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Optimization of PCR conditions to detect susceptibility genes, *KCNQ1*, *TCF7L2*, and *DPP4*, for type 2 diabetes mellitus in Filipinos

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Type 2 diabetes mellitus (T2DM) is a chronic disease that is increasing at an alarming rate in Asian countries including the Philippines. The aim of this study is to optimize PCR conditions to detect SNPs within the susceptibility genes *KCNQ1*, *TCF7L2*, and *DPP4* and to verify if mutations within these genes are targeted. PCR conditions were optimized for each of the genes using gene specific primers. These conditions were then used to detect single nucleotide polymorphisms (SNPs) in each of the susceptibility genes of the selected samples. Gene specific primers were designed based on the translated regions of the target genes. For *TCF7L2*, F/R (GGCTTTCTCTGCCTCAAAACC/ACTAAGGGTGCCTCATACGG); for *DPP4* - F/R (CCCAGGTTCGCTGACAAATC/TCATTCCACGGTTGCAGGTG) and for *KCNQ1*- F/R (GTAAGCAGATGACAGGGCAGT /TAAAGGTCCT GACCCCCACC). Template DNA was obtained from patients of the University of Santo Tomas Hospital (USTH). The final concentration of the PCR components are: 1X 2X Taq Master Mix, 10 μM forward and reverse primers, and 2ng DNA template. For the PCR conditions: initial denaturation, 3 min; 35 cycle of denaturation, 1 min, annealing, 1 min, elongation, 1 min and; final elongation of 5 min. The annealing temperatures were optimized at 58.3°C for *KCNQ1* and 54.5°C for *TCF7L2*. The genes were successfully amplified giving the correct fragment lengths. The designed primers and PCR conditions for *KCNQ1* and *TCF7L2* were effectively used to verify the SNPs within the susceptibility genes.

Keywords: T2DM, PCR, SNPs, primers, *KCNQ1*, *TCF7L2*, *DPP4*

Introduction

Type 2 diabetes mellitus (T2DM) is a serious metabolic disease associated with an increased risk of premature death and substantial disability, largely mediated through its adverse effects on the vasculature. The prevalence of the disease is increasing, and the World Health Organization estimates suggest that by 2025 there will be 300 million affected individuals worldwide [1].

The disorder is characterized by a combination of impaired insulin secretion and insulin action, both of which precede and predict the onset of disease [2]. In recent years, molecular approaches have been used in the diagnosis and prediction of susceptibility to diseases such

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as in the different forms of cancers, chronic obstructive pulmonary as well as T2DM. Single nucleotide polymorphisms (SNPs) is one such approach for which candidate genes have been strongly associated in disease occurrence in various races and populations. SNPs occur normally throughout a person's DNA, once in every 300 nucleotides on average. They are utilized as genetic markers, helping scientists locate genes that are associated with disease [3].

The use of polymerase chain reaction (PCR) and its downstream applications were employed to detect the presence of candidate genes in order to establish the association with T2DM. Association studies using the genes, *DPP4*, *KCNQ1* and *TCF7L2* have already been strongly implicated with increased risk of T2DM in Malaysian, Chinese, and Japanese individuals [8, 15, 17]. In this study, the detection of these genes in Filipino diabetic and non-diabetic patients is optimized.

Dipeptidyl peptidase 4 (DPP4), which is also known as CD26, is a ubiquitously expressed glycoprotein of 110 kDa, which was first characterized by Hopsu-Havu and Glenner [4]. The human DPP4 gene is located on chromosome 2, spans 70 kb, and consists of 26 exons. The importance of *DPP4* for the scientific and medical community raised substantially since the approval of *DPP4* inhibitors for the treatment of T2DM [5].

The transcription factor-7-like-2 (*TCF7L2*) gene encodes the transcription factor 4 (TCF4) which is involved in Wnt signaling. In the nucleus, stabilized β-catenin binds to TCF transcription factors to regulate the transcription of Wnt target genes. The Wnt/β-catenin signaling pathway affects pancreatic β-cell development and function, and thus affects glucose metabolism. *TCF7L2* gene has been consistently associated with type 2 diabetes mellitus (T2D) in different ethnic groups [6].

Potassium voltage-gated channel subgamily Q member 1 (*KCNQ1*) gene has a total of 17 exons, spans 404 kb of chromosome sequence and is located on chromosome 11p15.5. It encodes a pore-forming subunit of a voltage- gated K+ channel (KvLQT1) and salt transport in epithelial tissues. *KCNQ1* was reported to be expressed in insulin-secreting cells and inhibition of this potassium channel has been shown to significantly increase insulin secretion [7].

The study, therefore, aims to optimize PCR conditions in the detection of the susceptibility genes *KCNQ1*, *TCF7L2*, and *DPP4* to establish their association with the development of T2DM.

Experimental

Samples and DNA extraction. DNA of whole blood samples was obtained from three diabetic and three nondiabetic patients of the USTH. The protocol was approved by the Faculty of Pharmacy Ethics Review Committee. Total genomic DNA was extracted using the Vivantis (Malaysia) GF-1 Blood DNA Extraction Kit and the DNA were kept at –20°C for storage.

Oligonucleotide primers. Primers were designed based on the translated regions of the target genes. The designed primers were synthesized by Integrated DNA Technologies, Singapore. The primer pairs Forward/Reverse (F/R) for each gene are as follows: For *TCF7L2*, F/R(GGCTTTCTCTGCCTCAAAACC/ACTAAGGGTGCCTCATACGG); for *DPP4* - F/R (CCCAGGTTCGCTGACAAATC/TCATTCCACGGTTGCAGGTG) and for *KCNQ1*- F/R (GTAAGCAGATGACAGGGCAGT / TAAAGGTCCT GACCCCCACC).

Polymerase chain reaction. PCR was carried out based on manufacturer's recommendation (Vivantis, Malaysia), using gradient PCR in Bio-Rad thermal cycler with DNA Template

concentration, 0.02–5 mg; primer concentration, 0.1–1.0 µM and; 2X *Taq* Master Mix concentration (1X). The primer concentrations used in PCR were optimized to 10 mM. The concentration of the 2X *Taq* master mix was kept constant. The DNA template concentrations used in the PCRs were optimized to 4ng per reaction. To obtain the optimum annealing temperature for each primer pair, gradient PCR was used as listed in Table 1.

Agarose gel electrophoresis. A Vivantis VC 100bp Plus DNA Ladder (Malaysia) was used and 3% agarose gel electrophoresis was run at 100 volts for 30 min. The gel was placed in a UV tray and viewed using Gel Doc Ez imager (California).

DNA purification and sequence analysis. PCR products of successful amplifications were sent to First Base Sdn. Bhd., Malaysia for DNA sequencing after purification using the Vivantis Purification Kit GF-1 Ambiclean Kit (Malaysia). The sequences were analyzed using the Basic Local Alignment Search Tool BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results and Discussion

Selection of target genes and SNP loci. Studies (Table 2) show that different races and populations have single nucleotide variation found strongly associated with T2DM. The choice for target genes and SNP loci (from dbSNP) were based on the strongest association studies: for *KCNQ1*, A/G in rs2237895 [12]; for *TCF7L2*, C/T in rs7903146 [17] and; for *DPP4*, A/G in rs4664443 [8].

Primer design. Primers for each of the T2DM susceptibility genes were designed based on the published sequences of the genes, considering the SNP loci from the SNP database. For *KCNQ1*, forward primer: 5'-GTAAGCAGATGACAGGGCAGT-3', reverse primer: 5'-TAAAGGTCCTGACCCCCACC-3', with a product length of 188bp. For *TCF7L2*, forward primer: 5'-GGCTTTCTCTGCCTCAAAACC-3', reverse primer: 5'- ACTAAGGGTGCCTCATACGG-3', with a product length of 109bp. For *DPP4*, forward: 5'-CCCAGGTTCGCTGACAAATC-3', reverse primer: 5'-TCATTCCACGGTTGCAGGTG-3' with a product length of 133bp. Primers were verified using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

BLASTn. The primer sequences subjected to the BLASTn, showed degree of alignment and complementarity of primers with target genes primarily in *homo sapiens* and other species as shown Fig. 1.

Table 1. Gradient PCR

PCR Conditions	Temperature (°C)	Time
Initial denaturation	94°C	2 min
Cycles: 35		
Denaturation	94°C	2 sec
Annealing	45, 50, 55, 60°C	1 min
Elongation	72°C	30 sec
Final extension	72°C	7 min
	4.0°C	∞

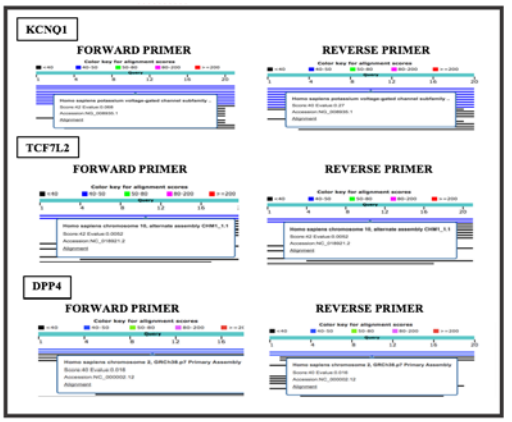

Figure 1. BLASTn

Table 2. Reference SNP for candidate genes associated with T2DM

Nationality	Gene	Chromosome	dbSNP	Location	Allele	Reference
Malaysia	DPP4	2	rs12617656 **rs4664443** rs7633162	45, 164	A>G	[10]
China	DPP4	2	**rs4664443** rs3788979 rs7608798 rs1558957	45, 164	A>G	[11]
China	KCNQ1	11	rs2074196 rs2237892 **rs2237895** rs2237897	390, 974	A>C	[12]
China	KCNQ1	–	rs151290			[13]
Malaysia-China	KCNQ1	11	rs2237892 rs2283228 **rs2237895**	390, 974	A>C	[14]
East Asia and Europe	KCNQ1	11	rs2283228 **rs2237895** rs2237897	390, 974	A>C	[15]
Korea	KCNQ1	–	rs1111875 rs2237892 rs13266634			[16]
China	KCNQ1	11	rs2237892 **rs2237895** rs2237897	390, 974	A>C	[17]
China	KCNQ1	11	rs2237892 **rs2237895**	390, 974	A>C	[18]
Japan	TCF7L2	10	rs12255372 rs7903146 **rs7901695** rs11196205	48, 372	C>T	[19]
China	TCF7L2	10	rs12255372 rs7903146 **rs7901695** rs11196205 rs7895340	48, 372	C>T	[20]
Northern Sweden	TCF7L2	10	**rs7901695** rs7903146 rs12255372	48, 372	C>T	[21]
Europe	TCF7L2	10	rs7903146 rs12255372 **rs7901695**	48, 372	C>T	[22]
North India	TCF7L2	10	rs4506565 **rs7903146**	48, 372	C>T	[23]
East Asia	TCF7L2	10	**rs7903146** rs12255372 rs11196205 rs290487	48, 372	C>T	[24]
United States	TCF7L2	10	rs12255372 **rs7903146**	48, 372	C>T	[25]
Finland	TCF7L2	10	rs12255372 **rs7903146**	48, 372	C>T	[26]
India	TCF7L2	10	**rs7903146** rs12255372 rs4506565	48, 372	C>T	[27]

Optimization

PCR components. The optimized PCR volume and concentration for each PCR component is presented in Table 3. Each reaction, with a total volume of 50 µL, contained 1X 2X *Taq* master Mix (Vivantis, Malaysia), 10 µM each of the forward and reverse primers, 2 ng template DNA.

PCR Conditions. Final PCR conditions showing optimum annealing temperature used for each gene is shown in Table 4. Gradient PCR was done using annealing temperature ranges (in C): 45, 50, 55 up to 60. The optimized annealing temperature for *KCNQ1* is 58.3°C and 54.5°C for *TCF7L2*. The initial denaturation was for 3 min, and 35 cycles of: denaturation at 94°C for 1 min; annealing at 58.3°C for *KCNQ1* for 1 min and at 54.5°C for *TCF7L2* for 1 min and; extension at 72°C for 1 min. The final elongation was at 72°C for 5 min.

Table 3. Optimized volume and final concentration of PCR components

PCR Components	Final Conc.
2X *Taq* master Mix	1X
Forward Primer	10 µM
Reverse Primer	10 µM
Template DNA	2 ng
Nuclease –free water	–

Table 4. Optimized temperatures, cycles, and duration of PCR conditions

PCR Conditions	Temperature (°C)	Time
Initial denaturation	94°C	3 min
Cycles: 35		
Denaturation	94°C	1 min
Annealing	KCNQ1: 58.3°C TCF7L2: 54.5°C DPP4: –	1 min
Elongation	72°C	1 min
Final elongation	72°C	5 min
Infinite hold	4.0°C	∞

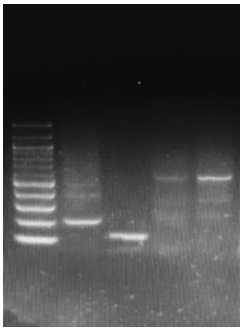

Figure 2. AGE Result Using the Optimized Procedures

Agarose gel electrophoresis of PCR products

PCR products of target genes sequences in Fig. 2 corresponding to genes *KCNQ1* (lane 2), *TCF7L2* (lane 3), and *DPP4* (lane 4) with 100 bp ladder (lane 1). Sizes in bp for each gene correspond to *KCNQ1*- 188 bp and for -*TCF7L2*- 133 bp. The amplification for *DPP4* gene did not initially give a distinct PCR product. The distinct bands were purified, sequenced and verified using BLAST to have targeted the genes specified.

Verification of the sequence results

Figure 3 shows the sequences of the diabetic and normal samples. For KCNQ1 control, as expected, there was no mutation seen and for the diabetic sample, the mutation was successfully targeted. For TCF7L2 control, the sequence results showed a normal sequence as well. However, for diabetic samples where the mutation must be present, there was no error seen. The DNA sequence results was viewed to BLASTn to confirm the presence of the sequence to the respective genes. Figure 4 shows that the control and diabetic sample sequences results were present to the genes accordingly. It was verified that susceptible genes were targeted using the designed primers and optimized conditions.

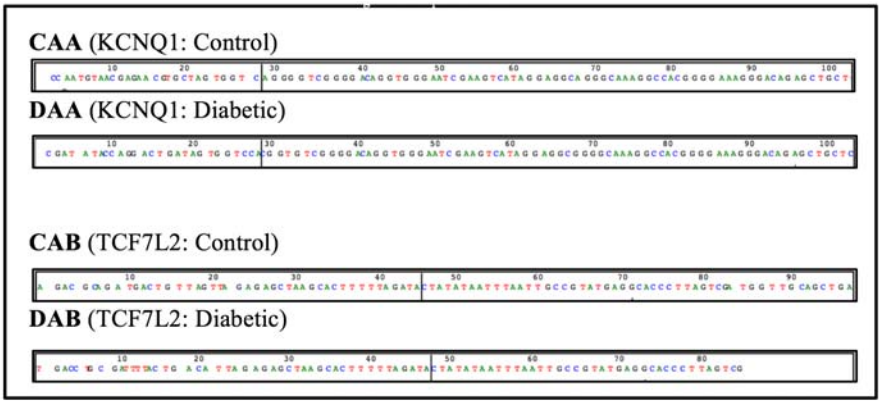

Figure 3. Sequence Results

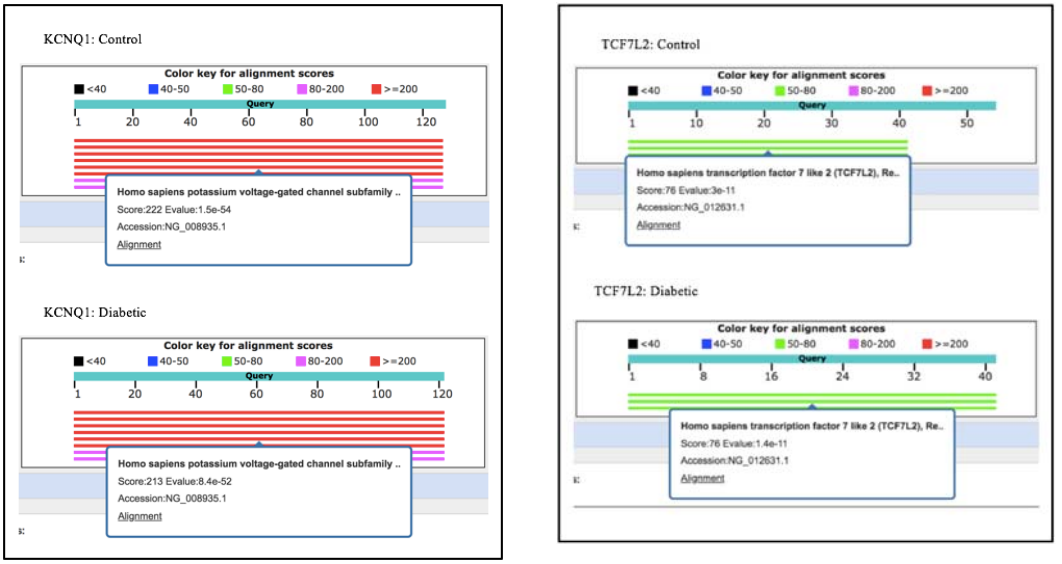

Figure 4. Confirmation of Sequence Results using BLASTn

Conclusion

Using the designed gene specific primers: F/R GGCTTTCTCTGCCTCAAAACC/ ACTAAGGGTGCCTCATACGG for *TCF7L2*, and for *KCNQ1*- F/R GTAAGCAGATGACAGGGCAGT/ TAAAGGTCCT GACCCCCACC, and the proper PCR conditions, the targets were amplified. The optimized PCR components are: 1X 2X *Taq* Master Mix, 10 µM forward and reverse primers, and 2 ng DNA template. PCR conditions are: initial denaturation, 3 min; 35 cycle of denaturation, 1 min, annealing, 1 min, elongation, 1 min and; final elongation of 5 min. The annealing temperatures were optimized at 58.3°C for *KCNQ1* and 54.5°C for *TCF7L2*. These conditions will be used for SNP detection and establish its association with T2DM in Filipino patients. The findings from Sanger sequencing

were verified using BLAST that showed the alignment with the respective target genes.

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