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Association between the Polymorphism of the Angiotensin-Converting Enzyme Gene and Tumor Size of Breast Cancer in Premenopausal Patients

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Yaren, A., Turgut, S., Kursunluoglu, R., Oztop, I., Turgut, G., Kelten, C. and Erdem, E. *Association between the Polymorphism of the Angiotensin-Converting Enzyme Gene and Tumor Size of Breast Cancer in Premenopausal Patients*. Tohoku J. Exp. Med., 2006, **210** (2), 109-116 —— The association between the polymorphism of the angiotensin-converting enzyme (ACE) gene and breast cancer risk has been extensively studied, however, the studies about the prognostic factors and ACE gene polymorphism are limited in number. Our aims were to analyze the distribution of the insertion/deletion (I/D) polymorphism of the ACE gene in Turkish premenopausal patients with breast cancer, which is more aggressive than the postmenopausal counterpart, and to assess whether DD genotype is associated with poor prognostic factors. The DD genotype has been shown to be associated with the increased serum and tissue levels of ACE, compared to those in II and ID genotypes. ACE genotypes were determined by polymerase chain reaction in 44 Turkish premenopausal patients with breast cancer and in 46 age-matched healthy premenopausal women. ACE genotypes are distributed in patients and control subjects as follows; DD is present in 25 (56.8%), ID in 17 (38.6%), and II in 2 (4.5%) patients, and DD in 28 (60.9%), ID in 12 (26.1%), and II in 6 (13.0%) healthy subjects, respectively. D and I alleles were found in 76.1% and 23.9% of the patients, while 73.9% and 26.1% in healthy subjects, respectively. In breast cancer patients, no significant association was observed between the ACE genotypes and poor prognostic factors, such as negative hormone receptor status, histological grade, lymph node involvement, higher number of lymph node metastases, and c-erb B2 overexpression, except that tumor size greater than 2 cm is associated with DD genotype ($p = 0.02$). Thus, ACE may influence the local tumor growth of breast cancer in premenopausal patients. ———— premenopause breast cancer; ACE gene polymorphism; poor prognostic factors

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Breast cancer in premenopausal women is more aggressive and is associated with many poor prognostic factors such as increasing tumor size, lymph node involvement, high histological grade, steroid receptor negativity and overexpression of HER2/neu, and also has a significantly higher local or distant relapse risk (Chung et al. 1996; Elkhuizen et al. 2000). Many studies on genetic predisposition have been reported in premenopausal breast cancer. Germ line mutations such as BRCA1 and BRCA2 take part in 5% to 10% of breast cancer (Shih et al. 2002). Recently, there is increasing evidence that polymorphisms in genes may have a role in altering the risk of breast cancer. In the literature, it has been found that the polymorphism of the angiotensin-converting enzyme (ACE) gene is associated with breast cancer risk (Koh et al. 2003, 2005; Ladd et al. 2005).

The polymorphism of the ACE gene is located on 17q23, consists of the I or D of a 287 base pair DNA fragment in intron 16, resulting in genotypes II, ID and DD. The DD genotype can exhibit about two-fold higher plasma and tissue ACE level than the II genotype, whereas ID genotype can exhibit an intermediate level (Rigat et al. 1990; Hubert et al. 1991). The angiotensin II (Ang II) is converted from angiotensin I by ACE, a zinc metalloprotease, which has a variety of functions. Several studies have suggested that Ang II has promitotic, proliferative and angiogenic effects on carcinogenesis (Le Noble et al. 1991; Lyall et al. 1992; Greco et al. 2002), and ACE inhibitors decreased tumor growth in experimental studies, including breast cancer cells (Volpert et al. 1996; Small et al. 1997; Hii et al. 1998).

Based on the earlier studies linking ACE activity and gene polymorphism in various malignancies, it has been reported that DD genotype increased the likelihood of prostate cancer in advanced stage (Medeiros et al. 2004), and the number of lymph node metastases was correlated with DD genotype in gastric cancer patients (Röcken et al. 2005). In breast cancer, it has been shown that women with DD genotype had a significantly increased risk of developing breast cancer, and cancer-free survival was significantly reduced (Koh et al. 2003, 2005; Ladd et al. 2005). In the literature, however, studies about the prognostic factors and ACE gene polymorphism are limited in number.

Based on these observations, we aimed to assess the distribution of the I/D polymorphism of the ACE gene in premenopausal patients with breast cancer and whether or not DD genotype associates with poor prognostic factors.

Materials and Methods

Subjects

Forty-four Turkish premenopausal patients with histologically confirmed invasive ductal carcinoma at early or locally advanced stage (mean age: 41.6 ±7.1 years) were enrolled in the present study. Forty-six healthy premenopausal women (mean age: 40.3 ± 5.3 years) were recruited as the control group. The patients who were using ACE inhibitors or any drug that affects ACE, who had metastatic disease and who had second malignancy were excluded from the study. Premenopausal breast cancer patients were admitted and treated in the Department of Medical Oncology, Pamukkale University, Denizli, Turkey. The prognostic factors including lymph node involvement, tumor size, tumor staging, histological grade, hormone receptors, c-erb B2 and type of the treatment like surgery, chemotherapy or radiotherapy were obtained from the medical records. The study protocol conforms to the ethical guidelines of the Declaration of Helsinki as reflected in a prior approval by the institution's human research committee. Additionaly, the study was approved by the Ethics Committee of Pamukkale University and written informed consent was obtained from each participant.

Genetic analysis

Genomic DNA from the patients and controls was prepared from peripheral blood samples by a standard phenol/chloroform extraction method (Poncz et al. 1982). PCR was used to detect the presence of I and D alleles in intron 16 of the ACE gene according to the method described by Rigat et al. (1990) using an upstream primer 5' –CTG GAG ACC ACT CCC ATC CTT TCT- 3' and downstream primer 5' –GAT GTG GCC ATC ACA TTC GTC AGAT-3'. Amplification was performed for 35 cycles with denaturation, extension and annealing temperatures of 94°C, 60°C and 72°C, respectively. Amplified fragments (490 bp for the I allele and 190 bp

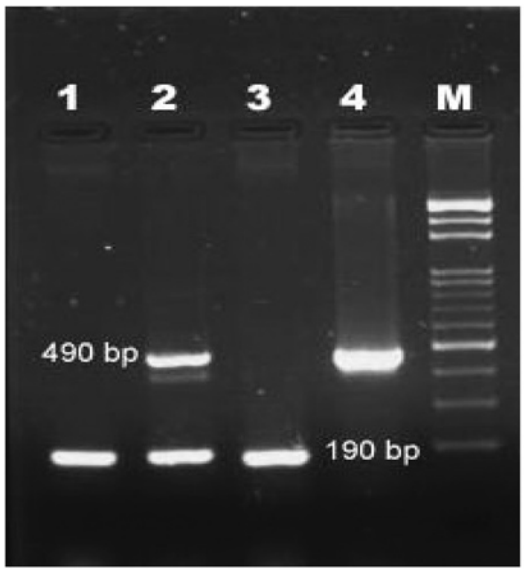

Fig. 1. Determination of ACE genotypes by PCR amplification. D and I alleles were identified by the presence of 190-bp and 490-bp fragments, respectively. DD (*lanes 1, 3*), ID (*lanes 2*), II (*lanes 4*) and M (Marker, 100 bp ladders).

for the D allele) were separated by 2% agarose gel electrophoresis and identified using the UVI Gel Documentation system. A representative sample from a breast cancer patient is shown in Fig. 1.

Statistical analysis

The frequency of ACE genotypes in premenopausal breast cancer patients and healthy premenopausal women as the control group and the association between the gene polymorphism and prognostic factors was assessed using the chi-square test or Fisher's exact test. A logistic regression model was used to calculate the odds ratio (OR) and the corresponding 95% confidence interval (CI). For the correlation analysis between variables, Spearman's correlation analyses was used. Kaplan-Meier method was performed for survival analyses and log-rank test was used to compare. All tests were two-sided and differences were considered significant when p value was $\leqq 0.05$. All analyses were performed with the SPSS for Windows (version 10.0).

Results

The frequencies of the ACE genotype and alleles in premenopausal breast cancer patients and control subjects are shown in Table 1. The distribution of ACE genotypes in patients and control group were as follows: 25 (56.8%) patients had DD, 17 (38.6%) had ID, and 2 (4.5%) had II genotype; DD was present in 28 (60.9%), ID in 12 (26.1%), and II in 6 (13.0%) healthy subjects (OR = 1.18, 95% CI = 0.51 - 2.74, p = 0.22). The frequencies of D and I alleles were 76.1% and 23.9% in the patient group, while 73.9% and 26.1% among the healthy subjects, respectively (OR = 1.12, 95% CI = 0.57 - 2.21, p = 0.43). No significant differences were found in the frequencies of ACE genotypes and alleles between patients and control subjects.

Of the forty-four patients, 22 (50.0%) had early stage disease (I, IIA and IIB), and 22 (50.0%) had locally advanced disease (stage III A, III B and III C). Fifty-nine percent of patients had lymph node involvement. The median number of positive lymph nodes was 6 (range 0-32). Fifteen (34.1%) patients had a tumor size of equal and smaller than 2 cm, and in 29 patients (65.9%) the tumor size was greater than 2 cm. Median tumor size was 4 cm (range 1.0-11 cm). Two patients (4.5%) had low histological grade (grade I), 24 (54.5%) had intermediate grade (grade II), and 18 (41%) had high grade (grade III) disease. More than half of the patients were estrogen receptor and/or progesterone positive (56.8%). Only eight patients (18.2%) had c-erb B2 overexpression. All patients were treated surgically (32 [72.7%] had mastectomy and axillary lymph node

Table 1. The genotype and allele frequencies of the ACE gene in study population.

	ACE genotypes			ACE alleles	
	DD (%)	ID (%)	II (%)	D (%)	I (%)
Breast cancer patients (n = 44)	25 (56.8)	17 (38.6)	2 (4.5)	67 (76.1)	21 (23.9)
Control subjects (n = 46)	28 (60.9)	12 (26.1)	6 (13.0)	68 (73.9)	24 (26.1)

dissection, 12 [27.3%] had breast conserving surgery), 29 (65.9%) were treated by adjuvant radiotherapy, all patients were treated by adjuvant chemotherapy.

The pathological parameters and ACE genotypes and alleles in patients with premenopausal breast cancer are shown in Table 2. The frequency of the DD genotype was higher in patients with negative hormone receptor status (11 patients [57.9%]) than those of ID and II genotypes (7 patients [36.8%] had ID genotype and only one patient [5.3%] had II genotype, respectively) (OR = 1.08, 95% CI = 0.32-3.60, $p = 0.57$). Similarly in patients with lymph node involvement, tumor size greater than 2 cm, high histological grade (grade III) and c-erb B2 overexpression, the percentage of DD genotype was higher than those of ID and II genotypes ([57.7%, 34.6% and 7.7%, $p = 0.30$]; [69%, 27.6%, and 3.4%, $p = 0.02$]; [61.1%, 33.3%, and 5.6%, $p = 0.75$]; [62.5%, 37.5%, 0%, $p = 0.65$], respectively); but the only the statistically significant difference was observed in patients with tumor size greater than 2 cm. However, no significant relation was found between tumor size and other poor prognostic factors. There was also no correlation between the number of lymph node metastases and ACE genotypes or alleles. Furthermore, DD genotype was observed in higher frequency in patients with disease recurrence and none of them had II genotype.

Similarly, the frequency of D allele tended to be higher in hormone receptor negative tumors (76.3%) ($p = 0.58$), and in tumors with high histological grade (73.3%) ($p = 0.48$), tumor size greater than 2 cm (80%) ($p = 0.16$), lymph node involvement (75%) ($p = 0.76$) or c-erb B2 overexpression (81.3%) ($p = 0.43$), although the differences were not statistically significant. Also, in patients with recurrent disease (only 12 patients), D allele was observed higher than I allele (83% vs 17%; OR = 1.81, 95% CI = 0.54 - 6.05, $p = 0.25$).

Median follow-up time for all patients was 32 (range: 4-131) months. Twelve (27.3%) patients had a recurrent disease while 3 cases were dead. The 5-year survival rate for this group was 78%. In a subgroup analysis based on the ACE genotypes, the disease-free survival (DFS) rate and median DFS time in patients with DD genotype were 68% and 62 months (95% CI: 51.3-73.6), respectively. For the II/ID genotype, DFS rate was 78% and median DFS was 74 months (95% CI: 45.4-121.6); but this DFS

TABLE 2. Correlation between ACE genotypes/alleles and the pathological parameters in breast cancer patients.

	ACE genotypes		*p* value	ACE alleles		*p* value
	DD	ID/II		D	I	
LN metastases (absent/present)	10/15	8/11	$p = 0.56$	28/39	8/13	$p = 0.48$
Tumor size (≦ 2 cm/ > 2 cm)	5/20	10/9	$p = 0.02^*$	19/48	9/12	$p = 0.16$
Histological grade (1-2/3)	14/11	12/7	$p = 0.43$	39/28	13/8	$p = 0.76$
ER and/or PR status (negative/positive)	11/14	8/11	$p = 0.57$	29/38	9/12	$p = 0.58$
c-erb B2status (scor 0-1/2-3)	20/5	16/3	$p = 0.51$	54/13	18/3	$p = 0.43$
Number of LN involvement (0-3/4-9/ ≥ 10)	14/7/4	11/7/1	$p = 0.20$	39/19/9	11/9/1	$p = 0.30$

LN, Lymph node.
$^*p < 0.05$.

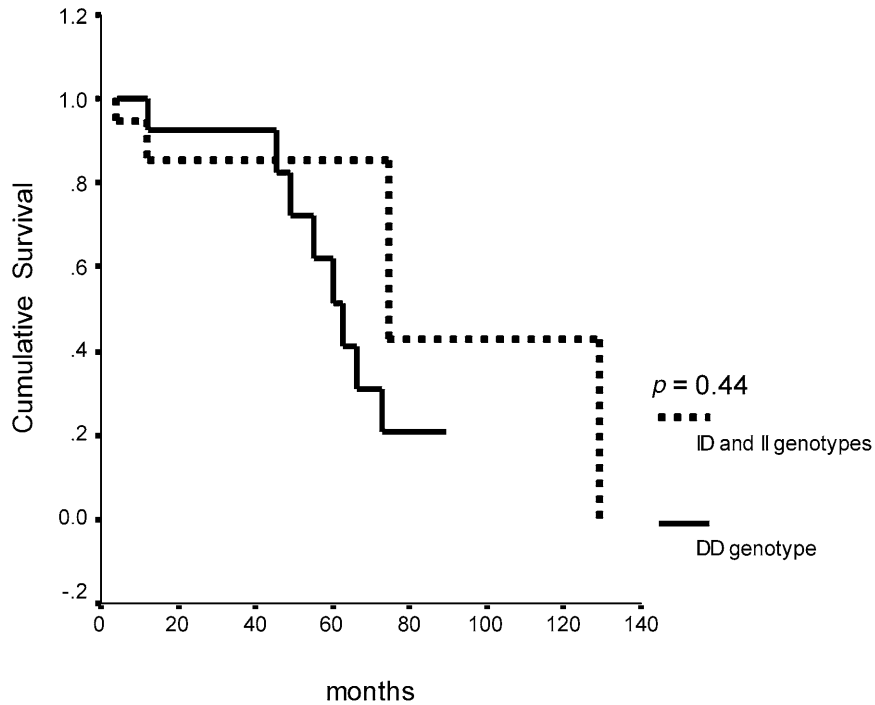

Fig. 2. The disease free survival rates of premenopausal breast cancer patients according to ACE gene polymorphism (DD genotype and ID/II genotypes).

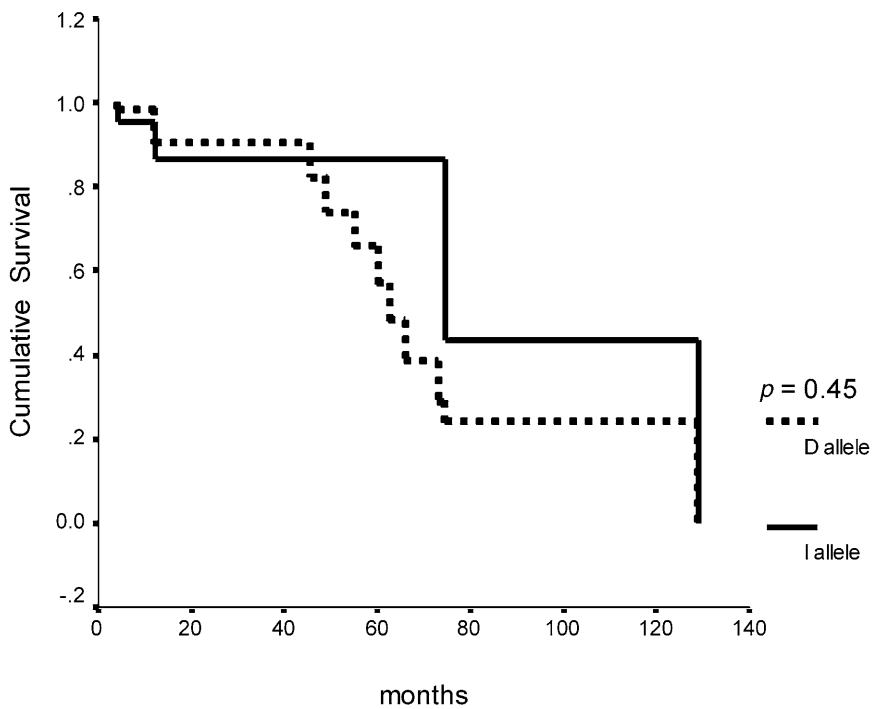

Fig. 3. The disease-free survival rates of premenopausal breast cancer patients according to ACE gene polymorphism (D and I alleles).

difference between DD and II/ID genotypes did not have any statistical significance ($p = 0.44$) (Fig. 2). For the D and I allele, DFS rates were 70% and 80%, respectively, and median DFS was shorter in D allele group than I allele group (62 months vs 74 months) (Fig. 3).

Discussion

In the present study, we studied the frequency of the ACE genotypes and alleles and the association between the ACE genotypes or alleles and poor prognostic factors in premenopausal breast cancer patients. The distribution of the ACE genotypes and alleles was similar in premenopausal breast cancer patients and in healthy controls. In breast cancer studies, it has been reported that DD genotype was seen more commonly. The DD carriers were shown to have a significantly increased risk, an odds ratio was 1.86 (Ladd et al. 2005). In contrast, low activity genotypes such as II genotype exhibited lower breast cancer risk, an odds ratio was 0.46 (Koh et al. 2003). In epidemiological studies, ACE gene polymorphisms, including I/D polymorphism were associated with increased breast cancer risk (Koh et al. 2003, 2005) except the Multiethnic Cohort Study (Haiman et al. 2003). The Multiethnic Cohort Study showed that in contrary to the expectations, women with II genotype had increased breast cancer risk (Haiman et al. 2003). Taken together, genetic polymorphisms may affect the development of breast cancer and may be helpful for a better understanding of the molecular epidemiology of breast cancer.

The poor prognostic factors in primary breast cancer in clinical practice are axillary lymph node involvement, large tumor size, high nuclear or histologic grade, negative hormone receptor status, overexpression of Her2/neu and high mitotic index. There are few studies about gene polymorphism and prognostic factors in breast cancer. The polymorphisms in the vascular endothelial growth factor (VEGF) gene (-634 CC genotype and -2578/-634 CC haplotype) were significantly associated with large tumor size and high histologic grade but not associated with regional or distant metastasis, stage at diagnosis, and hormonal receptor status (Jin et al. 2005). It was also found that BRCA2, microsatellite instability, and interleukin 6 gene polymorphisms were associated with tumor size, lymph node involvement, stage of disease, hormonal status and metastatic disease in breast cancer (Tomita et al. 1999; DeMichele et al. 2003; Ishitobi et al. 2003). In our study, there was no significant association between the ACE gene polymorphism and poor prognostic factors, except large tumor size. A possible explanation for our findings may be the role of ACE in the development of breast cancer, because this gene is associated with other important genes involved in the process of oncogenesis. High level of ACE and Ang II increased microvessel density, promoted angiogenesis and tumor cell proliferation due to VEGF gene overexpression (Yoshiji et al. 2001, 2002b). In addition, it has been suggested that Ang II can enhance transforming growth factor-beta 1, platelet derived growth factor and basic fibroblast growth factor (Itoh et al. 1993). On the other hand, it was known that estradiol was a more potent regulator of free VEGF levels, one mechanism involved in breast carcinogenesis; therefore, angiogenesis increased in premenopausal status (Dabrosin 2005). A significant correlation between the ACE genotype and large tumor size may indicate that ACE influences the local tumor growth in premenopausal breast cancer.

In various cancer types, it has been reported that ACE gene polymorphism was associated with some prognostic parameters. In gastric cancer patients, the number of the lymph node metastases correlated with DD genotype but there were no correlation between tumor type, tumor location, local tumor growth, distant metastasis and I/D gene polymorphism (Röcken et al. 2005). The patients with prostate cancer who had DD genotype had increasingly presented with advanced disease (Medeiros et al. 2004).

Survival analysis performed by Kaplan-Meier method in our study showed that the disease free survival was reduced in DD genotype or D allele compared with ID/II genotype or I

allele. It was reported that cancer-free survival was significantly reduced in patients with DD genotype compared with II genotype in a breast cancer study (Ladd et al. 2005). In the other studies with prostate cancer (Medeiros et al. 2004) and leukemia patients (Hajek et al. 2003), decreased survival time was also reported in patients who had DD genotype. Therefore, I/D gene polymorphism of the ACE might have an important role in survival of the breast cancer patients; but unfortunately we are unable to reach definite conclusions with short follow-up.

In conclusion, the present study showed that premenopausal breast cancer patients did not differ from the healthy subjects in terms of the I/D polymorphism of the ACE gene, but the DD genotype may account for large tumor size, which is poor prognostic factor in breast cancer. Recently, it was shown that ACE inhibitors had a protective effect against breast cancer (Meier et al. 2000; Friis et al. 2001; Grossman et al. 2001; Abali et al. 2002; Li et al. 2003) and had an inhibitory effect on tumor growth and angiogenesis in experimental studies (Small et al. 1997; Yoshiji et al. 2002a, b). These studies suggest the possibility that ACE inhibitors may be useful as additional treatment strategies in breast cancer. In any case, hormone positive patients used hormonal treatment such as tamoxifen and aromatase inhibitors principally based on the menopausal status. In hormone negative patients, no drugs were used to prevent disease recurrence. Based on these preliminary results, we considered the possibility that ACE inhibitors might have a role in premenopausal breast cancer patients. Confirmation of this possibility with further studies may provide the opportunity of preventing the recurrence in patients with breast cancer.

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