Serum miR-29a and miR-122 as Potential Biomarkers for Non-Alcoholic Fatty Liver Disease (NAFLD)

Kanisa Jampoka¹, Puth Muangpaisarn², Kritsada Khongnomnan¹, Sombat Treeprasertsuk²,³, Pisit Tangkijvanich¹,³ and Sunchai Payungporn¹,*

¹Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand; ²Department of Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand; ³Center of Excellence in Hepatitis and Liver Cancer, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

Abstract: Background: Non-Alcoholic Fatty Liver Disease (NAFLD) is an over accumulation of triglyceride in the liver without alcohol consumption. Its major cause is insulin resistance. Patients with NAFLD can develop liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC). MicroRNAs (miRNAs) are non-coding RNAs that regulate post-transcriptional gene silencing. Previous research reported that miR-29 family (a, b and c) and miR-122 have an important role in regulating insulin resistance related to NAFLD.

Objective: The purpose of this study was to investigate that miR-29 and miR-122 can be possible biomarkers for non-invasive diagnosis of NAFLD.

Method: Serum samples were collected from 58 NAFLD patients and 34 healthy controls. MiRNAs were extracted from serum by using microRNA purification kit followed by polyuridylation, reverse transcription and quantitative real-time PCR. Also, we analyzed the correlation between miR-29 and miR-122 and level of liver inflammation in NAFLD patients.

Results: We found that the serum miR-29a levels in NAFLD patients were significantly lower (P = 0.006) than the control group, while miR-29c levels were unchanged, and miR-29b levels were undetectable. However, we found that serum miR-122 levels in NAFLD patients were significantly higher (P < 0.001) than those found in the control group. For miR-29a, the area under curve (AUC) was 0.679 (P = 0.0065) with 60.87% sensitivity and 82.35% specificity. For miR-122, the AUC was 0.831 (P < 0.0001) with 75.00% sensitivity and 82.35% specificity. Interestingly, the levels of serum miR-122 were significantly different between patients without steatohepatitis (NAS < 4) and steatohepatitis (NAS ≥ 4), indicating that the levels of miR-122 were related to the severity of NAFLD.

Conclusion: The levels of miR-29a and miR-122 might be beneficial and compelling as possible biomarkers for non-invasive diagnosis of NAFLD.

Keywords: Biomarkers, microRNA, miR-122, miR-29a, Non-Alcoholic Fatty Liver Disease (NAFLD), nucleus.

1. INTRODUCTION

Non-Alcoholic Fatty Liver Disease (NAFLD) has become one of the important health problems worldwide. The cause of NAFLD is an over accumulation of triglycerides within hepatocytes in the absence of excessive alcohol consumption. This accumulation is strongly associated with obesity and metabolic syndrome [1-3]. The emerging evidence is related to epigenetic factors, which also include microRNAs (miRNAs) in the development of NAFLD [4]. Previous studies suggested that level of miRNAs in serum and plasma samples can be used as perfect biomarkers because they are simple to be accessible in a non-invasive manner [5, 6]. MicroRNAs (miRNAs) are small noncoding RNAs approximately 22 nucleotides (nt) in length that regulate about 30% of human genes via miRNAs degradation or translational repression mechanisms [7].

During miRNA biogenesis, miRNAs are post-transcriptional regulated, and the clarification of these mechanisms will enhance our understanding of miRNA in the disease progression [8, 9]. MiRNAs are transcribed by RNA polymerase II or III in the nucleus to generate primary miRNAs (pri-miRNAs) which will be consequently processed to become precursor miRNAs (pre-miRNAs). The pre-miRNAs
are exported to the cytoplasm by exportin-5 before being cleaved by the Dicer to yield mature miRNA duplex and bound to the RNA-induced silencing complex (RISC). After that, the miRNAs will bind to 3’untranslated region (3′UTR) of target mRNA(s). The consequence of miRNA-specific binding can lead to mRNA degradation or translational repression [8, 10].

In cancer context, the role of miRNAs has been extensively studied [11, 12]. However, in other diseases such as NAFLD, the involvement of miRNA has been scarcely analyzed. Previous research found that miR-29 family (a, b and c) and miR-122 have the most common role in regulating genes in many liver diseases such as fatty liver disease, metabolic syndrome, and liver cancer [13]. The human miR-29 family including hsa-miR-29a, hsa-miR-29b (hsa-miR-29b-1 and hsa-miR-29b-2) and hsa-miR-29c, is highly conserved in human, mouse and rat. They share the same seed sequences (position 2nd - 8th from the 5’t end of miRNA) which determine the similar target sequences. MiR-29 family is expressed aberrantly in multiple cancer types and involved in the complex regulatory process, indicating that it can play critical roles in apoptosis, metastasis, immune regulation and cell proliferation processes by targeting numerous genes such as p85 alpha and CDC42, TGF-beta, extracellular matrix, and CDK6, respectively [9, 14]. Former research revealed that up-regulation of miR-29 family blocks insulin-stimulated glucose uptake via the Akt pathway by preventing insulin signaling [15]. In 2011, researchers found that miR-29 family in HepG2 cells is up-regulated, leading to insulin resistance that links all components from the metabolic syndrome, which is the most probable risk factor for the development of NAFLD [16]. Moreover, down-regulation of Ten-Eleven Translocations 1 (TET1) which is the target gene of miR-29, influences cancer development such as hepatocellular carcinoma (HCC), indicating that miR-29 can be employed as prognosis marker and therapeutic target for HCC [17].

MiR-122 is the most abundant miRNA for nearly 70% of all miRNAs in the liver, which is found in mouse, woodchuck, human livers, primary hepatocytes, and cultured liver-derived cells, such as mouse Hepa 1-6 cells and human HuH7 cells [18]. MiR-122 can be detected in the circulation and serum. The miR-122 plays a central role in liver development, differentiation, homeostasis and functions, cholesterol and fatty acid metabolism in the adult liver while it is also a biomarker of liver injury in chronic hepatitis B or C, NAFLD and drug-induced liver damage [19, 20]. Consequently, for miRNA involved in liver diseases, miR-122 deserves to be a dominant alternative [18]. The previous study showed the relationship between serum level of circulating miRNAs and NAFLD, and also the correlation between miR-122 and the severity of liver steatosis. These serum miRNAs, especially miR-122 can be considered as a beneficial biomarker for NAFLD [21, 22]. The hepatic and serum miR-122 levels are associated with hepatic steatosis and fibrosis. The inverse correlation between liver fibrosis and a decrease in miR-122 expression are also associated with advanced fibrosis stage [23]. As same as in the study in 2011, there may be the advantage of using miR-122 to identify the patients who have NAFLD in which they developed significant liver fibrosis [24]. Another study reported that the silence of miR-122 would affect the stimulation of hepatocarcinogenesis [25]. Moreover, miR-29 was interested to be the candidate for circulating biomarker for non-alcoholic fatty liver disease [26]. The detection of miR-122 and miR-29 can be effective in evaluating the liver injury and fibrosis associated with HBV infection [13, 27]. The expression of miR-122 may be associated particularly with the occurrence of liver damage in Chronic Hepatitis B (CHB) also in liver cancer while low miR-29 expression could correlate to liver fibrosis [13]. In contrast, another research reported that in NAFLD patient’s levels of serum miR-29a and miR-122 were not different from the controls [28]. In this study, we aimed to investigate the association between the levels of miR-29 (a, b and c) and miR-122 in serum the degree of liver inflammation in Thai patients with NAFLD, which can be crucial to be candidate biomarkers for NAFLD diagnosis.

2. MATERIALS AND METHODS

2.1. Serum from Healthy Controls and NAFLD Patients

The control group included 34 healthy individuals without a history of significant alcohol consumption, the absence of serum hepatitis B antigen and anti-hepatitis C antibody (anti-HCV), and Body Mass Index (BMI) <25 kg/m2. All controls underwent liver stiffness measurement and showed transient elastography (TE) of 0-6 kPa and a controlled attenuation parameter <200 dB/m. NAFLD patients were recruited at the King Chulalongkorn Memorial Hospital, Bangkok, Thailand. NAFLD was defined as the presence of hepatic steatosis in at least 5% of the hepatocytes without secondary hepatic fat accumulation. The degree of liver inflammation was graded for NAFLD Activity Score (NAS), which is based on the sum of three components, steatosis grade (0-3), lobular inflammation (0-3), and hepatocyte ballooning (0-2), and therefore, the total NAS ranges from 0-8 [29, 30]. Additionally, severity of fibrosis was graded by fibrosis score (F0-F4) [29-30]. Exclusion criteria were the presence of other liver diseases (hepatitis virus infection or autoimmune hepatitis), significant alcohol consumption (more than 140 g/week in men and 70 g/week in women), decompensated cirrhosis or Child-Pugh score ≥7, receiving steatogenic medications within 6 months of enrollment, human immunodeficiency virus infection, malignancy and pregnancy. All participants provided written informed consent prior to study enrollment. The study protocol was approved by Institutional Review Board (IRB No. 357/57), Faculty of Medicine, Chulalongkorn University. Baseline characteristic data and blood samples were obtained from the participants. Laboratory evaluation included liver function tests, metabolic profile (total & HDL cholesterol and triglyceride). In addition, 10 mL of blood was drawn and prepared for serum miRNA analysis. MiRNAs were extracted from 100 μL of serum by using microRNA purification kit (Norgen) according to the manufacturer’s instruction.

2.2. Preparation of Positive Control miRNAs

Nucleotide sequences of miR-29 (a, b and c) and miR-122 were designed by using data obtained from miRBase (http://www.mirbase.org). RNA positive controls for each miRNA were generated by in vitro transcription. DNA templates for in vitro transcription composed of short (top
Table 1. Primers and oligonucleotides used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-29a</td>
<td>F</td>
<td>TAGCACCATCTGAAATCGG</td>
</tr>
<tr>
<td>miR-29b</td>
<td>F</td>
<td>GCACCATTGAAAATCGT</td>
</tr>
<tr>
<td>miR-29c</td>
<td>F</td>
<td>TAGCACATTGAAAATCGT</td>
</tr>
<tr>
<td>miR-122</td>
<td>F</td>
<td>TGGAGTGTGACAATGGT</td>
</tr>
<tr>
<td>U6</td>
<td>F</td>
<td>CTCGCTTCCGACGAC</td>
</tr>
<tr>
<td>qPCR</td>
<td>R</td>
<td>GCAGGGTCCGAGGTATTC</td>
</tr>
<tr>
<td>T7</td>
<td>TS</td>
<td>TAATAGAATATCTAGCCCCCA</td>
</tr>
<tr>
<td>T7+miR-29a</td>
<td>BS</td>
<td>CCGATTTCAGATGGTGCTACCTTATAGTGAGTGTATTACCA</td>
</tr>
<tr>
<td>T7+miR-29b</td>
<td>BS</td>
<td>ACACGTATTACAAATGGTGCCCTATAGTGAGTGTATTA</td>
</tr>
<tr>
<td>T7+miR-29c</td>
<td>BS</td>
<td>ACACGTATTACAAATGGTGCCCTATAGTGAGTGTATTA</td>
</tr>
<tr>
<td>T7+miR-122</td>
<td>BS</td>
<td>ACACGTATTACAAATGGTGCCCTATAGTGAGTGTATTA</td>
</tr>
<tr>
<td>SL-poly A</td>
<td>RT</td>
<td>GTCGTATCCAGTGCGGATCGGATCGGATCACGACACACAAAAAAAGAAA</td>
</tr>
</tbody>
</table>

F: Forward Primer; R: Reverse Primer; TS: Top Strand; BS: Bottom Strand; RT: for Reverse Transcription

Serum miR-29a and miR-122 as Potential Biomarkers

The short oligonucleotide was synthesized based on T7 promoter sequence whereas the long oligonucleotides were synthesized based on the complementary sequence (antisense) of the T7 promoter linked to the 5'-end of each miRNA. The sequences were described in Table 1. After that, duplex DNA templates (top and bottom strands) were prepared by mixing 10 µM of each oligonucleotide in a total volume of 10 µl then incubate at 90°C for 1 minute and followed by 37°C for 1 hour. In vitro transcription was performed by using RibopMax™ Large Scale RNA Production System T7 (Promega) according to the manufacturer’s instruction. Briefly, the reaction mixture (20 µl) composed of 4.0 µl of 5X T7 transcription buffer, 6.0 µl of 25mM rNTPs mix (ATP, CTP, GTP, UTP), 5.0 µl of DNA template (5 µg), 40 units of T7 RNA polymerase and 3.0 µl of nuclease-free water. After that, the transcription reaction was occurred by incubating at 37°C for 4 hours followed by adding 5.0 µl of RQ1 RNase-free DNase and incubate at 37°C for 15 minutes. The in vitro transcribed miRNAs were extracted by microRNA purification kit (Norgen) following the instruction protocol from the company. The concentration of each miRNA was measured by NanoDrop 1000 spectrophotometer (Thermo Scientific) and then calculated regarding copies/µl. These miRNAs were then used as a positive control and standard miRNA for quantitation of miRNA.

2.3. Reverse Transcription

The technique for miRNA quantification used in this study was derived from the protocol described previously [27]. Briefly, mature miRNAs were polyuridylation by poly (U) polymerase and then reverse transcribed by using Stem-Loop (SL) poly A primer and reverse transcriptase. After that, real-time PCR based on SYBR Green dye was performed by using miRNA-specific forward primer and universal reverse primer. Polyuridylation reaction included 2.5 µl of 10x NE buffer, 0.25 µl of 50 mM UTP, 40 units of RNase inhibitor, 2 units of poly (U) polymerase (New England BioLabs Inc.), 100 pmol of miRNA and nuclease-free water in a final volume of 25 µl then incubate at 37°C for 10 minutes. After that reverse transcription was performed by using 12.3 µl of polyuridylated miRNA, 4.0 µl of 5x RT reaction buffer, 0.2 µl of 10 µM stem-loop (SL) poly A primer, 2 µl of 10 mM dNTPs mix, 20 units of RNase inhibitor, 200 units of RevertAid Reverse Transcriptase (Thermo Scientific) and nuclease-free water in a final volume of 20 µl.

2.4. Quantitative Real-Time PCR

Quantitation of miRNA was carried out in StepOnePlus real-time PCR (Applied Biosystems) based on SYBR Green dye. Real-time PCR reaction mixture composed of 6.25 µl of 2x Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific), 0.4 µl of 10 µM of each primer, one µl of cDNA and nuclease-free water in a final volume of 12.5 µl. The sequence of primers used in this study was summarized in Table 1. Thermal profiles were optimized for each miRNA as the following: initial denaturation at 95°C for 3 minutes and then 50 cycles of amplification including 95°C for 15 seconds and 60°C (miR-122) or 62°C (miR-29 family) for 30 seconds.

2.5. Construction of Standard Curve

For each miRNA, serial 10-fold dilutions of the standard in vitro transcribed miRNAs ranging from 10^8 to 10 copies/µl were detected by real-time PCR assay and used to prepare the standard curve for quantitation of miRNAs from patient specimens. A standard curve was constructed by plotting the Ct value against the amount of each serially diluted standard miRNA. Then the standard curve was also used for determi-
2.6. Statistical Analysis

Statistical analysis was calculated using SPSS Statistics version 22.0 (USA). Unpaired Student’s t-test was used to compare clinical parameters between groups. The stages of NAFLD and serum miRNA levels were compared by One-way ANOVA. At P < 0.05 was considered as statistically significant. The receiver operating characteristic (ROC) curve analysis was performed by GraphPad version 6.01.

3. RESULTS

3.1. Detection of miR-29 and miR-122 Based on Real-time PCR

In this study, the assays based on real-time PCR for detection and quantitation of miR-29 family (a, b & c) and miR-122 were successfully developed. The designed primers and optimized conditions yielded effective and specific amplification of the miRNAs without any non-specific amplification or primer dimers. In addition, the specificity of the assay was evaluated by using miRNA specific primers to test against the in vitro transcribed miRNA positive control (10^6 copies/µl). The results revealed that miRNA specific primers provided impressive specificity under the optimized thermal profile without any cross-amplification with other miRNAs (Supplementary Table 1). Moreover, the assay was also validated regarding capability for detecting miRNA from clinical samples. Serum miRNAs were extracted from NAFLD patients (N=58) then followed by polyuridylation, reverse transcription and real-time PCR detection. The result showed that the assay could be used to detect miR-29 family and miR-122 extracted from the serum of NAFLD patients. However, the capability of detection was differently observed for each miRNA.

3.2. Performances of the Real-Time PCR Assay

For each miRNA, a standard curve was constructed by using 10-fold serial dilutions of the standard in vitro transcribed miRNAs ranging from 10^8 to 10 copies/µl (Fig. 1). Then, the standard curves were used for analysis of the overall performance of the real-time PCR assay regarding detection limit and amplification efficiency. The results were summarized in Table 2.

3.3. Comparison of Serum miRNA Levels Between Healthy Controls and NAFLD Patients

The expression levels of miR-29 family and miR-122 in the serum of healthy controls and NAFLD patients were determined via absolute quantitation by comparing the Ct value of each sample with the standard curves. The result revealed that miR-29a could be detected in all healthy controls (34/34) and most of the NAFLD patients (46/58). Beside, miR-29c could be quantified in most of the healthy controls (31/34) and NAFLD patients (53/58). Similarly, miR-122 could be detected in all healthy controls (34/34) and almost all of the NAFLD patients (56/58). However, the level of miR-29b was undetectable in both healthy controls and NAFLD patients. Comparison of the serum miRNA levels between healthy controls and NAFLD patients revealed that miR-29a levels were significantly lower in NAFLD patients.
(P = 0.006), while miR-122 levels were significantly higher in NAFLD patients (P < 0.001) than those found in the control group. However, the serum miR-29c levels were not significantly different between healthy controls and NAFLD patients (Fig. 2).

### 3.4. Serum miRNA Levels and NAFLD Activity Score (NAS)

In general, NAFLD activity score (NAS) was developed as a tool to measure changes in NAFLD patients during treatment. NAS can be used to distinguish between steatohepatitis (NAS ≥ 4) and simple steatosis (NAS < 4) [25, 26]. In this study, the level of serum miR-122 was significantly different between patients with NAS < 4 and patients with NAS ≥ 4 (P = 0.001). In contrast, the levels of serum miR-29 family were not significantly associated with the NAS score (Fig. 3).

### 3.5. Sensitivity and Specificity of Serum miRNA Levels for NAFLD Diagnosis

The receiver operating characteristic (ROC) curve analysis was performed to evaluate the levels of serum miR-29a, miR-29c and miR-122 could be used as the diagnostic biomarkers for NAFLD (Fig. 4). For miR-29a, the area under curve (AUC) was 0.679 (P = 0.0065) with 60.87% sensitivity and 61.29% specificity. For miR-29c, the area under curve (AUC) was 0.529 (P < 0.0001) with 43.40% sensitivity and 61.29% specificity. For miR-122, the area under curve (AUC) was 0.831 with 83.29% sensitivity and 82.35% specificity.
3.6. Relationship Between Serum miRNA Levels and Clinical Parameters of NAFLD Patients

Baseline demographic and clinical parameters of patients with NAFLD were summarized in Table 3. In NAFLD patients, serum miR-122 levels were significantly associated with age, body mass index (BMI), NAFLD activity score (NAS), alkaline phosphatase (ALP) and fibrosis stage (P < 0.05). Serum miR-29a was significantly correlated with only triglyceride (Table 3).

### Table 3. The relationship between serum miRNA levels and clinical parameters of NAFLD patients.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values</th>
<th>miR-29a (r)</th>
<th>P Value</th>
<th>miR-122 (r)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>49 (18-70) b</td>
<td>0.100</td>
<td>0.507</td>
<td>0.312</td>
<td>0.019</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>30.45 (20.3-98.1) b</td>
<td>-0.145</td>
<td>0.337</td>
<td>-0.356</td>
<td>0.007</td>
</tr>
<tr>
<td>NAS &lt;4/≥4</td>
<td>45/55 a</td>
<td>0.205</td>
<td>0.172</td>
<td>0.306</td>
<td>0.022</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>43.5 (17-153) a</td>
<td>0.039</td>
<td>0.792</td>
<td>0.135</td>
<td>0.320</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>61.5 (18-233) a</td>
<td>-0.032</td>
<td>0.830</td>
<td>0.152</td>
<td>0.265</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>70.5 (16-131) a</td>
<td>-0.041</td>
<td>0.789</td>
<td>0.306</td>
<td>0.021</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>192.5 (138-311) a</td>
<td>0.091</td>
<td>0.548</td>
<td>0.192</td>
<td>0.156</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>147 (62-271) a</td>
<td>0.144</td>
<td>0.048</td>
<td>0.171</td>
<td>0.208</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>42.5 (24-91) a</td>
<td>-0.035</td>
<td>0.816</td>
<td>0.201</td>
<td>0.138</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>114.5 (71-232) a</td>
<td>-0.051</td>
<td>0.735</td>
<td>0.116</td>
<td>0.393</td>
</tr>
<tr>
<td>Inflammation activity: ≤1/≥2</td>
<td>78/22 a</td>
<td>0.185</td>
<td>0.236</td>
<td>0.198</td>
<td>0.155</td>
</tr>
<tr>
<td>Steatosis grade: ≤1/≥2</td>
<td>44/56 a</td>
<td>-0.023</td>
<td>0.883</td>
<td>0.145</td>
<td>0.301</td>
</tr>
<tr>
<td>Balloon hepatocytes: ≤1/≥2</td>
<td>76/24 a</td>
<td>0.291</td>
<td>0.058</td>
<td>0.244</td>
<td>0.078</td>
</tr>
<tr>
<td>Fibrosis stage: ≤1/≥2</td>
<td>76/24 a</td>
<td>0.184</td>
<td>0.220</td>
<td>0.399</td>
<td>0.002</td>
</tr>
</tbody>
</table>

BMI: Body Mass Index; NAS: NAFLD Activity Score; AST: Aspartate Aminotransferase; ALT: Alanine Aminotransferase; ALP: Alkaline Phosphatase; TG: Triglycerides; HDL: High-Density Lipoprotein; LDL: Low-Density Lipoprotein; *significant (P < 0.05); a (%); b mean (range); r: Pearson’s correlation test

3.7. Prediction of Target Genes of miRNAs

The prediction of target genes was performed by using computational methods including the miRTarbase (http://miRTarbase.mbc.nctu.edu.tw) for identifying common targets of microRNAs and the PANTHER (Protein ANalysis Through Evolutionary Relationships) (http://pantherdb.org) for finding the pathway of each target. Several target genes related to metabolic pathways that influence NAFLD were identified as summarized in Table 4.

### Table 4. MiRNAs and target genes related to metabolic pathway.

<table>
<thead>
<tr>
<th>miRNAs</th>
<th>Target Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>miRNA-29a</td>
<td>CDK6, RAN, BACE1, S100B, IMPDH1, GLUL, PPM1D, PIK3R1, LPL, CPEB3, CPEB4, ADAMTS9, TRIM63, MYCN, SERPINB9, DICER1, TNFAIP3, CDC42, PXD1, ITIH5, PTEN, ABL1</td>
</tr>
<tr>
<td>miRNA-29b</td>
<td>HDAC4, SP1, CDK6, BACE1, SFPQ, DNAJB11, S100B, ESR1, NCOA3, MMP15, Mmp24, MMP2, HMGA2, BMP1, PTEN, CDC42, GSK3β, PIK3CG, NKIRAS2, RAX, TXB21, DUSP2, FOS, IMPDH1, MYCN</td>
</tr>
<tr>
<td>miRNA-29c</td>
<td>GAPDH, CDK6, SRSF10, CTSK, MMP15, Mmp24, TFAP2C, CDC42, MYCN, BACE1</td>
</tr>
<tr>
<td>miRNA-122</td>
<td>CYP7A1, SRF, RAC1, RHOA, CCNG1, GTF2B, GYS1, NFATC2IP, ENTPD4, ANXA11, FOXP1, MECP2, NCAM1, TBX19, AACS, DUSP2, ATP1A2, MAPK11, AKT3, GALNT10, G6PC3, SLC7A1, FOX3, SLC7A11, TRIB1, DSTYK, PRKAB1, ACVR1C, PRKRA, PTP1B, P4HA1, ZNF395, SOCS1, HMOX1, CDK4</td>
</tr>
</tbody>
</table>

DISCUSSION AND CONCLUSION

Quantitative real-time PCR is one of the potential techniques for investigating gene expression regarding quantitative and qualitative manners. This technique has been used in life sciences and medical aspects because of its high sensitivity, specificity, and accuracy. Moreover, it is widely used for identifying common targets of microRNAs and the PANTHER (Protein ANalysis Through Evolutionary Relationships) (http://pantherdb.org) for finding the pathway of each target. Several target genes related to metabolic pathways that influence NAFLD were identified as summarized in Table 4.
adapted to quantify miRNAs in general research [31]. Some studies also used real-time PCR for miRNAs related to NAFLD detection including miR-122 and miR-29a [11, 12]. In this study, the assay based on real-time PCR was developed for detection and quantitation of miR-29 family and miR-122. The result from real-time PCR revealed that the limit of detection for miR-29a, miR-29c and miR-122 was approximately 100 copies/µl, indicating that in order to detect those miRNAs, one microliter of sample needed to contain at least 100 copies of the miRNA. On the contrary, in case that if there are less than 100 copies of miRNA, it yielded undetectable results. The detection limit of miR-29b was around 1000 copies/µl, indicating the lower efficiency of the primers for detection. That may be one reason to support that we cannot detect the expression of miR-29b in the clinical samples. Furthermore, serum miR-29 family and miR-122 from NAFLD patients were also detected and quantitated by real-time PCR assay. The results suggested that the assay may be used to detect and quantify miR-29 family and miR-122 extracted from the serum of NAFLD patients.

In our study, miR-29a could be detectable whether it was NAFLD serum or not. The result implied that miR-29a can be used as a diagnostic biomarker for NAFLD with 60.87% sensitivity and 82.35% specificity. However, the severity of NAFLD could not be indicated. The expression of miR-29a levels significantly correlated with triglyceride that is consist with the pathogenesis of NAFLD. MiR-29a levels in serum were significantly lower only in NAFLD female patients. MiR-29c levels were unchanged whereas miR-29b levels were undetected. The several miRNA-29 family precursors led to the different expression of each miRNA level indicating that we cannot detect levels of miR-29b [9]. Another report suggested that miR-29c was significantly down-regulated from the control group in C57BL/6J mice treated with methyl-deficient diet [32]. There is limited research which described the relationship between miR-29 family and NAFLD. Most of the research study linked miR-29 family through metabolic pathways [15, 16, 20].

MiR-122 levels in serum NAFLD patients were significantly higher than those found in the control group which related to both sexes and correlated with the severity of NAFLD indicating that the levels of miR-122 were related with the severity of NAFLD in our study. Similarly, the serum levels of miR-122 in NAFLD patients in both sexes are up-regulated compared to the control group (P < 0.001) and correlated with the severity of liver steatosis [21]. The comparison between healthy controls and NAFLD patients stated that miR-122 levels were increased by 7.2-fold (P <0.0001) [24]. The expression of miR-122 levels in serum correlated significantly with age, BMI, NAS, ALP and fibrosis stage are consistent with the pathogenesis of NAFLD. The result implied that miR-122 was an effective biomarker for NAFLD with 75.00% sensitivity and 82.35% specificity.

The prediction of targeted miRNA pointed out that one miRNA controls several target genes which are connected to different pathways such as metabolic pathway, apoptotic process, biological regulation, cellular process or immune system process. For example, the proposed mechanism between miR-122 and CYP7A1 (Cholesterol 7-alpha-monooxygenase) is a rate-limiting step by which enzyme converts cholesterol into 7α-hydroxycholesterol to produce cholic acid in humans [33, 34]. In this case, we found that the serum levels of miR-122 upregulated the effects of inhibiting CYP7A1 function in human, causing hypercholesterolemia which lead to liver injury apoptosis and necrosis in the liver, respectively [35].

AUTHOR’S CONTRIBUTION

Kanisa Jampoka performed microRNA extraction, quantitative real-time PCR and data analysis. Puth Muangpaisarn and Sombat Treeprasertsuk performed serum sample collections and provided clinical data. Kristadsa Khongnomnan assisted in manuscript preparation and revision. Pisit Tangkijvanich coordinated the project. Sunchai Payungporn designed the study, analyzed data and revised the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study protocol was approved by Institutional Review Board (IRB No. 357/57), Faculty of Medicine, Chulalongkorn University.

HUMAN AND ANIMAL RIGHTS

No Animals were used for this study. The reported experiments on human were in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2008.

CONSENT FOR PUBLICATION

All participants provided written informed consent prior to study enrollment.

CONFLICT OF INTEREST

The authors hereby declare no personal or professional conflicts of interest with any aspect of this study.

ACKNOWLEDGEMENTS

Funding was supported by the 90th Anniversary of Chulalongkorn University Fund; National Research University Project, Office of Higher Education Commission (NRU59-029-9R); the Thailand Research Fund (TRF) (RSA6180035); the Ratchadapiseksompotch Fund (Faculty of Medicine); the Asahi Glass Foundation; the Research Chair Grant, National Science and Technology Development Agency (NSTDA); Chulalongkorn Academic Advancement into Its 2nd Century Project.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher’s website along with the published article.

REFERENCES


Pandey AK, Verma G, Vig S, Srivastava S, Srivastava AK, Datta M. miR-29a levels are elevated in the db/db mice liver and its overexpression leads to attenuation of insulin action on PEPCK gene expression in HepG2 cells. Mol Cell Endocrinol 2011; 322(1-2): 125-33.
