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RESEARCH

Association of P53 (−16ins-Pro) Haplotype with the Decreased Risk of Differentiated Thyroid Carcinoma in Iranian-Azeri Patients

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Abstract Association of P53 polymorphisms with the increased risk of various cancers has been investigated in numerous studies. However, the results were conflicting and no polymorphism has been determined as a definite risk factor. It is likely that the study of P53 combined genotypes and haplotypes may be more useful than individual polymorphisms. Thus, in this study, we analyzed the associations of intron 3 Ins16bp and exon 4 Arg72Pro polymorphisms, as well as their combined genotypes and haplotypes with the risk of differentiated thyroid carcinoma in Iranian-Azeri patients. This case–control study was performed on 84 Iranian Azeri patients with differentiated thyroid carcinoma and 150 healthy subjects. Intron 3 genotype was determined using PCR products analysis on polyacrylamide gels and AS-PCR was used for genotyping Arg72Pro polymorphism. The javastat online statistics package software and *SHEsis* program were applied for data analysis. *There was no significant difference in genotype frequencies* of both two polymorphisms between cases and controls. However, the (−16ins/−16ins) (Arg/Pro) genotype combination had a noticeable but not significant association with decreased risk of thyroid cancer development (OR= 0.497 95%CI: 0.209–1.168 P=0.080) and also the frequency of (−16ins-Pro) haplotype was significantly higher in controls rather than patients (OR=0.543 95%CI: 0.326–0.903 P=0.018). In our study, there was association between (−16ins-Pro) haplotype with decreased risk of differentiated thyroid carcinoma development in Iranian-Azeri patients.

Keywords Thyroid · Neoplasms · P53 tumor suppressor protein · Polymorphism · Haplotype · Molecular marker

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Introduction

P53 functions as a transcription factor and regulates the expression of more than 2,500 genes. This protein contributes to cellular response to various stresses such as hypoxia, DNA damage and oncogene activation, resulting in cell cycle arrest or apoptosis depending on the severity and duration of stress [1]. These features make P53 a crucial tumor suppressor gene that is mutated in about half of the cancers and it seems that the other half is developed by alterations in upstream or downstream signaling pathways or regulating factors of P53 [2]. The P53 status of a tumor could significantly affect the prognosis of patients and response to treatment, thus P53 could be an important clinical marker [2]. Besides mutations, several polymorphisms have been identified in coding and non-coding regions of P53 that some of them have been associated with increased risk of cancer [3]. Two important polymorphisms that have been well studied are codon 72 polymorphism in exon 4 and 16 bp duplication in intron 3.

Intron 3 16 bp duplication of P53 may alter gene expression through affecting pre-RNA alternative splicing or DNA-protein interactions. An in vitro study on lymphoblastoid cell lines affected by radiation showed that the P53 mRNA level, capacity for DNA repair and apoptotic indices were reduced in

the cells containing the duplicated allele [4]. In this regard, Gemignani and et al. showed the role of 16 bp duplication in increased risk of colorectal cancer and reduced level of the P53 mRNA in lymphoblastoid immortal cells [5]. The role of 16 bp duplication in intron 3 of P53 has been reported in association with other cancers like lung [4], breast [6, 7] and ovary [8], However there were studies that failed to confirm these associations [9, 10].

The codon 72 Arg/Pro polymorphism in exon 4 is the most studied polymorphism of P53. The codon 72 is within a polyproline domain that is important for the apoptotic function of P53. It has been shown that 72Arg, the more common variant, induces apoptosis more rapidly and preventing stressed cells from neoplastic transformation more effectively in comparison with 72Pro [11]. However in some studies, instead of 72 pro, 72Arg variant has been reported to be involved in cancer development and it is thought that codon 72 polymorphism association with neoplasia can be variable depending on cancerous cell lines or ethnicity [12, 13]. For example, one study has shown that individuals with Arg/Arg genotype are more prone to HPV-associated cervical cancer, because HPV-E6 oncoprotein can inactive 72Arg variant more effectively than 72Pro [13]. Despite extensive studies, there is no definite consensus on the role of codon 72 polymorphism in the increased risk of cancer [14, 15].

Thyroid cancer is the most common malignancy of the endocrine system and in recent decades, its incidence has been shown a steady increase in many countries [16]. Most of thyroid cancers originate from follicular cells that *constitute* the epithelium of follicles inside thyroid and are divided into three major groups: differentiated, Poorly differentiated and undifferentiated (anaplastic) carcinomas.

Various genetic alterations have been observed in thyroid neoplasms that P53 inactivation is one of them [17]. Several studies have been indicated that P53 mutations are mostly found in anaplastic and poorly differentiated carcinoma and rarely attend in benign and differentiated tumors. In fact it seems that P53 mutations occur as a secondary alteration, resulting in dedifferentiation and more aggressiveness of differentiated cancers [18, 19]. Besides mutations, there are some important polymorphisms in P53 that have been studied less in thyroid cancers and their association with thyroid neoplasia is mostly unknown.

In this study we have focused on differentiated thyroid cancers that constitute 98 % of all thyroid cancers and include papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC) and Hürthle cell thyroid carcinoma (HCC) [17]. We have analyzed the association of P53 intron 3 Ins16bp and exon 4 Arg72Pro polymorphisms, as well as their combined genotypes and haplotypes, with increased risk of these carcinomas in Iranian -Azeri patients.

Material and Methods

Sample Collection and Clinical Data

Peripheral blood and tumor tissue samples were obtained from 84 Iranian-Azeri patients with their consent. All of the patients had undergone thyroidectomy at Imam Reza or Noor-E-Nejat hospitals in Tabriz, Iran between 2010 and 2012. According to clinicopathological information, all of cases had differentiated thyroid cancer and the mean age of them was 37.7±12.08. The blood samples also were obtained from 150 Azeri controls with no history of cancer among their relatives and a mean age of 48.06±13.08. Blood samples were collected in falcons containing EDTA and fresh tumor tissues were snap frozen in liquid nitrogen and transported to laboratory immediately.

Genotyping P53 Polymorphisms

DNA was extracted from blood and tissue samples using proteinase K and salting out method. PCR reaction was used as basic procedure for genotyping of both polymorphisms. Each PCR reaction was performed in a total volume of 25 μl containing 1 to 2 μl DNA (with an average concentration of 200 ng), 2.5 ml of 10 X PCR buffer, 1 μl MgCl2, 0.5 μl dNTP, 0.75 μl of each forward and reverse primers (10 pM) and 0.15 μl of Taq DNA polymerase enzyme. The primers sequences, annealing temperatures and amplicon sizes for each reaction were given in Table 1.

For the Intron 3 polymorphism (rs17878362), primers were designed in a way to encompass the duplicated region (Table 1). Length of the PCR products for duplicated and non-duplicated alleles was 195 bp and 179 bp, respectively. Two different alleles were identified by electrophoresis of

Table 1 Primers used for genotyping of codon 72 and Intron 3 polymorphisms

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

PCR products on 8 % non-denaturing polyacrylamide gel and silver staining.

Genotyping of codon 72 (rs1042522) was carried out by AS-PCR method and using three pairs of primers. ArgF and arginine specific ArgR primers produced an amplicon of 144 bp and proline allel specific ProF Coupled with the ProR were applied to yield a 177 bp product. B globin gene amplication was used as internal control, for this purpose a fragment of 861 bp was amplified with a pair of primers designed for B globin.

Statistical Analysis

Pearson's chi-square test or Fisher's exact test, if there was any cell with expected count less than 5, were used to assess the relationship between polymorphisms and increased risk of cancer development. The javastat online statistics package (http://statpages.org/ctab2x2.html) was applied to perform these statistical tests and calculate odds ratios (with 95 % CI) for each genotype, allele and genotype combination. The frequencies of intron 3 and exon4 pairwise haplotypes were estimated based on the EM algorithm and using the *SHEsis* platform, available on http://analysis.bio-x.cn/myAnalysis.php. The *SHEsis* program was also applied to *calculate the linkage disequilibrium* and check Hardy–Weinberg equilibrium in controls based on pearson's chi-square test. *For all tests*, p value<0.05 was considered significant.

Results

A total of 84 patients with differentiated thyroid cancer and 150 healthy subjects were participated in this study. The mean age was 37.7±12.08 for cases and 48.06±13.08 for controls. 71.4 % of cases was female and 28.6 % was male. Among controls, 76.0 % was female and 24.0 % was male. According to clinicopathological information, 73 patients had PTC, 9 of them have been presented with FTC and only 2 had HCC. Based on TNM staging, the tumor stage in 75 patients was I or II and 9 had stage III tumor (Table 2).

The genotypes of 84 patients and 150 controls were determined at two P53 polymorphic sites: 16 bp insertion and Arg72Pro substitution. For both polymorphisms in control group, there was no deviation from Hardy–Weinberg equilibrium (P=0.075 and P=0.202 for intron 3 and exon 4 respectively).

For intron 3, the frequency of homozygous genotype for 16 bp duplicated allele in controls was higher than in cases (63.4 % and 57.1 % respectively). In contrast, the heterozygous genotype frequency in patients was about 6 % higher than controls (OR=1.349; 95%CI: 0.725–2.509; P=0.310), however the frequency distribution of genotypes were not significantly different between cases and controls.

Table 2 Characteristics of controls and patients with differentiated thyroid carcinoma

	cases	controls
Mean age	37.7±12.08	48.06±13.08
gender		
Female	60 (71.4 %)	114 (76.0 %)
Male	14 (28.6 %)	36 (24.0 %)
Cancer type		
PTC	73 (86.9 %)	–
FTC	9 (10.7 %)	–
HCC	2 (2.4 %)	–
Stage		
I or II	75 (89.3 %)	–
III	9 (10.7 %)	–

For exon 4, the frequency of homozygous genotype for 72Pro allele in controls was 8.2 % more than cases (OR=0.511; 95%CI: 0.210–1.226; P=0.100) and Arg/Arg genotype was more frequent in patients, however like intron 3, the overall difference was not significant. Allelic frequencies were also calculated for each polymorphism. The frequencies of exon 4 72Arg and intron 3 duplicated alleles were higher in patients rather than controls but the difference was not significant (Table 3).

The combinations of different genotypes of two polymorphisms were studied as combined genotypes. A total of 8 combinations were found in cases and controls. The (−16ins/−16ins) (Arg/Arg) genotype combination was the most frequent one in both groups, with the frequency of 38.1 % in patients and 32.7 % in controls. The next frequent combination in cases was (−16ins/+16ins) (Arg/Pro) that had the third frequency in controls (Δ=10.6 %; OR=1.361; 95%CI: 0.631–2.937; P=0.393). Instead, the (−16ins/−16ins) (Arg/Pro) genotype combination which had the third frequency in patients, was the second most frequent one in controls (Δ=10.4 %; OR=0.497; 95%CI: 0.209–1.168; P=0.080) (Table 4).

Pairwise haplotypes frequencies were estimated and D' was calculated using SHEsis software (Table 5). The frequency distribution of intron3-exon4 diplotypes were noticeably different between cases and controls (P=0.086) and (−16ins-Pro) had significantly a higher frequency in controls rather than cases (OR=0.543; 95%CI: 0.326–0.903; P=0.018).

Discussion

Exposure to ionizing radiation is an established risk factor for thyroid cancer. This can be indicator of the fact that thyroid is hypersensitive to neoplasia development induced by cellular stress. That is why the statues of P53, as a guardian of genome against cellular stress could be important in thyroid

Table 3 Genotypes and allelic frequencies of p53 polymorphisms in cases and controls

polymorphisms	controls	cases	Odds ratio (95 % CI)	P value
Intron 3 16 bp insertion				
−16ins/−16ins	95 (63.4 %)	48 (57.1 %)	reference	–
−16ins/+16ins	44 (29.3 %)	30 (35.7 %)	1.349 (0.725–2.509)	0.310
+16ins/+16ins	11 (7.3 %)	6 (7.2 %)	1.080 (0.331–3.412)	0.887
(−16ins/+16ins) + (+16ins/+16ins)	55 (36.0 %)	36 (42.9 %)	1.295 (0.724–2.317)	0.351
−16ins	234 (78.0 %)	126 (75.0 %)	reference	–
+16ins	66 (22.0 %)	42 (25.0 %)	1.182 (0.740–1.885)	0.460
Codon 72 Arg/Pro				
Arg/Arg	52 (34.7 %)	35 (41.7 %)	reference	–
Arg/Pro	66 (44.0 %)	38 (45.2 %)	0.855 (0.457–1.602)	0.601
Pro/Po	32 (21.3 %)	11 (13.1 %)	0.511 (0.210–1.226)	0.100
(Arg/Pro) + (Pro/Po)	98 (65.3 %)	49 (58.3 %)	0.743 (0.414–1.335)	0.288
Arg	170 (56.7 %)	108 (64.3 %)	reference	–
Pro	130 (43.3 %)	60 (35.7 %)	0.726 (0.483–1.093)	0.107

cancer development and prognosis. Several studies have been indicated that P53 mutations are mostly found in anaplastic and poorly differentiated carcinoma and rarely attend in benign and differentiated tumors. However besides mutations, p53 has several polymorphisms that some of them could affect its function and be associated with increased risk of cancer. In this case–control study, we investigated the association of two probable functional polymorphisms of P53 and their Haplotypes with the development of thyroid differentiated carcinomas.

In vitro studies have suggested that 16 bp duplication of intron 3 has a negative impact on the apoptotic function of P53 [4, 5]. Alternative splicing of P53 intron 2 leads to the production of two different isoforms: wild type P53 and N-terminal truncated D40P53. A G-quadruplex structure has been recognized in P53 intron 3 that affects the splicing in favor of the wild type P53 enhancement. It is probable that the 16 bp duplication in intron 3 could alter G-quadruplex stability and increase D40P53 isoform, which is thought to be a negative regulator of wild type P53 [20]. until now, there has been no study on the relationship between intron 3 16 bp polymorphism and increased risk of thyroid cancer. However, several case control studies have been analyzed the association of duplication with many of other cancers. Most of them have been indicated that duplicated allele has a higher frequency among patients rather than controls, but in some others there was no association or even contrastingly, the duplicated allele frequency in controls was more than patients [4–10]. Sange and et all, in a meta analysis including 26 study, indicated that the association of intron 3 polymorphism with increased risk of cancer may be variable based on tumor type and population of study and therefore, these two factors may be an explanation for different results in different studies [10]. In our study, the frequency of duplicated allele and heterozygous genotype was noticeably higher in thyroid tumor patients in comparison with controls but the differences were not significant.

Many case control studies have been examined the association of codon 72 Arg/Pro polymorphism with predisposition to cancer development. While some authors have been reported associations with various cancers like breast [12], lung [21] and bladder [22], some others failed to find a significant relationship [14, 15, 23]. It has been shown that homozygosity for proline allele increases the sensitivity for thyroid cancer development in Brazilian and Turkish people [24, 25]. Boltze

Table 4 The frequency distribution of P53 genotype combinations in cases and controls

Intron 3	Exon 4	Cases (n=84)	Controls (n=160)	OR (95%CI)	P value
−16/−16	Arg/Arg	32 (38.1 %)	49 (32.7 %)	reference	–
−16/−16	Arg/Pro	12 (14.3 %)	37 (24.7 %)	0.497 (0.209–1.168)	0.080
−16/−16	Pro/Pro	4 (4.8 %)	9 (6.0 %)	0.681 (0.160–2.715)	0.547
−16/+16	Arg/Arg	3 (3.6 %)	3 (2.0 %)	1.531 (0.228–10.321)	0.681
−16/+16	Arg/Pro	24 (28.6 %)	27 (18.0 %)	1.361 (0.631–2.937)	0.393
−16/+16	Pro/Pro	3 (3.6 %)	14 (9.3 %)	0.328 (0.069–1.365)	0.102
+16/+16	Arg/Pro	2 (2.4 %)	2 (1.3 %)	1.531 (0.144–16.267)	0.527
+16/+16	Pro/Pro	4 (4.8 %)	9 (6.0 %)	0.681 (0.160–2.715)	0.760

Table 5 The frequency distribution of P53 pairwise haplotypes in cases and controls

Pairwise haplotypes	controls	cases	OR (95%CI)	P value
(−16ins-Arg)	0.546	0.608	1.288 (0.877–1.891)	0.196
(−16ins-Pro)	0.234	0.142	0.543 (0.326–0.903)	0.018
(+16ins-Arg)	0.020	0.020	1.737 (0.552–5.471)	0.340
(+16ins-Pro)	0.196	0.200	1.099 (0.691–1.748)	0.691
D’	0.816	0.782		

and et al. did not find an association between genotypes of codon 72 and differentiated thyroid cancers in German patients, but indicated that proline homozygous genotype is associated with poor prognosis and an increased risk of development of non-differentiated carcinomas [26]. Recently, a meta-analysis study showed that Pro/Pro genotype increases the risk of thyroid cancer only under a recessive model [27]. Our results were consistent with Boltze study and we could not find a significant association between codon 72 polymorphism and the risk of thyroid well differentiated carcinoma in any genetic model.

We also studied the role of Ins16bp and Arg72Pro polymorphisms in cancer development as P53 genotype combinations. Based on our results, The (−16ins/+16ins) (Arg/Pro) combination was significantly more frequent in patients rather than controls and in contrast the (−16ins/−16ins) (Arg/Pro) combined genotype had a higher frequency in healthy subjects. These data suggested that in (Arg/Pro) people, genotype of intron 3 may be determinant in the risk of thyroid cancer development. however there are several complicated molecular pathways that alter in thyroid neoplasia and in addition to analysis of p53 polymorphisms, their statues should also be studied to support our results.

Several case control studies have been performed to assess the association of p53 intron 3 and exon4 haplotypes with the risk of cancer development in different ethnic groups. However, the results have been controversial and indefinite. The study of breast cancer in Tunis [28] and oral cancer in India [29] did not revealed a significant difference in intron3-exon4 haplotypes frequencies between cases and controls. But in Portuguese women the (+16ins-Arg) haplotype was associated with increased risk of familial breast cancer [30]. Turkish group’s study showed that in breast cancer patients (+16ins-Arg) and (−16ins-Arg) haplotypes were significantly more frequent in comparison with controls [31]. In Sweden patients this was the same about (−16ins-Arg), but the frequency of (+16ins-Arg) was higher in controls [32]. In our study on thyroid cancer, the (+16ins-Arg) and (−16ins-Arg) haplotypes had a higher frequency in cases rather than controls, but the difference was not significant. In addition, there was a significantly higher frequency of (−16ins-Pro) haplotype in controls rather than patients. These results suggested that −16ins allele in cis with Pro allele may protect from cancer development in thyroid organ. However, because of the limitations of this study, we are not able to investigate the influence of this haplotype on cancer development in molecular level. It could not be assessed that whether (−16ins-Pro) haplotype enhance functions of p53 or its interaction with other factors in favor of cancer suppression or simply is in a linkage disequilibrium with other anti-cancerous genes.

To our knowledge, there was no similar study on intron3-exon4 haplotypes in thyroid cancer to make a comparative analysis, but among mentioned studies on other cancers, our results had the most similarity with the study of Turkish group on breast cancer that may be a result of ethnical, geographical and sociocultural proximity of the Azeri people to Turkish people.

In this study, we investigated the association of p53 intron 3 and codon 72 polymorphisms with the risk of differentiated thyroid carcinoma. We also analyzed the pairwise and combined genotypes of these polymorphisms in cases and controls. Our data did not show a significant association between P53 polymorphisms with the risk of thyroid cancer. However, There were noticeable associations between (−16ins/+16ins) (Arg/Pro) combined genotype and (−16ins-Pro) haplotype with decreased risk of carcinoma development. If these results were confirmed in large studies and supported by in vitro assays, they could be useful in determination of relative risk of thyroid differentiated cancer development, especially in susceptible individuals with a positive family history.

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Declaration of interest There is no conflict of interest for this paper.

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