The in silico design of stem-loop Real-time PCR for detection of hsa-miR-140-3p expression on human Osteoarthritis

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ABSTRACT

The deregulation of microRNA-140 (miRNA-140) has been reported to be involved in the human pathogenesis of Osteoarthritis (OA). However, in Vietnam, to our knowledge, there is a limited study of evaluation of a miRNA-140 expression. The commercial kits for the quantitation of miRNA-140 are more expensive. Thus, for the aim to establish the less cost assay for quantitation of miRNAs in developing countries, we designed the set of specific primers to quantify the expression of microRNA for further application on the local population. The sequence of hsa-miR-140-3p was input from the miRNA database using the accession number MI0000456. Following bioinformatics tools, including RNAfold Web Server, BioEdit, IDT Oligo analyzer, were used to design the set of specific primers. As a result, we successfully designed the set of specific primers, including stem-loop primer, named as StL-miR-140-3p, which is specific to the target sequence of hsa-miR-140-3p, and specific forward primer and reversed primer, named as StL-F-miR-140-3p, and StL-F-miR-140-3p, respectively, which designed for the target to the sequence of hsa-miR-140-3p. For further study, it is required to conduct the assay, which is based on those designed primers, on the clinical samples to evaluate the specificity, sensitivity, limit of detection, as well as carried on the local population.

1. Introduction

Osteoarthritis (OA) is a common degenerative joint disease and a leading cause of pain disability, affecting more than 25% of the adult population (Chen et al., 2017; Lao & Le, 2021). The etiology of OA has been reported including both exogenous, such as knee events, obesity, lifestyle factors, nutrition, occupation, sports, etc., and endogenous factors, such as age, gender, ethnicity, genetics, epigenetic, etc. (Lao & Le, 2021; Michael, Schlüter-Brust, & Eysel, 2010). Tepigenetic modification, which refers to the changes in gene expression without any changes in DNA sequence, includes DNA methylation, histone modification, and microRNAs (miRNAs) (Chuang & Jones, 2007; Lao & Le, 2021; Yao, Chen, & Zhou, 2019). MicroRNAs (miRNAs) are the abundant class of evolutionarily conserved single-stranded non-coding RNA molecules, typically represent ~20 nucleotides in length, which can bind to the 3'-untranslated region of their target mRNAs, resulting from being down regulated (Felekkis, Touvana, Stefanou, & Deltas, 2010; O'Brien, Hayder, Zayed, & Peng, 2018). Resulting in the affections on numerous

cellular processes, including cell proliferation, differentiation apoptosis, and metabolism. In recent years, numerous remarkable studies demonstrated that the dysregulation of miRNAs has emerged as important modulators in the pathogenesis of OA (Beyer et al., 2015; Karlsen, Jakobsen, Mikkelsen, & Brinchmann, 2014; Lao & Le, 2021; Ntoumou et al., 2017). Among miRNAs, putative roles of miRNA-140, located at chromosome 16 (nucleotide: 69933081-69933180, [+]), in the pathogenesis of OA has emerged. In the previous study reported by Miyaki et al. (2009) miRNA-140 was expressed in the normal human articular cartilage. The reduction of miRNA-140 expression was observed in the OA cartilage and in response to IL-1β, a cytokine classically involved in the pathogenesis of OA, thus, may contribute to the pathogenesis of OA (Miyaki et al., 2009). The expression was decreased in osteoarthritic cartilage compared with healthy cartilage (Beyer et al., 2015; Ntoumou et al., 2017). Also, several genes, including *ADAMTS5*, *MMP-13*, *IGFBP5*, and *RALA* have been identified as the target gene of miRNA-140, which play important roles in mediating the degradation of cartilage matrix, modulating the availability of IGF-1 in joint (Karlsen et al., 2014; Liang et al., 2012; Miyaki et al., 2009). The transgenic mice with up-regulated expression of miRNA-140 pointed out to be resistant to antigen-induced arthritis via the regulation of ADAMTS5, a major cartilage matrix-degrading protease in OA (Miyaki et al., 2009). Based on these findings, miR-140 was required for the development of skeletal as well as cartilage homeostasis and protected against OA-like pathophysiology via the regulation of numerous target genes; therefore, quantitation of miRNA-140 might be highlighted its possible application as the biomarker for the pathogenesis of OA.

Real-time PCR is extensively used to quantify nucleic acids. However, due to the length of microRNAs being too short, only an average size of 22 - 24 nucleotides, it leads to the difficult task of designing an adequate primer or probe for the level of specific amplification using standard Real-time PCR methods. Therefore, many methods, including poly(A)-tailing RT-PCR, primer-extension RT-qPCR, stem-loop Real-time PCR, etc., have been applied to increase the length of miRNA, which create a long sequence template sufficient to design a set of primers (Rachh & Desai, 2017). Among them, stem-loop Real-time PCR with a specific set of primers has the benefit that is good for additional flexibility for primer design and better specificity (Rachh & Desai, 2017). Here, we took the miRNA-140 (hsa-miR-140) for designing a set of specific primers to detect as well as quantify its expression for further application on its abilities served as the biomarker for the pathogenesis of OA on the local population.

2. Materials and methods

2.1. Sequence input

The sequence of hsa-miR-140 was input from miRNA database: miRBase (MiRBase: The microRNA database, n.d.) using the accession number of MI0000456. The selection of the desired sequence of hsa-miR-140 is hsa-miR-140-3p, which is the precise sequence of the miRNA-140 of interest.

2.2. Primers design for hsa-miR-140-3p

The set of specific primers includes (1) stem-loop primer for the first stage, which creates a cDNA - a long sequence template, which is used as a template for the second stage; (2) forward primer and reverse primer, which used to amplify the sequenced cDNA. The secondary structure of stem-loop primer was analyzed by RNAfold Web Server (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). The characteristic of a set of primers was evaluated by using the IDT Oligo analyzer (https://www.idtdna.com/pages/tools/oligoanalyzer).

3. Results

3.1. Sequence of hsa-miR-140-3p input

The sequence of hsa-miR-140 was collected from the miRNA database (Accession number: MI0000456). The length of *hsa-miR-140* is 100 nucleotides, contains two strands: passenger strand (hsa-miR-140-5p) and mature strand (hsa-miR-140-3p) (**Figure 1, Table 1**). Among hsa-miR-140-5p and hsa-miR-140-3p, the sequence of hsa-miR-140-3p is reported as the precise sequence, which was chosen as target miRNA for primer designing.

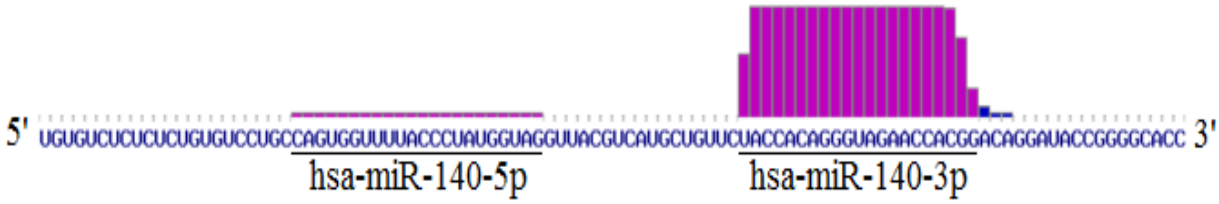


Figure 1. The sequence of hsa-miR-140 (Accession number: MI0000456) contains hsa-miR-140-5p and hsa-miR-140-3p

Table 1

Sequences of hsa-miR-140, hsa-miR-140-5p and hsa-miR-140-3p

Sequences (5'-3')		**Length (nucleotide)**
hsa-miR-140	UGUGUCUCUCUCUGUGUCCUGCCAGUGG UUUUACCCUAUGGUAGGUUACGUCAUGC UGUUCUACCACAGGGUAGAACCACGGAC AGGAUACCGGGGCACC	100
hsa-miR-140-3p	UACCACAGGGUAGAACCACGG	21

Source: miRNA database (Accession number: MI0000456) (MiRBase: The microRNA database, n.d.)

3.2. Designing the set of specific primers for amplifying hsa-miR-140-3p

Output designing primers contains a stem-loop primer, named StL-miR-140-3p, which is specific to a target sequence of hsa-miR-140-3p, and specific forward primer and reversed primer, named as StL-F-miR-140-3p, and StL-F-miR-140-3p, respectively, which designed for the target to the sequence of cDNA. The sequences of designed primers are shown in Table 2.

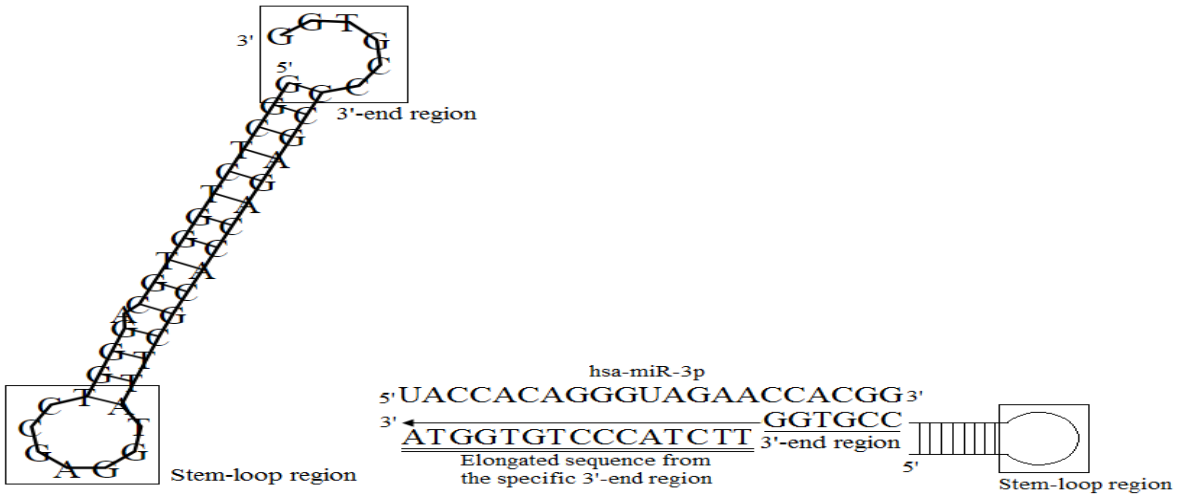
Table 2

Sequences of StL-miR-140-3p, StL-F-miR-140-3p, and StL-F-miR-140-3p

Sequences (5'-3')		**Length (nucleotide)**
StL-miR-140-3p	GGCTCTGGTGCAGGGTCCGAGGTATT CGCACCAGAGCCCCGTGG	44
StL-F-miR-140-3p	TCTGGTGCAGGGTCCGAGGTA	21
StL-R-miR-140-3p	TACCACAGGGGAGAACCACGG	21

Source: Data analysis result of the research

The secondary centroid structure of StL-miR-140-3p was predicted and analyzed by using RNAfold Web Server (**Figure 2**). According to the centroid secondary structure of StL-miR-140-3p, it formed the hair-spin structure (ΔG = -23.20 kcal.mol^{-1}) within following part: (1) non-complementary stem-loop region; and (2) non-complementary 6 nucleotides at the 3' end region (**Figure 2A**). The sequence at the 3'-end region of (5'-CCGTGG-3') of the StL-miR-140-3p is a specially complementary to mature hsa-miR-140-3p at the sequence of 3'-GGCACC-5', and the elongated sequence from the specific 3'-end region of 5'-TTCTACCCTGTGGTA-3' was double-underlined (**Figure 2B**). The StL-F-miR-140-3p and StL-R-miR-140-3p primer were designed for Real-time PCR and yielded the product of 56 bps in length, and Tm of the yielded product was 74.7ºC (**Figure 2C**). Moreover, nucleotide BLAST for the determination of the specificity of each primer showed that no complement was to any other sequences, except its pertaining target hsa-miR-140-3p. The characteristic of StL-miR-140-3p and hsa-miR-140-3p were analyzed by using the IDT Oligo analyzer. The results of the analysis of StL-F-miR-140-3p and StL-R-miR-140-3p are shown in **Table 3**. Based on the analysis, it was concluded that a set of specific primers was successfully designed to detect the expression of hsa-miR-140-3p based on the Real-time PCR method.



(A) (B)

Figure 2. The structure of StL-miR-140-3p. (A) The secondary structure of StL-miR-140-3p. (B) Schematic representation of complementary sequence between StL-miR-140-3p and StL-R-miR-140-3p. The elongated sequence from the specific 3' end is double-underlined

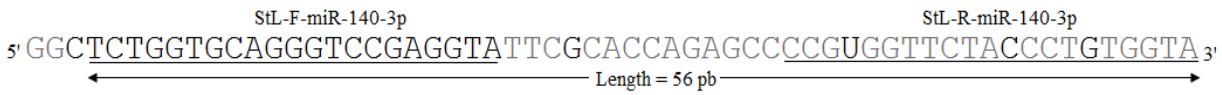


Figure 3. The product yield by StL-F-miR-140-3p and StL-R-miR-140-3p

Table 3

The characteristic of StL-miR-140-3p and hsa-miR-140-3p

Primers	Length (Nucleotide)	C+G percent (%)	Melting temperature (ºC)	ΔT_m
StL-F-miR-140-3p	21	61.9	62.3	1.3
StL-R-miR-140-3p	21	61.9	61.0	

Source: Data analysis result of the research

4. Discussion

Several RT-qPCR methods, such as poly(A)-tailing RT-PCR, primer-extension RT-qPCR, stem-loop RT-qPCR, miQPCR, etc., for miRNAs detection, including miRNA-140, has been developed (Androvic, Valihrach, Elling, Sjoback, & Kubista, 2017; Rachh & Desai, 2017). These commercial kits for the quantitation are more expensive; thus, at a less cost it's possible to perform the assay for quantitation of miRNAs in developing countries, we tried to develop the stem-loop Real-time qPCR method to apply in detection as quantifying its expression in the local population. The abnormal expression of miRNA-140 has been found in the human pathogenesis of OA. However, up to date, no in Vietnam, no study about miRNA-140 detection in Vietnamese human OA patients has been reported. For this purpose, miR-140, which has been demonstrated as the various roles in the human pathogenesis of OA, was chosen to be the miRNA target for designing the set of specific primers.

The primer design for the detection of miRNAs has to face many challenges due to the average size of miRNAs of only 22 - 24 nucleotides (Rachh & Desai, 2017). This problem could be solved by increasing the length of a sequence by adding a tail to the miRNA. In this study, the length of hsa-miR-140-3p is 21 nucleotides. For this solution, a stem-loop sequence was added to the sequence of hsa-miR-140-3p to increase its length. In the current study, stem-loop Real-time PCR was designed through designing the set specific primers, including stem-loop primer: StL-miR-140-3p, specific forward primer: StL-F-miR-140-3p, and reverse primer: StL-F-miR-140-3p. In detail, the StL-miR-140-3p was designed to target specifically reversed transcriptase of hsa-miR-140-3p based on the non-complementary 6 nucleotides (5'-CCGTGG-3' targeting to 3'-GGCACC-5'). After the reverse transcription PCR assay, the size of the cDNA sequence, which was amplified by the specific StL-miR-140-3p, was elongated to 59 nucleotides. The last stage of stem-loop Real-time PCR was performed with the couple-specific primer to the 59-nucleotide cDNA obtained from the previous step. Using the StL-F-miR-140-3p and StL-R-miR-140-3p in the Real-time PCR amplification yielded the amplified product within 56-bps in length. Additionally, the ΔT_m of StL-F-miR-140-3p and StL-R-miR-140-3p was 1.3^{o}C, which was less than 4.0^{o}C, making the choice of designing Ta to be feasible, increasing the possibility of successful assay design. In general, we succeeded in developing the stem-loop Real-time PCR method for detection of hsa-miR-140-3p expression based on the designed principles of this method. It will be easy to expand to develop each specific set primer for each interested miRNAs.

5. Conclusion

In this study, we successfully described the designed stem-loop Real-time PCR method for the detection of hsa-miR-140-3p expression. A set of primers of current stem-loop Real-time PCR method includes stem-loop primer: StL-miR-140-3p, specific forward primer: StL-F-miR-140-3p, and reversed primer: StL-F-miR-140-3p. For further study, it is required to conduct the assay, which is based on those designed primers, on the clinical samples to evaluate the specificity, sensitivity, limit of detection.

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Conflicts of Interest

The authors declare no conflict of interest.

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