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Original Article

COMPARISON OF GENES EXPRESSION; MIRNA 146 A, MIR-103, MIR-423-3P, MIR-21, MIR-16 IN CELL LINES HEP-G2 SERIES 1886 AND PLC5

ASEP SUKOHAR[1], HENING HERAWATI[2], ARIEF B WITARTO[2], HENDRA T SIBERO[3], SUTYARSO[4]

[1]Department of Pharmacology and Therapy, Faculty of Medicine, Lampung University, Post Code 35145, Bandar Lampung, Indonesia. [2]Department of Research and Development, Dharmais Cancer Hospital, Jakarta, Indonesia. [3]Department of Dermatology, Faculty of Medicine, Lampung University, Post Code 35145, Bandar Lampung.[4]Department of Biology, Faculty of Medicine, Lampung University, Post Code 35145, Bandar Lampung.

Email: asepsukohar@gmail.com

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ABSTRACT

Objective: Detection of hepatocellular cancer cells(HCC) *in vitro* has reached the smallest level, that is miRNA(micro RNA) in particular miRNA146A. This study was conducted from February to May2013 at the Department of Molecular Biology, Dharmais Cancer Hospital. The purpose of this study was to compare the value of Cq and expression ofmicroRNA(miRNA/mir) on Hep-G2 and PLC5.

Methods: *In vitro* study were performed in Hep-G2 series 1886 and PLC5. The study used on 33 samples consisting of 5 groups of mir (miRNA146A, miR-423-3p, miR-103, miR-21and miR-16) in Hep-G2 and 5 groups of mirinPLC5 with 3 times repetition, 1 NTC and 2 spike.

Results: MiRNA146 A expressionin Hep-G2against mir-423-3p, mir-103, mir-21, mir-16resulted in values as follows in consecutive order: (0.05), (0.03), (0.01), and (0.55). The highest expression was: miRNA146 Aagainst mir-16 and the smallest expression miRNA146 Aagainst mir-21in Hep-G2. MiRNA146 A expressionincell linesPLC5against mir-423-3p, mir-103, mir-21, mir-16resulted in values as follows: (0.01), (0.00), (0.00), and(0.02). The highest expression: miRNA146 A against mir-16, and the smallest expression: miRNA146 Aagainstmir-103 and mir-21 in PLC5.

Conclusion: MiRNA 146 A, mir-423-3p, mir-103, mir-21, and mir-16 were expressed in Hep-G2 series 1886 and PLC5. The highest gene expression in miRNA146 A against mir-16 was found inHep-G2 andPLC5. The lowest gene expression in miRNA 146 A against mir-103 andmir-16 was found in PLC5.

Keyword: Cell lines Hep-G2, miRNA 146 A, mir-423-3p, mir-103, mir-21, mir-16, PLC5.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth highest cancer in men (7.9%) and seventh in women (6.5%) in the world, as much as 85% HCC incidence in developing countries. Hepatocellular carcinoma is the third highest cause of death from cancer in the world with a mortality ratio of 0,93 [1].

Early detection of cancer, rapid management and development of new drugs are required research, *in vivo* and in vitro. Along with the advancement of science and technology in medicine, detection of hepatocellular cancer cells in vitro has reached a cellular level,called miRNA(micro RNA) especially miRNA 146 A.

Micro RNA (miRNA) is a new non-protein-coding ribonucleotide acid, micro size, consisting of 19-25 paired base which is functional in inhibiting gene expression. Mi RNA was discovered by Victor Ambros and had been developed since 2000 [2].

Micro RNA is transcribed from DNA, but it is not processed into a protein/ polynucleotide. RNA polymerase II helps it turn into pri-miRNA (primary MicroRNA). Pri-miRNA with coding was cut in the nucleus and becomes shorter in shape and turned to be miRNA precursor. This reaction is done by a complex protein called microprocessor by involving Drosha which has an RNase III enzyme inside [3].

Pre-miRNA is a short loop with ~70 nucleotides. Pre-miRNAs release from the nucleus, assisted by Exportin-5, splitting in the cytoplasm using the enzyme RNase III, resulting in a 19-25 nucleotide matures double miRNA. MiRNA strands with an unstable partner (guide strand), divided into the RISC (RNA-induced silencing complex), which consists of the TRBP (TAR RNA Binding Protein) and protein Ago2 (Argonaute) [3].

MiRNA target is poly (A) at the end of 3′mRNA [3-4]. MiRNA 146 A gene target is C-Myc (Innate immunity response) and ERK5 (tumor-suppressor gene). C-Myc pathway is an innate immune response pathway through the NF - KB pathway. NF-KB activates miRNA 146 AthroughP65 and P50 gene. WhenNF-KB activity increases, so does miRNA 146 A. Moreover, miRNA 146 A will inhibit down regulation of IRF5 (Interferon Regulatory Factor-5), STAT-1(Signal Transducer and Activator of Transcription-1) and inhibit up regulation of IRAK-1 (Interleukin-1 Receptor-Associated Kinase 1), IRAK-2 (Interleukin-1 Receptor-Associated Kinase 2) andTRAF-6 (TNF Receptor Associated Factor). TRAF 6 gene targets are TGF-beta activated kinase 1/MAP3K7 binding protein 2, TAB ½ and TAK 1 (Triticum aestivum kinase) [5-7].

The target of TAB 1/2 and TAK 1 gene is to inhibit IKKe gene (inhibitor of kappa light polypeptide gene enhancer in B-cells/ kinase epsilon). The IKKe gene target is to inhibit IKb (Inhibitor kappa beta). Another pathway TRAF 6 gene can directly activate MAPK and then activate ERK5 cascade [5-7].

We want to compare between the miRNA146 A gene expression,mir-423-3p, mir-103, mir-21, mir-16 in cancer cells Hep-G2 1886 and PLC5. In addition, we would like to assess the target gene (gene expression of miRNA146A) and the reference genes (mir-423-3p, miR-103, mir-21, and mir-16). Mir-423-3p, mir-103, mir-21 and mir-16 are often used for reference genes [8-11].

MATERIALS AND METHODS

Descriptive and experimental studies were conducted on Cell Lines (Hep-G2,No: 1886). In this research Hep-G2 which were obtained from the Riken Cell Bank-Tohoku University, proliferated at Dharmais Cancer Hospital Molecular Biology Laboratory with inclusion criteria: Cell Lines Hep-G2 is taken from the same origin, grew on cultured media until it reached 60-80% were confluent.

Cell lines Hep-G2 1886 were cultured using Dulbecco's Modified Eagle Medium (DMEM) from Gibco 11965, 10% fetal bovine serum

(FBS) from Gibco 26140 and 1% penicillin-streptomycin from sigma P4333. The cell was cultured in the incubator on 37°C temperature, 5% humidity of CO2 and medium alteration every 2 days.

The samples were divided into 2 groups (Cell Lines Hep-G2 and PLC5), every group consists of 5 wells and 3 times repetition (15 wells). This research had 33 groups in total, including 2 spikes and 1 NTC. It was tested using RT-PCR. The RNA total isolation used Exicon protocol with 203300 product codes, then made cDNA by using Universal kit 203300.

The purpose of the research was to analyze gene expression of miRNA 146 A, mir-423-3p,mir-103, mir-21 and mir-16 on cell lines Hep-G2 and PLC5.

Optimization of miRNA 146 A reference gene candidate has been done previously and published [10]. Primer reverse and forward miRNA 146 A used Exicon: 204688, mir-423-3p, mir-103, mir-21, mir-16 Exicon: 204488, 204063, 204230 and 204409.

Gene expression was analyzed using Livaks method, and normality test was analyzed using the Shapiro-Wilk. The fifth corelation of miRNA: miRNA 146 A, mir-423-3p, mir-103, mir-21 and mir-16 was analyzed using Pearson correlation test [12-13].

RESULTS

MiRNA 146 A gene expressions were analyzed using Livak method with formula: $2^{-\Delta\Delta Cq}$. Normalization was done by lessening each Cq target value with the Cq reference value resulting in ΔCq. ΔCq was determined by decreasing ΔCq target value upon ΔCq reference[12].

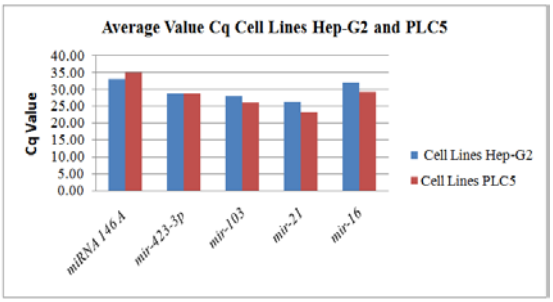

Fig. 1: The average of Cq miRNA 146 A, mir-423-3p, mir-103, mir-21, mir-16 on cell lines Hep-G2 and PLC5 shows an average Cq target of miRNA 146 A with 4 references mir such as: mir-423-3p(28.76), mir-103(27.96), mir-21(26.34) and mir-16(32.17) on cell lines Hep-G2. The average of Cq target miRNA 146 A(35.07) with 4 references mir are: mir-423-3p(28.66), mir-103(26.14), mir-21(23.16) and mir-16(29.23) on cell lines PLC5.

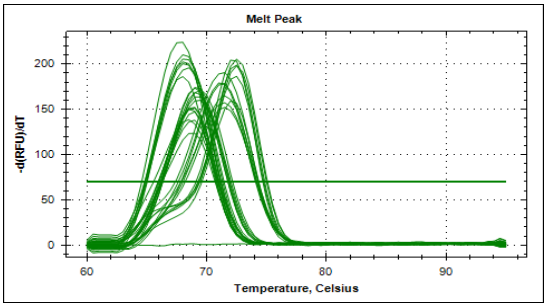

Fig. 2: Melt peak between Hep-G2 1886 and PLC5 with RT PCR CFX-96 shows information about the highest temperature that reached 68°C with Relative Fluorescence Unit (RFU) 220 on mir-21 cell lines Hep-G2 and the second peak is on 72°C with RFU 200 on mir-16 cell lines PLC5.

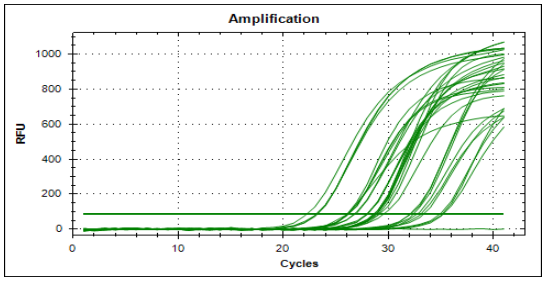

Fig. 3: Amplification of miRNA 146 A, mir-423-3p, mir-103, mir-21 and mir-16 on Hep-G2 1886 and PLC5 with RT-PCR CFX-96 showsinformation about amplification that began at the twentieth cycle,which started from cell lines PLC5 mir-21 and ended at cell lines PLC5 miRNA 146 A on the thirty fourth cycle.

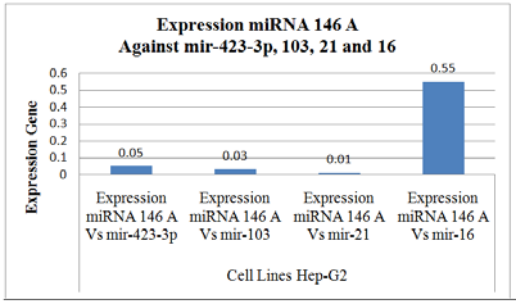

Fig. 4: Expression of miRNA 146 A against mir-423-3p, mir-103, mir-21 and mir-16 on cell lines hep-G2 shows expression in cell lines Hep-G2, miRNA 146 A to mir-423-3p (0.05), miRNA 146 A to mir-103 (0.03), miRNA 146 A to mir-21 (0.01) and miRNA 146 A tomir-16 (0.55). Based on fig. 4, the highest expressionwasmiRNA 146 A to mir-16 and the lowest expressionwasmiRNA 146 A to mir-21.

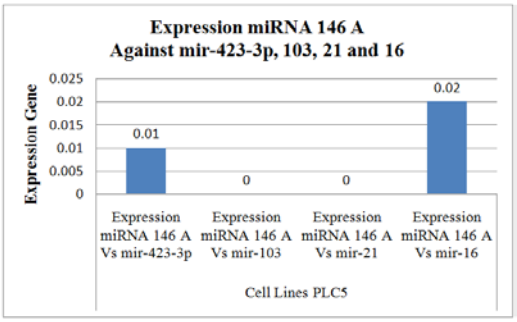

Fig. 5: MiRNA 146 A expression against mir-423-3p, mir-103, mir-21 and mir-16 on PLC5 shows expression on cell lines PLC5, miRNA 146 A to mir-423-3p (0,01), miRNA 146 A to mir-103 (0.00), miRNA 146 A to mir-21 (0.00) and miRNA 146 A to mir-16 (0.02). Based on fig. 5, the highest expressionwasmiRNA 146 A againstmir-16, and the lowest expressionwasmiRNA 146 A to mir-103 and mir-21.

Annotation: using pearson correlation test

Correlation grading standard between miRNA gene was determined based on a range from; ≤ 0. 2 very low and ≥ 0. 9 very strong. The moderate correlation is between 0.2 and 0.7, and between 0.7 to 0.9 is the strong correlation [13].

Table 1 shows the poor negative correlation among miRNA 146 A andmir-103 and mir-423-3p. The strong negative correlation is between miRNA 146 A and mir-16 on cell lines Hep-G2. The strong positive correlation is between miRNA 146 A and mir-21cell lines Hep-G2 [10].

The poor negative correlation was found between miRNA 146 A and mir-103. The strong positive correlation between miRNA 146 A and mir-16 and the poor positive correlation among miRNA 146 A, mir-423-3p and mir-21 on cell lines PLC5 [10-11].

Table 1: Correlation of miRNA 146 A on Cell Lines Hep-G2 and PLC5 Against mir-423-3p, mir-103, mir-21, mir-16

Correlation	mir-423-3p	mir-103	mir-21	mir-16
miRNA 146 Acell lines Hep-G2	-0.679	-0.253	0.745	-0.735
miRNA 146 Acell lines PLC5	0.430	-0.500	0.038	0.799

DISCUSSION

PCR real-time quantification can be done using two procedures. First, by using dye fluorescence, which tightened with double stranded DNA. Second, by using probes, DNA oligonucleotides modification that emitted fluorescence when DNA complements hybridization. The result was analyzed by computer software that connected with the thermal cycle to calculate the number of SYBR Green DNA as dye fluorescence. This method was easier to detect the PCR product,more economical, more sensitive compared to probe usage and ethidium bromide coloration on agarosa gel electrophoresis. SYBR Green works by binding to double-stranded DNA and excites rays. A ray fluorescence increases in proportion to the accumulation of PCR product.

Amplification of the same sample with the same primary will have the same melting temperature (the temperature when double stranded DNA changed into single stranded DNA). Melting values can be used to establish the specificity of the test and can only be read on the melt curve. Melt curve is a graph that compare the temperature and RFU derivative due to temperature in discovering sample specificity. To obtain a melt curve, amplification performed at 95°C in 10 seconds, continued with 60°Cin 1 minute. Fig. 2, giving us information about the highest temperature that reached 68°C with RFU 220 on mir-21 cell lines Hep-G2 and the second peak on 72°C with RFU 200 on mir-16 cell lines PLC5. The cell lines Hep-G2 melt peak miRNA 146 A, mir-423-3p, mir-103, mir-16 in respective order 69°C, 71.5°C, 69°C and 72.5°C. While at cell lines PLC5 melt peak miRNA 146 A, mir-423-3p, mir-103, mir-21 in respective order 69°C, 71.5°C, 69.5°C and 68°C. Melting peak cell lines Hep-G2have the same value asour previously published study [10-11].

RT-PCR amplification stage was preceded in thermal cycle and was shown in graph to monitor with software Bio-Rad CFX Manager 2.1 (Hercules, California). Graphs were obtained in the form of amplification graphs, a standard curve, melting curve (melt curve) and melting peak curve. Graphs were used to evaluate the performance of RT-PCR amplification. Amplification curve was formed when the amplification process has begun and the use of this curve is to determine whether it contains the occurrence of amplification or not in a thermal cycler.

Amplification was determined based on the intensity of fluorescence. The more amplification products were formed, the greater accumulation of fluorescent would be legible. The fluorescence rise was characterized by the formation of sigmoid graphs. Sigmoid graphs would intersect with the baseline threshold that was determined automatically by the program / software. The intersection point between sigmoid graph and baseline threshold reflected on the x-axis (cycle) which was previously called the threshold cycle (Ct), but which is now called cycle quantification (Cq). Cq value is the quantification cycle of the product accumulation (valued 2n, n is the number of repetition cycles of amplification) which was initiallyread at the exponential phase. The exponential phase ends into the plateau phase and the same time the reactants in the PCR reaction mix. Cq values are used in the standard curve as a function of the concentration log of Hep-G2. Fig. 3 shows data about amplification which is started on the 20th cycle with cell lines PLC5 mir-21 and ended at the 34th cycle of cell lines PLC5 miRNA 146 A. These cycles in this study are different from previous studies of active anticancer compounds in which the last cycle occurred close to 40 [10-11].

There are two methods to analyze the results of RT-PCR (qPCR): absolute quantification method (measure Cq sample to standard curve) and relative quantification (compare Cq sample to Cq reference gene). In this study, the latter method was used. The result show that the average of Cq mir-423-3p, mir-103, mir-21 and mir-16 are higher on cell lines Hep-G2 than PLC5. The average of miRNA 146 A Cq is higher on cell lines PLC5 than cell lines Hep-G2 as shown on fig. 1.

The miRNA 146 A, mir-423-3p, mir-103, mir-21 and mir-16 gene expression analyses used relative quantification method with Livak formula; $2^{-\Delta\Delta Cq}$. The way to normalize it is by subtracting Cq target value with Cq reference value (mir-423-3p, mir-103, mir-21 and mir-16). As a result, Δ Cq data are obtained from RT-PCR were processed software Bio-Rad CFX Manager 2.1 (Hercules, California). The data were from the amplification result of RT-PCR in numerical that describes cycle quantification (Cq) then analyzed using software excels [11].

The highest gene expressions in cell lines Hep-G2resulted; miRNA 146 A to mir-16, miRNA 146 A to mir-423-3p, miRNA 146 A to mir-103, miRNA 146 A to mir-21 are 0.55, 0.05, 0.03 and 0.01 respectively. For comparison, Peltier HJ which was conducted to lung cancer resulted in expression on mir-16 (-1,576) [9, 10, 14].

The highest gene expressions in cell lines PLC5 resulted; miRNA 146 A to mir-16, miRNA 146 A to mir-423-3p, miRNA 146 A to mir-103, miRNA 146 A to mir-21 are 0.02, 0.01, 0.00 and 0.00 respectively. The expression of miRNA 146 A on cell lines PLC5 which was conducted Akira Tomokuni was1,00[10-11, 15].

According to the data above, the expression of miRNA 146 A to mir-16 is the highest expression on both cell lines Hep-G2 and PLC5. The expression value of miRNA 146 A to the 4th reference mir in PLC5 was lower than in cell lines Hep-G2**.**

The expression of 146 miRNAs expressed in a Hep-G2 cell line has been published by Sukohar and Gang Chen[10-11, 14]. So does, mir-423-3p, mir-103, mir-21 and mir-16. Those are well-expressed in cell lines Hep-G2 and PLC5.

After the expression of miRNA 146 A, mir-423-3p, mir-103, mir-21 and mir-16 was expressed in cell lines Hep-G2 and PLC5, so it is necessary to hold a correlation test for these five miRNAs. As can be seen in table 1, there is apoor negative correlation between miRNA 146 A and mir-103, mir-423-3p. The strong negative correlation is found between miRNA 146 A to mir-16 in cell lines Hep-G2. The strong positive correlation is found betweenmiRNA 146 A to mir-21cell lines Hep-G2[10-16].

There is a poor negative correlation between miRNA 146 A to mir-103. The strong positive correlation between miRNA 146 A to mir-16 and the poor positive.

CONCLUSION

MiRNA 146 A, mir-423-3p, mir-103, mir-21 and mir-16were expressed in Cell Lines Hep-G2 series 1886 and PLC5.

The highest value of gene expression in the miRNA 146 A with mir-16 as the reference gene was found in cell lines Hep-G2 and PLC5.

The lowest value of gene expression in miRNA 146Awith mir-103 and mir-16 as a reference gene was found in PLC5.

There is a strong negative correlation between the expression of miRNA146 A with mir-16 as a reference gene in Hep-G2.

There is a strong positive correlation between the expression of miRNA 146 A with mir-16 as a reference gene in PLC5.

CONFLICT OF INTERESTS

Declared None

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