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ORIGINAL PAPER

Analysis of *p53* Gene Polymorphisms and Protein Over-expression in Patients with Breast Cancer

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Abstract *p53* polymorphic variants play an important role in the determination of tumor phenotype and characteristics in breast cancer. In this study, we examined three common polymorphisms in *p53* gene and their haplotype combinations to assess their potential association with inherited predisposition to breast cancer development, in relations with the protein over-expression and patients' demographic data. A total of 99 patients with breast cancer and 107 age-matched healthy controls were included in the study. Genotypes were determined using PCR-RFLP and DNA sequencing techniques. Evaluation of *p53* protein over-expression was also examined by immunohistochemistry. Among three polymorphisms, increased codon 72 Pro allele frequency ($p=0.0067$) and the presence of Pro allele were found to be significantly associated with breast cancer ($p=0.013$). A significant risk was also found in subjects with combinations of specific haplotypes and genotypes. Most of breast cancer women especially younger than 50 years carry at least one *p53* polymorphism ($p=0.001$). There was no any association between these three *p53* polymorphisms and the protein over-expression, separately or in interaction, with breast cancer. In conclusion, presence of proline allele at codon 72 alone, and its special combinations with other two polymorphisms appear to be a significant risk factor for breast cancer. Determination of well-known *p53* polymorphisms might be a good predictor for breast cancer development especially in women younger than 50 years.

Keywords p53 polymorphisms · Haplotype · Breast cancer

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Introduction

Gene variants have a potential role in breast cancer development [1]. The susceptibility is frequently modulated by polymorphisms in tumor suppressor and DNA repair genes as well as oncogenes. Although the polymorphisms were shown to be in low-penetrance, they are highly common in general population. *p53* (*geneID: 7157*) is a critical tumor suppressor gene for cell growth control and genomic stability maintenance [2]. Mutations of *p53* gene are associated with more than 50% of all human cancers [3], and particularly, its specific point mutations within the exons 5 to 8 were found to be related with breast cancer-specific death [4]. Besides these mutations, more than 20 different single nucleotide polymorphisms in *p53* gene have also been identified [5, 6]. Although the role of *p53*

mutations in breast cancer development is well defined, the role of its polymorphisms is not fully verified.

Codon 72 variant (Arg>Pro) is the most common coding-region polymorphism of *p53* gene. Although the results of the epidemiological studies, which assessed the association of the polymorphism with the risk of different cancer types, remained inconclusive, some studies revealed a strong association with arginine allele [7–9], while others showed an association with proline allele [10]. Experimental studies have demonstrated that some but not all polymorphisms affect *p53* function. It was demonstrated that Pro72 allele variant induced less *in vitro* apoptosis than Arg72 allele did [11]. However, another study showed enhanced effects of *p53* mutants when the mutation occurred at Arg72 allele [12]. They also reported that in several malignant tumors including breast cancer, *p53* mutations on the Arg72 allele are more frequent than those on Pro72 allele.

Other cancer—related polymorphisms of *p53* gene are 16 bp duplication at intron 3 and MspI (G>A) polymorphism at intron 6. Both polymorphisms occur at non-coding regions of the gene. Studies showed that 16 bp duplication at intron 3 is a risk factor for both breast [7] and colorectal cancers [13]. In a study with a limited sample size, a weak association between cancer predisposition and MspI polymorphism at intron 6 polymorphism was reported [14].

Considering ethnic differences and potential association between genetics variants and carcinogenesis, haplotype analysis of several variants of polymorphisms on the same chromosome provides more information than single polymorphism. It was proposed that inheritance of specific germline haplotypes based on three biallelic polymorphisms of *p53* is a better predictor for breast, colorectal and lung cancer development [7, 10, 15–17]. Therefore, in this retrospective case-control study we aimed to assess each polymorphism individually as well as to analyze the haplotype of all polymorphism variants in both breast cancer patients and healthy subjects.

Aim of the Study

The main objectives of this study were to measure the frequencies of overall *p53* polymorphism and three site-specific (codon 72, 16 bp duplication at intron 3 and MspI at intron 6) polymorphisms in breast cancer patients and in age-matched healthy women. We further aimed to assess the frequency of different allele carriers for each polymorphism and their genotype combinations in both groups. The secondary objective was to assess the association of demographic and histopathological factors with the overall *p53* gene polymorphisms in Turkish breast cancer patients. The third objective was to evaluate relationship between these polymorphisms and protein over-expression in breast cancer patients.

Material & Methods

The study was approved by the local research ethics committee of the Marmara University School of Medicine.

Study Population

Cases The consecutive female patients, who underwent curative surgical treatment between 1999–2002 for early stage breast cancer in a university hospital setting, were within the scope of this study. Demographic data (i.e. age at diagnosis, family history regarding breast and/or ovarian cancer, menopausal status), clinical and pathological findings (i.e. tumor size, presence of lymph node involvement, tumor grade, presence of vascular and lymphatic invasion, ER and PR status) of cancer patients were recorded from their files.

Patients' age was defined as the age at cancer diagnosis. Regarding age, patients were grouped whether they are 50 years-old or younger versus over 50. Menopausal status of women was defined as postmenapousal when their last menses was elapsed more than 1 year. Otherwise they were regarded premenopausal. Patients with any first and/or second degree relative with breast and/or ovarian cancer were regarded as a case with positive family history. Tumor histological grading was reported according to Elston-Ellis modification of Scarff-Bloom-Richardson grading system [18]. At least 1% immunoexpression was regarded as hormone receptor positivity for both estrogen and progesterone. Tumor size and lymph node involvement were classified according to the latest AJCC/TNM revision [19]. Vascular and lymphatic invasion was determined as present or absent by conventional morphological assessment following hematoxylin—eosin staining.

Controls For each index cancer patient, one age-matched healthy woman with similar race from the same region was asked to participate the study as a control (1:1). Women with a previous history of any type of in-situ or invasive cancer or healthy women who have a first or second degree family member with a breast and/or ovarian cancer were not included in the study.

DNA Extraction

In cancer patients, DNA samples were retrieved from paraffin embedded tumor and adjacent normal tissues, whereas blood samples were withdrawn from healthy

women to obtain DNA. DNA were then isolated from paraffin embedded tissue by standard proteinase K treatment [20] and from peripheral leukocytes by standard phenol-chloroform method [21], accordingly.

Genotype Analysis

PCR amplifications of intron 3 (16 bp duplication) and codon 72 were performed using the same touch-down protocol to increase the reaction specificity. Amplifications were carried out in 50 μL reaction mixture, containing 12.5 pmole of each specific primer pairs (Table 1), 1x PCR Buffer, 100 μM dNTPs, 1.5 mM $MgCl_2$ and 0.5 U of *Taq* polymerase. Samples were denatured for 5 min at 94°C, followed by 34 cycles, where the annealing temperature was gradually decreased from 69°C to 67°C. The final extension was allowed to proceed for 7 min.

The third polymorphic site (*Msp*I) was amplified with specific primers (Table 1). The reaction was carried out by 3 min initial denaturation at 94°C followed by 35 cycles of amplification at 94°C for 1 min, 59°C for 1 min and 72°C for 1 min.

The codon 72 and *MspI* polymorphisms were genotyped by restriction fragment length polymorphism (RFLP). Amplification products were digested for 16 h at 37°C in a 20 μL reaction containing 10 U of *Bsh*1234I and *Msp*I enzymes, respectively. The 16 bp duplication polymorphism was evaluated by presence of 156 or 172 bp fragments.

The amplified and digested products were separated on a gel containing of 1% NuSieve and 3% basica agarose. They were stained with ethidium bromide.

DNA Sequencing Analysis

DNA sequencing was performed to confirm the results of PCR-RFLP analysis. Forward primers used for sequencing of p53 polymorphic sites were the same as those utilized in the RFLP technique. The PCR products were purified (High Pure PCR Product Purification Kit, Roche) and sequenced on ABI Prism™ 310 fluorescent sequencing analyzer (DYEnamic™ ET Terminator Cycle Sequencing Kit, Amersham Biosciences).

Definitions

Codon 72 (Arg>Pro) Polymorphisms

Polymorphisms of codon 72 (Arg>Pro) were defined as presence of three allele probabilities; Arg/Arg, Arg/Pro and Pro/Pro. Polymorphisms are grouped as Arg/Arg and non-Arg/Arg (Arg/Pro and Pro/Pro alleles) allele carriers. "Arg/Arg" allele is regarded as the normal variant which also define as A1/A1 allele (absence of the restriction sites).

16 bp Duplication Polymorphisms at Intron 3

Polymorphisms of 16 bp duplication were defined as presence of three allele probabilities; A1/A1, A1/A2 and A2/A2. Polymorphisms are grouped as "duplicated" allele carriers (A2/A2 and A1/A2) and "non-duplicated" allele carriers (A1/A1). "Non-duplicated" allele is regarded as the normal variant.

MspI Polymorphisms at Intron 6

Polymorphisms of *Msp*I at intron 6 were defined as presence of three allele probabilities; A1/A1, A1/A2 and A2/A2. Polymorphisms are grouped as "absence of restriction enzyme site" allele carriers (A1/A1 and A1/A2) and "presence of restriction enzyme site" allele (A2/A2), which is regarded as the normal variant.

p53 Immunohistochemistry

Consecutive 5-μm sections were cut from the paraffin blocks. Sections underwent histologic evaluation to control the quality of histologic materials and to select tumor areas. *p53* immunohistochemical staining was performed by the avidin-biotin peroxidase technique using DO-seven monoclonal antibody (NeoMarkers, cat # MS-186-R7, ready-to-use) as primary and diaminobenzidine (DAB) as the chromogen, according to manufacturers' instructions (Lab Vision), running in parallel with the known positive and negative controls. *p53* protein immunoexpression was evaluated by counting the number of stained nuclei in at

Table 1 Primer pairs for PCR and expected bands

Polymorphisms	Primer sequences (5′ to 3′)	PCR Products (bp)	Detection of polymorphisms
16 bp dup	GCAGAGACCTGTGGGAAGCGA GAGCAGTCAGAGGACCAGGTC	156 or 172	16 bp duplicated band (172 bp)
Codon 72	TTGCCGTCCCAAGCAATGGATGA TCTGGGAAGGGACAGAAGATGAC	199	Restriction with *Bsh1234I* (113 and 86 bp)
MspI	AGGTCTGGTTTGCAACTGGG GAGGTCAAATAAGCAGCAGG	107	Restriction with *MspI* (63 and 44 bp)

least 500 tumor cells in five different tumor fields. If the percentage of positive tumor nuclei to the total number of counted tumor nuclei was 10% or more, the slide was scored as positive. If 10% or less of the nuclei were stained, the slide was scored as negative. The staining was evaluated by two observers simultaneously and a consensus was reached for each sample.

Statistics

Comparisons were made between cases (breast cancer patients) and controls (healthy women). Any polymorphic allele combination other than normal variants at codon 72 (Arg/Pro and Pro/Pro), intron 3 (A1/A2 and A2/A2) or intron 6 (A1/A1 and A1/A2) was regarded as "overall *p53* polymorphism" regardless of their site and allele combination. Any polymorphic allele combination other than normal variants at each genomic site (codon 72, intron 3 or intron 6) was regarded as "site-specific polymorphism" regardless of their allele combination (i.e. codon 72 polymorphism). For statistical comparison, codon 72 polymorphism was grouped as Arg/Arg and non-Arg/Arg (Arg/Pro and Pro/Pro; polymorphic variants) allele carriers. 16 bp polymorphism at intron 3 were grouped as duplicated allele carriers (A2/A2 and A1/A2; polymorphic variants) and non-duplicated allele carriers (A1/A1). *MspI* polymorphism at intron 6 were grouped as absence of restriction enzyme site allele carriers (A1/A1 and A1/A2; polymorphic variants) and presence of restriction enzyme site allele carriers (A2/A2).

χ^2 analyses, followed by Fisher's exact test wherever required, was used to compare the frequencies of polymorphisms between cases and controls as well as for univariate analysis of comparing demographic and clinicopathologic factors between positive and negative polymorphic cancer patients. The test was also applied for identifying the deviations from the Hardy-Weinberg proportion (χ^2HW). Haplotype frequencies and linkage disequilibrium were analyzed by the estimating haplotype (EH) software program [22]. Multivariate analysis was further applied for the variables found to be significant in univariate tests in order to define independent factors which are associated with the presence of polymorphisms in cancer cases. Odds ratios (OR) with 95% confidence intervals (CI) were given wherever appropriate. A difference was determined significant when $p < 0.05$. SPSS 11.0 statistical software was used for all analysis.

Results

Ninety-nine consecutive early stage (Stage I–II) breast cancer patients, who underwent curative surgery at Marmara University Hospital, Breast Center between 1999 and 2002, were included in this study retrospectively. In addition, 107 age-matched healthy women were included in the study as controls. All cases and controls were Turkish Caucasian women. Mean age of cancer patients was 59.8 (36–82) years. All loci for cases and controls showed a generally good fit to Hardy-Weinberg equilibrium. Due to technical problems, all of the 99 samples from cancer cases could not be assessed for polymorphisms at all genomic sites.

We performed RFLP and DNA sequencing at tumor and its adjacent normal tissues to see whether there are any difference between these two tissues in the same cancer patients concerning about p53 polymorphic status. We found that there was absolute genotype concordance between tumor and its adjacent normal tissues in the same cases. We also confirmed all RFLP results by DNA sequencing and found complete correlations between these two methods. Figure 1 shows representative images for RFLP and Sequencing results.

Overall p53 Polymorphisms

The genotypes and allele frequencies of *p53* polymorphisms at three genomic sites at breast cancer patients and controls are given in Tables 2 and 3.

The number of carriers of polymorphic variant of any *p53* polymorphisms was significantly increased in breast cancer group when compared to that of healthy women (OR %95 CI: 1.97 [1.08–3.63]; $p = 0.03$). We further analyzed the whole cohort by classifying according to age (50 years below vs. above) and presence of polymorphisms (Table 4). The analysis revealed that there was a strong correlation between only for the group below 50 years of age and the presence of at least one *p53* polymorphisms in breast cancer patients when compared to the same age group of the controls ($p = 0.001$).

Codon 72 (Arg>Pro) Polymorphisms

Ninety-five samples from breast cancer patients were assessed for codon 72 polymorphisms. The frequencies of each allele combination are given in Table 3. The frequency of non Arg/Arg allele was significantly higher in the breast cancer patients (n=70; 74%) than in healthy controls (n=61; 57%; OR 95% CI: 2.11 [1.16–3.83]; $p = 0.02$).

16 bp Duplication Polymorphisms at Intron 3

Ninety-seven samples from breast cancer patients were assessed for 16 bp duplication polymorphisms. The frequencies of each allele combination are given in Table 3. The frequency of duplicated allele was similar in breast cancer patients (n=38; 39%) when compared to that of

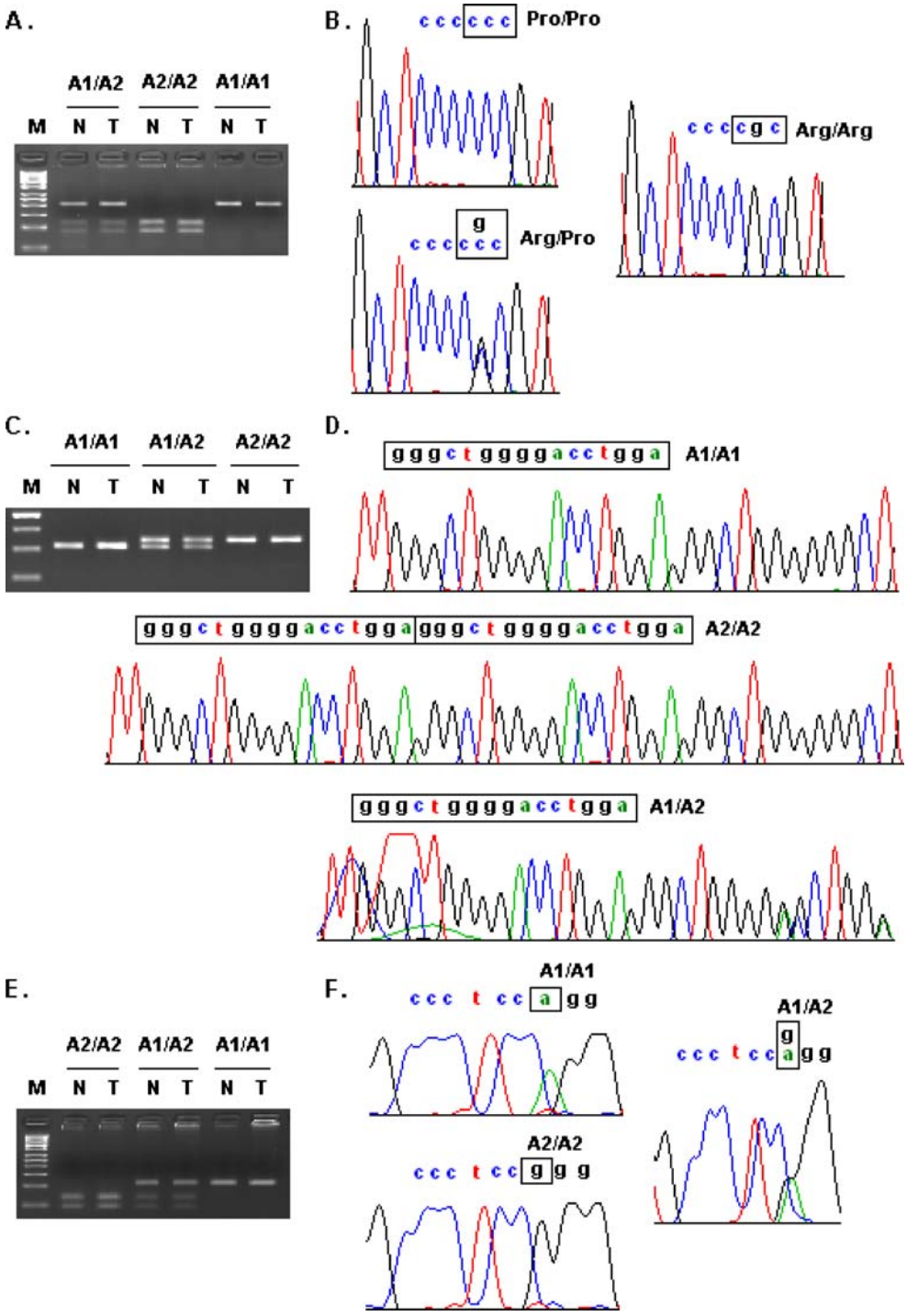

Fig. 1 Representative RFLP and sequencing analysis of p53 polymorphisms. **a**. **c**. and **e**. 3% agarose and 1% NuSieve gel from RFLP for codon 72, 16 bp duplication and MspI polymorphisms analysis, respectively. M: 50 bp ladder marker, N: adjacent normal; T: tumor tissues from the same cases. **b**. **d**. and **f**. DNA sequencing electropherograms showing nucleotide changes in codon 72, intron 3 (16 bp duplication), and intron 6 (MspI G>A), respectively. Nucleotides in square represent polymorphic sites, and A1 allele for codon 72, 16 bp duplication and MspI polymorphisms describe Arg, non-duplicated and polymorphic allele respectively

healthy controls (n=46; 43%; OR 95% CI: 0.85 [0.48–1.49]; $p= 0.58$).

MspI Polymorphisms at Intron 6

Samples from all breast cancer patients were assessed for *MspI* polymorphisms. The frequencies of each allele combination are given in Table 3. The frequency of absence of restriction enzyme allele was similar in breast cancer patients (n=48; 48%) when compared to that of healthy controls (n=46; 43%; OR 95% CI: 1.25 [0.72–2.16]; $p= 0.43$).

p53 Haplotype Analysis

Haplotype frequencies estimated by EH program indicated that the haplotype A1-A1-A2 was higher in cases than in controls (0.23 vs 0.07, respectively; df=1, $p= 0.0001$).

Table 2 The presence of overall and site-specific polymorphisms in breast cancer patients and in healthy women

	Cases[a] *n(%)*	*Controls*[b] *N(%)*	*OR 95% CI*	*P*
Overall p53 polymorphism[c];			**1.97 (1.08–3.63)**	**0.03**
Variant carriers	76(77)	67(63)		
Wild-type carriers	23(23)	40(37)		
Codon 72 polymorphism;			***2.11 (1.16–3.83)***	***0.02***
Non Arg/Arg carriers[d]	70(74)	61(57)		
Arg/Arg carriers	25(26)	46(43)		
16 bp polymorphism;			***0.85 (0.49–1.49)***	***0.67***
Duplicated allele carriers[e]	38(39)	46(43)		
Non-duplicated allele carriers[f]	59(61)	61(57)		
*Msp*I polymorphism;			***1.25 (0.72–2.16)***	***0.49***
Absence of R.E[gh]	48(48)	46(43)		
Presence of R.E. allele carriers[i]	51(52)	61(57)		

[a] 95, 97 and 99 cases were assessed for codon 72, 16 bp duplication and MspI polymorphisms, respectively;
[b] 107 controls were assessed for all polymorphisms;
[c] total case number (n=99);
[d] includes Arg/Pro and Pro/Pro allele carriers;
[e] includes A2/A2 and A1/A2 allele carriers;
[f] includes A1/A1 allele carriers;
[g] restriction enzyme;
[h] includes A1/A1 and A1/A2 allele carriers;
[i] includes A2/A2 carriers.

Genotype Combinations

The genotype combinations of three *p53* polymorphisms were analyzed in each individual (Table 5). The six most prevalent genotypic combinations were presented separately and the remaining rare combinations were pooled together.

The number of samples retrieved from cancer cases which revealed "Arg/Pro-A1/A1-A2/A2" genotype combination was found to be significantly higher than that of samples from healthy controls (OR 2.55 95% CI [1.15–5.61], *p= 0.025*).

On the other hand, "Arg/Pro-A1/A2-A2/A2" genotype combination was found to be significantly lower in cancer cases when compared to controls (OR 0.16 95% CI, [0.02–1.29], *p= 0.044*).

Clinical Aspects of the p53 Polymorphisms and Protein Over-expression

p53 Immunohistochemistry was evaluated in only 77 breast cancer samples because of insufficient tissue sections. Among these, 35 cases (45%) were detected to have *p53* overexpression based on the 10% cut off level for *p53* overexpression in immunohistochemical analysis. We

Table 3 The frequencies of *p53* polymorphisms

	Cases	Controls
Codon 72 polymorphism; *n (%)*		
Arg/Arg carriers	25(26)	46(43)
Arg/Pro carriers	50(53)	49(46)
Pro/Pro carriers	20(21)	12(11)
16 bp polymorphism; *n (%)*		
A1/A1 carriers	59(61)	61(57)
A1/A2 carriers	35(36)	43(40)
A2/A2 carriers	3(3)	3(3)
MspI polymorphism; *n (%)*		
A1/A1 carriers	9(9)	8(7)
A1/A2 carriers	39(39)	38(36)
A2/A2 carriers	51(52)	61(57)

Table 4 Correlation between presence of *p53* polymorphisms and diagnosed age of the disease

	p53 polymorphisms		Total, n (%)	*p* value
	No any *p53* polymorphism, n (%)	At least one *p53* polymorphism carriers, n (%)		
<50 age				
Control	17 (47)	19 (53)	36 (100)	0.001
Case	3 (9)	28 (91)	31 (100)	
Total	20 (30)	47 (70)	67 (100)	
>51 age				
Control	23 (32)	48 (68)	71 (100)	0.607
Case	19 (28)	48 (72)	67 (100)	
Total	42 (30)	96 (70)	138 (100)	

Table 5 The frequency of genotypic combinations of all site-specific *p53* polymorphisms in breast cancer patients and in healthy women

Genotype combinations[a]						
Arg72Pro	*16 bp dup*	*Msp*I	Controls	Cases	OR;95% CI	*p*
Pro/Pro	A1/A1	A2/A2	40 (37)	23 (25)	0.55; 0.30–1.02	0.055
Arg/Pro	A1/A2	A1/A2	28 (26)	21 (23)	0.82; 0.43–1.58	0.556
Arg/Pro	A1/A1	A2/A2	11 (10)	21 (23)	2.55; 1.15–5.61	**0.025**
Arg/Arg	A1/A2	A1/A2	3 (3)	5 (5)	1.97; 0.46–8.48	0.354
Arg/Arg	A1/A1	A1/A2	1 (1)	5 (5)	6.02; 0.70–52.52	0.066
Arg/Pro	A1/A2	A2/A2	7 (7)	1 (1)	0.16; 0.02–1.29	**0.044**
Remaining combinations			17 (16)	17 (18)		
Total			107	93		

[a] Combinations were listed according to their frequencies from most common to least.

didn't find any significant correlation between *p53* over-expression levels and all three p53 polymorphisms. Statistical correlations with p53 polymorphisms and clinical parameters are shown in Table 6.

On the other hand, when we assessed the relationship between the *p53* polymorphisms and *p53* expression levels with the demographic, clinical and pathological parameters of these patients, we found only one significant correlation

Table 6 Correlations with *p53* polymorphisms and patient's clinical parameters

	Codon 72 Arg/Pro			*16 bp duplication, PIN3*			*Intron 6 G>A, MspI*		
Clinical variability	A2/A2 (n)	Non-A2/A2 (n)	Total (n) / *p*	A1/A1 (n)	Non-A1/A1 (n)	Total (n) / *p*	A2/A2 (n)	Non-A2/A2 (n)	Total (n) / *p*
Age									
<50	4	26	(94)/	18	12	(96)/	17	14	(98)/
>51	20	44	0.078	40	26	0.955	33	34	0.607
ER status									
Negative	6	13	64/	8	11	65/	9	10	65/
Positive	9	36	0.327	27	19	0.222	24	22	0.724
PR status									
Negative	6	15	62/	10	11	63/	11	10	63/
Positive	9	32	0.568	25	17	0.371	22	20	1.00
Family history									
No	19	52	82/	43	29	83/	38	534	83/
Yes	1	10	0.279	6	5	0.746	6		1.00
Tumor grade									
I	6	17	84/	14	9	85/	12	11	85/
II	8	28	0.872[a]	23	13	0.601[a]	23	13	0.454[a]
III	6	19		14	34		11	15	
Menopause									
Pre	4	19	86/	14	9	87/	14	9	87/
Post	18	45	0.405	38	26	0.900	33	31	0.441
Type of invasion									
Vascular									
No	8	37	62/	22	23	63/	21	24	63/
Yes	7	10	0.063	11	7	0.378	11	7	0.299
Lymphatic									
No	7	31	63/	19	19	64/	20	18	64/
Yes	8	17	0.219	15	11	0.544	13	13	0.836
p53 IHC (10% cut off)									
Negative	10	24	76/	18	17	77/	18	17	77/
Positive	9	33	0.425	24	18	0.616	21	21	0.901

[a] Linear-by linear associations have been used for *p* value.

between age (younger than 50 years vs. older than 50 years) and the presence of *p53* polymorphisms when we consider all breast cancer cases. Almost all cancer women younger than 50 years (90%) carry at least one *p53* polymorphism ($p = 0.001$). Correlation between the presence of *p53* polymorphisms and diagnosed age of the disease is shown in Table 4.

Discussion

Single nucleotide polymorphisms in cancer-related genes are potential molecular markers for inherited predisposition for malignant tumors. Therefore, we aimed to assess the relationship between *p53* gene polymorphisms (Arg72Pro-16 bp dup-*Msp*I) and breast cancer susceptibility in a group of Turkish women. We found that significantly a greater number of breast cancer patients carry non-Arg/Arg allele at codon 72, when compared to healthy women. Moreover, relatively a higher number of breast cancer patients who are younger than 50 were shown to carry significantly more polymorphic alleles of *p53* gene when compared to relatively older patients. When analyzing different allele combinations of three genomic sites, it was found that significantly higher numbers of breast cancer patients carry "Arg/Pro-A1/A1-A2/A2" combination as compared to healthy women. On the other hand, "Arg/Arg-A1/A2-A2/A2" combination was found to be significantly less in breast cancer patients when compared to healthy women.

The present study is the first study to assess the *p53* polymorphisms at three genomic sites in Turkish women. In order to determine the risks for breast cancer development in a relatively large number of samples by means of *p53* polymorphisms, our study included both the breast cancer patients and healthy control women in its scope. Moreover, this study is the only study which gives data about the determinants of *p53* polymorphisms and the protein overexpression in Turkish breast cancer patients.

Arg72Pro (Arg/Pro) variant at codon 72 was previously studied in different types of malignancies including breast cancer. However, inconsistent findings have been shown. Some studies have revealed that Arg allele was associated with an increased breast cancer risk [7–9], whereas others have shown the association with the Pro allele [10]. Recently published two case-control studies have shown that there is no association between codon 72 polymorphism and breast cancer in Iranian and Slovakian populations [23, 24]. The inconsistent results in these studies may be due to different ethnicity and geographical distribution. Therefore, our results are important in reflecting the general ethnicity of Turkish population, because none of the cases were from ethnic minorities or groups in Turkey. To date, there is only a single case-control study, which has also assessed Turkish population for breast cancer susceptibility by analyzing different *TP53* gene polymorphisms [25]. In contrary to their results, we found that Pro allele is significantly associated with increased breast cancer risk in Turkish women. It was suggested that the occurrence of polymorphisms in a gene might be triggered by the same or other gene's variants or mutations. Arg allele coexists with *p53* mutations in breast cancers [26], but not in colorectal cancers. It could be possible that tumorigenic effect of Arg72 only occurs when combined with a somatic mutation at the *p53* gene in breast carcinomas [9].

Our results demonstrated that being a carrier of any one allele of 16 bp duplication or MspI polymorphism in *p53* gene was not associated with increased risk of breast cancer development. Wang-Gohrke *et al.,* showed that 16 bp duplication at intron 3 polymorphism increased breast cancer risk by age 50 in German women with a family history of breast cancer in a first-degree relative [7]. However, this result was not confirmed by a large population-based study in Russia [27]. In another study with a small sample size, a statistically significant association between the intron 6 and the risk of breast cancer development were demonstrated [14].

Another study from Slovakia showed that *p53* codon 72 and MspI polymorphisms might play a critical role in breast cancer development especially in women younger than 50 years old [24]. We also found that coexistence of one and more than one well-known *p53* polymorphisms is a significant risk factor for breast cancer development especially before 50 years of age. It could be possible that tumorigenic potential of Pro allele is affected by the presence of other *p53* polymorphisms especially in women who are less than 50 years-old.

Haplotypic and genotypic combinations of these three polymorphisms in *p53* gene can provide more information about an individual's susceptibility for breast cancer. In our study, one of the most common three genotypic combinations, 1-2, 1-1, 2-2, showed an increased frequency and 1-1-two haplotype was found to be associated with an increased breast cancer risk. Although our results are consistent with the findings of breast cancer patients reported by Sjalander *et al.*, [10], in some studies, higher frequency of haplotype 2-2-2 was found in cancer cases when compared to controls [28].

We want to check whether there are any difference between normal (constitutive) and tumor DNA concerning about *p53* polymorphic status in the same breast cancer patients. LOH (loss of heterozygocity) at *p53* locus is frequently observed in different types of tumor. To find whether our cases harbor LOH at these *p53* loci, we carried out genotyping both tissue samples from all cases. Our results show that there were complete genotype matches between these two tissues in the same cases. We can say

that our cases do not contain LOH at these common polymorphic sites of *p53* gene.

Distribution of three polymorphisms within the *p53* gene may affect its function and the response to treatment. The relationship among three polymorphisms, apoptotic index and DNA repair capacity has been shown [17]. Experimental studies supported that Pro72 variant exhibits a decreased ability for inducing apoptosis as compared to Arg72 variant [11]. Codon 72 polymorphism might be a predictor of response to different therapies in breast cancer [29]. Recently, it has been found that this polymorphism is associated with acute side-effects of radiotherapy in breast cancer patients [30] and also correlated with lymph node metastases [31].

We also examined the impact of these common *p53* polymorphisms on p53 expression levels in patients with breast cancer. Detection of *p53* protein by immunohistochemistry (IHC) has been widely used as a surrogate marker for *p53* mutations; however there is a not complete match between immunohistochemical positivity with the presence of gene mutations. On the other hand, some studies show that potential use of *p53* gene mutations in clinical practice of breast cancer [4]. But we can not rule out importance of IHC analysis as a useful indicator of prognosis [32, 33], therapy [34–36], and environmental exposure [37] in clinical applications of breast cancer. So, we checked for possible association between *p53* polymorphisms and overexpression of the gene, but there were no correlations between these parameters. It could be possible that neither *p53* mutational status nor *p53* protein stability is affected by these polymorphisms.

In conclusion, our data indicated that the codon 72 polymorphism in *p53* gene alone is a statistically significant risk factor for breast cancer development in Turkish women. In order to achieve an adequate assessment for a woman's inherited predisposition for breast cancer development, analysis of haplotypic and genotypic combinations of these three polymorphisms seems to be more useful than studying single nucleotide polymorphism especially in women under 50 years old. Further studies in this field might provide more data to understand breast cancer development, progression and therapeutic response in these patients.

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