Quantitative evaluation of PTPN22 copy number variation by digital droplet PCR and association with type 2 diabetes risk

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Abstract. Type 2 diabetes (T2D) is a chronic endocrine disorder with rapidly increasing prevalence worldwide. Genetic instability leading to metabolic dysfunction plays an important role in T2D susceptibility and progression. Structural alteration in genome, that is, copy number variation (CNV) is emerging as the inherent marker for disease identification. Previous genomic CNV array revealed that protein tyrosine phosphatase non-receptor type 22 (PTPN22) gene was overlapped with a CNV region, however, whether this CNV affected T2D risk remains to be further elucidated. In this study, we first identified divergent distributions of the PTPN22 copy number (CN) between T2D patients and healthy controls in Chinese population ($p < 0.01$). Risk assessment analysis revealed that the CN gain (OR = 3.28, $p < 0.001$) was the promising risk factor for T2D. Also, significantly positive correlations of the PTPN22 CNV with fasting plasma glucose and glycated hemoglobin were demonstrated in T2D patients. Statistical association analysis investigated that the T2D individuals carrying CN gain showed higher plasma glucose and lower insulin levels than those carrying CN normal and loss at 60 min/120 min/180 min during an OGTT test. In addition, the PTPN22 CNV had an effect on total cholesterol, and the CN gain presented higher values than the other two CN types. These results suggested that the CN gain types of the PTPN22 gene accompany with the glycometabolism dysregulation, and finally predispose their carriers to T2D; therefore, the PTPN22 CNV may be a promising biomarker for predicting T2D risk, or a clinical target for T2D diagnosis and therapy.

Key words: Copy number variation, PTPN22 gene, Digital droplet PCR, Type 2 diabetes

COPY NUMBER VARIATION (CNV) belongs to sub-microscopic chromosomal rearrangement across the whole genomic architecture, and refers to a size larger than 1 kb DNA segment variation supplementary to single nucleotide polymorphism (SNP) and deletion/insertion (Indel) [1]. Genetic CNVs including sequential duplications, deletions, insertions, and complex multisite polymorphisms contribute to the alterations of gene structure, gene dosage, and even phenotypic diversity [2]. While SNPs and Indels constitute the most commonly investigated genomic variants, previous studies revealed that CNVs are emerging as one kind of the inherent mutations leading to a variety of population evolution and phenotypic characteristics [3]. In addition, CNVs also play important roles in modeling complex human diseases. Extensive CNVs evaluation studies have identified significant associations between CNVs and diseases such as schizophrenia [4], tumor [5, 6], and endocrine metabolism disorders [7, 8] using the candidate-gene approaches.

Type 2 diabetes mellitus (T2D) is a serious metabolic disorder which is characterized by chronic hyperglycemia, insulin resistance and impairment of β cell function [9]. Epidemiological investigations revealed that T2D has been considered as a major health problem due to the increasing disease morbidity worldwide. Elbein et al. previously found that the incidence of T2D was concentrated and reached a higher rate in the familial population, suggesting that genetic factors may contribute to the pathogenesis and development of the disease [10]. In recent years, the great progress of high throughput sequencing and chip array technologies has promoted the discovery of T2D-susceptible genetic loci. CNVs have been proved to significantly associate with the risk and susceptibility of T2D [11]. Grayson et al. identified 39 CNVs presented different distribution (with enriched or
Association studies revealed that the susceptibility SNP disease progression of non-obese diabetic mice [18].

Compliance with Ethics Guidelines

All the procedures on human subjects have been carried out in accordance with the guidelines in the World Medical Association Declaration of Helsinki [21]. The experimental protocols involved human samples were approved by the Ethics Committee of affiliated Tianyou Hospital of Wuhan University of Science and Technology, and diagnosed as defined by American Diabetes Association (ADA) criteria when the venous plasma glucose concentration was ≥200 mg/dL 2 h after a 75 g oral glucose load, the fasting plasma glucose was ≥126 mg/dL [22]. Importantly, we included the T2D patients who received metformin monotherapy for at least 6 months in our study. For the patients accompanied by dyslipidemia, they were also treated with atorvastatin (20 mg/d) to control the clinical lipid index. Patients with other morbidities such as malnutrition, pancreatitis, anemia and malignant cancer were excluded from this study. In addition, the individuals that undergo combination therapy of insulin, acarbose, rosiglitazone, and pioglitazone were also excluded from the case population. The controls were recruited from the Hospital of Wuhan University, diagnosed by fasting glucose <126 mg/dL and glycated hemoglobin <6.0%, no history of diabetes or other autoimmune disease in the degree relatives, and no hypertension. All cases and controls were belonging to Chinese ethnicity, and no bias in geographic distribution was identified for all subjects. Genomic DNA from whole blood was obtained from peripheral blood leukocytes samples by phenol-chloroform extraction.

Clinical characteristics report

Each subject was interviewed to complete a systemic questionnaire including the information about demographic variables, disease duration, medical history and current medication. For T2D patients, no limitations and alterations were conducted to the pharmacological therapy program to control diabetes or its complications throughout the duration of the study. Height and weight were measured for all recruited individuals. In addition, biochemical indexes including fasting plasma glucose, levels of glycated hemoglobin, total cholesterol, triglycerides, low-density lipoprotein cholesterol and high-density lipoprotein cholesterol were detected using Hitachi 912 auto-analyzer (Roche, Basel, Switzerland). For an oral glucose tolerance test (OGTT) assay, the T2D patients were adjusted diet for maintaining weight about 3 days, and then the patients underwent an overnight fasting and ingested 75 g glucose; blood was drawn before ingesting the glucose and at 60, 120, and 180 min, respectively. Plasma glucose concentrations were meas-
ured by the glucose oxidase-peroxidase method using commercial kits (Shanghai Biological Products Institution, Shanghai, China). Insulin levels were measured by radioimmunoassay (Linco Research, St Charles, MO, USA). All clinical characteristics were recorded and shown in Supplementary Table 1.

**Determination of PTPN22 copy number variation using ddPCR**

Copy number of *PTPN22* was quantified by droplet digital PCR (ddPCR) methods using the QuantStudio® 3D Digital PCR System (Life Technologies). Primers and specific fluorescent MGB-FAM labelled probes for *PTPN22* copy number quantification were designed by Primer premier 5.0. A fluorescent HEX-TAMRA probe was used for detecting the reference gene *AP3B1* which has two copies for normalization as described by a previous study [23]. The sequences of primers and probes were shown in Supplementary Table 2. A total of 15 μL of reaction volume was prepared with 2× Quantstudio® 3D digital PCR master mix, 900 nM of *PTPN22_fwd* primer, 900 nM of *PTPN22_reverse* primer, 100 nM of *PTPN22_MGB* probe, 900 nM of *AP3B1_fwd* primer, 900 nM of *AP3B1_reverse* primer, 100 nM of *AP3B1_HEX* probe and 50 ng target DNA. The mixture was loaded onto the chips using the QuantStudio® 3D Digital PCR Chip Loader, and then the chips were sealed and loaded onto a GeneAMP® PCR system 9700 (Applied Biosystems®) and cycled according to the following procedures; 96°C for 10 min, followed by 39 cycles of 60°C for 2 min and 98°C for 30 s, and a final extension at 60°C for 2 min. After cycling, the end-point fluorescence of the partitions on the chips was measured by transferring the chips to the measurement unit (application version 1.1.3, algorithm version 0.13). Concentration (copies/μL) was calculated and normalized to the reference gene. Copy number values for *PTPN22* gene were subsequently obtained from the ratio of these two concentrations.

**Association of the PTPN22 copy number with type 2 diabetes risk**

Binary logistic regression was used to evaluate the odds ratio (OR) and 95% confidence interval (CI) of different CNV types to T2D risk. Bonferroni adjustment was applied to examine the p value, adjusted for confounding variables including age, sex, and BMI. The *PTPN22* copy number was fitted as a continuous variable. SPSS software (version 20.0, Illinois, USA) was used to analyze the associations of *PTPN22* CNV with clinical traits in T2D patients and healthy controls by the linear regression model.

**Statistical analysis**

Statistical calculations were performed with STATA statistical software (version 10.0; StataCorp, College Station, TX, USA) and SPSS software (version 20.0, Illinois, USA). All studies were repeated at least three times and the data was expressed as mean value ± standard error. The correlation of *PTPN22* copy numbers and fasting plasma glucose or levels of glycated hemoglobin levels were analyzed using the R 2.15.0 software. Statistical significance of the copy number differences of the *PTPN22* gene between the T2D patients and the control subjects were tested by the two-sided Student’s t-test. Probability value less than 0.05 was considered to be significant.

**Results**

**Detection and verification of the PTPN22 CNV in Chinese population**

Whole genome-wide array has been widely used as an available and effective high-resolution method for CNV detection. Previous study using SNP array for establishing linkage disequilibrium patterns of genomic CNVs and utility in genetic disease revealed that a CNV region located in chr1: 112,869,177-116,453,414 was overlapped with *PTPN22* gene, which was also proved and identified in the database of genomic variations. To further evaluate the relation of *PTPN22* CNV with the susceptibility to diabetes mellitus, we first detected and verified the existence of the *PTPN22* CNV in Chinese T2D patients and healthy controls. As shown in Fig. 1, three MGB probes (CNV1-Exon7, CNV2-Exon12, and CNV3-Intron17) were designed to examine the consistency of the different hybridization results. Respective ten individuals from T2D and control groups were randomly chosen for CNV validation. As shown in Supplementary Fig. 1A and B, the copy numbers of the *PTPN22* gene were detected by Exon7-probe1, Exon12-probe2, and Intron17-probe3, and the same tendency of the copy number variations was investigated between the T2D patients and controls. Furthermore, quantitative PCR (qPCR) method was used to confirm the copy number as described by our previous study [8]. The CN types were classified as gain (>0.5), loss (<–0.5) and normal (<|±0.5|) based on the log2 ΔΔCt values. We also found the consistent copy numbers of the *PTPN22* gene in T2D and control individuals among the three CNV regions (Supplementary Fig. 1C–E). In addition, we analyzed the coefficient of variation (CV) to test the accuracy and reproducibility within-run among the three probes, and lower within-run CV values were detected for probe1 (CNV1-Exon7) in both T2D and controls comparing to CNV2-Exon12 and CNV3-Intron17 (Supplementary Fig. 1F).
Thus, in our subsequent study, the CNV1-Exon7 probe was used to detect the CNV distribution and evaluate the association with T2D risk.

**Divergent distributions of the PTPN22 CNV**

Given that genetic variations are exploring as molecular markers in the diagnosis and therapy of human disease, the divergent genotype distribution between two population sets may be an important precondition for the precise distinguish. Therefore, in this study, we detected the copy number (CN) of the PTPN22 gene of each individual from T2D (n = 209) and non-diabetic control (n = 223) groups using ddPCR. The result showed that copy number range of the PTPN22 gene presented obviously different between T2D (CN mean = 4.2) and controls (CN mean = 2.1) (Fig. 2). Further analysis revealed that the CNV distribution reached a highly significant difference (p < 0.01) (Table 1). According to the identification of array/sequencing-based technologies, the CNV genotypes were classified as gain (CN > 2), normal (CN = 2) and loss (CN < 2). Genotypic frequencies of the CN gain, normal, and loss were 61.7%, 11.5%, and 26.8% in T2D group, while the frequencies were 26.4%, 35.9%, and 37.7% in control individuals, respectively (Fig. 3A). The copy number distributions of each CNV genotype between T2D patients and controls were illustrated in Fig. 3B, we found that the PTPN22 copy number of the T2D was higher than that of the control populations in CN gain group, however, similar copy number distributions between T2D and control were identified in CN normal and loss groups. Fig. 3C displayed the copy number of the PTPN22 gene that ranged from 0 to 9 in T2D and healthy controls per diploid genome, respectively. In addition, the individuals with CN (0–2) owned a percentage of 38.3% in T2D patients (CN = 0, 8.1%; CN = 1, 18.7%; CN = 2, 11.5%), while the percentage climbed up to 73.6% in controls (CN = 0, 10.8%; CN = 1, 26.9%; CN = 2, 35.9%) when compared to those with CN > 2 (Fig. 3D), suggesting that the CN gain is the dominant copy number genotype in T2D group.

**Effects of the PTPN22 CNV on clinical parameters**

Before performing the association analysis, we analyzed the relationship between the PTPN22 CNV (CN gain, CN normal, and CN loss) and several clinical characteristics such as gender, age, BMI, duration of diabetes, and medical drug, while non-significant differences were found in T2D and control subjects (Supplementary Table 3 and Supplementary Table 4), suggesting that the clinical effects of the PTPN22 CNV have not been affected by the response to these factors. Next, to further investigate the association of the PTPN22 CNV with T2D susceptibility and determine the causative at-risk genotypes, we established the risk assessment to compare the estimated PTPN22 CNV genotype frequency with T2D, and found that the CN gain (OR = 3.28, 95%CI = 2.076–5.182, p < 0.001) was significantly associated with increased risk of diabetes, even after adjusting for the potential confounders including age, gender and BMI (adjusted OR = 3.35, 95%CI = 2.181–5.092, p < 0.001) (Table 1). In addition, correlation analysis between the PTPN22 CNV and clinical parameters revealed that the copy number of the PTPN22 gene was significantly positive related with the value of fasting plasma glucose (r² = 0.241, p < 0.0001) (Fig. 4A) and glycated hemoglobin (r² = 0.1390, p < 0.0001) (Fig. 4B). However, the PTPN22 CNV showed non-significant
correlation with the fasting plasma glucose and glycated hemoglobin in non-diabetic control subjects ($p > 0.05$, Supplementary Fig. 2).

The levels of plasma glucose and insulin are two important factors to reflect the gluconic metabolism function. Based on the significant association of the PTPN22 CNV with fasting plasma glucose, we hypothesized that the insulin level may be also affected by different PTPN22 copy numbers. Therefore, the association analysis was performed to explore the evidence for linkage between CNV and gluconic characteristics in T2D and control populations. For T2D cases with an OGTT test, the individuals carrying CN gain showed significantly higher levels of plasma glucose than those carrying CN normal at 60-min ($p < 0.05$), 120-min ($p < 0.05$), and 180-min ($p < 0.05$) and CN loss at 60-min ($p < 0.05$)

**Fig. 2** PTPN22 copy number of each individual from the T2D patients and healthy controls. A, the copy number of the PTPN22 gene in T2D individuals ($n = 209$). B, the copy number of the PTPN22 gene in healthy control individuals ($n = 223$).

| CNV type   | T2D (n) | Control (n) | OR (95% CI)   | p-value | Adjusted ORb (95% CI) | p-value |
|------------|---------|-------------|---------------|---------|-----------------------|---------|
| CN Loss    | 56      | 84          | 1             |         | 1                     |         |
| CN Normal  | 24      | 80          | 0.45 (0.255–0.794) | 0.06    | 0.51 (0.287–0.806)    | 0.13    |
| CN Gain    | 129     | 59          | 3.28 (2.076–5.182) | <0.001  | 3.35 (2.181–5.092)    | <0.001  |

*CN represented copy number of the PTPN22 gene.
*a p value was assessed by Yates’ correction of Chi-square test.
*b After adjustment for age, gender and BMI.

Table 1 Association analysis of the PTPN22 CNV types with the type 2 diabetes risk
120-min (p < 0.01), and 180-min (p < 0.01), however, the insulin levels were significantly higher in the cases with CN loss than those with CN normal at 60-min (p < 0.05) and 120-min (p < 0.01) and CN gain at 120-min (p < 0.01) (Fig. 5A and B). The effects of the PTPN22 CNV on total cholesterol, triglycerides, low-density lipoprotein cholesterol and high-density lipoprotein cholesterol were investigated in T2D patients. Significant difference was found between the PTPN22 CNV and total cholesterol, and the CN gain presented higher total cholesterol than the other two genotypes (Fig. 5C). The PTPN22 CNV genotypes were not significantly associated with triglycerides, low-density lipoprotein cholesterol and high-density lipoprotein cholesterol (Fig. 5D, E, F). Also, no significant associations were observed between the PTPN22 CNV and the four clinical parameters in control group (Supplementary Table 5).

**Discussion**

T2D is a complicated disease with high rates of diabetes-related complications [24]. In addition to the environmental factors such as behavioral characteristics, dietary structure and regional differences, genetic com-
ponent also plays an important role in the T2D pathogenesis [25], therefore, identification of the disease specific genetic loci is the crucial step for clinical diagnosis. Our candidate gene approach to disease risk assessment revealed that the PTPN22 CNV was significantly correlated with increased risk of T2D in Chinese population, and the individuals carrying CN gains demonstrated more reactive susceptibility to disease morbidity and progression. Further CNV association analysis with clinical parameters confirmed the positive correlation results. To our knowledge, this is the first case-control study to examine the association of the PTPN22 CNV with T2D risk in Chinese people, which could provide evidence to utilize the PTPN22 CNV as a novel susceptibility marker for T2D diagnosis and a promising target for drug exploration.

CNVs have been identified to provide valuable insights into the genetic basis of many diseases, including colorectal cancer, gastric cancer, congenital heart disorder, myeloma and endocrine diabetes [26-30]. For instance, Arakawa et al. assessed copy number alterations for differentiated-type gastric cancers using a BeadChip array, and found that many CNV loci were associated with disease progression, in which increasing CN numbers were demonstrated from early gastric cancer to advanced gastric period [31]. Further efforts have been made to link DNA copy numbers to endocrine disorders, and the E2 DNA CNV of LEPR gene had a sig-
nificant effect on diabetes-related phenotypes and total cholesterol level that exhibited a negative correlation between copy number and the metabolic traits [14]. These studies suggested that the CNV distribution differences between case and control, at least in part, is necessary for determination of the disease risk loci. Moreover, the divergent CNV locus of the functional gene was considered to be associated with the alternative transcription and translation due to gene dosage effects. The PTPN22 copy number changes may contribute to different metabolic regulations which is account for different susceptibility to T2D. In our study, we found that CN gain of the PTPN22 gene was predominant in T2D patients, while the CN loss accounted for the most in healthy controls, which revealed that the individuals carrying the increasing PTPN22 copy number may be high susceptible population to T2D risk.

Previous studies reported that glucose metabolism, insulin sensitivity and pancreatic β cell function are the most important relevant etiologies in T2D pathogenesis [32-34]. Insulin signaling is critical for physiological processes of glucose utilization, however, the disruption of molecular pathways causes declining insulin sensitivity which underpins many metabolic disorders including T2D. A number of mechanisms have been suggested to aggravate this process and even result in pancreatic β cell dysfunction, such as oxidative stress, insulin receptor mutations, and inflammation [35]. For example, the expression levels of the inflammatory cytokines and cytokine-induced endoplasmic reticulum stress play central roles in β cell demise [36, 37]. The PTPN22 gene is located at chromosome 1p13 encoding for lymphoid-specific phosphatase (LYP), and participates in the regulation of inflammatory response [38]. Numerous studies revealed that the PTPN22 gene was implicated in a number of metabolic and inflammatory diseases. A missense mutation (C1858T SNP) of the PTPN22 gene contributed to altered inflammatory cytokine profiles and progression of rheumatoid arthritis [39]. In addition, this SNP has also been reported to be correlated with several diseases [40], as well as the T2D in an Estonian population [20]. As a structural variability, CNV can directly or indirectly influence gene expression by altering dosage effects and distance position interaction, while the regulatory relation between the PTPN22 CNV and T2D pathophysiology is rarely established. Our case-control study found that the PTPN22 CNV significantly affected the levels of plasma glucose and insulin; as a result, carriers with the CN gain were prone to T2D susceptibility. Scott et al. demonstrated a positive correlation of the PTPN22 gene expression with oral glucose area in a metabolic syndrome mouse model [41]. Inflammation is considered as a link between obesity and T2D [42]. In our study, the PTPN22 CNV was significantly associated with the total cholesterol value of the T2D patients, suggesting the important regulatory role of lipid metabolism in T2D etiology. Based on these data, we hypothesized that the copy number increment of the PTPN22 gene may intensify the inflammatory response and disrupt pancreatic cell function, subsequently result in glucose metabolic disorder and insufficient insulin secretion, especially for the CN gain carriers, finally lead to abnormal cholesterol level. However, the exact molecular mechanism of the PTPN22 CNV on gene expression and the involved functional cell signaling networks on T2D pathogenesis may require further interpretation.

In conclusion, the present study first examined the association of the PTPN22 copy number variation with T2D patients in Chinese population. There was statistically significant difference between the T2D cases and control group with regards to CNV frequencies. We also found that the PTPN22 copy number was positively correlated with fasting plasma glucose and glycated hemoglobin, suggesting that the PTPN22 gene contributed to the regulation of T2D pathogenesis. In addition, for T2D patients with the OGTT test, the PTPN22 CN gain was associated with elevated glucose content and declining plasma insulin in comparison to CN loss and normal. These findings revealed that the PTPN22 CNV could be considered as a new biomarker for susceptibility of T2D. For implication of the CNV locus in clinical diagnosis, further studies with larger cohorts of Chinese and other ethnic populations are needed to clarify the physiological and pathological role of PTPN22 CNV and its molecular mechanisms on T2D pathogenesis.

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Disclosure

None of the authors have any potential conflicts of interest associated with this research.
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