ISSN: 0067-2904

Association of rs965513 Polymorphism Near *FOXE1* Gene with Papillary Thyroid Carcinoma in a sample of Iraqi patients

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Received: 10/12/2022 Accepted: 23/1/2023 Published: 30/12/2023

Abstract

Thyroid carcinoma incidence is increasing yearly and ranks second among the top ten cancers in Iraq, especially among women. The single nucleotide polymorphism (rs965513, A>C) near *FOXE1* gene was found to be associated with papillary thyroid carcinoma risk in several Genome-wide association studies. Therefore, this is a case-control study aimed at identifying whether this variation is associated with the risk of papillary thyroid carcinoma in the Iraqi population. Association of rs965513 was investigated in one hundred and one papillary thyroid carcinoma Iraqi patients and one hundred and two controls using quantitative PCR-high resolution melting technique. The results of genotype and allele frequencies showed that there is a strong association between rs965513 and papillary thyroid carcinoma. The allele C was significantly associated with the disease as a risk factor ($P < 0.0003$, odd ratio (OR): 2.1014, 95% confidence interval (CI): 1.3987-3.1570), while the allele A represents a protective factor (OR=0.4759). In conclusion, the allele C in rs965513 near *FOXE1* gene is strongly associated with papillary thyroid carcinoma in the Iraqi population.

Keywords: rs965513; *FOXE1*; papillary thyroid carcinoma; Case-control study; HRM

علاقة تعدد اشكال النيوكليوتيدة الاحادية rs965513 بالقرب من المورث *FOXE1* مع سرطان الغدة الدرقية الحليمي في عينة من المرضى العراقيين

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الخلاصة

يتزايد معدل الإصابة بسرطان الغدة الدرقية عامًا بعد عام حتى اصبح يحتل المرتبة الثانية بين العشرة سرطانات الاولى في العراق، وخاصة بين النساء. وجد بأن تعدد أشكال النيوكليوتيدة الاحادية (rs965513، A>C) بالقرب من المورث *FOXE1* يرتبط بمخاطر الإصابة بسرطان الغدة الدرقية الحليمي في العديد من دراسات الارتباط على نطاق الجينوم، وبالتالي، فهذه دراسة من النوع مرضى– سيطرة تهدف إلى تحديد ما إذا كان هذا التنوع مرتبطًا بخطرالاصابة بسرطان الغدة الدرقية الحليمي في المجتمع العراقي. تم التحري عن rs965513 في مائة وواحد من مرضى سرطان الغدة الدرقية الحليمي العراقيين ومائة واثنين من عينات السيطرة باستخدام

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تفاعل البلمرة المتسلسل المرتبط بتقنية الانصهار عالية الدقة. أظهرت نتائج الطرز الوراثية وتكرارات الأليل أن هناك ارتباطاً وثيقاً بين rs965513 وسرطان الغدة الدرقية الحليمي، وكان الأليل C مرتبطًا بالمرض ارتباطا وثيقا كعامل خطر (الاحتمالية <0.0003، النسبة الفردية: 2.1014، ثقة الفاصل الزمني 95٪ (1.3987- 3.1570) ، بينما يمثل الأليل A عامل وقائي (النسبة الفردية: 0.4759). ومن ذلك تم الاستنتاج بأن الأليل C الموجود في rs965513 قريبا من المورث *FOXE1* يرتبط ارتباطًا وثيقًا بسرطان الغدة الدرقية الحليمي في المجتمع العراقي.

Introduction

Thyroid carcinoma (TC) is a disease in which thyroid gland cells grow abnormally and may spread, if not treated, to other body parts. It is classified as differentiated thyroid cancer (involving papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma) and undifferentiated thyroid cancer (anaplastic) according to histology, in which 75% of thyroid carcinoma cases belong to PTC [1], [2], and when diagnosed early, are mostly associated with a good prognosis and rate of survival [3]. In Iraq, in the last few years, TC ranked second among the top ten cancers in women. The incidence trend of all the top ten cancers in females decreased for the last few years, except for thyroid gland cancer. The incidence trend was increasing [4]. The studies of molecular association have identified multiple genes variants for differentiated thyroid carcinoma, and this indicates that there is a high percentage of genetic heterogeneity and the development of this carcinoma tumor represents a multifactorial process in which predisposing genomic variants interact with environmental risk factors which are incompletely understood [5]. Several genetic factors were associated with thyroid disorders worldwide, such as *BRAF* [6], *LARP7* gene [7], and *CTLA*-4 gene [8]. One of the genes that known to have an effect on thyroid function is *FOXE1*, that expresses the Forkhead box protein E1 [9], which is a protein that belongs to one of the transcription factors families (the forkhead family), which has a distinct forkhead domain. One of these functions is that it is a thyroid transcription factor that plays a crucial role in the morphogenesis of the thyroid [10]. One single nucleotide polymorphism (SNP: a variation in a single nucleotide in a specific locus in the genome, which has an important role in the molecular diagnosis) near *FOXE1* region (rs965513) has been revealed to associate with various etiologies related to the thyroid in a number of ethnics [11], such as Caucasians [12], and East Asians [13]. For the reason that SNPs effects differ from one race to another, every race should have its study. Because of the increasing number of PTC in Iraq, especially in women, and because there is no published study on the association of rs965513 with the PTC in Iraq, this study was conducted.

Material and Methods

Participants recruitment

Consent was obtained from both the PTC Iraqi patients and controls, and all the participants were informed about the details and consequences. Some personal information was collected from patients and controls using structured questionnaire. This study was adhered to the tenets of the Declaration of Helsinki and approved by the College of Science Research Ethics committee (Ref.: CSEC/1022/0125) in the University of Baghdad, Iraq, and the Iraqi Ministry of Health. Blood samples were collected from 203 participants (101 PTC patients: 90 women and 11 men, of ages (22-65) years who underwent thyroidectomy and were diagnosed and histologically confirmed at the Baghdad Center for Radiation Therapy and Nuclear Medicine, City of Medicine, Baghdad, Iraq according to the criteria of the Iraqi Ministry of Health and 102 controls: 86 women and 16 men, of age ranged from (21-67) years. The blood samples were randomly collected from May 2021 to September 2021, while the control samples were collected from unrelated apparently healthy people from various regions in Iraq to be matched

with patients in gender and age, who were used to perform a periodic health examination and who did not have a medical history with any type of cancers.

Inclusion and exclusion criteria

The selection criteria were: Patients diagnosed with PTC after thyroidectomy, while the cases excluded were: a. Patients with PTC treated with radioiodine capsules, chemotherapy, or hormonal therapy. b. Patients with other types of TC (follicular, medullary, and anaplastic), c. Patients with PTC who have a history with cancers other than TC. d. patients with other types of thyroid diseases, and e. Pregnant women.

Thyroid hormones testing (T3, T4 and TSH)

Three thyroid hormones (T3, T4, and TSH) were investigated in the serum of both PTC patients and controls to evaluate thyroid function status using the ELISA technique (TOSOH, Japan).

DNA extraction

Genomic DNA was extracted using *EasyPure*® Genomic DNA Kit (TransGen Biotech, China) from blood samples conserved in EDTA tubes.

Quality and quantity analysis of DNA

DNA concentrations and purities were estimated by Nanodrop spectrophotometer (Nanovau, China) and gel electrophoresis was carried out for the detection of DNA integrity [14], [15].

Primers design

The primers were designed using Beacon Designer 8, shown in Table 1 (Bioneer, Korea).

Table 1: Designed primers sequences

Genetic locus	SNP	Primer Sequences (5' – 3')	Annealing Temperature (°C)	HRM (°C)
Near *FOXE1* gene region	**rs965513**	ADPRT2 **F** GGTAATGAGTGGCTGGAATGGAACA 25 ADPRT2 **R** CCCAGGCTCAGGTTATGTCTTTGT 24	58	Wild 74 Mutant 74.4

DNA genotyping using Qualitative PCR-High Resolution Melting (qPCR-HRM)

All PTC patients and controls in this case-control study were genotyped by using qPCR-HRM technique (Rotor-Gene Q Series Software 2.1.0 QIAGEN, Germany). The specific region of rs965513 near *FOXE1* was amplified using the designed primers (Table 1) and qPCR-HRM conditions (Cycling profile) summarized in Table 2. Amplification reactions were carried out in a total volume of 20 µl containing TransStart® Tip Green qPCR SuperMix (TransGen Biotech, China), 200 nm from each of the forward and reverse primers (Bioneer, Korea), 60 ng of genomic DNA, and the volume was completed to 20 µl using deionized D.W. (Bioneer, Korea). The patient's samples were then compared with control samples.

Table 2: Cycling profile of qPCR-HRM

Step	Temperature (^{O}C)	Duration	Cycle no.
Hold	94	1 min.	1
Denaturation	94	5 sec.	35
Annealing	58	15 sec.	
Extension	72	20 sec.	
HRM	60-90	0.1˚C/sec	

Statistical analysis

The G*Power 3 software was used to estimate the sample size's power. This study has 95% power [16]. Pearson Chi-square test was used and the significance was evaluated based on the minimum *p*-value ($p \leq 0.05$). The normal distribution of serum thyroid hormones was tested using Kolmogorov-Smirnov and Shapiro-Wilk normality testing to ascertain whether the data deviated from normality, The data subjected to the normal distribution (Parametric variables) were given as mean ± SD. The significant differences between patients and control groups were tested using a T-test. In contrast, data that significantly deviated from a normal distribution, were given as median and range. Thus, the significant differences between medians were assessed by Mann–Whitney U test. Hardy-Weinberg Equilibrium (HWE) was used to compare observed and expected values to evaluate genotype distribution in the population. The odd ratios were calculated using the software (https://www.medcalc.org/calc/odds_ratio.php) to check if the alleles and genotypes represent risk factors or protective factors, with a confidence of interval (CI estimate at 95%). The statistical analysis was carried out using IBM SPSS Statistics 22.

Results and Discussion

One hundred and one PTC patients and one hundred and two control participants have been recruited for this study. The control group samples were collected to be gender matched with the patients' group with no statistically significant difference in the distribution of gender between the two groups ($p \leq 0.3$). The percentage of females suffering from PTC was significantly higher than males; thus, the total percentage of females (in both patients and controls groups) was 86,70%. In comparison, the total percentage of males was 13,30% as shown in Figure 1. That could be according to the role of sex hormones in cancer, which is very important and well documented for a number of cancers such as prostate and breast. The effect of sex hormones is mediated by hormone-specific nuclear receptors that regulate gene expression and the biology of tumor cells [17]. The effect of estrogen is mediated by estrogen receptors α- and β-, which are expressed in PTC [18]. Researchers found that the polymorphism in estrogen receptors can be a risk factor for TC [19]. The cell proliferation in TC cell lines can adequately increase by estrogen hormone compared to male sex hormones [20]. It also modifies the estrogen receptor subtypes expression in TC cell lines. The estrogen increases the levels of estrogen receptor-α in PTC. At the same time, there are no significant alterations in anaplastic and follicular carcinomas, because the effects of estrogen on TC cell lines are type-dependent [21]. There is an association between estrogen and increased adherence, invasion and migration in TC cell lines [22]. These observations could play a role in the disparity of gender observed among PTC patients, and can serve as an important and potential target in the treatment of PTC.

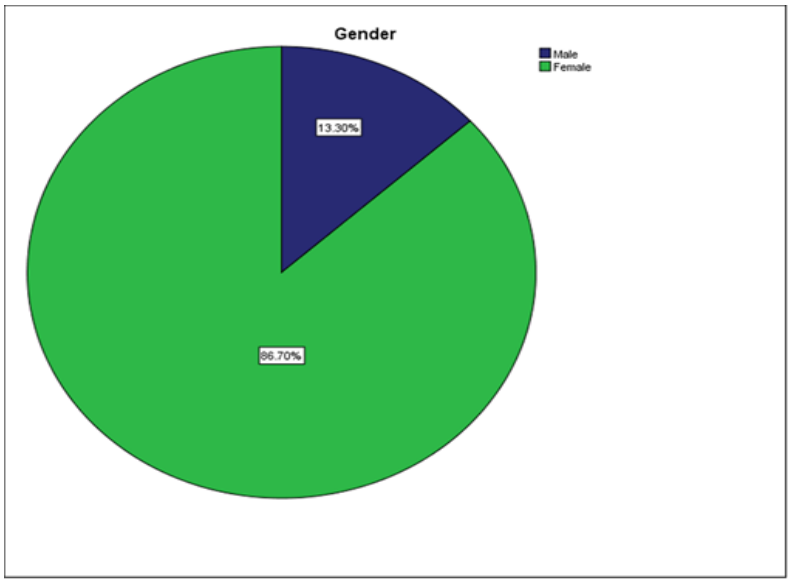

Figure 1: Distribution of subjects according to gender, the total percentage of females (in both patient and control groups) was 86,70%, and the total percentage of males was 13,30%.

The mean age ± SD of PTC patients (40.76 ± 10.79) years was higher than controls (37.11 ± 9.72) years, but the difference was not significant ($p \leq 0.08$) as shown in Figure 2. Papillary thyroid carcinoma is popular in various groups of age, but it shows a higher relative frequency in ages between 31 and 50 years [23] and this may be according to that younger individuals own a higher rate of proliferation in the cells of thyroid tissue [24]. The normality test for thyroid hormones revealed that the serum levels of T3 in both patients and control groups were subjected to the normal distribution, while serum levels of T4 and TSH hormones significantly deviated from the normal distribution. The results of thyroid hormonal levels in patients and controls were shown in Table 3.

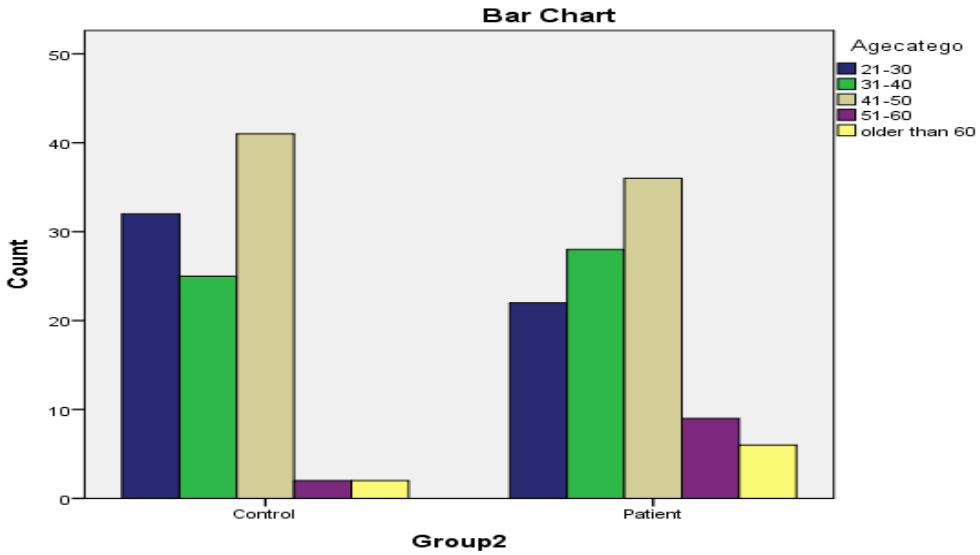

Figure 2: Distribution of subjects according to age categories

The thyroid hormonal levels were altered after surgery, and this come to an agreement with [25] who reported that T4 levels were higher and T3 levels were lower in individuals who underwent total thyroidectomy in comparison to control group matched by TSH level. It is suggested that the variable kinetics hypothesis of the thyroid hormonal levels is associated with

thyroid gland manipulation during surgery, but these alterations do not result in clinical effects, including thyrotoxicosis [26].

Table 3: Thyroid hormones levels in patients and controls

Group	T3(Mean±SD)	T4Median(range)	TSH Median (range)
Patients	1.39±0.53	89.85±(23.70-139.90)	3.30±(0.71-16.40)
Controls	1.63±0.54	8.10±(7.65-8.30)	3.87±(2.39-4.48)
***p*-value**	0.025	0.009	0.968

The deviations of the differences of the alleles and genotypes frequencies for the patients and controls were tested using HWE and both of them deviated from the equilibrium. The genotype distribution of rs965513 in the patients and controls groups was shown in Table 4.

Table 4: Hardy-Weinberg equilibrium (HWE) analysis of SNP rs2275913 near *FOXE1* gene among PTC patients and controls.

Genotype	Thyroid carcinoma patients (N=101)		Controls (N=102)	
	N (%)		N (%)	
	Observed	**Expected**	**Observed**	**Expected**
AA	38 (37.63%)	27.29 (27.02%)	60 (58.82%)	50.82 (49.83%)
AC	29 (28.71%)	50.42 (49.92%)	24 (23.53%)	42.35 (41.52%)
CC	34 (33.66%)	23.29 (23.06%)	18 (17.65%)	8.82 (8.65%)
HWE *p* value=	p<0.0001		p<0.0001	

the genotype distribution frequencies in both of the control and patient groups deviated from HWE, because the SNP is located in a functional region near *FOXE1*, and the deviations from HWE in parental and unaffected sibling (controls) genotype could be due to an association with the regulatory regions (functional loci) [27]. The results of the association of alleles and genotypes with PTC revealed that the C allele of rs965513 was a risk factor for the disease, and its frequency in the patients has a highly significant difference from that in the controls ($p < 0.0003$, odd ratio (OR): 2.1014, 95% confidence interval (CI): 1.3987 to 3.1570), while the allele A was a protective factor with OR of 0.4759. The genotypes odd ratio for the heterozygotes (AC) was 2.9310 and the rare homozygotes (CC) odd ratio was 2.9825, which means that both genotypes are risk factors for the disease. The normalized graph of rs965513 generated by qPCR-HRM software is shown in Figure 3. Forkhead box E1 gene is located on chromosome 9q22.23 and contains one exon only, it encodes the thyroid transcription factor 2 (TTF-2), which is a DNA-binding protein, and a member of the forkhead/winged helix family of the transcription factors that are evolutionarily conserved [28]. The *FOXE1* is a key player in the organogenesis of the thyroid and the migration and differentiation of the thyrocyte precursors, with an expression onset of the thyroid primordium at the Carnegie stage 15 in humans [29]. Our finding that rs965513 SNPs near *FOXE1* were associated with PTC susceptibility supports findings of the studies in other ethnics, which also pointed out a strong association between rs965513 and PTC in the other ethnics, but the C allele was the risk factor in the Iraqi population and the A allele was the protective factor, while the A allele represents the risk factor for all of the other studied populations such as Caucasian [12], German population [30], and East Asians [13], in which the A allele was the risk factor.

Conclusion

In conclusion, There is a strong association between rs965513 and PTC in Iraqi patients, and because it represents a risk factor for several other ethnics, and because of the potential role of *FOXE1* in thyroid gland function, additional association studies should be independently carried out for other races because of their significant impact in the molecular diagnosis of the PTC, which is increasing day after day, especially among women in the world, especially with the limitations of the diagnosis and the lack of prognostic factors accuracy, which are important clinical challenges.

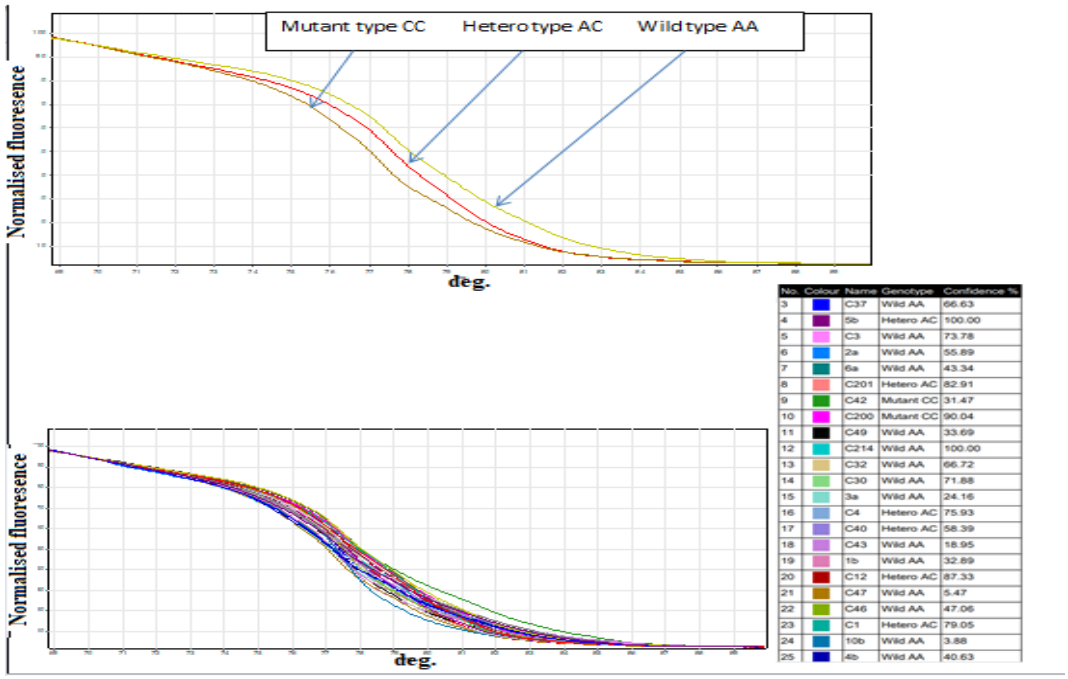

Figure 3: Normalized graph of qPCR-HRM profile result of the rs965513 near *FOXE1* gene

Acknowledgements

We would like to thank all of the participants and their families for providing this study with blood samples, and the staff members of Baghdad Center for Radiation Therapy and Nuclear Medicine, City of Medicine, Baghdad, Iraq for providing us with the clinical information of papillary thyroid carcinoma patients. We would also like to thank the staff members of the Genetic Engineering Laboratory in the Department of Biotechnology, College of Science, University of Baghdad, Iraq for their excellent technical assistance, and Prof. Dr. Ahmed A. Suleiman, University of Anbar, Anbar, Iraq for helping in primers designing and Prof. Dr. Ali H. Ad'hiah, University of Baghdad, Baghdad, Iraq for helping in statistical analysis and for their kind support and helpful discussion.

Conflict of Interest:

The authors declare that they have no conflicts of interest.

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