Detection of Non-coding RNA (hsa_circ_0003416) in Pulmonary Arterial Hypertension Patients' Plasma by qRT-PCR Analysis

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Abstract. Circular RNA refers to a type of non-coding RNA molecule and possesses a circular conformation. Circular RNA has greater stability compared to linear RNA due to its resistance to RNAse activity. hsa_circ_0003416 is a circular RNA reported to have potential as a biomarker in patients with pulmonary arterial hypertension (PAH). This study aims to detect hsa_circ_0003416 using qRT-PCR in the plasma of PAH patients. The sample was taken from RSUP Dr. Sardjito Yogyakarta. The miRNeasy Serum/Plasma Advanced kit was used to isolate total RNA. Following that, cDNA was generated with an Applied Biosystems thermal cycler and the ExcelRT™ Reverse Transcription Kit II reagent, and qPCR was performed with an Applied Biosystems™ 7500 Real-Time PCR equipment and the SensiFAST™ SYBR® Lo-ROX Kit reagent. Specific divergent primers were utilized. Melting curve analysis and visualization of the amplified products were performed using gel electrophoresis. The qRT-PCR technique achieved a single amplification, resulting in a melting curve value observed at 81.8°C. Examination by visualizing the gel electrophoresis results showed a single band that matched the target size, specifically 124 base pairs. In conclusion, the qRT-PCR analysis successfully identified the presence of hsa_circ_0003416 in the plasma of PAH patients.

Keywords: Circular RNA, hsa_circ_0003416, Pulmonary arterial hypertension, Non-coding RNA, qRT-PCR

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INTRODUCTION

Circular RNA or circRNA is a unique form of RNA molecule that does not code for proteins (non-coding RNA) [1]. It is conserved across different species and exhibits a strong preference for specific tissues and cells [1,2]. Additionally, circRNA can be identified in the peripheral blood [3]. Circular RNA has enhanced stability compared to other RNA types due to its closed-loop structure, resulting in an extended half-life [4]. The attributes above make circRNA a promising candidate for future use as noninvasive and liquid biopsy biomarkers.

Circular RNAs can modulate gene expression both during and after transcription by interacting with microRNAs (miRNAs) or RNA-binding proteins [5]. They play a part in a diverse array of biological processes. Newly available data uncovers complex connections between several forms of RNA, including protein-coding messenger RNAs and non-coding RNAs [4]. The presence of this circRNA-miRNA-mRNA regulatory pathway is associated with the pathogenesis of numerous diseases, including pulmonary arterial hypertension (PAH) [6,7].

Dysregulated circRNA expression may lead to the development of PAH. A study found that hsa_circ_0003416 was markedly reduced in children with PAH associated with congenital heart diseases (CHD) and has potential as a biomarker [8]. An in-silico study has discovered that hsa_circ_0003416 is a circRNA molecule that is 124 base pairs in length. It originates from exon three of the thymosin beta 4 X-linked gene (TMSB4X) [8]. It plays a crucial function in regulating the process of angiogenesis [8]. There is a scarcity of studies on the expression of hsa_circ_0003416 in PAH associated with CHD. Furthermore, the utilization of qRT-PCR for the analysis of circular RNA remains unfamiliar, particularly in Indonesia. This study aims to detect hsa_circ_0003416 using qRT-PCR approaches from the plasma of PAH patients.

SUBJECT AND METHOD

The study was conducted in the Integrated Research Laboratory of the Faculty of Medicine, Public Health, and Nursing at Universitas Gadjah Mada (UGM). The specimens utilized were plasma samples obtained from patients diagnosed with PAH associated with CHD, who had an examination at RSUP Dr. Sardjito Yogyakarta and were subsequently enrolled in the Congenital HeARt Disease in adult and Pulmonary Hypertension (COHARD-PH) registry [9]. Medical And Health Research Ethics Committee (MHREC) Faculty of Medicine, Public Health And Nursing UGM approved this study (number KE/FK/0429/EC/2023).

The RNA isolation processed using the miRNeasy Serum/Plasma Advanced kit from Qiagen [217204]. The sample volume utilized was 200 µl. The isolation technique is executed in accordance with the manufacturer's protocol. The isolation results were further analyzed using a nanodrop to ascertain the concentration and purity of RNA.

The cDNA synthesis was performed using the ExcelRT™ Reverse Transcription Kit II from Smobio [RP1400], following the manufacturer's instructions. The RNA templates utilized were 50 ng. The cDNA synthesis method was conducted using an Applied Biosystems thermal cycler, the protocol is as follows: incubation step at 25°C

(10 minutes), reverse-transcription step at 42ºC (50 minutes), and inactivation of reaction 85ºC (5 minutes).

For qPCR hsa_circ_0003416, the SensiFAST™ SYBR® Lo-ROX Kit [BIO-94005] was employed in accordance with the manufacturer's instructions, utilising the Applied Biosystems™ 7500 Real-Time PCR instrument. The cDNA product was diluted in a ratio of 1:5 before being used as a template. The primers used are Forward divergent CCCCTTTCACACATCAAAGAAC and Reverse divergent ATTTAAACTTGATCCAACATGC [8]. The primer concentration used was 400nM with a total reaction volume of 20 µl. The qPCR protocol is as follows: polymerase activation at 95ºC (2 minutes), 40 cycles of denaturation step at 95ºC (5 seconds), and annealing/extension step at 60ºC (30 seconds). The amplicons were subjected to melting curve analysis and visualization using gel electrophoresis when they were completed.

RESULTS

A study was done using the protocol described in the methods section. The findings from the hsa_circ_0003416 analysis are presented in Figure 1. According to the qRT-PCR technique employed, a single amplification was achieved, resulting in a melting curve value observed at a temperature of 81.8ºC.

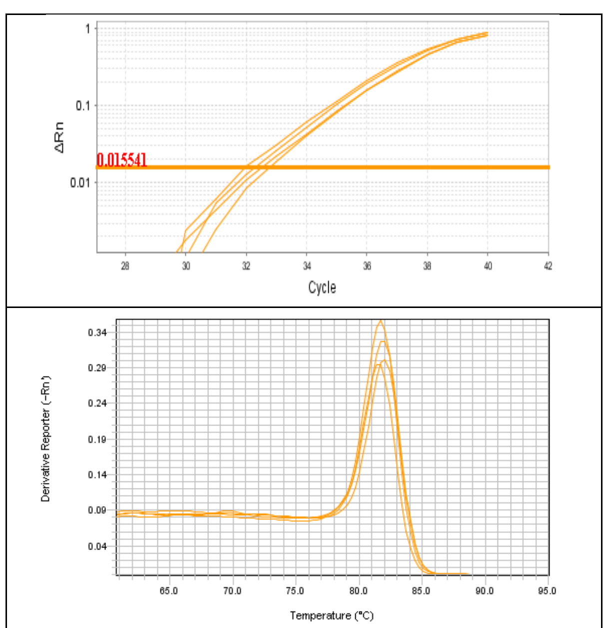

Figure 1. qPCR amplification (top) and melting curves (bottom) of hsa_circ_0003416.

Subsequently, gel electrophoresis was conducted to verify the amplification product. The visualization results with gel electrophoresis are shown in Figure 2. There is a visible band within the sample that is about 124 base pairs in length.

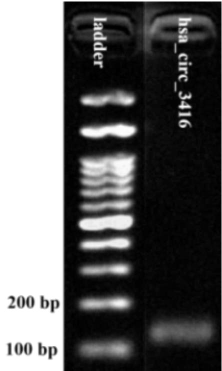

Figure 2. Visualization with 2% gel electrophoresis.

DISCUSSION

A qRT-PCR study was performed to amplify the hsa_circ_0003416 target in the plasma of patients diagnosed with PAH associated with CHD at Dr. RSUP. Sardjito Yogyakarta. Circular RNA is a circular-shaped non-coding RNA. Unlike linear RNA, the analysis of circRNA requires the utilization of random primers during the cDNA synthesis step [10]. The subsequent phase involves the utilization of a unique primer known as a divergent primer. Divergent primers are designed to amplify a specific portion of circRNA, known as the backsplice junction, by working in the opposite manner as shown in Figure 3 [11]. Amplification will take place if the cDNA template utilized includes the desired circRNA.

The approach we employ, while able to identify the target circRNA, has limitations, specifically that the target circRNA is detected at a relatively high cycle threshold value (CT Value) of approximately 32. These results suggest a low abundance of circRNA targets in the cDNA template. Therefore, additional steps, such as the circRNA purification procedure, must be considered. The RNase R treatment might enhance the presence of circular RNAs in an RNA sample. This strategy can effectively eliminate linear RNAs [12].

A single peak in the melting curve analysis signifies the existence of a distinct qPCR product. Subsequently, the qPCR product can be observed by gel electrophoresis. Essentially, the amplicons generated by qPCR will migrate across the medium and come to a halt at a specific location based on their size. A band will be formed at the location where these amplicons accumulate, which will be visible [13]. Thus, the size of the amplicon generated by the qPCR reaction can be determined using gel electrophoresis. The investigation revealed that the length of hsa_circ_0003416 was 124 base pairs. The acquired gel electrophoresis results showed a solitary band within the 100-150 bp range, confirming the findings. Nevertheless, a sequencing analysis is required to validate this result [11].

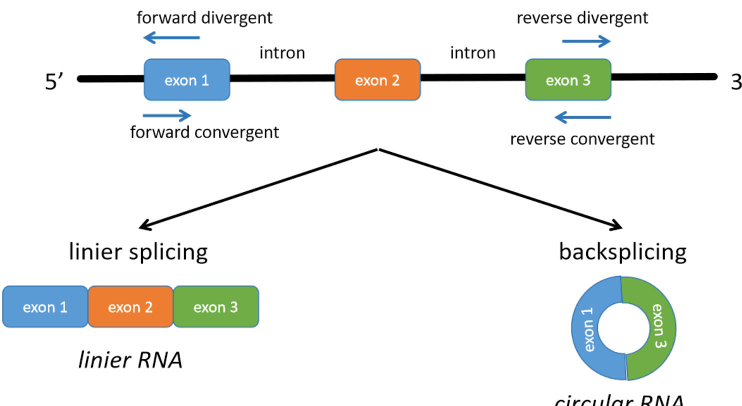

Figure 3. The linear RNA that is made by linear splicing is shown on the left, and the circRNA that is made by back splicing is shown on the right. These two types of primaries (convergent and divergent) work in different directions

This study aims to verify the qRT-PCR method for assessing the expression of the hsa_circ_0003416 gene in patients with PAH at RSUP Dr. Sardjito Yogyakarta. It is a preliminary research report. Subsequently, all research samples will undergo examination in order to acquire expression patterns. Patients with PAH may have an aberrant regulation of hsa_circ_0003416 expression, which could play a role in the development of the disease.

CONCLUSION

The qRT-PCR approach, as described in the protocol, is capable of detecting hsa_circ_0003416 in the plasma of patients diagnosed with PAH associated with CHD at RSUP Dr. Sardjito Yogyakarta. Additional research, utilizing a sufficient number of samples, is required to ascertain the expression pattern of hsa_circ_0003416.

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