The influence of serum HER-2 levels and HER-2 codon 655 polymorphism on breast cancer outcome

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HER-2 (human epidermal growth factor receptor-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. Alterations of HER-2 have been associated with carcinogenesis and poor prognosis of breast cancer. The present case-control study was conducted to clarify the predictive and prognostic significance of serum HER-2 protein in breast cancer patients in relation to Ile655Val single nucleotide polymorphism (SNP) of this gene. Fifty-six consecutive patients with primary breast cancer and 45 healthy women were prospectively included and evaluated. Serum levels of HER-2 were significantly increased in breast cancer patients compared to healthy controls (p=0.035). The optimal cut-off point of 1.98 ng/ml, which was determined to classify breast cancer patients, yielded sensitivity of 54%, specificity of 73% and accuracy of 62%. Significantly elevated serum HER-2 levels were associated with lymphovascular invasion (p=0.022), poor differentiation (p=0.011), advanced clinical stages (p=0.001), lymph node metastasis (p=0.011), higher number of positive lymph nodes (p=0.007) and the immunohistochemical overexpression of HER-2 protein (p=0.016). Regarding to HER-2 Ile655Val SNP, Ile-Val and Val-Val genotypes exhibited highly significant serum HER-2 elevation compared to homozygous Ile-Ile (both p<0.001). In multivariate analysis advanced stages (p=0.003) and Val-containing genotypes (p=0.009) remained the two significant independent determinants of high HER-2 levels. Survival analysis demonstrated an independent prognostic significance of homozygous Val-Val genotype for reduced survival (p=0.045), but not of serum HER-2 (p=0.181). Our findings confirm that serum HER-2 could be used clinically as a useful tumor marker for the diagnosis and the progression of breast cancer. Furthermore, they provide clinical evidence that HER-2 Ile655Val SNP does affect serum HER-2 levels and it can be regarded as a predictive biomarker for breast cancer patients with poor prognosis.

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

The transformation of normal epithelial cells into a cancer is a multi-step process associated with the progressive accumulation of abnormalities in DNA repair genes, tumor suppressor genes, oncogenes, cellular growth factors, surface receptors and cellular adhesion molecules [1]. Structural and functional alterations of human epidermal growth factor receptor-2 (HER-2), also known as c-erbB-2 or neu proto-oncogene, have been reported in different steps of carcinogenesis including initiation, promotion and progression [2]. HER-2 is a member of the epidermal growth factor (EGF) receptor family, mapped on chromosome 17q21-q22 that encodes a 185-kDa transmembrane glycoprotein growth factor receptor (p185-HER-2/neu) that contains an extracellular domain and has intracellular tyrosine kinase activity [3-5]. Binding of extracellular growth factor ligands to HER-2 leads to the formation of dimers with other molecules of HER-family receptors. This dimerization results in phosphorylation of tyrosine residues on the cytoplasmic domain of the receptor and subsequent activation of intracellular signaling pathways

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that regulate diverse biologic responses, including proliferation, differentiation, cell motility and survival [6].

Overexpression of HER-2 has been reported in a wide range of cancer types, including breast, ovarian, lung, salivary gland, colon, prostate and pancreatic [7, 8]. HER-2 DNA amplification is suggested to be the principal mechanism of HER-2 protein activation and is nearly always accompanied by its protein overexpression in breast cancer. HER-2 gene amplification and/or protein overexpression occur in 10-40% of all breast cancer cases [9-11] and is associated with rapid tumor growth, increased risk of recurrence after surgery, poor response to conventional chemotherapy and shortened survival [12]. The use of trastuzumab, a function blocking monoclonal antibody against the extracellular domain of HER-2, as a treatment option in HER-2-overexpressing advanced breast cancer patients underlines once again the importance of this protein and its downstream signaling cascade(s) in the pathogenesis of the above-mentioned cancers.

There are at least seven general categories of methods to evaluate HER-2. These methods measure either gene amplification, overexpression of RNA, or overexpression of the protein product. Although each method provides an indication of HER-2 status, substantial variability among different methods and different laboratories have been reported [13, 14]. In addition to the full-length transmembrane HER-2 protein, the truncated product of the receptor that is released into the circulation can be detected in sera of patients with breast cancer [15]. Serum HER-2 has been associated with tumor progression or tumor recurrence of the breast [16], but controversy continues in terms of its correlation with breast cancer clinicopathological characteristics (17). Moreover, its prognostic value in breast cancer has not been confirmed, because most studies were analyzed retrospectively and the sample size was small.

One common variant, a single nucleotide polymorphism (SNP) in the transmembrane coding region of the HER-2 gene at codon 655 (Ile655Val) was identified by Papewallis et al. [18], encoding either isoleucine (Ile: ATC) or valine (Val: GTC), has been reported in different cancer types. 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 [19]. Xie et al. [20] 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 [21-24]. One reason for these contradictory results might be the substantial differences in genetic polymorphism in the HER-2 codon 655 between ethnic groups [25]. We previously 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 [26].

The aim of the present study was to clarify the possible relationship of serum levels of HER-2 with breast cancer development, to examine their association with clinicopathological characteristics and overall survival, and to evaluate the utility of serum HER-2 as a predictive and prognostic tumor marker in breast cancer patients. The possible relation of HER-2 Ile655Val polymorphism with the serum HER-2 levels was also investigated and the prognostic potential of this SNP was assessed.

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 biopsy or surgical resection. Tumors were graded according to the criteria described by Bloom and Richardson [27], and tumor stage was assigned according to the TNM classification defined by the International Union Against Cancer [28]. 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. [20] 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 contains 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 HER-2. 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-ß1, 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 HER-2 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 HER-2 levels between two or more groups of patients. Post hoc analyses was performed using Bonferroni's correction. A multivariate stepwise linear regression analysis was constructed to explore the independent effect of patient's and disease characteristics on serum HER-2 levels. The logarithmic transformation of HER-2 levels was used. For the evaluation of the diagnostic significance of HER-2 levels, the area under the receiver operating characteristic (ROC) curve (AUC) was calculated. Sensitivity, specificity, positive and negative predictive values were also calculated. 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 HER-2 levels and HER-2 SNP on overall survival. Patients' age, tumor size, lymphovascular invasion, histological type, clinical stage, histological grade, lymph node status, and ER and PR status 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 breast cancer 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%) were moderately differentiated (G2) and 36 (64.3%) were poorly differentiated carcinomas. Twenty seven cases (48.2%) were of stage II, while in 30 patients (53.6%) lymph node metastases were

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)	

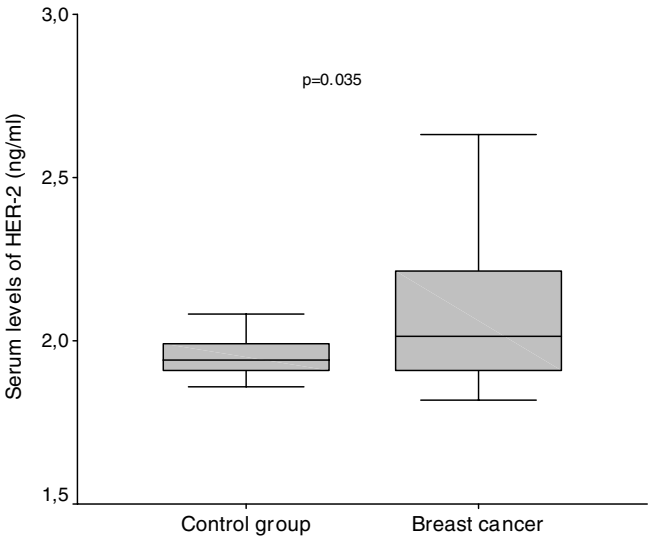

Figure 1. Serum level of HER-2 of 56 breast cancer patients and 45 healthy controls

detected. ER, PR and HER-2 positivity was detected in 66.1%, 44.6% and 55.4% of the patients, respectively.

Serum levels of HER-2 among patients with primary breast cancer were significantly higher as compared to control group (2.02 ng/ml, IQR = 1.91 to 2.22 ng/ml vs 1.94 ng/ml, IQR = 1.91 to 1.99 ng/ml, p=0.035; Fig. 1). For the evaluation of the diagnostic significance of HER-2 for breast cancer, the area under the ROC curve was 0.622 (95%CI = 0.513 to 0.732, p=0.035; Figure 1). The optimal cut-off point of 1.98 ng/ml was determined to classify breast cancer patients, which yielded high sensitivity of 54% (95% CI = 40% to 67%), specificity of 73% (95% CI = 58% to 85%), positive predictive value of 71% and negative predictive value of 56%. The overall accuracy of women's classification according to this cut-off point was 62% (63 of 101 cases).

The association of serum levels of HER-2 with the clinicopathological parameters are presented in Table 2. Statistically significantly elevated levels of HER-2 were found in invasive (p=0.022) and poorly differentiated (p=0.011) tumors, in advanced stage carcinomas (p=0.001), among patients with positive lymph nodes (p=0.007) and among patients with more than three positive lymph nodes (p=0.048). No significant association was found between the serum levels of HER-2 and patient's age (p=0.369), menopausal status (p=0.301), BMI (p=0.152) and ethnicity (p=0.285), tumor's size (p=0.662) and histological type (p=0.877), and the immunohistochemical expression of ER (p=0.341) and PR (p=0.179). Overexpression of HER-2 protein was associated with increased serum levels of HER-2 (p=0.016).

To evaluate the association between serum levels of HER-2 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

Table 2. Serum levels of HER-2 in patients with breast cancer according to clinicopathological characteristics

	Levels of HER-2 (ng/ml)		
	Median	Interquartile range	p value
Age			0.369
≤ 45 years	2.04	1.93 – 2.22	
> 45 years	2.00	1.91 – 2.26	
Menopausal status			0.301
Premenopausal	2.00	1.94 – 2.51	
Postmenopausal	2.02	1.90 – 2.20	
Body mass index (BMI)			0.152
≤ 25 Kg/m^2	1.96	1.92 – 2.05	
>25 Kg/m^2	2.04	1.91 – 2.61	
Religious/ethnic group			0.285
Greek Christians	2.04	1.91 – 2.31	
Greek Muslims	1.94	1.93 – 2.15	
Histological type			0.877
Lobular	2.04	1.90 – 2.31	
Ductal	1.99	1.93 – 2.19	
Lymphovascular invasion			0.022
No	1.90	1.83 – 1.96	
Yes	2.04	1.93 – 2.31	
Tumor size			0.662
T1	1.96	1.91 – 2.22	
T2 – T3	2.04	1.91 – 2.58	
Histological grade			0.011
I-II	1.96	1.88 – 2.03	
III	2.07	1.93 – 2.31	
Clinical stage			0.001
I-II	1.96	1.91 – 2.05	
III-IV	2.31	2.14 – 3.19	
Lymph node status			0.007
Negative	1.96	1.91 – 2.04	
Positive	2.15	1.93 – 2.61	
No of positive lymph nodes			0.048
≤ 3 lymph nodes	1.94	1.92 – 2.27	
>3 lymph nodes	2.26	1.99 – 3.08	
Estrogen receptors			0.341
Negative	1.96	1.93 – 2.15	
Positive	2.04	1.91 – 2.45	
Progesterone receptors			0.179
Negative	1.96	1.91 – 2.22	
Positive	2.04	1.91 – 2.23	
HER-2 protein			0.016
Negative	1.93	1.89 – 2.07	
Positive	2.05	1.94 – 2.58	
Ile655Val HER-2 polymorphism			<0.001
Ile-Ile	1.89	1.85 – 1.99	<0.001[a]
Ile-Val	2.08	1.96 – 2.31	0.647[b]
Val-Val	2.09	1.94 – 3.01	<0.001[c]
Ile-Val or Val-Val	2.09	1.94 – 2.60	<0.001[c]

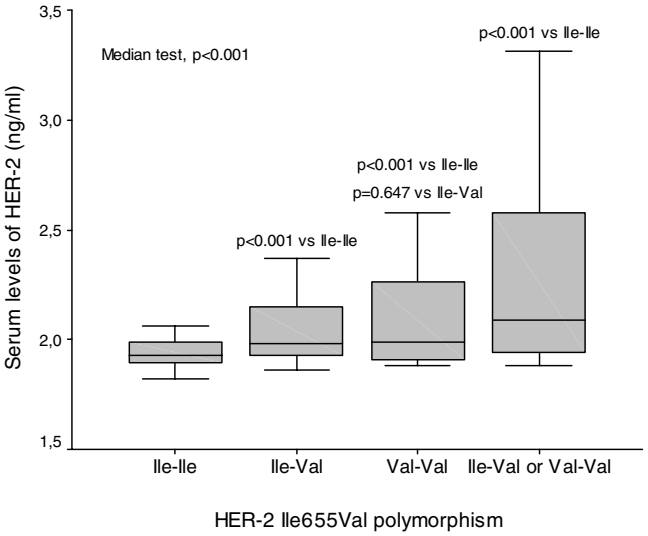

Figure 2. Serum level of HER-2 of breast cancer patients according to HER-2 Ile655Val polymorphism

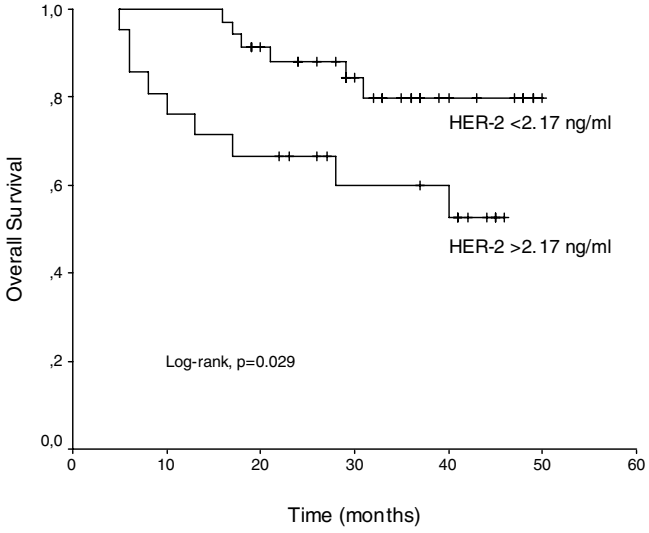

Figure 3. Overall survival of breast cancer patients according to serum levels of HER-2 (≤ 2.17 ng/ml vs >2.17 ng/ml)

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 [26]. A statistically significant association was also found between serum levels of HER-2 and HER-2 SNP (p<0.001). In this regard, Ile-Val and Val-Val genotypes exhibited highly significant HER-2 elevation compared to homozygous Ile-Ile (both p values <0.001). No significant difference in HER-2 levels was found between Ile-Val and Val-Val patients (p=0.647) (Fig. 2).

Multivariate stepwise linear regression analysis revealed that the advanced stages III-IV and Val-containing genotypes (p=0.009) remained the two significant independent determinants of high levels of HER-2 in breast cancer patients.

For the evaluation of the prognostic significance of HER-2 for advanced breast cancer (stages III or IV) and lymph node metastasis, the areas under the ROC curves were 0.807 (95%CI = 0.660 to 0.954, p=0.001) and 0.733 (95%CI = 0.603 to 0.864, p=0.003), respectively. The optimal cut-off point of 2.17 ng/ml was determined to predict more advanced stages and positive lymph nodes, which indicated sensitivities of 64% (95% CI = 36% to 86%) and 43% (95% CI = 26% to 62%), specificities of 83% (95% CI = 68% to 93%) and 88% (95% CI = 69% to 97%), positive predictive values of 56% and 81%, negative predictive values of 88% and 56% and accuracy of 79% (44 of 56 patients) and 64% (36 of 56 patients), respectively.

Therefore, the serum HER-2 level of 2.17 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 HER-2-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 45 ± 2 months (95% CI = 41 to 49 months) in patients with low levels of HER-2 (≤2.17 ng/ml; n=35) and 32 ± 4 months (95% CI = 25 to 40 months) in patients with high levels of HER-2 (>65.15 ng/ml; n=21). The log-rank test revealed a statistically significant difference between survival rates over time (p=0.029), with patients with high levels of HER-2 having worse prognosis (Fig. 3). Mortality rate was significantly greater in patients with high levels of HER-2 compared to patients with low levels of HER-2 (42.9% vs 17.1%, p=0.035), where patients with high levels of HER-2 were 3 times more likely to die of cancer than those with low levels of HER-2 (Hazard ratio = 3.0, 95% CI = 1.1 to 8.5, p=0.038). Regarding to the prognostic significance of HER-2 SNP on overall survival, mean survival time was 48 ± 2 months (95% CI = 44 to 52 months) in Ile-Ile patients, 40 ± 3 months (95% CI = 34 to 46 months) in Ile-Val patients and 34 ± 4 months (95% CI = 26 to 41 months) in Val-Val patients. Homozygous Val-Val genotype was associated with reduced survival compared to Ile-Ile genotype (p=0.011), while its difference with heterozygous Ile-Val was of borderline statistical significance (p=0.092) (Fig. 4a). There was no significant difference in survival rates between Ile-Ile and Ile-Val patients (p=0.194). Moreover, when Ile-Ile or Ile-Val patients were compared to homozygous Val-Val patients, the latter exhibited significantly lower mean survival time than the other patients (34± 4 months vs 44 ± 2 months, p=0.014; Fig. 4b). Mortality rate was significantly increasing as the number of Val allele in the genotype was increasing (6.7%,

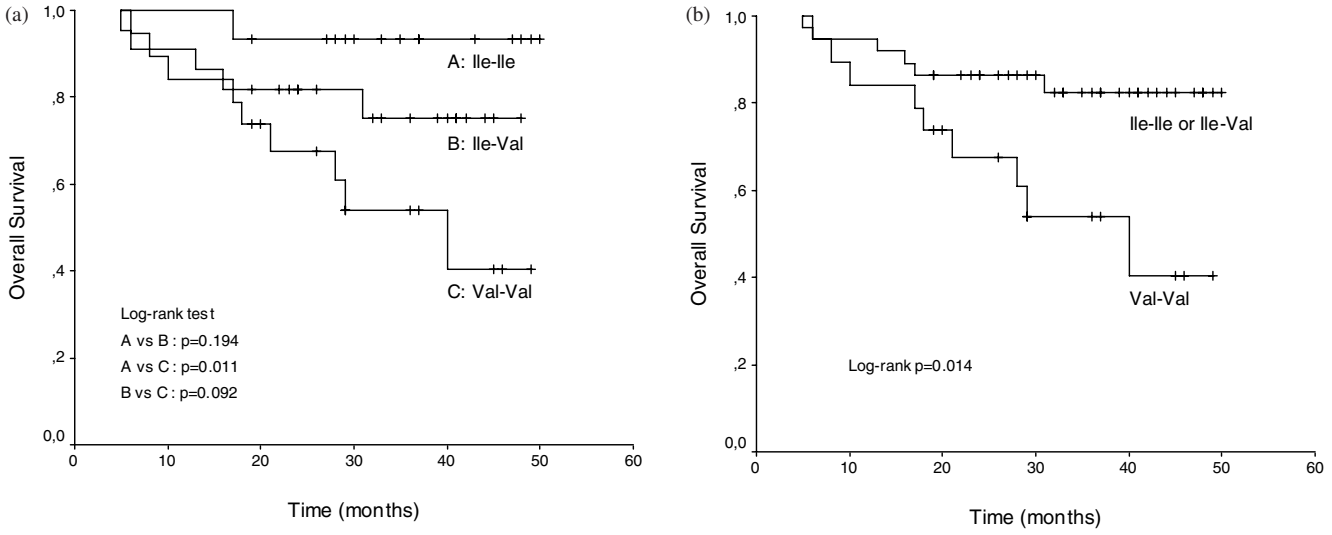

Figure 4. Overall survival of breast cancer patients according to the HER-2 Ile655Val polymorphism. (a) Comparison between the three genotypes and (b) comparison between Ile-Ile or Ile-Val genotypes and Val-Val genotype

22.7% and 47.4% in Ile-Ile, Ile-Val and Val-Val genotypes, respectively; p=0.008 for linear trend).

Further investigation with multivariate Cox proportional hazards regression analysis, showed that serum HER-2 levels failed to retain an independent relation with overall survival (Hazard ratio = 2.3, 95% CI = 0.7 to 8.1, p=0.181). However, the presence of homozygous Val-Val genotype in HER-2 Ile655Val polymorphism was the only independent prognostic factor of worse overall survival (Hazard ratio = 9.2, 95% CI = 1.1 to 80.1, p=0.045), along with lymph node metastasis (Hazard ratio = 5.0, 95% CI = 1.1 to 22.5, p=0.035).

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 [29]. Alterations of HER-2 have been associated with carcinogenesis and poor prognosis of breast cancer. We conducted this study to clarify the possible relationship between serum levels of HER-2 and breast cancer development, to examine their association with clinicopathological characteristics and Ile655Val polymorphism of HER-2, and to investigate any possible relation of serum HER-2 and this SNP with overall survival.

The current study demonstrated that serum levels of HER-2 were significantly higher in breast cancer patients compared to healthy women. These findings are consistent with previous studies which showed elevated serum HER-2 levels [30, 31]. Two methods are usually used to determine a patient's HER-2 status: immunohistochemistry (IHC), which measures protein expression, and fluorescence in situ hybridization (FISH), which detects gene amplification. Both show low specificity and FISH is a costly procedure [32,33]. Additionally, both methods require high quality tissue samples, which are not always available. Although different methods to detect the same process, such as amplification, are reasonably concordant, the results are still conflicting [14]. The extracellular domain of the HER-2/neu oncoprotein (105kDa) is shed into the blood and may be detected in the serum [15]. Bayer Diagnostics has developed an automated ELISA assay (Bayer Immuno 1) for measuring circulating concentrations of the HER-2 oncoprotein. Some of the advantages of this method are that it is reproducible, quantitative, and objective and blood is routinely collected in the physician's office before administration of chemotherapy. In addition, blood collection provides a real-time analysis of the patient's serum HER-2 status at the time when clinical decisions are made concerning therapy for metastatic disease. In the present study, we have also found a strong positive association between serum HER-2 levels and the immunohistological expression of this protein in the tumor. This is in concordance with previous studies, which demonstrated that although various methods have been used to determine HER-2 expression, similar results have been reported regarding HER-2 overexpression in tumors and sera from several types of cancer patients [15, 16, 34-36]. Furthermore, the current study demonstrates a high diagnostic significance of serum HER-2 levels for breast cancer (AUC, 0.622). The optimal cut-off value of 1.98 ng/ml for predicting breast cancer yielded high sensitivity of 54%, specificity of 73% and accuracy of 62%. Our results are in keeping to those reported for other tumor markers such as CEA, CA15-3 or EGFR. These results indicate that serum HER-2 levels merit to be an independent biomarker for predicting breast cancer.

There is increasing evidence that serum levels of HER-2 reflect tumor maintenance and aggressiveness in breast cancer patients. In this regards, recent studies have demonstrated an association between high serum levels of HER-2 and other clinicopathological characteristics indicative of tumor progression. The current study indicates that serum levels of HER-2 associated with lymphovascular invasion, poor differentiation, advanced clinical stages, lymph node metastasis and higher number of positive lymph nodes in breast cancer patients. Multivariate analysis showed that advanced clinical stages (III or IV) remained a strong independent prognostic factor for higher HER-2 levels. Our findings are more consistent with the reports of [16, 37, 38] demonstrating that serum HER-2 contributes not only on the development, but also on the progression and dissemination of breast cancer. On the other hand, in the recent study by Kong et al. [17], neither HER-2 overexpression nor raised serum HER-2 levels were significantly associated with any of the clinicopathological characteristics of breast cancer patients. The diversity of these results may in part be due to the use of various EIA with different antibodies of HER-2 protein, different cutoff criteria for HER-2 in tissues and sera, different patients background and retrospective statistical analyses based on a small sample size. In this study, no correlation could be established between the expression of serum HER-2 and steroid hormone receptors content. This is consistent with other reports [15, 16, 39], who did not find any correlation between HER-2 levels in serum and sex steroid receptor content either.

Lymph node status and clinical stage are the most significant prognostic factors in breast cancer. Therefore, any factor associated with these is likely to be associated with survival. Another major finding of the present study was that serum HER-2 levels were predictive of more advanced stages (AUC, 0.807) and lymph node metastasis (AUC, 0.733) in breast cancer patients. The optimal probability value of 2.17 ng/ml may be more suitable for predicting advanced stages and lymph node metastasis in this cohort with sensitivities of 64% and 43%, and specificities of 83% and 88% respectively; overall accuracy was 79% 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 serum HER-2 appears to be a useful predictive marker of advanced stages and lymph node metastasis.

Molina et al. [40] have shown that cleavage of the extracellular domain of HER-2 receptor leads to increased phosphorylation of the intracellular tyrosine kinase. This suggests that circulating antigen concentrations are not only a marker of tumor overexpression of HER-2, but they may also be indicative of the degree of receptor activation. This affects 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 [41].

Alterations of HER-2 proto-oncogene have been associated with carcinogenesis and poor prognosis of breast cancer. A single nucleotide polymorphism (SNP) 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 [20-22]. However, several studies have shown that this association is controversial [23, 24]. An explanation for these contradictory results might be the considerable differences in the HER-2 codon 655 genetic polymorphism between ethnic groups [25]. 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 this HER-2 SNP with more advanced clinicopathologic characteristics and immunohistochemical HER-2 overexpression, which may indicate its possible implication on the more aggressive phenotype [26]. The relationship between Ile655Val HER-2 SNP and HER-2 amplification has also been suggested in previous studies [42-45], which showed that the presence of Val allele is associated with HER-2 amplification and/or protein overexpression, although the number of cases was small and the results were not always statistically significant. Furthermore, our present study showed that patients who were either heterozygous Ile-Val or homozygous Val-Val exhibited significantly elevated serum HER-2 levels compared to homozygous Ile-Ile patients. Multivariate linear regression analysis revealed that the Val-containing genotypes remained significant independent determinants of high levels of serum HER-2 in breast cancer patients. Our findings may support the suggestion of Fleishman et al. [46] about the functional implication for this SNP by enhancing active dimeric conformations of HER-2 protein that result in increased autophosphorylation and tyrosine kinase activation, even under conditions of HER-2 overexpression. Moreover, the presence of the Val allele, considering that isoleucine to valine change might alter the hydrophobicity of residues responsible for the conformational stability of the hydrophobic transmembrane domains [19], could explain the altered function of HER-2 receptor on the cell surface (ligand binding, dimerization, signal transducing, receptor degradation). Therefore, further study is clearly warranted to elucidate the relationship between genetic polymorphisms and HER-2 gene amplification.

Several investigators demonstrated that higher preoperative serum HER-2 was associated with a poor prognosis in breast carcinoma patients [16, 34, 47-49], with a median survival time for patients with the overexpression of serum HER-2 ranging from 21 to 27 months. On the other hand, in a study by Molina et al. [50], serum HER-2 did not have any prognostic significance in patients with locoregional breast carcinoma. In our study, although the duration of follow-up was short (median, 30 months), preliminary results have shown that higher serum HER-2 levels were significantly associated

with poor prognosis, with mean survival time of 32 months. When the relation of Ile655Val HER-2 SNP with overall survival was assessed, homozygous Val-Val genotype was found to be associated with reduced survival compared to Ile-Ile genotype or heterozygous Ile-Val. In multivariate Cox regression analysis, serum HER-2 failed to retain an independent prognostic value, while homozygous Val-Val genotype remained a significant independent predictor for worse overall survival. These results are consistent with a resent report of Pinto et al. [51], who demonstrated that homozygous Val-Val ovarian cancer patients presented a lower overall survival. Although, longer follow-up time is required to confirm the results on survival association, our results indicate the significant role of Ile655Val HER-2 SNP in the outcome of breast cancer patients demonstrating its implication in the formation of HER-2 receptors, with a more aggressive phenotype.

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