Genome-wide association study identifies a novel susceptibility gene for serum TSH levels in Chinese populations

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Thyroid-stimulating hormone (TSH) is a sensitive indicator of thyroid function. High and low TSH levels reflect hypothyroidism and hyperthyroidism, respectively. Even within the normal range, small differences in TSH levels, on the order of 0.5–1.0 mU/l, are associated with significant differences in blood pressure, BMI, dyslipidemia, risk of atrial fibrillation and atherosclerosis. Most of the variance in TSH levels is thought to be genetically influenced. We conducted a genome-wide association study of TSH levels in 1346 Chinese Han individuals. In the replication study, we genotyped four candidate SNPs with the top association signals in an independent isolated Chinese She cohort (n = 3235). We identified a novel serum TSH susceptibility locus within XKR4 at 8q12.1 (rs2622590, Pcombined = 2.21 × 10^{-10}), and we confirmed two previously reported TSH susceptibility loci near FOXE1 at 9q22.33 and near CAPZB at 1p36.13, respectively. The rs2622590_T allele at XKR4 and the rs925489_C allele near FOXE1 were correlated with low TSH levels and were found to be nominally associated to patients with papillary thyroid carcinoma (PTC) (OR = 1.41, P = 0.014 for rs2622590_T, and OR = 1.61, P = 0.030 for rs925489_C). The rs2622590 and rs925489 genotypes were also correlated with the expression levels of FOXE1 and XKR4, respectively, in PTC tissues (P = 2.41 × 10^{-4} and P = 0.02). Our findings suggest that the SNPs in XKR4 and near FOXE1 are involved in the regulation of TSH levels.

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INTRODUCTION

Impinging on virtually every tissue of the body, thyroid hormones affect a variety of metabolic and developmental processes in humans (1). They are vital to brain development and skeletal maturation in fetuses and infants, and they influence protein synthesis, fat and carbohydrate metabolism, bone deposition, metabolic rate, temperature regulation and blood pressure in adults (2–4).

Thyroid function is regulated by a homeostatic negative-feedback loop involving the pituitary–thyroid axis and is evaluated by measuring circulating concentrations of thyroid-stimulating hormone (TSH), free thyroxine (FT4), and free triiodothyronine (FT3) (5). Secreted by the pituitary gland, TSH interacts with the TSH receptor on thyroid cells to control the release of T4 and T3 through a series of signal transduction pathways (6). TSH and FT4 have an inverse, log-linear relationship such that small changes in FT4 levels result in dramatic changes in TSH secretion. Hence, the serum TSH concentration is a very sensitive indicator of the function of the thyroid gland (7).

The variability of serum TSH concentration is more dramatic among healthy individuals than within healthy individuals (measured multiple times in the same individual) (8), suggesting that the regulation of the thyroid-hormone axis is exact and varies among individuals. Evidence from studies of healthy Danish twins has shown that genetic factors can account for 64% (57–70%) of the variance in circulating TSH levels (9). Another heritability study of Mexican Americans showed that non-genetic covariates only accounted for 2–18% of the total phenotypic variation, whereas genetic factors accounted for 26–64% of the total phenotypic variation (10).

Previous studies have identified many putative genetic susceptibility variants for serum TSH levels, but unequivocal replication has been limited to a few loci (such as PDE8B at 5q13.3, CAPZB at 1p36.13, MAF at 16p23.2 and NR3C2 at 4q31.23) (11–17). Moreover, more recent GWAS studies in large samples recruited from Caucasian by Gudmundsson et al. (18) and Porcu et al. (17), identified 22 novel susceptibility loci for TSH level. All the 26 TSH risk loci only explained about 4.3–5.6% of the inter-individual variability in serum TSH levels, suggesting that as-yet-unknown variants may also be important.

To identify novel serum TSH susceptibility loci, we conducted a GWAS of 1346 healthy Chinese Han individuals and an independent replication study of 3235 healthy Chinese She individuals. We identified a new susceptibility locus (rs2622590, near the XKR4 region at 8q12.1; \( P_{\text{combined}} = 2.21 \times 10^{-10} \)) and confirmed two previously reported loci (near FOXE1 at 9q22.33 and near CAPZB at 1p36.13).

RESULTS

Genome-wide association study of serum TSH levels

The clinical and demographic characteristics of the study participants are shown in Table 1. After stringent QC filters, a total of 483 947 genotyped SNPs and 8 019 905 imputed SNPs were analyzed for associations with serum TSH levels among 1346 individuals whose serum TSH levels ranged from 0.35 and 4.94 mU/l (Fig. 1). The results for the typed and imputed SNPs (\( P < 10^{-5} \)) (http://www.dropbox.com/s/q32p5yrldlacc3a/Data%20for%20TSH.zip), organized by chromosome location in a Manhattan plot, are shown in Figure 2. A quantile–quantile plot of TSH levels is shown in the Supplementary Material (Supplementary Material, Fig. 1). The estimated inflation factor was modest (\( \lambda = 1.004 \)), and thus the distribution of \( P \)-values for the association tests shows no evidence of systematic bias. A principal component analysis (PCA) and a multidimensional scaling (MDS) analysis, both described in our previous study (18), showed that all of the subjects clustered around Chinese and Japanese lines of descent.

In our initial GWAS, 10 genotyped SNPs located in five different chromosomal regions had \( P \)-values \( <1 \times 10^{-5} \) (Table 2). From those 10 SNPs, we identified the four SNPs, located in three different chromosomal regions, with the strongest (\( P < 10^{-6} \)) serum TSH associations: rs134827 at 11q22.1 (\( P = 1.09 \times 10^{-7} \)), rs925489 and rs7850258 at 9q22.3 (\( P = 3.19 \times 10^{-7} \) and \( P = 3.19 \times 10^{-7} \), respectively), and rs2622590 at 8p12.1 (\( P = 6.49 \times 10^{-7} \)). The minor allele frequencies (MAFs) for the four SNPs ranged from 0.05 to 0.46.

Analyses of replication and combined data

Given that the sample size of the initial GWAS data set in the current study is underpowered to detect common variants associated with TSH levels, the three SNPs (rs1348271, rs925489 and rs2622590) with the strongest serum TSH associations, and one SNP (rs6683419, \( P_{\text{GWAS}} = 1.33 \times 10^{-7} \)) in lp36.13 region previously reported to be associated with serum TSH levels (13), were selected and genotyped in an independent sample of the Southern Chinese She population, who is one of the important ethnic minorities, amounts to 0.7 million people in China (in the 2010 census). They mainly work in agriculture, forestry, animal husbandry, fishing and water industry, and have their own living customs. The marriage between Han and She were prohibited 60 years ago. The replication sample was recruited from the She population residing in Ningde city of Fujian Province and consisted of 3235 individuals with serum TSH levels between 0.35 and 4.94 mU/l (19). Three of the four selected SNPs (rs925489, \( P = 9.13 \times 10^{-9} \); rs2622590, \( P = 5.62 \times 10^{-6} \); and rs6683419, \( P = 5.86 \times 10^{-4} \)) showed evidence of replication in the tested data set (Table 3).

In the combined samples, two SNPs (rs925489 at 9q22.33, \( P = 1.02 \times 10^{-13} \); and rs2622590 at 8q12.1, \( P = 2.21 \times 10^{-10} \)) showed unequivocal evidence of association with serum TSH levels, with a genome-wide significance threshold of \( P = 5 \times 10^{-8} \); and one more SNP (rs6683419 at 1p36.13) nearly met the genome-wide significance level for association with serum TSH levels (\( P = 2.90 \times 10^{-7} \)) (Table 3). These three SNPs included one new serum TSH susceptibility locus (the XKR4 region at 8q12.1) and two previously reported TSH susceptibility loci (Table 3).

Notably, we identified a novel serum TSH susceptibility locus, rs2622590 (\( P = 2.21 \times 10^{-10} \)) in the 8p12.1 region. In the initial GWAS, we analyzed 2966 imputed SNPs and 164 genotyped SNPs in a ~900 kb linkage disequilibrium (LD) block containing XKR4 and SBFI1P1, TMEM68, TGS1 and LYN located at 8p12.1 (Fig. 3A). We found that all of the SNPs with \( P < 10^{-5} \) within the LD block were located within a
higher than those among individuals with the homozygous-
"CC" genotype were 1.52 ± 2.19 for the CC genotype, 1.81 ± 0.84 for the CT genotype, and 1.50 ± 0.73 for the TT genotype. Adjustment for the relevant covariates, the serum TSH levels for the CC genotype, 1.52 ± 2.19 for the CC genotype, 1.81 ± 0.84 for the CT genotype, and 1.50 ± 0.73 for the TT genotype. The combination analysis (GWAS + replication) reached the genome-wide significance level in the combined analysis (6.31 × 10^-6; Supplementary Material, Table S3) and in the replication cohort (1.67 ± 0.85 for the CC genotype, 1.47 ± 0.79 for the TT genotype; P = 3.44 × 10^-6; Supplementary Material, Table S3). Rs2622590 is located in intron 2 of XKR4, 3.5 kb downstream of the pseudogene SBF1P1 (Fig. 3A). XKR4 is expressed abundantly in the tissues of the brain, esophagus and stomach and relatively sparsely in the tissues of the thyroid, kidney and pancreas (Fig. 4C). To further test whether the TSH associated SNPs regulate the expression of XKR4, we inspected three cis-gene expression quantitative trait loci (cis-eQTL) databases from European Caucasian population (20–22) and found that out of the 327 TSH associated SNPs (P < 0.05) in a ~900 kb region on 8q12.1 (Supplementary Material, Table S2), no SNPs were correlated with the expression of XKR4. Furthermore, a ~40 kb region surrounding the rs2622590 on 8q12.1 was analyzed to identify regulatory elements by inspection the UCSC Genome Browser. Interestingly, a ~5 kb fragment near to rs2622590, which contained four TSH strongly associated SNPs (rs2975987, rs2939632, rs2975986 and rs2975985), was conserved from zebrafish to human (Fig. 5). These four SNPs were in complete LD with TSH strongly associated SNPs (rs2975987, rs2939632, rs2975986 and rs2975985), leaving 652,029 SNPs for further analysis. We further excluded SNPs with a low call rate (< 98%), an MAF < 1% within the population, or a significant deviation from Hardy-Weinberg equilibrium (P ≤ 10^-6). Samples were excluded if they had a high missing call rate (>98%), gender inconsistencies, cryptic relatedness, ab- normal TSH (TSH > 4.94 or < 0.35 mU/l) or high TPO-Ab (TPO-Ab ≥ 5.61 U/ml). In the replication study, three SNPs associated with TSH (P < 10^-6) were selected as candidate loci for replication. Additionally, one SNP that was previously reported to be associated with TSH was also chosen for genotyping in the replication samples.

35 kb region of intron 2 of XKR4 (Fig. 3A; Supplementary Material, Tables S1 and S2). After conditioning on rs2622590 (P_wat = 6.49 × 10^-10; Table 3), none of the other SNPs at 8p12.1 remained significantly (P < 0.01) associated with serum TSH levels, suggesting that rs2622590 might be an independent SNP associated with serum TSH levels. Therefore, we selected rs2622590 for further genotyping in the replication sample. The replication study confirmed the serum TSH association of rs2622590 (P_repl = 5.62 × 10^-10; Table 3), and the association reached the genome-wide significance level in the combined analysis (P_combined = 2.21 × 10^-10; Table 3). After adjustment for the relevant covariates, the serum TSH levels among individuals with the rs2622590 CC genotype were higher than those among individuals with the homozygous-susceptible TT genotype in the GWAS cohort (1.79 ± 0.82 for the CC genotype, 1.52 ± 0.75 for the TT genotype; P = 6.31 × 10^-6; Supplementary Material, Table S3) and in the replication cohort (1.67 ± 0.85 for the CC genotype, 1.47 ± 0.79 for the TT genotype; P = 3.44 × 10^-6; Supplementary Material, Table S3). Rs2622590 is located in intron 2 of XKR4, 3.5 kb downstream of the pseudogene SBF1P1 (Fig. 3A). XKR4 is expressed abundantly in the tissues of the brain, esophagus and stomach and relatively sparsely in the tissues of the thyroid, kidney and pancreas (Fig. 4C). To further test whether the TSH associated SNPs regulate the expression of XKR4, we inspected three cis-gene expression quantitative trait loci (cis-eQTL) databases from European Caucasian population (20–22) and found that out of the 327 TSH associated SNPs (P < 0.05) in a ~900 kb region on 8q12.1 (Supplementary Material, Table S2), no SNPs were correlated with the expression of XKR4. Furthermore, a ~40 kb region surrounding the rs2622590 on 8q12.1 was analyzed to identify regulatory elements by inspection the UCSC Genome Browser. Interestingly, a ~5 kb fragment near to rs2622590, which contained four TSH strongly associated SNPs (rs2975987, rs2939632, rs2975986 and rs2975985), was conserved from zebrafish to human (Fig. 5). These four SNPs were in complete LD with TSH strongly associated SNPs (rs2975987, rs2939632, rs2975986 and rs2975985), leaving 652,029 SNPs for further analysis. We further excluded SNPs with a low call rate (< 98%), an MAF < 1% within the population, or a significant deviation from Hardy-Weinberg equilibrium (P ≤ 10^-6). Samples were excluded if they had a high missing call rate (>98%), gender inconsistencies, cryptic relatedness, abnormal TSH (TSH > 4.94 or < 0.35 mU/l) or high TPO-Ab (TPO-Ab ≥ 5.61 U/ml). In the replication study, three SNPs associated with TSH (P < 10^-6) were selected as candidate loci for replication. Additionally, one SNP that was previously reported to be associated with TSH was also chosen for genotyping in the replication samples.

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69 kb upstream of FOXE1, and 87 kb upstream of XPA (Fig. 3B).

Notably, FOXE1 is specifically expressed in human thyroid tissues (Fig. 4C).

Rs6683419, located in the 1p36.13 region harboring CAPZB, MINOS1, NBL1 and other genes, was previously reported to be associated with serum TSH levels in European population (13,15–17). Our initial GWAS provided the genotypes of 1300 imputed SNPs and 86 genotyped SNPs in a ~400 kb LD block in the 1p36.13 region. Among all genotyped SNPs, rs6683419 had the most significant association with TSH levels ($P_{\text{GWAS}} = 1.33 \times 10^{-5}$) (Supplementary Material, Table S5). We therefore analyzed rs6683419 in the replication study (Fig 3C and Table 3). After the results of the discovery and replication studies were combined, rs6683419 showed a suggestive serum TSH association that nearly met the genomewide significance level ($P_{\text{replication}} = 5.86 \times 10^{-4}$; $P_{\text{combined}} = 2.90 \times 10^{-7}$; Table 3). Rs10917469, rs10799824 and rs10917477 in the 1p36.13 region, which were previously reported as the best SNPs associated with serum TSH levels in European cohort, respectively (13,15–17), were further analyzed in our GWAS. Although rs10917469 and rs10799824 were only nominally associated with TSH levels ($P_{\text{GWAS}} = 0.015$), we did find rs10917477 was strongly associated with TSH levels in our Chinese Han population ($P_{\text{GWAS}} = 4.19 \times 10^{-4}$) (Supplementary Material, Table S5). Moreover, we found that rs10917469 and rs10799824 were not in complete LD with rs6683419 (both $r^2 = 0.056$ in the 1000 Genomes ASN samples), otherwise rs10917477 was strongly correlated with rs6683419 ($r^2 = 0.744$ in the 1000 Genomes ASN samples) (Table 4).

Though our GWAS implicated XKR4, FOXE1 and CAPZB gene with a significant effect on variation of serum TSH levels, other variants also contribute to this quantitative trait. Therefore, we further analyzed the 45 SNPs in the 26 loci, which were reported to be associated with TSH levels in previous studies, and found that out of the 45 SNPs, 27 in 15 risk loci were associated with TSH levels in our GWAS data ($P < 1 \times 10^{-3}$ in our GWAS data, which included four loci reported to be associated with TSH in previous studies. However, only nine out of 280 loci associated with TSH level at $P < 1 \times 10^{-3}$ in our GWAS data, were confirmed to be the susceptibility loci for TSH in previous studies (Table 4) (11–17).

Association of the TSH candidate SNPs with thyroid diseases and expression patterns in target tissues

Serum TSH levels were previously reported to be correlated with the risk of thyroid cancer and could be a marker to predict thyroid cancer relapse (24–26). Hence, we investigated whether the four
serum-TSH candidate SNPs identified in our initial GWAS were associated with papillary thyroid carcinoma (PTC) in a sample of 108 patients with PTC and 1490 control individuals from the Chinese Han population. Two of the SNPs, rs2622590 and rs925489, were nominally associated with PTC (rs2622590, \( P = 0.030 \), OR = 1.61, 95% CI = 1.04–2.47; Table 5).

Although we apply a Bonferroni correction for multiple testing of association between SNPs (rs2622590, rs1348271, rs6683419 and rs925489) and PTC (\( P < 0.005/4 = 0.00125 \)) and PTC (\( P = 0.05/4 = 0.0125 \)), rs965513 near \( XKR4 \) on 9q22.33 (\( P = 0.030 \)) and rs2622590 in \( XKR4 \) on 8p12.1 (\( P = 0.014 \)) were no longer associated with PTC. However, we found that rs965513 in \( FOXE1 \) was significantly associated with PTC in Chinese population in a previous report (\( P = 1.8 \times 10^{-4} \), OR = 1.53, 95% CI = 1.23–1.90; Supplementary Material, Table S6) (27). Because rs965513 was in complete LD with rs925489 (\( r^2 = 0.97 \) in our Chinese Han samples), we performed a meta-analysis and found that rs925489 or rs965513 was significantly associated with PTC (\( P_{\text{meta}} = 1.28 \times 10^{-5} \), OR = 1.54, 95% CI = 1.27–1.88) in combined Chinese PTC population (Supplementary Material, Table S6). We have neither more PTC samples nor reported data about the genotypes of SNPs in \( XKR4 \) region to further confirmed the association of rs2622590 with PTC. Therefore, the relation of \( XKR4 \) SNP to PTC remains to be determined, and further studies based on larger PTC samples to confirm these two findings were needed.

The PTC risk alleles of the two SNPs (the TT genotype for rs2622590 and the CC genotype for rs925489) were also associated with low serum TSH levels (Table 5 and Supplementary Material, Table S3). None of the four candidate SNPs were associated with Graves’ disease (GD), however, in a sample of 1442 patients with GD and 1468 control individuals collected from the Chinese Han population in our previous GWAS (18) (Table 5).

We used quantitative real-time PCR to measure the expression of \( XKR4 \), \( FOXE1 \) and \( CAPZB \) in 66 PTC tissues and 40 normal thyroid tissues adjacent to PTC tissues. We found that the expression levels of \( XKR4 \), \( FOXE1 \) and \( CAPZB \) in the PTC tissues were significantly higher than those in the normal thyroid tissues adjacent to PTC tissues (\( P = 3.85 \times 10^{-7} \), \( 3.24 \times 10^{-4} \) and \( 2.67 \times 10^{-15} \), respectively; Fig. 4A). Moreover, the rs2622590_T and rs925489_C alleles, which were the risk alleles for PTC and were correlated with lower serum TSH levels, were also correlated with high expression levels of \( XKR4 \) and \( FOXE1 \), respectively, in the thyroid tissues of patients with PTC (\( P_{\text{ANOVA}} = 2.41 \times 10^{-4} \) for rs2622590; \( P_{\text{ANOVA}} = 0.02 \) for rs925489; Fig. 4B). The rs6683419 genotypes, however, were not correlated with the expression of \( CAPZB \) in the PTC tumor tissues (\( P_{\text{ANOVA}} = 0.65 \); Fig. 4B).

**DISCUSSION**

The identification of susceptibility loci and genes related to serum TSH levels may provide important insight into the regulation of thyroid hormones, and may also be valuable for designing new preventative and therapeutic strategies for the disorders influenced by TSH, such as hypothyroidism, dyslipidemia, cardiovascular disease, myocardial infarction and atrial fibrillation (28–34). Through a two-stage GWAS of circulating TSH levels in individuals from the Chinese populations, we identified a novel susceptibility locus in \( XKR4 \) at 8p12.1, and we confirmed two previously reported susceptibility loci near \( FOXE1 \) at 9q22.33 and near \( CAPZB \) at 1p36.13 (12–17).

This study is the first to associate rs2622590, located in intron 2 of \( XKR4 \) at 8p12.1, with serum TSH levels. The \( XKR4 \) gene encodes a member of the XK, Kell blood group complex subunit-related protein family. Kell and XK are two distinct red blood cell-membrane proteins that form the Kell blood-group complex. XK is considered to be a membrane transport protein, and the absence of XK can lead to McLeod syndrome, a rare X-linked hereditary disease characterized by red blood cell acanthocytosis and late-onset central nervous system and neuromuscular abnormalities (35). More recently, a few SNPs
Table 3. Association results for the selected SNPs in the GWAS and replication phases

<table>
<thead>
<tr>
<th>SNP</th>
<th>Position</th>
<th>A1/A2</th>
<th>GWAS (n = 1340)</th>
<th>Replication (n = 1235)</th>
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<td>0.039</td>
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<td>4.43</td>
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<tr>
<td>rs925489</td>
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<td>0.51</td>
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<td>2.01</td>
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<td>FOXE1</td>
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**SNP**: single-nucleotide polymorphism; **Chr**: chromosome; **A1/A2**: minor allele/MAX-allele; **MAF**: minor allele frequency.

- **p-values** are from linear regression models adjusted for age, gender, geographic region and race.

Regrettably, the FT4 levels have not been collected in our current data, rs7045138, but not rs7045138 was significantly associated with lower TSH levels, leading to the association of the C allele of rs925489 with lower TSH levels. Indeed, all the four PTC risk alleles except rs965513 near to FOXE1 were associated with the lower TSH levels and trended to increased FT4 levels. Moreover, for rs966423 on 2q35 and rs116909374 on 14q13.3, the lower-TSH alleles were significantly associated with increase FT4 at the P < 0.05 level (15). Although the PTC risk allele of rs965513 in FOXE1 was associated with lower TSH and FT4 levels, the risk allele appeared to be associated with the increase FT3 level (P < 0.05) (15). Therefore, all the data suggested that the PTC risk allele trended to lower the TSH and increase the physiological function of thyroid as well as supported our hypothesis. With regard to the SNPs near to FOXE1 at 9q22.33, ours and previous reports supported that rs925489, a proxy rs965513 (r² = 0.97 in our Chinese Han samples), was associated with TSH concentration at the GWAS significant level (12,15). However, the more recent study from Porcu et al. (17) found that rs7045138 and rs965513 near FOXE1 were associated with FT4 level (P = 1.5 × 10⁻¹¹ and 3.45 × 10⁻⁸, respectively), but not associated with the TSH level. Interestingly, the rs965513 was weakly linked with rs7045138 in Caucasian, but not linked with rs7045138 in Chinese Han population (r² = 0.409 in the 1000 Genomes CEU samples and 0.001 in the 1000 Genomes ASN samples, respectively). In our GWAS data, rs965513, but not rs7045138 was significantly associated with TSH level (P = 1.26 × 10⁻⁶ and 0.70, respectively). These data suggested that two SNPs near FOXE1 region were independently associated with the TSH or FT4 levels, respectively. Regrettfully, the FT4 levels have not been collected in our current

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- **p-values** are from linear regression models adjusted for age, gender, geographic region and race.

Regrettably, the FT4 levels have not been collected in our current...
Figure 3. Regional plots of association results at 8p12.1, 9q22.33 and 1p36.13. Regional plots of association results in the GWAS samples at 8p12.1 (A), 9q22.33 (B) and 1p36.13 (C). The color of each SNP spot, ranging from red to blue, reflects the magnitude of the $r^2$ between the SNP spot and the candidate SNP (purple circle) within each candidate locus for serum TSH levels. Genetic recombination rates, estimated using the 1000 Genomes ASN samples, are plotted in the background in cyan. The vertical axis represents the $-\log_{10}P$-value. Physical positions are based on NCBI build 36/hg19 of the human genome.
study, the association study of the SNPs in FOXE1 region with FT4 and TSH levels in the Chinese population in further to unlock the perplexed was expected.

The rs2622590_T allele, which was associated with lower serum TSH levels, was also correlated with the increased expression of XKR4 in thyroid tumor tissues; however, the relationship between serum TSH levels and XKR4 expression in thyroid tissues remains unknown. Consistent with the results of our study, the alleles of four other SNPs associated with lower serum TSH levels were found to increase the risk of PTC in previous studies (12,15). All of the available data suggest that low serum TSH levels are correlated with an increased risk of PTC.

In summary, we identified a new serum TSH susceptibility locus in the XKR4 region at 8q12.1 and confirmed two previously reported susceptibility loci near FOXE1 at 9q22.33 and CAPZB at 1p36.13, respectively. The rs2622590_T allele in XKR4 and

Figure 4. The expression analyses of the candidate genes XKR4, FOXE1 and CAPZB at 1p36.13, 8q12.1 and 9q22.33. (A) The mRNA levels of XKR4, FOXE1 and CAPZB in the tumor tissues (n=66) and adjacent normal thyroid tissues (n=40) of patients with PTC. Data are shown as means ± SD. The mRNA levels of XKR4, FOXE1 and CAPZB are normalized to that of GAPDH. (B) The correlation of XKR4, FOXE1 and CAPZB mRNA levels in PTC tumor tissues with the genotypes of rs2622590 in XKR4 (CC, n=14; TC, n=34; TT, n=18), rs925489 near FOXE1 (TT, n=59; CT, n=7) and rs6683419 near CAPZB (AA, n=28; GA, n=30; GG, n=8), respectively (normalized to GAPDH). (C) The expression patterns of CAPZB, XKR4 and FOXE1 in human tissues resolved by real-time PCR. *P < 0.05; **P < 0.01; ***P < 0.001. Error bars, ± SD.
the rs925489_C allele near FOXE1, both associated with lower serum TSH levels, were correlated with the increased expression of XKR4 and FOXE1, respectively, in the thyroid tumor tissues, and possibly conferred a predisposition to PTC. These findings provided new insights into the mechanisms regulating serum TSH levels and suggested that low serum TSH levels were correlated with susceptibility to PTC.

MATERIALS AND METHODS

GWAS and replication cohorts
The GWAS cohorts included in this study were collected from the Chinese Han population through collaborations with two hospitals in northern China (Xuzhou and Linyi) (18,45,46). The replication cohort was collected from the Chinese She population, who is one of the important ethnic minorities, residing in the Ningde City, Fujian Province in southern China. The marriage between Han and She were prohibited 60 years ago, suggesting the She ethnic is an independent isolated population in China (19). Subjects were excluded if they had thyroid nodules, autoimmune thyroid disease (AITD) or a family history of AITD or other autoimmune disorders.

During a physical examination, 5 ml blood samples were collected from each participant and stored at \(-80^\circ\text{C}\) for DNA extraction and measurements of serum TSH sensitivity and TPO-Ab levels. TSH and TPO-Ab were measured using a chemiluminescence immunoassay (CLIA) according to the manufacturer’s instructions in our laboratory (Siemens ADVIA Centaur CP). To exclude subjects with clinical or subclinical AITD and subjects taking medications likely to influence serum thyroid function, we measured the serum levels of TPO-Ab among the 1816 GWAS subjects and the 4233 replication subjects. Based on those measurements, we excluded 1420 individuals with TPO-Ab levels <5.60 U/ml and TSH levels <4.93 mU/l or <0.36 mU/l. Ultimately, we included 1394 and 3235 subjects for the GWAS and replication studies, respectively (Fig. 1). The median TSH level was 1.62 mU/l and the interquartile range was 1.02–2.06 mU/l. We also included 108 patients with PTC recruited from the First hospital affiliated to Bengbu Medical College, in northern China to evaluate whether selected loci were associated with PTC. The sample sizes, sex distributions, mean ages, BMIs and serum TSH levels of the GWAS and replication participants are summarized in Table 1.

All of the participating cohorts were granted approval by the local Ethics Committee from Ruijin Hospital, the Central Hospital of Xuzhou, Linyi People’s Hospital, the Fujian Province Hospital Affiliated to Fujian Medical University and the First Hospital Affiliated to Bengbu Medical College. All of the subjects in this study provided written informed consent using protocols approved by the local Ethics Committees.

GWAS genotyping and initial quality control
DNA was extracted from the blood samples using a FUJIFILM QuickGene-610L system. Genome-wide genotyping was performed using the Illumina Human Omni-Express BeadChip platform on the Illumina Human660-Quad BeadChips at the Chinese National Human Genome Center in Shanghai, China (18). Genotype clustering was conducted using Illumina GenomeStudio V2011.1 software based on the 660W-Quad_v1_H manifest files, which converted the fluorescence intensities into SNP genotypes. The mean call rate across all 1394
The association of the top SNPs in 26 previously reported TSH risk loci with TSH in our GWAS data

<table>
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<tr>
<th>Chr.</th>
<th>Marker name</th>
<th>Annotated Gene</th>
<th>Tested SNP</th>
<th>P-value</th>
<th>r²</th>
<th>Top SNP</th>
<th>P-value</th>
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<td>1p36.13</td>
<td>rs10799824</td>
<td>CAPZB</td>
<td>rs10799824</td>
<td>1.51 × 10⁻²</td>
<td>0.056</td>
<td>rs2314146</td>
<td>5.80 × 10⁻⁶</td>
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<td>rs10917469</td>
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<td>0.056</td>
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<td>5.80 × 10⁻⁶</td>
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<td>1p36.13</td>
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<td>NA</td>
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<td>NFIA</td>
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<td>2p25.3</td>
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<td>NR3C2</td>
<td>rs10028213</td>
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<td>0.266</td>
<td>rs9968300</td>
<td>1.77 × 10⁻⁵</td>
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<td>4q13.23</td>
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<td>NR3C2</td>
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<td>rs4704397</td>
<td>PDE6B</td>
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<td>0.001</td>
<td>rs10055027</td>
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<td>VEGFA</td>
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<td>NA</td>
<td>NA</td>
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<td>SASH1</td>
<td>rs9497965</td>
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<td>rs58884869</td>
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<td>PDE1A</td>
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<td>rs58885758</td>
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<td>NRG1</td>
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<td>rs11991469</td>
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<td>rs11997971</td>
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<td>rs965513</td>
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<td>rs10818050</td>
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<td>rs7913750</td>
<td>1.97 × 10⁻¹</td>
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<td>PRDM11</td>
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<td>rs2790072</td>
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<td>rs12814360</td>
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<td>rs1537424</td>
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<td>SIVAI</td>
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<td>15q21.2</td>
<td>rs73389284</td>
<td>FGFI7</td>
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<td>rs74406736</td>
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<tr>
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<td>MIR1197</td>
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<td>0.007</td>
<td>rs74027996</td>
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<td>rs7190187</td>
<td>MAF</td>
<td>rs7190187</td>
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<td>rs4575545</td>
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<td>17q24.3</td>
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<td>0.108</td>
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<td>0.609</td>
<td>rs4499503</td>
<td>3.26 × 10⁻²</td>
</tr>
</tbody>
</table>

Chr., chromosome; SNP, single-nucleotide polymorphism; NA, not assessed.

*P*-values were from linear regression models adjusted for age, gender, and geographic region.

r² in the 1000 Genomes ASN samples.

Top SNP, the top association signal in each TSH risk locus in our data (recombination rate ≤ 0.3 in the 1000 Genomes ASN samples).

Genotype imputation
To infer the genotypes of SNPs in the GWAS cohort that were not previously genotyped, we used the IMPUTE2 software (48) with the 1000G phase-1 interim impute data (March 2012) as a reference. We used an estimated probability ≥ 0.90 to call imputed genotypes. Taking imputation uncertainty into account, the association analysis was carried out using the SNPTEST2 software (frequentist) association tests with score method (49).

SNP selection and genotyping in the replication study
We selected three SNPs (rs1348271, rs925489 and rs2622590) that were strongly associated with serum TSH levels (P < 1 × 10⁻⁶).

Genotyped samples was 99.8%. No individuals were excluded from the GWAS analysis. Quality control was performed on the SNPs and samples before analysis using the PLINK software to ensure robust association tests (47).

Of the 655 214 markers assayed, 3185 were removed because they were from the Y or mitochondrial chromosomes or were CNV-related. We discarded 168 082 markers because of deviation from Hardy–Weinberg equilibrium P ≤ 10⁻⁶, genotype call rates below 98%, or MAFs < 0.01, leaving 483 947 SNPs for subsequent analyses. Next, we excluded 48 samples with call rates < 98%, gender inconsistencies or cryptic relatedness. Ultimately, 1346 samples were available for the GWAS analysis (Fig. 1).
Table 5. Association of the selected SNPs with PTC risk and GD

<table>
<thead>
<tr>
<th>SNP</th>
<th>PTC association analysis (108 versus 1490)</th>
<th>GD association analysis (1442 versus 1468)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Risk allele</td>
<td>Case MAF</td>
</tr>
<tr>
<td>rs2622590</td>
<td>T</td>
<td>0.519</td>
</tr>
<tr>
<td>rs1348271</td>
<td>G</td>
<td>0.046</td>
</tr>
<tr>
<td>rs6683419</td>
<td>G</td>
<td>0.315</td>
</tr>
<tr>
<td>rs925489</td>
<td>C</td>
<td>0.120</td>
</tr>
</tbody>
</table>

SNP, single-nucleotide polymorphism; PTC, papillary thyroid carcinoma; GD, Graves’ disease; OR, odds ratio for the risk allele; CI, confidence interval.

as candidate loci for the replication study (rs7850258 was in complete LD with rs925489, $r^2 = 1.00$ in our Chinese Han samples). We also selected SNP rs6683419 ($P = 1.33 \times 10^{-5}$) at 1p36.13, which was previously reported (13) to be associated with serum TSH levels. In the replication study, the DNA concentrations of all samples were standardized to 50 ng/μl in 96-well plates, and one negative control (DNase-free and RNase-free water) was included in one of the 96 wells at random. Four SNPs were genotyped using the ABI ViiATM 7 Real-Time PCR System according to the manufacturer’s protocol by technicians who were blinded to the sample status. The average call rate was >95%.

Statistical analysis

To reduce potential bias caused by deviation of TSH levels from a normal distribution, we applied the natural-log transformation to normalize the distributions of all phenotypic data before testing for associations. A standard linear regression model was used to analyze the association of each SNP with quantitative traits, assuming an additive genetic model, which was implemented in the PLINK software package (47). For typed or imputed genotypes, the allelic dosage at each SNP was the independent variable, adjusted for primary covariates of age, gender, geographic region and race. A $P$-value of $5 \times 10^{-8}$ was used as the cutoff for genome-wide significance. The effects of selected SNPs on PTC risk were further evaluated for the case-control samples using logistic regression models.

PCA and MDS analysis were implemented using the EIGENSTRAT (50) and PLINK (47) software, respectively, to evaluate population stratification in the GWAS samples. The Haploview software version 4.2 was used to generate the genome-wide plot of $P$-values, and regional plots were generated using the LocusZoom Version 1.1 software (51). Quantile–quantile plots were constructed by plotting the observed distribution of the $P$-values for the given SNPs against the theoretical distribution of the expected $P$-values. The calculations and the plots were implemented using the PLINK and R statistical packages.

mRNA expression analyses

In the mRNA expression analysis, 66 PTC samples and 40 para-neoplastic tissue samples were surgically collected from patients. Total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer’s instructions and then treated with DNase I at room temperature for 10 min to degrade possible contaminating genomic DNA. cDNAs were made from 1 μg RNA templates using reverse transcriptase and oligo(dT) primer (Takara). Quantitative real-time PCR for a series of genes was performed in triplicate using the SYBR Green and ABI ViiATM 7 Real-Time PCR System. Data were analyzed and presented relative to the expression of the GAPDH housekeeping gene. The primer sequences used for real-time PCR are shown in the Supplementary Material, Table S7. We performed statistical analysis of the expression data using an ANOVA and an unpaired Student’s $t$-test (the two-tail $P$-values are indicated on the figures).

URLs

PLINK v1.07, http://pngu.mgh.harvard.edu/~purcell/plink/
R statistical environment version 2.10.0, http://www.r-project.org/
LocusZoom Version 1.1, http://csg.sph.umich.edu/locuszoom/
IMPUTE2, http://mathgen.stats.ox.ac.uk/impute/impute_v2.html/
SNPTEST v2 software, https://mathgen.stats.ox.ac.uk/genetics_software/snpset/snpset.html/
ENCODE database, http://genome.ucsc.edu/ENCODE/

AUTHOR CONTRIBUTIONS


SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

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Conflict of Interest statement. None declared.
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REFERENCES


**APPENDIX**

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