# Allelic imbalance of HER-2 codon 655 polymorphism among different religious/ethnic populations of northern Greece and its association with the development and the malignant phenotype of breast cancer

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Alterations of c-erbB-2 (neu or 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. This study aims to determine the prevalence of the HER-2 genotype and its association with breast cancer in the Greek Christian and Greek Muslim population of Thrace, Greece.

In this case-control study, we genotyped 56 patients (43 Christians and 13 Muslims) with primary breast cancer and 45 healthy women (32 Christians and 13 Muslims) for the Ile655Val polymorphism, with the PCR-RFLP method.

The Val allele and the Val-containing genotypes were significantly more frequent in Muslims than in Christians (p=0.020 and p=0.008, respectively). Among the Greek Christian population, a 5-fold and a 3.1-fold increase in risk of breast cancer was associated with the Val-Val genotype and the Ile-Val or Val-Val genotypes (95% CI, 1.3-18.4; p=0.017 and aOR, 3.1; 95% CI, 1.2-8.3; p=0.025; respectively) compared to homozygous Ile-Ile. No significant association was found in the Muslim population. Among the entire cohort, 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). This effect was even more pronounced in younger women. Among breast cancer patients, invasive carcinomas, low differentiation tumors, advanced stages, positive lymph nodes, high number of lymph nodes and HER-2 overexpression were more frequent in patients with allele Val than those with allele Ile.

Our study proposes the allelic imbalance of Ile655Val polymorphism between Greek Christian and Greek Muslim populations of Thrace contributes to the inconsistent association between this SNP and breast cancer risk across these two different ethnic groups. The association of the HER-2 genotype with clinicopathologic characteristics and HER-2 expression may indicate its possible implication on the more aggressive phenotype.

*Keywords: HER-2, c-erbB-2, polymorphism, breast cancer, relative risk*

HER-2 (also known as c-erbB-2 or neu) proto-oncogene, a member of the epidermal growth factor receptor family, located at chromosome 17q12-q21, encodes a transmembrane glycoprotein (p185) with tyrosine kinase activity [1,2]. HER-2 is believed to be the preferred hetero-dimerization partner for the other members of the family, leading to tyrosine kinase activation and subsequent signalling cascades [3-5]. Amplification and/or overexpression of this receptor has been found in breast, ovarian, stomach, prostate and bladder carcinomas [6-10]. HER-2 overexpression occurs in 20-30% of breast cancers, resulting in ligand-independent activation and more aggressive growth behaviour, reduced response to chemotherapy and hormonal therapy, as well as a poor prognosis

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**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) | |
| HER-2 protein | | |
| Negative | 25 (44.6) | |
| Positive | 31 (55.4) | |

[7, 11,12]. HER-2 is also a target for therapy. Trastuzumab, a humanized monoclonal murine antibody directed against the extracellular domain of HER-2, was introduced for the treatment of patients with HER-2-overexpressing advanced breast cancer, with improved patient outcomes including survival [13-16]. Therefore, since HER-2 is clearly an established prognostic factor, its encoding gene is therefore a natural target for investigation regarding polymorphisms that might indicate resistance or susceptibility to breast cancer development.

Sequence analysis of the human HER-2 gene identified 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 [17]. Recently, in a population-based case-control study of this polymorphism, Xie et al. [18] reported that the variant Val allele was associated with an increased risk of breast cancer in a Chinese population, particularly among younger women. Subsequent studies also showed positive association between this SNP and breast cancer risk among Slovak [19], Portuguese [20] and Ashkenazim [21] populations, as well as in Australian women younger than 40 years of age [22] and in African-American and white women from North Carolina aged 45 or younger with a family history of breast cancer [23]. However, several studies have shown that this association is controversial [24-31]. Regarding other human cancers, a positive association was also found in gastric cancer [32], but not in bladder [33], ovarian [27], cervical [35] or prostate [36, 37] cancers. Furthermore, the presence of Val allele has been associated with cancer invasion, progression and metastasis in gastric cancer [32], and with more advanced stages in breast [24] and bladder [33] cancer, although other studies have shown conflicting results [20,23,33,36,37]. One reason for these contradictory results might be the considerable differences in the HER-2 codon 655 genetic polymorphism between ethnic groups [38].

This study was conducted in Thrace, an area in northern Greece. On the basis of the last population survey in 2001, 71% of the population of Thrace are Christians and 29% are Muslims. Although both Muslims and Christians are Greek citizens they constitute distinct cultural/ethnic groups, with different diet and lifestyle characteristics. The aim of the present study was to assess the genetics of HER-2 polymorphism at codon 655 in Greek Christian and Greek Muslim women of Thrace and evaluate the association of this alteration with the development and the malignant phenotype of breast cancer.

## 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 (n=43) and Greek Muslims (n=13). 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), reli-

gion/ethnicity (p=0.517), menopausal status (p=0.453) and BMI (p=0.250) between patients and controls. Almost all of the Christians were residing in urban areas of Thrace and they belonged to middle class families. On the contrary, all Muslims were farmers, residing in rural areas, and all belonged to low class families.

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 [39], and tumor stage was assigned according to the TNM classification defined by the International Union Against Cancer [40]. Histological type, lymphovascular invasion, tumor size, histological grade, clinical stage, lymph node status, number of positive lymph nodes, immunohistochemical expression of HER-2 proteins were recorded for each patient (Table 1). The expression of HER-2 protein was considered positive when at least 10% of the cancer cells showed staining. Regarding to histological type, 43 (76.8%) were ductal and 13 (23.2%) lobular carcinomas. More than 80% were invasive carcinomas, and based on the largest diameter of the tumor, the majority of them (60.7%) had size between 2 and 5 cm. Nine (16.1%) were well-differentiated (G1), 11 (19.6%) were moderately differentiated (G2) and 36 (64.3%) were poorly differentiated carcinomas. Regarding clinical stage, 27 (48.2%) of the tumors were of stage II, while in 30 patients lymph node metastases were found. HER-2 overexpression was observed in 55.4% of the patients. 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 venous blood was collected by standard venipuncture technique in ethylenediaminetetraacetic acid (EDTA)- containing tubes. Genomic DNA was isolated from whole blood specimen 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. [18] 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%).

*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 chi-square test was used to assess differences of genotype frequencies between Christian and Muslim Greek women, and between breast cancer patients and controls. It was also used to evaluate any potential association between HER-2 polymorphism and tumor characteristics and to compare the observed frequency of each genotype with that expected for a population in Hardy-Weinberg equilibrium. Unconditional logistic regression analysis was used to estimate odds ratios (OR) and 95% confidence intervals (CI) as the measure of association of HER-2 Ile655Val polymorphism with the development of breast cancer and the clinicopathologic characteristics of the tumor. Multivariate stepwise logistic regression models were separately constructed, adjusted for age, menopausal status, BMI and religion/ethnicity to evaluate the independent effect of HER-2 polymorphism on the development of breast cancer, clinical stage, lymph node status and the expression of HER-2 protein. All tests were two tailed and statistical significance was considered for p values less than 0.05.

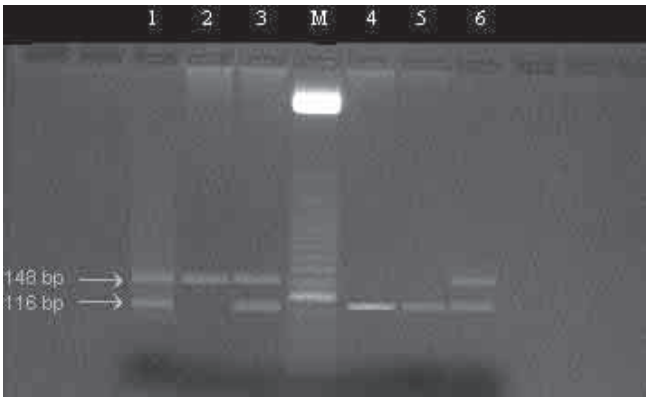


**Figure 1: Detection of the isoleucine (Ile) to valine (Val) polymorphism at codon 655 in the HER-2 gene by the PCR-RFLP technique. Restriction fragments treated by BsmAI are 148 bp for the Ile homozygote (lane 2), a band of 116 bp for the Val homozygote (lanes 4 and 5) and two bands of 148 bp and 116 bp, respectively, for Ile/Val heterozygote (lanes 1, 3 and 6). M, molecular size marker (125 bp).**

## Results

*HER-2 polymorphism and risk of breast cancer.* First we analyzed the Ile655Val genotypes in 56 healthy women in order to characterize the genetics of the studied population. Restriction analysis for 6 representative samples is shown in Figure 1. The valine allele was identified by the presence or absence of the 116 bp fragment on gel electrophoresis after enzyme restriction, because the 32 bp fragment was too small to be clearly visualized. Valine homozygotes showed a band of 116 bp without a 148 bp band and isoleucine/valine heterozygotes presented two separate bands of 148 bp and 116 bp.

**Table 2. Distribution of HER-2 genotypes among breast cancer patients and controls**

| | Patients* | Controls* | OR | 95% CI | P value |
|---|---|---|---|---|---|
| **Greek Christians** | | | | | |
| Genotype Ile-Ile | 12 (27.9) | 17 (53.1) | 1.0† | | |
| Ile-Val | 16 (37.2) | 10 (31.3) | 2.2 | 0.7-6.8 | 0.170 |
| Val-Val | 15 (34.9) | 5 (15.6) | 5.0 | 1.3-18.4 | 0.017 |
| Ile-Val or Val-Val | 31 (72.1) | 15 (46.9) | 3.1 | 1.2-8.3 | 0.025 |
| Allele | | | | | |
| Ile | 40 (46.5) | 44 (68.8) | | | |
| Val | 46 (53.5) | 20 (31.2) | | | |
| **Greek Muslims** | | | | | |
| Genotype | | | | | |
| Ile-Ile | 3 (23.1) | 2 (15.4) | 1.0† | | |
| Ile-Val | 6 (46.2) | 6 (46.2) | 0.5 | 0.1-5.3 | 0.562 |
| Val-Val | 4 (30.8) | 5 (38.5) | 0.3 | 0.1-3.7 | 0.338 |
| Ile-Val or Val-Val | 10 (76.9) | 11 (84.6) | 0.4 | 0.1-3.1 | 0.429 |
| Allele | | | | | |
| Ile | 12 (46.2) | 10 (38.5) | | | |
| Val | 14 (53.8) | 16 (61.5) | | | |
| **Total** | | | | | |
| Genotype | | | | | |
| Ile-Ile | 15 (26.8) | 19 (42.2) | 1.0‡ | | |
| Ile-Val | 22 (39.3) | 16 (35.6) | 2.0 | 0.8-5.2 | 0.170 |
| Val-Val | 19 (33.9) | 10 (22.2) | 2.6 | 0.9-7.4 | 0.076 |
| Ile-Val or Val-Val | 41 (73.2) | 26 (57.8) | 2.2 | 0.9-5.1 | 0.079 |
| Allele | | | | | |
| Ile | 52 (46.4) | 54 (60.0) | | | |
| Val | 60 (53.6) | 36 (40.0) | | | |

NOTE: Statistical significance for differences in genotype, Val-containing genotype and allelic frequencies between Greek Christians and Greek Muslims: p=0.053, p=0.020 and p=0.008;

Statistical significance for differences in genotype, Val-containing genotype and allelic frequencies between patients and controls: p=0.056, p=0.027 and p=0.007 among Greek Christians; p=0.856, p=0.619 and p=0.575 among Greek Muslims; p=0.218, p=0.103 and p=0.055 among the entire cohort.

* Data are number of subjects and percentage (%);

† Adjusted for age, menopausal status and body mass index;

‡ Additionally adjusted for religious/ethnic group.

The Ile-Ile, Ile-Val and Val-Val genotypes were found in 53.1%, 31.3% and 15.6% of healthy Greek Christians and in 15.4%, 46.2% and 38.5% of healthy Greek Muslims, respectively (Table 2). Although the difference of genotype frequencies between the two religious populations were of borderline statistical significance (p=0.053), the Val allele and Val-containing genotypes were significantly more frequent in Muslims than in Christians (p=0.008 and p=0.020, respectively). Healthy Muslims were 3.5-fold and 6.2-fold more likely to have the Val allele and a Val-containing genotype, respectively, than healthy Christians (OR, 3.5; 95% CI, 1.4-9.1; OR, 6.2; 95% CI, 1.2-32.7). The genotype distribution in both groups was in Hardy-Weinberg equilibrium (p=0.254 for Christians; p=0.993 for Muslims).

Among women with breast cancer, the Ile-Ile, Ile-Val and Val-Val genotypes were found in 27.9%, 37.2% and 34.9% of Christian patients and in 23.1%, 46.2% and 30.8% of Muslims patients, respectively (Table 2). Allele and genotype frequencies were similar in Christian and Muslim patients (p=0.974 and p=0.844, respectively). The genotype distribution in both patient groups was in Hardy-Weinberg equilibrium (p=0.254 for Christians; p=0.962 for Muslims). Among Christians, the Val allele and the Val-containing genotypes were more prevalent in patients compared to healthy women (p=0.007 and p=0.027, respectively), while no such differences were observed among Muslim women (p=0.575 and p=0.619, respectively).

To evaluate the risk of breast cancer according to the HER-2 genotype, logistic regression analysis was conducted with an adjustment for age, menopausal status and BMI. In the Christian population, the presence of two copies of the Val allele was associated with a 5-fold increase in risk of breast cancer compared to the Ile-Ile genotype (OR, 5.0; 95% CI, 1.3-18.4; p=0.017). Heterozygous Ile-Val genotypes showed an intermediate risk of 2.2 (95% CI, 0.7-6.8), which did not reach the statistical significance level (p=0.170). The presence of the Val-containing genotypes (Ile-Val or Val-Val) yielded an odds ratio for breast cancer of 3.1 (95% CI, 1.2-

**Table 3. Distribution of HER-2 genotypes among breast cancer patients and controls according to subject's age**

| | Patients | Controls | OR* | 95% CI | P value |
|---|---|---|---|---|---|
| Age ≤ 45 years | | | | | |
| Ile-Ile | 2 (18.2) | 7 (58.3) | 1.0 | | |
| Ile-Val | 4 (36.4) | 4 (33.3) | 7.6 | 0.5-29.0 | 0.136 |
| Val-Val | 5 (45.5) | 1 (8.4) | 15.6 | 1.4-138.2 | 0.031 |
| Ile-Val or Val-Val | 9 (81.8) | 5 (41.7) | 9.9 | 1.1-57.8 | 0.045 |
| Age > 45 years | | | | | |
| Ile-Ile | 13 (28.9) | 12 (36.4) | 1.0 | | |
| Ile-Val | 18 (40.0) | 12 (36.4) | 1.6 | 0.5-4.9 | 0.421 |
| Val-Val | 14 (31.1) | 9 (27.3) | 1.7 | 0.5-5.2 | 0.456 |
| Ile-Val or Val-Val | 32 (71.1) | 21 (63.7) | 1.6 | 0.6-4.4 | 0.374 |

NOTE: Statistical significance for differences in genotype and Val-containing genotype frequencies between patients and controls: p=0.067 and p=0.049 among women ? 45 years; p=0.782 and p=0.485 among women >45 years.
* Adjusted for religious/ethnic group and body mass index.

8.3; p=0.025) compared to the Ile-Ile genotype. There was no statistically significant association between this HER-2 polymorphism and breast cancer risk in the Muslim population. Among the entire cohort, with additional adjustment for religious/ethnic groups, the Val-Val genotype and the Ile-Val or Val-Val genotypes were associated with a marginally increased risk of breast cancer (OR, 2.6; 95% CI, 0.9-7.6; p=0.076; aOR, 2.2; 95% CI, 0.9-5.2; p=0.079; respectively) compared to the Ile-Ile genotype (Table 2).

When we stratified the analysis according to women's age, we observed that the positive association between the number of Val allele and the development of breast cancer was more pronounced in women younger than 45 years than in women older than 45 years. Among younger women, an increased risk of breast cancer with an odds ratio of 15.6 (95% CI, 1.4-138.2; p=0.031) was associated with the Val-Val genotype and of 9.9 (95% CI, 1.1-57.8; p=0.045) was associated with the Ile-Val or Val-Val genotypes. The risk for women older than 45 years was also increased by 60-70%, but these increments were not statistically significant (Table 3).

*HER-2 polymorphism and tumor characteristics.* HER-2 polymorphism was analyzed in relation to the following clinicopathologic characteristics: histological type, lymphovascular invasion, tumor size, histological grade, clinical stage, lymph node status, number of positive lymph nodes, and the expression of HER-2 protein (Table 4). Ductal carcinomas tended to be more frequently found among patients with the Val-Val genotype than those with the Ile-Ile or Ile-Val genotype (p=0.059). Similarly, differences in the prevalence of invasive carcinomas among the three genotypes were of borderline statistical significance (p=0.080). No significant association was found between HER-2 polymorphism and tumor size (p=0.636). A positive correlation was found between HER-2 polymorphism and the histological grade of tumor (p=0.028). In this regard, poorly differentiated tumors were 2.6 (95% CI, 0.7-10.1; p=0.161) and 8 (95% CI, 1.6-40.0; p=0.011) times more likely to be found in patients with Ile-Val and Val-Val, respectively, compared to homozygous Ile-Ile. The prevalence of advanced stage carcinomas was statistically significantly stepwise increased as the number of the Val allele in the genotype increased (p=0.004), while positive lymph nodes where significantly more frequent in patients with Val-containing genotypes than those with the Ile-Ile genotype (p=0.050). In particular, positive lymph nodes were 4.8 (95% CI, 1.2-20.3; p=0.032) and 4.7 (95% CI, 1.1-20.6; p=0.039) times more likely to be found in patients with Ile-Val and Val-Val, respectively, compared to homozygous Ile-Ile. A statistically significant positive association was found between the number of positive lymph nodes and the number of the Val allele in the genotype (p=0.029), with more than three positive lymph nodes being 4.3 (95% CI, 0.4-47.6; p=0.236) and 22.5 (95% CI, 1.6-314.6; p=0.021) times more likely to be found in patients with Ile-Val and Val-Val, respectively, compared to homozygous Ile-Ile. Furthermore, patients with Val-Val tended to have more lymph node metastases than those with the Ile-Val genotype (OR, 5.3; 95% CI, 0.8-34.4; p=0.084). Immunohistochemically HER-2 positive rates tend to increase with the presence of the Val allele, but this apparent trend did not reach the statistical significance level (p=0.112).

When the Ile-Val and Val-Val genotypes were compared to the Ile-Ile genotype similar results about the association of HER-2 polymorphism with tumor characteristics were obtained (Table 4). In this regard, the Val-containing genotypes were associated with higher rates of invasive carcinomas (OR, 4.6; 95% CI, 1.1-20,5; p=0.033), poorly differentiated tumors (OR, 4.1; 95% CI, 1.2-14.2; p=0.022), advanced stage carcinomas (p=0.009), positive lymph nodes (OR, 3.5; 95% CI, 1.0-12.9; p=0.015), more than three positive lymph nodes (OR, 8.3; 95% CI, 0.9-83.2; p=0.044) and positive HER-2 (OR, 3.5; 95% CI, 1.3-12.1; p=0.045) compared to the Ile-Ile genotype.

Multivariate stepwise logistic regression analyses were separately performed to evaluate the independent effect of

**Table 4. Association between HER-2 genotypes and tumor characteristics in breast cancer patients**

| | Genotypes | | | | Val-containing | |
|---|---|---|---|---|---|---|
| | Ile-Ile | Ile-Val | Val-Val | P value | genotypes | P value* |
| Histological type | | | | 0.059 | | 0.711 |
| Lobular | 4 (26.7) | 8 (36.4) | 1 (5.3) | | 9 (22.0) | |
| Ductal | 11 (73.3) | 14 (63.6) | 18 (94.7) | | 32 (78.0) | |
| Lymphovascular invasion | | | | 0.080 | | 0.033 |
| No | 5 (33.3) | 3 (13.6) | 1 (5.3) | | 4 (9.8) | |
| Yes | 10 (66.7) | 19 (86.4) | 18 (94.7) | | 37 (90.2) | |
| Tumor size | | | | 0.636 | | 0.342 |
| T1 | 6 (40.0) | 6 (27.3) | 5 (26.3) | | 11 (26.8) | |
| T2 – T3 | 9 (60.0) | 16 (72.7) | 14 (73.7) | | 30 (73.2) | |
| Histological grade | | | | 0.028 | | 0.022 |
| G1-G2 | 9 (60.0) | 8 (36.4) | 3 (15.8) | | 11 (26.8) | |
| G3 | 6 (40.0) | 14 (63.6) | 16 (84.2) | | 30 (73.2) | |
| Clinical stage | | | | 0.004 | | 0.009 |
| 0-I-II | 15 (100.0) | 17 (77.3) | 10 (52.6) | | 27 (65.9) | |
| III-IV | 0 (0.0) | 5 (22.7) | 9 (47.4) | | 14 (34.1) | |
| Lymph node status | | | | 0.050 | | 0.015 |
| Negative | 11 (73.3) | 8 (36.4) | 7 (36.8) | | 15 (36.6) | |
| Positive | 4 (26.7) | 14 (63.6) | 12 (63.2) | | 26 (63.4) | |
| No. positive lymph nodes | | | | 0.029 | | 0.044 |
| ≤ 3 lymph nodes | 5 (83.3) | 7 (53.8) | 2 (18.2) | | 9 (37.5) | |
| >3 lymph nodes | 1 (16.7) | 6 (46.2) | 9 (81.8) | | 15 (62.5) | |
| HER-2 protein expression | | | | 0.112 | | 0.045 |
| Negative | 10 (66.7) | 9 (40.9) | 6 (31.6) | | 15 (36.6) | |
| Positive | 5 (33.3) | 13 (59.1) | 13 (68.4) | | 26 (63.4) | |

NOTE: Data are number of patients and percentage (%);
* indicates statistical significance compared to Ile-Ile genotype.

HER-2 polymorphism on clinical stage, lymph node status and the expression of HER-2 protein. As confounders in these multivariate models, all statistically significantly ($p<0.10$) associated clinicopathologic characteristics with the above dependent variables, were included. The presence of two copies of Val allele remained an independent risk factor for more advanced stages (OR=9.8; 95% CI, 1.6-58.5; p=0.013), while the Val-containing genotypes had an independent effect on lymph node involvement (OR, 9.4; 95% CI, 1.4-65.1; p=0.023) and HER-2 expression (OR, 5.7; 95% CI, 1.2-22.8; p=0.029).

## Discussion

The HER-2 proto-oncogene as a member of the EGFR family 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 [41]. Overexpression of HER-2 is detected in a large proportion of breast cancers, indicating that activation of this gene is an important step mediating breast carcinogenesis [6-10,42].

Genetic polymorphisms may affect not only cancer development but also cancer progression, and as a result could influence cancer phenotypes. Sequence analysis of human HER-2 complementary DNA clones identified a single nucleotide polymorphism in the transmembrane coding region at codon 655 [17], which encodes either isoleucine (Ile; ATC) or valine (Val; GTC). The critical role of the transmembrane domain, makes this genetic polymorphism a strong candidate as a human cancer susceptibility factor. In a primary study, Xie et al. [18] reported the association of this SNP of the HER-2 gene with an increased risk of breast cancer. Furthermore, Kuraoka et al. [32] suggested that it could be associated with development of gastric carcinoma and may serve as a risk predictor of a malignant phenotype of gastric cancer. By contrast, no evidence of such association has been found in ovarian, bladder, cervical or prostate cancer [27,33-37].

Regarding breast cancer, Xie et al. [18] reported that the HER-2 Ile655Val SNP was associated with an increased risk of breast cancer among Chinese population. Subsequent studies demonstrated an equal importance of this polymorphism in the development of breast cancer among Slovak [19], Portuguese [20] and Ashkenazim [21] populations. In two case-control studies, elevated risk of breast cancer was associated with the presence of the Val allele among Australian women with breast cancer diagnosed before the age of 40 years [22], and among African-American and white women in North Carolina aged 45 or younger with a family history of breast cancer [23]. However, these findings were not confirmed by similar studies among other populations [24-31]. These conflicting reports might be attributed partly to the small

number of subjects with the homozygous Val genotype, leading to a decreased statistical power to detect the association between the SNP and cancer risk. Another possibility could be a variable influence of environmental factors with respect to cancer etiology in different populations. In addition, this polymorphism may be in linkage disequilibrium with the other polymorphic allele of functional significance [43] and there may be a difference to the extent of the disequilibrium among different ethnic groups. In fact, the allele frequency of this HER-2 polymorphism appears to vary considerably among the different ethnic groups [38]. In our study, the frequency of the valine allele in Greek Christian controls was 31.2%, slightly higher than frequencies previously reported in other Caucasian populations from Germany [25,44], England [26,27] and USA [23,38,45,46], ranging from 23% to 27%. This rate was higher than that reported in a recent publication including Greek participants from another region [29]. This regional variability is not unusual and indicates the need of selecting controls from the same area with patients [30]. The even higher frequency of the valine allele found among the Greek Muslim controls (61.5%) might be partially attributed to the small sample size of this group, to different diet and life-style characteristics or to their exposure to pesticides, since all Muslims were farmers. These results confirmed the high variability in the occurrence of the Val allele among various ethnic groups. Although sample sizes were small for individual religious groups in our case-control study, the association of HER-2 SNP with breast cancer in each individual group was evaluated. Our results showed an elevated risk of breast cancer development associated with the presence of the Val allele among Greek Christian population, but not among Greek Muslim population, in whom the valine allele appeared to be protective. Among the entire cohort, the 2.6-fold and 2.2-fold increased risk for the Val-Val and Val-containing genotypes, respectively, was of borderline statistical significance. These findings contribute to the recent published literature [18-21] and suggest that the different ethnic groups have different genetic backgrounds and there is a possibility that this difference may influence the occurrence of the disease.

Several investigators have demonstrated that the natural history of breast cancer in young women, compared to their older counterparts, may vary in terms of pathological features and clinical outcomes. Moreover, age has been shown to be an independent prognostic factor, suggesting that early- and late- onsets of breast cancers have different biological origins [47,48]. It is possible that some unknown genetic factor may also contribute to these different propensities with age. Based on previous observations, HER-2 polymorphism has been associated with a younger age of onset of breast cancer [18,21,22,23]. This association between SNP and breast cancer risk is controversial [19,20,25]. Our results showed a 15.6-fold and 9.9-fold increased risk for the Val-Val and Val-containing genotype respectively, among younger patients (≤45 years of age), compared to a 1.6-fold and 1.7-fold risk among older patients respectively, and suggest that this HER-2 alteration might affect the occurrence of cancer in younger patients, whereas other factors may have a stronger influence in carcinogenesis in older subjects. Although the difference between age groups regarding exposure to carcinogens could explain the difference in cancer risk and tumor characteristics according to age of onset, our observation is consistent with the notion that genetic factors have greater influence on earlier-onset diseases.

Our present study showed the association of this SNP not only with the development, but also with the progression of breast cancer. In this regard, this genotyping was associated with lymphovascular invasion, poor differentiation, advanced clinical stages, lymph node metastasis and higher number of positive lymph nodes in breast cancer patients. These results were concordant with those previously published on gastric cancer patients [32], where the Val allele has been associated with cancer invasion, progression and metastasis, and with two reports on breast [24] and bladder [33] cancer patients, in which this genotype has been associated with more advanced stages. However, other studies have shown conflicting results [23,34,36,37]. 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. In our study, multivariate logistic regression models separately conducted for stage of the disease and lymph node metastasis revealed that the Val-Val genotype remained an independent risk factor of more advanced stage (OR, 9.8) and Val-containing genotypes of more lymph node metastases (OR, 9.4). Our findings may support the suggestion [24] of the possible implication of HER-2 SNP genotype on the more aggressive phenotype.

The mechanistic role of the HER-2 Ile655Val polymorphism has not been fully clarified. Although there are no published data on the in vivo or in vitro consequences of the Ile to Val amino change at codon 655 of the HER-2 receptor, Fleishman et al. [49] recently found two stable conformations, active or inactive; a Val to Ile variant at codon 655 of the transmembrane region will destabilize the formation of the active dimeric form, resulting in reduced protein tyrosine kinase activity, even under conditions of HER-2 overexpression.

The clinical implications of breast cancer in relation to HER-2 overexpression are well documented. The fact that only 20-40% of the patients undergoing therapy with the humanized monoclonal antibody (trastuzumab) against the HER-2 receptor achieved significant improvement is a stimulus to uncover other mechanisms which modify the effectiveness of the therapy [50]. The observation that some patients with immunohistochemical overexpression of HER-2 do not present amplified HER-2 gene, or in other patients a strong gene amplification is accompanied with weak protein expression, is considered together with other factors to be the reason of insufficient therapeutic response [19]. The presence of the Val allele, considering that the isoleucine to valine change might alter the hydrophobicity of residues responsible for the conformational stability of the hydrophobic transmembrane

domains [51], could explain the altered function of HER-2 receptor on the cell surface (ligand binding, dimerization, signal transducing, receptor degradation). In our study the presence of the Val-containing genotypes have been independently associated with HER-2 overexpression (OR, 5.7). Such a trend has been also found by Millikan et al. [23] and Kamali-Sarvestani et al. [31] in patients with breast cancer. Our findings may support the suggestion of the possible impact of this SNP on the effectiveness of trastuzumab therapy [19] and provide a foundation for future studies to examine the prevalence of the HER-2 Val allele in women with HER-2 overexpressing breast cancers.

In conclusion, our study suggests the allelic imbalance of Ile655Val polymorphism in the transmembrane domain-coding region of HER-2 between Greek Christian and Greek Muslim populations of Thrace, which contributes to the inconsistent association between this SNP and breast cancer risk across these two different ethnic groups. It was also shown that, at least among the Christian population, the Val allele could be associated with development of breast cancer and may serve as an indicator of genetic susceptibility to this disease. The association of the HER-2 genotype with clinicopathologic characteristics and HER-2 expression may indicate its possible implication on the more aggressive phenotype. These findings demonstrate the feasibility of further extensive studies to confirm the real meaning of HER-2 genetic polymorphisms in breast cancer.

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