ORIGINAL ARTICLE

Quantification of Beta-Defensins (DEFB) Gene Copy Number Variations in Relation to Inflammation in Type 2 Diabetes Mellitus and Diabetic Nephropathy Patients

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ABSTRACT

Introduction: Association studies between single nucleotide polymorphisms (SNPs) and type 2 diabetes mellitus (T2DM) have been abundant. However, there are limited reports on copy number variations (CNVs) of beta-defensins (*DEFB*) gene in relation to T2DM. In this study, *DEFB* copy numbers were quantified in T2DM with nephropathy, T2DM without nephropathy and non-diabetic control groups to investigate its influence in chronic inflammation in Malaysian individuals. **Methods:** *DEFB* copy number in Malaysian individuals were quantified by using paralogue ratio tests (PRT) which allow direct quantification of gene copy number by using PRT107A and HSPD21 PRT primers. The copy number generated was then validated from insertion/deletion ratio measurement 5DEL (rs5889219) and two microsatellite analyses (EPEV-1 and EPEV-3). **Results:** *DEFB* copy number was found extending from 2 to 8 copies in the non-diabetic group (n=146), while in T2DM group (n=392), copy numbers were more extensive, ranging between 1 and 12 copies; with 1, 10 and 12 copies detected in T2DM with nephropathy group (n=202). Statistically, there is no significant difference in *DEFB* copy number between T2DM and the non-diabetic group (p=0.209) as well as between diabetic nephropathy and without nephropathy of the T2DM group (p=0.522). However, significant white blood cell (WBC) count was found between T2DM groups with and without diabetic nephropathy (p=0.000). **Conclusion:** Extreme *DEFB* copy numbers in T2DM with nephropathy group suggest future studies with bigger sample size are necessary to elucidate the true impact of CNVs of *DEFB* gene in promoting early onset of nephropathy in T2DM.

Keywords: Beta-defensins, CNVs, Diabetes, Nephropathy, Paralogue ratio test

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INTRODUCTION

Diabetes mellitus affects 422 million adults globally in 2014, compared to 108 million in 1980, causing prevalent morbidity and mortality cases in middle- and low-income countries (1). In Malaysia, the prevalence of type 2 diabetes mellitus (T2DM) showed alarming increase from 11.6% in 2006 to 15.2% in 2011 and further escalates to 17.5% in 2015 which involved 3.5 million adult population of 18 years and above (2). Moreover, 40-60% of T2DM individuals in Malaysia developed diabetic nephropathy (DN) which may progress into end-stage renal disease (ESRD) and leading to death (3).

Numerous studies have been conducted to investigate the role of genetic variants in increasing individual's risk of T2DM. Significant evidence suggested strong genetic influence underlies this this multifactorial disease as monozygotic twins shares 96% concordance rate for genetic contribution towards T2DM (4). The advancement in technology enable genome-wide association scans (GWAS) which confirmed nine loci (*PPARG, KCNJ11, TCF7L2, CDKAL1, CDKN2A/B, IGF2BP2, HHEX/IDE, FTO* and *SLC30A8*) with susceptibility towards T2DM from the aspect of single nucleotide polymorphisms (SNPs) (4). However, recent studies suggest that common variants also have compelling effect on pathophysiology of the disease, apart from the rare variants which could only be found in certain individuals or populations (4). As (GWAS) catered for large-scale genotyping, there is limited knowledge on the true potential of common variants involving copy number variations (CNVs) in

disease and complications prediction. (4, 17).

The role of beta-defensins in inflammatory conditions has been described in previous studies. Defensins are small, cationic, cytotoxic, antimicrobial and immunoregulatory peptides that function as the innate defence mechanism by inducing production of pro-inflammatory cytokines and activation of complement systems (5). Beta-defensins exhibit copy number variations (CNVs) which commonly ranging between 2 and 7 copies in healthy Caucasian population (6) and has been associated with susceptibility towards inflammatory conditions including psoriasis (7), Crohn's disease (8, 9), HIV (10), adenocarcinoma (11) as well chronic obstructive pulmonary disease (COPD) (12) through alteration of gene dosage.

Nevertheless, accurate copy number quantification of *DEFB* is technically demanding due to the complex and extensive duplication of *DEFB* gene (13, 14). Therefore, paralogue ratio test (PRT) was used in this study as a mean of robust and accurate copy number quantification of the multiallelic *DEFB* (15, 16) which overcame the limitations reported for several measurement techniques, including quantitative real-time PCR (qPCR) (14).

As most studies on CNVs of *DEFB* investigated complex diseases, efforts in unveiling the association with common diseases are still needed. Thus, it is important to further characterize this variant especially in Malaysian population, which has not been genotyped before. In this study, we aimed to quantify CNVs in *DEFB* gene by using PRT system validation by indel and microsatellite analysis to ensure final copy number were inferred with high confidence. At the same time, the role of CNVs in T2DM and diabetic nephropathy were further elucidated.

MATERIALS AND METHODS

Ethical Approval and Subjects Recruitment

The study protocol obtained approval from Ministry of Health Malaysia (NMRR 13-1614-16772). Informed consent was obtained from all study subjects. A total of 146 non-diabetic controls were recruited at health check-up centre Hospital Tuanku Jaafar and Universiti Putra Malaysia (UPM) were comprised of 51 Malay, 53 Chinese and 42 Indian individuals. On the other hand, T2DM individuals were recruited from Hospital Tuanku Jaafar, Seremban, Negeri Sembilan were consist of 137 Malay, 124 Chinese and 131 Indian individuals. Out of 392 T2DM samples, 202 individuals have been diagnosed with diabetic nephropathy while 190 individuals were free of it at the time of sample collection. All subjects enrolled in this study are Malaysian and includes both male and female (Table I).

DEFB copy number from Human Random Control (HRC) DNA Panel 1 and 2 from European Collection of Authenticated Cell Cultures (ECACC) samples of Caucasian origin were directly used as the copy number were known from previous typing (14, 16). In this study, the copy number was used to construct copy number distribution for Caucasian population. At the same time, several samples were selected as internal control (reference samples) to infer copy number for the unknown samples by deriving a linear regression equation from each PRT systems (7, 16).

Table I Distribution of T2DM and non-diabetic control samples based on gender and age

	Number (Age range, years)		
	T2DM with nephropathy	**T2DM without nephropathy**	**Non-diabetic controls**
Female			
Malay	27(45-84)	22 (47-70)	25 (23-41)
Chinese	33 (37-83)	31 (36-83)	26 (23-35)
Indian	37 (45-75)	28 (45-86)	21 (22-50)
Male			
Malay	46 (46-80)	41 (44-78)	26 (23-47)
Chinese	27 (42-86)	33 (48-89)	27 (24-41)
Indian	32 (47-76)	33 (46-82)	21 (24-55)
Total	202	188	146

N = 536

Genomic DNA extraction

QIAamp DNA Mini Kit (QIAGEN, Germany) was used to extract DNA from 200 uL of peripheral blood collected in BD Vacutainer® K2 EDTA tubes according to manufacturer's protocol. The concentration and purity of the extracted DNA were quantified by using Nanodrop ND-1000 spectrophotometer (Thermo Scientific, USA).

Paralogue ratio test (PRT) systems

Paralogue ratio test (PRT) is a robust technique designed to reliably genotype multiallelic CNVs using small quantity of genomic DNA in large sample size (7, 15). In order to determine *DEFB* copy number, PRT systems were carried out as described by Aldhous et al. (14) and Abu Bakar et al. (16) which can simultaneously amplify the test and reference loci in a single PCR reaction. Since one of the PRT primer (either forward or reverse) were fluorescently-label, it enables the test and reference amplicons to be separated by capillary electrophoresis, generating peaks of the amplified PCR products. The peak heights and values were then compared for copy number determination (7, 15). Due to the nature of *DEFB* gene cluster (consisting of seven genes), the segmental duplication (SD) that span this region theoretically means that each *DEFB*-specific gene exists in same number of copies. Therefore, 2 PRT primers (PRT107A and HSPD21) were previously designed to measure gene copy number at specific *DEFB* gene (*DEFB*107 and *DEFB*4) at each end of the *DEFB* repeat unit to ensure precise copy number quantification for each sample (14, 16).

PRT107A amplifies a test locus downstream of *DEFB*107 on chromosome 8 and its reference paralogue on chromosome 11, whereas HSPD21 amplified a target locus upstream of *DEFB*4 and its reference paralogue on chromosome 21 (14, 16). The test and reference paralogues were determined using PRT primer design tool (PRTPrimer, University of Leicester). The test and reference paralogues shared similar sequences but only to differ by a single nucleotide change and the reference paralogue should not map on a copy-variable region to ensure relative comparison of copy number between paralogues can be obtained in a single amplification reaction (7, 15).

The PRT primers were fluorescently labelled: FAM/HEX for PRT107A and FAM/TAMRA for HSPD21, to allow duplication as well as aiding in calibration of the experiment. Multiplex PCR was conducted by preparing a total of 10 uL reaction mixture consisting of 1 uL (10 ng) DNA, 5 uL of 2X Prime Taq Premix (Genet Bio, Korea), 0.5 ul (10 uM) of each of forward and reverse primers for PRT107A and HSPD21 and 2 uL of double-distilled water (ddH2O). DNA samples were amplified in a pre-denaturation step at 95°C for 5 minutes and followed by repeated cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 70°C for 1 minute for 25 cycles. This cycle was followed by another annealing step at 58°C for 30 seconds and final extension at 70°C for 40 minutes.

5DEL (rs5889219) system

An indel known as 5DEL (rs5889219) was adapted (14, 16) to validate integer copy number quantified by PRT systems. DNA samples were amplified in 28 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 70°C for 1 minute, followed by another annealing step at 58°C for 1 min and final extension at 70°C for 20 minutes. A single PCR reaction was prepared in a total of 10 uL of 1 uL (10 ng) DNA, 5 uL of 2X Prime Taq Premix (Genet Bio, Korea), 0.5 ul (10 uM) of each of forward and reverse 5DEL primers and 3 uL of ddH2O.

Triplex assay

Amplification by both PRT systems was carried out in a duplex PCR reaction, while 5DEL was run in a separate reaction. In a Triplex assay, amplification products from PRT107A, HSPD21 and 5DEL were combined. 1 µl of each PCR products was taken from each plate and mixed with ROX marker and HiDi formamide in a separate 96-well plate before being resolved by capillary electrophoresis. Amplification by PRT107A resulted in amplicons sized at 155 bp (chromosome 11) and 157 bp (chromosome 8) while HSPD21's amplicons sized at 180 bp (chromosome 21) and 172 bp (chromosome 8). Peak heights and values from 155 bp and 157 bp and 180 bp and 172 bp, were compared respectively for each PRT primer. Mean ratio from dual fluorescent for each PRT primer were used during the analysis. A total of four HRC DNA samples were used to calibrate each experiment resulting in linear regression to infer *DEFB* copy number from this assay. On the other hand, amplification by 5DEL resulted in amplicons sizing at 125, 128 and 130 bp length. The genomic ratio of each peaks to the smallest peak were taken resulting in estimated copy number (Fig. 1).

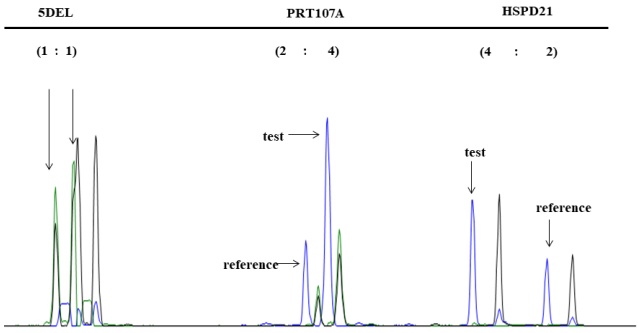

Figure 1: Electropherogram as recorded by Peak ScannerTM for a 4-copy sample typed by Triplex assay. Test and reference peaks for PRT systems for FAM-labelled products are shown by arrows. For HEX–labelled 5DEL system, ratio of each peak to the smallest peak (1:1) was taken and multiplied by a factor of 2 to reflect copy number of 4 as typed by PRT systems.

Microsatellite analysis

Two sets of primers; EPEV-1 (6) and EPEV-3 (16) were used to amplify the microsatellite alleles. The inferred copy number from Triplex assay and microsatellite analysis were analysed based on the single system analysis. Both primers were fluorescently labelled with HEX to perform the capillary electrophoresis. DNA was amplified in 25 cycles of denaturation at 95°C for 1 minute, annealing at 58°C for 1 minute and extension at 72°C for 1 minute was carried out. 1 µl of each PCR products was taken from each plate and mixed with ROX marker and HiDi formamide before being resolved in capillary electrophoresis. EPEV-1 yielded alleles of different lengths ranging between 171 bp – 193 bp while alleles from EPEV-3 amplification ranged between 133 bp – 147 bp. The peak height ratios of all alleles to the smallest allele were taken and total allelic ratio resulted in integer copy number which further used to validate inferred copy number from PRT systems. The primer sequences used in this study are listed in Table II.

Statistical analysis

In this study, student's t-test were used to compare *DEFB* mean copy number for four assessments: 1) between HRC ECACC (Caucasian) and non-diabetic control (Malaysian), 2) between non-diabetic control and T2DM groups, 3) between T2DM with and without nephropathy and 4) between male and female groups. Next, one-way ANOVA was used to compare mean copy number among the three major ethnicities of Malaysian individuals for both control and T2DM groups. In addition, the relationship between *DEFB* copy number and white blood cell (WBC) count T2DM group was tested by using Pearson correlation.

Table II Primer sequences for DEFB copy number quantification.

Validation	Primer name	Sequences
Paralogue ratio test (PRT)	PRT107A	FAM- or HEX-labelled forward: 5′-AGCCTCATTTAACTTTGGTGC-3′ Reverse: 5′-GGCTATGAAGCAATGGCCTA-3′
	HSPD21	Forward: 5′-GAGGTCACTGTGATCAAAGAT-3′ FAM- or TAMRA- labelled reverse: 5′-AACCTTCAGCACAGCTACTC-3′
Indel	5DEL	HEX- or TAMRA- labelled forward: 5′-AAACCAATACCCTTTCCAAG-3′ Reverse: 5′-TTCTCTTTTGTTTCAGATTCAGATG-3′
Microsatellite analysis	EPEV-1	HEX-labelled forward: 5′-GGCAGTATTCCAGGATACGG-3′ Reverse: 5′-GAACAATTAGATATCCCTATGC-3′
	EPEV-3	HEX-labelled forward: 5′-GATACTGTGAACTACAGATCAC-3′ Reverse: 5′-CTGCCCTGATTCAGTATTGAAC-3′

RESULTS

Typing analysis of DEFB copy number in non-diabetic control group

DEFB copy number were successfully typed in a total of 146 non-diabetic control. The copy number for non-diabetic controls were first established in this study prior to T2DM and diabetic nephropathy to compare the distribution between both groups. By using single system analysis, 71% (104/146) samples was recorded for measurement of unrounded copy number by PRT107A and HSPD21 in non-diabetic control group with 29% of the samples were rejected due to the stringent criteria in PRT measurement. A linear adjustment was applied to PRT107A measurement due to the shifting involving PRT107A measurement which were also reported in previous studies (14, 16). The unrounded copy number were compared in a histogram (Fig. 2A). A mean copy number of 4.19 was observed in a subset of 104 non-diabetic samples. The unrounded copy number was transformed to the closest integer which caused a substantial difference in frequency for copy number 2, 3, 4, 6 and 7 between both PRTs recorded with 58.65% concordance rate (Fig. 2B). Quantification by 5DEL and microsatellites were carried out to carefully analyse the peak traces in all 146 non-diabetic samples inclusive of the rejected samples from PRT system measurement. Thus, a distribution of copy number for a total of 146 non-diabetics produced a distribution between 2 and 8 copies with a modal diplotype of 4 recorded in the non-diabetic group (Fig. 2C).

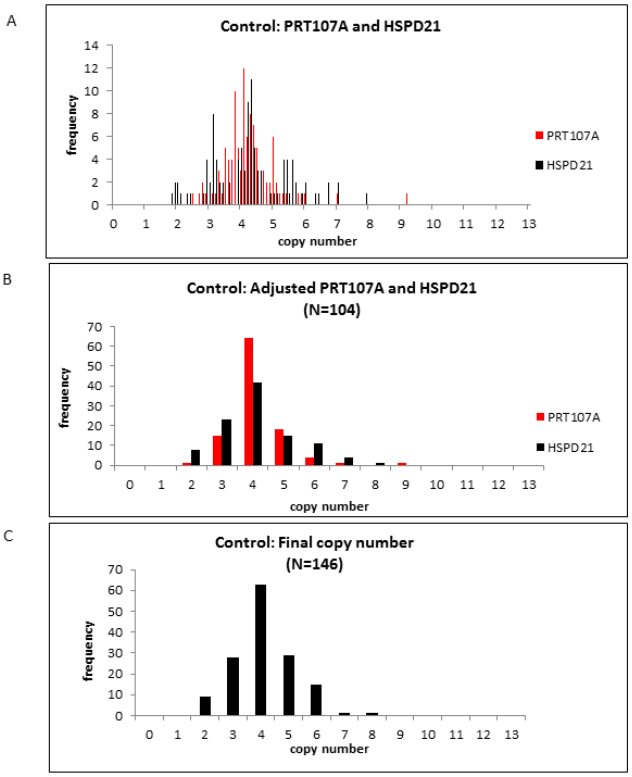

Figure 2: Copy number distribution in non-diabetic control group. A) Histogram of unrounded copy number showed copy number measurement yielded by both PRT systems through comparison of test and reference amplification products. B) Histogram of integer copy number using both systems were obtained when the unrounded copy number were rounded. Both measurements agreed at modal copy number of 4, with overestimation depicted by PRT107A measurement. C) Distribution of final copy number was obtained after validation of PRT systems with 5DEL and microsatellite analysis.

Based on our findings, there was more individuals with >4 copies (31.5%) than <4 copies (23.5%) of *DEFB* gene. As this is the first study in Malaysia which involved typing of the multiallelic *DEFB* gene copy number, the distribution obtained was compared to a different population to check if differences exist in distribution of *DEFB* copy number. Therefore, the copy number of the non-diabetic group was compared to ECACC samples (an established copy of Caucasian healthy population) which was readily available from previous typing and has been published (14, 16). Distribution of *DEFB* copy number was compared in a histogram to assess the extent of copy number in healthy groups from different populations. Based on our analysis, both groups (European Caucasian; 2 to 9 copies) and non-diabetic control (Malaysian; 2 to 8 copies) shared similar pattern of distribution with similar modal copy number of 4 ($p=0.377$, t-test) (Fig. 3).

We also analysed the comparison among the ethnicities to investigate if different ethnicities may have different distribution pattern. Based on our findings, the copy number distribution among the three major ethnicities from Malaysian non-diabetic control samples shared a

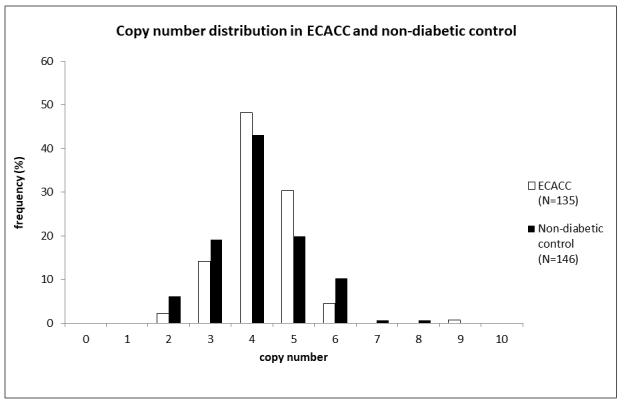

Figure 3: Histograms of integer copy number between ECACC (European Caucasian) and non-diabetic (Malaysian) control groups. This step is taken to validate the distribution of DEFB copy number as the first typing made in Malaysian control population.

modal copy number of 4. Despite the increased mean copy number in Chinese (4.19) compared to Indian (4.14) and Malay (4.08), there is no significant difference in mean copy number distribution among ethnics from the non-diabetic population (p=0.878, ANOVA). Our analysis on copy number distribution on gender factor also showed that male have higher mean copy number (4.23) than female (4.04), yet no significant difference was observed (p=0.303, t-test).

Typing analysis of DEFB copy number distribution in T2DM group

DEFB copy number were quantified in 392 T2DM samples which was further divided based on their nephropathy status (202 with nephropathy, 188 without nephropathy and 2 samples with unknown nephropathy status). Unrounded copy number by PRT107A and HSPD21 was successfully produced in 90.8% (356/392) T2DM samples using PRT systems with 9.18% rejections after the criteria have been applied. Histogram of unrounded copy number by both PRT systems demonstrated clusters for copy number 3, 4, 5 and 6 regardless of the loose clustering pattern which caused overlapping between copy number 4, 5 and 6. Besides that, the distribution of copy number was widely disseminated involving samples with higher copy number. However, an overestimation by 1.5 copies was recorded in PRT107A after linear adjustment was applied compared to HSPD21 for copy number 2 and 3, while loose cluster pattern remained for copy number 6 and above (Fig. 4A).

PRT measurement showed that T2DM groups shared similar copy number of 4 with mean copy number of 4.38 in 356 T2DM individuals with 38.52% concordance (Fig. 4B). Similar approach was applied on T2DM cohort by validating PRT-inferred copy number from 356 samples as a part of Triplex assay. Through careful analysis, a subset of 53 samples required clarification by microsatellite analysis to confirm final copy number. In addition, 36 samples which had been previously

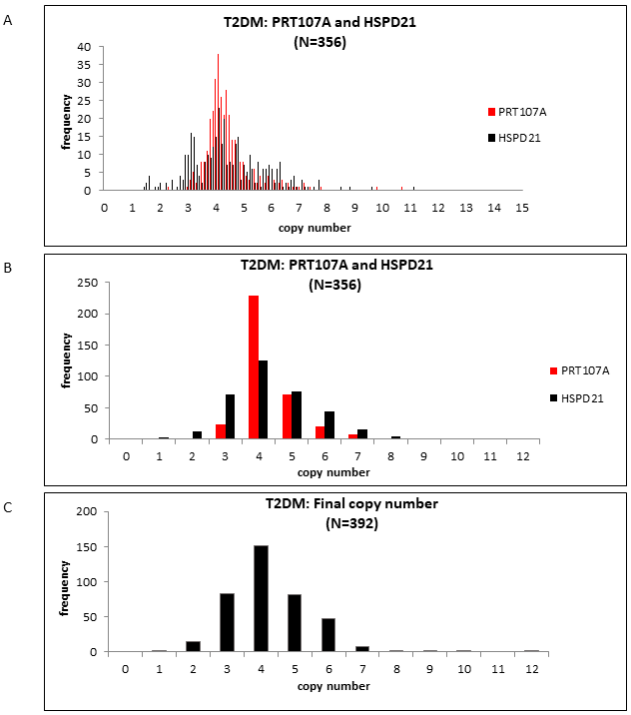

Figure 4: Copy number distribution in T2DM group. A) Histogram of unrounded copy number showed copy number measurement yielded by both PRT systems through comparison of test and reference amplification products. B) Histogram of integer copy number using both systems were obtained when the unrounded copy number were rounded. Both measurements agreed at modal copy number of 4. Again, overestimation of 4 copies can be observed in PRT107A measurement. C) Distribution of final copy number was obtained after validation of PRT systems with 5DEL and microsatellite analysis.

removed during PRT measurement were included again and the peak traces from the electrophoresis were carefully analysed. Hence, a distribution of copy number for a total of 392 T2DM individuals was produced in Malaysian individuals, showing that *DEFB* copy number varies extensively between 1 and 12 copies with a modal copy number of 4 (Fig. 4C).

There were more individuals with >4 copies (36.2%) than <4 copies (25.2%) of *DEFB* gene. Nevertheless, copy number distribution between non-diabetic controls and T2DM were not significantly different in integer (p=0.209, t-test) copy number measurement (Fig. 5A).

Although there is an increased mean unrounded copy number observed in Malay (4.46), followed by Indian (4.21) and Chinese (4.18), there was no significant difference in mean copy number distribution observed among these ethnicities from T2DM group (p=0.132, ANOVA). Likewise, male with T2DM showed increased mean copy number (4.31) than female (4.26). Yet, there was no significant difference observed (p=0.740, t-test).

We also found a more extreme variation between 1 and 12 copies in T2DM with nephropathy compared to 2 and 9 copies in T2DM group without nephropathy (Fig. 5B). Individuals with nephropathy showed a

higher white blood cell (WBC) count and mean copy number (10.204x109 cell/L; mean copy number 4.33) compared to those without nephropathic complications (8.487x109 cells/L, mean copy number 4.24). However, the mean copy number does not significantly differ between these two groups (P=0.522, t-test) and there was no correlation found between WBC count and copy number among T2DM individuals (r=0.047, p=0.367) despite the significant increase in mean WBC count in individuals with diabetic nephropathy (p=0.000, t-test).

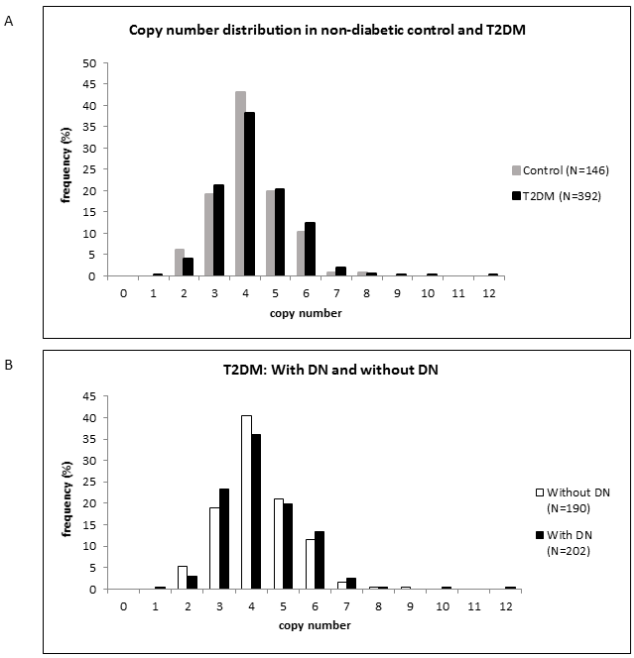

Figure 5: Histogram of integer copy number involving T2DM group. A) Copy number distribution in non-diabetic control and T2DM group, and B) in T2DM group with and without diabetic nephropathy (DN). Note that the copy number is more extensive in T2DM group, particularly the group with DN with extremely low copy number (1 copy, denoting deletion in one of the gene copy) and high copy numbers (10 and 12 copies).

DISCUSSION

Current understanding highlights the importance of *DEFB* copy number influence in inflammatory diseases. However, there are limited studies conducted to explore its influence in T2DM as well as diabetic nephropathy as most studies were revolving around alpha-defensins (DEFA) CNV which has established association with the kidney. Apart from that, we are also hampered by the limited references in Asian populations, particularly on Malaysian population.

In this study, we quantified *DEFB* copy number from genomic DNA extracted from peripheral blood to investigate *DEFB* copy number distribution in both non-diabetic and T2DM Malaysian individuals. By using a combination of Triplex assay and microsatellite analyses, a careful analysis was conducted to carefully assign the copy numbers, yielding a distribution of 2 to 8 copies in the non-diabetic samples. Thus, our results support the previously detected beta-defensins CNV loci through SNP array to detect CNV in Singapore population of Malay, Chinese and Indian (17). However, the range of copy number generated from Malaysian population show consistency with UK's British population even though the distance between these countries is 10,576 km apart. It is still unclear whether the similar pattern is due to heterogeneous sampling of combining Malay, Chinese and Indian or due to the small sample size (18). Meanwhile, our typing showed an extreme variation in T2DM samples which is considerably different than the non-diabetic individuals, yet not significant enough. From our observation, the number of T2DM individuals having >4 copies of *DEFB* is higher than those having <4 which may hint that high copy number of *DEFB* might predispose our population to T2DM which is also increasing every year. Mean copy number is also higher in individuals with diabetic nephropathy compared to those without. Therefore, this is consistent with previous findings of high copy number in chronic inflammatory diseases as seen in psoriasis (7), systemic lupus erythematosus (SLE) and antineutrophil cytoplasmic antibodies (ANCA)-associated small vasculitis (19), higher HIV load (10) and acne inversa (20). There is also evidence of inducible human beta-defensins 2 (HBD-2) gene expression in chronic bacterial infection which is absent in normal kidney condition (21) which may highlight the unfamiliar role of *DEFB* in inflamed kidney tissues.

The signal transduction of white blood cell for recruitment towards inflammatory sites is heavily influenced by HBD antimicrobial peptide which modulate secretion of cytokines (5). Based on this principle, we hypothesized that *DEFB* copy number might be correlated with WBC count screened from T2DM individuals. However, there was no correlation between WBC and *DEFB* copy number found, despite the significant increase of WBC count in nephropathic individuals. For future studies, quantification of HBD2 concentration is necessary to study the correlation with *DEFB* copy number as well as WBC count.

Based on the study that have been conducted, there are several limitations that were encountered. Firstly, the small sample size for non-diabetic populations may not be able to truly represent the Malaysian population. As the copy number of *DEFB* copy is already extensively variable, inclusion of more samples might be able to reflect the true genetic makeup by including not only the major ethnicities, but also the minorities, including the aborigines. Bigger sample size may increase possible inclusion of the less encountered individuals with high copy numbers.

Secondly, the establishment of non-diabetic sample was made by comparison against the Caucasian sample as there is a lack of complete reference copy number available from another heterozygous population. However, the study proceeded considering that both

populations were typed using similar PRT method.

Thirdly, there is a gap in direct correlation between *DEFB* CNV and HBD2 which was not assessed in this study. We would recommend further investigations into HBD2 dosage through common techniques such as enzyme-linked immunosorbent assay (ELISA) or a gene expression study to judge the level of functional mRNA, in relation to *DEFB* CNV.

CONCLUSION

From this study, PRT has once again proven to be a robust and simple method in accurate quantification involving complex, multiallelic and extensive copy number as seen in *DEFB* gene for huge sample size. The precise quantification is convincing to develop it as one of the diagnostic tools in targeting risk factors for T2DM and diabetic nephropathy. Hence, intervention measures can be taken in high risk populations. As we study the *DEFB* gene, we also see that there is a possibility that HBD can be a biomarker of inflammation, which can be a channel towards specific treatment in controlling the chronic inflammation, not only in T2DM, but also in other inflammatory disorders. Although there was no significant association was established between *DEFB* CNV and T2DM from this study, extreme *DEFB* copy numbers found in T2DM with nephropathy group suggest that future studies with bigger sample size are necessary to elucidate the true impact of CNVs of *DEFB* gene in promoting early onset of nephropathy in T2DM.

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