Int J Clin Exp Med 2016;9(6):11756-11763
www.ijcem.com /ISSN:1940-5901/IJCEM0023460

Original Article

TS 1494del6 polymorphism and increased risk of developing breast cancer

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Received January 7, 2016; Accepted May 4, 2016; Epub June 15, 2016; Published June 30, 2016

Abstract: Thymidylate synthase (TS) is a critical enzyme involved in folate metabolism and catalyses reductive methylation of deoxyuridylate to thymidylate, which is the essential precursor for DNA. Polymorphisms in genes involved in folate metabolism may influence DNA methylation, DNA synthesis and repair. These functions may provide insight individual susceptibility to cancer. We investigated the probable effects of TS 1494del6 polymorphism on the risk of developing breast cancer. Genotypes of 298 cases and 300 controls were determined by use of PCR-RFLP. Variant -6 bp allele frequency was significantly higher in women with breast cancer than in controls (0.554 vs. 0.467; OR: 1.42, 95% CI: 1.13-1.78; P=0.003). The frequency of the -6 bp/-6 bp genotype was 12% in control group, while increased to 26.8% in women with breast cancer. Similarly, increased risk was observed for breast cancer of the -6 bp/-6 bp genotype compared with the +6 bp/+6 bp genotype (OR 2.59, 95% CI: 1.49-4.49; P=0.001). The risk increased in women with -6 bp/-6 bp genotype as well as age ≥55 (OR, 3.06; 95% CI: 1.15-8.07; P=0.024), BMI ≥25 kg/m^2 (OR, 2.81; 95% CI: 1.45-5.46; P=0.002), early menarche (before age 13 years) (OR, 3.52; 95% CI: 1.37-9.04; P=0.009), late age at first pregnancy (OR, 2.87; 95% CI: 1.77-4.64; P=0.0001), postmenopausal status (OR 5.44; 95% CI: 2.23-13.24; P=0.001), and negative smoking history (OR=3.09; 95% CI: 1.87-5.09; P=0.001). In conclusion, the TS 1494del6 polymorphism gene may contribute to an increased risk for breast cancer development, particularly in the presence of 55 years of age or older, high BMI, early age at menarche, late age at first pregnancy, postmenopausal status, and negative smoking history.

Keywords: Breast cancer, thymidylate synthase, TS 1494del6, polymorphism

Introduction

Breast cancer (BC) is the most common form of cancer among women worldwide, accounting for 37% of the cancer related deaths in females between the ages of 25-64 [1]. In recent years risk factors for BC have been identified, although the causes of breast cancer aren't fully understood. However, it is generally believed that hereditary, hormonal, reproductive, and environmental factors play an important role in the development and progression of this cancer [2].

Folate is an essential cofactor for DNA synthesis, repair and methylation [3]. When levels of 5,10-methylene-tetrahydrofolate, which is required to convert deoxyuridylate to thymidylate, are inadequate, misincorporation of uracil for thymidine may occur during DNA synthesis, possibly increasing aberrant DNA methylation, micronucleus formation and chromosomal breaks [4]. These genetic changes can further result in chromosomal instability and increase the frequency of mutation events [5].

Thymidylate synthase (TS), encoded by the TS gene located on chromosome 18p11.32, is known to be involved in folate metabolism [6]. The TS gene has several genetic polymorphisms (rs34743033, rs2853542 and rs34489327) in the 5'- and 3'-untranslated regions (UTRs) that can influence TS transcription and expression [7]. Polymorphism rs34743033 is a 28 base pairs (bp) variable number tandem repeat (VNTR) in 5'-UTR immediately up-

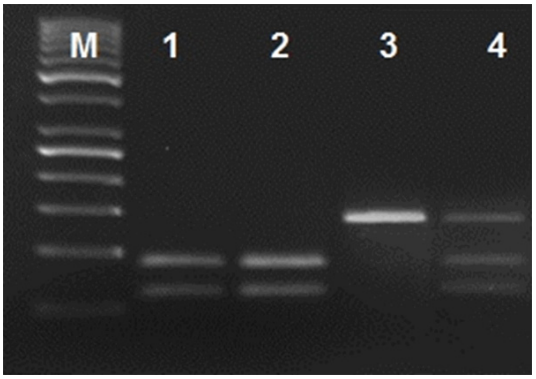

Figure 1. TS 1494del6 genotyping by PCR-RFLP (+6: 70 and 88 bp, -6: 152 bp). Lane 1-2: +6/+6, Lane 3: -6/-6 and Lane 4: +6/-6. M: 50-bp ladder.

stream of the ATG codon initiation site that contains triple (TSER 3R) or double (TSER 2R) repeats of a 28-bp sequence as well as several rare alleles containing 4, 5 or 9 repeats [8]. The presence of the triple (TSER 3R) versus double (TSER 2R) 28-bp repeat sequence has been shown to enhance TS transcription and TYMS expression level in in vitro and in vivo studies [8, 9]. A common G->C polymorphism (rs2853542) in the second repeat of 3R alleles that alters translation of TS gene [10]. Another TS polymorphism (rs3448-9327), a 6-bp deletion/insertion (TAAAGT) in the 3'-UTR, may modulate TS expression levels. Previous one study suggested that a deletion of 6 bp in the 3'-UTR affects a region of TS premessenger ribonucleic acid (mRNA) that contains cis adenylateuridylate-rich elements (AREs). These elements bind to a trans AU-rich factor 1 (AUF1). AUF1, also named hnRNPD (heterogeneous nuclear ribonucleoprotein D), has a destabilizing effect on ARE-mRNAs. The 6-bp deletion at position 1494 in the TS affects mRNA stability or secondary mRNA structure and could thus ultimately affect protein levels of TS. Specifically, increased TS activity may might affect the levels of homocysteine, which is associated with cancer risk [11-14].

Germline BRCA1 and BRCA2 mutations are responsible for 5-10% of all cases of breast cancer, whereas most of breast cancer are sporadic, resulting from polymorphic low-penetrance genes. The primary aim of the current study was to determine whether there was association between TS 1494del6 polymorphism and risk of breast cancer for the Turkish population in the Mersin sample. The secondary aim of the study was to evaluate any role of putative risk factors, such as BMI, age at first pregnancy, age at menarche, family history, menopausal status, and smoking status in breast-cancer risk in Turkish women.

Materials and methods

Study subjects

A total of 598 participants consisting of 298 women with breast cancer and 300 controls matched for region, sex, and age were enrolled in this study. Breast cancer cases comprised women who had been diagnosed with breast cancer at the Department of Medical Oncology, Mersin University Faculty of Medical and Kahramanmaraş Necip Fazil City Hospital, Turkey, between 2010 and 2015. Healthy volunteer women were randomly chosen from the general population of the same area and 10% of the controls had a history of personal or familial malignancy. Interview response-rates among eligible case and control participants were 96.9% and 93.1%, respectively. This study had received Ethical Approval from the Mersin University Medical Faculty Ethics Committee and informed consents were given. Detailed information on risk factors including age, reproductive risk factors (age at menarche, age at first pregnancy, menopausal status), family history of breast cancer (first-degree relatives) and smoking status were obtained with a baseline questionnaire. The body-mass index (BMI) was calculated from the height and weight of the women by use of the formula BMI=(kg/m^2).

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes by using a standard (phenol-chloroform) method [15]. The polymorphism was analyzed by using of PCR/RFLP. The primary sequences for the TS 1494del6 forward and reverse primers were (5'-CAA ATC TGA GGG AGC TGA GT-3') and (5'-CAG ATA AGT GGC AGT ACA GA-3'), respectively. Genomic DNA was amplified in a 25 μL reaction mixture containing 100 ng DNA, 0.3 nM of the primers, 0.2 nM dNTP mix, 10× PCR buffer, 2.5 mM $MgCl_2$, and 1 U Taq polymerase (MBI Fermentas, Vilnius, Lithuania). The samples were initially denatured for 5 min at 94°C followed by 30 cycles of denaturation for 30 s at 94°C, 45 s at 58°C and extension for 45 s at 72°C, with a

Table 1. Comparison of cases and controls according to demographic factors and putative risk factors for breast cancer

Variables	Cases (n=298)	Controls (n=300)	P
Age (years [mean ± SD])	50.64±10.27	49.14±8.37	0.510
Age at menarche (years [mean ± SD])	13.31±3.38	13.35±1.23	0.830
Age at menopause (years [mean ± SD])	48.17±6.19	46.65±5.28	0.036
Body-mass index (years [mean ± SD])	28.81±5.28	26.84±5.12	0.001
Age at first pregnancy (years [mean ± SD])	22.38±3.33	22.49±4.87	0.753
Family history (%)			
No	77.2	90	0.001
Yes	22.8	10	
Smoking status			
Never smokers (%)	76.5	77.3	0.811
Current smokers (%)	23.5	22.7	

Table 2. Multivariate logistic regression results of genotypes among breast cancer cases and controls

	Cases (n=298)	Controls (n=300)	OR (95% CI)[a]	P
Genotype frequencies				
+6/+6	48 (16.1)	56 (18.7)	1	
+6/-6	170 (57.0)	208 (69.3)	0.95 [0.61-1.47]	0.830
-6/-6	80 (26.8)	36 (12.0)	2.59 [1.49-4.49]	0.001
Allele frequencies				
+6	266 (44.6)	320 (53.3)	1 (Reference)	
-6	330 (55.4)	280 (46.7)	1.42 [1.13-1.78]	0.003

[a]ORs and CIs are adjusted according to BMI and family history.

final extension for 5 min at 72°C [11]. Amplicons (152 bp) were digested with 4 U *Dra*I (Thermo Scientific Fermentas) by overnight incubation at 37°C. Restriction fragments were analyzed following electrophoresis in 3% agarose gel stained with ethidium bromide (0.5 μg/mL). The expected fragment sizes were 70 and 88 bp for the wild-type allele (+6 bp/+6 bp) and 152 bp for the variant allele (-6 bp/-6 bp) (**Figure 1**).

Statistical analysis

Differences in demographic characteristics, selected variables and frequencies of the genotypes, alleles of the TS 1494del6 polymorphism between the two groups were evaluated using the Chi-square test (for categorical variables) and Student's t test (for continuous variables). Hardy-Weinberg equilibrium was tested by using Chi-square test. We calculated odds ratios and 95% confidence intervals using multivariate logistic regression. Statistical analyses were performed with SPSS for windows version 22. *P* value <0.05 is accepted as statistically significant.

Results

The distribution of demographic factors and putative risk factors for breast cancer are shown in **Table 1**. The mean age of the patients was 50.64±10.27 years and the mean age of the control group was 49.14± 8.37 years. Compared with control subjects, breast cancer patients had a higher BMI (P=0.001) and higher age at menopause (P=0.036). There were 68 (22.8%) breast cancer cases and 30 (10%) controls reported a family history of cancer, which was associated with a significantly increased risk for breast cancer (P= 0.001). However, no significant differences were found between cases and controls for late age at first pregnancy (P= 0.753), early age at menarche (P=0.83), and smoking status (P=0.811).

The distribution of genotypes and the prevalence of alleles in the cases and controls were shown in **Table 2**. The -6 bp allele frequency was higher in the cases than in the controls (P=0.003). TS 1494del6 genotype frequencies were 16.1% (+6 bp/+6 bp), 57% (+6 bp/-6 bp), and 26.8% (-6 bp/-6 bp), respectively, in cases and 18.7% (+6 bp/+6 bp), 69.3% (+6 bp/-6 bp), and 12% (-6 bp/-6 bp), respectively, in control subjects, and these differences were statistically significant (P=0.001). Compared with the wild-type variant (+6 bp/+6 bp), a significantly increased risk was associated with the homozygous variant (-6 bp/-6 bp) (adjusted OR=2.59, 95% CI=1.49-4.49), but it was not related with (+6 bp/-6 bp) heterozygous genotype (OR=0.95, 95% CI=0.61-1.47). We observed significant

Table 3. Distribution of TS 1494del6 genotypes (case/control) and ORs (95% CI) for breast cancer according to putative risk factors

Risk factors		Genotype		
		(+6 bp/+6 bp)	(+6 bp/-6 bp)	(-6 bp/-6 bp)
Age	≤44	1.0 (Ref.)	0.66 (0.29-1.52)	2.54 (1.17-5.52)
		14/14	48/72	22/12
	45-54	1.0 (Ref.)	1.41 (0.70-2.83)	2.81 (1.50-5.27)
		16/28	68/84	38/18
	≥55	1.0 (Ref.)	0.96 (0.44-2.08)	3.06 (1.15-8.07)
		18/16	54/50	20/6
BMI	<25	1.0 (Ref.)	0.91 (0.39-2.12)	2.57 (0.91-7.21
		10/20	42/92	18/14
	≥25	1.0 (Ref.)	1.12 (0.67-1.88)	2.81 (1.45-5.46)
		38/38	128/114	62/22
Age at menarche	<13	1.0 (Ref.)	0.65 (0.25-1.65)	3.52 (1.37-9.04)
		18/8	56/38	34/6
	≥13	1.0 (Ref.)	1.31 (0.67-1.88)	2.32 (1.40-3.85)
		30/50	114/168	46/30
Age at first pregnancy	<20	1.0 (Ref.)	0.77 (0.33-1.80)	0.90 (0.25-3.19)
		12/18	32/62	4/8
	≥20 or no birth	1.0 (Ref.)	1.06 (0.64-1.76)	2.87 (1.77-4.64)
		36/40	138/144	76/28
Family history	No	1.0 (Ref.)	1.11 (0.69-1.80)	2.61 (1.66-4.11)
		36/56	128/178	66/36
	Yes	1.0 (Ref.)	NC	NC
		12/2	42/28	14/0
Menopausal status	Premenopausal	1.0 (Ref.)	1.21 (0.61-2.41)	2.35 (1.34-4.11)
		14/34	66/132	34/30
	Postmenopausal	1.0 (Ref.)	0.99 (0.54-1.81)	5.44 (2.23-13.24)
		34/24	104/74	46/6
Smoking status	Never smokers	1.0 (Ref.)	1.01 (0.59-1.71)	3.09 (1.87-5.09)
		30/38	134/168	64/26
	Current smokers	1.0 (Ref.)	1.05 (0.48-2.30)	1.71 (0.71-4.11)
		18/20	36/38	16/10

NC: Not calculated.

deviation from Hardy Weinberg equilibrium in both control and case groups (Respectively P=0.001, P=0.001).

Associations between breast cancer and putative risk factors by TS 1494del6 genotypes are presented in **Table 3**. Women 55 years of age and older than in those younger than 55 have demonstrated that homozygosity for the -6 bp allele was associated with an increased risk for breast cancer (OR=3.06; 95% CI: 1.15-8.07; P=0.024). The OR was elevated for the -6 bp/-6 bp genotype and a BMI ≥25 kg/m^2 (OR=2.81; 95% CI: 1.45-5.46; P=0.002). Women carrying the -6 bp/-6 bp genotype with menarche at age younger than 13 had 3.52-fold increased risk for breast cancer (OR=3.52; 95% CI: 1.37-9.04; P=0.009). An increased risk for breast cancer was found in women who carried the -6 bp/-6 bp genotype and who were also more susceptible to estrogen [no history of pregnancy or older (≥20 years) at first full-term pregnancy] (OR=2.87; 95% CI: 1.77-4.64; P=0.0001). There was statistically significant evidence that postmenopausal status was associated with an increased breast cancer risk in women carrying the -6 bp/-6 bp genotype (OR=5.44; 95% CI: 2.23-13.24; P=0.001).

Never smokers carrying the -6 bp/-6 bp genotype had significant increased breast cancer risk compared with smoking women with the -6 bp/-6 bp genotype (OR=3.09; 95% CI: 1.87-5.09; P=0.001).

Discussion

In this population-based case-control study, we examined the association of TS 1494del6 polymorphism in the 3'-UTR of the TS gene with risk of breast cancer in a Turkish population. Furthermore, we investigated whether putative risk factors influence breast-cancer development.

In the study of Akisik and co-workers [16], the -6 bp genotype incidence was 19.85% in a Turkish population. In our study, prevalence of the -6 bp genotype was 12.0% in a Turkish population. While the prevalence of the -6 bp allele was found as 36.4% [17], we detected it as 46.7% in a Turkish population. The -6 bp allele frequency in Asians was 71.0% (95% CI=67.7-74.3%), significantly higher than that in Caucasians (33.5%, 95% CI=31.1-35.8%, P< 0.001) [18]. The -6 bp allele frequency found in our controls (n=280) was slightly higher at 0.335 than Caucasians. As given above, there are significant ethnic differences in the frequency of the TS 1494del6 polymorphism. This discrepancy may reflect the limited sample size included in some studies. Genotyping of large populations is being used to evaluate disease and gene associations.

The deletion of 6 bp in the 3'-UTR may influence TS mRNA transcription, mRNA stability or protein expression levels. Altered enzyme levels could affect cancer susceptibility as well as treatment efficacy [11]. One study shows that TS 3'-UTR 6 bp insertion was associated with better clinical outcome by FU-based chemotherapy in advanced gastric cancer (OR=0.41; 95% CI, 0.22-0.76; P=0.01) [19], another study showed that -6 bp carriers were associated with better survival at 12 months when compared to +6 bp homozygotes lung cancer patients (P=0.006) [20]. On the other hand, one of the study, authors did not observe an association between the deletion polymorphism at the TS 3'-UTR and survival of colorectal cancer patients [21].

In the current study, we observed a strong association between the heterozygotes for the -6 bp allele and breast cancer risk factors among women with breast cancer (OR=1.42; 95% CI; 1.13-1.78; P=0.003). To date, many epidemiological studies have evaluated the polymorphisms in the 5'-UTR region of the TS gene with the risk of cancer such as blood cancers [22, 23], lung cancer [24], gastric cancer [25] and colorectal cancer or adenoma [26-28] but the results are conflicting. It was also found that homozygosity for the TS 1494del6 polymorphism (-6 bp/-6 bp) was associated with an increased risk of ALL (OR=1.46; 95% CI; 1.02-2.08; P=0.04), B-lineage ALL (OR= 1.44; 95% CI, 1.00-2.08; P=0.05), and AML (OR=2.04; 95% CI; 1.03-4.03; P=0.04) [23]. Similarly, results of the other study shows that the -6 bp allele was associated with an increased risk of gastric cancer [25]. However, in another study it was observed that the highest risk (OR=1.52) for lung cancer was associated with the presence of the 6 bp/0 bp + 6 bp/6 bp genotypes [24]. On the other hand, in some studies no differences in the distribution of genotypes between cases and controls were observed [26-28]. Several studies have been conducted to evaluate the association of the TS gene polymorphism and breast cancer risk. The results, however, are conflicting. One study showed that the TS 3'-UTR +6 bp/+6 bp genotype was associated with a significantly reduced risk of breast cancer in a Chinese population [29], another study showed that the homozygous variant genotype (-6 bp/-6 bp) was associated with an decreased risk for breast cancer in Turkish population [16]. A meta-analysis conducted in 2011, which included 10 eligible studies, concluded that the -6 bp/-6 bp genotype might be a risk factor for breast cancer susceptibility in Asian individuals [30]. This result was similar to those of our study. On the other hand, one study has investigated the association between TS polymorphism and TS protein expression in normal and tumour tissue specimens from 49 lymph node-positive breast cancer patients. The frequency of -6 bp/-6 bp genotype of the TS 1494del6 polymorphism was found to be higher in the tumour (27%) than the normal tissue specimens (4%). The level of TS protein expression has been observed higher in cancer than in normal tissue specimens, regardless of the TS 3'-UTR genotype. Moreover, the TS protein expression in cancer tissue specimens was significantly higher in tumors with the +6 bp/+6 bp genotype

than did those with other genotypes (P<0.05). In agreement with others [12, 13] these results may be explained by a 6-bp deletion at nucleotide 1494 in the TS is associated with decreased mRNA stability and lower intratumoural TS expression [31].

In this study, the -6 bp/-6 bp genotype was associated with higher breast cancer risk in women but significantly higher in women 55 years of age and older than women aged <55 years. Additional to our study, a case-control study was conducted among 432 cases and 473 controls in a Chinese population and reported a higher risk of breast cancer among older women with the -6 bp/-6 bp genotype [29]. There is substantial evidence that estrogens play a role in the etiology of breast cancer. Several mechanisms have been suggested to account for this, including: (i) after the estrogen binds to its receptors in a cell, it stimulates breast cell proliferation through direct and indirect actions on the enhanced production of growth factors; (ii) estrogen metabolites, catalyzed mainly by cytochrome P450 enzymes, exert direct genotoxic effects that might increase mutation rates; and (iii) estrogen is effective inducer of aneuploidy [32]. Our study detected an association between the -6 bp/-6 bp genotype and breast cancer risk among premenopausal and postmenopausal women but significantly higher in postmenopausal than premenopausal. One study also reported an increased risk of breast cancer among postmenopausal women with -6 bp/-6 bp genotype [29]. On the contrary, in other study it wasn't observed significant modification of ORs for -6 bp/-6 bp genotype by menopausal status [33]. One of the reproductive risk factors, such as late age at first pregnancy was associated with breast cancer risk with an odds ratio of 2.87 (95% CI, 1.77-4.64). The risk was higher among women with the -6 bp/-6 bp genotype who had early menarche age (<13 years). In contrast, one study observed association between reproductive factors, including older age at menarche and younger age at first-live birth and breast cancer risk [29]. Obesity, an abnormal white adipose tissue accumulation, is related to the increased peripheral conversion of androgenic precursors to estradiol due to increased aromatase enzyme activity from large amounts of adipose tissue and is also related to decrease sex hormone-binding globulin [34]. Changes in endogenous sex steroid hormone levels may play a role in the development of breast cancer. Our results showed an increase in the risk for breast cancer in individuals -6 bp/-6 bp genotype with women BMI ≥25 kg/m^2. The association of breast cancer risk and the -6 bp/-6 bp genotype with women BMI ≥25 kg/m^2, is in consistent with one of the study [33]. Cigarette smoking, which decreases age at menopause, appears to have anti-estrogenic effect and might protect against breast cancer [35]. In the present study, we found that the -6 bp/-6 bp genotype was associated with a 3-fold increased risk of breast cancer among women who have never smoked.

In conclusion, we confirmed the observation that TS 1494del6 polymorphism in the 3'-UTR of the TS gene may be a risk factor for developing breast cancer in women with age ≥55, postmenopausal status, BMI ≥25 kg/m^2, negative smoking history, late age at first full-term pregnancy and early menarche age. The result of this study emphasizes the importance of assessing both genetic and putative risk factors for breast carcinogenesis.

Acknowledgements

The author thanks Seval KUL for assistance with statistical analysis.

Disclosure of conflict of interest

None.

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