RESEARCH ARTICLE

The TP53 intron 6 G13964C Polymorphism and Risk of Thyroid and Breast Cancer Development in the Iranian Azeri Population

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Abstract

    <u>Background</u>: *TP53* mutations are the most common genetic alterations in human cancers. There are also several polymorphisms in both exons and introns of *TP53* that may influence its anti-tumor functions and increase the risk of cancer development. Associations of the *TP53* intron 6 G13964C polymorphism with increased risk of development of several cancers have been investigated in numerous studies, but the results were controversial and conflicting. In this study, we aimed to investigate the probable association of this polymorphism with risk of both thyroid and breast cancers among the Iranian-Azeri population. <u>Materials and Methods</u>: We performed two separate case control studies on associations of the intron 6 polymorphism with two different kinds of cancer. In one case-control study, a total of 75 patients with thyroid carcinoma and 180 controls were analyzed and the other study included 170 patients with breast cancer and 135 healthy women. The intron 6 genotype was determined by RFLP-PCR and the SPSS 16 program was applied for data analysis. <u>Results</u>: For thyroid cancer, the frequencies of GG genotype were 96.0% in patients and 93.3% in controls. The GC genotype had a frequency of 4.0 % in patients and 6.7% in controls. In the study on breast cancer, the frequency of GG and GC genotypes in patients were 95.3% and 4.7%, respectively. In breast related control group, the frequency of GG genotype was 93.3 % and the frequency of GC genotype was 6.7%. None of the cases and controls had the CC genotype. <u>Conclusions</u>: There was no significant association between the *TP53* intron 6 G13964C polymorphism and risk of development of both thyroid and breast cancer in Iranian-Azeri patients.

Keywords: Thyroid - breast - cancer - P53 tumor suppressor protein - polymorphism - molecular marker

Introduction

    TP53 gene encodes a crucial transcription factor that is activated in response to various stresses such as hypoxia, DNA damage and oncogene activation and protects cells from neoplastic transformation through cell cycle arrest or apoptosis (Stegh et al., 2012; Pouladi et al., 2013). This tumor suppressor protein is mutated in about half of the cancers and it seems that the other half is developed by alterations in P53 related genes or pathways (Machado-Silva et al., 2010; Sedaie-Bonab et al., 2014). The *TP53* status of a tumor could significantly affect the prognosis of patients and serve as an important diagnostic marker to distinguish the different variants of a tumor (Radha et al., 2014; Shin et al., 2014).

    Breast cancer is the most common cancer of women in the world. Public health schemes in the developed countries have led to the stabilization or even decrease in the incidence of breast cancer, nevertheless in most developing countries, the incidence rate is certainly rising

(Pathy et al., 2011; American cancer society, 2013; Pouladi et al., 2014). Molecular changes, including the P53 gene mutations, are related to increased risk of breast cancer development. In females with p53 mutations, the breast cancer is the most common malignancy and in comparison with general population, p53 mutation carriers have from an 18- to 60-fold increased risk for early onset breast cancer (diagnosed before the age of 45) (Olivier et al., 2003; Walsh et al., 2006).

    Unlike breast, the thyroid cancer incidence is low and it is estimated that it only comprises approximately 1% of all malignancies. Nevertheless, thyroid cancer is the most prevalent malignancy of endocrine system and in recent decades; epidemiological studies have been shown a steady increase in its incidence in many countries (Sipos et al., 2010; Akbari et al., 2011). Various genetic alterations have been observed in thyroid tumors that *TP53* inactivation is among them (Legakis et al., 2009). Several studies have been cleared that *TP53* mutations are mostly common in anaplastic and poorly differentiated carcinomas and

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rarely found in benign and differentiated tumors. In fact, it seems that P53 mutations occur as secondary alterations and leads to more dedifferentiation and aggressiveness of differentiated carcinomas (Parameswaran et al., 2010; Montagna et al., 2011; Dehghan et al., 2014).

Besides mutations, there are some important polymorphisms in p53 that their association with the risk of cancer development has been reported in some studies; however the results have been conflicting (Whibley et al., 2009). Intronic mutations and polymorphisms can occur within regulatory sequences like promoters, enhancers, silencers and regulatory miRNA or affect gene expression by altering RNA splicing (Malkinson et al., 1994). G13964C substitution (rs17880604) is one of the well-studied intronic polymorphisms of P53 that is not within splice site consensus sequences or enhancers. However, some studies have been suggested that 13964G variant contributes to the stability of P53 mRNA and protein (Lehman et al., 2000). The association of the 13964C variant has been investigated in increased risk of various cancers like breast (Trifa et al., 2010; Alawadi et al., 2011; Surekha et al., 2011), ovarian (Liu et al., 2004) and colon (Buyru et al., 2005), but the results have not been completely consistent.

In this study, we have analyzed the association of P53 G13964C polymorphism with the increased risk of thyroid and breast cancers development among Iranian-Azeri population.

Materials and Methods

Sample collection

A total of 75 Iranian-Azeri patients with thyroid cancer and 170 ones with breast cancer have been participated in this study with their consent. All of the patients had undergone thyroidectomy or mastectomy surgery at Imam Reza or Noor-E-Nejat hospitals in Tabriz, Iran between 2010 and 2013. The blood samples also were obtained from 180 Azeri controls with no history of cancer among their relatives. This group included 135 women and 45 men that all of them were analyzed as controls in relation to thyroid cancer but only the women participated as control group in association study of G13964C polymorphism with breast cancer.

Genotyping of P53 polymorphism:

The proteinase K and salting out method were used to extract DNA from both blood and tissue samples. The genotype Intron 6 G13964C polymorphism was determined using PCR-RFLP technique. For this purpose, the polymorphism containing fragment was amplified using the following primers:

5'-GCCCTCCCCTGCTTGCC-3'
5'-CCGCCCATGCAGGAACT-3'

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 131 bp PCR amplicon was digested with 2 units of *Hin*6I (Fermentase) enzyme at 37°C overnight

and RFLP products electrophoresed on a 3% agarose gel. Digestion resulted to two 98 and 33 bp fragments for the G allele and a single 131bp fragment for the C allele (Figure 1).

Statistical analysis

The online HWE calculator, http://www.oege.org/software/hardy-weinberg.html, was applied to assess Hardy– Weinberg equilibrium based on a pearson's chi-square test for both control and case groups. Pearson's chi-square test or Fisher's exact test (if there was any cell with an expected count less than 5) were used to analyze the relationship between the polymorphism and increased risk of neoplasia development, as well as clinicopathological characteristics. Odds ratios with 95% confidence intervals were calculated to determine the relationship between the each genotype and allele with thyroid neoplasia development. pearson's chi-square or Fisher's exact tests and Odds ratio calculation were performed using SPSS 16 software and the Javastat online statistics package (http://statpages.org/ctab2x2.html). The p value<0.05 was considered significant.

Results

Thyroid cancer

To investigate the association of P53 G13964C with increased risk of thyroid cancer, we genotyped a total of 75 thyroid cancer patients with the mean age of 38.45±12.96 and 180 healthy subjects with the mean age of 46.53±12.19. 73.3% of cases were female and 26.7% were male. Among 180 controls, 75.0% were female and 25.0% were male. According to clinicopathological information, among thyroid patients, 66 patients presented papillary thyroid carcinoma, 4 patients had follicular thyroid carcinoma and 2 had medullary thyroid carcinoma. Based on TNM staging, the tumor stage in 88% of patients was I or II and 12% had stage III tumor.

Pearson chi-square test was performed to compare observed genotype and allele frequencies with those that are expected in a population with Hardy-Weinberg equilibrium. The frequencies of genotypes and alleles did not show a deviation from Hardy-Weinberg equilibrium in both patient and control groups (Table 1). The frequencies of GG genotype were 96.0% in patients and 93.3% in controls. The GC genotype had a frequency of 4.0% in cases and 6.7% in controls. The CC

Table 1. Allele and Genotype Frequencies of p53 Intron 6 Polymorphisms in Thyroid Cancer Patients and Controls

	Controls	Thyroid patients	Odds ratio (95%CI)	*P* value
Intron 6 G13964C polymorphisms				
GG	168 (93.3%)	72 (96.0%)	reference	-
GC	12 (6.7%)	3 (4.0%)	0.583 (0.126-2.313)	0.564
CC	0	0	-	-
G	348 (96.7%)	147 (98.0%)	reference	-
C	12 (3.3%)	3 (2.0%)	0.592 (0.131-2.292)	0.57
HWE*	0.643	1	-	-

*Hardy–Weinberg Equilibrium *P* value

Table 2. Allele and Genotype Frequencies of p53 intron 6 Polymorphisms in Breast Cancer Patients and Controls

	Controls	Breast patients	Odds ratio (95% CI)	P value
Intron 6 G13964C polymorphisms				
GG	126(93.3%)	162 (95.3%)	reference	-
GC	9 (6.7%)	8 (4.7%)	0.691 (0.235-2.021)	0.458
CC	0	0	-	-
G	261 (96.7%)	332 (97.6%)	reference	-
C	9 (3.3%)	8 (2.4%)	0.699 (0.242-2.005)	0.465
HWE*	0.688	0.753	-	-

*Hardy–Weinberg Equilibrium P value

Table 3. Association of p53 Intron 6 Polymorphism with Clinicopathological Characteristics among Patients with Thyroid Cancer

	GG	GC	Total	P value
Age				1
≤0	45(95.7%)	2(4.3%)	47	
<40	27(96.4%)	1(3.7%)	28	
Gender				0.565
Female	52(94.5)	3(5.5)	55	
Male	20(100%)	0	20	
Tumor type				0.258
PTC*	66(97.1%)	2(2.9%)	68	
FTC**	4(80.0%)	1(20.0%)	5	
MTC***	2(100%)	0	2	
Iymph node metastasis				1
Positive	36(94.7%)	2(3.3%)	38	
Negative	36(97.3%)	1(3.7%)	37	
Tumor stage				0.999
I	57(95.0%)	3(5.0%)	60	
II	6(100%)	0	6	
III	9(100%)	0	9	

*Papillary Thyroid Carcinoma, **Follicular Thyroid Carcinoma, ***Medullary Thyroid Carcinoma

Table 4. Association of p53 Intron 6 Polymorphism with Clinicopathological Characteristics among Patients with Breast Cancer

	GG	GC	Total	P value
Age				0.725
≤40	81(94.2%)	5(5.8%)	86	
<40	81(96.4%)	3(3.6%)	84	
Tumor type				0.188
IDC*	151(95.6%)	7(4.4%)	158	
ILC**	9(100%)	0	9	
DCIS***	2(96.7%)	1(3.3%)	3	
Tumor size				1
≤3.5	80(95.2%)	4(473%)	84	
<3.5	82(95.3%)	4(4.7%)	86	
Iymph node metastasis				1
Positive	100(95.2%)	5(4.8%)	105	
Negative	62(95.4%)	3(4.6%)	65	
Tumor stage				0.154
stage 0	2(66.7%)	1(33.3%)	3	
Early(I&II)	78(95.1%)	4(4.9%)	82	
Late (III&IV)	72(96.5%)	3(3.5%)	75	

*Invasive Ductal Carcinoma, **Invasive Lobular Carcinoma, ***Ductal Carcinoma In Situ

Discussion

For the first time, Buller and et al found the 13964C variant of intron 6 in three ovarian cancer patients with a history of metachronous breast cancer. They considered it as a polymorphism, because it was located in the intronic region and did not show an association with *TP53* overexpression in tumor samples obtained from patients carrying C allele (Buller et al., 1995). Later, Lehman and et al found this variant in 7.1% of 42 American patients with familial breast cancer, but not in any of 171 sporadic breast cancer cases. In their in vitro studies on lymphoblastoid cell lines, derived from patients with 13964C variant, it was revealed that G to C substitution in intron 6 is associated with a long-term cell survival after DNA damage and reduced apoptosis after chemotherapy (Lehman et al., 2000). In addition, strong nuclear P53 immunoreactivity was observed in samples derived from familial breast cancer patients with 13964C variant that indicated the involvement of this variant in the *TP53* gene overexpression. Based on these results, Lehman and et al considered 13964C variant as a specific germ-line mutation in familial breast cancer (Lehman et al., 2000).

In the years that followed, several other groups have been studied the importance of G13964C polymorphism in cancer predisposition and most of them were in contrast with Leman group's findings. In one study in the UK, on 19 patients with Li-Fraumeni and Li-Fraumeni like syndromes, the C allele was not found in any patient (Varley et al., 2001). In other studies on German and Australian women, C allele was found in both sporadic and familial breast cancer patients and there was no association between G13964C polymorphism and increased risk of cancer development (Marsh et al., 2001; Liu et al., 2004). Also, lack of association has been found in other case control studies, including HPV associated colon cancer in Turkish people and sporadic breast cancer in Kuwait and India (Buyru et al., 2005; Alawadi et al., 2011; Surekha et

genotype was not observed in any group. There was no significant association between *TP53* intron 6 G13964C polymorphism and increased risk of thyroid neoplasia development (p>0.05). Also, No association was observed between intron 6 polymorphism and any clinicopathological characteristic (Table 3).

Breast cancer

Among breast patients, 158 people had invasive ductal carcinoma, 9 had invasive lobular carcinoma and 3 of them presented with ductal carcinoma in situ. The mean age of breast patients was 46.26±11.04. The tumor stage in 48.2% of patients was I or II and 50.0% had stage III or IV tumor. The frequency of stage 0 was only 1.8%. The other clinicopathological information are given in table 4. The frequency of GG and GC genotypes in patients were 95.3% and 4.7%, respectively. In breast related control group, the frequency of GG genotype was 93.3 % and the frequency of GC genotype was 6.7%. None of the cases and controls had the CC genotype. Like as thyroid cancer, no association has been found between intron 6 G13964C polymorphism and the risk of breast cancer development as well as clinicopathological characteristics.

al., 2011). Recently, GC genotype has been reported to be involved in the increased risk of familial but not sporadic breast cancer in Tunisian women (Trifa et al., 2010).

In Iran, thyroid cancer is the 7th most prevalent cancer in females, 14th in the males and the 11th in both sexes, with the worst survival among Iranian-Azeri population (Akbari et al., 2008; Khayamzadeh et al., 2011). Exposure to ionizing radiation is one of the established risk factors for thyroid cancer (Duntas et al., 2006). This can be indicative of the fact that thyroid is a sensitive organ to tumor development induced by cellular stress and therefore, the status of This work has been financially supported by Azarbaijan Shahid Madani University under the grant number 401/461. gene, as a guardian of the genome against cellular stress, could be important in the thyroid cancer development and prognosis. Several studies have been shown that P53 mutations are mostly found in undifferentiated and poorly differentiated carcinoma and rarely attend in benign and differentiated tumors (Parameswaran et al., 2010; Montagna et al., 2011). However, besides mutations, This work has been financially supported by Azarbaijan Shahid Madani University under the grant number 401/461. has several polymorphisms that some of them could affect its function and be associated with increased risk of cancer. In this case-control study, for the first time, we have investigated the association of intron 6 G13964C substitution, a probable functional polymorphism of This work has been financially supported by Azarbaijan Shahid Madani University under the grant number 401/461., with the development of thyroid cancer (follicular adenoma and differentiated carcinoma). In our study, we could not find any association between G13964C polymorphism and the risk of thyroid cancer development among Iranian-Azeri population. Also, the C allele was not associated with clinicopathological characteristics, including stage, tumor type, lymph node metastasis, gender and age of diagnosis in thyroid patients.

Breast cancer is the first cause of cancer-related death and the first cancer to have been diagnosed in Iranian women (21.4% of all cancers) (Babu et al., 2011). Therefore, it is crucial that we improve our understanding of this problem among Iranian women, both on the subject of genetic susceptibilities and environmental risk factors. Molecular changes, including the This work has been financially supported by Azarbaijan Shahid Madani University under the grant number 401/461. gene mutation and polymorphism, are related to increasing the risk of breast cancer development. This work has been financially supported by Azarbaijan Shahid Madani University under the grant number 401/461. mutations occur in 15-30% of breast tumors that most of them lay in exons 4-9 (Olivier et al., 2001; Langerod et al., 2007). These mutations can occur as an early or late events and could be helpful as an prognostic factor in clinical practice (Olivier et al., 2006). Also, some of p53 polymorphisms may influence the susceptibility to breast cancer, however this relationship has not been confirmed in all of studies. In this study, we aimed to investigate whether the G13964C polymorphism is responsible for susceptibility to breast cancer in Iranian-Azeri patients. No association has been found

between intron 6 G13964C polymorphism and the risk of breast cancer development as well as clinicopathological characteristics. The C allele frequency was even slightly less in patients rather than controls and like as many of the other similar investigations, G13964C substitution seemed to be a neutral polymorphism.

Based on our findings, we concluded that G13964C polymorphism lack a functional importance on anti-tumor activity of p53 and also is not in linkage disequilibrium with other susceptibility sites on p53. Therefore, G13964C polymorphism could not serve as a molecular marker for determining the risk of thyroid or breast cancers among Iranian-Azeri population.

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