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A Pilot Study on the Development of a PCR-Sanger Sequencing Method for Detecting the NOTCH1 c.17_18delinsTT Variant in Individuals with Aortopathy History

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Abstract

Aortopathy encompasses various structural and degenerative disorders of the aorta, posing significant clinical challenges. Among the genetic variants reported in individuals with cardiovascular disorders, the c.17_18delinsTT variant in the *NOTCH1* gene has attracted interest due to its potential biological relevance. This pilot study aimed to develop and evaluate a PCR-Sanger sequencing workflow for detecting the *NOTCH1* c.17_18delinsTT variant in members of a Malaysian family with a history of aortopathy. DNA samples from six family members were analysed using polymerase chain reaction (PCR) and Sanger sequencing for mutation detection. Optimized dimethyl sulfoxide (DMSO) concentration significantly improved amplification, yielding a DNA fragment size of 893 bp. The mutation exhibited both a homozygous normal genotype (CG/CG) and a heterozygous genotype (CG/TT). Further studies are required to determine whether this variant affects NOTCH1 protein function. The developed workflow successfully amplified and identified the target *NOTCH1* variant in selected samples, demonstrating its potential utility for future genetic screening and research applications. However, due to the limited sample size and the variant's classification as a variant of uncertain significance (VUS), no conclusions regarding its causal role in aortopathy can be drawn from this pilot study.

Keywords: Aortopathy; *NOTCH1* gene; Polymerase chain reaction; Sanger sequencing

INTRODUCTION

Understanding the genetic basis of aortopathy is crucial in unravelling its molecular mechanisms. Aortopathies involve structural abnormalities and degenerative changes in the

aorta, presenting clinical challenges. Among genetic contributors, the *NOTCH1* gene plays a vital role in cardiovascular integrity with mutations linked to various cardiovascular diseases, including aortopathy. A specific mutation, c.17_18delinsTT alters the *NOTCH1* gene sequence and may impact protein function, potentially contributing to aortopathy (Tessler et al., 2021). However, its exact role remains unclear. This study investigates the c.17_18delinsTT mutation using Polymerase Chain Reaction (PCR) to provide insights into its genetic implications. Research on this mutation is limited, though *NOTCH1* mutations are associated with conditions like bicuspid aortic valve (BAV) and thoracic aortic aneurysm (TAA). Adams-Oliver syndrome 5, linked to this mutation results in the p.Ala6Val substitution, but direct evidence connecting it to aortopathy is scarce. Additionally, population-based data on its incidence remain limited. Given *NOTCH1*'s role in cardiovascular diseases, further study is needed to determine its prevalence and impact.

This pilot study aims to develop and evaluate a PCR-based approach for detecting the *NOTCH1* c.17_18delinsTT variant among family members with a history of aortopathy in Malaysia. The study focuses on establishing a reliable molecular workflow comprising primer design, PCR amplification, and Sanger sequencing confirmation. Rather than determining disease causality, this work serves as a preliminary methodological framework that may support future investigations involving larger cohorts and comprehensive genotype-phenotype analyses. Objectives include obtaining the *NOTCH1* sequence from NCBI for primer design, performing mutation-specific amplification, and confirming its presence through sequencing. The null hypothesis states the mutation is either absent or undetectable due to methodological limitations, while the alternate hypothesis posits that it is present and identifiable. Given the rarity of this mutation, a targeted approach is necessary to assess its presence and develop reliable detection methods (Mahdieh & Rabbani, 2013). By studying this family, the research advances genetic diagnostics for aortopathy. Since aortopathy has a strong genetic component, yet many mutations remain poorly understood (Garg et al., 2005), this study aims to enhance knowledge of the c.17_18delinsTT mutation, improving genetic screening for early detection and risk assessment. Additionally, refining PCR-based detection methods could facilitate future diagnostic advancements. Conducting this study in a Malaysian family also contributes valuable regional genetic data. Limited genetic research exists in Southeast Asian populations, and this study helps bridge the knowledge gap, informing genetic counselling and public health strategies. Findings will enhance understanding of *NOTCH1* mutations in aortopathy and broader medical genetics research.

METHODOLOGY

This study was approved by the Medical Research and Ethics Committee (MREC) of the Ministry of Health Malaysia (MOH) under reference number NMRR-21-1854-60563-IIR. Six buccal swab samples were collected from family members with a history of aortopathy at Hospital Kuala Lumpur (HKL). DNA extraction was carried out according to the protocol outlined in the DNA Genotek manual (PD-HB-00002). Primers were designed using Primer-BLAST, with the forward primer sequence 5' TGCAAATTTCAGTCGCCAGT-3' and the reverse primer sequence 5' CACACCGGCCTCCTAACTC-3'. PCR amplification was

performed with 5% (v/v) DMSO using an initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 5 min, and the resulting amplicons were visualized by agarose gel electrophoresis. Two amplification procedures, PCR 1 and PCR 2, were employed. PCR 1 utilized the extracted genomic DNA sample, while PCR 2 used the amplicon from PCR 1 as a DNA template to increase DNA copies due to limited sample availability. The final PCR 2 product was subjected to Sanger sequencing to determine the nucleotide sequence.

RESULTS AND DISCUSSION

Polymerase Chain Reaction

Figure 1 presents the agarose gel electrophoresis results of PCR 1. Lane 1 contained a DNA ladder used as a molecular size marker, while lanes 2–7 contained PCR products from six family member samples (A–F). Distinct bands of approximately 893 bp were observed in all samples, confirming successful amplification of the target *NOTCH1* gene fragment. The consistent amplicon size across all samples indicates the specificity and effectiveness of the designed primers. Lane 8 contained a negative control lacking DNA template, and the absence of amplification in this lane further confirmed the specificity of the PCR reaction and the absence of contamination.

Figure 2 presents the agarose gel electrophoresis results of PCR 2 for samples A–F. Distinct bands of approximately 893 bp were observed in all samples, confirming successful amplification of the target *NOTCH1* gene fragment. Samples A and C exhibited stronger bands, indicating higher DNA yield and amplification efficiency. In contrast, samples B, D, E, and F produced less intense bands, suggesting lower DNA abundance. As band intensity generally correlates with DNA quantity, these differences may reflect variations in template concentration among the samples (Arslan et al., 2021). The lower DNA abundance observed in samples B, D, E, and F may have contributed to the reduced quality of the sequencing templates, potentially explaining the unsuccessful Sanger sequencing results obtained for these samples. To facilitate downstream Sanger sequencing, individual wells were used to allow the isolation and purification of specific PCR products while minimizing sample loss and maintaining amplification consistency across reactions.

Dimethyl sulfoxide (DMSO) was incorporated into the master mix for both PCR 1 and PCR 2 to enhance specificity and efficiency. By reducing the formation of secondary structures in DNA templates and primers, DMSO minimized nonspecific amplification and improved primer annealing (Varadharajan & Parani, 2020). This facilitated double-stranded DNA (dsDNA) separation, leading to more efficient amplification (Kim et al., 2023). During gel electrophoresis, the expected bands were observed, with stronger signals indicating improved DNA yield. The addition of DMSO optimized PCR conditions by enhancing polymerase access and increasing product reliability, underscoring its crucial role in achieving accurate and high-yield amplification (Kim et al., 2021).

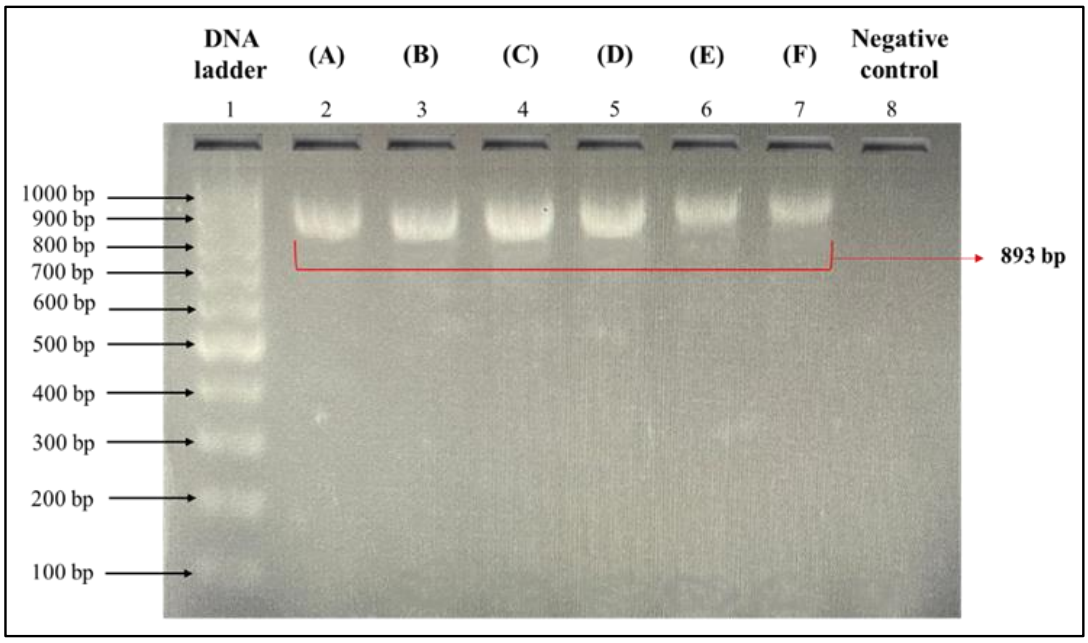

Figure 1 The result of gel electrophoresis from PCR 1 containing the six samples (A to F)

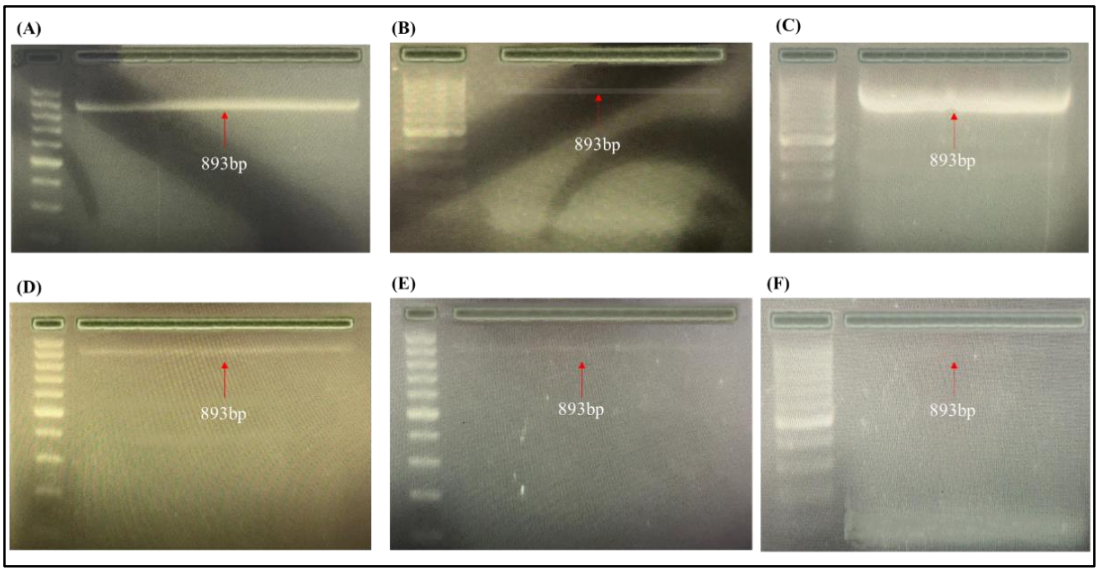

Figure 2 The results of gel electrophoresis from PCR 2 displaying bands for sample A to F

Sanger Sequencing

DNA sequencing was performed on samples A to F, but only samples A and C yielded successful results. The electropherogram of sample A showed a heterozygous mutation, indicated by overlapping peaks of "C" and "T". This suggests that one allele carries a mutation (T), leading to a C to T substitution, while the other allele retains the normal nucleotide (C). The overlapping peaks, represented by distinct colours, indicate the presence of two different sequences at this location in the *NOTCH1* gene (CG/TT). This heterozygous mutation may have clinical significance depending on the gene's nature, with dominant mutations potentially leading to disease phenotypes and recessive mutations resulting in asymptomatic carriers (Karger & Guttman, 2009). In contrast, sample C's electropherogram displayed sharp, distinct peaks, corresponding to (C) and (G), confirming a homozygous wild-type genotype (CG/CG). The absence of overlapping peaks suggests both alleles share the same nucleotide sequence, indicating no mutation at this site. The integrity of the genetic material was validated by minimal baseline noise in the electropherogram, ensuring reliable sequencing results (Al-

Shuhaib & Hashim, 2023). Overall, sample A demonstrated a heterozygous mutation, while sample C displayed a wild-type genotype, highlighting the importance of high-quality PCR products in obtaining accurate Sanger sequencing data (Figure 3). Sanger sequencing is particularly effective for detecting small insertions or deletions in DNA sequences (Carmody et al., 2016).

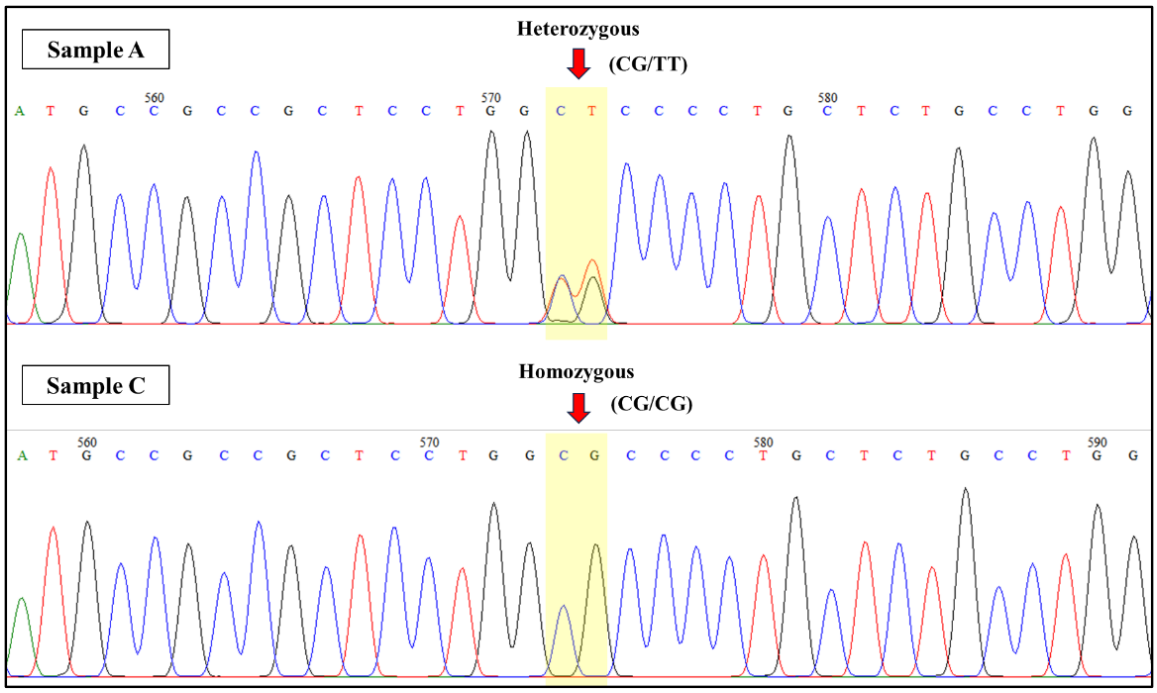

Figure 3 Electropherogram sequencing result for sample A and C displaying heterozygosity and homozygosity

CONCLUSION

This study aimed to investigate the presence of the c.17_18delinsTT mutation in the *NOTCH1* gene among family members with a history of aortopathy. By utilizing PCR and DNA sequencing techniques, the research sought to determine the mutation's inheritance pattern and its potential role in disease development. The findings provide valuable insights into the genetic basis of aortopathy, contributing to future diagnostic advancements and genetic screening approaches. This pilot study successfully developed and applied a PCR-Sanger sequencing workflow for detecting the *NOTCH1* c.17_18delinsTT variant in individuals from a family with a history of aortopathy. The method generated a target amplicon of 893 bp and enabled the identification of both heterozygous and homozygous genotypes in successfully sequenced samples. While the findings demonstrate the feasibility of the developed approach, the limited sample size and incomplete sequencing success restrict conclusions regarding the clinical significance of the variant. Future studies involving larger populations and functional analyses are required to further evaluate the role of this variant in aortopathy and to validate the utility of the proposed detection method.

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