DETERMINING KCNJ11 E23K POLYMORPHISM IN A GROUP OF VIETNAMESE POPULATION BY RESTRICTION FRAGMENT LENGTH POLYMORPHISM METHOD

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Abstract. The potassium inwardly rectifying channel, sub-family J, member 11 (KCNJ11), which is a pore-forming sub-unit of ATP sensitive potassium channels in pancreatic β-cells, plays a critical role in the regulation of insulin secretion. A non-synonymous E23K variant (SNP rs5219) in the KCNJ11 gene, which results from C to T transition and substitutes glutamate for lysine at position 23, is identified as a SNP associated with prediabetes and type 2 diabetes. The study aimed to apply the restriction fragment length polymorphism (RFLP) method to identify the KCNJ11 E23K polymorphism in Vietnamese population. The forward and reverse primers for PCR were 5'-GACTCTGCAGTGAGGCCCTA-3' and 5'-ACGTTGCAGTTGCCTTTCTT-3', respectively. The BanII restriction enzyme was selected to incubate PCR products and distinguish the C or T allele after electrophoresis in 3% agarose gel or 12% polyacrylamide gel. The frequency of T minor allele of 542 random subjects from the Vietnamese population was 34.6%. The genotype distribution was in Hardy-Weinberg equilibrium. Among CC, CT and TT genotypes, there were a significant difference in cholesterol ($P = 0.041$) and a trend of difference in LDL-C ($P = 0.059$). The RFLP-PCR is very useful method to identify the KCNJ11 E23K polymorphism in a numerous groups of Vietnamese population.

Keywords: KCNJ11 E23K, genotyping, restriction fragment length polymorphism, Vietnamese.

1. Introduction

Glucose metabolism stimulates insulin secretion from pancreatic beta cells by closing KATP channels, thereby inducing beta cell depolarization, calcium influx, and insulin exocytosis [1] conversely, opening of KATP channels which prevents insulin secretion by causing beta cell hyper-polarization. The potassium inwardly rectifying channel, sub-family J, member 11 (KCNJ11) is a pore-forming sub-unit of ATP sensitive potassium (KATP) channels in pancreatic β-cells [2]. The KATP channels which consist of four sub-units of the inwardly-rectifying potassium channel Kir6.2, and four sub-units of the sulfonylurea receptor 1 (SUR1), play a critical role in the regulation of insulin secretion. Variations of KCNJ11 can contribute to the decreasing sensitivity of the ion channel to ATP, leading to more ATP consumption, which further
contributes to insulin-release impairment and causes hyperinsulinemia. KCNJ11 gene was proven to have an association with hypertension, obesity, dyslipidemia, and glucose intolerance [3].

A non-synonymous E23K variant (E23K) in the KCNJ11 gene, which results from a C to T transition and substitutes glutamate for lysine at position 23, is identified as a SNP associated with pre-diabetes and type-2 diabetes [4]. Frequency of genotypes and allele are different from each ethnic group. Several methods are used to genotype rs5219 polymorphism in populations: PCR-SSCP [5]. TaqmanPCR [6], Mass ARRAY [7], PCR-RFLP [8]. The polymerase-chain reaction–restriction fragment-length polymorphism, a popular technique for genotyping, is based on the principle that DNA product resulted from polymerase-chain reaction is broken into pieces digested by restriction enzymes, and the resulting restriction fragments are separated according to their lengths by gel electrophoresis [9]. Despite the fact that it is less widely-used now, the PCR-RFLP method has several advantages including low costs and a requirement of basic molecular equipment. It is a suitable method for genotyping rs5219 polymorphism in Vietnam. Thus, the study aimed to apply the PCR-RFLP method to determine KCNJ11 rs5219 polymorphism in Vietnamese population.

2. Content

2.1. Materials and methods

* Samples

From 2710 subjects in the previous cross-sectional study in Ha Nam province (2011) [10], we selected 542 subjects (20%) recruited randomly from the total samples. This study was conducted with the approval of the Ethics Committee of the National Institute of Hygiene and Epidemiology, Vietnam, and a writtenly-informed consent was obtained from all study subjects. Genomic DNA was extracted from 300 µL peripheral blood leukocytes, using Wizard® Genomic DNA Purification Kit (Promega Corporation, USA). Purity and concentration of DNA were measured by Nano Drop device.

* Amplification of KCNJ11 rs5219 sequences

The RFLP-PCR is a technique that exploits variations in homologous DNA sequences. This technique involves fragmenting sample of DNA by a restriction enzyme, which can recognize and cut DNA at a particular sequence, in a process known as a restriction enzyme digestion. The rs5219 polymorphism was genotyped by PCR on genomic DNA with the forward and reverse primers according to Nielsen et al. (2003) [8]. The forward and reverse primers were 5’-GACTCTGCAGTGAGGCCCTA-3’ and 5’-ACGTTGCAGTTGCCTTTCTT-3’, respectively. PCR amplification was carried out in a volume of 12 µL containing 2 µL nuclease-free water, 2 µL primers (10 pmol each primer), 2 µL genomic DNA and 6 µL GoTaq® Green PCR master mix 2 X (GoTaq® DNA Polymerase is supplied in 2X Green GoTaq® Reaction Buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP and 3 mM MgCl₂ (Promega Corporation, USA). The thermal cycling was a denaturing step at 94 °C for 3 min followed by 32 cycles of 94 °C for 30 s, annealing at 56 or 58 or 60 or 62 °C for 30 s, and elongation at 72 °C for 30s, with a final elongation step at 72 °C for 10 min, using an Eppendorf’s Master cycler. Five µL PCR products containing 210 bp bands were stained with RedSafe and electrophoresis on 3% agarose
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gel for 30 min at 100 v in 0.5 X TBE buffer, with ΦX174 DNA HaeIII Digest ladder. The DNA bands were detected using Gledoc-It™ gel camera.

* Digestion of PCR product by BanII enzyme

The BanII restriction enzyme was selected (New England Bio-labs, Beverly, MA) to digest PCR product, using restriction mapper database [11]. According to manufacturers’ guidelines, the components of a total 15 µL volume per restriction enzyme incubated reaction included 8.3 µL nuclear-free water, 1.5 µL 10 X CutSmart 10 X digest buffer, 0.2 µL fast digest BanII (NEB), and 5.0 µL PCR product. The mixed solution was incubated at 37 °C for 2 hours. Alleles were visualized as fragments by electrophoresis through stained with RedSafe on 3% agarose gel for 30 min at 100v in 0.5 X TBE buffer or 12% polyacrylamide gel for 2 hours. Distribution patterns of the rs5219 polymorphism were AA (2 bands: 178 bp and 32 bp), AG (4 bands: 178 bp, 150 bp, 32 bp and 28 bp), and GG (3 bands: 150 bp, 32 bp and 28 bp).

* Determination of KCNJ11 rs5219 polymorphism

The representative 542 subjects were genotyped to determine rates of genotype and allele of KCNJ11-rs5219 polymorphism. Genotype frequencies were compared and tested for Hardy-Weinberg Equilibrium (HWE) by chi-square test. Characteristics of anthropometric, glucose and lipid profiles among the three rs5219 genotypes were compared in middle-aged Ha Nam people.

2.2. Results and discussion

2.2.1. Selection appropriate protocol for polymerase chain reaction

According to recommendations, the melting temperature of the primer was approximately 60 °C [12]. Therefore, to select the appropriate annealing temperature (Ta) for PCR, we conducted a test with 3 samples and a negative control (H₂O) at 4 following annealing temperatures: 56 °C, 58 °C, 60 °C, and 62 °C. The result is shown in Figure 1.

![Figure 1. Electrophoresis result of 210 bp products by PCR at 4 annealing temperatures](image)

Among 4 annealing temperatures, the bands of PCR product visualized the thickest at Ta = 62 °C. So we selected the Ta = 62 °C for further experiments. The thermal cycling was a denaturing step at 94 °C for 3 min followed by 32 cycles of 94 °C for 30 s, annealing at 62 °C for
30 s, and elongation at 72 °C for 30 s, with a final elongation step at 72 °C for 10 min, stopped by chilling at 15 °C.

The forward and reverse primers to determine this polymorphism were used in this research according to Nielsen et al. (2003) [8]. However, in the cycling program, the annealing temperature was 60 °C for 30 s. In Hansen’s research, the reverse primer was the same, yet, the forward one was different with 5’- CGAGGAATACGTGCTGACAC-3’ and the annealing temperature in PCR was 62 °C for 45 sec [13]. Based on the experimental result, we agreed that the appropriate annealing temperature was 62 °C for 30 sec.

2.2. Selection of appropriate gel to determine distribution patterns

We used BanII to digest the amplified PCR products to determine genotypes of samples because BanII enzyme cut the sites:

\[
5'\ldots G R G C Y C \ldots 3' \quad 3'\ldots C Y C G R G \ldots 5'
\]

According to the manufacturer’s advice, we conducted an experiment using 0.2 µL/reaction of BanII. While using 3% agarose gel to identify bands’ length, the position of the bands was not appeared to be in lines of 178-bp band or 150-bp band (Figure 2a). Thus, we checked the digested products using electrophoresis in 12% polyacrylamide gel. As a result, the PCR products were digested completely and the position of the digested products was seen in appropriate lines as compared to the standard ladder (Figure 2b).

![A. 3% agarose gel](image1.png)  ![B. 12% polyacrylamide gel](image2.png)

**Figure 2. Electrophoresis result PCR products before and after digestion by 0.2 µL/reaction of BanII enzyme**

* M: phiX174 DNA/BsuRI (HaeIII) Marker, Sample 1 - 3: sample No 1 - 3 incubated with 0.2 µL of BanII enzyme, PCR product before incubated with 0.2 µL of BanII enzyme.
* AG (178 bp and 150 bp), GG (150 bp), AA (178 bp)

All products of both reference studies used BanII (New England Bio-labs, Beverly, MA) at 37 °C to digest and the resulting DNA digestion fragments were resolved on 3% agarose gel [8, 13]. In our experiment, when using 3% agarose gel according Nielsen et al. (2003) [8] to identify the length of bands, the position of the bands was not appeared to be in lines of 178bp band or 150bp band. Instead, we used electrophoresis in 12% polyacrylamide gel, the position of the digested products was seen in appropriate lines as compared to the standard ladder. It was also confirmed by the DNA sequencing analysis. This shows that the KCNJ11-rs5219 genotypes obtained by
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using 3% agarose gel are similar to those by using 12% polyacrylamide gel. Thus, it is recommended that using 3% agarose gel is believable and convenient to identify the genotypes in large samples.

2.2.3. Genotype and allele frequencies, and characteristics of prediabetes related traits in genotypes of rs5219 gen KCNJ11 in middle-aged Ha Nam people

Results of genotype and allele frequencies of rs5219 polymorphism in the representative 275 samples (10%) are shown in table 1. Frequencies of GG, AG, AA genotypes were 52.7, 36.7 and 10.5%, respectively, and frequencies of G and A allele were 71.1 and 28.9%, respectively. Genotype frequencies in this population were in Hardy–Weinberg equilibrium (\( P > 0.05 \)).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Allele</th>
<th>( P ) value for Hardy-Weinberg Equilibrium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>232</td>
<td>697</td>
</tr>
<tr>
<td>CT</td>
<td>233</td>
<td>369</td>
</tr>
<tr>
<td>TT</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>Percentage</td>
<td>(%)</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>43.5</td>
<td>65.4%</td>
</tr>
<tr>
<td>CT</td>
<td>43.7</td>
<td>34.6%</td>
</tr>
<tr>
<td>TT</td>
<td>12.8</td>
<td></td>
</tr>
</tbody>
</table>

The 542 subjects in this study were recruited randomly from the total samples in the previous cross-sectional study in Ha Nam province. Thus, the distribution of alleles and genotypes of KCNJ11-rs5219 can be interpreted for the Kinh group in Ha Nam province. Frequencies of rs5219 genotypes were 43.5% (CC genotype), 43.7% (CT genotype) and 12.8% (TT genotype). And A-minor allele frequency was 34.6% in this group of Vietnamese population. The minor allele frequency was similar to that in Japan (34%) [5], and higher than that in Arabic group of population from Tunisia (29%) [13] but lower than that in Mexico (36.7%) [14], China (39%) [15], and Sweden (40%) [16]. This indicates the specific genetic background of the Vietnamese population.

Table 2 shows characteristics of anthropometric, glucose and lipid profiles in CC, CT and TT genotypes of KCNJ11-rs5219 polymorphism in middle-aged Ha Nam people. Results showed that there was a difference of cholesterol among the three genotypes which ranged the highest concentration in TT genotype (4.6 mmol/L), the CT genotype (4.43 mmol/L), and the lowest concentration in CC genotype (4.3 mmol/L) \( (P = 0.041) \). Besides, LDL-C tended to have differences between the three genotypes \( (P = 0.059) \). No differences were found among CC, CT and TT genotypes in sex, BMI, waist-hip ratio, fat percentage, systolic blood pressure, diastolic blood pressure, HDL-C, triglyceride, fasting plasma glucose and 2h-glucose. The KCNJ11-rs5219 polymorphism has been reported to be associated with type-2 diabetes, pre-diabetes, and hypertension in many Caucasian and East Asian populations [2-4, 8]. So, it is necessary to conduct the study to identify an association of CC, CT and TT genotypes with blood pressure, blood lipids and glucose in Vietnamese population. The present study found the difference in LDL-C among the three genotypes with limited size of samples. Thus, this study deserves the primary finding.
Besides, it is important to carry out further analyses on the association between the KCNJ11-rs5219 polymorphism and chronic diseases in Vietnamese population.

Table 2. Characteristics of several traits in genotypes of rs5219 gen KCNJ11 in middle-aged Ha Nam people

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CC</th>
<th>CT</th>
<th>TT</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female n (%)</td>
<td>155 (66.8%)</td>
<td>155 (66.5%)</td>
<td>49 (72.1%)</td>
<td>0.674</td>
</tr>
<tr>
<td>BMI (type of gene/m²)</td>
<td>21.3 ± 2.5</td>
<td>21.5 ± 2.7</td>
<td>21.8 ± 3.0</td>
<td>0.49(^a)</td>
</tr>
<tr>
<td>Waist hip ratio (cm)</td>
<td>0.84 ± 0.05</td>
<td>0.84 ± 0.7</td>
<td>0.85 ± 0.06</td>
<td>0.61(^a)</td>
</tr>
<tr>
<td>Fat percentage (%)</td>
<td>26.9 ± 6.5</td>
<td>27.2 ± 6.4</td>
<td>28.0 ± 7.0</td>
<td>0.48(^a)</td>
</tr>
<tr>
<td>Maximum blood pressure (mmHg)</td>
<td>110.5 (100 - 130)</td>
<td>115 (100 - 130)</td>
<td>120 (110 - 130)</td>
<td>0.436(^b)</td>
</tr>
<tr>
<td>Minimum blood pressure (mmHg)</td>
<td>70 (60 - 80)</td>
<td>70 (60 - 80)</td>
<td>70 (60 - 80)</td>
<td>0.874(^b)</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.19 (0.97 - 1.60)</td>
<td>1.2 (0.97 - 1.61)</td>
<td>1.12 (0.96 - 1.40)</td>
<td>0.222(^b)</td>
</tr>
<tr>
<td>LDL-C (mmol/ L)</td>
<td>2.80 ± 0.79</td>
<td>2.96 ± 0.9</td>
<td>3.05 ± 0.91</td>
<td>0.059(^a)</td>
</tr>
<tr>
<td>Triglyceride (mmol/ L)</td>
<td>1.25 (1.0 - 1.91)</td>
<td>1.42 (1.0 - 1.91)</td>
<td>1.70 (1.02 - 2.28)</td>
<td>0.201(^b)</td>
</tr>
<tr>
<td>Cholesterol (mmol/ L)</td>
<td>4.30 ± 0.79</td>
<td>4.43 ± 0.85</td>
<td>4.60 ± 0.95</td>
<td>0.041(^a)</td>
</tr>
<tr>
<td>Fasting plasma glucose (mmol/ L)</td>
<td>4.5 (4.1 - 5.2)</td>
<td>4.6 (4.2 - 5.0)</td>
<td>4.35 (4.0 - 5.0)</td>
<td>0.072(^b)</td>
</tr>
<tr>
<td>2h-Glucose (mmol/ L)</td>
<td>5.3 (4.63 - 6.18)</td>
<td>5.2 (4.75 - 6.30)</td>
<td>5.5 (4.6 - 6.0)</td>
<td>0.787(^b)</td>
</tr>
</tbody>
</table>

Note. 2h-Glucose: glucose concentration after 2 hours using oral glucose tolerance test
\(^a\) Variables are expressed by mean ± standard deviation. \(P\) are received from ANOVA test
\(^b\) Variables are expressed by median (25th - 75th percentile); \(P\) are received from Kruskal-Wallis H test

3. Conclusion

The study has reported the designed protocol for genotyping KCNJ11-rs5219 polymorphism using the PCR-RFLP method in Vietnamese samples. The forward and reverse primers were 5’-GACTCTGAGTGAGGCCCC-TA-3’ and 5’-ACGTTGCAGTTGCCTTCTT-3’, respectively. The standard protocol included four steps: 1) using PCR with annealing at 62 °C to amplify the KCNJ11 gene region containing SNP rs5219; 2) incubating PCR products with BanII restriction enzyme; 3) using 3% polyacrylamide gel to determine digested products; and 4) identifying genotypes of KCNJ11 rs5219 based on electrophoresis fragments. Frequencies of T minor allele of 542 random subjects from the Vietnamese population were 34.6%. There was a difference in cholesterol among three genotypes \((P = 0.041)\), and LDL-C tended to have differences between three genotypes \((P = 0.059)\). To conclude, the PCR-RFLP method is very useful for laboratories to identify the KCNJ11-rs5219 polymorphism in large groups of population in Vietnam.
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