recruited as controls. They were frequency matched to breast cancer patients based upon age (± 5 years) and religion/ethnicity. Controls' age ranged from 31 to 82 years, with a median age of 59 years (mean age ± SD, 57.20 ± 12.04 years). The demographic characteristics of breast cancer patients and controls are summarized in Table 1. There were no significant differences in age (p=0.176), religion/ethnicity (p=0.517), menopausal status (p=0.453) and BMI (p=0.250) between the patients and the controls.

The diagnosis of breast cancer was confirmed by histological examination, using specimens obtained from aspiration biopsy or surgical resection. Tumors were graded according to the criteria described by Bloom and Richardson [23] and tumor stage was assigned according to the TNM classification defined by the International Union Against Cancer [24]. The expressions of estrogen receptor (ER), progesterone receptor (PR) and HER-2 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.

Genotyping the HER-2 codon 655. Approximately 10ml of peripheral blood samples were collected with a standard venipuncture technique in ethylenediaminetetraacetic acid (EDTA) – containing tubes. Genomic DNA was isolated from whole blood using Puregene-DNA Purification kit of Gentra System (Inc., Minneapolis, MN). The genotype of the HER-2 gene was determined by using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) – based assay. The primers, based on the protocol used by Xie et al. [14] were as follows: Forward: 5'-AGA GCG CCA GCC CTC TGA CGT CCA T- 3'; Reverse: 5'-TCC GTT TCC TGC AGC AGT CTC CGC A- 3'. DNA was amplified in a MJ Research PTC-100 Thermal Cycler. The PCR reaction was carried out in 50 μl of reaction mixture, containing 500 ng of genomic DNA, 300 nM of each primer, 350 μM of each dNTP, PCR buffer which contained 1.75 mM $MgCl_2$ and 2.5 U of Taq Polymerase (Roche). The reaction mixtures were heated to 94 °C for 30 sec followed by 35 cycles at 94 °C for 30 sec, 62 °C for 30 sec and 72 °C for 30 sec. A final extension step was included at 72 °C for 7 min. The PCR products (148 bp) were digested with 5 U BsmAI (Invitrogen, USA) at 37 °C, overnight, and separated by agarose gel electrophoresis (agarose concentration 3%).

Measurement of serum 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 -70 °C until biochemical assessment. Quantitative sandwich enzyme immunoassay (ELISA) was performed for measuring concentrations of serum 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 13.0 (SPSS, Inc., Chicago, IL, USA). The normality of quantitative variables was tested with Kolmogorov-Smirnov test. Serum TGF-β1 levels were expressed as the median and interquartile range. Categorical variables were expressed as frequencies (and percentages). The chi-square test was used to evaluate any potential association between categorical variables. Median test was used to assess differences of TGF-β1 levels between two or more groups of patients. A multivariate stepwise linear regression analysis was constructed to explore the independent effect of patient's and disease characteristics on serum TGF-β1 levels. The logarithmic transformation of TGF-β1 levels was used. For the evaluation of the diagnostic significance of TGF-β1 levels, the area under the receiver operating characteristic (ROC) curve (AUC) was calculated. Sensitivity, specificity, positive and negative predictive values were also calculated, while Cohen's kappa was used to assess agreement. 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, using a backward selection approach, were performed to explore the independent effect of TGF-β1 levels and its combination with HER-2 SNP on overall survival. Patients' age, tumor size, lymphovascular invasion, histological type, clinical stage, histological grade, lymph node status, ER and PR status, and HER-2 SNP were included as potential confounders in the multivariate model. All tests were two tailed and statistical significance was considered for p values <0.05.

Results

Histological type, lymphovascular invasion, tumor size, histological grade, clinical stage, lymph node status, immunohistochemical expression of estrogen receptor (ER), progesterone receptor (PR) and HER-2 proteins were recorded for each BC patient (Table 1). Regarding to histology, 43 (76.8%) were ductal and 13 (23.2%) lobular carcinomas. More than 80% of cases were invasive carcinomas and the 60.7% had size between 2 and 5 cm (T2). Nine (16.1%) were well-differentiated (G1), 11 (19.6%) moderately differentiated (G2) and 36 (64.3%) poorly differentiated carcinomas. Twenty seven cases (48.2%) were of stage II, while in 30 patients (53.6%) lymph node metastases were detected. ER, PR and HER-2 positivity was detected in 66.1%, 44.6% and 55.4% of the patients, respectively.

Serum levels of TGF-β1 among patients with primary breast cancer were significantly higher as compared to the control group (66.68 ng/ml, IQR = 31.39 to 97.30 ng/ml vs 18.60 ng/ml, IQR = 10.19 to 30.20 ng/ml, p<0.001; Figure 1). For the evaluation of the diagnostic significance of TGF-β1 for BC, the area under the ROC curve was 0.804 (95%CI = 0.715 to 0.893, p<0.001). The optimal cut-off point of 30.86 ng/ml was determined to classify BC patients, which yielded

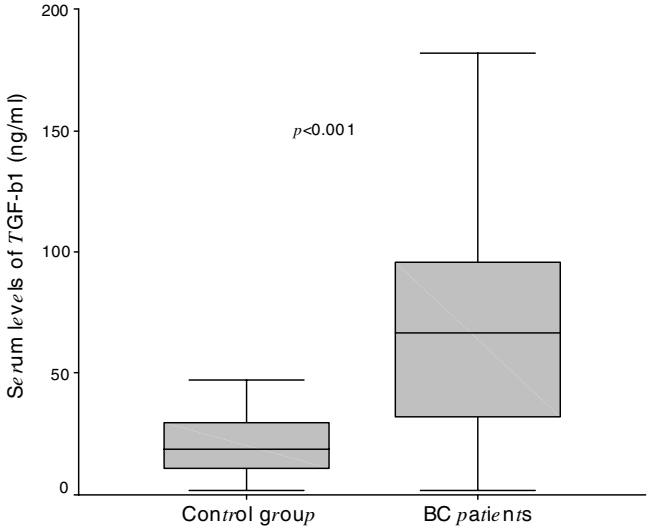

Figure 1. Serum level of TGF-β1 of 56 breast cancer (BC) patients and 45 healthy controls

high sensitivity of 77% (95% CI = 63% to 87%), specificity of 78% (95% CI = 63% to 88%), positive predictive value of 81% and negative predictive value of 73%. The overall accuracy of women's classification according to this cut-off point was 77.3% (78 of 101 cases), while Cohen's kappa indicated a substantial agreement (k=0.542, p<0.001).

The association of serum levels of TGF-β1 with the clinicopathological parameters are presented in Table 2. Statistically significantly elevated levels of TGF-β1 were found in advanced stage carcinomas (p=0.023), among patients with positive lymph nodes (p=0.019) and among postmenopausal women (p=0.031). A marginal trend towards higher values of TGF-β1 was found among patients older than 45 years compared to younger patients (p=0.093). Regarding to tumor size and histological type, increased levels of TGF-β1 were found in larger tumors (T2 or T3) and in poorly differentiated tumors (G3), but none of these differences were statistically significant (p=0.115 and p=0.161, respectively). No significant association was found between the serum levels of TGF-β1 and patient's BMI (p=0.767) and ethnicity (p=0.764), histological type of the tumor (p=0.793), lymphovascular invasion (p=0.798), the number of positive lymph nodes (0.846) and the immunohistochemical expression of ER (p=0.311), PR (p=0.902) and HER-2 (p=0.760) proteins.

To evaluate the association between serum levels of TGF-β1 and HER-2 SNP, we analyzed the Ile655Val genotypes in all breast cancer patients. The Ile-Ile, Ile-Val and Val-Val genotypes were found in 15 (26.8%), 22 (39.3%) and 19 (33.9%) patients, respectively. This genotype distribution was in Hardy-Weinberg equilibrium (p=0.273). The frequency of the Val allele among the breast cancer patients was 53.6%. We have recently shown that the Val allele confers a modest increase in breast cancer risk (OR, 2.6; 95% CI, 0.9-7.6; p=0.076, for Val-Val and OR, 2.2; 95% CI, 0.9-5.2; p=0.079 for Ile-Val or Val-Val), while, among breast cancer patients, invasive carcinomas, low differentiation tumors, advanced stages, positive lymph nodes, high number of lymph nodes and immunohistochemical HER-2 overexpression were more frequent in patients with allele Val than those with allele Ile [22]. Serum

Table 2. Serum levels of Transforming Growth Factor (TGF) – η1 in patients with breast cancer according to clinicopathological parameters

	Levels of TGF-η1 (ng/ml)		
	Median	Interquartile range	p value
Age			0.093
Ω45 years	44.64	24.64-74.70	
> 45 years	71.88	31.86-100.36	
Menopausal status			0.031
Premenopausal	41.88	15.28-70.20	
Postmenopausal	75.44	34.83-109.04	
Body mass index (BMI)			0.767
Ω25 Kg/m^2	52.04	18.40-100.36	
>25 Kg/m^2	68.16	33.12-91.88	
Religious/ethnic group			0.764
Greek Christians	68.16	32.80-92.20	
Greek Muslims	51.44	26.14-127.31	
Histological type			0.793
Lobular	71.88	51.74-89.14	
Ductal	64.68	24.64-111.72	
Lymphovascular invasion			0.798
No	65.10	10.16-100.00	
Yes	68.16	32.80-92.20	
Tumor size			0.115
T1	51.44	12.10-78.56	
T2 - T3	74.70	33.12-101.00	
Histological grade			0.161
I-II	48.10	12.07-97.76	
III	71.43	39.57-97.30	
Clinical stage			0.023
I-II	54.37	17.36-91.96	
III-IV	78.56	61.76-174.94	
Lymph node status			0.019
Negative	52.04	12.10-82.30	
Positive	78.11	40.92-156.00	
No of positive lymph nodes			0.846
Ω3 lymph nodes	81.72	36.12-161.91	
>3 lymph nodes	76.56	48.81-124.44	
Estrogen receptors			0.311
Negative	74.70	24.64-156.00	
Positive	64.68	31.86-85.76	
Progesterone receptors			0.902
Negative	71.88	18.36-99.00	
Positive	56.70	32.96-93.06	
HER-2 protein			0.760
Negative	65.10	32.96-95.60	
Positive	68.16	21.36-111.72	
Genotype			0.183
Ile-Ile	51.44	8.16-86.40	
Ile-Val	70.02	40.47-84.26	
Val-Val	75.00	32.80-156.54	
Ile-Val or Val-Val	71.88	37.88-99.36	0.094

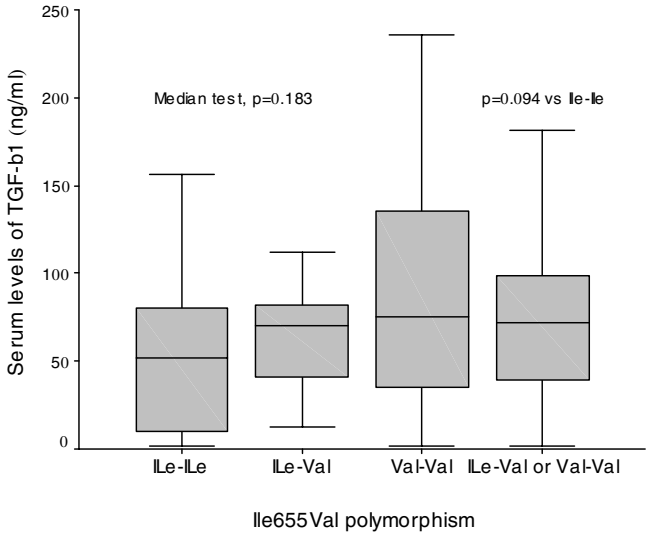

Figure 2. Serum level of TGF-β1 of breast cancer patients according to Ile655Val HER-2 polymorphism

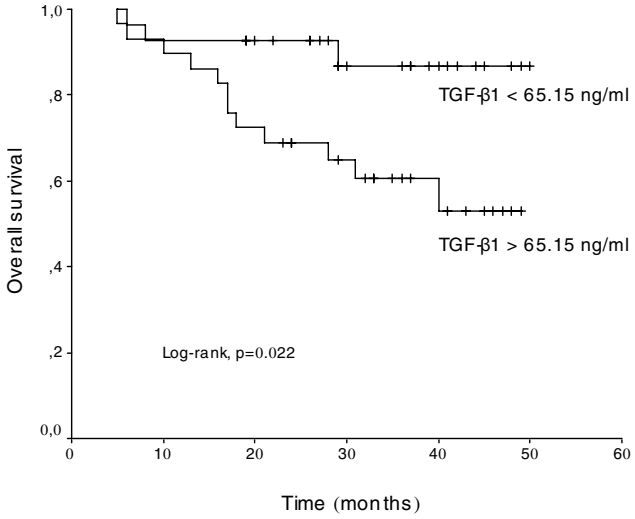

Figure 3. Overall survival of breast cancer patients according to serum levels of TGF-β1 (L65.15 ng/ml vs >65.15 ng/ml)

levels of TGF-β1 were stepwise increased as the number of the Val allele in the genotype was increased, but this apparent trend did not reach the statistical significance (p=0.183). On the contrary, a marginal trend towards higher values of TGF-β1 was found among patients with Val-containing genotypes compared to homozygous Ile-Ile (p=0.094) (Figure 2).

Multivariate stepwise linear regression analysis revealed that the presence of lymph node metastases (p=0.009) remained the only significant independent determinant of high levels of TGF-β1 in breast cancer patients.

For the evaluation of the prognostic significance of TGF-β1 for advanced breast cancer (stages III or IV) and lymph node metastasis, the areas under the ROC curves were 0.704 (95%CI = 0.557 to 0.852, p=0.023) and 0.683 (95%CI = 0.543 to 0.823, p=0.019), respectively. The optimal cut-off point of 65.15 ng/ml was determined to predict more advanced stages and positive lymph nodes, which indicated sensitivities of 86% (95% CI = 56% to 97%) and 67% (95% CI = 46% to 83%), specificities of 60% (95% CI = 43% to 74%) and 62% (95% CI = 42% to 79%), positive predictive values of 41% and 62%, negative predictive values of 93% and 67% and accuracy of 66% (37 of 56 patients) and 64% (36 of 56 patients), respectively.

Therefore, the serum TGF-β1 level of 65.15 ng/ml was selected as the cut-off point to subdivide breast cancer patients into two groups, in order to assess preliminary results about the TGF-β1-dependent overall survival. Follow-up was available for all patients. Median follow-up time was 30 months (range, 5 to 50 months). Fifteen patients (26.8%) died during follow-up. Mean survival time was 46 ± 2 months (95% CI = 41 to 50 months) in patients with low levels of TGF-β1 (L65.15 ng/ml; n=27) and 36 ± 3 months (95% CI = 30 to 42 months) in patients with high levels of TGF-β1 (>65.15 ng/ml; n=29). The log-rank test revealed a statistically significant difference between survival rates over mean survival time (p=0.022), with patients with high levels of TGF-β1 having worse prognosis (Figure 3). Mortality rate was significantly greater in patients with high levels of TGF-β1 compared to patients with low levels of TGF-β1 (41.4% vs 11.1%, p=0.011), where patients with high levels of TGF-β1 were almost 4 times more likely to die of cancer than those with low levels of TGF-β1 (Hazard ratio = 3.9, 95% CI = 1.1 to 13.9, p=0.034). Regarding to the prognostic significance of HER-2 SNP on overall survival, in a recent paper, we have shown that Val-Val genotype was independently associated with poor prognosis (Hazard ratio = 9.2, 95% CI = 1.1 to 80.1, p=0.045) [25]. A combined analysis of TGF-β1 levels and HER-2 SNP revealed that the subgroup of patients with simultaneous presence of the Val-Val genotype and high levels of TGF-β1 had significantly reduced survival not only compared to the most favourable group (low TGF-β1 levels and Ile-Ile or Ile-Val genotypes; p<0.001), but also compared to patients with high levels of TGF-β1 only (p=0.047) (Figure 4). In particular, mean survival time was 48 ± 2 months (95% CI = 44 to 52 months) in patients with low levels of TGF-β1, 40 ± 4 months (95% CI = 33 to 47 months) in patients with high levels of TGF-β1 only, 31 ± 4 months (95% CI = 24 to 39 months) in Val-Val patients and 29 ± 5 months (95% CI = 20 to 38 months) in homozygous Val-Val patients with high levels of TGF-β1. There were no other statistically significant differences in survival rates between these groups of patients.

Further investigation with multivariate Cox proportional hazards regression analysis revealed that independent impact of serum levels of TGF-β1 on overall survival was of border-

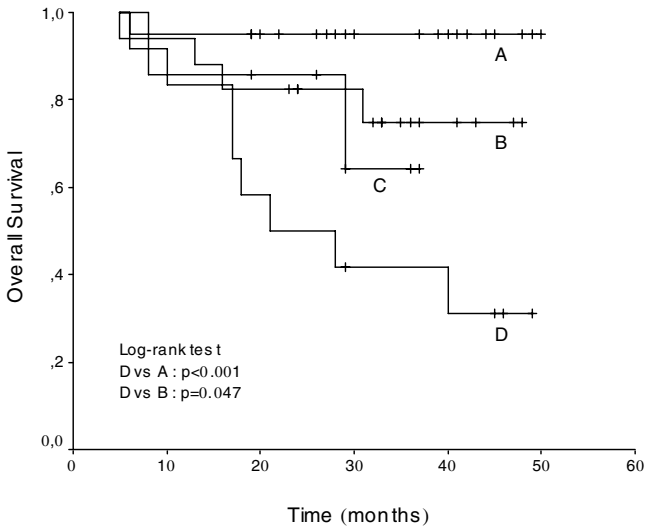

Figure 4. Overall survival of breast cancer patients according to the combined presence of serum levels of TGF-β1 (L65.15 ng/ml vs >65.15 ng/ml) and Ile655Val HER-2 polymorphism : A, low levels of TGF-β1 and Ile-Ile or Val-Val genotype; B, high levels of TGF-β1 only; C, Val-Val genotype only; D, high levels of TGF-β1 and Val-Val genotype. Only statistically significant differences are shown.

line statistical significance (Hazard ratio = 3.5, 95% CI = 0.9 to 13.5, p=0.072). When the combination of TGF-β1 and HER-2 SNP was entered in the regression analysis, the simultaneous presence of the Val-Val genotype with high levels of TGF-β1 remained an independent determinant for poor survival (Hazard ratio = 12.8, 95% CI = 1.4 to 89.2, p=0.025 compared to most favorable subgroup; Hazard ratio = 3.4, 95% CI = 1.1 to 13.7, p=0.041 compared to patients with high levels of TGF-β1 alone).

Discussion

Transforming growth factor TGF-β1 is involved, in normal mammary biology as a potent inhibitor of mammary epithelial proliferation and regulator of mammary ductal and alveolar development. The TGF-β1 signaling pathway is involved early in mammary carcinogenesis and functions as a tumor suppressor, with cytostatic and apoptotic action [26]. Resistance to the growth-inhibitory effects of TGF-β1 is common in human breast cancer and elevated levels of TGF-β1 are associated with decreased incidence of mammary cancer in mouse models and decreased breast cancer incidence in humans [27]. However, at later stages of mammary carcinogenesis, levels of TGF-β1 increase with tumor progression and confer a poorer prognosis for human breast cancer patients [6, 28]. It seems that this cytokine has a diverse role in growth control, depending on the conditions, acting either as an inhibitor of cellular proliferation or as a mitogene [27].

HER-2 receptor has become an important target in oncology research and it is considered a prognostic and predictive factor in breast cancer. A single nucleotide polymorphism at codon 655 (Ile655Val), in the transmembrane domain-coding region of HER-2 gene, could be associated with the development of breast cancer and may serve as an indicator of genetic susceptibility to this disease. We conducted this study to clarify the possible relationship between serum levels of TGF-β1 and breast cancer development and to examine their association with clinicopathological characteristics and overall survival; also, to investigate, for the first time, the effect of HER-2 Ile655Val SNP on serum TGF-β1 of breast cancer patients and to analyze the prognostic impact of the combination of serum TGF-β1 with this SNP.

In the study herein, we demonstrated that serum TGF-β1 levels were significantly higher in breast cancer patients compared to healthy women. These findings are consistent with previous studies which showed elevated TGF-β1 levels not only in breast cancer patients [6, 29], but also in other human cancers, such as stomach, liver, colorectal, lung, bladder, kidney, prostate and pancreas [5, 30–36]. However, since TGF-β1 is produced by a variety of cells and distributed widely throughout the body, the origin of increased TGF-β1 production is unclear; whether it is the result of increased production from tumor cells or increased secretion from other cells is unknown. With respect to production of TGF-β1 by tumor cells, increased secretion of TGF-β1 mRNA and TGF-β1 protein have been reported in numerous cell lines [37]. Overexpression of TGF-β1 mRNA has also been reported in tumor cells from breast cancer, stomach, colon and hepatocellular carcinoma [38–40], while a correlation between mRNA in tumor tissue and plasma TGF-β1 has also been found [41]. Furthermore, tumors have an ability to activate latent TGF-β1 derived from other sources, such as surrounding tissues [42]. Therefore the increased serum levels of TGF-β1 might correlate with the presence of breast cancer.

Furthermore, the current study demonstrates a very high diagnostic significance of serum TGF-β1 levels for breast cancer (AUC, 0.804). The optimal cut-off value of 30.86 ng/ml for predicting breast cancer yielded high sensitivity of 77%, specificity of 78% and accuracy of 77%. These results are in keeping to those reported for other tumor markers such as CEA, CA15-3 and EGFR, and indicate that serum TGF-β1 levels merit to be an independent biomarker for predicting breast cancer.

Although there is increasing evidence that TGF-β1 secretion by tumor cells and/or stromal cells within the peritumoral microenvironment can contribute to tumor maintenance and progression, the reports demonstrating the role of TGF-β1 expression in breast cancer are conflicting [6, 28, 43–45]. Some authors have claimed that the immuno-histochemical staining intensity for TGF-β1 is in close relationship with the rate of disease progression. Sheen-Chen [6] have shown that high serum concentrations of TGF-β1 measured by quantitative sandwich enzyme assay are indicative of an aggressive tumor behaviour. On the contrary, Auvinen et al. found that evlevated values of expression of TGF-β1are related to favorable prog-

nostic factors [28]. Also, Murray [45] showed that patients with high levels of TGF-β1 mRNA expression have a longer disease-free interval. The diversity of these results may in part be due to the detection methods applied, or to the different antibodies used [6]. Some times, TGF-β1 proteins and TGF-β1 mRNA are found in the same cells, however, differences exist in the location of cells where TGF-β1 proteins or TGF-β1 mRNA expressions have been observed [46]. Although semiquantitative evaluation is sufficient enough to differentiate negative from positive reactions, it may not be accurate enough to evaluate the intermediate staining patterns. The use of quantitative analysis in our study could possibly avoid the above-mentioned disadvantages of a semiquantitative analysis of immunohistochemical staining.

The current study indicates that serum levels of TGF-β1 are associated with postmenopausal status, advanced clinical stages and lymph node metastasis. Multivariate analysis showed that lymph node involvement remained a strong independent prognostic factor for higher TGF-β1 levels. Our findings are more consistent with the reports of Gorsch et al. [44], Walker and Dearing [43] and Sheen-Chen et al. [6] demonstrating that TGF-β1 contributes not only on the development, but also on the progression and dissemination of breast cancer. Several other studies have reported that a significant augmentation of TGF-β1 production in tumor tissue and in the circulation was associated with clinicopathological features of aggressive disease such as lymphovascular invasion, higher grade, advanced stage and metastasis in patients with bladder, prostate and colorectal cancer [33, 41, 47]. In the present study, no significant correlation between serum TGF-β1 levels and tumor size was detected, which confirm the hypothesis that in early stages of cancer TGF-β1 protects, in a way, and acts as a tumor suppressor [27]. Similarly, no correlation could be established between TGF-β1 levels and steroid hormone receptors (ER and PR). These results are in keeping with previous studies on TGF-β1 mRNA and also in vitro studies in which TGF-β1 expression was irrelevant from tumor progression, while other authors suggest that TGF-β1 may be hormonally regulated and may function as a mediator to antioestrogen therapy [28, 45].

Another major finding of the presented study was that serum TGF-β1 levels were of predictive value for of more advanced stages (AUC, 0.704) and lymph node metastasis (AUC, 0.683) in breast cancer patients. The optimal probability value of 65.15 ng/ml may be more suitable for predicting advanced stages and lymph node metastasis in this cohort with sensitivities of 86% and 67%, and specificities of 60% and 62% respectively; accuracy was 66% and 64%, respectively. These characteristics are not inferior to those reported for other tumor markers, such as CEA and CA15-3. Therefore, these results indicate that determination of TGF-β1 appears to be a useful predictive marker of advanced stages and lymph node metastasis.

Transforming growth factor-β1 has been reported to be overexpressed in several tumors, including breast cancer and is thought to be related to tumor transformation and progression, for which some mechanisms have been suggested: 1) produced TGF-β1 by tumor cells can enhance tumor growth by angiogenesis and evading immune surveillance [48]. 2) TGF-β1 can promote accumulation of extracellular matrix glycoproteins and cell adhesion molecules, which may enhance the metastatic potential of cancer [49], 3) secreted TGF-β1 may increase the cellular motility and the production of proteases, enhancing the invasive potential of fibrosarcoma [50] and 4) 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 [51].

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 [8]. Alterations of HER-2 proto-oncogene have been associated with carcinogenesis and poor prognosis of breast cancer. A single nucleotide polymorphism at codon 655 resulting in a G to A transition (Ile655Val) in the transmembrane domain-coding region of this gene has been associated with an increased risk of breast cancer [12]. However, several studies have shown that this association is controversial [14–20]. An explanation for these contradictory results might be the considerable differences in the HER-2 codon 655 genetic polymorphism between ethnic groups [21]. In a recent paper, we revealed the allelic imbalance of Ile655Val polymorphism of HER-2 between Greek Christian and Greek Muslim populations of Thrace and we showed that the Val allele could be associated with development of breast cancer and may serve as an indicator of genetic susceptibility to this disease. Moreover, we demonstrated the association of the HER-2 genotype with more advanced clinicopathologic characteristics and immunohistochemical HER-2 overexpression and high serum HER-2 levels as well, which may indicate its possible implication on the more aggressive phenotype. The current study indicates that serum TGF-β1 levels tend to increase with the presence of Val allele. Our findings may support the suggestion that a functional interaction between TGF-β1 and this SNP of HER-2 gene may contribute to the progression of breast cancer.

Results of Seton-Rogers et al. [52] support the role of TGF-β1 in the progression of breast cancers with activated ErbB2 and suggested that activation of the Erk and EGFR pathways are the key in mediating these events. HER-2 and the ras/MAPK pathway have been previously reported to synergize with TGF-β1 signals to promote invasive behaviour and metastasis [53]. The cooperation between TGF-β1 and HER-2 tyrosine kinase (RTK), may occur through at least four possible mechanisms: 1) transcriptional modulation that targets the same downstream genes through TGF-β-induced transcription factors Smads, 2) activation of the Smad-independent signaling pathway, 3) inhibition of TGF-β-induced antiproliferative effects through the up-regulation of the inhibitory Smad7 and 4) autocrine induction of TGF-β1 and ligands that activate RTKs. Upon ligand

binding, activated TGF-β receptors phosphorylate Smad2/3, which assemble with Smad4, followed by the translocation of Smad2/3/4 complexes into the nucleus. These complexes interact with other transcription factors to selectively bind to and to transactivate TGF-β1 target gene promoters [53].

Many studies have reported a relation between survival and the TGF-β1 expression. However, these results should be interpreted with caution because of the different methods used. Many studies have found a relationship between high levels of TGF-β1 and increased disease free survival or overall survival [44, 45, 54], while other studies have shown that overexpression of TGF-β1 is significantly associated with poor prognosis or has no prognostic significance in several types of carcinomas [3, 28, 55–57]. In our study, although the duration of follow-up was short (median, 30 months), preliminary results have shown that high serum TGF-β1 levels were significantly associated with reduced overall survival. In multivariate Cox regression analysis, TGF-β1 was a borderline independent predictor of poor prognosis, even after including other well-established prognostic factors. The combined analysis of TGF-β1 levels and Ile655Val HER-2 polymorphism gave additional prognostic information regarding survival compared to serum TGF-β1 alone. The simultaneous presence of high TGF-β1 levels with the Val-Val genotype defined a high-risk subgroup of patients, which were independently associated with worse survival compared to patients with high serum TGF-β1 levels alone.

Our findings suggest that serum TGF-β1 is involved in tumor malignancy and lymph node metastasis and could be used clinically as a useful tumor marker for grade diagnosis of the extension and the outcome of the disease. They also provide clinical evidence for a significant association between HER-2 Ile655Val SNP and serum TGF-β1, which confirms the hypothesis that the pathways of TGF-β1 and HER-2 proteins, may interact and collaborate to signal transduction, resulting to more aggressive phenotype of the tumor and poor prognosis. Longer follow-up time is required to confirm the results on survival association.

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