Detection of CAPN10 copy number variation in Thai patients with type 2 diabetes by denaturing high performance liquid chromatography and real-time quantitative polymerase chain reaction

Nattachet Plengvidhya1, Kanjana Chanprasert2, Watip Tangjittipokin2, Wanna Thongnoppakhun3, Pa-thai Yenchitsomanus4*

1Division of Endocrinology and Metabolism, Department of Medicine, 2Department of Immunology, 3Division of Molecular Genetics, Department of Research and Development, and 4Division of Molecular Medicine, Departments of Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

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*Correspondence
Pa-thai Yenchitsomanus
Tel.: +662-4192759
Fax: +662-4110169
E-mail address: ptyench@gmail.com

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ABSTRACT
Aims/Introduction: A combination of multiple genetic and environmental factors contribute to the pathogenesis of type 2 diabetes. Copy number variations (CNVs) are associated with complex human diseases. However, CNVs can cause genotype deviation from the Hardy–Weinberg equilibrium (HWE). A genetic case–control association study in 216 Thai diabetic patients and 192 non-diabetic controls found that, after excluding genotyping errors, genotype distribution of calpain 10 (CAPN10) SNP44 (rs2975760) deviated from HWE. Here, we aimed to detect CNV within the CAPN10 SNP44 region.

Materials and Methods: CNV within the CAPN10 SNP44 region was detected using denaturing high-performance liquid chromatography, and the results confirmed by real-time quantitative polymerase chain reaction with SYBR Green I.

Results: Both methods successfully identified CNV in the CAPN10 SNP44 region, obtaining concordant results. Correction of genotype calling based on the status of identified CNVs showed that the CAPN10 SNP44 genotype is in good agreement with HWE (P > 0.05). However, no association between CNV genotypes and risk of type 2 diabetes was observed.

Conclusions: Identified CNVs for CAPN10 SNP44 genotypes lead to deviation from HWE. Furthermore, both denaturing high-performance liquid chromatography and real-time quantitative polymerase chain reaction are useful for detecting CNVs.

INTRODUCTION
Type 2 diabetes is a complex disease that involves participation of several genetic and environmental factors. Recent genetic research has identified several susceptibility genes. Discovery of type 2 diabetes genes is expected to aid development of novel therapeutic interventions or disease prevention. Calpain 10 (CAPN10), a type 2 diabetes susceptibility gene, was identified from a genome-wide scan. CAPN10 encodes an intracellular cysteine protease that plays a physiological role in insulin-mediated glucose metabolism and insulin production from pancreatic β-cells. It is expressed in various tissues including pancreatic islets, adipose tissue, skeletal muscle and the liver. CAPN10 is located on chromosome 2q37.3, and consists of 15 exons spanning 31 kb. Four single nucleotide polymorphisms (SNPs), SNP44 (rs2975760), SNP43 (rs3792267), Indel19 (rs3842570) and SNP63 (rs5030952), and/or their haplotypes have previously been associated with type 2 diabetes, insulin action and insulin secretion. However, the impact of CAPN10 variants on type 2 diabetes had not been investigated in the Thai population;
Deviations from HWE commonly indicate genotyping errors, show increased homozygosity and decreased heterozygosity. High-performance liquid chromatography (DHPLC) from HWE. In the present study, we used DHPLC to provide evidence supporting the presence of CNVs in the Indel19 marker region, and further evidence supporting the presence of CNVs in the CAPN10 SNP44, and confirmed the results by real-time quantitative polymerase chain reaction (PCR) to ensure accuracy and reliability. In addition, we investigated the role of this CNV and the type 2 diabetes risk in our population.

**MATERIALS AND METHODS**

**Study Participants**
A total of 216 patients with type 2 diabetes and 192 non-diabetic control subjects were included in the present study. Patients with type 2 diabetes were recruited from the Diabetic Clinic, Siriraj Hospital, Mahidol University, Bangkok, Thailand. Diabetes was defined according to the American Diabetes Association. Non-diabetic controls were selected from participants at the health check-up center at the Department of Preventive and Social Medicine, Siriraj Hospital. Inclusion criteria were: (i) fasting plasma glucose <100 mg/dL (6.1 mmol/L); (ii) glycated hemoglobin A1c (HbA1c) <5.7%; (iii) no family history of type 2 diabetes in the first degree relatives; (iv) age ≥50 years; (v) no hypertension; and (vi) body mass index range 18.50–24.99 kg/m². Study approval was granted from the institutional ethics committee. The patients and control participants provided informed consent before participating in the study.

**PCR-Restriction Fragment Length Polymorphism Analysis**
Genomic deoxyribonucleic acid (DNA) was extracted from peripheral blood leukocytes (obtained from ethylenediaminetetraacetic acid anticoagulated blood) using the standard phenol chloroform method. All participants were genotyped for SNP44 by PCR-restriction fragment length polymorphism (PCR-RFLP). The primers are listed in Table S1. PCR products were digested using MwoI restriction endonuclease (Fermentas, Vilnius, Lithuania). Digestion products were separated on 10% acrylamide and silver stained. Allele T was detected as a 183-, 118- and 74-bp fragments, and allele C as 183-, 102-, 74- and 16-bp fragments.

**DHPLC Analysis**
To detect CNVs in the CAPN10 SNP44 region by multiplex PCR and DHPLC, multiplex PCR was carried out using three primer pairs, specifically, co-amplified CAPN10 SNP44 region, cytotoxic T lymphocyte associated protein-4 (CTLA4), chromosome 2q) and dystrophin (DMD, chromosome X) genes. CTLA4- and DMD were used as two and single copy (observed only in males) internal control genes, respectively. Primer sequences are summarized in Table S1. Multiplex PCR was carried out in a 25-µL total volume containing 125 ng genomic DNA; 4.5, 3.0 and 3.0 µmol/L of each primer for CAPN10 SNP44, DMD and CTLA4, respectively, 2 nmol/L dNTPs, 0.5 units IMMOLASE™ DNA polymerase (Bioline Inc., London, UK), 2.5 mM Imobuffer and 1.5 mM MgCl₂, provided by the manufacturer. PCR for linear amplification was carried out in a T gradient Thermocycler (Biometra, Goettingen, Germany) with an initial denaturation step at 95°C for 10 min, followed by 27 cycles consisting of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s and a final extension step at 72°C for 5 min. Multiplex PCR products were analyzed by gel electrophoresis through 2.5% LE-agarose and visualized after ethidium bromide staining. For quantification, 10 µL amplified products were processed through non-denaturing conditions at 50°C using the WAVE™ Nucleic Acid Fragment Analysis System (Transgenomic Inc., San Jose, CA, USA). The mobile phase was carried out in 0.1 mol/L triethylammonium acetate solution (buffer A). PCR products were then eluted using an acetonitrile gradient (49–58%). The gradient was created by mixing buffer A and 0.1 mol/L triethylammonium acetate solution in water, with 25% acetonitrile, pH 7 (buffer B). Each sample was then separated by DHPLC and quantified by fluorescence detection. Data were analyzed using appropriate software (Transgenomic Inc.). The results clearly show discrete chromatography profiles peaks: the x-axis represents elute time (min) and the y-axis represents fluorescence intensity. Relative peak intensity of each sample directly reflects copy number. For SNP44, the expected ratio of peak height compared with internal control genes should be 1 for samples without CNVs (genotypes T/T, T/C and C/C), 0.5 for samples with deletions and 1.5 for samples with duplications. Triplicate assays were carried out to check analysis accuracy.

**Real-Time Quantitative PCR**
Presence of CNVs was confirmed using specific primers for the target sequence (SNP44) and reference sequence (GAPDH), designed using Primer 3 (http://frodo.wi.mit.edu). Primer...
sequences and gene locations are listed in Table S2. Real-time quantitative PCR was carried out using the Light Cycler 480® system (Roche Diagnostics GmbH, Mannheim, Germany). GAPDH was simultaneously co-amplified with SNP44 and served as an internal standard control. Amplification reactions (20 μL) were carried out using 50 ng template DNA, 1xLightCycler® 480 SYBR Green I Masterkit (Roche), and 0.25 μmol/L of each forward and reverse primer. Thermal cycling was one cycle with pre-denaturation at 95°C for 10 min, followed by 45 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. In each assay, two normal control DNA and non-template controls were included. For data analysis, SNP44 gene copy number was determined by comparing the target SNP44 sequence to the reference (GAPDH). The relative gene copy number for each sample was determined using the comparative threshold cycle number (Ct) method. Each sample was run in triplicate in separate tubes to allow SNP44 quantification relative to GAPDH. Ct was determined for all PCR reactions. ΔCt represents the mean Ct value of each sample, and was calculated for SNP44 and GAPDH. By using a calibrator sample of normal control DNA, the gene copy number of unknown samples was estimated based on the following formula:

\[
\Delta\Delta Ct = (\Delta Ct \text{ GAPDH \ (calibrator sample)}) - \Delta Ct \text{ SNP44 \ (calibrator sample)}) - (\Delta Ct \text{ GAPDH \ (unknown sample)}) - (\Delta Ct \text{ SNP44 \ (unknown sample)})
\]

Relative SNP44 copy number was determined using the formula \(2 \times 2^{(\Delta\Delta Ct)}\) as described, previously.13

**Statistical Analysis**

Genotype distribution was tested for deviation from HWE using the Chi square-test from a web-based program (http://ihg.gsf.de/cgi-bin/hw/hwal.pl). P-values for comparing cases and controls, and determining type 2 diabetes association were obtained using Pearson’s chi-square test. Odds ratios (ORs) were calculated for one copy genotypes either (T/–) or (C/–) by comparing with the normal two copies genotype (TT + TC + CC). After excluding the identified CNVs (1 copy), the CAPN10 SNP44 genotype was determined according to genetic models: TT vs TC vs CC (co-dominant model); (CC + TC) vs TT (dominant model); and CC vs (TC + TT; recessive model). For each genetic model, ORs were estimated by comparing each genotype with the other genotypes.

The linear regression model was used to analyze continuous variables adjusted for covariate variables of age, sex, body mass index and drugs where appropriate. P-values ≤0.05 were considered statistically significant. Statistical power calculation for SNP44 was carried out using the Power and Sample size program (PS program version 3.0; http://mc.vanderbilt.edu/wiki/Main/PowerSampleSize). To detect an association with the traits examined in the present study, 28.6% power (α = 0.05) was calculated.

**RESULTS**

Clinical characteristics and laboratory parameters of the study participants are shown (Table 1). Type 2 diabetic patients had higher body mass index, waist-to-hip ratio, waist circumference and blood pressure. Fasting plasma glucose, serum triglyceride, serum total cholesterol and serum low-density lipoprotein (LDL)-cholesterol levels were also significantly higher in diabetic patients. Non-diabetic controls had higher serum high-density (HDL)-cholesterol levels.

We previously investigated the association of CAPN10 Indel19 and SNP44 with type 2 diabetes. The genotypes obtained for these SNPs showed deviation from HWE (Table S2). We initially examined CAPN10 Indel19, and successfully confirmed the presence of CNVs in the CAPN10 region by DHPLC13. Here, using the same cohort comprising 216 type 2 diabetes patients and 192 non-diabetic controls, we further examined the presence of CNVs in the SNP44 region using DHPLC, and confirmed the results by real-time quantitative PCR with SYBR Green I. First, we carried out DHPLC analysis. CAPN10 SNP44 chromatograms clearly showed the different patterns between male and female individuals harboring two copies or one copy of the variant (Fig.

### Table 1 | Clinical characteristics of study participants

| Clinical characteristics | Type 2 diabetes | Non-diabetic controls | P-value |
|--------------------------|----------------|-----------------------|---------|
| n (male/female)          | 202/144        | 188/135               | –       |
| Age (years)              | 53.75 ± 11.00  | 53.34 ± 9.09          | NS      |
| Age at diagnosis (years) | 49.39 ± 10.87  | ND                    | ND      |
| Weight (kg)              | 68.57 ± 12.46  | 59.25 ± 9.89          | <0.001  |
| BMI (kg/m²)              | 27.84 ± 4.95   | 23.91 ± 3.17          | <0.001  |
| Waist circumference (cm) | 87.99 ± 10.90  | 82.21 ± 9.37          | <0.001  |
| Hip circumference (cm)   | 9982 ± 9.73    | 9562 ± 6.69           | <0.001  |
| Waist-to-hip ratio       | 0.89 ± 0.07    | 0.86 ± 0.06           | <0.001  |
| Systolic BP (mmHg)       | 132.13 ± 16.16 | 116.14 ± 15.12        | <0.001  |
| Diastolic BP (mmHg)      | 81.42 ± 10.40  | 70.31 ± 9.90          | <0.001  |
| Fasting glucose (mmol/L) | 11.34 ± 5.09   | 4.88 ± 0.35           | <0.001  |
| HbA1c (%)                | 8.34 ± 2.58    | 5.36 ± 0.26           | <0.001  |
| Total cholesterol (mmol/L)| 5.86 ± 1.19    | 5.32 ± 0.93           | <0.001  |
| Triglyceride (mmol/L)    | 463 ± 233      | 268 ± 1.11            | <0.001  |
| LDL (mmol/L)             | 3.70 ± 1.08    | 3.23 ± 0.86           | <0.001  |
| HDL (mmol/L)             | 1.27 ± 0.31    | 1.58 ± 0.42           | <0.001  |

Data are shown as mean ± standard deviation. P-values compare clinical and laboratory data between 202 type 2 diabetes patients and 188 non-diabetic controls with completely available data, using either Mann-Whitney U-test or Student’s unpaired t-test. BMI, body mass index; BP, blood pressure; HbA1c, glycated hemoglobin A1c.; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ND, not determined; NS, not significant.
ure 1). Relative \( \text{CAPN10 SNP44} \) copy number was determined from DHPLC peak heights of amplified products by comparing with the reference internal control genes (\( \text{DMD} \) and \( \text{CTLA-4} \)). Chromatogram peak heights from individuals carrying two normal copies of \( \text{CAPN10 SNP44} \) were equal to \( \text{CTLA-4} \) peak heights (Figure 1a,b). Conversely, chromatogram peak heights reflecting heterozygous deletion (1 copy) of SNP44 were only half \( \text{CTLA-4} \) peak heights (Figure 1a,b).

We carried out real-time quantitative PCR using SYBR Green in the same cohort to confirm our DHPLC results. PCR amplification plots of normal control samples showed nearly identical Ct values for \( \text{CAPN10 SNP44} \), compared with \( \text{GAPDH} \) (Figure 2a). Individuals with \( \text{CAPN10 SNP44} \) deletions showed increased Ct values of nearly 0.5 cycles compared with \( \text{GAPDH} \) (Figure 2b). The copy number status of \( \text{CAPN10 SNP44} \) among non-diabetic controls and type 2 diabetes participants is shown (Figure 2c). The results obtained from the two methods were blinded for CNV calling genetic results, and the concordance rate between them was 98%. We next investigated the relationship between \( \text{CAPN10 SNP44} \) gene copy number and the risk of type 2 diabetes. There was no difference in the one copy genotype frequency either (\( T/– \)) or (\( C/– \)), when compared between case and control groups (Table 2). After excluding CNVs, re-analysis showed that SNP44 genotypes fitted well within HWE in both non-diabetic controls and type 2 diabetes patients (\( P = 0.742 \) and 0.771, respectively; Table 3). We then re-examined the association, finding that \( \text{CAPN10 SNP44} \) was not associated with type 2 diabetes under co-dominant, dominant or recessive models (Table 2). We also found no association between clinical characteristics and laboratory parameters of the studied participants and CNV genotypes (Tables S3 and S4).

**DISCUSSION**

Genetic association studies assess the association between phenotypic traits and genetic variants within a population. Genotyping data from case–control studies must first be tested for deviation from HWE to better understand the genetic characteristics of the population. The HWE test is carried out using the Chi square testing procedure to compare observed with expected genotype frequencies within a population. The genotype frequencies should be in agreement with HWE in cases and controls. Various reasons for deviation from HWE have been proposed, including natural selection, non-random mating, mutation and migration\(^{14}\). However, in humans, these factors seldom occur in the general population. A significant deviation might indicate genotyping errors and sampling bias, which, by far, are the most common sources of deviation\(^{15}\). An additional theoretical concern is that the result of a genetic association study could be spurious if the genotype distribution...
Figure 2 | Amplification plots of **CAPN10** SNP44 copy number status determined by real-time quantitative polymerase chain reaction, including (a) a normal control of **CAPN10** SNP44, and (b) an individual with a gene deletion of gene. Quantification analysis of **CAPN10** SNP44 copy number in non-diabetic controls and diabetic patients. In each group, copy numbers are shown as one copy or two copies of genes (x-axis). Analysis of gene copy number was carried out using the formula $2 \times 2^{-\Delta\Delta C_t}$ (y-axis). (c) The copy number mean after $2 \times 2^{-\Delta\Delta C_t}$ calculation in each group is represented by the horizontal line. T2D, type 2 diabetes.
in controls does not fulfill HWE. Data must therefore be analyzed carefully for all the aforementioned reasons.

In our previous report, we investigated the association of the \textit{CAPN10} variants, \textit{Indel19} and \textit{SNP44}, with type 2 diabetes in the Thai population. Genotype distribution of these SNPs significantly deviated from HWE, with homozygotes excess and reduced heterozygotes in the same direction (Natattachment P, Kanjana C, Watip T, Yenchitsomanus PT, unpublished data). We then re-genotyped these SNPs using different methods, obtaining concordant results, and carefully selected case and control subjects according to our inclusion criteria.

There is evidence that structural DNA variations, such as CNVs, influence genomic variability and risk of human diseases. Furthermore, recent studies have shown that the frequency of SNPs residing in CNV regions (CNVRs) might deviate from HWE\textsuperscript{16}. \textit{CAPN10} variant genotypes are reported to deviate from HWE in several ethnic groups: for example, Korean, Spanish, British and Finnish\textsuperscript{17-19}. However, these studies did not adequately describe why deviation of genotypes from HWE was observed. To address this, we used DHPLC and showed that CNV presence in the \textit{CAPN10} \textit{Indel19} region leads to deviation of genotypes from HWE. In the present study, our aim was to ratify the presence of CNVs in the \textit{CAPN10} \textit{SNP44} region using two independent techniques, DHPLC and real-time quantitative PCR, which increases the accuracy and reliability of our experimental results. Both methods produced highly concordant outcomes, and were equally effective in CNV detection in the \textit{CAPN10} \textit{SNP44} region.

DHPLC is powerful, simple, time saving and cost-effective, enabling CNV detection in the gene of interest. However, some limitations were proposed in our previous study\textsuperscript{13}. Therefore, we also examined \textit{CAPN10} \textit{SNP44} genotyping results obtained by DHPLC using real-time quantitative PCR. This method is suitable for CNV detection in targeted regions. Two detection chemistries are generally used for this technique: the double-stranded DNA-intercalating agent, SYBR Green I dye, or sequence-specific TaqMan probes. We used SYBR Green I, because it is relatively simple and inexpensive compared with TaqMan probes. In addition, a previous study showed that gene copy number analysis using DHPLC and real-time quantitative PCR are compatible\textsuperscript{10}.

| Model | Genotype | Genotype frequency (%)\textsuperscript{†} | P-value | OR (95% CI) |
|-------|----------|------------------------------------------|---------|-------------|
|       |          | Control (n = 192) | Case (n = 216) |       |
| 1 copy |          |                                 |         |             |
| T/–   | 64 (33.33) | 83 (38.42) | 0.313\textsuperscript{*} | 1.23 (0.81–1.86) |
| C/–   | 8 (4.17) | 7 (3.24) | 0.732\textsuperscript{*} | 0.83 (0.29–2.36) |
| 2 copies |          |                                 |         |             |
| TT    | 88 (73.33) | 99 (78.57) | 0.776 | 1 (reference) |
| TC    | 29 (24.17) | 25 (19.84) | 0.77 (0.42–1.41) | 0.77 (0.42–1.41) |
| CC    | 3 (2.50) | 2 (1.59) | 0.59 (0.10–3.63) | 0.59 (0.10–3.63) |
| Co-dominant | |                   |           |             |
| TT    | 88 (73.33) | 99 (45.83) | 0.336 | 1 (reference) |
| TC + CC | 31 (25.83) | 27 (21.43) | 0.75 (0.42–1.35) | 0.75 (0.42–1.35) |
| CC + TC | 117 (97.50) | 124 (98.41) | 0.956 | 1 (reference) |
| Recessive |          |                                 |         |             |
| TT + C | 3 (2.50) | 2 (1.59) | 0.59 (0.09–3.62) | 0.59 (0.09–3.62) |

\textsuperscript{*}P-value assessed by comparing either (T/–) or (C/–) genotype and (TT + TC + CC) genotype between cases and controls. \textsuperscript{†}\textit{CAPN10} \textit{SNP44} was genotyped by denaturing high-performance liquid chromatography and real-time quantitative polymerase chain reaction. CI, confidence interval; OR, odds ratio.

### Table 2 | Genotype distribution and association analysis of \textit{CAPN10} \textit{SNP44} copy number variation genotypes with type 2 diabetes risk

### Table 3 | \textit{CAPN10} \textit{SNP44} genotype distribution with or without consideration of identified copy number variations

- **P-value** reflects Hardy Weinberg Equilibrium (HWE) before\textsuperscript{*} and after\textsuperscript{**} consideration of identified copy number variations (CNVs).
The Database of Genomic Variants contains CNV information from many ethnic groups; for example, Caucasian, African-American and East Asian. The CNVs reported in Database of Genomic Variants to cover CAPN10 were shown by Redon et al. Wang et al. Jakobsson et al. and Dogan et al. (Figure S1). Several studies have attempted to identify CNVs specific for certain populations, such as European, Korean and Chinese. Nevertheless, investigation of CNVs in the Thai population is very limited. We were not able to accurately extend the CNV breakpoints, which could partly be explained by the fact that only CAPN10 Indel19 and SNP44 were investigated. It is also possible that the CNVs identified in the present study were Thai-specific and differentially distributed among other populations. Future work should clarify this issue.

Type 2 diabetes is a metabolic disorder characterized by insulin resistance and relative insulin deficiency. Most current studies attempting to discover genes causing type 2 diabetes focus only on SNPs as genetic markers, whereas studies using CNVs as newly discovered genetic markers are relatively scarce. Recently, reports have shown that several CNVRs are significantly associated with type 2 diabetes risk in various ethnic groups. The present study failed to find an association between the CAPN10 CNV genotype and diabetes or any clinical parameter, possibly because of the small sample size preventing detection of a significant association. Nevertheless, association of CAPN10 CNVs with type 2 diabetes has not yet been reported. A further study using a larger sample size might be required to establish an association between these CNVs and disease risk in Thais. The present study adds to accumulating evidence that CNVRs might affect precise genotype calling of SNPs, leading to disagreement of the HWE assumption. Additionally, combining DHPLC and real-time quantitative PCR will confirm CNV presence in genetic studies, particularly when SNPs are not in perfect HWE.

In conclusion, the present study shows that genotype distribution of certain SNPs, that display deviation from HWE, might result from CNVs in proximity to these SNP markers. Furthermore, using different genotyping methods is crucial to confirm the presence of CNVs in a genomic region of interest. Investigations regarding precise locations, (including breakpoints), together with molecular characteristics of these CNVs are required to obtain more insight into the role of CNVs in pathogenesis of type 2 diabetes.

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DISCLOSURE

The authors declare no conflict of interest.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article:

Table S1 | Polymerase chain reaction primer sequences for denaturing high-performance liquid chromatography and real-time quantitative polymerase chain reaction analysis.

Table S2 | CAPN10 SNP44 genotype distribution obtained by Polymerase chain reaction-restriction fragment length polymorphism analysis.

Table S3 | Association between CAPN10 SNP44 copy number variations and clinical parameters in patients.

Table S4 | Association between CAPN10 SNP44 copy number variations and clinical parameters in controls.

Figure S1 | Copy number variations location harboring CAPN10 obtained from the Database of Genomic Variants (DGV).