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# Association between FOXP3, FOXE1 Gene Polymorphisms and Risk of Differentiated Thyroid Cancer in Chinese Han Population

**Weichao Jiang[1]*, Lei Zheng[1], Lijuan Xu[2,3], Yang Zhang[1], Xingxin Liu[1], Lihua Hu[1] and Xiaobei Wang[1]**

[1]*Department of Clinical Laboratory, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, P. R. China*
[2]*Department of Clinical Laboratory, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, 430022, P. R. China; 2*
[3]*Department of Clinical Laboratory Medicine, Chinese People's Liberation Army General Hospital and Postgraduate Medical School, Beijing, P. R. China*

## Abstract

**Background:** Thyroid transcription factor gene (FOXE1) and Foxp3 play important roles in autoimmune and inflammatory disease as well as human malignancies. We aimed to investigate the association of FOXE1 and Foxp3 polymorphism with the susceptibility to differentiated thyroid cancers (DTC).

**Methods:** Genotyping was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) in 350 DTC patients and 306 healthy controls.

**Results:** AA/AG genotype of FOXE1-rs1867277 and AA/AC genotype of Foxp3-rs3761548 were associated with a higher risk of DTC. The frequency of Foxp3-rs2280883 CC/CT genotype was lower in DTC patients. Besides, the AA/AC genotype of rs3761548 was more frequent in female DTC than the male. We further analyzed the association between 3 single nucleotide polymorphisms (SNPs) and DTC. We found that rs3761548 AA/AC genotype was more frequent in severe DTC patients (tumor diameter >1 cm) compared with the relative tender DTC patients (tumor diameter <1 cm). On the contrast, the frequency of rs2280883 CC/CT genotype was lower in severe DTC patients.

**Conclusion:** Our findings suggested that FOXE1 and Foxp3 polymorphism were associated with the risk of DTC in Chinese Han population. Besides, rs3761548 AA/CC genotype was a potential risk factor for the susceptibility to DTC and rs2280883 CC/CT was a protective factor.

**Keywords:** Single nucleotide polymorphism; Differentiated thyroid cancer; FOXE1; FOXP3

## Introduction

Thyroid carcinoma is the most common malignant thyroid tumor, accounting for about 1% of all malignant tumors [1]. Besides medullary carcinoma, most of thyroid cancer originated in the follicular epithelial cells. The differentiated thyroid cancers (DTC) include papillary (PTC, 80-90% of cases) and follicular (FTC, 10% of cases) subtypes and their etiology are still not very clear [2]. It is no doubt that thyroid carcinoma is the result of interaction between genetic and environmental factors [3,4]. There are a lot of reports that single nucleotide polymorphisms (SNPs) are associated with DTC risk. However, most of these associations have not been investigated in different populations. Association of thyroid specific genes (such as TSHR, TPO, TG, PDS, TTF1, FOXE1) and non-thyroid specific genes with the risk of thyroid carcinoma is investigated [5,6].

As a gene with a single exon located at chromosome 9q22.33 [7], FOXE1 (Forkhead Box E1, also known as Thyroid transcription factor 2), is a thyroid-specific forkhead transcription factor that is essential for thyroid gland development, as well as for the maintenance of the thyroid differentiated state in adults [8] FOXE1 is highly expressed in thyroid follicular cells [9] and lots of study data showed that this gene is crucial in the initiation of specific tumors, such as pancreatic cancer, cutaneous squamous cell carcinoma and thyroid neoplasms [10]. Some studies also supposed that upregulation of FOXE1 could play a role in the malignant behaviour of thyroid cells [11], and some case-control studies have been performed to investigate the role of SNP of FOXE1 (rs1867277) in the genetic susceptibility of both PTC and FTC. In addition, a meta-analysis indicated that common variations of FOXE1 were associated with increased thyroid carcinoma susceptibility [12].

FOXP3 (Forkhead Box Protein3), a member of transcription factor winged-helix family, is involved in regulating the immune system development and function by playing an indispensable role in the generation of regulatory T cells (Tregs) [13]. Expression of FOXP3 is crucial for Tregs which may influence its function in several different mechanisms [14]. A number of studies have been performed to investigate the polymorphisms of Foxp3 promoter (rs3761548 and rs2280883) in various diseases including breast cancer in different populations [15]. However, there are no reports of Chinese DTC patients for these three SNPs.

According to the above data, we speculated that the gene polymorphisms of FOXE1 and FOXP3 would be related to the risk of DTC. The change of genotypes and allele frenquency could affect the transcription process, as well as the expression of specific genes which are involved in tumor initiation and immune system, as result of increasing or decreasing the risk of DTC. To test our hypothesis, we conducted the following experiments.

In this study, we investigated roles of two polymorphisms (rs3761548 and rs2280883) within the promoter of FOXP3 gene and one polymorphism (rs1867277) within the promoter of FOXE1 in DTC patients. As knowledge have been obtained, this represents the first report on association between FOXP3 and DTC worldwide and it is also the first study on association between FOXE1 and DTC in Chinese population.

***Corresponding author:** Weichao Jiang, Department of Clinical Laboratory, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, No. 1227 Jiefang Road, Wuhan, Hubei, 430022, P. R. China, E-mail: jwc027@hotmail.com

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## Material and Methods

### Subjects

In total, 350 patients (281 females and 69 males) with pathologically confirmed diagnosis of DTC were recruited from the Department of Breast and Thyroid surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei Province, People's Republic of China. Of these patients, 303 were papillary (241 females and 62 males) and 47 were follicular (38 females and 9males). All the patients were recruited when they were first diagnosed as thyroid cancer without any treatments. A cohort of 306 healthy controls (HC, 228 females and 78 males), were selected as control group, with a routine healthy examination in the same hospital. The volunteers of our healthy control had no evidence of thyroid and autoimmune diseases, without radiation exposure or occupational exposure risks in the past. The demographic information of both cases and controls was carried out from self-administered questionnaires. All subjects were Chinese Han ethnic origin and from the Central China.

Whole blood samples were collected by venipuncture. Genomic DNA was extracted from EDTA-treated peripheral blood cells by TIAN combi DNA Lyse and Det PCR Kit (TIANGEN Biotech, Beijing), according to the manufactures introduction. Our study had been approved by the Wuhan Union Hospital Ethics Committee and all the subjects had informed consent.

### Genotyping

Genotyping for the FOXE1 (rs1867277), FOXP3 (rs3761548), FOXP3 (rs2280883) in the promoter region were performed with polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The primers, restriction enzymes (New England Biolabs, Beverly, MA, USA) and product sizes were listed in the Table 1.The PCR conditions for FOXE1 (rs1867277) were as follows: initial denaturation at 95°C for 5 min, 35 cycles of denaturing at 94°C for 30 sec, annealing at 68°C for 30 sec, extension at 72°C for 1 min and a final extension at 72°C for 10 min. The PCR conditions for FOXP3 (rs3761548 and rs2280883) were: denaturation at 94°C for 5 min, 38 cycles of denaturing at 94°C for 30 sec, annealing at 61°C for 15 sec and 59°C for 15sec, extension at 72°C for 30 sec and a final 5 min extension at 72°C.

The PCR products were purified by PCR purification kit (Takara, Japan).The system for the restriction enzyme reaction included 5.5 μl PCR product, 1 μl Buffer, 1 μl restriction enzyme, 2.5 μl $H_2O$. After incubating at 37°C for 30 minutes, the digested products were analyzed by 2% agarose gel electrophoresis containing SYBR green at 100 V for 20 minutes. Besides, the PCR products were sent to the company of Sangon Biotech (Shanghai) for direct sequencing, using an ABI 3730XL DNA Analyzer (Applied Biosystems).

### Statistical analysis

The mean age in patients and HC was compared using student's test. Chi-square ($\chi^2$) test or Fisher exact test was used to compare the allele and genotype frequencies between patients and controls and various subgroups. By SPSS17.0, unconditional univariate and multivariate logistic regression analyses were performed to obtain the crude and adjusted odds ratio (OR) and their 95% confidence intervals (CIs).

In order to prove FOXE1 and FOXE3 as susceptibility genes of DTC in Chinese population, the comparison of the genotype/allele frequencies between cases and controls were analyzed for each cancer group separately (DTC/PTC/FTC vs. HC respectively). Furthermore, patients were divided into subgroups according to their sizes (<1 cm or >1 cm), and sites (unifocal and bilateral PTC). In addition, patients with PTC were classified according to calcification state and lymph node metastasis based on the pathological findings. Data analysis was carried out by SPSS version 17.0. Statistical significance was defined as a two-sided p-value <0.05.

## Results

### General characteristics

In total, we collected 350 patients (281 females and 69 males) and 306 healthy controls (228 females and 78 males), the average age was 40.6 ± 13.6 yr. and 40.1 ± 9.9 yr., respectively. There was no significant difference of gender and age between the two groups (P=0.944; P=0.60). The distributions of males and females were 19.71% and 80.29% respectively in DTC, 19.93% and 80.07%, respectively in HC. We also collected information about tumor features and disease progression, including tumor location, tumor size, lymph node metastases, and calcification state.

### Genotypes and allele frequencies of FOXE1 and Foxp3 polymorphisms between DTC and HC

The genotypes and allele frequencies of rs1867277 A/G, rs3761548 A/C and rs2280883 C/T in the patients and controls were shown in Table 2, as well as their associations with the risk of DTC.

For FOXE1-rs1867277, the frequencies of AA, AG and GG were 4.9%, 20.0% and 75.1% in DTC, 3.3%, 13.7% and 83.0% in HC, respectively. The difference was statistically significant (P=0.049). When we used the GG genotype as the reference, we found that the AG genotype was associated with a higher risk of DTC (OR, 1.610; 95%CI, 1.058-2.449). Additionally, A allele was observed more frequent in DTC than in HC. These differences in the genotypes and allele distributions between patients and controls were more pronounced in the DTC histological PTC subtype (P=0.024, P=0.023 respectively) than FTC (P=0.079, P=0.024, respectively). Although the genotypes of rs1867277 had no significant difference between DTC and HC, the frequency of A allele was higher in PTC and FTC than HC (14.4% vs 10.1%; 18.1% vs 10.1% respectively). We also found that the AG genotype was associated with a higher risk of PTC (OR, 1.611; 95%CI, 1.046-2.281).

As shown in Table 3, the frequencies of Foxp3 rs3761548 were significantly different between the DTC and HC (P=0.042). The frequency of A allele was higher in DTC than HC (21.0% vs. 15.5%, P=0.011). Consistent with the A allele distribution, the frequency of A carriers (AA+AC) was higher in DTC (36.6%) than HC (27.5%, P=0.013) too. When we used the CC genotype as the reference, we found that the AC genotype was associated with a higher risk of DTC (OR, 1.493; 95%CI, 1.052-2.119). In addition, the A variant was associated with an increased risk of DTC (OR, 1.524; 95%CI, 1.093-2.124). The difference in allele distributions between patients and HC was only found in the PTC subtype (P=0.017), not in the FTC (P=0.097). The

| SNP | Forward primer (5'→3') | Reverse primer (5'→3') | Restrict enzyme | Product size |
|---|---|---|---|---|
| FOXE1-rs1867277 | GCCGCCCCACGCTTCGTGGCCCCTTTAA | CCTGCTGCCGTCTCGCCGCGCTCTTCCT | NruI | 820bp |
| FOXP3-rs3761548 | CCTCTCCGTGCTCAGTGTAG | GGGTGTTACAAGGAAAGGTTGGGAC | PstI | 473bp |
| FOXP3-rs2280883 | GCCTCAGCCTTCGCCAATA | ACCTAACCCTCTCCTGGACCCATA | MspI | 225bp |

**Table 1:** The primers, restriction enzymes (New England Biolabs, Beverly, MA, USA) and product size.

| | HC(n=306) | DTC(n=350) | P[a] | OR(95% CI)[b] | PTC(n=303) | P | OR(95% CI) | FTC(n=47) | P | OR (95% CI) |
|---|---|---|---|---|---|---|---|---|---|---|
| | N (%) | N (%) | | | N (%) | | | N (%) | | |
| rs1867277 | | | | | | | | | | |
| GG | 254(83.0) | 263(75.1) | | 1 | 229(75.6) | | 1 | 34(72.3) | | 1 |
| AG | 42(13.7) | 70(20.0) | **0.049** | 1.610(1.058-2.449) | 61(20.1) | 0.075 | 1.611(1.046-2.281) | 9(19.1) | 0.166 | 1.601(0.716-3.577) |
| AA | 10(3.3) | 17(4.9) | | 1.642(0.738-3.654) | 13(4.3) | | 1.442(0.620-3.352) | 4(8.5) | | 2.988(0.888-10.056) |
| AA+AG | 52(17.0) | 87(24.9) | **0.014** | 1.616(1.100-2.373) | 74(24.4) | **0.024** | 1.578(1.061-2.348) | 13(27.6) | 0.079 | 1.868(0.923-3.781) |
| A allele | 62(10.1) | 104(14.9) | **0.010** | | 87(14.4) | **0.023** | | 17(18.1) | **0.024** | |
| rs3761548 | | | | | | | | | | |
| CC | 222(72.5) | 222(63.4) | | 1 | 192(63.4) | | 1 | 30(70.2) | | 1 |
| AC | 73(23.9) | 109(31.2) | **0.042** | 1.493(1.052-2.119) | 96(31.7) | 0.052 | 1.521(1.060-2.181) | 13(23.4) | 0.278 | 1.318(0.653-2.660) |
| AA | 11(3.6) | 19(5.4) | | 1.727(0.803-3.714) | 15(4.9) | | 1.577(0.707-3.515) | 4(12.8) | | 2.691(0.805-8.990) |
| AA+AC | 84(27.5) | 128(36.6) | **0.013** | 1.524(1.093-2.124) | 111(36.6) | **0.015** | 1.528(1.084-2.153) | 17(36.2) | 0.218 | 1.498(0.785-2.857) |
| A allele | 95(15.5) | 147(21.0) | **0.011** | | 126(20.8) | **0.017** | | 21(22.3) | 0.097 | |
| Rs2280883 | | | | | | | | | | |
| TT | 227(74.2) | 288(82.3) | | 1 | 251(82.8) | | 1 | 37(78.7) | | 1 |
| CT | 69(22.5) | 49(14.0) | **0.017** | 0.560(0.373-0.840) | 42(13.9) | **0.021** | 0.550(0.360-0.841) | 7(14.9) | 0.325 | 0.622(0.266-1.459) |
| CC | 10(3.3) | 13(3.7) | | 1.025(0.441-2.380) | 10(3.3) | | 0.904(0.370-2.213) | 3(6.4) | | 1.841(0.484-7.002) |
| CC+CT | 79(25.8) | 62(17.7) | **0.012** | 0.619(0.425-0.900) | 52(17.2) | **0.009** | 0.595(0.402-0.882) | 10(21.3) | 0.505 | 0.777(0.369-1.634) |
| C allele | 89(14.5) | 75(10.7) | **0.036** | | 62(10.2) | **0.022** | | 13(13.8) | 0.855 | |

[a]Value was determined by $\chi^2$ test.

[b]Odds ratios (ORs) were obtained from a logistic regression model with adjustment for age and gender; 95% CI, 95% confidence interval.

**Table 2** Genotypes and allele frequencies of the FOXE1 and Foxp3 polymorphisms in the DTC and HC.

| Variables | rs1867277 [n (%)] | | P[a] | rs3761548 [n (%)] | | P | rs2280883 [n (%)] | | P |
|---|---|---|---|---|---|---|---|---|---|
| | AA/AG | GG | | AA/AC | CC | | CC/CT | TT | |
| Gender | | | | | | | | | |
| Male | 16(23.2) | 53(76.8) | | 18(26.1) | 51(73.9) | | 14(20.3) | 55(79.7) | |
| Female | 71(25.3) | 210(74.7) | 0.720 | 110(39.1) | 171(60.9) | **0.044** | 48(17.1) | 233(82.9) | 0.532 |
| PTC | | | | | | | | | |
| Unilateral | 53(24.1) | 167(75.9) | | 81(36.8) | 139(63.2) | | 38(17.3) | 182(82.7) | |
| Bilateral | 21(25.3) | 62(74.7) | 0.827 | 30(36.2) | 53(63.8) | 0.914 | 14(16.9) | 69(83.1) | 0.934 |
| Tumor diameter | | | | | | | | | |
| >1 cm | 36(25.0) | 108(75.0) | | 62(43.1) | 82(56.9) | | 18(12.5) | 126(87.5) | |
| <1 cm | 51(24.8) | 155(75.2) | 0.959 | 66(32.0) | 140(68.0) | **0.035** | 44(21.4) | 162(78.6) | **0.033** |
| Calcification | | | | | | | | | |
| positive | 14(16.7) | 70(83.3) | | 31(36.9) | 53(63.1) | | 12(14.3) | 72(85.7) | |
| negative | 73(27.4) | 193(72.6) | **0.046** | 97(36.5) | 169(63.5) | 0.942 | 50(18.8) | 216(81.2) | 0.345 |
| lymph node metastasis | | | | | | | | | |
| yes | 34(26.2) | 96(73.8) | | 46(35.4) | 84(64.6) | | 23(17.7) | 107(82.3) | |
| no | 53(24.1) | 167(75.9) | 0.666 | 82(37.3) | 138(62.7) | 0.723 | 39(17.7) | 181(82.3) | 0.993 |

[a] Value was determined by $\chi^2$ test or Fisher probabilities test (when one or more variables were <5 and >1.

**Table 3** Analysis of association between FOXE1, Foxp3 genotypes and DTC clinical pathology variables.

difference of genotypes distributions was not significant in PTC and FTC (P=0.052, P=0.278, respectively).

Similarly, for the Foxp3 rs2280883 polymorphism, the frequencies of TT, CT, CC genotypes were 82.3%, 14.0%, 3.7 respectively in the DTC, and 74.2%, 22.5%, 3.3% respectively in the HC. The difference was statistically significant (P=0.017) and the frequency of C variant (CT+CC) was lower in DTC than HC (17.7% vs. 25.8%, P=0.012). When we used the TT genotype as reference, we found the C carriers were associated with a lower risk of DTC (OR, 0.619; 95%CI, 0.425-0.900). Consistent with the C carriers, the frequency of C allele was significantly lower in the DTC than the HC (10.7% vs. 14.5%, P=0.036). These differences of genotypes and allele distributions were only found in the DTC histological PTC subtype (P=0.021, P=0.022, respectively).

## Association between FOXE1 and Foxp3 polymorphisms and clinical characteristics of DTC

To explore the relationship between FOXE1, Foxp3 SNPs and general characteristics of DTC, a stratified analysis was further performed. The data were shown in Table 3.

At the FOXE1-rs1867277 site, the frequency of AA/AG genotype was lower in the DTC patients with positive calcification than the

patients with negative calcification (16.7% vs. 27.4%, P=0.046). According to the gender, the DTC patients were subdivided. The AA/AC genotype of Foxp3-rs3761548 in female DTC patients was more frequent than the male (39.1% vs. 26.1%, P=0.044). We also analyzed the SNPs according to the tumor diameter of DTC patients. The frequency of Foxp3-rs3761548 AA/AC genotypes was significantly higher among severe DTC patients (tumor diameter >1cm) than the relative tender DTC patients (tumor diameter <1cm). (43.1% vs. 32.0%, P=0.035). Meanwhile, the CC/CT genotype of Foxp3-rs2280883 was less frequent in severe DTC patients than the relative tender DTC patients (12.5% vs. 21.4%, P=0.033).

## Discussion

The development and migration of the thyroid gland in the early stage, as well as the maintenance of the differentiated state throughout lifetime could be dependent on the interaction between genetic and environmental factors.

Genetic alterations of FOX family genes as well as binding proteins and target genes of FOX family transcription factors should be roundly investigated to develop novel therapeutics and preventives for human diseases [16]. It is confirmed that FOXE1 and FOXP3 genes are mutated in human congenital disorders [17,18].

Among all the genetic factors, FOXE1 plays an important role in the morphogenetic process which is regulated by both cell-autonomous and mesoderm-derived mechanisms acting in concert to promote growth and survival of progenitor cells [19,20]. In other studies, the FOXE1 variants were associated with the risk of DTC in some populations, including Spain, Belarusian, Portuguese, et al [7,11,21,22]. Consistent with these findings, the frequency of FOXE1 (rs1867277) A allele was much higher in DTC patients than the HC in our study. The patients carried A allele were associated with a significantly higher risk of DTC.

Studies revealed high nuclear Foxp3 expression in papillary carcinoma and follicular carcinoma [23]. Both in thyroid tissue and peripheral blood, the number of Foxp3+ Treg was significantly higher in patients with PTC plus multinodular non-toxic goiter (MNG) compared to patients with MNG alone [24]. These all suggested that a strong activation of oncogenic processes would exist in this lesion. The Foxp3-rs3761548 polymorphisms had been reported to be associated with the risk of various cancers [25,26]. In addition, the Foxp3 gene polymorphisms at rs2280883 and rs3761549 sites may be associated with hepatitis B-related hepatocellular carcinoma [27].

In our study, the evaluation of these polymorphisms of Foxp3 gene has revealed the fact that they were significantly associated with the susceptibility to DTC. We demonstrated that A allele of rs3761548 was more frequent in DTC patients than HC, and the frequency of rs2280883 C allele was lower in DTC patients compared with HC. The patients with AC genotype of rs3761548 were at a higher risk of DTC than with the other genotypes. On the contrary, the patients with CT genotype of rs2280883 were at a lower risk of DTC.

Recently, the significant difference of rs3761548 AA/AC genotype between the male and female cases was found in several studies [28,29]. In our study, the frequency of rs3761548 AA/AC genotype was much higher in female DTC patients than the male. Moreover, the frequency of rs2280883 CC/CT genotype was not significantly different between the male and female DTC patients. The results may be explained by the location of Foxp3, which is located on Xp11.23, leading to the difference between male and female DTC patients.

In terms of tumor diameter, the rs3761548 AA/AC genotype was more frequent in severe DTC patients (tumor diameter >1cm) compared with the relative tender DTC patients (tumor diameter <1cm) and the frequency of rs2280883 CC/CT genotype was lower in severe DTC patients. The above results indicated that rs3761548 AA/CC genotype was a potential risk genotype for DTC and rs2280883 CC/CT was a protective genotype. Therefore, these two SNPs may be used as an indicator of DTC general aggressiveness.

In conclusion, the FOXE1 and Foxp3 polymorphisms were associated with the susceptibility to DTC. AA/AG genotype of FOXE1-rs1867277 and AA/AC genotype of Foxp3-rs3761548 were associated with a higher risk of DTC in Chinese Han population. Moreover, the frequency of rs2280883 CC/CT genotype was lower in DTC patients. The AA/AC genotype of rs3761548 was more frequent in female DTC than the male. In addition, rs3761548 AA/AC genotype was more frequent in severe DTC patients. Conversely, the frequency of rs2280883 CC/CT genotype was lower in severe DTC patients. These results suggest that the FOXE1 and Foxp3 seem to be associated with the risk of DTC. Larger population-based studies with multi-regional and multi-center are needed to confirm our findings. Furthermore, the specific molecular mechanisms of FOXE1 and Foxp3 in the development of DTC need to be investigated.

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