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Association of P53 (+16ins-Arg) Haplotype with the Increased Susceptibility to Breast Cancer in Iranian-Azeri Women

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Abstract

Background: Many case-control investigations have showed the correlation of *TP53* gene polymorphisms with the risk of breast cancer. However, the findings are not consistent. It has been suggested that the investigation of *P53* genotype combinations and haplotypes may be more helpful than the detection of single polymorphisms. In the present study, we investigated the association of *P53* intron 3 and codon 72 polymorphisms, as well as their Haplotypes and genotype combinations, with the development of breast cancer among Azeri women of Iran.

Methods: A total of 143 Iranian-Azeri females suffering from breast cancer and 160 ethnically and age-matched healthy females participated in this study. Intron 3 genotype was indicated by length analysis of PCR amplicon on polyacrylamide gels and allele specific–polymerase chain reaction (AS-PCR) was applied for genotyping Arg72Pro variation. Data analysis was performed using the JavaStat online statistics package and SHEsis online program.

Results: Our findings did not show a significant association of *P53* intron 3 and codon 72 polymorphisms with the risk of breast neoplastic tumors among Iranian-Azeri women. However, the (-16ins/+16ins) (Arg/Arg) combined genotype and (+16Ins-Arg) haplotype had a higher frequency in patients in comparison with the control group (OR=3.816; 95%CI: 0.906-18.459; *P*=0.047 and OR=3.941; 95%CI: 1.583-9.812; *P*=0.002, respectively).

Conclusion: In our study, (-16ins/+16ins) (Arg/Arg) genotype combination and (+16ins-Arg) haplotype showed significant correlation with the increased susceptibility to breast cancer development in Iranian-Azeri females.

Introduction

Breast cancer is the most frequent malignancy, as well as a predominant cause of death among females worldwide. Based on epidemiological studies, the occurrence rate of breast malignancy and its related deaths are increasing in Iran (1). Early detection of this disease and knowing its related risk factors are critical for reducing the mortality of females in populations at risk. Molecular changes, including alteration of genes in the *ATM-CHK2-TP53* cell-cycle checkpoint pathway are related to breast cancer development (2).The recent next generation sequencing studies have showed that *TP53* mutations are the most frequent genetic variations in breast malignances and account for 30% of them. These mutations can affect tumor progression and prognosis. Also, their

distribution is highly associated with tumor subtypes (3). The importance of *TP53* gene variations in breast cancer is also emphasized by the frequent incidence of this malignancy type in the Li-Fraumeni syndrome, a hereditary cancer predisposition condition that is correlated with germline mutations of *P53* tumor suppressor gene. Besides mutations, there are also several polymorphisms in coding and non-coding positions of *P53*. Given the established character of P53 in response to cellular stress, such as DNA damage, it is probable that these polymorphisms affect the susceptibility to breast cancer and its severity (4, 5).

The codon 72 polymorphism (rs1042522), located in exon 4, is the most considered polymorphism of *P53*. It involves a CGC to CCC substitution, leading to arginine (Arg) to prolin (Pro) amino acid exchange. The codon 72 is within a polyproline region that is crucial for growth suppression and apoptosis mediated by P53 (6). Different structural and biological properties between two Arg and Pro variants have provided incentives for researchers to study largely the correlation of the codon 72 polymorphism with the risk of malignancy development. It has been shown that, in comparison with 72Pro, 72Arg allele induces programmed cell death more rapidly and is more influential in preventing neoplastic alteration of stressed cells (7). Recently, Ozeki et al. demonstrated significant decrease of Mdm2-mediated degradation of *P53*-72Arg in comparison with *P53*-72Pro, which could be due to the increased phosphorylation at Ser-20 in *P53*-72 Arg. They also revealed enhanced P53-dependent P21 translation in cells expressing P53-72Arg, which is associated with phosphorylation at Ser-6 (8). To date, numerous case-control investigations have studied the codon 72 polymorphism association with all major malignancies, including breast, cervical, lung and colorectal malignancies. However, the results are inconsistent and have failed to yield a clear conclusion (9).

The intron 3 16 bp duplication (rs17878362) is one of the other polymorphisms of *P53* that has been extensively investigated in regard to cancer susceptibility. The intronic position of the polymorphism has led researchers to suspect its role in gene expression through altering pre-RNA alternative splicing or interactions between DNA and protein (10). It has been shown that the alternative splicing of *P53* intron 2 leads to two distinct isoforms, including wild type P53 and N-terminal truncated D40P53. There is a G-quadruplex assembly within *P53* intron 3 that could affect the splicing in favor of the wild type P53 development. Given the overlap of 16 bp duplication polymorphism with this structure, Marcel et al. suggested that the duplicated allele could change the G-quadruplex stability and subsequently increase D40P53 isoform, which is supposed to have a damaging impact on wild type P53 tumor suppressor function (11). Nevertheless, the most recent study by Morten et al. showed that, in contrary to Marcel et al. hypothesis, the duplicated allele is related with a low Δ40P53:P53 ratio in breast cancer and has a better consequence (12).

An in vitro investigation on lymphoblastoid cell lines exposed to radiation indicated that the P53 mRNA expression level, capability for DNA repair and apoptotic indices were diminished in the cells with the duplicated variant (10). Also, Gemignani et al. demonstrated the correlation of 16 bp duplication with the increased risk of colorectal malignancy and reduced level of the P53 mRNA in immortal lymphoblastoid cells (13). However, Woelfelschneider et al. could not show a correlation between the *TP53* 16 bp duplication polymorphism and P53 mRNA translation levels in primary blood lymphocytes of cases with prostate cancer

(14). Several case-control investigations have reported the correlation of the intron 3 polymorphism genotypes with cancers like colorectal and breast cancer. However, there are also investigations that have failed to approve these correlations (13, 15). These different results may be explained to some extent by the tumor types and populations that have been examined in different studies (16, 17).

Considering the conflicting results of the various studies on intron 3 Ins16 bp and exon 4 Arg72Pro polymorphisms, some authors have focused on the association of genotype combinations and haplotypes of these polymorphisms with different kinds of cancers. In the present investigation, we have studied the association of the two polymorphisms, as well as their genotype combinations and haplotypes, with increased susceptibility to breast cancer among Iranian-Azeri patients.

Method

Samples and clinical data

All of 143 Iranian-Azeri females who had undergone mastectomy surgery at Imam Reza or Noor-E-Nejat hospitals in Tabriz, Iran between 2008 and 2012, participated in this study. Their peripheral blood and tissue samples were collected after obtaining their informed consent, as well as ethic code of 5.4.3259/13.3.92 (2013) from the 13th Ethics committee of Tabriz University of Medical Sciences Research Center. The relevant clinicopathological information was provided by a qualified pathologist. The investigation protocol conformed to the 4 of the 1975 Declaration of Helsinki. The patients had been histologically diagnosed to have in situ or invasive breast carcinoma, with the mean age at the time of diagnosis being 46.90 (range 25-83) years. A total of 160 ethnically-and age-matched healthy women with no history of malignancy among their families and relatives and mean age of 46.5 (range 14-94) years constituted the control group. Blood samples of both patients and controls were obtained in falcons containing EDTA and fresh tumoral tissue samples were snap frozen in liquid nitrogen and transported to the laboratory immediately.

Genotyping P53 polymorphisms

Genomic DNA was obtained from bloods and tissues using SDS/proteinase-K based DNA extraction procedure and stored at -20º C until further studies with PCR- based methods. Each PCR reaction was done in a total volume of 25 µl, including 1 to 2 µl DNA (with an average concentration of 200 ng), 2.5 ml of 10 X PCR buffer, 1 µl $MgCl_2$, 0.5 µl dNTP, 0.5 µl of each forward and reverse primers (10 pM) and 0.2 µl of Taq DNA polymerase. The primer sequences, annealing temperatures and size of products for each polymorphism have been shown in Table 1.

For genotyping the codon 72 polymorphism (rs1042522), we used an AS-PCR method, including three pairs of primers. ArgF and arginine specific ArgR primers resulted in a 144 bp amplicon. Proline allele specific ProF together with the ProR primer were used to yield a target amplicon of 177 bp in length. The B globin gene was amplified as internal control using a pair of specific primers leading to a 861 bp amplicon (Table 1).

For genotyping the Intron 3 polymorphism, primers were designed in a way to include and amplify the duplicated position (Table 1). The size of PCR products was 195 bp and 179 bp for duplicated and non-duplicated alleles, respectively. Two distinct alleles were identified by electrophoresis of PCR products on 8% non-denaturing polyacrylamide gel and silver staining method.

Table 1. Primers applied for genotyping of codon 72 and intron 3 SNPs

Primer name	Primer sequence	PCR product (bp)	Annealing Temperature (°C)
ArgF	5′-TCCCCCTTGCCGTCCCAA-3′	144	61
ArgR	5′-CTGGTGCAGGGGCCACGC-3′		
ProF	5′-GCCAGAGGCTGCTCCCCC-3′	177	62
ProR	5′-CGTGCAAGTCACAGACTT-3′		
BetaF	5′-CAATGTATCATGCCTCTTTGCACC-3′	861	60
BetaR	5′-GAGTCAAGGCTGAGAGATGCAGGA-3′		
Int3F	5′-TGGGACTGACTTTCTGCTCTT-3′	179 or 195	61
Int3R	5′-TCAAATCATCCATTGCTTGG-3′		

Statistical analysis

Pearson's chi-square and Fisher's exact test, if there was any information with an expected count < 5, were applied to study the association between the polymorphisms and increased risk of malignancy. The Java Stat online statistics package (http://statpages.org/ctab2x2.html) was used to carry out mentioned statistical tests and to analyze odds ratios (with95% confidence interval, CI) for genotypes, alleles and combined genotypes. The SHEsis online program, accessible at http://analysis.bio-x.cn/myAnalysis.php was used to estimate the frequencies of intron 3 and exon 4 pairwise haplotypes according to the EM algorithm. This program was also applied for checking the Hardy-Weinberg equilibrium in control group using Pearson's chi-square test. In all calculations p value less than 0.05 was considered as significant.

Results

A total of 143 Iranian-Azeri females suffering from breast cancer and 160 ethnically-and age-matched healthy females were enrolled in this case-control investigation. The mean age was 47.55 ± 10.85 years for cases and 48.03±13.07 years for controls. Based on clinicopathological data, 133 cases had invasive ductal carcinoma (IDC), 6 of them had been presented with ductal carcinoma in situ (DCIS) and only 4 ones had invasive lobular carcinoma (ILC). According to TNM staging, the tumor stages were III or IV in 76 patients, I or II in 61 patients and stage 0 in 6 patients (Table 2).

Table 2. Clinicopathological characteristics of patients involved in this study

Parameters	n (%)
Age (years)	
≤47	77 (53.8%)
>47	66 (46.2%)
Tumor type	
DCIS	6 (4.2%)
IDC	133 (93%)
ILC	4 (2.8%)
Grade	
I	14 (9.8%)
II	119 (83.2%)
III	10 (7%)
Tumor size	
≤3.5	79 (55.2%)
>3.5	64 (44.8%)
Lymph node metastases	
Positive	91 (63.6%)
Negative	52 (39.4%)
Tumor stage	
Stage 0	6 (4.2%)
Early (I & II)	61 (42.7%)
Late (III & IV)	76 (53.1%)

The genotypes of 143 patients and 160 controls were determined for two *P53* polymorphisms: 16 bp duplication and Arg72Pro exchange. The statistical analysis represented no deviation from Hardy-Weinberg equilibrium in the controls, (P= 0.057 and P= 0.470 for intron 3 and exon 4, respectively).

For intron 3, the frequencies of all three possible genotypes showed just a little difference between patient and control groups. Also, the frequency distribution of duplicated and non-duplicated alleles was not significantly different between the two groups (OR=1.111; 95%CI: 0.741-1.664; P=0.594).

For exon 4, allelic frequencies calculation showed a non-significant higher frequency of exon 4 72Arg variant in patients rather than control subjects (OR=1.288; 95%CI: 0.917-1.809; *P*=0.129). The frequency of homozygous genotype for Arg allele in cases was higher than controls (OR=1.546; 95%CI: 0.945-2.530; *P*=0.066) and contrarily Arg/Pro genotype had more frequency in control group (OR=0.705; 95%CI: 0.434-1.146; *P*=0.135) (Table 3).

Table 3. Genotypes and allelic frequencies of P53 gene SNPs in patient and control groups

polymorphisms	cases	controls	Odds ratio (95 % CI)	*P* value
Intron 3 16 bp insertion				
−16ins/−16in	88 (61.5%)	104 (65.0%)	0.862 (0.525-1.414)	0.532
−16ins/+16in	45 (31.5%)	45 (28.1%)	1.173(0.696-1.980)	0.525
+16ins/+16in	10 (7.0%)	11 (6.9%)	1.018 (0.386-2.678)	0.968
(−16ins/+16ins) + (+16ins/+16ins)	55 (38.5%)	56 (35.0%)	1.161 (0.707-1.905)	0.532
−16ins	221 (77.3%)	253 (79.1%)	0.900 (0.601-1.349)	0.594
+16in	65 (22.7%)	67 (20.9%)	1.111 (0.741-1.664)	0.594
Codon 72 Arg/Pro				
Arg/Arg	63 (44.0%)	54 (33.8%)	1.546 (0.945-2.530)	0.066
Arg/Pro	54 (37.8%)	74 (46.3%)	0.705 (0.434-1.146)	0.135
Pro/Po	26 (18.2%)	32 (20.0%)	0.889 (0.481-1.641)	0.688
(Arg/Pro) + (Pro/Po)	80 (56.0%)	106 (66.3%)	0.562 (0.344-0.917)	0.014*
Arg	180 (64.7%)	182 (56.9%)	1.288 (0.917-1.809)	0.129
Pro	106 (35.3%)	138 (43.1%)	0.777 (0.553-1.091)	0.129

*P<0.05

The combinations of various genotypes of two polymorphisms were investigated and totally 9 combined genotypes were shown in patients and controls. The (-16ins/-16ins) (Arg/Arg) combined genotype showed the most frequency in both cases (34.3%) and controls (31.9%). The (-16ins/+16ins) (Arg/Arg) genotype combination had a higher frequency in cases rather than control group (Δ=5.8%; OR=3.816; 95%CI: 0.906-18.459; *P*=0.047) (Table 4).

Table 4. The frequency distribution of P53 genotype combinations in patients and control groups

Intron 3	Exon 4	Cases (n=143)	Controls (n=160)	OR (95%CI)	*P* value
−16/−16	Arg/Arg	49 (34.3 %)	51 (31.9 %)	reference	-
−16/−16	Arg/Pro	30 (20.1 %)	44 (27.5 %)	0.710 (0.369-1.362)	0.285
−16/−16	Pro/Pro	11 (7.7 %)	9 (5.6 %)	1.154 (0.401-3.342)	0.770
−16/+16	Arg/Arg	11 (7.7 %)	3 (1.9 %)	3.816 (0.906-18.459)	0.047
−16/+16	Arg/Pro	26 (18.2 %)	28 (17.5 %)	0.966 (0.472-1.977)	1.000
−16/+16	Pro/Pro	8 (5.6 %)	14 (8.7 %)	0.540 (0.188-1.522)	0.199
+16/+16	Arg/Arg	2 (1.4 %)	0	Inf [a] (0.221-Inf)	0.496*
+16/+16	Arg/Pro	0	2 (1.2 %)	0.000 (0.000-4.028)	0.243
+16/+16	Pro/Pro	6 (4.2 %)	9 (5.6 %)	0.630 (0.183-2.118)	0.582

*P<0.05

[a]Inf: Infinity

The pairwise haplotypes frequencies analysis results showed a significantly more frequency of (+16ins-Arg) haplotype in cases in comparison with control group (OR=3.941; 95%CI: 1.583-9.812; *P*=0.002). In contrast, the (-16ins-Pro) showed a higher frequency in controls rather than cases, but the difference could not be considered as a significant result (Table 5).

Table 5. The frequency distribution of P53 gene haplotypes in case and control groups

Pairwise haplotypes	cases	controls	OR (95%CI)	*P* value
(−16ins -Arg)	0.557	0.549	1.032 (0.749-1.422)	0.849
(−16ins -Pro)	0.216	0.241	0.865 (0.591-1.265)	0.454
(+16ins -Arg)	0.072	0.019	3.941 (1.583-9.812)	0.002*
(+16ins -Pro)	0.155	0.190	0.782 (0.511-1.196)	0.256

*P<0.05

Discussion

The tumor suppressor gene, *TP53* has a key role in preventing tumor growth and development through a variety of mechanisms, including cell cycle arrest, programmed cell death, DNA repair, promoting genomic stability, controlling tumor inflammation and immune response, etc (18). Therefore, it is not surprising that *TP53* mutations have been found in about 50% of all human malignancies and 20-30% of breast cancers. Besides mutations, there are also several polymorphisms in coding and non-coding positions of *TP53*, some of which may affect *P53* function and be correlated with increased susceptibility to cancer development (3, 5). In the present case-control investigation, we studied the association of two probable functional single nucleotide polymorphisms (SNPs) of *TP53* and their Haplotypes with the risk of breast malignancy.

Until now, some case-control investigations have analyzed the association of *TP53*intron 3 16 bp duplication polymorphism with the increased risk of breast cancer development. In most of them the duplicated allele had a higher frequency among cases in comparison with control group. However, in some others, the results showed no association or even a negative association between the duplicated allele and cancer development (15-17). In a meta-analysis, including 19 case-control investigations from different ethnicities, Wu et al. indicated the association of intron 3 polymorphism with the increased susceptibility to breast neoplasms (19). This was consistent with the prior meta-analysis of pooled 15 studies based on breast cancer (20). Similarly to a previous study on thyroid cancer (21), the duplicated variant and heterozygous genotype had noticeably, more frequency in breast cancer cases rather than control group.

Various case-control investigations have studied the association of codon 72 Arg/Pro exchange with susceptibility to breast cancer development. However, the results were not consistent even in meta-analysis studies. A meta-analysis of 39 case-control investigations showed that Arg/Pro and Pro/Pro genotypes were correlated with a reduced risk of breast malignancy among Europeans, but not among Asians (OR=0.89 and OR=1.04, respectively) (22). However, Hou et al. meta-analysis suggested that the *P53* codon 72 SNP

genotypes were not correlated with breast cancer risk in neither Asian nor Caucasian subjects (23). In the present study, the frequency distribution of homozygous genotype for Arg variant in cases was higher than control subjects, but the differences showed no significance. Also, based on our results, the (-16ins/+16ins) (Arg/Arg) genotype combination showed a higher frequency in cases rather than controls. Given the approximately equal frequency of (-16ins/+16ins) genotype between patients and controls, the difference was assumed to be mostly resulted from higher frequency of (Arg/Arg) genotype in cases in comparison with controls.

Several case-control studies have analyzed the correlation of *P53* intron 3 and exon 4 haplotypes with the risk of breast neoplasms. However, the results were not completely consistent and definite. Trifa et al. reported that the intron3-exon4 haplotypes frequencies did not show a significant difference between the two groups of cases and healthy controls among Tunisian women (24). However, the (+16ins-Arg) and (-16ins-Arg) haplotypes had significantly more frequency in Turkish cases rather than controls (25). Similarly, in Sweden group's study, the frequency of (-16ins-Arg) haplotype was significantly higher in patients with breast carcinoma in comparison with control subjects, but in contrast with Turkish women, the (+16ins-Arg) was more frequent in control group (26).In our investigation, the (+16ins-Pro) and (-16ins-Pro) haplotypes showed a higher frequency in controls in comparison with breast cancer patients, but the differences were not significant. We also found a significantly more frequency of (+16ins-Arg) haplotype in cases rather than controls. These findings were mostly similar to those obtained by Turkish group, which could be explained by similarities in terms of the ethnicity and geographical area between Azeri and Turkish populations.

In the present study, we did not obtain a significant association of *TP53* intron 3 and codon 72 polymorphisms with the risk of breast carcinomas among Iranian-Azeri women. However, there were significant associations between (-16ins/+16ins) (Arg/Arg) genotype combination and (+16ins-Arg) haplotype with increased susceptibility to breast cancer development. If these findings will be approved by larger investigations, determination of *P53* intron 3 and exon 4 haplotypes would be helpful in the estimation of relative risk of breast carcinomas, especially insusceptible persons with cancerous cases in their families and relatives. However, because of the limitations of our study, we were not able to assess that whether (+16ins-Arg) haplotype interfere with the tumor suppression functions of P53 or is in linkage disequilibrium with other oncogenic variants in other genes.

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