Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 8, 2014

Original Article

MIR-423-3P USED AS REFERENCE GENE FOR MIRNA 146 A IN CELL LINES HEP-G2

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Received: 09 Jun 2014 Revised and Accepted: 05 Aug 2014

ABSTRACT

Objective: We explored the stable reference genes for miRNA 146 A by RT-PCR in the cell lines Hep-G2 that were treated chlorogenic acid. We must do a series of tests in order to get a reference genes. Based on the literature there are 7 candidates recommended reference genes; mir-423-3p, 423-5p, 191, 103, 21, 16, and let-7a. We conducted using four candidates reference gene; mir-423-3p, 103, 16, and 21.

Methods: *In vitro* study was performed in Hep-G2 cells. The samples were divided into control group and experiment group treated with 727µM chlorogenic acid. Samples were analyzed at 0, 2, 8, 18, and 24 hours after being treated with chlorogenic acid.

Total RNA was isolated from Hep-G2 with RNA extraction kit (miRCURYTM RNA Isolation Kit-Cell and plant Exiqon, Code Number 300110) and reverse transcribed to cDNA with Primerscript RT Reagent Kit (miRCURY LNATM Universal RT microRNA PCR, Polyadenylation and cDNA synthesis kit Exiqon, Code Number 203300). The primers for miRNA 146 A were Code Number 204688 from Exiqon (forward and reverse), and quantitative real time reverse transcription polymerase chain reaction was performed using SYBR Master Mix with Code Number 203450 (Exicon). MiRNA profiling was performed in four pairs of mir, consist of; mir-423-3p, 103, 21, and mir-16. By using the mean expression value of all expressed mir, we identified the most stable candidate reference genes for subsequent validation with normfinder software.

Results: Data from RT-PCR were analyzed using Normfinder software. We found out that mir expression with good stablity is mir-103 and the best combination from two genes are mir-103 and mir-423-3p. Finally mir-423-3p is found more stable than mir-16, 21, and 103.

Conclusion: We conclude the stable reference genes for miRNA 146 A treated with chlorogenic acid is mir-423-3p.

Keywords: Chlorogenic acid, Hep-G2, Mirna 146 A, Mir-423-3p, Reference genes.

INTRODUCTION

Micro RNA (miRNA) is a new ribonucleotide acid of non protein coding, containing 19-25 pairs of bases and plays a role in inhibiting gene expression. MiRNA was discovered by Victor Ambros and had been develoved since year 2000 [1, 2].

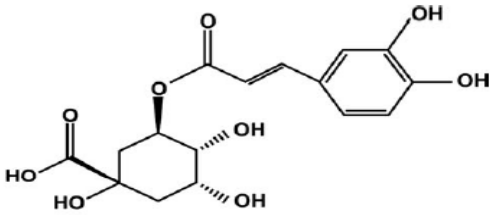
We interested in studying miRNA because it can be used as cancer diagnosis and therapy. To get the best results, the study should determined miRNA 146 A as the target gene, looking for reference genes and optimization of protocols. Use of unreliable reference genes for normalisation may lead to inaccurate quantification of miRNAs [3, 4] and the use of a single reference genes for normalization leads to large errors and inappropriate result [5, 6].

RT-PCR at this time has become a necessity to support molecular biology research and before doing experiment we perform optimization. To optimize reference gene of microRNA there are several approaches or optimization that recommended by previous researchers. Some miRNAs that have been recommended are hsa-mir-103, hsa-mir-423-3p, hsa-mir-191, hsa-mir-16, hsa-mir-423-5p, and hsa-let-7a [2, 4, 7-8]. Based on the data that has been collected, we selected four candidates for reference genes those are mir-16, mir-21, mir-103, and mir-423-3p.

Chlorogenic acid can be isolated from various traditional medicines; strawberry, betel leaves and coffee. Chemopreventive chlorogenic acid in cell lines Hep-G2 model is expected to increase the repertoire of natural products/traditional medicine which is used as a complementary alternative medicine and expected to be used as basic data in experimental animals [9]. In this study we wanted to find a reference genes for miRNA 146 A treated by chlorogenic acid.

MATERIALS AND METHODS

Chlorogenic acid



Chlorogenic acid
(5-o-caffeoylquinic acid)

Fig. 1: Formula of chlorogenic acid

Structurally, chlorogenic acid/CGA (chlorogenic acid) are esters formed between certain cinnamic acid (trans) and acid (L)-quinic (1L-1 (OH), tetrahydroxycyclohexanecarboxylic 3.4/5 acid. Isomerization of chlorogenic acid have been reported with 3 quinic acid isomerizations at position 3, (3-CQA), 4 (4-CGA) and 5 (5-CQA). Isomerization at positions 1 and 6 have not been reported.

Chlorogenic acid molecular weight is 354, it is important to know for the calculation of the therapeutic dose to be used for cell lines Hep-G2. Chlorogenic acid as a potent anti cancer, antioxidant in vitro and therefore contribute to the prevention of Type 2 diabetes mellitus, cardiovascular disease, antiviral, antibacterial, antifungal, and chemopreventive.

Cultured hep-G2

Hep-G2 cell obtained from SCI-Kalgen Jakarta, culture performed with medium (DMEM/F12, Gibco) containing 10% Fetal Bovine Albumin (Sigma) and antibiotics penicillin-streptomycin (100 mg / L). Cells were incubated at 5% CO2 and 37°C. Passage was performed on day 3.

Hep-G2 cell viability

Performed by culturing Hep-G2 cells with medium (DMEM/F12, Gibco) containing 10% Fetal Bovine Albumin (Sigma) and antibiotics penicillin-streptomycin (100 mg / L), the number of cells per well 0.5 x 10^4 and planted in 96 well with DMSO different concentrations of 1%, 0.5% and 1%. Were analyzed using Elisa Reader and in DMSO concentration of 0.1% Hep-G2 cells can live 100%.

MTT assays

We used MTT product from sigma. Principle of MTT method was the separation of tetrazolium MTT salt (3 - (4, 5 dimethyl-thiazole-2-yl) - 2,5-diphenyltetrazolium bromid) by reductase succinate tetrazolium enzyme system which was in mitochondria living cells so that purple formazan crystal was formed. The color intensity could be read with ELISA Reader. A living Cell still have active metabolism activity so that MTT will be disparted by reductase succinate tetrazolium enzyme in cell's mitochondria and formed purple formazan crystal. The result data from toxicity test of chlorogenic acid was analyzed using excel. The results was in the form of living cells percentage (cell viability) with the equation Y=189.81-193.67X. IC_{50} doses of chlorogenic acid: 727 µM.

Chlorogenic acid intervention

Chlorogenic acid interventions was performed at 48 hours after Hep-G2 cell cultured and 60-80% confluent. Assessment was done at the 0, 2^{nd}, 8^{th}, 18^{th}, and 24^{th} hours.

Total RNA isolation

Isolation of total RNA was performed by Exiqon protocol, using miRCURY™ RNA Isolation Kit with code of product 300110. Isolates obtained from 1 ml of Hep-G2 cell culture which had been resuspension, then made pellet by centrifugation at 1500 rpm for 5 min. Pellets stored at -70°C. Concentration and purity of RNA was assessed using the Gene Quant RNA/DNA with code 80-2105-20.

Preparation of cDNA

Total Isolation of RNA is performed by Exiqon protocol, using RNA Isolation Kit™ miRCURY with code of products 203300 from Exiqon (stage 1 to stage 5).

After having obtained total isolate RNA, conversion into cDNA was performed using a cDNA synthesis kit Universal protocol 203300.

Determination of mir-16

Mir-16 (product code number 204409) obtained from Exiqon through PT.Scienwerke.

Determination of mir-21

Mir-21 (product code number 204230) obtained from Exiqon through PT.Scienwerke.

Determination of mir-103

Mir-103 (product code number 204063) obtained from Exiqon through PT.Scienwerke.

Determination of mir-423p

Mir-423-3p (product code number 204488) obtained from Exiqon through PT. Scienwerke.

Validation RT-PCR

RT-PCR performed with Exiqon protocol, using RNA Isolation Kit™ miRCURY code number 203300 from Exiqon (stape 6 to stape 11) and using SYBR Green from Exiqon with code number 203450.

RT-PCR machine used Bio Rad CFX96, with protocol number 10203300. Data were analyzed with Bio Rad CFX Manager (Bio Rad, Jakarta). RT-PCR performed with a final total volume of 10 µl consisting of; a) 5 µl SYBR Green master mix, b) 1 µl PCR primer mix, c) 4 µl cDNA template. Followed by initial denaturation at 95°C for 10 minutes. The miRCURY LNA™ Universal RT-PCR microRNA protocol is a two part protocol consisting of; strand cDNA synthesis and Real time PCR amplification.

RT-PCR amplification of miRNA 146 A and mir-423-3p from Hep-G2 was performed using the CFX-96 system with the SYBR Green kit (Exiqon, Denmark, USA). Data were analyzed using the Bio-Rad CFX manager 2.1 software (Applied Bio Rad, Denmark, USA). RT-PCR was carried out in a 20 µl final volume containing; a) 5x Reaction buffer 4 µl, Nuclease free water; b) 9 µl, Enzyme mix 2 µl; c) Synthetic spike in, optional replace with H2O if omitted 1 µl; d) Template total RNA (5 ng/µl) 4 µl. Following Incubate for 60 minutes at 42°C, heat inactivate the reverse transcriptase for 5 minutes at 95°C, immediately cool to 4°C and store at 4°C or freeze.

RT-PCR amplification was carried out in a 10 µl final volume containing; a) SYBR Green master mix 5 µl; b) PCR primer mix 1 µl; c) Diluted cDNA template 4 µl. Following initial denaturation at 95°C for 10 minutes, temperature cycling was initiated. Each cycle consisted of denaturation at 95° for 10 second, annealing at 60°C for 1 minute. A total of 40 cycles was carried out.

Data analysis

Data was analyzed using Bio-Rad CFX Manager 2.1 software (Hercules, California). The relative expression of reference genes (mir-16, 21, 103 and 423-3p) was normalized using Norm Finder software [10] and universally applicable quantification model based on efficiency correction, error propagation and multiple reference gene normalization [11-12].

Average values of triplicate Cq were converted to relative quantities for Norm Finder [10].

RESULTS

To examine the effect of chlorogenic acid on cell morphology, HepG2 cells were treated with 727 µM chlorogenic acid for 24 hours and morphological changes were observed by microscopy. The images showed that the chlorogenic acid induced severe morphological changes of cell death including rounding and shrinkage of cells (Figure 3). The morphological difference with Hep-G2 control that attach to the plate (figure 2).

The MTT test is a cytotoxic test to assess effect of chlorogenic acid against Hep-G2. The optimization of the test was done with variety of time and dose to obtain the optimal concentration of IC_{50} and inhibition time of growing. Based on the previous research, it is concluded that chlorogenic acid could inhibit growth of Hep-G2 as much as 50% as shown in figure 4 [13]. Furthermore, we determined the value of IC_{50} chlorogenic acid which is obtained on the concentration of 727 µM. After IC_{50} was determined, we proceed to assess the viability of Hep-G2 with 3 concentrations of DMSO are 0.1, 0.5 and 1%. The best concentration for viabiliy cell was DMSO 0,1%.

To determine the control gene from target genes of miRNA, we do a series of preliminary test based on the literature that has been published. The results are shown in figure 5, 6, 7, 8 and analyzed using Normfinder software.

Figure 5 shows mir-423-3p Cq values, the red line (control) and the blue line (chlorogenic acid) with dose 727 µM. Based on the results of the t test with P=0.419 higher than 0.05, there is no difference between the value of Cq mir-423-3p control and mir -423-3p treated with 727 µM dose chlorogenic acid.

Figure 6 shows mir-16 Cq values, the red line (control) and the blue line (chlorogenic acid) with dose 727 µM. Based on the results of the t test with P=0.956 higher than 0.05, there is no difference between the value of Cq mir-16 control and mir-16 treated with 727 µM dose chlorogenic acid.

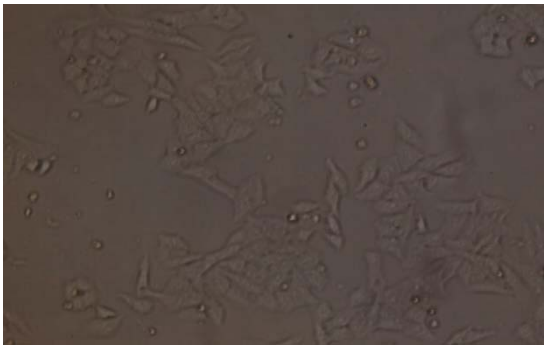


Fig. 2: Cells hep-G2 at 0 hour (without chlorogenic acid)

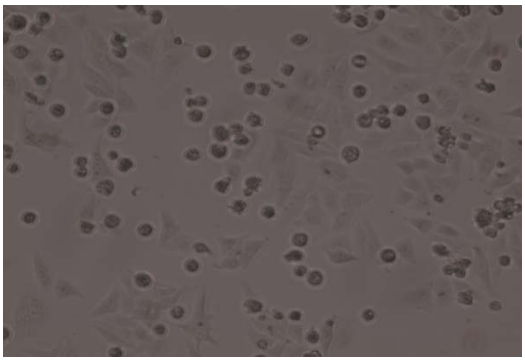


Fig. 3: Cells hep-G2 at 24 hours after treated chlorogenic acid

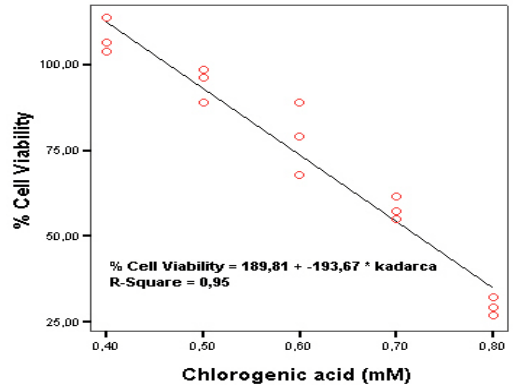


Fig. 4: Percentage of hep-G2 viability and linear regression equation to determine IC_{50} doses of chlorogenic acid

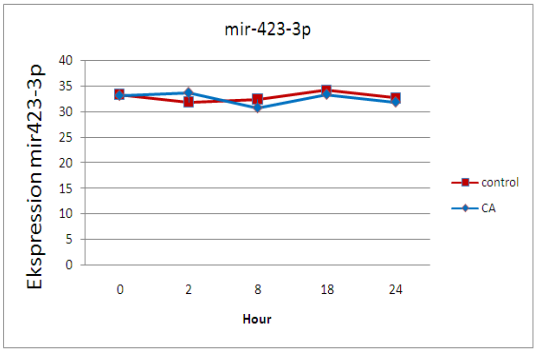


Fig. 5: The expression mir-423-3p after treated with 727 µM chlorogenic acid, red line for chlorogenic acid and blue line for control

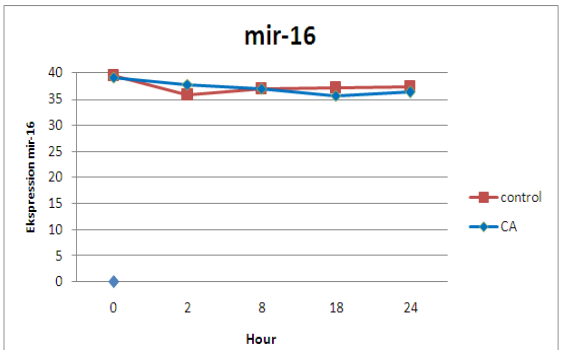


Fig. 6: The expression mir-16 after treated with 727 µM chlorogenic acid, red line for chlorogenic acid and blue line for control

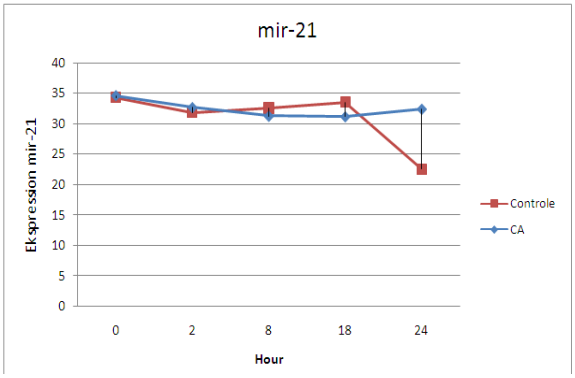


Fig. 7: The expression mir-21 after treated with 727 µM chlorogenic acid, red line for chlorogenic acid and blue line for control

Figure 7 shows mir-21 Cq values, the red line (control) and the blue line (chlorogenic acid) with dose 727 µM. Based on the results of the t test with P=0.246 higher than 0.05, there is no difference between the value of Cq mir-21 control and mir-21 treated with 727 µM dose chlorogenic acid.

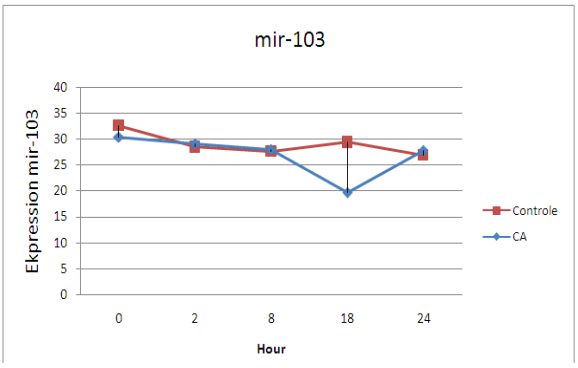


Fig. 8: The expression mir-103 after treated with 727 µM chlorogenic acid, red line for chlorogenic acid and blue line for control

Figure 8 shows mir-103 Cq values, the red line (control) and the blue line (chlorogenic acid) with dose 727 µM. Based on the results of the t test with P=0.440 higher than 0.05, there is no difference between the value of Cq mir-103 control and mir-103 treated with 727 µM dose chlorogenic acid.

Table 1: The data candidate reference genes

Gene Name	Genbank Acession No	Exiqon Assay ID	Amplicon length
mir-16	NR_029486.1	204409	89
mir-21	NR_029738	204230	92
mir-103	NR_029520.1	204063	23
mir-423-3p	NR_029945.1	204488	23

Table 1 shows data for investigation the most stable reference genes, we need some data were code genbank, spesific product and amplicon length.

Table 2: The rangking of values stability four candidate reference genes

Rank	NormFinder	
	Gene name	Stability value
1	mir-103	0,049
2	mir-21	0,067
3	mir-423-3p	0,073
4	mir-16	0,19

Table 2 shows the values of mir stability analyzed with Normfinder software. Based on the table the smallest value is mir-103 and the largest value is mir-16.

Table 3: The stability value single and combination mir

Stability of Cq	Value of mir
Stability value mir-103	0,049
Stability value mir-423-3p	0,073
Best combination of two genes	Mir-103 and mir-423-3P
Stability value for best combination of two genes	0.045

Table 3 shows the smallest values of mir stability 0.049 for mir-103 and 0.073 for mir 423-3p. Determination of candidate reference genes were based on the stability and linearity of Cq value. Based on the 2 consideration the best candidate reference gene was mir-423-3p.

DISCUSSION

With the discovery of miRNA as regulators of gene expression, it can be understood that the miRNA roles both in normal gene regulation and cancerous. MiRNA has ability of regulating some genes in the same pathway, express biologically and clinically significant [12]. This is the first report detailing identification and validation of reference genes for normalization of miRNA RT-PCR in Hep-G2 cells with the active compound chlorogenic acid. We looked for reference gene candidates which were not affected after chlorogenic acid aadministration at different temperature levels and on the run in the RT-PCR. There are four candidate of reference gene; mir-16, 21, 103 and 423-3p, which was checked on the machine CFX96 RT-PCR. Samples were taken from cDNA Hep-G2 cells that have been given intervention with chlorogenic acid at 2, 8, 18 and 24 hours. Amplification of 4 genes be the basis on fluorescent by measuring RFU, data from Normfinder software transferred in the form of excel. The final value of the cycle (40^{th} cycle) was removed and the new data was created with entering reference gene candidates, the number of samples and the RFU value at the end of the 40^{th} cycle. Data from RT-PCR results were analyzed using Normfinder software, which is used as the basis of reference of genes selection is mir stability (table 3) and the smallest results are as shown in table 2 namely mir-103. However, if we us compare figure 5 with Figure 8, it is clearly seen that figure 5 shows the graphs are more stable. Thus, mir-423-3p can be used as reference genes for miRNA 146 A treated chlorogenic acid.

CONCLUSION

These results are very useful for researchers who will conduct the study using Hep-G2 cells with an active compound chlorogenic acid. Based on the data that has been discussed, we conclude the reference genes candidate for miRNA 146 A treated chlorogenic acid is mir-423-3p.

CONFLICT OF INTERESTS

Declared None

ACKNOWLEDGEMENT

The authors gratefully acknowledge Prof Dolara, DR Cristina Luceri from department pharmacology Florence University, Prof Sahidin from Haluoleo University, Team of Center of Cancer Dharmais Hospital and PT Sciencewerke for continued technical assistance.

Author's contribution

Conception and design, obtaining of funding, provision of study materials, collection, assembly, analysis, and interpretation of the data, statistical expertise, drafting of the article: AS, HH, HT. Critical revision and final approval of the article: ST, HS, FW.

REFERENCES

1. Ambros V. The Functions of Animal MicroRNAs. J Nat 2004;431:350-55.
2. Bargaje R, Hariharan M, Scaria V, Pillai B. Consensus MiRNA Expression Profiles Derived from Interplatform Normalization of Microarray Data. J RNA 2010;16(1):16-25.
3. Davoren PA, McNeill RE, Lowery AJ, Kerin MJ, Miller N. Identification of Suitable Endogenous Control Genes for MicroRNA Gene Expression Analysis in Human Breast Cancer. J BMC Mol Bio 2008;9(76):1-11.
4. Peltier HJ, Latham GJ. Normalization of MicroRNA Expression Levels in Quantitative RT-PCR Assays:Identification of Suitable Reference RNA Targets in Normal and Cancerous Human Solid Tissues. J RNA 2008;14(5):844-52.
5. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate Normalization of Real Time Quantitative RT-PCR Data by Geometric Averaging of Multiple Internal Control Genes. J Gen Bio 2002;3(7):1-12.
6. Tricarico C, Pinzani P, Bianchi S, Paglierani M, Distante V, Pazzagli M, Bustin SA, Orlando C. Quantitative Real Time Reverse Transcription Polymerase Chain Reaction: Normalization to rRNA or Single Housekeeping Genes is Inappropriate for Human Tissue Biopsies. J Anal Biochem 2002;309:293-300.
7. Chang KH, P. Mestdagh, Vandesompele J, Kerin, MJ, Miller N. MicroRNA Expression Profiling to Identify and Validate Reference Genes for Relative Quantification in Colorectal Cancer. J BMC Gen 2010;10(173):1-13.
8. Liang Y, Ridzon D, Wong L, Chen C. Characterization of MicroRNA Expression Profiles in Normal Human Tissues. J BMC Gen 2007;8(166):1-20.
9. Ramos S. Cancer Chemoprevention and Chemotherapy:Dietary Polyphenols and Signalling Pathways. J Mol Nutr Food Res 2008;52:507-26.
10. Andersen CL, Jensen JL, Orntoft TF. Normalization of Real Time Quantitative Reverse Transcription PCR Data:A Model Based Variance Estimation Approach to Identify Genes Suited for Normalization, Applied to Bladder and Colon Cancer Data Sets. J Can Res 2004;64:5245-50.
11. Hellemans J, Mortier G, Paepe AD, Speleman F and Vandesompele J. QBase Relative Quantification Framework and Software for Management and Automated Analysis of Real Time Quantitative PCR Data. J Gen Bio 2007;8:R19.
12. Calin GA, Croce CM. MicroRNA Cancer Connection:The beginning of a new tale. J Can Res 2006;15:7390-94.
13. Sukohar A, Herawati H, Witarto AB, Setiawan, Wirakusumah FF, Sastramihardja HS. Role of Chlorogenic Acid From Lampung Robusta Coffee Against Gene Expression of Mirna 146 A on Hepatocellular Carcinoma Cells. Int J of Res in Phar and Nan Sci 2013;2(6):776-84.