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Mir-125a Rs12976445 Is Associated With Susceptibility to Thyroid Cancer: A Case-Control Study

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1. Abstract

Thyroid cancer is the fifth communal cancer type in females. Latest data have demonstrated mir-125 is down-regulated in various cancer types. We conducted a case-control (179 cases, 165 controls) study in order to explore the association of mir-125 rs12976445 with the risk of thyroid cancer in Iranian population. rs12976445 C/T polymorphisms were investigated using PCR–RFLP. Logistic regression analyses were done to find the association of mir-125 rs12976445 C/T polymorphisms with thyroid cancer and its stages. The genotype frequencies for patients were [(TT: 81(45.2%), CT: 75(41.9%), CC: 23 (12.9%)], and for controls [(TT: 100 (60.1%), CT: 53(32.2%), CC: 12 (6.7%)]. The T allele distribution was significantly altered between patients and controls (P=0.002) with the odds ratio of 1.68. In the dominant model, there was a significant difference between CT vs TT genotypes (adjusted OR = 1.69, 95% CI= 1-2.8, P = 0.026), and between CC vs TT genotypes (adjusted OR = 2.18, 95% CI= 1-4.7, P = 0.047). We compared CT/CC genotype to TT genotype and found a highly significant difference (adjusted OR = 1.78, 95% CI= 1.15-2.74, P = 0. 0.009). Our findings suggest that miR-125a rs12976445 is a possible prognostic biomarker for thyroid cancer.

2. Introduction

Thyroid cancer is the fifth communal cancer type in females and its occurrence rate continues to rise rapidly worldwide, typically through increased use of diagnostic imaging and observation (Abdolahi et al. 2015; Cabanillas et al. 2016). In 2017, more than 56,870 novel cases were detected in the United States including 3.4% of all new cancer cases (Saini et al. 2018). it is the 7th most prevalent cancer in females, 14th in males and the 11th most frequent malignancy in both genders in Iranian population (Abidi et al. 2017). Its etiology seems to be multifactorial and mostly triggered by the interfaces between genetic elements and environmental exposures (such as exposure to radiation and lack of iodine in the diet) (Ashouri et al. 2012). Thyroid cancer is classified as differentiated (Papillary Thyroid Cancer: PTC and Follicular Thyroid Cancer: FTC) and undifferentiated or poorly differentiated (Medullary Thyroid Cancer: MTC and Anaplastic Thyroid Cancer: ATC) (Dong et al. 2015). PTC, as the most public type of thyroid cancer, accounts for nearly 80% of all cases (Haghshenas et al. 2017; Liu and Xing 2016). PTC has emerged to be even more dominant in recent years (Liu and Xing 2016). Latest data demonstrated that besides genetic alterations in PTC, like other tumors, which is identified by unfamiliar expression of microRNAs (miRNAs), a class of small noncoding RNAs modulates the gene expression at post-transcriptional level (Minna et al. 2016). Previously, several studies have examined miRNA dysregulation in PTC, and their usefulness as diagnostic and prognostic markers has already been endorsed (He et al. 2005).

MicroRNAs (miRNAs) are endogenous, single-stranded, small

non-coding RNAs with about 21-23 nucleotides in length. MiRNA modulates the gene expression by binding to the complementary sequences of target mRNAs and typically functions as a negative regulator at the post-transcriptional level through translational repression or degradation of mRNA targets (Carthew and Sontheimer 2009; de la Chapelle and Jazdzewski 2011). It is estimated that miRs altogether regulate around 30% of the human genome, emphasizing their crucial role as global regulators of gene expression. miRs participate in the biological processes such as development, apoptosis, cell proliferation, immune response, and hematopoiesis (Croce and Calin 2005).

MiR-125a is recognized to be a negative regulator of Kruppel-like factor 13 (KLF13), and the tumor necrosis factor-a-induced protein 3 (TNFAIP3) prevents the production of regulated activation of normal T cell and secreted (RANTES), and stimulates the nuclear factor kappa B (NFkB) pathway. The MiR125A gene, which encodes miR-125a, is located on chromosome 19q13.41 (Inoue et al. 2014).

miR-125a expression in the peripheral blood mononuclear cells (PBMCs) is associated with autoimmune thyroid disease (AITD) development and prognosis (Cai et al. 2017). In addition, miR-125a is down-regulated in systemic lupus erythematosus (SLE) (Zhao et al. 2010), breast cancer (Iorio et al. 2005), gastric cancer (Nishida et al. 2011), ovarian cancer (Cowden Dahl et al. 2009) and verrucous carcinoma (Odar et al. 2012) although the roles of miR-125a in thyroid cancer still remain unclear. In the nucleus, the Drosha system converts pri-miR-125a into mature hsa-miR-125a (Lehmann et al. 2013a). rs12976445 is located in the sequence of pre-mir-125 and disrupts a potential GATA-1 site (Cai et al. 2017). A previous study showed that this single nucleotide polymorphism links to the mature hsa-miR-125a serum concentration in breast cancer cells (Lehmann et al. 2013a). In this study, we hypothesized that miR-125a may contribute to the risk of thyroid cancer, especially PTC. Thus, we directed a case-control study in order to explore the association of rs12976445 with the risk of thyroid cancer, especially PTC in the Iranian population.

3. Materials and Methods

3.1. Study Design and Subjects

In the present research, we conducted a case-control study on 179 patients with thyroid cancer including papillary, follicular, medullary, and undifferentiated types, and 165 healthy donors. Cases were newly diagnosed subjects with histological confirmation. The inclusion criteria for the control group were no family history of malignancy and autoimmune disease. Clinical data including the tumor stage and type were obtained from medical records. Blood samples were collected from the subjects for genotyping after receiving an informed written consent from the participants. This study has been reviewed and permitted by the Ethics local committee at Shiraz University of Medical Sciences, Shiraz, Iran and have therefore been performed in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The informed consent was obtained for experimentation with human subjects.

3.2. SNP Selection Criteria

The mir-125 gene single-nucleotide polymorphisms (SNPs) were selected according to the following criteria:

1-it was located in the pre-mir-125 sequence which affects its maturation and leads to an increased risk of autoimmune thyroid conditions. 2. retrospective pathological studies suggested the association between autoimmune thyroid disease and PTC (Dailey et al. 1955; Okayasu et al. 1995; Ott et al. 1987). 3- it was a common polymorphism with the minor allele frequency (MAF) of more than 0.1. 4- previous literature indicated its association with several types of cancer. 5- until now, its association with PTC was not investigated. 6. Mir-125a regulates the nuclear factor kappa B (NFkB) pathway which control many aspects of thyroid cancer biology (Pacifico et al. 2004; Starenki et al. 2004). Based on the evidence mentioned above, we studied the association between rs12976445 polymorphism and risk of PTC in Iranian population.

3.3. DNA Extraction and Genotyping

DNA extraction from the peripheral blood leukocytes was performed using salting out method according to Miller' instructions (Miller et al. 1988). In order to investigate rs12976445 C/T polymorphisms, polymerase chain reaction restriction–fragment length polymorphism (PCR–RFLP) was done. The primers nucleotide sequences were as follow:

Forward primer 5′- TCTCTGTGGCTTCTGTGTCTCTT-3′,

Reverse primer 5′- CTCCGCATCCCAACAAACATCT-3′.

PCR settings were 94◦C for 5 min (pre-denaturation), 35 cycles of 95◦C for 30 (denaturation), 55◦C for 30s (annealing), and 72◦C for 60s (extension), and finished with a 5-minute final extension at 72◦C. After PCR, amplified fragments were digested using BaeGI fast digest restriction enzyme (Fermentas).

3.4. Statistical Analysis

Mean and standard deviations and frequencies of the basic characteristics were calculated. SNP distribution was tested by $\chi 2$ test to meet the Hardy–Weinberg equilibrium. Variations in the distribution frequencies of age, sex and genotypes concerning cases and controls were calculated by Student t-test or $\chi 2$. Logistic regression analyses were done to assess adjusted odds ratios (ORs) for the potential interfering factors (age and sex), and 95% confidence intervals (95% CIs) between total patients with thyroid malignancy and controls, and between patients with papillary thyroid malignancy and controls. The major homozygote and allele for the rs12976445 were set as a reference. The statistical analysis was performed by SPSS software package (version 22; SPSS Inc, Chicago, IL, USA). $P < 0.05$ was considered as significant.

4. Results

4.1. Subject Characteristics

The sex distribution in patients and controls was [male: 49 (27.9%), Female: 119 (72.1%)], and [male: 42 (23.5%), female: 137 (76.5%)], respectively. The mean age in patients and controls was 43.08±14.70 and 39.48±15.2, respectively. 120 out of 179 patients were PTC, and 67% of the patients were in stage I of thyroid cancer. The clinical and pathological features of the patients with thyroid cancer are presented in (Table 1). There was no significant difference between cases and controls in age (P=0.1) and sex (P=0.35).

Table 1: Clinic and pathological characteristics of the patients with thyroid cancer

Variables	Patients (n=179)	Controls (n=165)
Age	43.08±14.70	39.48±15.2
Sex		
Male	42(23.5%)	46 (27.9%)
Female	137(76.5%)	119 (72.1%)
Tumor type		
Papillary	158 (64.2%)	
Follicular	9 (5.0%)	
Medullary	10 (5.6%)	
Undifferentiated	2 (11.2%)	
Papillary tumor subtype		
Classic	142 (89.8%)	
Follicular variant	14 (8.8%)	
Columnar	2 (1.4%)	
Tumor stage		
I	120 (67%)	
II	27(15%)	
III	18 (10%)	
IV	12 (6.7%)	
Missing	2 (1.3%)	

4.2. Genotype Frequency

Genotypes and allele frequencies of rs12976445 gene polymorphisms in patients with thyroid cancer are reported in (Table 2). Patients and controls' genotype frequencies were consistent with Hardy–Weinberg equilibrium (P value for patients=0.39, P value for controls=0.18). The genotype frequencies for patients were [(TT: 81(45.2%), CT: 75(41.9%), CC: 23 (12.9%)], and for controls [(TT: 100 (60.1%), CT: 53(32.2%), CC: 12 (6.7%)]. The T allele distribution was significantly altered between patients and controls (P=0.002) with the odds ratio of 1.68.

Table 2: Genotype and allele frequencies of rs12976445 C/T gene polymorphisms in patients with thyroid cancer in comparison with controls

SNP	Genotype/allele	Patients with thyroid cancer (n=179)	Controls (n=165)	Odds ratio (95% CI)	*P* value
rs12976445 C/T	TT	81	100	(0.170-0185)	0.177
	CT	75	53	(0.060-0.070)	0.065
	CC	23	12	(0.086-0.097)	0.091
	T	237 (66.2%)	253 (76.6%)	1.68 (1.2-2.3)	0.002
	C	121 (33.8%)	77 (23.4%)		

4.3 The Association Between Mir-125 Rs12976445 C/T Polymorphisms and The Risk of Thyroid Cancer

The association between mir-125 rs12976445 C/T polymorphisms and the risk of thyroid cancer was illustrated in (Table 3). We first compared the effects of mir-125 rs12976445 C/T polymorphisms on the risk of all types of thyroid cancer in three inheritance models adjusted for age and sex. In the co-dominant model, TT genotype was set as reference and compared with CT, and CC genotypes. There was a significant difference between CT vs TT genotypes (adjusted OR = 1.69, 95% CI= 1-2.8, P = 0.026), and slightly significant differences between CC vs TT genotypes (adjusted OR = 2.18, 95% CI= 1-4.7, P = 0.047). In the dominant model, we com-

pared CT/CC genotype to the reference genotype (TT) and found a highly significant difference (adjusted OR = 1.78, 95% CI= 1.15-2.74, P = 0. 0.009). In the recessive model, TT/CT genotypes were compared to the CC genotype, and no significant variance was observed (adjusted OR = 0.57, 95% CI= 0.27-1.2, P = 0. 0.14). We then compared the association between mir-125 rs12976445 C/T polymorphisms and risk of papillary thyroid cancer. In the co-dominant and recessive models, no significant association was detected, while in the dominant model we identified that CT/CC genotypes significantly (P=0.04) increased the risk of papillary thyroid cancer when compared to CC genotype with the odds ratio (95% CI) of 1.6 (1-2.5).

Table 3: Association of mir-125 rs12976445 C/T polymorphisms with thyroid cancer

mir-125 rs12976445 C/T	Total patients with thyroid cancer (n=179)		Patients with papillary thyroid cancer (n=158)	
	Odds ratio (95% CI)	*P* value	Odds ratio (95% CI)	*P* value
Co-dominant				
TT	1			
CT	1.69 (1-2.8)	0.02	1.5 (0.94-2.4)	0.08
CC	2.18 (1-4.7)	0.04	1.9 (0.88-4.3)	0.1
Dominant				
TT	1		1	
CT+CC	1.78 (1.15-2.74)	0.009	1.6 (1-2.5)	0.04
Recessive				
TT+CT	1		1	
CC	0.57 (0.27-1.2)	0.14	0.6 (0.28-1.3)	0.26

4.4 Association of Mir-125 Rs12976445 C/T Polymorphisms with Thyroid Cancer and Its Stages

The association of mir-125 rs12976445 C/T polymorphisms with thyroid cancer stages is shown in Table 4. We first merged the patients with stages 1 and 2 in one group, and stages 3 and 4 in another group. Then, we performed a logistic regression analysis in three models to evaluate the association of mir-125 rs12976445 C/T polymorphisms between these groups. No significant association was found between the patients with stages 1/2 and those with stages 3/4 according to the mir-125 rs12976445 C/T polymorphisms.

Table 4: Association of mir-125 rs12976445 C/T polymorphisms with thyroid cancer stages

mir-125 rs12976445 C/T	Patients with thyroid cancer (n=179)	
	Odds ratio (95% CI)	*P* value
Co-dominant		
TT	1	
CT	0.62 (0.21-1.8)	0.4
CC	3.1 (0.57-16)	0.2
Dominant		
TT	1	1
CT+CC	0.85 (0.32-0.26)	0.75
Recessive		
TT+CT	1	1
CC	0.27 (0.05-1.38)	0.11

5. Discussion

To the best of our knowledge, this is the first study to explore the correlation of mir-125 rs12976445 C/T with the risk of thyroid cancer. Initially, we hypothesized that T allele was the risk factor of thyroid cancer in patients when compared to the controls. We found that the rs12976445 CT and CC genotypes increased the risk of thyroid cancer (all types), 1.69-fold and 2.18-fold, respectively. We also found that CT/CC genotypes had a 1.6-fold increased risk of papillary thyroid cancer. Furthermore, regression analysis demonstrated that miR-125a rs12976445 was an independent risk factor for thyroid cancer. In addition, there was no correlation between mir-125 rs12976445 C/T polymorphisms and thyroid cancer stages.

miR-125a plays a critical role both in organ development and in the adult tissues (Jiao et al. 2014). miR-125a functions as an oncogene or a tumor suppressor depending on the cellular background (Jiao et al. 2014). Gathering evidence has suggested that miR-125a is associated with the pathogenesis of human cancers, such as lung, gastric, and breast cancer (Li and Lei 2015). Furthermore, the effect of miR-125a on the growth, migration and apoptosis of multiple myeloma cells is p53-dependant (Guo et al. 2010). The expression level of miR-125a not only is correlated with prognosis of cancer patients, but also makes an influence on the response of cancer cell lines to anticancer drugs (Ufkin et al. 2014). The downregulation of miR-125a was reported in some cancers, such as leukemia (Shaham et al. 2012) and lung cancer (Zhu et al. 2014), where the increased expression suppressed the proliferation of cancer cells and promoted apoptosis. Recent evidence showed that mature miRNA processing and mature miR-125a expression may be interrupted by the T allele of rs12976445, resulting in augmented production of target genes, including LIFR and ERBB2 (Hu et al. 2011). A SNP (rs12976445) is present in the pre-miR-125a gene, and it is involved in the processing of mature miRNA, which leads to an increased risk of autoimmune thyroid disease. It has been stated that miR-125a concentration is reduced

in Graves' disease (GD) (Inoue et al. 2014). Diminished levels of hsa-miR-125a were detected in correlation with the rs12976445 variant (Hu et al. 2011). The mechanism of the rs12976445 gene polymorphisms' effect on the level of miR-125a remains undecided and it is not clear whether it is the effect of transcriptional regulation, maturation of hsa-miR-125a or transport of hsa-miR-125a (Lehmann et al. 2013b).

de la Chapelle et al. reviewed the role of mir in thyroid cancer (de la Chapelle and Jazdzewski 2011). They showed that mir-125a is a suppressor of the main oncogenes involved in prognosis and susceptibility of thyroid cancer including BRAF and RAS, and also MAPK/ERK pathway (de la Chapelle and Jazdzewski 2011). BRAF serine-threonine kinase is a member of the family of RAF proteins, which are intracellular effectors of the MAPK signaling cascade. After activation generated by RAS binding and protein recruitment to the cell membrane, these kinases phosphorylate and activate MEK, which sequentially activates ERK and resultant effectors of the MAPK cascade (Nikiforov 2008). In the present study, we observed that subjects with at least one mutant allele (CT or CC) had increased risk of thyroid cancer. The association of C allele with increased risk of cancer was also found in patients with papillary thyroid cancer. Since point mutations of the BRAF gene occur in ~45% of thyroid papillary carcinomas which result in constitutive activation of BRAF kinase, and mir-125 is a negative regulator of BRAF, it seems that changing T to C in mir-125 results in augmentation of production and activation of its target genes like BRAF in thyroid cancer cells and also cause continuous phosphorylation of MEK (Nikiforov 2008) which finally leads to cell proliferation, differentiation, and survival. Nishida et al. showed that miRNA-125a-5p had an anti-proliferative effect through decreasing the proliferation of the related genes, including ERBB2, a member of the EGF receptor family of receptor tyrosine kinases, which maintains a key initiator of phosphoinositide-3 kinase (PI3K)-AKT and RAS/RAF/mitogen-activated protein kinase signaling (Nishida et al. 2011). Therefore, we speculate that this may be an explanation for the role of this polymorphism in the prognosis of thyroid cancer.

6. Conclusion

As concluding remarks, our findings suggest that miR-125a rs12976445 is a possible prognostic biomarker for thyroid cancer patients. Further larger-scale and well-designed clinical investigations are necessary to confirm these outcomes. Also, more studies are needed to evaluate the effect of this polymorphism on mir-125 expression in thyroid cancer and its influence on expression of target genes related to the pathways involved in prognosis of thyroid cancer to indicate the exact mechanism of mir-125a in thyroid cancer.

7. Statements and Declarations

Compliance with Ethical Standards: "All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards." This study has been reviewed and permitted by the Ethics local committee at Shiraz University of Medical Sciences, Shiraz, Iran and have therefore been performed in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The informed consent was obtained for experimentation with human subjects.

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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