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## **The *FOXE1* and *NKX2-1* loci are associated with susceptibility to papillary thyroid carcinoma in the Japanese population**

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## ABSTRACT

**Background:** *FOXE1* and *NKX2-1* are two known genetic risk factors for the predisposition to sporadic papillary thyroid carcinoma (PTC) in Europeans, but their association in other ethnicities is still unknown.

**Objective:** We aim to examine the association of the two genes with Japanese sporadic PTC, which exhibits high *BRAF*<sup>V600E</sup> mutation rate.

**Methods:** 507 Japanese sporadic PTC cases and 2,766 controls were genotyped for rs965513 (*FOXE1*) and rs944289 (*NKX2-1*). PTC cases were also examined for their *BRAF*<sup>V600E</sup> mutational status.

**Results:** We confirmed the association of both rs965513 ( $p=1.27\times 10^{-4}$ , OR=1.69, 95% CI: 1.29-2.21) and rs944289 ( $p=0.0121$ , OR=1.21, 95% CI: 1.04-1.39) with the risk of sporadic PTC. Subgroup analysis based on the *BRAF* mutational status showed strong association of rs965513 with *BRAF*<sup>V600E</sup>-positive cases ( $p=2.26\times 10^{-4}$ , OR=1.72, 95% CI: 1.29-2.29), but not with *BRAF*<sup>V600E</sup>-negative cases ( $p=0.143$ , OR=1.52, 95% CI: 0.87-2.65). However, there was no difference in the observed effect size between both subgroups. For rs944289, both subgroups showed marginal association ( $p=0.0585$ , OR=1.17, 95% CI: 0.99-1.37 for *BRAF*<sup>V600E</sup>-positive cases, and  $p=0.0492$ , OR=1.35, 95% CI: 1.00-1.81 for *BRAF*<sup>V600E</sup>-negative cases).

**Conclusions:** Both *FOXE1* and *NKX2-1* were associated with the increased risk of sporadic Japanese PTC. No clear associations were observed for either SNP with *BRAF*<sup>V600E</sup> status.

## INTRODUCTION

Papillary thyroid carcinoma (PTC) is the most common malignant tumor in endocrine organs, and the only well-known environmental risk factor is exposure to ionizing radiation. Genetic polymorphisms have been shown to contribute to individual susceptibility to PTC.[1] The identification and further assessment of the relevant genetic variations are important for understanding the potential mechanisms involved in thyroid carcinogenesis.

Recently, two genome-wide association (GWA) studies on thyroid cancer have been achieved. The first study dealt with sporadic thyroid cancer in Icelandic population using 192 and 37,196 cases and controls, respectively, followed by a replication study in individuals of European descent.[2] The *FOXE1* (*TTF2*) gene on 9q22.33 ( $p=1.7\times 10^{-27}$ , odds ratio [OR]=1.75, 95% confidence interval [CI]: 1.59-1.94 for rs965513) and *NKX2-1* (*TTF1*) on 14q13.3 ( $p=2.0\times 10^{-9}$ , OR=1.37, 95% CI: 1.24-1.52 for rs944289) showed the strongest association signals. Both genes encode thyroid-specific transcription factors and appear to contribute to an increased risk of both PTC and follicular thyroid carcinoma (FTC). The second GWA study focused on radiation-related PTC using 667 young patients exposed to radioiodine fallouts during childhood and 1,275 age-matched control subjects residing in the radiocontaminated regions of Belarus at the time of the Chernobyl accident.[3] In this study, the *FOXE1* gene showed strong association with radiation-related PTC ( $p=4.8\times 10^{-12}$ , OR=1.65, 95% CI: 1.43-1.91 for rs965513) whereas no association was found with *NKX2-1* ( $p=0.17$ , OR=1.13, 95% CI: 0.95-1.36 for rs944289). These results demonstrate that *FOXE1* is a major genetic determinant of predisposition to thyroid carcinoma regardless of etiology and age. In contrast, *NKX2-1* may be associated only with adult sporadic PTC. Furthermore, the involvement of these genes in thyroid carcinogenesis is yet to be examined in non-European populations.

Thyroid carcinoma in the Japanese population shows distinct characteristics: higher incidence of PTC than in European populations, the vast majority of PTCs are low-risk tumors with classic papillary morphology, and higher *BRAF*<sup>V600E</sup>-positive rate (~80%) than in European populations (~50%).[4-8] Similar findings have also been observed in the Korean population.[9] These characteristics are presumably due to high iodine intake, since distribution of thyroid carcinoma type seems to be related to the intake of iodine: more aggressive follicular and anaplastic carcinomas in iodine-deficient areas and more papillary carcinomas in iodine-rich areas.[10, 11] A recent study demonstrated significant association between the prevalence of *BRAF*<sup>V600E</sup> mutation and high iodine intake.[12] The *BRAF*<sup>V600E</sup> mutation is the most prevalent genetic alteration in adult sporadic PTC and is related to aggressive clinicopathological characteristics including extrathyroidal invasion, lymph node metastasis, advanced tumor stages, and poorer prognosis.[8] This mutation is also observed in micropapillary carcinomas and may thus be an

early event in thyroid carcinogenesis.

In this study, we aimed to examine the association of the former-identified genetic loci, namely, *FOXE1* and *NKX2-1*, with PTC in a Japanese case-control series. In addition, we have evaluated if the two genes are associated with the presence of the *BRAF*<sup>V600E</sup> mutation in PTC.

## **METHODS**

### **Subjects**

A total of 509 patients with sporadic PTC (mean±SD age 51.3±16.0 years, range 13-87 years; 84.4% women) were recruited from Kuma Hospital (Kobe, Japan). Histological diagnosis was performed by a thyroid pathologist (MH). 2,766 Japanese individuals were collected as population controls at Kyoto University. All patients and controls have no history of radiation exposure. The protocol was approved by the ethics committees of Nagasaki University, Kuma Hospital, and Kyoto University.

### **DNA extraction and *BRAF* status screening**

DNA from PTC subjects was extracted from formalin-fixed paraffin-embedded (FFPE) primary tumor tissues using QIAamp DNA mini kit (QIAGEN, Tokyo, Japan) according to the manufacturer's protocol. DNAs with sufficient quality for genotyping and sequencing were obtained from 507 out of 509 PTC specimens. *BRAF* status was screened by direct DNA sequencing. Primer sequences used for PCR and sequencing were: BRAFi14F, 5'-ACATACTTATTGACTCTAAGAGGAAAGATGAA-3' and BRAFi15R, 5'-GATTTTTGTGAATACTGGGAACTATGA-3'. PCR products were treated with ExoSAP-IT PCR clean-up reagent (GE Healthcare Japan, Tokyo, Japan), and sequencing was performed with Big Dye Terminator sequencing kit version 3.1 (Life Technologies, Foster City, CA) on an ABI3100 automated sequencer (Life Technologies). We prepared five negative controls (w/o tissue section) per 96 samples to ensure contamination-free amplifications.

### **Genotyping**

Genotyping was performed for PTC cases using the ABI TaqMan SNP assays (Life Technologies) in accordance with the manufacturer's guidelines. A pre-designed and functionally tested probe for rs965513 (C\_1593670\_20) and rs944289 (C\_1444137\_10) were used. The conditions were denaturation at 95°C for 10 minutes followed by 50 cycles of 92°C for 15 seconds and 60°C for 1 minute for both probes.

As control subjects, genotypes of rs965513 and rs944289 were extracted from the genome scan results using Illumina Human610-Quad BeadChip of 2,766 healthy Japanese individuals (mean±SD age 50.1±15.4 years, range 20-79 years; 63.1% women).

### **Statistical analysis**

A case-control association in each study was examined using trend exact test to compare genotypic distributions between cases and controls.[13] A subtype analysis was performed based on the *BRAF* mutational status (*BRAF* mutant versus control, and *BRAF* wild-type versus control), and the homogeneity of the odds ratios between the two studies was examined with the

Breslow-Day test.[14] For each case-control study, p-values and ORs adjusted for age and sex were calculated using multiple logistic regression analyses. Haplotype frequency was calculated using haplo.stats R package, and linkage disequilibrium (LD) between SNPs was estimated with gap package.[15]

## RESULTS

DNA samples extracted from 507 PTC tissues were genotyped by TaqMan assay for rs965513 and rs944289 (Table 1). Genotyping success rates for cases were 479/507 (94.5%) and 467/507 (92.1%) for rs965513 and rs944289, respectively. As for controls, the genotyping results that could be extracted from the genome scan data were 2,764/2,766 (99.9%) and 2,766/2,766 (100%) for rs965513 and rs944289, respectively. The genotype distributions of the two SNPs conformed to Hardy-Weinberg equilibrium both in cases and in controls. A case-control association was examined using trend exact test to compare the genotypic distributions. Significant associations were obtained for both SNPs (rs965513:  $p=1.27\times 10^{-4}$ , OR=1.69, 95% CI: 1.29-2.21; rs944289:  $p=0.0121$ , OR=1.21, 95% CI: 1.04-1.39) (Table 2). Our results confirmed the previously reported risk alleles, namely, allele A for rs965513 and allele T for rs944289.

We next screened for *BRAF* mutation in PTC tissues by DNA sequencing. Out of 507 samples, 492 (97.0%) were successfully genotyped, of which 388 were found to carry a heterozygous *BRAF*<sup>V600E</sup> mutation, and 104 were negative for the mutation (Table 1). Subgroup analysis based on the *BRAF* mutational status showed a strong association for rs965513 between 381 *BRAF*<sup>V600E</sup>-positive cases and 2,766 controls ( $p=2.26\times 10^{-4}$ , OR=1.72, 95% CI: 1.29-2.29) (Table 2). On the other hand, no statistically significant association was found between 95 *BRAF*<sup>V600E</sup>-negative cases and controls ( $p=0.143$ , OR=1.52, 95% CI: 0.87-2.65), although there was no reversal of the risk allele and a similar trend was observed (Table 2). There was no difference in the observed effect size between the *BRAF*<sup>V600E</sup>-positive and *BRAF*<sup>V600E</sup>-negative groups ( $p=0.615$ ).

For rs944289, both analyses showed marginal association ( $p=0.0585$ , OR=1.17, 95% CI: 0.99-1.37 for *BRAF*<sup>V600E</sup>-positive cases, and  $p=0.0492$ , OR=1.35, 95% CI: 1.00-1.81 for *BRAF*<sup>V600E</sup>-negative cases) (Table 2). Again, there was no reversal of the risk allele, and there was no difference in the observed effect size between the *BRAF*<sup>V600E</sup>-positive and *BRAF*<sup>V600E</sup>-negative groups ( $p=0.455$ ).



## DISCUSSION

Here, we report for the first time an association between the *FOXE1* gene and PTC in the Japanese population by genotyping rs965513 located 57-kb upstream to the *FOXE1* gene. The association of the *FOXE1* gene has been previously demonstrated by a GWA study for sporadic PTC[2] as well as for radiation-related PTC[3] in European populations. Furthermore, a recent study using an SNP panel of 97 genes related to thyroid cell differentiation and proliferation identified rs1867277, a causal SNP within the *FOXE1* 5' UTR, functioning as a genetic risk factor associated with susceptibility to PTC.[16] The sequence containing the risk allele was demonstrated to recruit the USF1/USF2 transcription factors which in turn increased *FOXE1* transcriptional activity. Indeed, animal model experiments have shown that mice lacking the *FOXE1* locus exhibit neonatal hypothyroidism that shows similarity to thyroid dysgenesis in humans.[17] We additionally genotyped rs1867277 in 64 randomly selected cases in our series to estimate LD with rs965513. There was no strong evidence for LD between these two SNPs ( $D'=0.23$ ), suggesting that the functional significance of rs965513 may be different from that of rs1867277.

The association between *NKX2-1* at chromosome 14q13.3 and sporadic PTC was also successfully reproduced in the Japanese population. However, the association was weaker for *NKX2-1* compared to *FOXE1* in our study in concordance with the results of the Icelandic study. Although rs944289 lies in a 249-kb LD region with no known genes, transcription units or predicted exons, *NKX2-1* is one of the closest genes residing in this region. *NKX2-1* is another thyroid specific transcription factor, and together with *FOXE1*, is expressed from early stages of thyroid morphogenesis and plays a major role in the development of the thyroid gland. Knock-out mice lacking the *NKX2-1* gene die at birth because they lack normal thyroid and lungs, demonstrating the essential role of the gene in embryonic differentiation of these organs.[18] Interestingly, rs944289 was strongly associated with sporadic PTC in the Icelandic population[2] as well as in our Japanese series, but not in the Belarusian radiation-related PTC, suggesting that this variant may be associated only with sporadic PTC.

The strong association of rs965513 with Japanese PTC was also found between *BRAF*<sup>V600E</sup>-positive cases and controls ( $p=2.26\times 10^{-4}$ , OR=1.72, 95% CI: 1.29-2.29) but not between *BRAF*<sup>V600E</sup>-negative cases and controls ( $p=0.143$ , OR=1.52, 95% CI: 0.87-2.65). However, the effect size is similar between the two groups ( $p=0.615$ ), and the statistical power is relatively low (0.31) in the latter analysis, suggesting that the lack of significance is due to the lower minor allele frequency (MAF) of the SNP in the Japanese population (0.090 in cases, 0.057 in controls) than in Europeans (0.462-0.490 in cases, 0.352-0.367 in controls),[2, 3] and/or the much smaller number of *BRAF*<sup>V600E</sup>-negative cases ( $n=95$ ). On the other hand, a marginal

association was observed for rs944289 in both subgroups ( $p=0.0585$ , OR=1.17, 95% CI: 0.99-1.37 for *BRAF*<sup>V600E</sup>-positive subgroup and  $p=0.0492$ , OR=1.35, 95% CI: 1.00-1.81 for *BRAF*<sup>V600E</sup>-negative subgroup). Again, the effect size is similar between the two groups ( $p=0.455$ ). However, for both SNPs, the number of *BRAF*<sup>V600E</sup>-negative cases needs to be increased to draw significant conclusions in the subtype analyses, especially for rs965513 for which the MAF in the Japanese population is so much lower compared to Europeans.

In conclusion, our study successfully confirms the association of both rs965513 and rs944289 with sporadic PTC in the Japanese population. Conceivably, *FOXE1* is likely to be the most important genetic determinant of susceptibility to PTC regardless of ethnicity. There was no clear difference in genetic impact for either of the SNPs with *BRAF*<sup>V600E</sup> status.

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**Table 1.** Specification of the DNA collections recruited for the study

Sample	Number	Age (Mean±SD)	Age range	%Male
Cases	507	51.3±16.0	13-87	15.6
<i>BRAF</i> <sup>V600E</sup> (+)	388	52.4±15.5	17-87	16.1
<i>BRAF</i> <sup>V600E</sup> (-)	104	48.1±17.2	13-81	13.6
Not available	15	-	-	-
Controls	2,766	50.1±15.4	20-79	36.9

**Table 2.** Association results for rs965513 and rs944289 in Japanese PTC

SNP [ref/var] <sup>1</sup>	Study Group	Genotyped samples		Allele frequency		HWE-Exact p-value <sup>3</sup>		p-value <sup>4</sup>	OR (95% CI) <sup>5</sup>	Power <sup>6</sup>
		Case <sup>2</sup>	Cont	Case	Cont	Case	Cont			
rs965513	All cases	479	2,764	0.090	0.057	0.255	0.721	1.27 x 10 <sup>-4</sup>	1.69 (1.29-2.21)	0.76
[A*/G]	<i>BRAF</i> <sup>V600E</sup> (+)	381	2,764	0.092	0.057	0.113	0.721	2.26 x 10 <sup>-4</sup>	1.72 (1.29-2.29)	0.69
	<i>BRAF</i> <sup>V600E</sup> (-)	95	2,764	0.079	0.057	1.000	0.721	0.143	1.52 (0.87-2.65)	0.31
rs944289	All cases	467	2,766	0.466	0.411	0.306	0.695	0.0121	1.21 (1.04-1.39)	0.60
[C/T*]	<i>BRAF</i> <sup>V600E</sup> (+)	373	2,766	0.458	0.411	0.118	0.695	0.0585	1.17 (0.99-1.37)	0.52
	<i>BRAF</i> <sup>V600E</sup> (-)	93	2,766	0.489	0.411	0.411	0.695	0.0492	1.35 (1.00-1.81)	0.19

<sup>1</sup>The reference (ref) and variant (var) alleles refer to NCBI Build 36.3, and the risk allele is indicated by an asterisk.

<sup>2</sup>*BRAF* mutational statuses were not available in three samples of 479 cases successfully typed for rs965513 and in one sample of 467 cases successfully typed for rs944289.

<sup>3</sup>The Exact p-values for Hardy-Weinberg equilibrium (HWE) are shown.

<sup>4</sup>The p-values using Trend Exact test adjusted for age and sex are shown.

<sup>5</sup>Odds ratios (OR) are calculated for the risk allele with a 95% confidence interval (CI).

<sup>6</sup>Statistical power is calculated by using power function in Hmisc package (<http://cran.r-project.org/web/packages/Hmisc/index.html>) of R.