Methodology

DEVELOPMENT OF A RESTRICTION FRAGMENT LENGTH POLYMORPHISM METHOD FOR GENOTYPING THE *ATP2B1* RS2681492 IN VIETNAMESE POPULATION

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Abstract

Aims: The rs2681492, a single nucleotide polymorphism in the *ATP2B1* gene, has been reported to be associated with cardiovascular diseases and traits including blood pressure, chronic heart failure, and hypertension. The study aimed to develop a PCR-RFLP genotyping method for rs2681492 among the Vietnamese population.

Methods and Results: The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method incorporated optimizations of primers, thermal cycles, and restriction enzymes in a novel restriction fragment length polymorphism (RFLP) approach. To evaluate the accuracy of the PCR-RFLP method, three different genotypes (AA, AG, and GG) were confirmed by gene sequencing. The experimental results determined the annealing temperature of 55°C for PCR and the minimum of 3 units of restriction enzyme HaeII for incubation with the PCR product.

Conclusion: This optimal PCR-RFLP method was successfully applied to genotype *ATP2B1* rs2681492 in large populations to investigate the association between the rs2681492 polymorphism and cardiovascular diseases.

Keywords: *ATP2B1 rs2681492, PCR-RFLP, hypertension, Vietnamese.*

I. INTRODUCTION

Cardiovascular disease represents an immense global threat, standing among the leading causes of death worldwide. Cardiovascular disease, responsible for nearly a third of the global death toll, tragically takes the lives of around 17 million people annually [1, 2]. Hypertension can increase your risk of several serious and potentially life-threatening health conditions, such as heart disease, heart attacks, strokes, peripheral arterial disease, aortic aneurysms, kidney disease, vascular dementia, and so on.

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The majority of people affected by hypertension (≈ 95%), have what is called essential hypertension, which is believed to be caused by a combination of genetic and environmental factors [3]. The Cohorts for Heart and Aging Research in Genome Epidemiology, through a groundbreaking genome-wide association study (GWAS), has uncovered the single nucleotide polymorphism (SNP) rs2681492 in the *ATP2B1* gene situated in the chromosome region 12q21−12q23, found to be significantly related to systolic blood pressure, diastolic blood pressure, and hypertension [4].

The *ATP2B1* gene is located on human Chromosome 12 (12q21.33). This gene belongs to the group of P-type primary ion transport ATPases (NM_001682). The gene measures 3660 nucleotides long, with a size range from nucleotide 89,588,049 to nucleotide 89,709,353. The structure of *ATP2B1* includes 20 exons with different splicing methods, creating different protein isoforms. The *ATP2B1* gene is responsible for producing isoform 1 of the plasma membrane calcium ATPase. These enzymes remove Ca^{2+} ions from eukaryotic cells, even when faced with extremely high concentration differences. This crucially contributes to maintaining the balance of calcium within the cells [5, 6].

In 2012, Yan Wang and their colleagues identified four specific spots in the *ATP2B1* gene (rs10858911, rs2681472, rs17249754, and rs1401982) that are definitively linked to hypertension, systolic blood pressure, diastolic blood pressure, and cervical artery pulse velocity. Genetic and hypertension association studies revealed a strong correlation with the findings concerning the rs2681492 in the introductory region of the *ATP2B1* gene. It's important to note that the link between the *ATP2B1* genotype at the SNP rs2681492 and hypertension varies across different populations [7]. Currently, in Vietnam, there is limited data on the distribution of genotypes and their correlation with hypertension, specifically concerning the SNP rs2681492. Therefore, it is crucial to research the association between the single nucleotide polymorphism of the rs2681492 and cardiovascular diseases.

To analyze the genetic makeup of samples, we utilized the restriction fragment length polymorphism (RFLP) method. This technique involves examining the variation in DNA segment lengths by analyzing the cutting points of restriction enzymes. In particular, restriction enzymes have the amazing ability to identify specific locations on genomic DNA. By combining them with a DNA fragment in an appropriate buffer solution, we can control the pH, temperature, and duration to achieve the desired results. This remarkable process results in the precise cutting of the fragment at a specific spot, creating a collection of DNA fragments with unique sizes. By analyzing the resulting segment sizes, we were able to determine the alleles and genotypes of the samples. This study aimed to develop a PCR-RFLP method for genotyping the rs2681492 among the Vietnamese population.

II. METHODS

2.1. Research materials

Blood samples were collected and centrifuged immediately in the morning after participants had fasted for 8 hours. Genomic DNA was extracted from peripheral blood leukocytes using the Wizard® Genomic DNA Purification Kit (Promega Corporation, USA). A nanodrop was used to determine the optical density (OD 260/280 ratio) and concentration of purified DNA then the concentration of DNA samples was normalized to ≥ 15 ng/µL for ready-to-use. The chemical and biological agents used for PCR were specific primers (10 pmol/µl, IDT, USA), Gotaq Green Master Mix 2X (Promega), and clean water Gibco® Ultrapure Distilled Water (Invitrogen). HaeII restricted enzyme (NEB) with 10X CutSmart® NEBBuffer was used for the digestion of the PCR products. The chemical and biological agents used for electrophoresis were agarose (Promega, Spain), Ultrapure TBE Buffer (Invitrogen), RedSafe™ stained (Intron Biotechnology), and 100 bp DNA Ladder (Invitrogen).

2.2. Experimental design

∗ ***Primer's design***

To obtain the SNP rs2681492 sequence and choose the best primers for bait design, we made use of the website https://www.ncbi.nlm.nih.gov/projects/snp/. The primers chosen have an ideal length of 18 to 22 nucleotides and a G and C content between 40% and 60%. This guarantees a temperature melting of primers around 50°C to 65°C. Following that, two sets of PCR primers were formulated using the online tools Primer3 (https://bioinfo.ut.ee/primer3-0.4.0/) and UCSC In-Silico PCR (https://genome.ucsc.edu/cgi-bin/hgPcr). The forward primer, ATP2B1_rs2681492F, was designed as 5'- CCCAAGTAACTGGGATTACAG -3', while the reverse primer, ATP2B1_rs2681492R, was formulated as 5'- ACACTGCAGCAGCATAAAC -3'. The melting temperature (Tm) of the primers was approximately 55°C according to the recommendations of the tools.

∗ ***Testing the PCR thermal recycling protocol***

Based on calculating the melting temperature, the equation used is: Tm = 64.9 + 41*(yG+zC-16.4)/(wA + xT + yG + zC), where w, x, y, and z are the numbers of the bases A, T, G, and C in the sequence, respectively. The annealing temperature is generally less than the primer melting temperature (Tm) by about 5°C–10°C. Then the PCR gradient method was used to determine the annealing temperature (Ta) with the following temperature range: 51°C, 53°C, and 55°C. The PCR protocol was carried out under the following conditions: 94°C for 3 min and 32 cycles of denaturation at 94°C for 30 sec, primer annealing at 51°C/53°C/55°C for 30 sec, extension at 72°C for 30 seconds; and a final elongation step at 72°C for 8 min (Fig. 1). 5µl PCR products were detected on Redsafe-stained 2.5% agarose gel at 337 bp band for 30 min at 100V in 0.5X TBE buffer. The DNA bands were detected using a Geldoc-ItTM gel camera system (Fig. 2).

∗ ***Selecting an appropriate restriction enzyme***

Then SNP rs2681492 was acquired from the esteemed NCBI database. Using the restriction mapper database, the restriction enzyme map for this sequence was identified. The restriction enzyme *Hae*II was chosen to distinguish A and G alleles at the cutting side RGCGC/Y (with R playing as A/G and Y playing as C/T). The process of digestion was conducted following the specific instructions provided by the manufacturers, ensuring that all steps and conditions were precisely adhered to.

∗ ***Confirming the polymorphism by sequencing method***

To verify the precision of the PCR-RFLP method, 3 samples were identified that represented 3 different genotypes: AA, AG, and GG. These samples were subjects for gene sequencing. If the sequences obtained were identical to the sequence downloaded from NCBI GenBank, these genotypes identified by PCR-RFLP were confirmed.

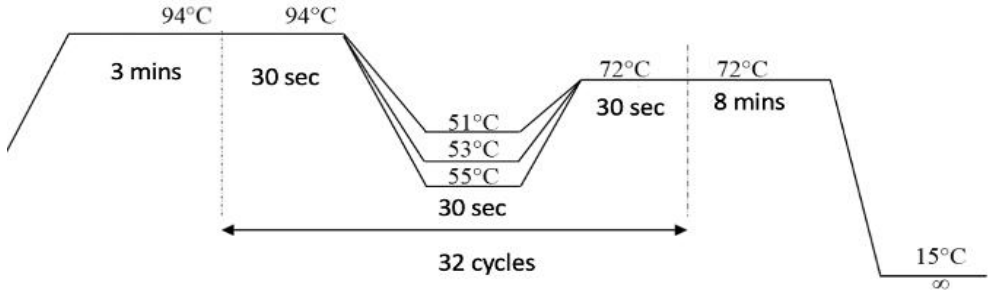

Figure 1. *Gradient thermal cycling for Restriction Fragment Length Polymorphism (RFLP) polymerase chain reaction.*

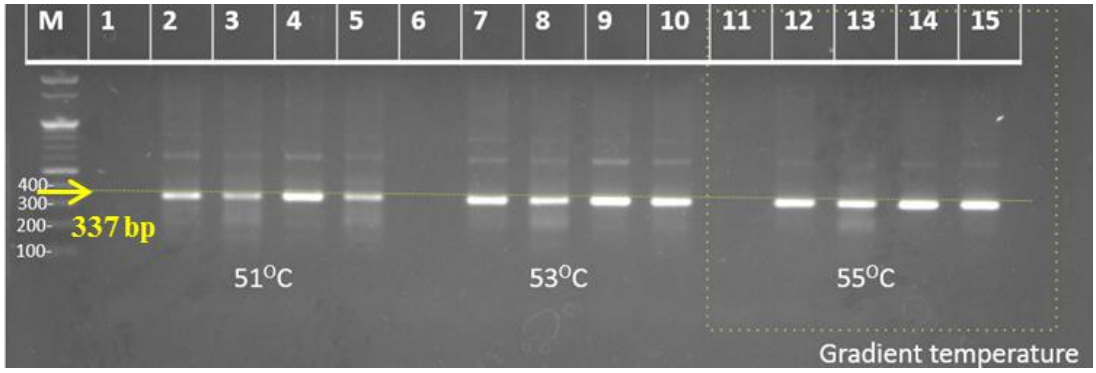

Figure 2. *Electrophoresis images of the optimization of the annealing temperature for RFLP.*

The PCR products were analyzed on a 2.5% gel electrophoresis at different temperatures of annealing (Ta), from left side to right side: Please take note of the following details: Lane M: Marker 100 bp DNA Ladder (Invitrogen), Wells 1, 6, and 11 are negative controls without a DNA template, The Ta was 51°C in wells 2, 3, 4, 5; The Ta was 53°C in wells 7, 8, 9, 10; The Ta was 55°C in wells 12, 13, 14, 15 respectively.

III. RESULTS

3.1. PCR protocol for rs2681492 amplification

After conducting empirical tests, it was determined that an annealing temperature of 55°C produced the brightest and clearest results for all PCR product bands, as illustrated in Figure 2A. In comparison, bands at 51°C and 53°C did not exhibit the same level of clarity. Consequently, the optimal annealing temperature for the thermal cycle was established as 55°C. The optimal PCR protocol for rs2681492 amplification was as follows: The reaction was carried out in a 15 µl volume consisting of 4.0 µl of nuclease-free water, 2.0 µl of primers (10 pmol each primer), 1.5 µl of genomic DNA, and 7.5 µl of GoTaq® Green PCR Master Mix 2X (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). The amplification process was carried out in 32 cycles, beginning with an initial denaturation at 94°C for 3 minutes. Each cycle then consisted of denaturation at 94°C for 30 seconds, followed by annealing at 55°C for 30 seconds and elongation at 72°C for 30 seconds. Finally, there was a concluding elongation step at 72°C for 10 minutes.

3.2. An appropriate restriction enzyme for recognizing the rs2681492 polymorphism

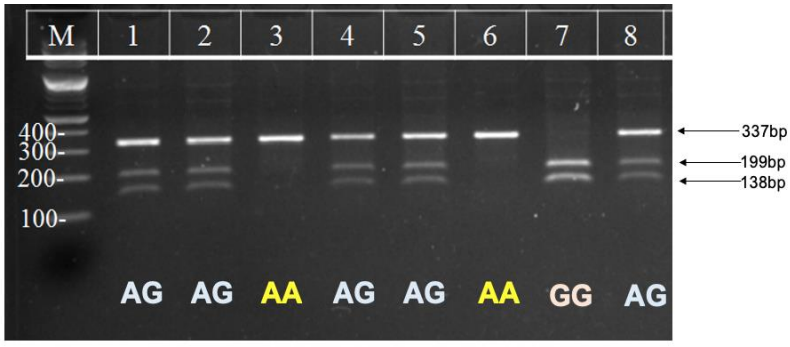

Figure 3: *Electrophoresis images of the PCR products digested by HaeII enzyme at 5.0 µL PCR products on 2.5% agarose gel electrophoresis.*

Three genotypes of *ATP2B1* rs2681492 were identified according to the length and size of digested products: length size of digested products: AA (337 bp band), AG (3 bands: 337 bp, 199 bp, and 138 bp), and GG (2 bands: 199 bp and 138 bp); M: Marker 100 bp DNA Ladder (Invitrogen).

In Figure 3, the presence of three genotypes (AA, AG, and GG) was identified based on the distinct band patterns observed following electrophoresis. The amplification products obtained from the polymerase chain reaction (PCR) were completely digested using the *Hae*II restriction enzyme. The digestion process involved adding 5 µL of PCR products to a total volume of 10 µL, consisting of 3.7 µL nuclease-free water, 1.0 µL CutSmart

NEB Buffer 10X (containing 10 pmol of each primer), and 0.3 µL of *Hae*II enzyme (equivalent to 0.3 fast digest units). The solution was incubated for 15 minutes at 37°C. Subsequently, the digested products were visualized as distinct fragments using 2.5% agarose gel electrophoresis stained with Redsafe in TBE 0.5X buffer.

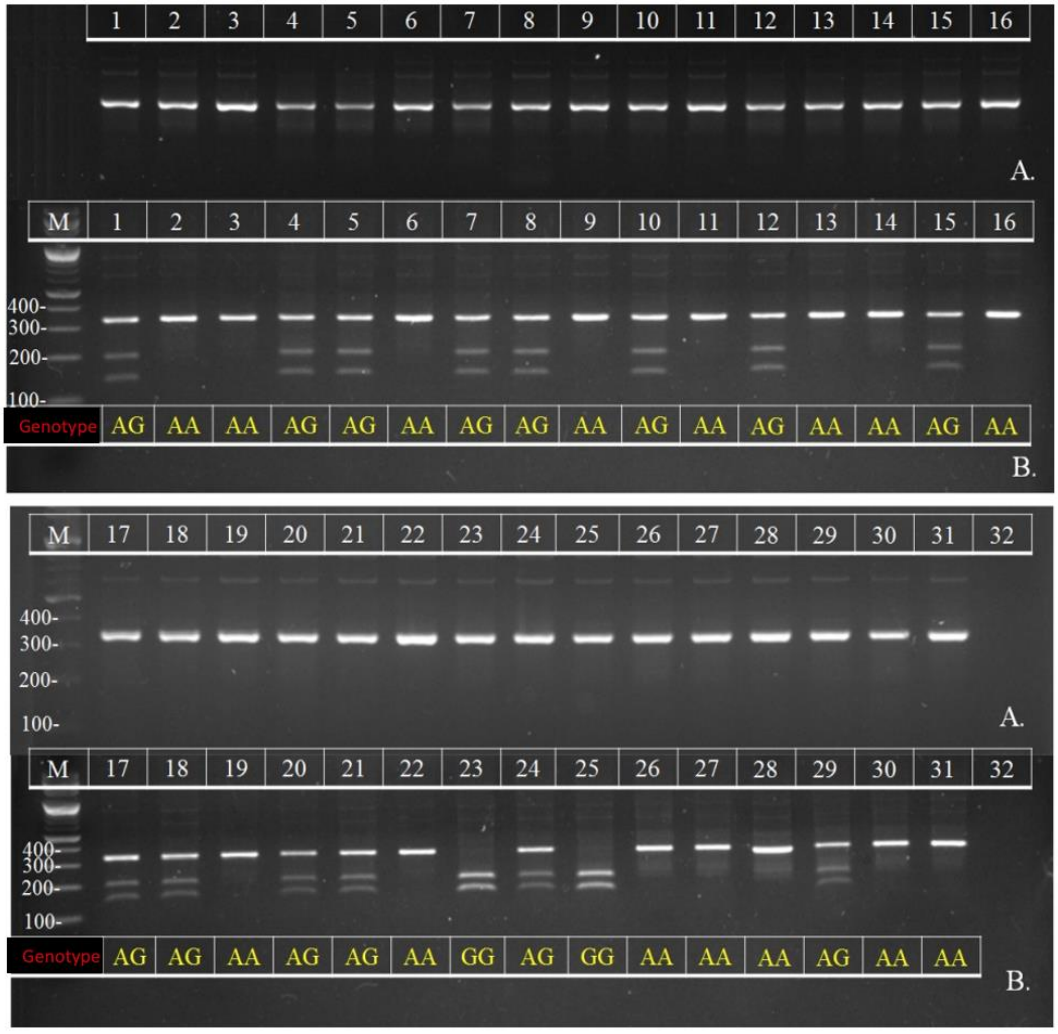

Figure 4. *PCR product of rs2681492 (A/G) SNP of ATP2B1 on 2.5% agarose gel electrophoresis.* Electrophoresis image of PCR products upper (A) and lower (B) digestion by the *Hae*II enzyme.

Three genotypes of *ATP2B1* rs2681492 were identified according to the length and size of digested products: AA (337 bp band), AG (3 bands: 337 b, 199 bp, and 138 bp), and GG (2 bands: 199 bp and 138 bp); M: Marker 100 bp DNA Ladder (Invitrogen), respectively.

Figure 4 provides evidence supporting the consistency and reliability of the protocol by successfully analyzing a set of 31 random samples. Notably, the band patterns observed on the gel were indicative of the distribution of the rs2681492 polymorphisms, with the AA genotype exhibiting a 337 bp band, the AG genotype demonstrating three bands (337 bp, 199 bp, and 138 bp), and the GG genotype displaying two bands (199 bp and 138 bp).

3.3. Validation by Sanger sequencing

The results of the sequence showed that at the SNP rs2681492 position (Fig. 5). The results of the genotype determination by the PCR-RFLP method coincided with the results of the *ATP2B1* rs2681492 using Sanger sequencing.

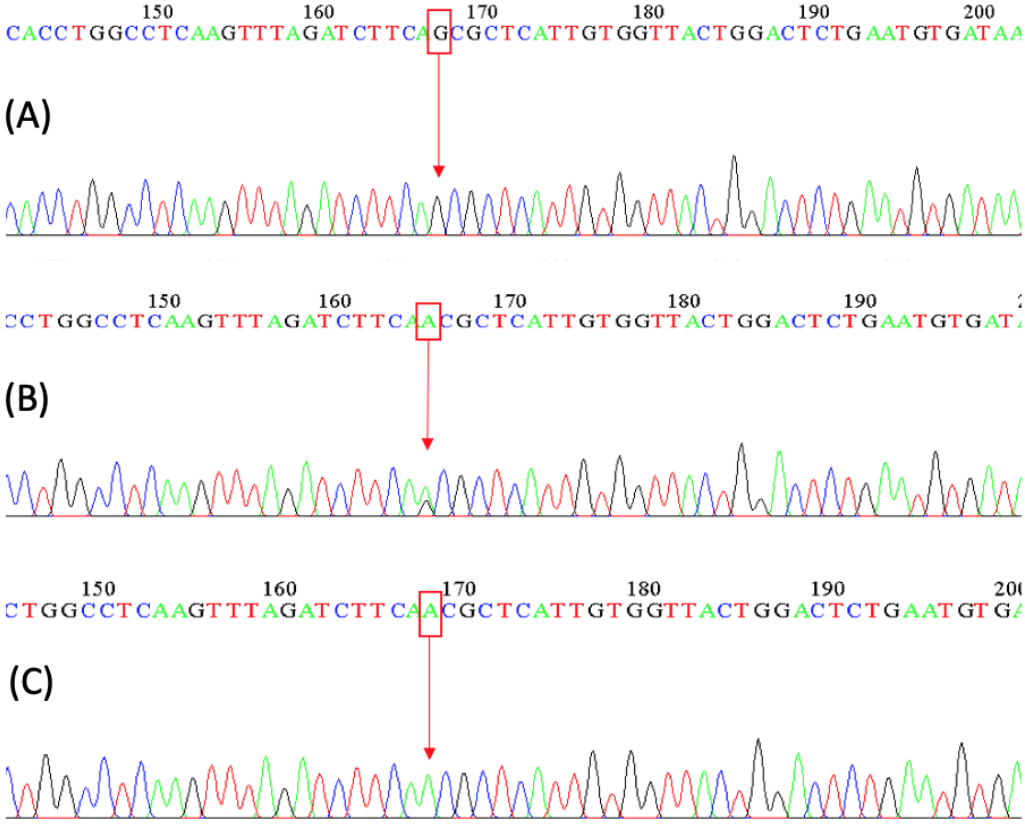

Figure 5. *Genotyping result of the rs2681492.* Taking partial samples as an example, (A) the GG genotype had black brightness intensity, (B) the AG genotype had 2 blue black intensity, and (C) the AA genotype had 1 blue light intensity, respectively.

IV. DISCUSSION

Genetics is an element of molecular biology, serving as an indispensable tool in comprehending the intricacies of biological processes. The genetic data is necessary for groundbreaking research into a specific set of genetic variations. This involves delving into personal heritage, evaluating health vulnerabilities, conducting investigations, or unraveling forensic inquiries. SNPs are the dominant genetic variants in the human population, with over 95% of all genetic variations being SNPs [10].

Numerous cost-effective methods have arisen over the last three decades for genotyping SNPs, including allele-specific polymerase chain reaction (AS-PCR), confronting two-pair primers-polymerase chain reaction (CTPP-PCR), the TaqMan method, gene chip technology, and sequencing. It is crucial to select a method that effectively

balances the precision of genetic information, pre-processing, post-processing, analysis, and financial constraints. Each method presents unique advantages and limitations. AS-PCR, for instance, is highly precise and expeditious, albeit requiring the execution of two concurrent PCR reactions, leading to increased overall expenditures. Conversely, CTPP-PCR offers a simple and cost-effective solution, necessitating only one PCR reaction and a single electrophoresis step similar to traditional PCR methods, thus significantly reducing the required time and resources for implementation. However, meticulous primer design is paramount for optimal outcomes in CTPP-PCR. Careful primer design is essential to mitigate potential issues related to primer pair competition. Employing this method substantially diminishes the likelihood of inconclusive and conflicting results. In the presence of uncertainties, it is imperative to validate results using genotyping techniques. While sequencing yields the most comprehensive genetic information, it is also costlier and demands greater computational resources. In contrast, less expensive techniques like RFLP or TaqMan PCR capture the same information. PCR-RFLP is an efficient technique used to determine fragment length polymorphism. The method involves amplifying a specific DNA fragment using PCR and digesting the resulting amplicon with a restriction enzyme. This enzyme cuts an allele at a diagnostic SNP, resulting in two fragments, while leaving the other allele intact, resulting in one fragment. This is due to a species-specific polymorphism in the enzyme's recognition site. Homozygous individuals for either allele and heterozygous individuals can be easily distinguished from each other through gel electrophoresis, which results in either two, one, or three fragments. Overall, PCR-RFLP is an excellent, fast, cheap, and reliable method for genotyping diagnostic markers.

ATP2B1 has been identified as a potential gene associated with blood pressure in multiple genome-wide association studies (GWA) in individuals of European and East Asian descent, including Japanese, Chinese, and Korean populations. This gene plays a role in maintaining calcium balance, which is crucial for normal blood vessel constriction and may be related to blood pressure control. Research on *ATP2B1* variations in East Asian populations through replication studies supports its involvement in hypertension. However, there is limited evidence of the association between *ATP2B1* variations and hypertension risk in other populations.

The 2021 study by Sami A. Althwab examined the association between *ATP2B1* gene polymorphism and hypertension in the Saudi population. The 246 hypertensive cases and 300 healthy human controls were genotyped. The results showed that genotypes rs2681472 (CT + TT) [p = 0.05; OR: 95% CI, 1.5 (1.0 to 2.4) and p = 0.006 OR: 95% CI, 2.0 (1.2 to 3.1) respectively] associated with the risk of hypertension. However, cases with body-mass-index (BMI)<25, carrying homozygous mutant genotypes (TT, rs2681472, p = 0.05; OR: (95%CI)

1.96 (1.03 to 3.72)] as well as T allele of rs2681472, p = 0.04, 1.43(1.03 to 1.98)] showed a significant association with high risk of hypertension [11]. In summary, there is a notable link between a certain variation in the *ATP2B1* gene and the likelihood of developing hypertension. Specifically, individuals with recessive genotypes are at a higher risk of developing hypertension compared to those with dominant genotypes. Additionally, individuals with high-risk BMI who also have *ATP2B1* variants may be particularly susceptible to developing hypertension.

The genotype of the *ATP2B1* rs2681492 was determined using the PCR-RFLP method in a sample of 615 individuals aged 40–64 years living in Ha Nam, Vietnam. The digestion of the PCR products (337 bp) was carried out using a 0.3 FDU HaeII restricted enzyme. Alleles were visualized as fragments by electrophoresis using RedSafe™ Nucleic Acid Staining Solution (20,000x) at a concentration of 2,5%, removing the necessity for expensive and intricate probes. The distribution patterns of the rs2681492 polymorphisms were AA (1 band: 337 bp band), AG (3 bands: 337 bp, 199 bp, and 138 bp), and GG (2 bands: 199 bp and 138 bp). This method provides a clear benefit: it is simple to implement and demands only a minimal amount of DNA material. Moreover, this method allows for easy interpretation of the results by directly examining the agarose gel during the application of an electrical current. The compatibility of results between the PCR-RFLP process in high-precision research and genetic sequencing of *ATP2B1* rs2681492. By applying the results of this study on a larger sample scale, we can analyze genetics and evaluate the relationship between SNP rs2681492 and hypertension. This analysis can help us make individualized predictions about prognosis, disease acceleration, and the best course of treatment.

V. CONCLUSION

The protocol of the PCR-RFLP was successfully developed with optimal primers, the melting temperature of primers, the components, and thermal cycling to identify *ATP2B1* rs2681492 polymorphism. The protocol should be applied to genotyping the *ATP2B1* rs2681492 polymorphism in large cohorts to investigate the association between the polymorphism and blood pressure in the Vietnamese population.

Acknowledgments

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