RESEARCH ARTICLE

Lack of Associations of the MDM4 rs4245739 Polymorphism with Risk of Thyroid Cancer among Iranian-Azeri Patients: a Case-Control Study

Ziba Mohammad Khanlou[1], Nasser Pouladi[2]*, Mohammadali Hosseinpour Feizi[1], Negar Pedram[3]

Abstract

Background and Aim: MDM4, a negative regulator of the p53 tumor suppression pathway, has been demonstrated to be overexpressed in a variety of human cancers. Research has revealed that the rs4245739 A>C polymorphism of MDM4 in the 3′-untranslated region makes it a miR-191 target site, leading to lower MDM4 expression. This study aimed to detect if the rs4245739 single nucleotide polymorphism (SNP) impacts on thyroid cancer (TC) development in Iranian-Azeri patients. **Materials and Method:** Blood samples were taken from 232 healthy controls and 130 TC patients of Iranian-Azeri ethnicity. For genotyping, Tetra-ARMS PCR was performed. SPSS for Windows (version 22.0, IBM SPSS Inc., USA) and the SHEsis online software were used for data analysis. **Results:** Alleles of MDM4 rs4245739 SNP demonstrated no significant different in frequencies between patients and controls ($p>0.05$). Additionally, genotypes of MDM4 rs4245739 SNP did not increase or decrease TC risk in patients compared with healthy subjects. **Conclusion:** Considering the lack of any observed association between the MDM4 rs4245739 polymorphism and TC, we conclude no significant role in the pathophysiology of the disease.

Keywords: Thyroid cancer- MDM4- SNP- rs4245739

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Introduction

A common disorder in the endocrine system, TC is a fast-growing malignancy, which is classified mainly into 4 groups of follicular thyroid carcinoma (FTC), papillary thyroid carcinoma (PTC), anaplastic thyroid cancer (ATC), and medullary thyroid cancer (MTC) (Mazzaferri, 1999). PTC is responsible for almost 80% of TC cases, however, FTC with 10%-15% occurrence, stands on the second rank (Faam et al., 2015). In spite of diet changes and improved health care services, along with economic development, an increment has been seen in the TC incidence (Davies and Welch, 2006). Like other malignancies, environmental and genetic factors have been observed to play role in the etiopathology of TC (Pierotti et al., 1998). To enumerate environmental contributing factors, there are lesions in thyroid (Galanti et al., 1995), thyroid-stimulating hormone (TSH) and its receptors (Boelaert, 2009), estrogen (Sakoda and Horn-Ross, 2002), malfunction in iodine uptake (Chow et al., 2003), ionizing radiation (Chow et al., 2003), neck ultrasound (Zheng et al., 1996), social and cultural factors (Pujol et al., 1996), and other disease (Aschebrook-Kilfoy et al., 2011; Kitahara et al., 2012).

On the other side, several investigations have documented association of genetic factors of oncogenes like CCND1, BRAF, MYC, RAS, and RET with TC (Motoi et al., 2000; Bièche et al., 2001; Fukushima et al., 2003; Rusinek et al., 2011; Huang and Yang, 2015). Furthermore, the genome-wide association studies (GWAS) emphasize on the direct genetic association of SNPs with TC risk (Maillard et al., 2015; Pereda et al., 2015). Therefore, studies showing novel potential SNPs associated with TC are worthwhile approaches to investigate the role of genetic variations in TC.

Mouse double minute 2 homolog (MDM2), a major negative regulator of tumor suppression pathway of p53, acts through direct binding to p53, which leads to ubiquitination and then degradation of P53 (Landers et al., 1997). Mdm4 p53 binding protein homolog (MDM4) which structurally has homology with MDM2, can collaborate with MDM2 to inhibit p53 activity when cell responses to DNA damage (Wade et al., 2010). Furthermore, MDM4 can interact with MDM2 protein and inhibit degradation of MDM2 (Linares et al., 2003). MDM4 becomes phosphorylated in response to DNA damages, which results in the shift from the degradation

*[1]Department of Biology, Faculty of Natural Science, University of Tabriz, [2]Department of Biology, Faculty of Science, Azarbaijan Shahid Madani University, [3]Division of Medical Genetics, School of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran. *For Correspondence: Nasser.pouladi@gmail.com*

of p53 to the degradation of MDM4. The consequence is stabilization of p53 and cell cycle suppression (Wang et al., 2007). It has been shown that transgenetic mice overexpressing MDM4 develop spontaneous carcinogenesis, which makes it more clear that MDM4 may act like an oncogene in vivo (Xiong et al., 2010). The rs4245739 A>C SNP, in the 3'-untranslated region (3'-UTR) of MDM4 has been shown as a putative target site for miR-191 (Wynendaele et al., 2010). miR-191 can selectively bind to C allele contained MDM4 mRNA but not A allele contained MDM4 mRNA, culminating in a lower level expression of the C allele contained MDM4 mRNA. This observation explains the significantly increased expression of MDM4 mRNA and protein in MDM4 rs4245739 A allele carriers in malignancies (McEvoy et al., 2012).

The P53 pathway malfunctions have pivotal roles in mammary tumorogenesis. Considering the role of the p53-MDM4 pathway in the preventing of tumor development, we hypothesized that rs4245739 A>C polymorphism of MDM4 might be involved in TC pathogenesis. In the present survey, 130 patients with TC and 232 healthy controls were recruited to investigate the association of MDM4 rs4245739 polymorphism with the risk of TC.

Materials and Methods

Subjects

In order to perform the genetic analysis, a total of 480 individuals, which comprised of 130 unrelated TC patients and 232 healthy individuals with no history of cancer, were recruited. Patients with histologically confirmed for TC, were chosen from Imam Reza Hospital, Tabriz, Iran. Clinical data of the cases was collected from 2014 till 2015 according to documentary files of patients diagnosed through physician and pathologist. All study patients were native of Azerbaijan to elicit valid results of race-related associations. Additionally, healthy subjects had the same race with the patients and were age and sex-matched with patients. The local Ethical Committee of Tabriz University approved the study protocol and written informed consent was collected through all subjects. Five ml of venous blood was taken from each patient and control through EDTA-containing venoject tubes. Afterwards, genomic DNA was extracted from peripheral blood through salting-out approach.

Genetic analysis

In the present study, Tetra-primer ARMS-PCR assay for allele and genotype detection was carried out. The primers were designed using Primer3Plus tool (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and were blasted in NCBI website: http://www.ncbi.nlm.nih.gov/tools/primer-blast/ (Table 1). The allele-specific (inner) primer was designed in opposed directions and, in combination with the common primers, can simultaneously amplify both the mutant allele and wild type alleles in a single-tube PCR. PCR-amplified DNA samples were electrophoresed through Agarose gel (2%). After that, a number of PCR products was assayed randomly by DNA sequencing in order to confirm if the results determined by T-ARMS-PCR were in concordance with those that determined by sequencing.

Genotyping was performed by Tetra-primer ARMS PCR using the Taq-PCR Master Mix (Cat. No: 5200350-0050, Lot. No: 13C18, amplicon, Denmark) and the thermocycler PCR System (SensoQuest, Germany). Each reaction mixture contained a total volume of 26.4µl (master mix 10µl, forward and reverse inner primer 1µl each, forward and reverse outer primer 0.2µl each, and H2O 12µl). The thermocycler conditions were: 95°C for 5 minutes, then 35 cycles of 95°C for 30 seconds 56°C for 30 seconds, and 72°C 40 seconds and final extension of 72°C for 10 minutes.

Statistical analysis

Genotype and allelic distribution between case and control groups were implemented by Fisher's exact test. Moreover, the odds ratio (OR) and 95% confidence interval (CI) were calculated. The genotype distributions of MDM4 rs4245739 SNP were tested for deviation from Hardy-Weinberg equilibrium in the control group. The Bonferroni correction test was exerted in multiple statistical testing (i.e. p-value was set to <0.01) to recognize a statistically significant result, adjusting the multiple comparison, and controlling the false discovery rate (FDR) (Benjamini and Hochberg, 1995). Also, several parts of statistical analysis were performed using the SPSS for Windows (version 22.0, IBM SPSS Inc., USA). Additionally, the SHEsis online software was exerted for analyzing the genotype and Hardy-Weinberg equilibrium (Yong and Lin, 2005).

Results

Allele frequency

The A allele of MDM4 rs4245739 (A/C) polymorphism was detected in 78.4% of the patients and healthy individuals. Furthermore, the C allele also showed an equal distribution between patients and controls (21.6% each). Hence, there was not any significant difference in the A and C allele frequencies of rs4245739 SNP between

Table 1. Primers Used in T-ARMS PCR, the Amplicon Size, and Melting Temperature (°C) of Each Reaction

SNP	Target	Sequence	Amplicon Size	Tm (°C)
MDM4 A/C	Forward inner primer (A allele)	5´GTAGTACGAACATAAAAATGCATTTATCCA3´	232	56
	Reverse inner primer (C allele)	5´ATTTTCAAATAATGTGGTAAGTGAGCG3´	275	56
	Forward outer primer	5´ACAGAGAACAGATACAGAAAACATGGAG3´	450	56
	Reverse outer primer	5´ACCTAACTATGTACCTGACTGCTGCATA3´	427	56

Table 2. Association of Clinical Manifestations of TC Patients with Three Genotypes Of MDM4

Clinical properties	Total	MDM4 Genotypes			P value
		AA	AC	CC	
Age	38.9±3.8	37.7±4.1	38.8±2.1	40.1±3.1	
Tumor Size (cm)	2.43±0.32	2.14±0.14	2.27±0.14	2.91±0.35	
Malignancy					0.034
Benign	24 (23.53%)	8 (33.3%)	9 (37.5.5%)	7 (29.16%)	
Malignant	78 (76.47%)	24 (30.76%)	26 (33.3%)	28 (35.9 %)	
Tumor Dimension					0.193
T1	40 (39.22%)	13 (32.5 %)	14 (35%)	13 (32.5%)	
T2	35 (34.31 %)	10 (28.57%)	13 (37.14%)	12 (34.28%)	
Other	27 (26.47 %)	9 (33.3%)	11 (40.74%)	7 (25.92%)	
Grade of Tumor					0.241
I	63 (61.76 %)	23 (36.5%)	18 (28.57 %)	22 (34.92 %)	
II	22 (21.57%)	7 (31.81%)	9 (40.9%)	6 (27.27%)	
III	17 (16.67 %)	5 (29.41 %)	8 (47.06%)	4 (23.52%)	
Involved Lobe					0.093
Left	35 (34.31%)	14 (40 %)	10 (28.57%)	11 (31.42%)	
Right	43 (42.16%)	17 (39.53%)	12 (27.90 %)	14 (32.55%)	
Both	24 (23.53 %)	10 (41.66 %)	8 (33.3%)	5 (20.83 %)	
Lymph Node Involvement					0.059
N0	47 (42.16 %)	19 (40.42 %)	16 (34.04 %)	12 (25.53 %)	
N1	36 (35.29 %)	15 (41.6%)	13 (36.11 %)	8 (22.2 %)	
N1a	10 (9.28%)	4 (40 %)	4 (40 %)	2 (20 %)	
N1b	4 (3.92 %)	2 (50%)	1 (25%)	1 (25 %)	
NX	5 (4.90 %)	3 (60%)	1 (20 %)	1 (20 %)	
Pathology					0.063
PTC	26 (25.49 %)	10 (38.46 %)	8 (30.76 %)	8 (30.76 %)	
FTA	20 (19.16 %)	7 (35 %)	8 (40 %)	5 (25 %)	
FTC	4 (3.92 %)	2 (50 %)	2 (50 %)	0 (0 %)	
HCC	3 (2.94 %)	1 (33.3 %)	2 (66.6 %)	0 (0 %)	
MTC	2 (1.96 %)	1 (50 %)	0 (0 %)	1 (50 %)	
PTC (classical)	28 (27.45 %)	10 (35.71 %)	11 (39.28 %)	7 (25 %)	
PTC (follicular)	7 (6.86 %)	4 (44.4 %)	2 (22.2 %)	3 (33.3 %)	
PTC (metastatic)	3 (2.94 %)	1 (33.3 %)	1 (33.3 %)	1 (33.3 %)	
Follicular Adenoma	6 (5.88 %)	3 (50 %)	1 (16.6 %)	2 (33.3 %)	
Hurthle Cell Adenoma	3 (2.94 %)	2 (66.7 %)	1 (33.3 %)	0 (0 %)	

PTC, papillary thyroid carcinoma; FTA, follicular thyroid adenoma; FTC, follicular thyroid carcinoma; HCC, hurthle cell carcinoma; MTC, medullary thyroid cancer

the patient and the control groups (p=0.99, OR=0.99, 95% CI: 0.67-1.49 vs. p=0.99, OR=1.001, 95% CI: 0.67-1.49, respectively).

Genotype frequency

Distributions of the rs3129882 (A/C) polymorphism genotypes in the healthy group disclosed no evidence of deviation from Hardy–Weinberg's equilibrium. The AA genotype was observed to be the most common genotype, which was represented in 61.8% of the patients and 62.1% of the controls (p=0.92, OR=0.98, 95% CI: 0.61-1. 59). Although the frequency of the AC genotype in the patients was higher than the control group, this difference was not significant (33.3% vs. 32.8%, respectively; p=0.92, OR=1.02, 95% CI: 0.62-1.63). Finally, there was no significant difference in respect of CC genotype distribution between the patient and the control groups (4.9% vs. 5.2%, respectively; p=0.92, OR=0.94, 95% CI: 0.32-2.75).

Demographic specifications and genotype distribution

Specifications of the study subjects according to genotype distribution are listed in Table 2. The patients with the mean age of 38.9±3.8 were age matched with healthy controls with that of 37.4±4.3, and did not show significant genotype distribution in respect of age. Tumor size of patients was 2.43±0.32 cm, and the mean tumor size of different genotypes were almost equal and

Table 3. Allele and Genotype Distribution of MDM4 in TC Cases and Healthy Controls

dbSNP	Alleles/ Genotypes	Case (n=102)N (%)	Control (n=232)N (%)	P	Adj. P[a]	OR (95% CI)
	C	44 (21.6%)	100 (21.6%)	0.996	0.99	1.001 (0.6707-1.4939)
	A	160 (78.4%)	364 (78.4%)	0.993	0.99	0.99 (0.6694-1.4909)
MDM4 rs4245739	CC	5 (4.9%)	12 (5.2%)	0.917	0.92	0.945 (0.3241-2.7559)
	AC	34 (33.3%)	76 (32.8%)	0.918	0.92	1.0263 (0.6258-1.6830)
	AA	63 (61.8%)	144 (62.1%)	0.957	0.92	0.9872 (0.6112-1.5944)
HWE		0.88	0.63			

[a]Adjusted p-value for multiple testing using Benjamini-Hochberg method; OR, odds ratio; CI, confidence interval

non-significant between three genotype groups. Among patients, 23.8% of patients had benign malignancy and tumor type of 76.15% of patients were malignant. The genotypes had significantly different frequencies in benign and malignant TC patients (p=0.034). No association was observed between Tumor dimension and genotype distribution (p=0.193). Tumor grade of 68.6% of patients was II, but 19% and 12.2 of the patients had tumor grade of I and III, respectively, and there was no association between grade of the tumor and genotypes (p=0.241). In respect of lymph node involvement, 46.1% of patients were N0, 35.3% were N1, 9.2% were N1a, 3% were N1b, and 6.1% of the patients were NX. The classification of tumor pathologically revealed that, 26.15%, 20.76%, 3.07%, 2.30%, 1.53%, 30%, 6.92%, 2.30%, 4.61%, and 2.30% of patients were PTC, follicular thyroid adenoma (FTA), FTC, hurthle cell carcinoma (HCC), MTC, PTC (classical), PTC (follicular), PTC (metastatic), Follicular Adenoma, and Hurthle Cell Adenoma, respectively.

Discussion

Several studies in the past two years have pointed on the pivotal importance of MDM4 in human cancers. It has been shown that MDM4 is overexpressed in roughly 10–20% of over 800 various tumors such as lung, colon, stomach, and breast cancers (Eischen and Lozano, 2014). The incidence of TC has been observed more in females than in males (Akslen et al., 1990) and is raising with aging (Albores-Saavedra et al., 2007). Several therapies have been implemented for treatment of TC like endocrine and radioactive therapies, surgery, and chemotherapy; nonetheless the outcomes were not satisfying (Lkhoyaali et al., 2015). GWAS revealed a role of genetic variants in the etiopathology of TC (Gudmundsson et al., 2009). Furthermore, interleukin 17 Receptor A (IL17RA) polymorphisms have been associated with both the development of PTC and bilaterality of involved sides in Korean population (Lee et al., 2015); the expression level of mir-149-5p was related to local progression and the susceptibility of PTC in Chinese patients (Wei et al., 2014). Chen et al., demonstrated that esophageal cancer-related gene 4 (ECRG4) had a role in the regulation of cell cycle in PTC cells through transiting them from the G1 phase to G2, resulting in tumor growth development (Chen et al., 2015). In this study, in order to make the results more reliable, standard confirmed safeguards were employed to decrease possible biases. All TC patients and healthy individuals were selected from a population native of the same geographic region (Iranian-Azeri race). Through present case-control study, we contemplated a hypothesis to examine if there is a potential association between MDM4 rs4245739 polymorphism and the TC risk.

The p53 pathway has been shown to be inactivated in almost all human cancers (Vousden and Lane, 2007). About half of human malignancies has been estimated to carry mutations in the TP53 gene itself, whereas the rest tumors with wild type TP53 have genetic alterations in other key regulatory genes in the p53 pathway (Marine and Jochemsen, 2004; Horn and Vousden, 2007). Genetic amplification of the MDM2 or MDM4 genes can result in the aberrant protein expression and suppression of the p53 response over the course of tumor development and progression (Marine et al., 2006; Wade et al., 2010). Furthermore, data suggests that polymorphisms at the MDM2 or MDM4 loci may contribute to the increased basal expression of these important p53 antagonists and increase cancer susceptibility (Bond et al., 2004; Atwal et al., 2009; Kulkarni et al., 2009). The MDM4 rs4245739 polymorphism in the 3'-UTR creates a potential target site for micro RNA-191 (Wynendaele et al., 2010). The miR-191 can selectively bind to MDM4-C allele mRNA but not MDM4-A allele mRNA, and therefore, result in a decreased expression of MDM4-C allele mRNA. This may somewhat explain the observation of a significantly increased expression of MDM4 mRNA and protein in MDM4 rs4245739 A allele carriers of ovarian cancer and retinoblastoma (Wynendaele et al., 2010; McEvoy et al., 2012).

The interaction of MDM4 and p53 was evaluated by Liu et al. and they reported that functional MDM4 rs4245739 SNP, alone and in combination with p53 Arg72Pro genetic variant, had association with a significantly decreased risk of breast cancer in Chinese women. Meanwhile, they postulated that perhaps genetic

variants modifying microRNA-mediated gene regulation could be regarded an important genetic regulation approach in breast cancer risk and mark the potential role of genes in the p53 tumor suppressor pathway in the initial steps of affecting as well during cancer development (Liu et al., 2013). In our investigation, the alleles and genotypes of MDM4 rs4245739 SNP were not significantly frequent in patients in comparison to controls. Therefore, none of the alleles could affect the risk of TC in our population.

The potential risk for TC is underlying to several clinical characteristics which can supposedly affect contracting, disease level, or being healthy. Regarding to research proven clinical manifestation of breast cancer, we attempted to correlate the age of onset, tumor size, being benign or malignant tumor type, tumor dimension, grade of tumor, involved thyroid lobe, number of involved lymph nodes, and pathology of cancer with various genotypes in MDM4 rs4245739 SNP towards TC risk. It was seen that none of the clinical characteristics were associated with three genotypes of MDM4 rs4245739 SNP. We found no statistically significant association between genotype distribution and specific prognostic predictors and clinical features for the TC outcome. Therefore, at least according to our data, MDM4 rs4245739 A>C SNP did not impress the clinical features of the TC. It seems that long time following-up of the patient's clinical changes will facilitate to elicit a more definitive conclusion pertaining to the influence of this polymorphism on TC manifestation.

All in all, to our best knowledge, this is the first study designed to assess the role of the MDM4 rs4245739 gene variant in a replicated case-control of Iranian-Azeri patients with TC. We could not identify significant differences in both allelic and genotype frequencies in the TC group in comparison to the control group. Last but not least, these results suggest the MDM4 rs4245739 (A/C) polymorphism may not be a risk factor for TC in Iranian-Azeri population and further studies are still needed to validate these data in other populations.

Disclosure of conflict of interest

None.

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