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Output จากโครงการวิจัย

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

1.Jongjaroenprasert W, Chanprasertyotin S, Butadej S, Nakasatien S, Charatcharoenwitthaya N, Himathongkam T, Ongphiphadhanakul B. Association of genetic variants in *GABRA3* gene and thyrotoxic hypokalaemic periodic paralysis in Thai population: Clin Endocrinol (Oxf). 2008 Apr;68(4):646-51

2. Manuscripts ผลงานวิจัย 2 เรื่องได้แก่

2.1 "Association between haplotyped tagging SNPs of GABRA3 and thyrotoxic hypokalaemic periodic paralysis in Thais.: Manuscript to be submitted to journal of Human Genetics

2.2 "A genome-wide association study identifies novel susceptibility loci for thyrotoxic hypokalemic periodic paralysis.: Manuscript to be submitted to Brief Communication report in Nature Genetics

3. การนำผลงานวิจัยไปใช้ประโยชน์

- เชิงสาธารณะ มีเครือข่ายความร่วมมือระหว่าง กรมวิทยาศาสตร์การแพทย์ และสถาบัน Riken Human Genomic Medicine
- เชิงวิชาการ มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่ โดยได้ส่งนักศึกษาปริญญาเอกไปเรียนรู้วิธีการทางห้องปฏิบัติการในการทำ Genotyping บน Microarrays, การคำนวณวิเคราะห์ทางสถิติ รูปแบบ Genome Wide Association Studyเรียนรู้เทคนิคการทำ PCR แบบMultiplex PCR ด้วย Invader assay

4. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)

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ภาคผนวก

1. Reprint ผลงานวิจัย 1 เรื่อง ได้แก่ เรื่อง “Association of genetic variants in *GABRA3* gene and thyrotoxic hypokalaemic periodic paralysis in Thai population”

2. Manuscripts ผลงานวิจัย 2 เรื่องได้แก่

2.1 “Association between haplotyped tagging SNPs of GABRA3 and thyrotoxic hypokalaemic periodic paralysis in Thais.”

2.2 “A genome-wide association study identifies novel susceptibility loci for thyrotoxic hypokalemic periodic paralysis.”

3. บทความสำหรับการเผยแพร่

Reprint ผลงานวิจัย 1 เรื่อง

Association of genetic variants in *GABRA3* gene and thyrotoxic hypokalaemic periodic paralysis in Thai population

ical Endocrinology (2008) 68, 646–651

doi: 10.1111/j.1365-2265.2007.03083.x

ORIGINAL ARTICLE

ssociation of genetic variants in *GABRA3* gene and hyrotoxic hypokalaemic periodic paralysis in Thai population

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ummary

ackground Genetic predisposition has been suggested to play le in the pathogenesis of thyrotoxic hypokalaemic periodic ralysis (THPP).

bjectives In this study, we assessed the differences of single-ucleotide polymorphisms (SNP) allelic frequency between THPP tients and well-characterized controls in order to find the suscep-ility genetic variants related to THPP using microarray-based sessments on pooled DNA.

ethods Fifty cases of THPP and 50 male hyperthyroid patients ithout hypokalaemia as controls were recruited. Equal amounts of dividual genomic DNA were pooled from each group. Estimated lele frequencies of SNPs were derived by averaging relative allele gnal score obtained by Affymetrix GeneChip® Mapping 10K Arrays.

esults Sixty-nine loci that display robust allele frequency differ-nces between THPP and controls were identified. SNP rs750841 A > T) in intron 3 of the gamma-aminobutyric acid (GABA) receptor 3 subunit (*GABRA3*) gene possessed the most significant dif-erence in allele frequency (27% in THPP case and 5% in controls, = 0·007). Actual allele frequencies obtained from genotyping in ach individual were very similar to the estimated frequency from ne pools (28% in THPP and 2% in controls, and $P = 0·0002$). Nearby DNA sequences of *GABRA3* were sequenced and an dditional two SNPs were found (A > C at exon 1 and G > T of s12688128). Allele A of rs750841 and allele G of rs12688128 in ntron 3 were predominantly found in THPP with significant enetic relative risk of 19 ($P < 0·0002$; 95%CI 2·4–151·6).

Conclusions Whole-genome scanning on pooled DNA provides n accurate, useful screening tool for elucidating genetic underpinnings f THPP. SNPs at intron 3 of *GABRA3* are found to be associated vith THPP.

Received 18 May 2007; returned for revision 14 June 2007; finally evised 23 August 2007; accepted 11 September 2007)

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Introduction

Thyrotoxic hypokalaemic periodic paralysis (THPP) is characterized by episodes of intracellular shift hypokalaemia and muscle weakness in thyrotoxic patients. The incidence is high in Asians including Chinese, Japanese Vietnamese, Filipino, Korean and Thai populations. It has occasionally been reported in American Indians and Latin Americans, and rarely found in Caucasians and Africans. This condition affects predominantly males with the male to female ratio ranging from 17 : 1 to 70 : 1.[1–3] It becomes lethal if the respiratory muscles are involved or cardiac arrhythmia develops. Various aetiologies of thyrotoxicosis have been reported to be related to THPP, for example, Graves' disease, thyrotrophin-producing pituitary adenoma and exogenous thyroid hormone abuse. The clinical features of thyrotoxicosis can be subtle. Importantly, weakness and hypokalaemia completely resolved once euthyroidism is restored by definitive therapy of hyperthyroidism.

Pathogenesis of this condition is still unclear, but it is believed to be genetically associated. Because of the similar clinical manifestations to familial form of hypokalaemic periodic paralysis (FHPP), some overlapping genetic determinants between FHPP and THPP has been hypothesized. For example, certain single-nucleotide polymorphisms (SNPs) of the *Cav1·1* gene which encodes L-type voltage-gated potassium channels, including nucleotide (nt) 476, intron 2 nt 57 and intron 26 nt 67, have been reported to be associated with THPP in southern Chinese.[4] One THPP patient of Portuguese descent has been found to carry an R83H mutation in the *KCNE* gene which encodes subunit MIRP2 of voltage-gated potassium channel.[5] In addition, the R672G mutation of the voltage-gated sodium channel Nav1·4 has been reported in one paediatric Caucasian patient with THPP.[6] The functional significance of the aforementioned genetic variants on the mechanism of THPP is unclear, although all are mutations that affect amino acid sequences.

Increasing activity of the Na^+/K^+ ATPase pump has been demonstrated in THPP patients. The pump causes potassium to move to the intracellular compartment and various factors that increase its activity can cause hypokalaemia and weakness, including: high carbohydrate, alcohol consumption, strenuous exercise, insulin and glucocorticoid-induced insulin resistance. Subsequently, genes coding for different subunits of the Na^+/K^+ ATPase were examined in southern Chinese THPP patients,[7] but no mutation was identified.

nilarly, polymorphisms in the β2-adrenergic receptor gene were eened and no mutation has been found in Korean patients.[8] Thus, ether THPP patients have a genetic predisposition to activation of Na^+/K^+ ATPase or various ion channels remains to be elucidated. As THPP does not follow the simple pattern of Mendelian inher- nce and its pathophysiology are still not well understood, study genetic association by a candidate-gene approach might not be propriate. A more efficient method is a genome-wide association sign by genotyping a large number of markers among a large mber of individuals using a high-throughput genotyping plat- rm.[9] Microarray-based platforms are one of the efficient platforms this kind of study. In order to make a genome scan feasible with eatly reduced genotyping costs, genotyping on pooled DNA has en proposed.[10] There are many studies demonstrating that the ele frequencies determined by quantitative analysis of PCR products om pooled DNA samples agree well with the analysis by individual notyping. Genome-wide association study on pooled DNA has en validated and applied in whole-genome association analysis in me complex diseases.[11–14] In the present study therefore we aimed find the genetic variants associated with THPP by performing a nall scale genome-wide scan using Affymetrix 10K GeneChip on ooled DNA.

Materials and methods

ubjects were 50 cases of thyrotoxic Thai patients with hypokalaemia nd paroxysms of proximal muscle weakness. They all had intra- ellular shifts of potassium as evidenced by hypokalaemia with low rine potassium excretion (urine $K^+ < 15$ mEq/dl). We excluded the ases with alcoholism, and none of them received any medications nat cause hypokalaemia during the attack. We recruited best-matched ontrols by selecting 50 hyperthyroid, male patients with normo- alaemia during their thyrotoxic states. The aetiology of hyper- hyroidism in both cases and controls was Graves' disease as confirmed y either clinical features of ophthalmopathy, and dermopathy, or ositive thyroid autoantibodies. The study has been approved by he Ethics Committee of Ramathibodi Hospital which has been erformed in accordance with the ethical standards laid down in the 964 Declaration of Helsinki. All the subjects gave their informed onsent prior to their inclusion in the study.

Genomic DNA was extracted from peripheral leucocytes, titrated nd quantified by fluorimetry (PicoGreen®, Cambridge Bioscience, Cambridge, UK) to a concentration of 50 ng/μl. Pooled DNA was eparately constructed for THPP cases and hyperthyroid control ubjects by mixing equal amounts of DNA from 50 individuals of ach group and then purified prior to hybridization on microarrays Quick PCR purification kit®, Qiagen, Valencia, CA). SNP genotyp- ng of pooled DNA was performed using a hybridization reaction vith the single probe technique on Affymetrix GeneChip® Mapping 10K Xba 142 2·0 Arrays following the manufacturer's protocol.[15] Two hundred and fifty nanograms of pooled genomic DNA were digested by *Xba*I, ligated to adaptor, amplified by PCR by using 3 min 95 °C hot start; 35 cycles of 20 s, 95 °C; 15 s, 59 °C; 15 s, 72 °C; and a final 7 min 72 °C extension. PCR products were purified (MinElute 96 UF kits, Qiagen), digested for 30 min with 0·04 unit/μl DNase I to produce 30- to 200-bp fragments, end-labelled by using terminal deoxynucleotidyl transferase and biotinylated dideoxynucleotides, and hybridized to 10K GeneChip arrays (Affymetrix, Santa Clara, CA), which were stained and washed as described by using biotinylated antistreptavidin antibody (Vector Laboratories, Burlingame, CA) and R-phycoerythrin streptavidin (Molecular Probes, Eugene, OR). Arrays were scanned and fluorescence intensities were quantified using an Affymetrix array scanner, as described. We duplicated 10K GeneChip genotyping for each group. GDAS program version 3·0 was used for analysing the GeneChips.

Statistical analysis

Estimated allele frequencies for each SNP in each DNA pool were assessed, and based on averaging hybridization intensity signals from two arrays. Allele frequency estimates were derived from relative allele signals (RAS) for the sense strand (RAS1) and the antisense strand of SNPs (RAS2). RAS scores should vary between 0 (for a BB homozygote) and 1·0 (for an AA homozygote), and heterozygotes should generate a RAS of about 0·5. As previously reported,[16,17] the average RAS scores derived from RAS1 and RAS2 is employed to estimate the SNP allele frequencies of the pools. Chromosomal positions of 11 482 SNPs, as well as previously linked and associated markers, and genes were determined by using National Center for Biotechnology Information and NetAffx data. Although there is no universally accepted method for analysing association genome scanning data, we calculated odds ratios and ranked the polymorphisms that related to THPP and then applied Pearson's χ^2 and Fisher extract with Monte Carlo permutation test to determine the differences of SNP frequencies between groups. We selected the SNP that provided the highest odds ratio and identified the gene and region within gene where this SNP is located. Then nearby tagged SNPs as defined by HAPLOVIEWER (http://www.hapmap.org) using data from Chinese population genotyping database were selected and genotyping was extended. Genetic relative risks were calculated for SNP that associated with THPP.

Results

Both THPP subjects and hyperthyroid patients without THPP had similar clinical baseline characteristics. They are males with similar age group (41·60 +1·73 years in controls *vs.* 39·65 +1·41 years in THPP) (Table 1). All THPP subjects and controls had Graves' disease

Table 1. Baseline clinical characteristics of both thyrotoxic hypokalaemic periodic paralysis (THPP) and hyperthyroid patients group without paralysis

	THPP ($n = 50$)	Hyperthyroid controls ($n = 50$)
Sex, male	100%	100%
Age (years)	39·65 ± 1·41 (20–67)	41·60 ± 1·73 (21–73)
Aetiology of hyperthyroidism	Graves' disease	Graves' disease

There was no statistical differences in the baseline clinical parameters (P = NS).

Table 2. The most 10 significant allele frequency differences from estimated SNP frequencies comparing between THPP pool and hyperthyroid control pool ($P \leq 0{\cdot}001$)

					THPP pool			Hyperthyroid control pool				
SNP	Chromosome	Chromosome position	Associated gene	Allele	RAS_{av} CHIP1	RA_{av} CHIP2	Estimated frequency	RAS_{av} CHIP1	RAS_{av} CHIP2	Estimated frequency	OR	*P* value
SNP1	X	151216374	*GABRA3*	A/T	0·28	0·27	0·28	0·00	0·05	0·03	12·57	3×10^{-6}
SNP2	20	57649420	*PHACTR3*	A/C	0·02	0·02	0·02	0·14	0·15	0·15	8·65	3×10^{-4}
SNP3	5	28372366	Upstream *CDH9*	C/T	0·16	0·17	0·17	0·05	0·01	0·03	6·62	6×10^{-4}
SNP4	1	230870522	Upstream *KIAA1383*	G/T	0·18	0·35	0·26	0·07	0·04	0·06	5·50	1×10^{-4}
SNP5	1	87384298	*HS2ST1*	C/T	0·16	0·19	0·17	0·03	0·04	0·04	4·92	3×10^{-4}
SNP6	11	49879899	*OR4C13*	C/T	0·29	0·11	0·20	0·57	0·52	0·55	4·89	1×10^{-4}
SNP7	18	27580619	Upstream *B4GALT6*	C/G	0·12	0·15	0·13	0·02	0·03	0·03	4·83	7×10^{-4}
SNP8	1	52007329	*OSBPL9*	A/T	0·28	0·29	0·29	0·04	0·12	0·08	4·70	9×10^{-4}
SNP9	5	18192076	Downstream *CDH18*	A/G	0·17	0·30	0·23	0·09	0·05	0·07	3·97	1×10^{-3}
SNP10	6	156595587	Upstream *NOX3*	C/T	0·08	0·10	0·09	0·35	0·19	0·27	3·74	$1{\cdot}1\times10^{-3}$

SNP, single-nucleotide polymorphism; THPP, thyrotoxic hypokalaemic periodic paralysis; RAS, relative allele signal; CHIP1/CHIP2, Affymetrix 10K SNP gene chip; OR, odds ratio.

Table 3. Estimated allele frequencies and actual allele frequencies of rs750841 in both groups

Phenotype	Estimated minor allele frequencies	Actual allele frequencies
THPP	0·28	0·28
Controls	0·03	0·02

THPP, thyrotoxic hypokalaemic periodic paralysis.

as the aetiology of their hyperthyroidism. Potassium levels of THPP subjects during their weakness were 1·7–3·3 mEq/l.

Estimated allele frequencies and validation

Successful genotyping was achieved in 10 204 SNPs across all chips. Signal detection for each chip was more than 99·9%, with the average SNP calling of 73·08% for THPP and 72·97% for hyperthyroid controls group. We excluded the genetic variants that had average RAS score below 0·05 or over 0·95. After odds ratios of SNPs that related to THPP were calculated, we chose the most interesting SNP by ranking the first 10 SNP with highest odds ratios as shown in Table 2.

The SNP which had the most significant difference in estimated allele frequency and greatest odds ratio ($P = 3 \times 10^{-6}$) was selected for further study. It is equivalent to rs750841 in dbSNP database. Individual genotyping was then performed in 50 cases and 50 controls by direct sequencing covering 300 nt nearby this SNP. As shown in Table 3, we demonstrated that the actual frequency was similar to estimated allele frequencies without any k-correction as described in a previous report.[16]

Extended sequencing and SNPs genotyping in GABRA3 gene

The SNP that we found associated to THPP was located in intron 3 between exons 3 and 4 region of *GABRA3*, which encodes GABA receptor α3 subunit. It contains 10 exons, but the first exon is not transcribed as shown in grey block in Fig. 1.[18] Nine exons of *GABRA3* transcribes to 1871 nts mRNA and translates to 492 amino acids.

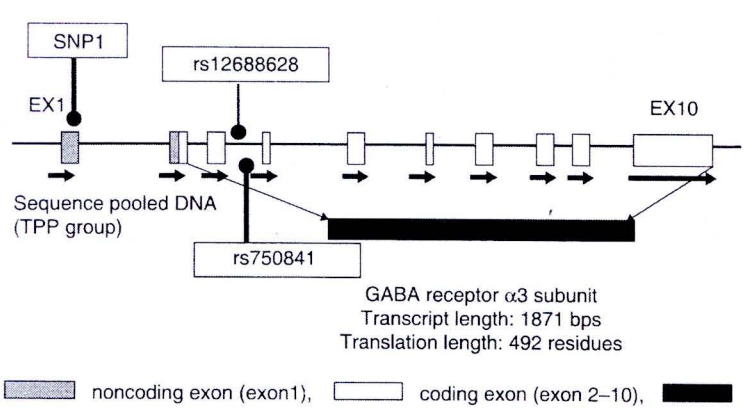

Fig. 1 Genomic characterization of *GABRA3* gene. GABRA3 gene is on chromosome X at location 151 085 362–151 370 993. It contains 10 exons. SNP rs750841 is located between exons 3 and 4. There were two additional SNPs nearby detected by direct sequencing method which are represented by SNP1 (a novel SNP) at exon 1and SNP rs12688628 at intron 3. SNP, single nucleotide polymorphism.

Table 4. Significant difference in allele frequencies of SNPs 2 and 3 ($P < 0{\cdot}001$)

SNP ID	Allele	Cases, *n* (%)	Controls, *n* (%)	Odds ratio	95%CI	*P* value
SNP1*	A	16 (32)	25 (50)	2·13	0·93–4·85	0·06
	C	34 (68)	25 (50)			
SNP2†	G	14 (28)	1 (2)	19	2·4–151·6	0·0002
	T	36 (72)	49 (98)			
SNP3‡	A	14 (28)	1 (2)	19	2·4–151·6	0·0002
	T	36 (72)	49 (98)			

CI, confidence intervals; SNP, single nucleotide polymorphism.
*SNP1 was a new SNP located in exon 1 which is not transcribed.
†SNP2 (rs12688628) was located at intron 3, 77 nucleotides apart from SNP3.
‡SNP3 (rs750841) was also at intron 3.

On the basis that all subjects were males and the location of *GABRA3* gene is on chromosome X, there is therefore only one allele of this gene for each subject. We performed direct sequencing on THPP pooled DNA for every exon to screen for new mutations in this gene. There were no new nonsynonymous variations detected in the encoding exonal region of *GABRA3* in THPP cases. However, two additional SNPs were found nearby the rs750841 in our population. As showed in Fig. 1, one new genetic variant of C/T at exon (SNP1 in Fig. 1) and rs12688628 (A/G) at intron 3 (SNP2 in Fig. 1) were identified.

We performed direct sequencing of the DNA segment covering these two additional SNPs individually in both THPP and hyperthyroid male controls groups. Allele C of SNP1 was predominantly found in THPP subjects, but it was not statistically significant (THPP = 68% *vs.* controls = 50%, $P = 0{\cdot}06$). For rs750841, allele A was significantly associated with THPP cases (28% *vs.* 2%, $P < 0{\cdot}001$). Moreover, we also demonstrated that allele G of rs12688628 was significantly related to THPP phenotype (28% *vs.* 2%, $P < 0{\cdot}001$). Genetic relative risk for allele A of rs750841 and allele G of rs12688628 for THPP subjects were 19 ($P < 0{\cdot}0002$; 95%CI 2·4–151·6) as demonstrated in Table 4.

We further searched 1200 bp of the 5′ flanking region of *GABRA3* for the presence of putative thyroid hormone responsive element (TRE). At 5′ upstream from the initiation codon (ATG), there are four different putative TREs identified; at nt −804 to −809 (AGGTCA), nt −624 to −632 (GGGAGA), nt −657 to −662 (GGGTTA) and nt −146 to −151 (AGGGAC). In addition, at intron 3, at 5 nt 5′ upstream to the SNP rs750841, a putative TRE ACTGGA is identified.

Discussion

Hypokalaemic periodic paralysis is a syndrome that was classified as channelopathies.[19] In the familial form of this syndrome (FHPP), mutations have been described in *CACNA1S*,[20,21] *SCN4A*[22,23] and *KCNE3* genes which encode voltage-gated L-type calcium channel, α subunit, voltage-gated sodium channel, α subunit and MinK-related peptide, respectively. Ion channel gene defects have been hypothesized to be the basic pathogenesis of THPP because of its similar clinical features to FHPP. Many authors[24–26] have screened these ion channel mutations with different methods but most of mutations reported in FHPP were not found in THPP. Only R83H at *KCNE3* as reported in one family of FHPP was demonstrated in one Portuguese case,[27] and R672G mutation of the voltage-gated sodium channel Nav1·4 was reported in one paediatric Caucasian patient.[6] However, Kung *et al.*[4] recently reported that three novel SNPs in Ca(v)1·1 found in patients with THPP have significant differences in genotype distribution compared with controls with Graves' disease and healthy controls. A genetic analysis on the skeletal muscle Na^+/K^+ ATPase has recently been extensively investigated by the same study group due to the indication by many clinical data of the important role of this pump.[28] However, they failed to detect any difference in the heterozygosity rates of the SNPs and haplotypes of the polymorphic SNPs between subjects with or without THPP. These findings suggested that candidate-gene approach to dissect genetic basis of diseases relies mainly on the prior biological hypotheses. When the fundamental physiological defects of a disease are uncertain like THPP, the candidate-gene association study will be inadequate to fully dissect the genetic basis of the disease. The method can at best identify only a fraction of genetic determinants even for diseases with relatively well understood pathophysiology. On the contrary, genome-wide association screens most of the genome for causal genetic markers without prior knowledge of hypothetical association. Our results support the efficiency of microarray-based pooled association genome scanning approach. We demonstrated that genetic polymorphisms located in intron 3 of *GABRA3* genes are strongly associated with THPP with the genetic relative risk of 19.

Hyperthyroidism itself is thought to be a precipitating factor of THPP, but it may not be the fundamental cause as evidenced by the disproportion of the incidence of hyperthyroidism and THPP. Therefore, to reduce the confounding factors as much as we can, we recruited best-matched controls in terms of the same sex, age and thyroid status. In the present study, we utilized DNA pooling in addition to genotyping based on high density microarray to elucidate genetic determinants of THPP. Quantitative analyses of allele frequencies in DNA pools has proved remarkably accurate when applied to simple tandem repeats or to SNPs using a variety of different genotyping technologies.[10] When using this method for the estimation of allele frequency differences between two pools, the mean error rate of pooled analysis is in the region of 1%–2%, and the statistical power is approximately the same as that obtained from individual genotyping of cases and controls[29] but at a much reduced cost. In keeping with previous results, we also demonstrated in the present study the accuracy of allele frequency estimation from pooled DNA. Altogether this suggests that pooled genotyping using Affymetrix 10K GeneChip arrays is a cost-effective approach in the genome-wide screening of SNPs likely to be associated with diseases of interest.

The most statistically significant SNP (rs750841) is located in the intronic region of *GABRA3* gene on chromosome X. Interestingly, it is known that THPP are predominantly found in males. Finding the associated polymorphisms on chromosome X may explain this clinical feature. The nearby SNP (rs12688628) was also significantly associated with THPP and is likely to be the effect of linkage disequilibrium as these two SNPs were only 77 base pairs apart. The

association of the *GABRA3* locus with THPP may be explained by two reasons. First, as in this study we use 10K GeneChip arrays which provided sparse SNPs interval, our results reveal an indirect association and the associated SNP may be in linkage disequilibrium with other causative alleles nearby, within or outside *GABRA3*. Second, this can be a direct association. The SNPs that we found associated with THPP may regulate *GABRA3* or nearby genes. There was a previous study which reported the association of polycystic ovary syndrome with genetic variant in intron of one gene which was subsequently shown to regulate the expression of another downstream gene. *GABRA3* gene encodes GABA receptor α3 subunit which is a member in the chloride channel family. The gene is highly expressed in neurone and neuromuscular junction tissue. Its mutation has been reported in Rett syndrome which is characterized by an X-linked dominant neurodevelopmental disorder that affects females.[31] From Gene Expression Omnibus (GEO), which is the NCBI high throughput gene expression resource, it was demonstrated that *GABRA3* also expressed in human skeletal muscle in various sites[32] (GEO accession number GSE4667). Different conditions affect the expression profiles of GABA receptor α3 subunit; for instance, it was downregulated in inflammatory disease like dermatomyositis (GSE5370 and GSE1551) and weight loss by 2 weeks of exercise training[33] (GSE1295).

It is possible for the influence of thyroid hormone on the regulation of *GABRA3* expression. In general, T3 and thyroid hormone receptor regulate target gene expression by binding to imperfect repeats of two or more TREs. The element consists of multiple copies of a hexameric sequence related to a consensus six-nucleotide core binding motif (A/G)GG(TCA/AGG) as direct and inverted repeats in a palindromic arrangement.[34,35] Sequences that are correspondent to putative motif for TRE were found in regulatory region of *GABRA3* and also one near the SNPs we studied, suggesting that thyroid hormone may modulate transcription or translation of the GABA receptor α3 subunit. There is some evidence from animal studies that thyroidal state has an affect on the number of GABA molecules and binding sites on GABA receptor on both neuronal tissue and the neuromuscular junction.[36] However, there is no *in vitro* study that directly assessed the effect of thyroid hormone on GABA receptor expression in myocyte. No clear explanation for the role of GABA receptor in THPP patients is readily apparent; therefore, further functional study is mandatory to elucidate the mechanism.

The 10K GeneChip mapping array used in this study provides a median SNP interval of 105 Mb which is considered to be of low coverage. Obviously, some SNPs within ion channel genes may be missed. Higher density array such as 100K or 500K GeneChip arrays should be further utilized in order to cover the whole genome and all haplotype blocks so that more associated genetic variants can be discovered, leading to an opportunity to understand more fully the basic pathophysiology of THPP. The other major limitation of the study was the small sample size which can provide inadequate power for the identification of susceptibility genes. Calculation of the statistical power was performed using sample size of 50 in both cases and controls, and type I error probability for a two-sided test at 0·000004 after adjustment for multiple comparisons of 11 482 SNPs. The result showed the study would have 80% power to detect SNPs if odds ratio were at least 49 and therefore could have insufficient power to detect SNPs with smaller genetic effects. For SNP rs750841 with an odds ratio of 19, the study would have 8·9% power for the detection. With the possibility of false association as a result of small sample size and multiple comparisons, our findings obviously need to be confirmed in different cohorts or ethnic groups.

Conclusions

This is the first report of the association between genetic variant in intron 3 of *GABRA3* and thyrotoxic hypokalaemic periodic paralysis. The results of our finding suggested that for searching susceptible genes in complex disease setting, whole-genome scan on pooled DNA using high-throughout genotyping technique is a cost effective, time saving and accurate tool for screening. With this method, many genetic loci associated to THPP are identified, and this provides an opportunity to understand the basic pathophysiology of THPP. New hypothesis about its pathogenesis can be generated.

Acknowledgements

This study was supported by the Thailand Research Fund.

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Manuscript 2 เรื่อง

1 “Association between haplotyped tagging SNPs of GABRA3 and thyrotoxic hypokalaemic periodic paralysis in Thais.”

2 “A genome-wide association study identifies novel susceptibility loci for thyrotoxic hypokalemic periodic paralysis.”

Association between haplotyped tagging SNPs of *GABRA3* and thyrotoxic hypokalaemic periodic paralysis in Thais.

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Abstract

Thyrotoxic state and genetic susceptibility are believed to be the underlying contributing factors to thyrotoxic hypokalaemic periodic paralysis (THPP). Genetic heterogeneity has become apparent, as more than one ion channel genes have been reported associated with THPP. We recently found the association of single nucleotide polymorphisms (SNPs) located in *GABRA3* with THPP. In this study, we aimed to confirm our previous study by examining the association of haplotype tagging SNPs (htSNPs) of the whole *GABRA3* gene and THPP. We also genotyped 2 additional sets of htSNPs of candidate genes; 1) *CACNA1S*, and *SCN4A* which have previously been demonstrated associated with THPP), and 2) *DPP6,* and *GLRA1* that we found related to THPP from our Affymetrix GeneChip Mapping 10K microarray analysis. Seventy five subjects of THPP and 81 male hyperthyroid patients without hypokalaemia were recruited. Totally 116 htSNPs was successfully assessed using multiplex-PCR-based method on the Invader assay; fifty- five of them were htSNPs of *GABRA3,* 37 , 8, 7, 9 SNPs for *CACNA1S, SCN4A, GLRA1* and *DPP6*, respectively. We identified 25 htSNPs located in the intron 3 of *GABRA3* strongly associated with THPP. The most significant *P*-value was 2.23×10^{-4}, with the odds ratio of 7.87 (95%CI; 2.23-27.76). However, no mutation was found in the adjacent exons (exon2-4). There was no association between htSNPs of *CACNA1S, SCN4A, DPP6,* and *GLRA1* and THPP.
Conclusions Our current study confirmed the previous report that genetic variants located in intron3 of GABRA3 are susceptible to THPP.

Key Words: Genetic association study, Hyperthyroidism, Hypokalemic Periodic Paralysis, GABRA3

Word Count: Text: 4987, **Abstract:** 242, **Tables:** 1, **Figures:** 1, **Supplementary table**: 2

Materials and Methods

Subjects

The study was approved by the Ethics Committee of Ramathibodi Hospital, Mahidol University and the Institute of Physical and Chemical Research, Yokohama Institute, (RIKEN). All subjects read and provided consent forms before participating in the study. Case group defined in our study were Thai, male patients with THPP and control group were Thai, male, thyrotoxic patients without episode of hypokalemia and weakness. All subjects had Graves' disease as their underlying hyperthyroidism and other possible causes of hypokalemia were excluded. None of our subjects was relative.

Methods

Genomic DNA from all subjects were extracted from peripheral blood leukocytes using the DNA extraction kit (PicoGreen®, Cambridge, Bioscience, UK), DNA degradation was then checked by running on 1% agarose gel electrophoresis. We measured DNA concentration using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA). The purity of the DNA was determined by the ratio of light absorbance at 260-280 nm. Final concentration of 5 ng/µl of genomic DNA was used for SNPs genotyping by the multiplex-PCR-based method on the Invader assay.

SNPs genotyping

A high throughput SNP genotyping was done using multiplex-PCR-based method[10] on the Invader assay[11] (Third Wave Technologies, Madison, WI, USA). The multiplex PCR amplification was performed according to the protocol by Ohnichi Y et al. Fifteen to twenty primer pairs for each nucleotide segment was designed for each PCR reaction.

Direct sequencing

Direct sequence of the interesting genes was performed using BigDye® Terminator v3.1 Cycle Sequencing Kit. The reaction was detected by a Applied Biosystems 96-capillary 3730xl DNA Analyzer (Foster City, CA, USA).

Statistical analyses

Hardy Weinberg Equilibrium (HWE) was tested for all SNPs. Odds ratio (OR) and confidence intervals (95% CI) were calculated using the risk genotypes as a reference. We used the Fisher extract's test by two-by-two contingency table to compare the allelic and genotypic frequencies between cases and controls in 3 different models.

Candidate genes and SNPs selection

As shown in table 1, we selected three genes which were reported associated with THPP from the 10K Affymetrix GeneChip Microarray analysis described in our previous study[9] (*GABRA3*, *GLRA1* and *DPP6*). Two additional candidate ion channel genes were also selected (*CACNA1S*, and *SCN4A*). The haplotype tagging single nucleotide polymorphisms (htSNPs) of each gene with the r^2 more than 0.8 and minor allele frequency (MAF) more than 0.05 from the International HapMap Project database were selected for individual genotyping.

Table 1 Candidate genes, map location and number of haplotype tagging single nucleotide polymorphisms (htSNPs).

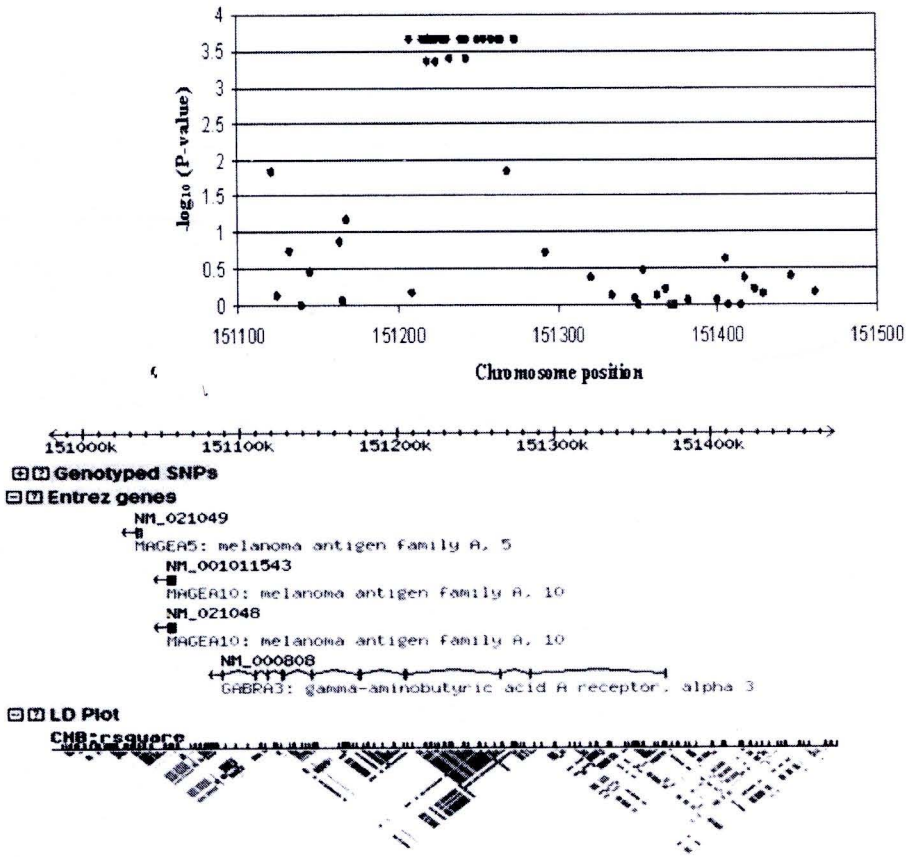
Gene Symbol	Gene Name	Map location	No. of tagged SNPs
GABRA3	Gamma-aminobutyric acid (GABA) A receptor, alpha3	Xq28	55
CACNA1S	Calcium channel, voltage-dependent, L type, alpha 1S subunit	1q32	37
SCN4A	Voltage-gated sodium channel subunit alpha Nav1.4	17q23.3	8
GLRA1	Glycine receptor, alpha1	5q32	7
DPP6	Dipeptidyl aminopeptidase-like protein6	7q36.2	9

Results

In the present study, we recruited 156 subjects, 81 of them were cases (Thai, male patients with THPP) and 75 of them were controls (Thai, male thyrotoxic patients without hypokalemia and weakness). All 55 htSNPs of *GABRA3*, 9 htSNPs of *DPP6*, 7 htSNPs of *GLRA1*, 37 htSNPs of *CACNA1S* and 8 htSNPs of *SCN4A* were successfully genotyped by a high throughput SNP genotyping was done using multiplex-PCR-based method on the Invader assay (Third Wave Technologies, Madison, WI, USA)[10] . We did not find the difference in allelic and genotypic frequencies of htSNPs of *DPP6, GLRA1, CACNA1S, and SCN4A* gene comparing between cases and controls (Data in table 2).

Table 3 showed the allelic and genotypic frequencies of 55 htSNPs closed to intron3 of *GABRA3* comparing between cases and controls. Among 55 SNPs, we also included 3 SNPs which were previously reported association with THPP (rs12688628, rs750841 and SNP1). In this study, we demonstrated significant association between 25 htSNPs near the intron3 of *GABRA3* gene including the rs12688628 and rs750841 and THPP. As shown in the table, the most significant p value was 2.3×10^{-4} with the odds ratio of 7.87 (95%CI; 2.23-27.76). All twenty-five SNPs were in the same LD block and located between intron2 to intron3 as shown in figure 1. However, the results of direct sequencing of exon 2, 3, and 4 of *GABRA3* showed no mutation in our THPP cases.

Figure 1 showed *P* value plots, genomic structure and LD map of the *GABRA3* gene on chromosome X. Blue and red dots represent p values in the fine mapping and included SNP in this study that shown significance in previous study, respectively.



Supplementary table 1 Summary results for an association of the *SCN4A*, *CACNA1S*, *GLRA1*, *DPP6* and THPP

SNP_ID	Position	Gene	Allele	Case				Control				Risk allele	*P*-value (Fisher's exact test)	OD (95%CI)
				Genotype				Genotype						
				11	12	22	MAF	11	12	22	MAF			
rs2532111	59371153	*SCN4A*	A/G	10	39	15	0.461	12	33	11	0.491	G	4.82E-01	1.47 (0.58-3.73)
rs4968604	59387054	*SCN4A*	G/A	23	33	8	0.383	18	27	11	0.437	G	3.24E-01	1.71 (0.63-4.61)
rs4968679	59400473	*SCN4A*	C/T	11	41	12	0.492	9	26	21	0.393	C	2.54E-02	2.59 (1.14-5.95)
rs2302236	59402199	*SCN4A*	A/G	2	28	34	0.250	1	13	42	0.134	A	1.46E-02	2.65 (1.21-5.77)
rs2070720	59372505	*SCN4A*	T/C	11	37	16	0.461	14	33	9	0.455	C	2.44E-01	1.74 (0.70-4.33)
rs2058194	59374080	*SCN4A*	T/C	9	39	16	0.445	10	34	12	0.482	C	6.05E-01	1.33 (0.50-3.55)
rs3785568	59377523	*SCN4A*	T/C	57	6	1	0.062	45	11	0	0.098	T	2.08E-01	1.99 (0.71-5.55)
rs2302237	59402439	*SCN4A*	C/T	33	28	3	0.266	30	22	4	0.268	C	7.04E-01	1.56 (0.33-7.31)
rs12566395	199278360	*CACNA1S*	G/T	53	11	0	0.086	43	11	2	0.134	G	2.16E-01	N/A
rs10494827	199280570	*CACNA1S*	G/C	24	36	4	0.344	21	29	6	0.366	G	5.12E-01	1.80 (0.48-6.74)
rs12407188	199282747	*CACNA1S*	G/C	49	15	0	0.117	46	10	0	0.089	C	5.05E-01	1.41 (0.58-3.45)
rs12065493	199285957	*CACNA1S*	C/T	38	26	0	0.203	34	19	3	0.223	C	9.87E-02	N/A
rs12032370	199291572	*CACNA1S*	C/T	50	12	2	0.125	45	9	2	0.116	T	8.24E-01	1.15 (0.47-2.78)

Introduction

Thyrotoxic hypokalaemic periodic paralysis (THPP) is characterized by episodes of hypokalaemia and weakness in thyrotoxic patients. The highest incidence of THPP worldwide are reported from Asia i.e. China, Japan, Philippines, including Thailand. Male gender predominance is observed which occurs at the male to female ratio range from 17:1 to 70:1 [1-3] Pathophysiology of THPP is unclear. The clinical features of THPP are similar to familial hypokalemic periodic paralysis (FHPP); an autosomal dominant inherited traits, suggesting defect in ion channels. However, there is some difference between the two. Most of FHPP was reported in Caucasian populations whereas most Asian countries reported THPP cases. THPP is not followed simple patterns of Mendalian inheritance. THPP could present with delay onset of the disease, comparing to FHPP cases. Hypokalemia and weakness will be restored once the patients become euthyroid. Therefore, other than genetic susceptibility, thyrotoxicosis may plays some role in the underlying pathophysiology.

Association between single nucleotide polymorphisms (SNPs) of ion channels and THPP have been reported in the previous literatures for instance 1) *CACNA1S* gene, which encodes for the L type voltage gated calcium channel [4], 2) *KCNE*3 gene, which encodes for the voltage gated potassium channel [5] and 3) the most recent new potassium channel *KCNJ18* [6]. These findings indicate underlying genetic heterogeneity in THPP. However the association between these genes and THPP has not been confirmed in other reports [7-8].

Our previous genetic association analysis [9] using Affymetrix GeneChip 10K microarray on pooled-DNA suggested association between set of SNPs locating near intron 3 of the *GABRA3* gene and THPP. The 10K GeneChip mapping array used in that study provided a median SNP interval of 113 Mb which is considered to be of low coverage distance between probes SNPs. In this study we aimed to find the most significant associated genetic loci, we therefore decided to perform higher density genotyping. Individual genotyping of additional htSNPs covering *GABRA3* gene was conducted and comparison of the allelic and genotype frequencies between cases and controls were assessed. Our second objective was to expand the association analyses to other 2 genes found in the top 10 ranked associated with THPP by affymetrix 10K microarrays, which were *DPP6* and *GLRA1*. And finally we would like to assess the association between htSNPs of known ion channel defects reported in FHPP and THPP; *CACNA1S* and *SCN4A,* and THPP.

SNP_ID	Position	Gene	Allele	Case				Control				Risk allele	*P*-value (Fisher's exact test)	OD (95%CI)
				Genotype				Genotype						
				11	12	22	MAF	11	12	22	MAF			
rs4915474	199293678	*CACNA1S*	C/T	5	37	20	0.379	7	29	20	0.384	T	5.46E-01	1.63 (0.49-5.46)
rs7556265	199295836	*CACNA1S*	T/C	58	6	0	0.047	50	6	0	0.054	T	1.00E+00	1.16 (0.35-3.83)
rs2297904	199297291	*CACNA1S*	G/A	26	29	9	0.367	26	25	5	0.312	A	4.11E-01	1.67 (0.52-5.31)
rs3767505	199301159	*CACNA1S*	T/C	37	25	2	0.227	37	18	1	0.179	C	4.23E-01	1.42 (0.68-2.99)
rs3820421	199301271	*CACNA1S*	A/T	33	22	9	0.312	30	22	4	0.268	T	8.56E-01	1.08 (0.53-2.22)
rs2297899	199303030	*CACNA1S*	G/C	50	12	2	0.125	42	13	1	0.134	G	8.29E-01	1.19 (0.51-2.78)
rs16847636	199303930	*CACNA1S*	A/G	49	13	2	0.133	44	11	1	0.116	G	8.30E-01	1.15 (0.48-2.66)
rs3767507	199308891	*CACNA1S*	C/T	50	14	0	0.109	47	8	1	0.089	T	4.67E-01	N/A
rs3767509	199310926	*CACNA1S*	G/A	48	14	2	0.141	39	14	3	0.179	G	4.80E-01	1.31 (0.59-2.92)
rs2296385	199321361	*CACNA1S*	G/A	60	4	0	0.031	47	9	0	0.080	G	1.39E-01	2.87 (0.83-9.91)
rs2147798	199328441	*CACNA1S*	C/G	28	25	11	0.367	24	23	9	0.366	G	1.00E+00	0.96 (0.47-1.99)
rs17454870	199333634	*CACNA1S*	G/A	49	12	3	0.141	42	11	3	0.152	G	8.56E-01	1.15 (0.22-5.95)
rs6704355	199332907	*CACNA1S*	A/G	18	27	19	0.492	14	25	17	0.473	A	7.97E-01	1.17 (0.52-2.65)

SNP_ID	Position	Gene	Allele	Case				Control				Risk allele	*P*-value (Fisher's exact test)	OD (95%CI)
				Genotype				Genotype						
				11	12	22	MAF	11	12	22	MAF			
rs16847726	199334548	*CACNA1S*	C/T	54	6	4	0.109	43	10	3	0.143	C	3.55E-01	1.63 (0.65-4.08)
rs1574408	199335410	*CACNA1S*	A/G	19	27	18	0.492	15	25	16	0.491	A	8.40E-01	1.15 (0.52-2.56)
rs1536129	199335814	*CACNA1S*	C/A	22	28	14	0.437	21	22	13	0.429	A	8.49E-01	1.15 (0.54-2.42)
rs16847737	199337023	*CACNA1S*	G/C	44	19	1	0.164	49	7	0	0.063	C	1.58E-02	3.18 (1.23-8.25)
rs12561765	199340314	*CACNA1S*	C/T	19	35	10	0.430	14	30	12	0.482	C	4.38E-01	1.47 (0.58-3.73)
rs998135	199342419	*CACNA1S*	T/C	3	25	36	0.242	4	22	30	0.268	C	6.59E-01	1.56 (0.33-7.31)
rs1325313	199342790	*CACNA1S*	C/T	5	17	42	0.211	2	22	32	0.232	T	3.54E-01	1.44 (0.68-3.00)
rs12132807	199343137	*CACNA1S*	T/C	51	13	0	0.102	43	13	0	0.116	T	8.25E-01	1.18 (0.50-2.83)
rs1325310	199345858	*CACNA1S*	C/T	11	27	24	0.395	9	29	18	0.420	T	5.64E-01	1.34 (0.62-2.85)
rs1546416	199276179	*CACNA1S*	A/G	38	26	0	0.203	34	19	3	0.223	A	9.87E-02	N/A
rs3850625	199282919	*CACNA1S*	G/A	58	6	0	0.047	48	8	0	0.071	G	5.70E-01	1.61 (0.52-4.97)
rs2297903	199297930	*CACNA1S*	A/C	2	31	31	0.273	1	21	34	0.205	A	2.02E-01	1.63 (0.80-3.40)
rs16847613	199300343	*CACNA1S*	G/A	43	18	3	0.187	36	18	2	0.196	G	8.47E-01	1.14 (0.53-2.42)

SNP_ID	Position	Gene	Allele	Case				Control				Risk allele	*P*-value (Fisher's exact test)	OD (95%CI)
				Genotype				Genotype						
				11	12	22	MAF	11	12	22	MAF			
rs2297901	199302230	*CACNA1S*	G/T	34	21	9	0.305	29	23	4	0.277	T	2.55E-01	2.13 (0.62-7.33)
rs3753960	199325707	*CACNA1S*	A/G	29	25	10	0.352	21	25	10	0.401	A	4.59E-01	1.38 (0.66-2.87)
rs10449267	199338413	*CACNA1S*	A/G	6	33	25	0.352	4	29	23	0.330	A	7.49E-01	1.34 (0.36-5.03)
rs10920115	199338507	*CACNA1S*	T/G	9	30	25	0.375	7	25	24	0.348	T	6.88E-01	1.17 (0.56-2.43)
rs12135240	199340178	*CACNA1S*	T/C	47	14	3	0.156	45	9	2	0.116	C	3.96E-01	1.50 (0.63-3.50)
rs2365293	199341868	*CACNA1S*	T/C	5	40	19	0.391	6	30	20	0.375	T	5.59E-01	1.32 (0.61-2.83)
rs6892117	151245178	*GLRA1*	A/G	27	28	9	0.359	22	28	5	0.345	G	5.70E-01	1.60 (0.51-5.21)
rs1549622	151246242	*GLRA1*	T/C	28	29	7	0.336	21	26	9	0.393	T	4.20E-01	1.56 (0.54-4.50)
rs890832	151250523	*GLRA1*	G/A	0	14	50	0.109	1	5	50	0.062	G	1.41E-01	2.33 (0.83-6.56)
rs1465555	151269891	*GLRA1*	T/C	5	29	30	0.305	9	25	22	0.384	C	2.21E-01	2.26 (0.71-7.20)
rs7709656	151270918	*GLRA1*	G/A	49	13	2	0.133	50	5	1	0.062	A	8.54E-02	2.58 (0.91-7.11)
rs2071221	151284499	*GLRA1*	G/A	14	33	17	0.477	14	25	17	0.473	G	6.88E-01	1.21 (0.54-2.67)
rs186217	151306772	*GLRA1*	G/T	25	33	6	0.352	18	27	11	0.437	G	1.23E-01	2.36 (0.81-6.88)

SNP_ID	Position	Gene	Allele	Case				Control				Risk allele	*P*-value (Fisher's exact test)	OD (95%CI)
				Genotype				Genotype						
				11	12	22	MAF	11	12	22	MAF			
rs12667032	154037514	*DPP6*	G/A	49	9	3	0.123	46	8	1	0.091	A	5.26E-01	2.79 (0.28-27.67)
rs937009	154032471	*DPP6*	T/C	10	23	31	0.336	3	25	28	0.277	T	8.41E-02	3.27 (0.85-12.55)
rs1488927	154033602	*DPP6*	C/G	10	23	30	0.341	3	21	32	0.241	C	8.19E-02	3.33 (0.87-12.80)
rs1488926	154033792	*DPP6*	C/T	10	23	31	0.336	3	25	28	0.277	C	8.41E-02	3.27 (0.85-12.55)
rs6960383	154012209	*DPP6*	G/A	4	19	41	0.211	1	9	46	0.098	G	2.08E-02	2.55 (1.10-6.06)
rs10267037	154020828	*DPP6*	G/A	8	23	33	0.305	2	17	37	0.187	G	5.16E-02	3.86 (0.78-18.99)
rs6975262	154034857	*DPP6*	A/C	10	23	31	0.336	3	25	28	0.277	A	8.41E-02	3.27 (0.85-12.55)
rs1080445	154035665	*DPP6*	G/A	52	11	1	0.102	43	13	0	0.116	G	6.54E-01	1.31 (0.54-3.17)
rs749120	154038740	*DPP6*	G/T	11	28	25	0.391	3	27	26	0.295	G	5.07E-02	3.67 (0.97-13.89)

CI confidence interval
From NCBI Genome build36.3
N/A Not Available

Supplementary table 2 Summary results for the 55 SNPs of *GABRA3* gene for the fine mapping

No.	dbSNP ID	Position[a]	Alleles	Case			Control			Risk allele	*P*-value (Fisher's exact test)	OR (95%-CI)
				Genotype			Genotype					
				1	2	MAF	1	2	MAF			
1	rs11796848	151167374	C/A	69	12	0.148	71	4	0.053	A	6.53E-02	3.09 (0.95-10.04)
2	rs5925155	151208574	G/A	67	14	0.173	63	10	0.133	A	6.58E-01	1.32 (0.55-3.17)
3	rs2194897	151163760	C/G	47	34	0.420	53	22	0.293	G	1.33E-01	1.74 (0.90-3.39)
4	rs6526084	151145136	A/G	57	23	0.288	59	16	0.213	G	3.55E-01	1.49 (0.71-3.10)
5	rs5970229	151139648	A/T	62	19	0.235	58	17	0.227	N/A	1.00E+00	N/A
6	rs6627549	151121380	A/G	74	7	0.086	56	17	0.227	A	1.47E-02	3.21 (1.25-8.27)
7	rs12688452	151268341	G/A	74	7	0.086	56	17	0.227	G	1.47E-02	3.21 (1.25-8.27)
8	**rs5970264**	151218561	C/G	60	20	0.250	70	3	0.041	G	4.49E-04	7.78 (2.20-27.46)
9	rs12007663	151165599	C/G	58	23	0.284	55	20	0.267	G	8.59E-01	1.09 (0.54-2.20)
10	rs6627550	151132182	A/G	67	14	0.173	55	20	0.267	A	1.78E-01	1.74 (0.81-3.76)
11	rs3947525	151124506	C/G	45	35	0.438	39	35	0.473	C	7.46E-01	1.15 (0.61-2.18)
12	**rs750841**[b]	151216374	T/A	61	20	0.247	72	3	0.040	A	2.23E-04	7.87 (2.23-27.76)
13	**rs12688628**[b]	151216451	T/G	61	20	0.247	72	3	0.040	G	2.23E-04	7.87 (2.23-27.76)
14	SNP1[b]	151370420	C/A	47	34	0.420	42	31	0.425	N/A	1.00E+00	N/A
15	**rs11094568**[c]	151207908	A/T	61	20	0.247	72	3	0.040	T	2.23E-04	7.87 (2.23-27.76)
16	**rs875478**[c]	151215734	T/G	61	20	0.247	72	3	0.040	G	2.23E-04	7.87 (2.23-27.76)
17	**rs5970265**[c]	151218653	A/G	61	20	0.247	72	3	0.040	G	2.23E-04	7.87 (2.23-27.76)
18	**rs5969888**[c]	151219413	G/A	61	20	0.247	72	3	0.040	A	2.23E-04	7.87 (2.23-27.76)
19	**rs6627574**[c]	151221892	C/T	61	20	0.247	72	3	0.040	T	2.23E-04	7.87 (2.23-27.76)
20	**rs12396220**[c]	151224227	T/C	61	20	0.247	72	3	0.040	C	2.23E-04	7.87 (2.23-27.76)
21	**rs12013373**[c]	151224400	A/G	61	20	0.247	71	3	0.041	G	4.56E-04	7.76 (2.20-27.38)
22	**rs5970267**[c]	151228525	G/A	61	20	0.247	72	3	0.040	A	2.23E-04	7.87 (2.23-27.76)

No.	dbSNP ID	Position[a]	Alleles	Case			Control			Risk allele	*P*-value (Fisher's exact test)	OR (95% CI)
				Genotype			Genotype					
				1	2	MAF	1	2	MAF			
23	**rs5970268**[c]	151230888	G/A	61	20	0.247	72	3	0.040	A	2.23E-04	7.87 (2.23-27.76)
24	**rs11795573**[c]	151231829	A/G	61	20	0.247	72	3	0.040	G	2.23E-04	7.87 (2.23-27.76)
25	**rs5970269**[c]	151233399	G/A	61	19	0.238	72	3	0.040	A	4.02E-04	7.48 (2.11-26.47)
26	**rs1565610**[c]	151240061	G/A	61	20	0.247	72	3	0.040	A	2.23E-04	7.87 (2.23-27.76)
27	**rs2201169**[c]	151240971	A/G	61	20	0.247	72	3	0.040	G	2.23E-04	7.87 (2.23-27.76)
28	**rs11094571**[c]	151243074	T/C	61	19	0.238	72	3	0.040	C	4.02E-04	7.48 (2.11-26.47)
29	**rs1492293**[c]	151243408	A/G	61	20	0.247	72	3	0.040	G	2.23E-04	7.87 (2.23-27.76)
30	**rs6526099**[c]	151250448	A/T	61	20	0.247	72	3	0.040	T	2.23E-04	7.87 (2.23-27.76)
31	**rs6653477**[c]	151254381	A/C	61	20	0.247	72	3	0.040	C	2.23E-04	7.87 (2.23-27.76)
32	**rs6653443**[c]	151259417	A/C	61	20	0.247	72	3	0.040	C	2.23E-04	7.87 (2.23-27.76)
33	**rs5970281**[c]	151263963	A/G	61	20	0.247	72	3	0.040	G	2.23E-04	7.87 (2.23-27.76)
34	**rs5970283**[c]	151265291	C/T	61	20	0.247	72	3	0.040	T	2.23E-04	7.87 (2.23-27.76)
35	**rs2201171**[c]	151273232	G/T	61	20	0.247	72	3	0.040	T	2.23E-04	7.87 (2.23-27.76)
36	**rs6526102**[c]	151274256	A/G	61	20	0.247	72	3	0.040	G	2.23E-04	7.87 (2.23-27.76)
37	rs389292	151292110	C/T	58	23	0.284	60	14	0.189	T	1.90E-01	1.70 (0.80-3.62)
38	rs5970294	151320857	C/T	62	19	0.235	62	13	0.173	T	4.28E-01	1.46 (0.66-3.22)
39	rs6526107	151333472	C/T	35	46	0.432	35	40	0.467	T	7.48E-01	1.15 (0.61-2.16)
40	rs3902802	151347857	A/G	73	8	0.099	66	9	0.120	A	7.99E-01	1.24 (0.45-3.41)
41	rs6526109	151350041	C/A	8	72	0.100	8	66	0.108	A	1.00E+00	1.09 (0.39-3.08)
42	rs6526110	151353914	A/G	40	41	0.494	31	44	0.413	A	3.38E-01	1.38 (0.74-2.61)
43	rs10482215	151362282	A/T	52	29	0.358	51	24	0.320	T	7.35E-01	1.19 (0.61-2.30)
44	rs6526113	151367997	A/G	53	28	0.346	52	22	0.297	G	6.06E-01	1.25 (0.63-2.46)
45	rs5970309	151373571	T/G	47	33	0.413	45	30	0.400	G	1.00E+00	1.05 (0.55-2.00)
46	rs6627597	151382065	A/G	53	28	0.346	51	24	0.320	G	8.65E-01	1.12 (0.58-2.19)
47	rs6627600	151399613	T/A	54	27	0.333	51	23	0.311	A	8.64E-01	1.11 (0.56-2.18)
48	rs5969898	151405698	A/G	22	59	0.272	28	47	0.373	G	2.29E-01	1.60 (0.81-3.15)

No.	dbSNP ID	Position[a]	Alleles	Case			Control			Risk allele	*P*-value (Fisher's exact test)	OR (95% CI)
				Genotype			Genotype					
				1	2	MAF	1	2	MAF			
49	rs12008828	151407087	C/T	76	5	0.062	70	5	0.067	C	1.00E+00	1.09 (0.30-3.91)
50	rs12353750	151414854	C/T	80	1	0.012	74	1	0.013	N/A	1.00E+00	N/A
51	rs4828697	151417383	C/T	37	44	0.457	29	46	0.387	C	4.19E-01	1.33 (0.70-2.52)
52	rs17326876	151423704	G/T	57	24	0.296	55	19	0.257	T	5.96E-01	1.22 (0.60-2.47)
53	rs2142466	151429146	G/A	18	63	0.222	19	55	0.257	A	7.07E-01	1.21 (0.58-2.53)
54	rs4379587	151446467	A/G	33	47	0.413	26	49	0.347	A	4.13E-01	1.32 (0.69-2.54)
55	rs4385609	151461027	C/T	68	13	0.160	61	14	0.187	C	6.78E-01	1.20 (0.52-2.75)

The significantly association study is shown in bold
CI confidence interval
N/A Not Available
[a] From NCBI Genome build 36.3_NT 011726.13 reference
[b] Included SNP in this study that shown significance in previous study
[c] Additional captured SNP by the rs5970264

Discussion

This study confirms the association of THPP and genetic variants closed to intron 3 of *GABRA3*. We recruited more subjects than our previous report, which provided greater power for statistical analyses. We demonstrated significant association of additional 25 htSNPs located in intron3 of *GABRA3* with THPP; however we could not demonstrate any mutation in the exon2, 3 and 4 of the gene.

On the basis of similar clinical features between FHPP and THPP, *CACNA1S* and *SCN4A;* which were reported as the genetic defects in FHPP, were selected for association screening in our study[12-14]. Consistent with the previous negative results [7-8, 15-16], we did not find any association between the htSNPs of *CACNA1S* and *SCN4A* and THPP. Candidate gene association analysis has been use to find the genetic susceptibility of multifactorial diseases. Mostly the selected candidate genes derive from the possible underlying pathogenesis. However this approach might not be applied in many situations of multifactorial diseases. As observed in the clinical manifestation, episodic paralysis in THPP occurs only when the patients are in thyrotoxic state, and it disappears once the euthyroidism restores. This symptom is quite different from episodic weakness in FHPP, which it occurs since childhood and cannot be cured. The difference in this major clinical feature and our negative genetic study results indicated the different underlying genetic susceptibility between FHPP and THPP.

Genome wide association approach using haplotype tagging SNPs has been recommended for complex diseases genetic analysis. In the previous study[9], we utilized DNA pooling genotyped on Affymetrix 10K GeneChip microarray to elucidate genetic determinants of THPP, as it is a cost effective approach in the genome-wide screening with valid results. Total of 10204 SNPs were designed on the Affymetrix GeneChip 10K 2.0 microarrays. The median intermarker distance was 113 Mb which provided sparse SNPs interval. We reported rs750841 and rs12688628 as the two most statistically significant SNP associated with THPP located in the intronic region of *GABRA3* gene on chromosome X. In this study fine-mapping for the most significant genetic variant associated to THPP was conducted by selecting more htSNPs cover *GABRA3* whole gene. Total 55 SNPs were selected and for high throughput genotyping, multiplex PCR with the Invader assay was used. The most significantly associated htSNPs were located in the intron 3 between position 151216374 and 151274256. According to the direct sequencing study, there was no mutation found in the exons nearby in THPP cases.

The *GABRA3* gene; encoding GABA receptor α3 subunit, is located on chromosome X (Xq28) at location 151,087,188-151,370,486 bp (Build 36.3). According to its location on X chromosome, the association of *GABRA3* and THPP may explain the predominant penetrance in male gender of THPP. How *GABRA3* gene involved in THPP is not clear, as the functional study of these genetic variants in THPP has not yet been studied. The GABA action has been proposed as an important excitatory system in the development of brain to promote the survival and differentiation of neuron [17]. Puia G and Losi G[18] demonstrated that T3 and T4 selectively affect GABAergic phasic and tonic neurotransmission in hippocampus. However the role of GABA on skeletal muscle and the interaction between potassium levels and GABA action need further study. As there was no mutation detected in the exon, and the most significant SNPs located in the intron 3. These SNPs may play the role of the intron-mediated regulation of gene expression[19]. It has been observed that the introns involved in intron-mediated regulation must be within transcribed sequences near the start of a gene. Interestingly, the associated SNPs we found they located in the intron 3, near the regulatory part of the gene. Another possibility is that these SNPs may control transcription of other genes as a role of long-range control of gene expression.

In this study we also selected additional 2 genes (*DPP6* and *GLRA1)* from the 67 SNPs that were found susceptible to THPP (p value < 0.01) from prior report for the genetic association screening. There was no difference in allelic and genotypic frequencies of the htSNPs of *DPP6* and *GLRA1* comparing between cases and controls. Our negative findings indicate the limitation of DNA pooling technique and small sample size in the previous report. Even the analyses of DNA pools have proved accurate using a variety of different genotyping technologies and the statistical power is approximately the same[20], regenotyping in the second cohort should be done to validate the results.

Conclusions

Our fine-mapping study confirmed the association between genetic variants located in intron 3 of *GABRA3* and THPP. The molecular mechanism of how these variants affect the gene needs to be explored.

Acknowledgements

This work was supported by the DMSc-RIKEN collaboration and the Thailand Research Fund. Additional support was provided by the Strategic Scholarships for Frontier Research Network for Ph.D. Programs from the Commission on Higher Education, Thailand. Finally we would like to thank all the subjects who participated in this study.

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A genome-wide association study identifies novel susceptibility loci for thyrotoxic hypokalemic periodic paralysis.

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Key Words: Genome-wide association study, Hyperthyroidism, Hypokalemic Periodic Paralysis, KCNJ2

Word Count: Text: 4046**, Abstract:** 215**, Tables:** 2**, Figures:** 4, Supplementary tables: 2

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Abstract

Several lines of evidence have pointed out that genetic components play roles in thyrotoxic hypokalemic periodic paralysis (THPP). From previous studies, genetic heterogeneity has been documented in different ethnicity of THPP. In this study, for the first time we performed genome-wide association study (GWAS) to find the susceptible loci to THPP in Thai male patients with Graves' disease. We genotyped 78 THPP cases and 74 controls with Illumina Human-Hap610 Genotyping BeadChip. Among the SNPs analyzed in the GWAS, rs312729 at chromosome 17 revealed the lowest P-value for association ($P = 2.09 \times 10^{-7}$). Fine mapping for linkage disequilibrium blocks surrounding the landmark SNP revealed that rs623011; located at about 75-kb downstream of *KCNJ2* gene, overcame the GWAS significance even after adjustment for multiple comparison, using a conservative Bonferroni correction for the 508,393 tests. ($P = 3.23 \times 10^{-8}$, odds ratio [OR] = 6.72; 95% confidence interval [CI] = 3.11-14.5). The association of rs623011 was replicated both in an independent set of samples consisting of 28 THPP patients and 48 controls ($P = 3.44 \times 10^{-5}$, OR = 5.13; 95% CI= 1.87-14.1; combined-analysis $P = 3.71 \times 10^{-12}$, OR = 5.46; 95% CI= 3.04-9.83). Our results suggest that the common genetic variation of *KCNJ2* may influence susceptibility to THPP in Thai male patients with Graves' disease.

Introduction

Thyrotoxic hypokalemic periodic paralysis (THPP) is a subtype of periodic paralysis disorder. It is a rare complication of thyrotoxicosis characterized by episodes of intracellular shift of potassium ions and muscle weakness. The hallmark clinical features of THPP are similar to familial form of hypokalemic periodic paralysis (FHPP). The muscle weakness involves mainly proximal part of extremities, and rarely respiratory and oropharyngeal muscles. To distinguish from FHPP, THPP occur during thyrotoxic stage only. Notably, THPP is prevalent in male Asians, occasionally reported in American Indians and Hispanics, and rarely found in Caucasians, and Africans. The ratio between male and female is 17:1 to 70:1[1-3], and the peak incidence occurs in young adult 20–39 yr of age[1,4]. Various etiologies of thyrotoxicosis are reported to be related to THPP, e.g. Graves' disease, thyrotropin producing pituitary adenoma, toxic adenoma, and exogenous thyroid hormone abuse. Mostly, the clinical signs and symptoms of thyrotoxicosis in THPP are subtle. Potassium supplement and propranolol [5-8] can reverse and prevent recurrent muscle weakness. Especially, this condition completely resolved once euthyroidism is restored.

As this condition affects primarily males and people of Asian descent, underlying genetic susceptibility and high thyroid hormone levels may play roles in the pathogenesis. Several candidate genes related to the hallmark clinical features have been examined in THPP patients. The specific human leukocyte antigen (HLA) was accessed and found to be present at a higher prevalence among THPP cases for instance B46, DR9, and DQB1*0303 in Hong Kong, Chinese THPP patients, HLA-A2, Bw22, AW19, B17, and DRW8 in Singapore Chinese and Japanese[9-12] . It is uncertain

whether these genes are independently related to THPP, as these HLA associations are also observed with Graves' disease. Different genes related to different biological pathways of THPP have been examined. Na^+/K^+-ATPase pump activity are found to be increased in THPP patients than hyperthyroidism alone. There are several lines of evidence that enhancing the pump activity by high carbohydrate, alcohol consumption, strenuous exercise, insulin therapy, and glucocorticoid administration may precipitate hypokalemia in THPP. As a results, the genes coding for α_1-, α_2-, β_1-, β_2-, and β_4-subunits of Na^+/K^+-ATPase were examined in southern Chinese THPP patients, but no mutation was identified in the promoter and coding sequences[13]. Because of the benefit of beta blocker for hypokalemia prevention, polymorphisms in the β_2-adrenergic receptor gene were tested in Korean THPP patients; however, the authors could not demonstrate any mutations[14]. The overlapping clinical features between thyrotoxic and familial form of hypokalemic periodic paralysis suggested that a likely genetic basis for FHPP may contribute to THPP. Various mutations in ion channel genes reported in FHPP i.e. *CACNA1S, SCN4A, KCNE3*, and *KCNJ2* have been screened in THPP patients. However, the results of these studies have rarely been confirmed and the functional significance of all of the aforementioned genetic variants is unclear.

Several lines of evidence have pointed out that genetic component play roles in THPP predisposition. However, THPP is not a Mendelian inheritance and genetic heterogeneity has been documented. The failure of genetic association analyses using selected candidate genes involving the biological pathway is mostly due to the narrow range of target genes, as the basic pathogenesis of THPP is still not well understood. Recently, genome-wide association studies (GWAS) have been proven to be a powerful

tool to identify susceptibility genes for common diseases, which are likely to enable us to identify as-yet-unknown genes[19]. In this study, we attempted the first GWAS to identify susceptibility gene(s) for THPP and here we described genetic loci near *KCNJ2* that was associated with THPP in Thais.

Materials and methods

Subjects

All the subjects that participated in this SNP association study were unrelated Thai male patients with graves' diseases from 4 regions in Thailand; The Northern, The Northeastern, The central, and The Southern part. We recruited thyrotoxic Thai patients who had intracellular shift of potassium as evidenced by hypokalemia (K^+<3.5 mEq/L) with low urine potassium excretion. Controls were recruited from hyperthyroid male patients without history of hypokalemia during their hyperthyroid states. We excluded subjects who had conditions predisposing to hypokalemia for examples; alcoholism, renal tubular acidosis, and diuretics use. For an initial screening, a total of 78 THPP patients and 74 control subjects (table 1) were recruited from 3 hospitals in Bangkok (Ramathibodi, King Chulalongkorn, and Theptarin Hospital). For a replication study, a second set of samples consisting of 28 THPP patients and 48 controls (table 1) were later recruited from 5 Hospitals Ramathibodi, Maharat Nakhonratchasima, Hospital, Prince of Songkla, Sappasitprasong , and Ratchaburi Hospital. The mean age of cases and controls were 40.5±2.0 years (range 22-64) and 44.3±2.1 years (range 15-77), respectively. All participants gave their written informed consent. The study was reviewed and approved by the Ethics Committee of Ramathibodi hospital, Mahidol University, and The Institutes of Physical and Chemical Research (RIKEN), Yokohama, Japan.

Genotyping and case-control association studies

A genome-wide analysis for 78 cases and 74 controls was conducted using Illumina Human-Hap610 Genotyping BeadChip according to the manufacturer's

protocols (San Diego, CA). A principal component analysis (PCA) was performed via an 'Eigen analysis' in the computer program smartpca, from the EIGENSOFT package [20]. Genotype data for the cases and controls and general population subjects for 89 East-Asian individuals (44 Japanese and 45 Han Chinese) from the International HapMap project[21] were analyzed for the PCA. PCA plots were obtained using the first two components (Eigenvectors 1 and 2). To validate the genotyping results, we performed genotyping by means of multiplex PCR-based Invader assays (Third Wave Technologies, Madison, WI)[22] and compared the data obtained by the two platforms.

To further analyze SNPs within the linkage disequilibrium (LD) regions including the landmark SNP (rs312729), 26 tag-SNPs (squared correlation coefficient between the two SNPs (r^2) > 0.8, minor allele frequency (MAF) > 0.05) were selected from International HapMap project database (http://www.hapmap.org/index.html.en) and genotyped on 77 cases and 72 controls by multiplex-PCR-based invader assay (Applied Biosystems). To draw an LD map, we applied Haploview software[23].

Statistical analyses

For association studies, the allele and genotype distributions in cases and controls were compared and evaluated in allelic, dominant- and recessive-inheritance models by two-tail Fisher's exact test. In the GWAS, SNPs were sorted according to the lowest P-value in one of these models. Significance levels were 9.83×10^{-8} (0.05/508,393) and 0.05 in the GWAS and the replication study, respectively.

Results

Case-control association studies

We first genotyped 78 THPP cases and 74 controls with Illumina Human-Hap610 Genotyping BeadChip. After excluding one case and two controls which were judged to be outliers in the PCA (figure 1), we applied SNP quality control (call rate of ≥0.99 in both cases and controls). Of 600,420 SNPs genotyped, 498,465 autosomal SNPs and 9,928 SNPs of X chromosome passed the quality control and were further analyzed. The genomic control inflation factor (λGC) was 1.085, indicating a low possibility of false-positive associations resulting from population stratification (figure 2). Among the SNPs analyzed in the GWAS, although no SNPs showed significant association with THPP after the correction of multiple testing, rs312729 at chromosome 17 revealed the lowest P-value for association ($P = 2.09 \times 10^{-7}$, figure 3, supplementary table 1).

To validate the genotyping results of the Illumina assay, we re-genotyped all subjects in the first stage with top 20 SNPs showing the smaller P-values in the GWAS by multiplex-PCR based Invader assay. We compared the genotype and allele frequencies for each of the 20 SNPs obtained from the two assays. The allele and genotype frequencies were comparable to those from Illumina assay (data not shown), indicating the reliability of these two genotyping platform.

Fine mapping

To further define a genomic region of interest, we genotyped 26 tag-SNPs and 31 SNPs captured by the tag-SNPs showing the minimal P-value < 0.01 in two LD blocks surrounding the landmark SNP (rs312729) identified from the GWAS

(supplementary table 1). We found that only rs623011 showed P-values which overcame the GWAS significance ($P = 3.23 \times 10^{-8}$, odds ratio [OR] = 6.72; 95% confidence interval [CI] = 3.11-14.5) even after adjustment for multiple comparison, using a conservative Bonferroni correction for the 508,393 tests (table 2, supplementary table 2). The rs623011 is located at about 75-kb downstream of *KCNJ2* gene (figure 4).

Replication study

After the fine mapping, we further evaluated the rs623011 in an independent set of samples consisting of 28 THPP patients and 48 controls by PCR-based Invader assay. The association of rs623011 was replicated ($P = 3.44 \times 10^{-5}$, OR = 5.13; 95% CI= 1.87-14.1; combined-analysis $P = 3.71 \times 10^{-12}$, OR = 5.47; 95% CI = 3.04-9.83) (table 2).

Discussion

This is the first GWAS for genetic susceptibility to THPP. We identified rs623011 as the most susceptible SNP to THPP. A combined result of the GWAS and the replication study strongly suggested the association with the P-value of 3.71×10^{-12}.

THPP was first described in Japan in 1957[24]; however, the pathogenesis of THPP has been unresolved. It is not a common manifestation in hyperthyroidism and is prevalent in young male Asians. Clinical signs of hypokalemia and episodic weakness and precipitating factors are similar to those found in FHPP. The previous studies showed massive intracellular shift of potassium ion and phosphate during development of weakness[25]. There are a number of evidence pointed out the role of ion channel defect in THPP for instance; increased skeletal muscle resting membrane potential to -78 mV in resting state, decreased membrane potential to -50 mV in depolarization phase during attack [26], and higher Na^+-K^+ ATPase pump activity[27-30] compared to hyperthyroid subjects without hypokalemia and healthy subjects.

However the association between mutation in ion channel genes causing FHPP and THPP were inconclusive. In 2002, Dias da Silva et al [31] reported R83H transition in *KCNE3* in 1 out of 15 Portuguese THPP cases, which was the same loci that discovered in FHPP reported by Abbott et al[32]. *KCNE3* encodes a regulatory peptide of voltage-gated potassium channel, MiRP2. In 2003 Sternberg et al[33] reported lack of association of the R83H variant of the MiRP2 with periodic paralysis. They found the same genetic variant in healthy subjects without hypokalemia, and the allele frequencies were not different from periodic paralysis cases. Their observations weakened the association between MiRP2-R83H and THPP. Lane et al[15] identified

R672S transition in *SCN4A* which encodes skeletal muscle voltage-gated sodium channel in a French family. The proband had both features of familial form of hypokalemic periodic paralysis and thyrotoxicosis, but his brother had paralysis without thyrotoxicosis. This was the first time FHPP and THPP reported in the same family. The weakness features in the proband could be resulted from the R672S mutation in *SCN4A* which segregated in the family or from thyrotoxicosis, as the authors did not describe the clinical features of the proband after thyrotoxicosis resolved.

Recently Ryan et al[18] described mutations in *KCNJ18*, a gene encoding the inwardly rectifying potassium (Kir) channel, Kir2.6. Candidate gene approach with a solid rationale was utilized by screening all genes encoding potassium channels expressed in skeletal muscle and containing thyroid hormone response elements (TREs) in its promoter region. With their approach, new potassium channel gene; *KCNJ18*, on chromosome 17 which shared 99% identity in their coding sequence with *KCNJ12* were identified. The authors sequenced the gene in 140 subjects from different ethnic groups. They reported six mutations in *KCNJ18* that resulted in five missense mutations (R205H, T354M, K366R, R399X, and Q407X) and one frameshift mutation, leading to a premature stop codon (I144fs) in Kir2.6. Five out of 30 THPP patients from Brazil, France, and US had mutations in *KCNJ18*. None from Thailand, one of 83 individuals from Hong Kong, and 7 of 27 Singaporean cases had the mutations.

Notably, even the authors have utilized the most rationalized candidate gene approach; the mutations were demonstrated only in 33% of THPP cases. Mostly Caucasian patients (French, US, Brazil), rarely in Thai and Hong Kong subjects had the

mutation, which suggests that other THPP genes probably exist in Asians.

In our study we performed GWAS comparing THPP cases and the controls. In order to minimize the confounding factors, the controls in this study were selected from male hyperthyroid patients without any history of hypokalemic paralysis. The marker SNP rs623011, which showed the most significant association by fine mapping, was located on chromosome 17 at 75-Kb downstream to *KCNJ2*. We also sequenced *KCNJ18* in our cases of both cohorts, and no mutation was found (data not shown).

KCNJ2 encodes the Inward-rectifying K^+ channel Kir2.1, which is highly expressed in cardiac and skeletal muscle. How rs623011 contributes to THPP susceptibility is needed to be elucidated. From the HapMap data, the frequencies of disease allele A of SNP rs623011 were lower in HapMap-CEU comparing to HapMap-CHB and HapMapJPT (0.26 vs 0.48 respectively), and the frequency was greatest in our THPP cases (RAF=0.77 from combined population) This finding may support the epidemiologic finding of the higher THPP prevalence in Asians. As the SNP is located downstream to *KCNJ2*, we then performed direct sequencing of the *KCNJ2* in all THPP cases and found no mutation, which was concordant with the negative result reported previously[34]. Mutations in *KCNJ2* cause Andersen-Tawil syndrome (ATS; also known as Andersen syndrome) which is an autosomal dominant disease. ATS consists of cardiac arrhythmia, periodic paralysis, and dysmorphic face. Our hypothesis was that rs623011 may alter *KCNJ2* expression in hyperthyroid patients that resulted in periodic paralysis as found in ATS patients. Recently Lior Dassau et al[35] demonstrated Kir2.6 regulates the surface expression of Kir2.1, Kir2.2 inward rectifier potassium channels both in cell culture and animal model. Kir2 channels work by forming tetrameric assemblies of

Kir2.x subunits. By immunocytochemistry studies, the authors found that Kir2.1 and Kir2.2 are localized at the plasma membrane and T-tubules in rodent skeletal muscle, whereas Kir2.6 is largely retained in the endoplasmic reticulum (ER). They demonstrated that Kir2.6 functions as a dominant negative, in which incorporation of Kir2.6 as a subunit in a Kir2 channel heterotetramer reduces the abundance of Kir2 channels on the plasma membrane. From this finding, we may confer that alteration of either Kir2.6 or Kir2.1 may affect the potassium channel function in skeletal muscle,

Further functional analysis is needed to clarify the roles of rs623011 on function of the potassium channels. There are some possibilities. Firstly, sequences located near rs623011 may be long range regulator to *KCNJ2* expression. The regulatory elements could be located long distance between the regulators and the target genes or on the different chromosomes[36]. As shown in transgenic mice study, a particular enhancer "H" was identified at 75-kb upstream of an olfactory receptor gene cluster[37]. Secondly, rs623011 may alter the tetrameric formation between Kir2.6 and Kir2.1, which affects the channel function. Finally, a new potassium channel gene may locate in this region. As demonstrated by Ryan et al[18], some genes may be excluded from annotation, especially for those with the high degree of similarity, resulted in non-homologous end-joining of non-overlapping BACs during the assembly and alignment of human genome sequences.

In summary, our data suggest that the genetic variant rs623011 is a new candidate susceptible locus for THPP in Thai population. Additional studies on other ethnic populations will also provide detailed information on the genetic etiology and heterogeneity of THPP.

ACKNOWLEDGMENTS

The work described in this manuscript was supported by grants from the Thailand Research Fund, Bangkok, Thailand.

AUTHOR CONTRIBUTIONS

W.J. recruited subjects, jointly wrote the manuscript.

T.P. performed genotyping by means of multiplex PCR-based Invader assays.

S.M. genome-wide association analysis and jointly wrote the manuscript.

N.H., T.S., P.T., S.S., P.R., S.M., T.H., B.O. recruited subjects.

S.C. performed DNA extraction, measured DNA concentration.

M.K. conducted whole-genome genotyping using Illumina Human-Hap610 Genotyping BeadChip.

N.K., A.T. performed PCA and genome-wide association analysis.

T.M. Y.N. provided logistical and intellectual support and jointly wrote the manuscript.

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Table 1 Characteristics of subjects

	Platform	No. of samples	Age[b]
First stage			
Case	Illumina HumanHap610	78	40.7 ± 1.4
Control[a]	Illumina HumanHap610	74	42.6 ± 1.7
Replication stage			
Case	Invader assay	28	40.2 ± 2.7
Control[a]	Invader assay	48	46.0 ± 2.4

[a] The control groups were Thai, hyperthyroid patients without history of hypokalemia and weakness.

[b] Mean ± standard error

Table 2 Associations of rs623011 with THPP in Thai male patients with Graves' disease for GWAS and the replication study

	Allele (1/2)	Case				Control				P-value			Odds ratio [a]	95% confidence interval [a]
		11	12	22	Freq of risk allele (A)	11	12	22	Freq of risk allele (A)	1 vs 2	11 vs 12+22	11+12 vs 22		
First stage	A / G	43	28	4	0.760	12	40	20	0.444	3.23E-08	4.76E-07	2.51E-04	6.72	3.11-14.5
Replication		19	7	2	0.804	14	16	18	0.458	3.44E-05	1.65E-03	5.89E-03	5.13	1.87-14.1
Combined		62	35	6	0.772	26	56	38	0.450	3.71E-12	4.95E-09	6.05E-07	5.47	3.04-9.83

Freq, frequency [a] Odds ratios and confidence intervals were calculated using the non-risk genotype (12+22) as reference.

Figure 1 Principle component analysis of substructure in a diverse set of East Asian descent (HapMap JPT, CHB and Thai THPP cases and controls)

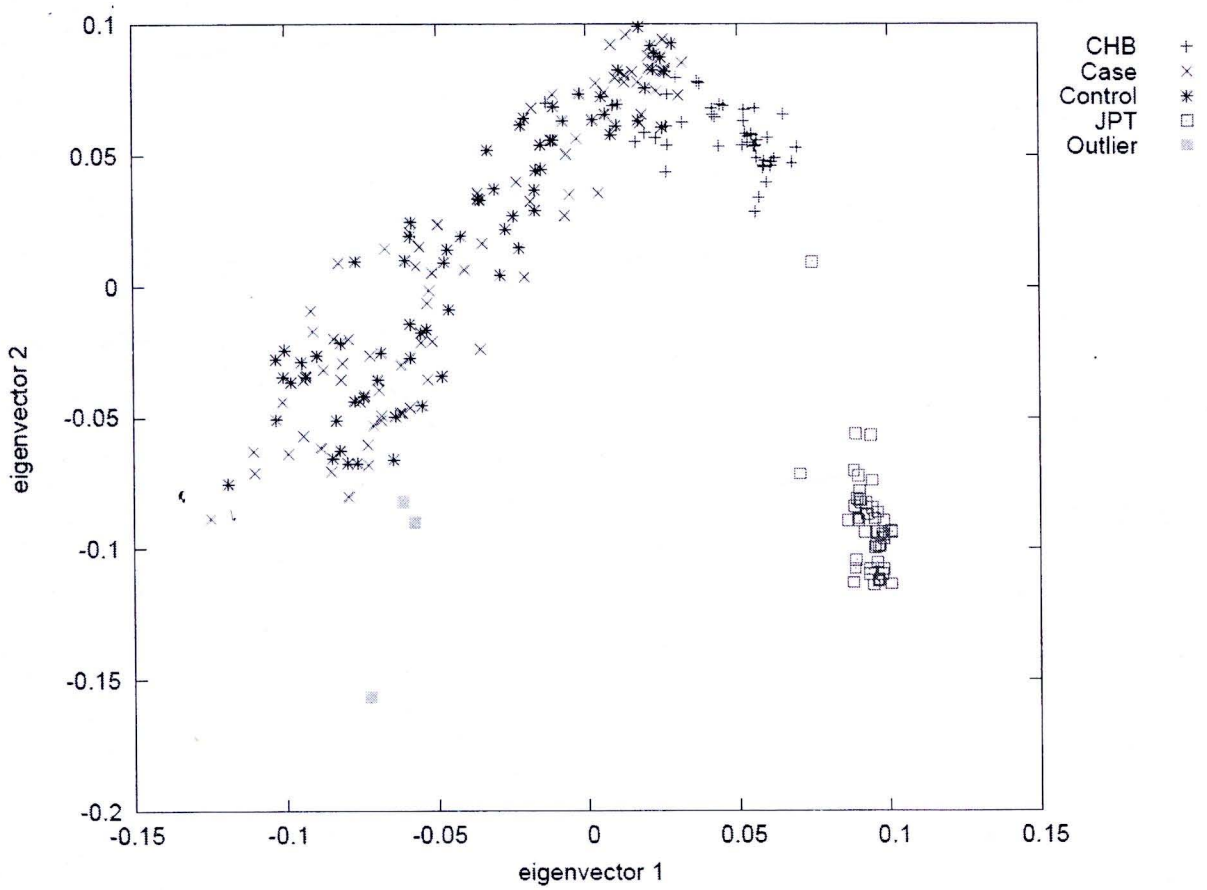


Figure 2 Log quantile-quantile P-value plot showing the distribution of observed statistics by allelic test for all utilized 498,465 SNPs from genome-wide association study of 77 THPP patients and 72 controls of Thai population. The diagonal line shows the values expected under the null hypothesis.

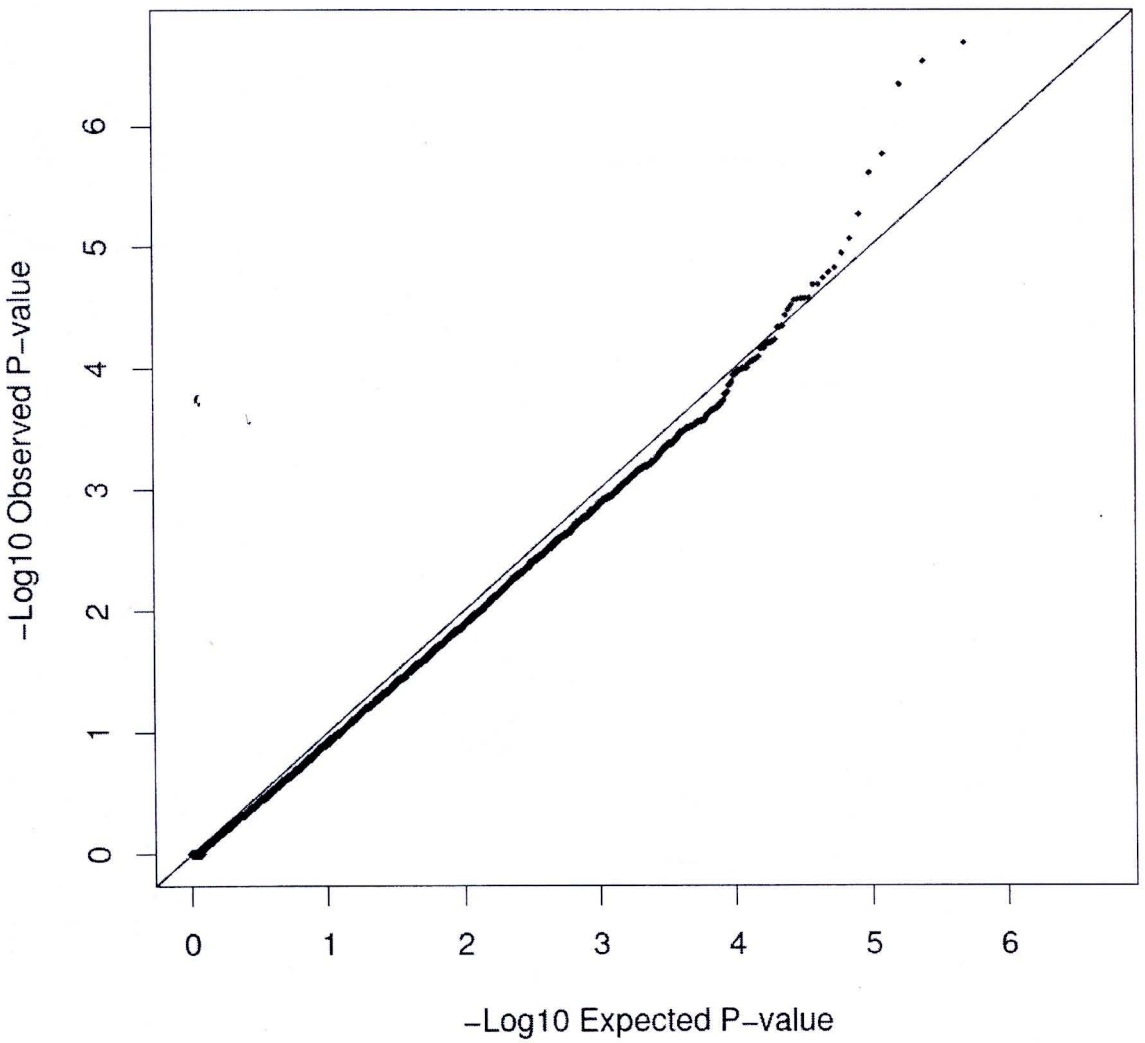


Figure 3 Log_{10} P-value plots from a genome-wide association study (GWAS). Each dot represents P-value obtained from the GWAS using 77 THPP cases and 72 controls in Thai male patients with Graves' disease. The Y axis represents the $-log_{10}$ of the minimal P-values calculated by Fisher's exact tests for three models: dominant, recessive and allele frequency model.

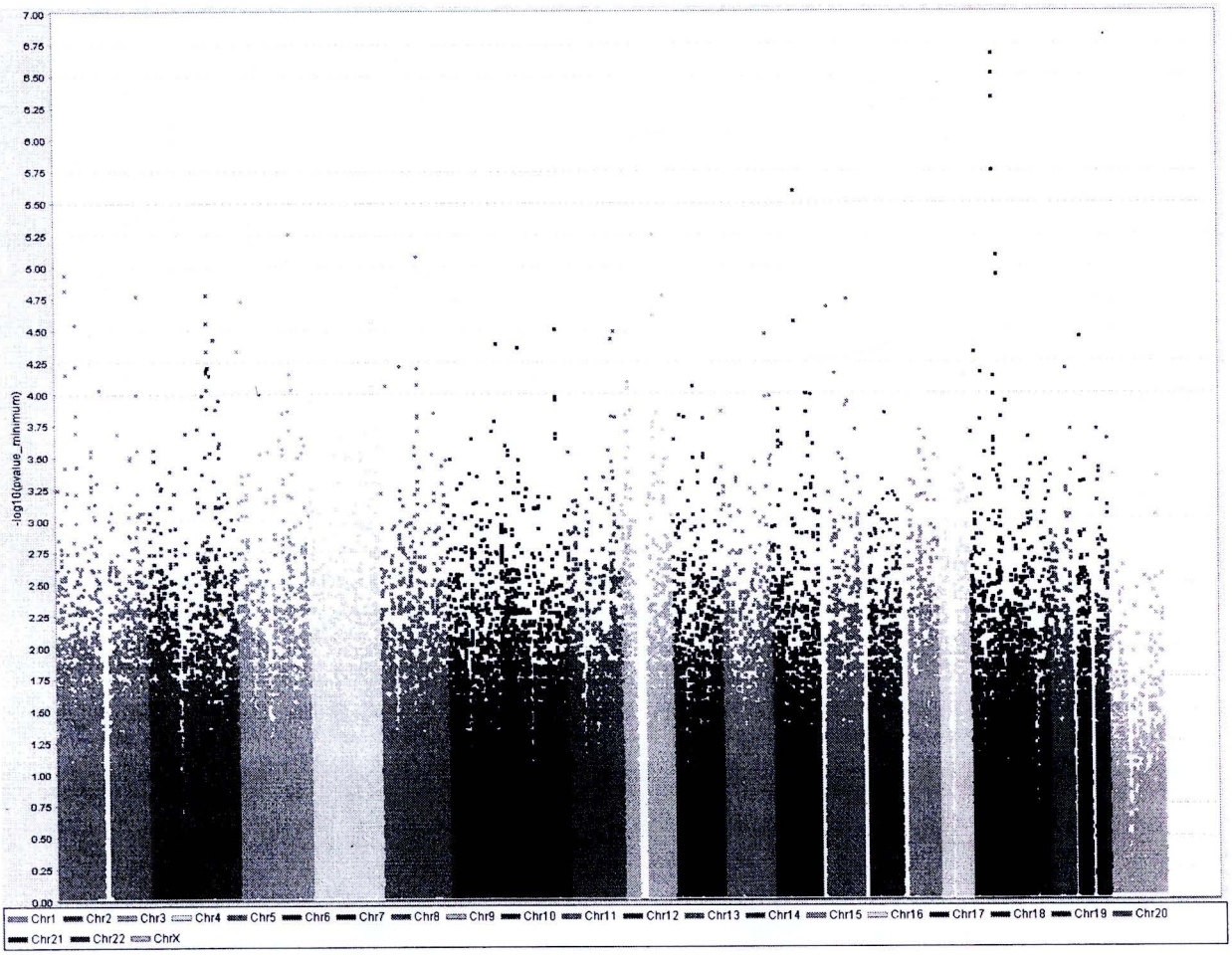
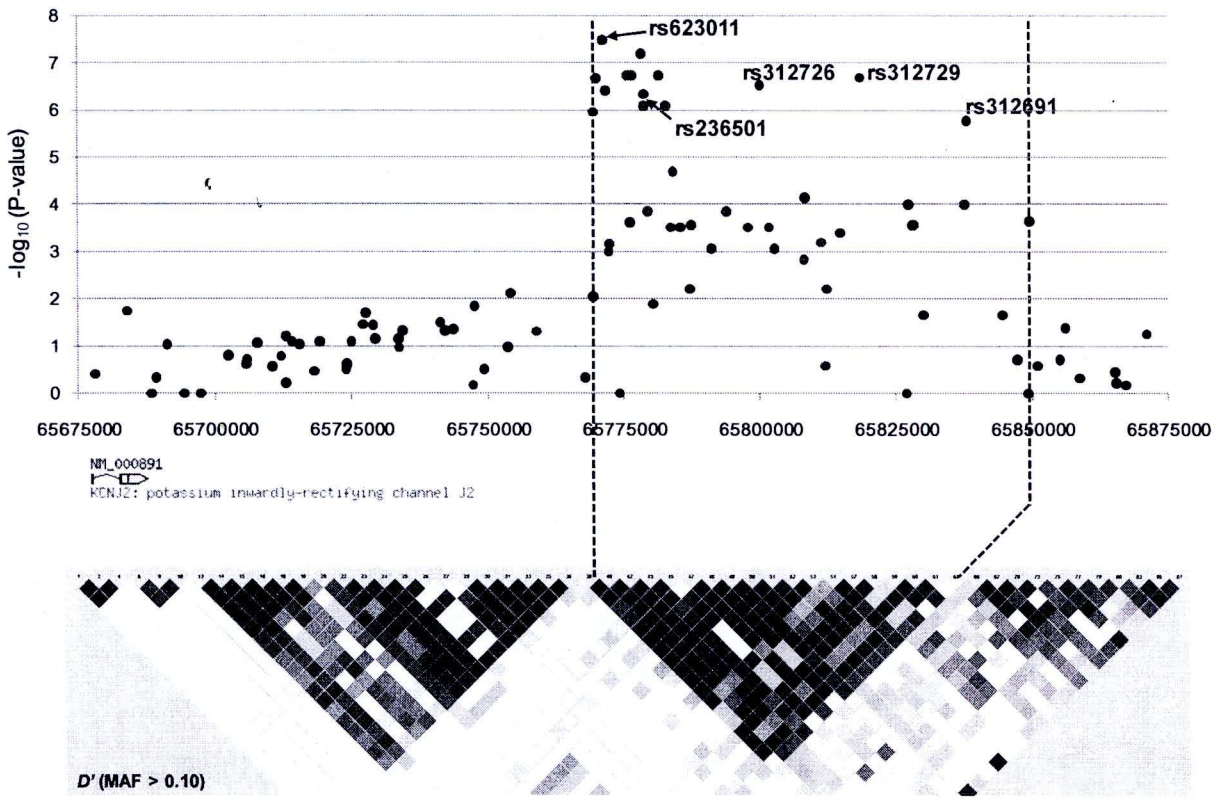


Figure 4 Log_{10} P-value plots, linkage disequilibrium (LD) map and genomic structure of the region in chromosome 17q24.3 near *KCNJ2*. Fine mapping was performed in the region from 65.65 to 65.85 Mb. Black and red dots represent $-log_{10}$P-value obtained from the GWAS and fine mapping using GWAS samples, respectively. Pairwise *D'* was based on the genotype data of the 77 THPP cases and 72 controls. An SNP rs623011 which is 75-kb downstream to *KCNJ2* shows the most significant association with THPP. MAF, minor allele frequency.



Supplementary table 1 Association of top 20 SNPs in genome-wide association study with THPP in Thai male patients with Graves' disease

SNP	Chr	Allele (1/2)	Case				Control				P-value		
			11	12	22	Freq of allele 1	11	12	22	Freq of allele 1	1 vs 2	11 vs 12+22	11+12 vs 22
rs312729	17	A / G	44	30	3	0.766	14	40	18	0.472	2.09E-07	2.29E-06	2.49E-04
rs312726	17	A / C	4	32	41	0.260	20	40	12	0.556	2.98E-07	2.30E-04	3.07E-06
rs236501	17	A / G	3	32	42	0.247	18	41	13	0.535	4.60E-07	2.49E-04	3.91E-06
rs312691	17	A / G	4	32	41	0.260	19	39	14	0.535	1.73E-06	4.56E-04	2.02E-05
rs12580112	12	A / G	39	32	6	0.714	16	32	24	0.444	2.47E-06	3.65E-04	1.60E-04
rs7647656	3	A / G	33	34	10	0.649	8	39	25	0.382	5.40E-06	1.67E-05	2.00E-03
rs10868609	9	A / G	16	28	33	0.390	27	38	7	0.639	1.80E-05	3.02E-02	5.45E-06
rs4969376	17	T / C	1	33	43	0.227	1	7	64	0.063	6.31E-05	1.00E+00	8.12E-06
rs386352	5	A / C	0	22	55	0.143	6	41	25	0.368	8.60E-06	1.14E-02	8.26E-06
rs12040932	1	T / C	2	23	52	0.175	14	31	27	0.410	1.13E-05	1.01E-03	2.98E-04
rs4969385	17	A / G	1	34	42	0.234	1	8	63	0.069	8.98E-05	1.00E+00	1.15E-05
rs4908382	1	T / C	2	25	50	0.188	16	29	27	0.424	1.49E-05	2.40E-04	1.02E-03
rs3754956	2	A / G	45	30	2	0.779	20	38	14	0.542	1.62E-05	2.50E-04	1.01E-03
rs12033057	1	A / C	11	26	40	0.312	16	43	13	0.521	2.69E-04	2.87E-01	1.65E-05
rs4979444	9	A / C	24	44	9	0.597	18	23	31	0.410	1.70E-03	4.68E-01	1.66E-05
rs696785	13	A / C	41	35	1	0.760	27	28	17	0.569	5.64E-04	7.02E-02	1.78E-05
rs905555	3	T / C	0	41	36	0.266	14	27	31	0.382	3.56E-02	1.84E-05	7.42E-01
rs10507300	13	A / G	61	16	0	0.896	33	34	5	0.694	2.05E-05	3.73E-05	2.45E-02
rs9316663	13	T / C	0	16	61	0.104	5	34	33	0.306	2.05E-05	2.45E-02	3.73E-05
rs10125534	9	T / G	15	28	34	0.377	27	36	9	0.625	2.75E-05	1.80E-02	2.34E-05

77 THPP cases and 72 controls

Chr, chromosome; Freq, frequency

Supplementary table 2 Association of 57 SNPs in fine mapping study with THPP in Thai male patients with Graves' disease

SNP	Chromosome position	Allele (1/2)	Case				Control				P-value		
			11	12	22	Freq of allele 1	11	12	22	Freq of allele 1	1 vs 2	11 vs 12+22	11+12 vs 22
rs6501379	65651635	T / C	55	21	1	0.851	56	16	0	0.889	3.91E-01	4.53E-01	1.00E+00
rs236512	65677948	C / G	34	36	7	0.675	28	34	10	0.625	3.96E-01	6.18E-01	4.43E-01
rs173135	65683921	C / T	53	23	1	0.838	62	10	0	0.931	1.83E-02	1.83E-02	1.00E+00
rs1544490	65688484	A / G	23	37	17	0.539	21	36	15	0.542	1.00E+00	1.00E+00	1.00E+00
rs4328485	65689360	C / A	20	39	18	0.513	21	37	14	0.549	5.63E-01	7.16E-01	6.90E-01
rs12949668	65694454	G / C	68	7	2	0.929	40	31	1	0.771	1.38E-04	7.92E-06	1.00E+00
rs17775970	65697514	C / T	56	18	3	0.844	53	16	3	0.847	1.00E+00	1.00E+00	1.00E+00
rs8079702	65702421	A / G	21	44	12	0.558	21	34	17	0.528	6.42E-01	8.56E-01	3.01E-01
rs3744486	65705638	G / A	26	44	7	0.623	32	29	11	0.646	7.19E-01	2.39E-01	3.17E-01
rs1468473	65712954	C / T	26	39	12	0.591	22	46	4	0.625	5.55E-01	7.27E-01	6.38E-02
rs236591	65718183	T / C	20	39	18	0.513	10	40	22	0.417	1.05E-01	1.01E-01	3.59E-01
rs236594	65719222	A / G	30	38	9	0.636	25	35	12	0.590	4.75E-01	6.14E-01	4.81E-01
rs8076345	65724145	G / A	61	14	2	0.883	51	21	0	0.854	4.95E-01	2.60E-01	4.97E-01
rs236524	65725094	G / A	24	37	16	0.552	13	38	21	0.444	8.19E-02	8.73E-02	2.60E-01
rs236530	65729066	C / T	17	53	7	0.565	11	46	15	0.472	1.31E-01	3.04E-01	6.32E-02
rs189323	65733612	A / G	44	28	5	0.753	30	36	6	0.667	1.25E-01	7.19E-02	7.60E-01
rs2366491	65733830	C / T	58	19	0	0.877	45	27	0	0.813	1.49E-01	1.11E-01	1.00E+00
rs12150382	65734367	G / A	59	15	3	0.864	41	29	2	0.771	4.99E-02	1.44E-02	1.00E+00
rs236550	65758966	C / T	47	26	4	0.779	32	35	5	0.688	8.78E-02	4.97E-02	7.39E-01
rs236562	65767890	A / G	37	33	7	0.695	30	34	8	0.653	4.60E-01	5.10E-01	7.88E-01
rs9905884	65769333	A / C	39	33	4	0.730	12	41	19	0.451	4.01E-06	1.11E-06	1.16E-05
rs992072	65769803	G / T	39	34	4	0.727	10	42	20	0.431	6.36E-07	2.11E-07	1.73E-06
rs623011	65771041	A / G	43	28	4	0.760	12	40	20	0.444	2.76E-07	3.23E-08	4.76E-07
rs9913349	65771665	C / T	39	34	4	0.727	11	41	20	0.438	1.47E-06	3.90E-07	4.95E-06
rs4968804	65772253	G / A	62	15	0	0.903	41	27	4	0.757	3.10E-03	1.00E-03	2.45E-03
rs11077484	65772311	G / A	66	11	0	0.929	46	22	4	0.792	3.92E-03	6.79E-04	2.41E-03
rs236511	65775691	G / C	41	32	4	0.740	12	40	20	0.444	1.19E-06	2.98E-07	3.07E-06
rs4968887	65776140	C / T	64	12	0	0.921	41	27	4	0.757	6.39E-04	1.15E-04	2.89E-04
rs2529681	65776392	T / G	41	32	4	0.740	12	40	20	0.444	1.19E-06	2.98E-07	3.07E-06
rs236499	65778101	A / G	42	33	1	0.770	12	43	17	0.465	1.06E-07	9.94E-08	1.20E-06

Supplementary table 2 Continued

SNP	Chromosome position	Allele (1/2)	Case				Control				P-value		
			11	12	22	Freq of allele 1	11	12	22	Freq of allele 1	1 vs 2	11 vs 12+22	11+12 vs 22
rs236500	65778619	T / A	42	32	3	0.753	13	42	17	0.472	1.94E-06	8.30E-07	3.91E-06
rs12453584	65779328	G / A	67	9	0	0.941	46	22	4	0.792	1.32E-03	2.25E-04	8.39E-04
rs236504	65780331	T / C	51	25	1	0.825	35	32	5	0.708	4.20E-02	1.97E-02	3.25E-02
rs7223705	65781307	C / A	42	32	2	0.763	13	40	18	0.465	5.46E-07	1.85E-07	3.62E-06
rs1399185	65782550	G / T	42	32	3	0.753	13	42	17	0.472	1.94E-06	8.30E-07	3.91E-06
rs1355915	65782640	G / C	42	32	3	0.753	13	42	17	0.472	1.94E-06	8.30E-07	3.91E-06
rs8075271	65783655	G / A	64	12	1	0.909	40	28	4	0.750	1.12E-03	3.03E-04	3.22E-04
rs8076950	65783673	A / C	64	12	1	0.909	40	28	4	0.750	1.12E-03	3.03E-04	3.22E-04
rs17714860	65783949	G / A	64	9	1	0.926	38	30	3	0.746	7.94E-05	4.76E-05	2.04E-05
rs11650230	65785348	C / T	64	12	1	0.909	40	28	4	0.750	1.12E-03	3.03E-04	3.22E-04
rs8074548	65787319	C / T	69	8	0	0.948	48	21	3	0.813	1.99E-03	2.75E-04	6.99E-04
rs2215027	65791032	G / A	66	10	1	0.922	45	23	4	0.785	4.66E-03	8.67E-04	1.36E-03
rs8068647	65793761	T / A	67	9	0	0.941	46	22	4	0.792	1.32E-03	2.25E-04	8.39E-04
rs4968890	65797863	C / T	67	10	0	0.935	46	22	4	0.792	2.19E-03	3.03E-04	1.12E-03
rs11077488	65801677	C / T	70	7	0	0.955	46	22	4	0.792	2.52E-04	1.68E-05	7.09E-05
rs6501384	65802728	C / T	66	11	0	0.929	43	26	3	0.778	1.02E-03	2.37E-04	4.11E-04
rs1606655	65808209	T / G	60	15	1	0.888	39	29	4	0.743	4.97E-03	1.47E-03	1.65E-03
rs1606656	65808380	C / T	65	12	0	0.922	39	33	0	0.771	1.00E+00	3.11E-04	7.26E-05
rs7222503	65811380	C / A	67	9	1	0.929	45	22	4	0.789	3.43E-03	6.35E-04	1.03E-03
rs7208007	65812076	G / C	75	2	0	0.987	67	5	0	0.965	1.00E+00	2.69E-01	2.64E-01
rs312707	65812231	C / A	52	24	1	0.831	33	34	5	0.694	1.44E-02	6.23E-03	8.47E-03
rs7225313	65814881	C / A	67	9	1	0.929	45	22	4	0.789	3.43E-03	6.35E-04	1.03E-03
rs17715938	65827243	T / C	67	10	0	0.935	43	29	0	0.799	1.00E+00	5.18E-04	1.74E-04
rs16975551	65828054	C / T	69	8	0	0.948	48	21	3	0.813	1.99E-03	2.75E-04	6.99E-04
rs11653587	65828273	G / A	69	8	0	0.948	48	21	3	0.813	1.99E-03	2.75E-04	6.99E-04
rs10775360	65837463	C / T	67	10	0	0.935	43	26	3	0.778	5.01E-04	1.00E-04	1.74E-04
rs10512574	65849559	T / C	68	9	0	0.942	48	19	5	0.799	2.65E-03	2.21E-04	1.63E-03

77 THPP cases and 72 controls

Freq, frequency

Chromosome positions of the SNPs were derived from dbSNP build 130.

บทความสำหรับการเผยแพร่

ผลงานวิจัยเรื่อง โครงการการศึกษาเพื่อหาสาเหตุทางพันธุกรรมของผู้ป่วยไทรอยด์เป็นพิษที่มีอาการกล้ามเนื้ออ่อนแรงและระดับโพแทสเซียมในเลือดต่ำด้วยวิธีการตรวจทั้งจีโนมด้วยไมโครอาเรย์ (Microarray based whole genome scan for genetic susceptibility of thyrotoxicperiodic paralysis).เป็นการศึกษาเพื่อหาสาเหตุทางพันธุกรรมของโรค Thyrotoxic hypokalemic periodic paralysis (THPP) ที่มีลักษณะอาการอ่อนแรงและระดับโปแทสเซี่ยมต่ำในผู้ป่วยไทรอยด์เป็นพิษ เป็นโรคที่พบน้อยในชาวตะวันตก แต่พบบ่อยในเอเชียมีหลักฐานในปัจจุบันทำให้เชื่อว่าเป็นโรคที่มีปัจจัยทางพันธุกรรมเป็นกลไกหื้นฐาน การศึกษาวิจัยนี้เป็นการวิจัยที่ใช้หลักการของ การหาสัมพันธ์ของยีนกับการเกิดโรค โดยเทคนิค genome-wide association study (GWAS) โดยทำการทดสอบยืนยันผลใน 2 กลุ่มประชากร และ ผลการวิจัยทำให้เราพบเป็นครั้งแรกถึงความเกี่ยวข้องของ Single nucleotide polymorphisms ที่ใกล้ ยีน *KCNJ2* และ ยีน *GABRA3* กับโรค THPP ในประชากรไทย