RESEARCH ARTICLE

Germ-line *MTHFR* C677T, *FV* H1299R and *PAI*-1 5G/4G Variations in Breast Carcinoma

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Abstract

Background: Various oncogenes related to cancer have been extensively studied and several polymorphisms have been found to be associated with breast cancer. The current report outlines analysis of germ-line polymorphisms for C677T, A1298C (*MTHFR*), Leiden, R2 (*FV*) and 5G/4G (*PAI*-1) in Turkish breast cancer patients. We studied 51 cases diagnosed with invasive ductal and operable with lymph node-positive breast cancer and 106 women as a control group. Materials and Methods: Peripheric blood-DNA samples were used for genotyping by StripAssay technique which is based on the reverse-hybridization principle and real-time PCR methods and results were compared statistically. Results: The frequency of the *MTHFR* gene 677T and 1298A alleles were significantly higher in cancer patients than in the healthy subjects. The T allele frequency in codon 677 was 2.3-fold and C allele frequency was 3.1-fold increased in BC when compared to the control group for the *MTHFR* gene. Both differences were statistically significant (OR: 2.295, CI: 1.283-4.106), p<0.006 and (OR: 3.131, CI:1.826-5.369), p<0.0001 respectively. The R2 allele frequency of *FV* gene was 5.1-fold increased in the current BC when compared to the control group and that difference was also statistically significant (OR: 5.133, CI: 1.299-20.28), p<0.02. Conclusions: The present data suggest that germ-line polymorphisms of C677T, C1298A for *MTHFR* and R2 for *FV* are associated in breast cancer and may be additional prognostic markers related to breast cancer survival. The results now need to be confirmed in a larger group of patients.

Keywords: MTHFR C677T and A1298C - FV Leiden and R2 - PAI-1 5G/4G; SNPs - breast carcinoma

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Introduction

Breast cancer (BC) is one of the most frequent diseases in women that influenced by environmental, genetic and epigenetic factors. Complex genetic and environmental components such as; diet composition, genotoxic and carcinogenic agents that effects the disease and most of them are still unknown. An important role of gene polymorphisms related to the risk of developing BC has been reported but results are still controversial. Approximately 25% of breast cancers are inherited by germ-line mutations in functional and/or oncogenes (Akbari et al., 2013; Carraro et al., 2013; Sohail et al., 2013). As claimed by Bonache et al there are various involvement of additional genetic susceptibility in BC (Bonache et al., 2013). Population based mutation type was reported in BRCA genes in BC (Akbari et al., 2013). In general, the tumoural samples include point mutations in BRCA1 and BRCA2 genes but Nomizu et al were also reported the germ-line deletions in both BRCA1 and BRCA2 genes in Japanese breast/ovarian cancer patients (Nomizu et al., 2012). Epigenetic alterations in cancer-related genes are recognized to play an important role in BC carcinogenesis. Epidemiological studies have consistently supported that cancer is related not only to mutations in functional genes but also related to the aberrant epigenetic modifications of various genes (Chang and Sharan, 2012; Jeong et al., 2013; Martin, 2013; Zheng et al., 2013). Altered DNA methylation profiles play crucial role in miRNAs process and this are important regulators of gene expression that are frequently deregulated in cancer. Vrba et al have identified altered miRNA counts in promoter regions of some tumour supressor genes and claimed that the promoter subunits are targets of aberrant DNA methylation in human breast tumour samples (Vrba et al., 2013). The *MTHFR* gene regulates DNA methylation by affecting synthesis of S-adenosylmethionine, which is a universal methyl donor for primer modification of the doughter strand DNA. Two SNP markers in the *MTHFR* gene (C677T and A1298C) have been associated with reduced enzyme activity, thereby making *MTHFR* polymorphisms a potential

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candidate cancer-predisposing factor due to genomic DNA hypomethylation, hyperhomocysteinaemia and atherosclerosis (de Cassia et al., 2012; Jiao and Lack, 2013; Muzeyyen, 2013). It was also reported that *MTHFR* C677T polymorphism effectes the C-erbB-2 methylation status and altered methylation profiles associated with some type of cancers (Henriquez et al., 2010). Literature findings have also addressed the association between some thrombophilic gene polymorphisms and BC, but the mechanism in the risk of developing breast cancer remains controversial (Yagmurdur et al., 2008; Hildenbrand et al., 2009; Eljuga et al., 2011). Common SNPs in three thrombophilic genes were studied in the current BC cohort. The 5G/4G promoter polymorphism of the plasminogen activator inhibitor-1 (*PAI*-1) is potentially involved in regulating gene transcription. Xu et al have claimed that polymorphic 4G SNPs in *PAI*-1 gene plays an important role in the pathogenesis of breast cancers (Xu et al., 2012). Descotes et al reported that *PAI*-1 is an independent prognostic factor in particular in pN0 breast ductal carcinoma (Descotes et al., 2008). High expression of gene due to 4G/4G polymorphism plays a crucial role in metastatic behavior by increasing cells' migratory, tumoral growth and angiogenesis by extracellular matrix remodeling (Meryet et al., 2007). A multidomain glycoprotein is encoded by *FV* gene that localised on chromosome 1q23 associated with active protein C resistance. The transition point mutation in codon 1691G>A causes to the mutated protein of Factor V and resistant to activated protein C (APC) cleavage (Ozturk et al., 2012; Valjevac et al., 2013). A common *FV*R2 haplotype has been associated with a reduced cofactor activity in activated protein C-mediated activated factor VIII inactivation (Martinelli et al., 2007) but association with cancer was not reported yet. The analysis of common thrombophilic germ-line polymorphisms as prognostic factors in patients with invasive ductal breast cancer was aimed in the current report. There are conflicting results that rangin from strong linkage and no association about germ-line functional gene variations and BC risk in different literature findings. In the present study, we aimed to investigate the *MTHFR* C677T, A1298C, *FV* Leiden and H1299R (R2) and *PAI*-1 gene polymorphisms in breast cancer patients and compare to the healthy subjects from the same population using PCR based StripAssay and Real-time techniques.

Materials and Methods

Patients, clinical diagnosis and laboratory assessment

Totally, 51 peripheral blood samples from patients with breast carcinomas (BC); 39 (76.5%) invasive ductal carcinoma (IDC) 6(11.8%) invasive lobular carcinoma (ILC), 5 (9.8%) medullary carcinoma (MC) and one (1.9%) tubular carcinoma (TC) were investigated for germ-line thrombophilic gene mutations. The mean age, min-max was [51.55±7.01(43-76)] for the current studied cohort. The solid tumoural tissue samples from each patients were used for profilling the transcriptome analyses for clinical follow-up and treatment (data not shown) and peripheral blood-EDTA samples were also used for germ-line thrombophilic gene analysis in the current cohort. The tumoural and peripheral blood samples were obtained during routine diagnosis from BC patients in Cumhuriyet University Training and Research Hospital by the collaboration of department of medical genetics and medical oncology between May 2008 and January 2010. Blood samples were used for genotyping for point mutations of *FV* leiden, *FV* R2, *MTHFR* C677T and *MTHFR* A1298C genes. Informed consent was obtained from all patients and the germ-line mutation profiles were compared to the healthy women from the same population that was used in our previous study (Ozdemir et al., 2012).

Mutation analysis

Peripheric blood tissues containing EDTA from patients and control group were used for genomic DNA isolation. The total genomic DNA was extracted by the MagnaPure Compact (Roche) and Invitek kit extraction techniques (Invitek®; Invisorb spin blood, Berlin, Germany). Target genes were simultaneously amplified in a biotin-labelled single multiplex amplification reaction (Viennalab®; CVD StripAssay, Vienna, Austria) which is based on the reverse-hybridization principle automatically and by Real Time PCR, LightCycler 2.0 methods(Roche). The multiple polymerase chain reaction (PCR) was performed in a Perkin Elmer 9600 and the profile consisted of an initial melting step of 2 min at 94°C; followed by 35 cycles of 30s at 94°C, 30s at 61°C, and 30s at 72°C; and a final elongation step of 7min at 72°C for stripAssay genotyping. High portion of samples(suspicious samples) were also analysed by real-time PCR technique (LightCycler 2.0, Roche). Briefly, LightCycler FastStart DNA Master HybProbes, master mix and DNA template were used for real-time amplification. The amplification conditions for 45 cycles were; denaturation in 95°C for 10 seconds, annealing for 5-20 seconds, extension in 72°C, melting curve step with denaturation in 95°C, annealing for 30 seconds, melting in 95°C and cooling step in 40°C for 30 seconds. Software programe (LightCycler 2.0, Roche) was used for detection of the mutated and normal genotype profiles of target genes in the current BC and healty controls.

Statistical analysis

Statistical analysis was performed using SPSS version 16 (SPSS, Chicago, IL, USA). The frequencies of homozygous and heterozygous thrombophilic gene mutations, the frequencies of allelic mutations were compared in patients of BC and control group by using chi-square analysis. A value of $P<0.05$ was considered as statistically significant.

Results

The polymorphism frequencies in the breast cancer and control groups were analyzed in the current study. Presented results showed the germ-line variations in *MTHFR* 677 C>T and1298 A>C, *FV* Leiden and R2 and *PAI*-1 5G/4G genes in BC patients. Significant differences were observed in both studied *MTHFR* SNPs and *FV* R2 but the difference was not statisticaly significant for

FV Leiden and *PAI*-1 5G/4G SNPs. The study group includes 51 BC of 39 IDC (76.5%), 6 ILC (11.8%), 5 MC (9.8%) and 1 TC (1.9%) patients who had undergone breast-preserving surgery (partial mastectomy/axillary dissection) and non-surgery. Seventeen (33.33%) patients had familial BC history in their first and/or second relatives and thirty-four patients (66.67%) were the first proband cases in their family in the current BC cohort (Table 1). Peripheral blood-EDTA samples from BC patients were used for genotyping in the current results and compared to the 106 healthy women (women who are not BC and no familial BC history) from the same population. The genotype prevalence of the variations for the studied genes for the current BC patients and control groups were showed Hardy–Weinberg equilibrium. Presented results showed increased germ-line T allele frequency of 677 C>T, C allele frequency of 1298 A>C for *MTHFR* and R2 SNP for *FV* genes that has been shown to be a risk factor for BC in the current cohort from Turkish population (Table 1). Results also confirms that *PAI*-1 is an independent prognostic factor in ductal breast carcinoma for the current studied BC cohort.

The prevelance of genotypes for *MTHFR* gene C677T SNP were; 54.9% for CC, 35.3% for CT and 9.8% for TT in patients and 71.7% for CC, 28.3% for CT and 0% for TT for healthy controls respectively. Genotypes for second SNP of A1298C were; 33.33% for CC, 56.87% for CT and 9.80% for TT in patients and 67.0% for CC, 33.0% for CT and 0% for TT for healthy controls respectively (Table 2). The T allele frequency for first SNP of C677T was 0.275 for BC patients and 0.142 for health individuals in the current results. The *MTHFR* 677TT (homozygous) genotype was found 9.8% and T allele frequency 2.3-fold increased in BC when compared to the control group. That difference was statisticaly significant when compared to the control group (Table 2), (OR: 2.295, CI: 1.283-4.106), p<0.006.The C allele frequency for second SNP of A1298C was 0.382 for BC patients and 0.165 for health individuals in the current results. The *MTHFR* 1298CC (homozygous) genotype was found 9.8% and C allele frequency 3.1-fold increased in BC when compared to the control group. That difference was also statisticaly significant when compared to the control group (Table 2), (OR: 3.131, CI:1.826-5.369), p<0.0001.

The prevelance of genotypes for *FV* Leiden gene were; 88.23% for GG, 11.77% for GA and 0% for AA in patients and 98.1% for GG, 1.9% for GA and 0% for TT for healthy controls respectively. The A allele frequency for *FV*L was 0.040 for BC patients and 0.010 for health individuals in the current results. Difference was not statisticaly significant when compared to the control group (Table 3), (OR: 3.281, CI: 0.905-11.9). Genotypes for second SNP of *FV* H1299R (R2) were; 83.78% for

Table 1. The Mean Age, Material Type and Some Other Clinical Characteristics of Current Breast Cancer Patients

Clinical Characteristics		BC Patients (n:51)
The mean age		51.55±7.01(43-76)
Material Type n (%)		Pheripheral blood/EDTA/51(100%)
	IDC	39 (76.5)
CancerType n (%)	ILC	6 (11.8)
	MC	5 (9.8)
	TC	1 (1.9)
Familial BC History n (%)	Yes	17 (33.33)
	No	34 (66.67)

*BC: Breast carcinoma; IDC:Invasive ductal carcinoma; ILC:Invasive lobular carcinoma; MC: Medullary carcinoma; TC:Tubular carcinoma

Table 2. Genotype and Allele Frequencies of *MTHFR C677T* and A1298C SNPs in the Current Patients with Breast Cancer and Healthy Controls

Gene/Genotypes	BC Patients (n=51) n	(%)	Control24 (n=106) n	(%)
MTHFR C677T				
CC	28	(54.9)	76	(71.7)
CT	18	(35.3)	30	(28.3)
TT	5	(9.8)	0	(0.0)
Alleles	P value 0.006;	OR 2295;	CI(95%) 1.283- 4.106	
C	74	(0.725)	182	(0.858)
T	28	(0.275)*	30	(0.142)
MTHFR A1298C				
AA	17	(33.33)	71	(67.0)
AC	29	(56.87)	35	(33.0)
CC	5	(9.80)	0	(0)
Alleles	P value 0.0001;	OR 3,131;	CI(95%) 1.826- 5.369	
A	63	(0.618)	177	(0.835)
C	39	(0.382)*	35	(0.165)

*BC: Breast carcinoma

Table 3. Genotype and Allele Frequencies of *FV* Leiden and R2 SNPs in Current the Patients with Breast Cancer and Healthy Controls

Gene/Genotypes FACTOR V	BC Patients (n=51) n	(%)	Control24 (n=106) n	(%)
G1691A Leiden				
GG	45	(88,23)	104	(98.1)
GA	6	(11,77)	2	(1.9)
AA	0	(0.0)	0	(0.0)
Alleles	P value 0.008;	OR 3,281;	CI(95%) 0.905-11.9	
G	96	(0.96)	210	(0.990)
A	6	(0.04)	4	(0.010)
H1299R (R2)				
HH	45	(83.78)	103	(97.2)
HR	5	(13.51)	3	(2.8)
RR	1	(2.70)	0	(0.0)
Alleles	P value 0.02;	OR 5133;	CI(95%) 1.299-20.28	
H	95	(0.910)	209	(0.986)
R	7	(0.090)*	3	(0.014)

*BC: Breast carcinoma

Table 4. Genotype and Allele Frequency of *PAI*-1 5G/4G Polymorphism in Current Patients with Breast Cancer and Healthy Controls

Gene/Genotypes	BC Patients (n=51) n	(%)	Control[24] (n=106) n	(%)
PAI-1 5G/4G				
5G/5G	13	(25.5)	34	(32.0)
5G/4G	30	(58.8)	62	(58.5)
4G/4G	8	(15.7)	10	(9.5)
Alleles	P value 0.141;	OR 1,302;	CI(95%) 0.807-2.1	
5G	56	(0.549)	130	(0.613)
4G	46	(0.451)	82	(0.387)

*BC: Breast carcinoma

HH, 13.51% for HR and 2.7% for RR in patients and 97.2% for HH, 2.8% for HR and 0% for RR for healthy controls respectively (Table 3). The R2 allele frequency was 0.090 for BC patients and 0.014 for health individuals in the current results. The R2 allele frequency 5.1-fold increased in BC when compared to the control group and that difference was statisticaly significant (Table 3), (OR: 5.133, CI: 1.299-20.28), p<0.02.

The prevelance of genotypes for *PAI*-1 5G/4G gene were; 25.5% for 5G/5G, 58.8% for 5G/4G and15.7% for 4G/4G in patients and 32.0% for 5G/5G, 58.5% for 5G/4G and 9.5% for 4G/4G for healthy controls respectively. The 4G allele frequency for *PAI*-1 was 0.451 for BC patients and 0.387 for health individuals in the current results. Difference was not statisticaly significant when compared to the control group (Table 4), (OR: 1.302, CI:0.807-2.1), p=0.141.

Discussion

BC is one of the most frequent diseases that occur in women through the worldwide. The environmental and dietary factors play a crucial role in the aetiology of breast cancers. The current results with some of previous literature findings have pointed out the importance of the germ-line functional genes mediated cancer in a BC cohort from Turkish population. Literature findings showed that, the risk factors are mainly derived from environmental carcinogens, hormonal, genetic parameters, and obesity in BC tumorigenesis (Li et al., 2013). The genetic parameters are focused on; oncogenes, epigenetic alterations in tumour supressor genes, oncogenic viruses and many other intrinsic and/or extrinsic environmental factors may directly play crucial role in BC inititiation and/or progression (Werner and Bruchim, 2012; Bhar et al., 2013). In contrast, limited findings are reported about failure of germ-line functional gene effect on familiar BC. Recent literature findings report the *MTHFR* C677T and A1298C polymorphisms and breast cancer risk in women in differen populations (Ericson et al., 2009; Gao et al., 2009; Eroglu and Akar, 2010; Qi et al., 2010; Sangrajrang et al., 2010; Eroglu and Akar, 2011; Hosseini et al., 2011; Akram et al., 2012; Diakite et al., 2012; Wu et al., 2012). The solid tumoural tissue samples from each patients were used for profilling the transcriptome analysis for the clinical follow-up and treatment and peripheral blood-EDTA samples were also used for germ-line thrombophilic gene analysis in the current cohort. The tumoural tissues have showed increased cDNA profiles for the studied target genes (Her2, brc1, p53, DAPK1 etc), (data not shown).

The current report includes the comparison of germ-line variations in *MTHFR* 677 C>T and1298 A>C, *FV* Leiden and R2 and *PAI*-1 5G/4G genes in BC patients and healthy controls. Significant differences were observed in both studied *MTHFR* SNPs and *FV* R2 but the difference was not significant in *FV* leiden and *PAI*-1 5G/4G polymorphisms when compared to the control group from the same population. Presented results showed the germ-line T allele frequency of *MTHFR* 677 C>T and *FV* R2 SNPs have been shown to be a risk factors for BC in the current cohort from Turkish population. Results also confirms that *FV* leiden and *PAI*-1 are independent prognostic factors in ductal breast carcinoma for the current studied BC cohort. The T allele frequency of C677T codon was 2.3-fold increased in BC patients and that difference was statisticaly significant when compared to the control group, (OR: 2.295, CI: 1.283-4.106), p<0.006. The C allele frequency for second SNP of A1298C was 3.1-fold increased and that difference was also statisticaly significant in BC when compared to the control group, (OR: 3.131, CI:1.826-5.369), p<0.0001.

The *PAI*-1, belonging to the urokinase plasminogen activation (uPA) system, is involved in cancer development and progression but results are still controversial (Yagmurdur et al., 2008). The meta-analysis that reported by Xu et al. (2012) have suggested that the *PAI*-1 4G/5G polymorphism most likely contributes to susceptibility to endometrial cancer, particularly in Caucasians. Offersen et al. (2008) have claimed that *PAI*-1 may play a role in angiogenesis and poor prognosis in breast cancer due to the plasma high levels of *PAI*-1 consantrations. Yagmurdur et al. (2008) have reported that the 4G allele in the PAI -1 gene had a negative impact on local recurrence and disease-free survival of patients with clinical T(1)-2N(0) M(0) invasive ductal breast carcinoma patients. Lee et al. (2013) have showed the prognostic significance of *PAI*-1 in patients with breast carcinoma in their long time follow-up study. Some other literature findings were also pointed out the strong association between *PAI*-1 5G/4G polymorphism and tumour severity, cell adhesion and motility in breast cell carcinomas (Jelisavac et al., 2011; Fersching et al., 2012; Tang and Han, 2013). In the current BC cohort of including high portion of IDC patients (76.5%) the *PAI*-1 promoter polymorphism was not significant when compared to the control group (Table 4), (OR: 1.302, CI:0.807-2.1), p=0.141. The 4G allele frequency for *PAI*-1 was 0.451 for BC patients and 0.387 for health individuals and it was not evaluated as an independent prognostic factor in breast carcinomas for current the BC cohort from Turkish population.

Factor V is the liver-synthesized multidomain glycoprotein encoded by a gene localised on chromosome 1q23. Various point mutations in this gene results in formation of an altered protein of V Factor resistant to activated protein C cleavage (Valjevac et al., 2013). *FV* R2 influences plasma *FV* concentration and associates with mild activated protein C resistance. This polymorphism was reported to have a trans inheritance with *FV* Leiden mutation (Ozturk et al., 2012). Activated protein C (APC) resistance is a major risk factor for venous thrombosis. Factor V (*FV*) gene mutations like *FV* (Leiden) (R506Q) and *FV* (R2) (H1299R) may cause APC resistance either by reducing the susceptibility of *FV*a to APC-mediated inactivation or by interfering with the cofactor activity of *FV* in APC-catalyzed *FV*IIIa inactivation. Yamazaki et al. (2002) have claimed that the existing of R2 haplotype in mutated *FV*, has the potential to result in quantitative *FV* deficiency, reduced plasma *FV* levels and increased *FV*1/*FV*2 ratios and mild APC resistance. Zaatari et al. (2006) have claimed that the HR2 polymorphism in factor V gene has been reported to be a possible risk factor for the

development of venous thromboembolism, with a high prevalence of 9.5-15.2% in patients of different ethnic groups in different parts of the world. In the presented results the A allele frequency for first SNP for *FV*L gene was 0.040 for BC patients and 0.010 for health individuals and that difference was not statisticaly significant (OR: 3.281, CI: 0.905-11.9). We calculated odds ratios in 51 BC patients and 106 age-matched controls for the second SNP of R2 allele frequency in *FV* gene. The odds ratio for the R2 allele in patients relative to controls was 5.1-fold increased in BC when compared to the control group and that difference was statisticaly significant, (OR: 5.133, CI: 1.299-20.28), p<0.02.

The frequency of the *MTHFR* gene 677 T and 1298 C alleles were significantly higher in cancer patients than in the healthy subjects. Results indicate that combined variant genotypes (677CT+TT), (1298AC+CC) significantly increased the risk of developing cancer for *MTHFR* gene than those with wild-type genotype. In this study of germ-line homogenous *MTHFR* TT individuals had 3.41-fold increased risk of developing BC due to the increased T allele frequency. The both homozygous and/or heterozygous A1298C SNPs were appear to be associated with BC risk in the current cohort. Literature findings showed the *FV* SNPs have a major risk factor for venous thrombosis but the current results showed that *FV* R2 polymorphism was also associated with BC risk in the current cohort fom Turkish population. The presented results showed the germ-line functional gene mediated cancer in BC cohort of high portion (33.3%) of familial BC history includes IDC, ILC and MC subtypes (Table 1). The *MTHFR* gene polymorphisms in both SNP markers affect enzymatic activity may be associated with DNA hypomethylation and cancer susceptibility in the current BC cohort. Results also provide support for the important role of folate and *MTHFR* metabolism in breast carcinogenesis.

In conclusion, the current results suggest that the *MTHFR* C677T and C1298A polymorphisms indicate the susceptibility to BC and germ-line homozygous mutated individuals has an additional risk factor for BC. It is also possible to assumed that parents with mutated 677T and 1298A profiles for *MTHFR* and R2 for *FV* genes may contribute BC risk in their offsprings by germ-lime mutated allele trnasmission.

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