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Brief communication (Original)

Serum microRNA-34a is potential biomarker for inflammation in nonalcoholic fatty liver disease

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Background: MicroRNA-34a (miR-34a) contributes to liver injury through an apoptosis pathway.

Objectives: To determine the correlation between serum miR-34a and liver inflammation as assessed by nonalcoholic fatty liver disease (NAFLD) activity score (NAS).

Methods: We included a cross-selectional study of 50 patients with NAFLD in this observational study and confirmed diagnosis by liver biopsy, with NAS grading. A control group comprised 23 healthy individuals without chronic liver disease. Serum miR-34a was assayed using a real-time quantitative PCR (Applied Biosystems).

Results: The mean age of NAFLD patients was 46.0 ± 13.7 years, and 52% were female. Metabolic syndrome was found in 76%. Liver histopathology showed that 54% of patients had NAS ≥4 and significant fibrosis (≥F2) was found in 22%. Serum levels of miR-34a were significantly correlated with NAS (r = 0.39, P = 0.005), and the degree of steatosis (r = 0.28, P = 0.049), ballooning (r = 0.30, P = 0.034), and fibrosis (r = 0.39, P = 0.005). Serum miR-34a in patients with NAS ≥4 was significantly higher than in those with NAS <4 (P = 0.011) and controls (P < 0.001). There was no significant correlation between serum miR-34a and other variables. The area under receiver operating characteristic curve for serum miR-34a comparing patients with NAS ≥4 and with NAS <4 was 0.67 (95% CI 0.52, 0.82).

Conclusions: Serum level of miR-34a has a significant fair to good correlation with NAS and may serve as a biomarker of liver inflammation and fibrosis in patients with NAFLD.

Keywords: Biomarker, microRNA-34a, non-alcoholic fatty liver disease

Currently, nonalcoholic fatty liver disease (NAFLD) has become a worldwide health concern because its prevalence is rising and it is an emerging etiology of chronic liver diseases [1-3]. Moreover, NAFLD is closely associated with obesity, metabolic syndrome, and cardiovascular diseases [4]. NAFLD can be divided into two groups: simple steatosis and nonalcoholic steatohepatitis (NASH) [5]. In particular, NASH increases the risk of death compared with the general population, and can progress to cirrhosis and hepatocellular carcinoma [5, 6]. Importantly, these serious complications are not found in patients with simple steatosis. Therefore, it is important to identify NASH in NAFLD patients to determine prognosis and to provide appropriate management. Liver biopsy remains the criterion standard for characterizing liver histology in patients with NAFLD [5]. Histopathological assessment of this liver disease consists of 2 major components: inflammation and fibrosis. NAFLD activity score (NAS) has been widely accepted for representing degree of inflammation in NASH and ranges from 0 to 8 [7]. NAFLD patient NAS scores of ≥5 correlate with a diagnosis of 'NASH' and NAS scores of <3 correlate with 'not NASH' [7]. NAS ≥4 has optimal sensitivity (85%) and specificity (81%) for predicting

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steatohepatitis [8]. Although liver biopsy is the only reliable method, it has limitations such as complications and sampling variation [9, 10]. A noninvasive test, such as a blood test, that both accurately differentiates NASH from steatosis and determines the stage of the liver inflammation and/or fibrosis should be developed [11].

MicroRNAs (miRNAs) are small noncoding RNAs and have been discovered to be crucial regulators of both mRNA and protein expression in diverse physiological and pathological processes, such as cell proliferation, inflammation, and apoptosis [12]. Numerous microRNAs have been implicated in the pathogenesis of many liver diseases, particularly NAFLD [13, 14]. For instance, miR-122 plays a major role in lipid metabolism, and miR-33 has effects on cholesterol and fatty acid homeostasis [15]. Interestingly, miR-34a has been studied in both animals and humans, and is a novel miRNA that explains the inflammatory process in NAFLD via the miR-34a/SIRT1/p53/apoptosis pathway and as a regulator of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR) [15-18]. The serum level of miR-34a in NAFLD patients has been reported to be higher than in healthy participants [19]. In addition, serum levels of miR-34a in patients with biopsy-proven NASH (NAS ≥5) were significantly higher than in those with simple steatosis (NAS ≤4) and controls [20]. Nevertheless, there is no report that directly examines the correlation between serum levels of miR-34a and degree of liver inflammation. Thus, we aim to determine the correlation coefficient between serum miR-34a and liver inflammation as assessed by NAS.

Materials and methods

Study designs

This is a cross-sectional study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (certificate of approval No. 301/2014) and conducted at King Chulalongkorn Memorial Hospital, Bangkok, Thailand from May to December 2014.

Patients

We recruited patients with biopsy-proven NAFLD. NAFLD was defined as the presence of hepatic steatosis in at least 5% of the hepatocytes without secondary hepatic fat accumulation. Exclusion criteria were the presence of other liver diseases (e.g., hepatitis B or C infection, autoimmune hepatitis), significant alcohol consumption (more than 140 g per week in men and 70 g per week in women), decompensated cirrhosis or Child–Pugh score ≥7, receiving steatogenic medications within 6 months of enrollment, human immunodeficiency virus infection, malignancy, pregnancy, and contraindication to liver biopsy. The control group included healthy individuals without history of significant alcohol consumption, absence of serum hepatitis B antigen and anti-hepatitis C antibody (anti-HCV), and body mass index (BMI) <25 kg/m^2. All controls underwent liver stiffness measurement and showed transient elastography (TE) of <6 kPa and a controlled attenuation parameter <200 dB/m. All study participants provided written informed written consent prior to study enrollment.

Materials and methods

Baseline characteristic data and blood samples were obtained on liver biopsy day. Laboratory evaluation included liver function tests, metabolic profile (fasting plasma glucose, hemoglobin A_{1C} (HbA_{1C}), total and HDL cholesterol, and triglyceride), complete blood count, prothrombin time and INR, and creatinine. Additionally, 10 mL of blood was drawn and prepared for miR-34a analysis. Liver biopsy was performed using an ultrasonography-guided technique.

Liver histological assessment

Each formalin-fixed liver tissue was stained with hematoxylin and eosin and Masson trichrome. All liver specimens measured at least 2 cm in length. The histological results were independently and blindly evaluated by an experienced pathologist (N.W.). 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 [7]. Additionally, severity of fibrosis was graded by fibrosis score (F0-F4) [7]. According to the latest AASLD guidelines [5], nonalcoholic steatohepatitis (NASH) is defined as “the presence of hepatic steatosis and inflammation with hepatocyte injury (ballooning) with or without fibrosis”. Conversely, nonalcoholic fatty liver or simple steatosis was defined as “the presence of hepatic steatosis with no evidence of hepatocellular injury in the form of ballooning of the hepatocytes or no evidence of fibrosis”.

MicroRNA extraction and reverse transcription

Serum from both groups was used for quantification of miR-34a. Serum miRs were extracted from 200 μL of serum using a miRNA purification kit (Norgen) according to the manufacturer's instructions. Mature miRNAs were polyuridylated using poly (U) polymerase and reverse transcribed by using a stem loop (SL) - poly A primer and reverse transcriptase. The polyuridylation reaction included 2.5 μL of 10× NEBuffer, 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 that was incubated at 37 °C for 10 min. Next, reverse transcription from RNA to DNA was performed using 12.3 μL of polyuridylated miRNA, 4.0 μl of 5× RT reaction buffer, 0.2 μL of 10 μM stem loop (SL) poly A primer (5′-GTCGTATCCAGTGCA GGGTCCGAGGTATTCGCACTGGATACGACAAA AAAAAAAAAAAAAAA VN-3′), 2 μL of 10 mM dNTPs mix, 20 units of RNase inhibitor, and 200 units of RevertAid reverse transcriptase (Thermo scientific) and nuclease-free water in a final volume of 20 μL.

Detection and quantitation of miR-34a by real-time PCR

Quantitation of miRNA was conducted using Step One Plus real-time PCR (Applied Biosystems, Foster City, CA) with SYBR Green dye. The real-time PCR reaction mixture consisted of 6.25 μL of 2× Maxima SYBR Green/ROX qPCR Master Mix (Thermo scientific), 0.3 μM of miR-34a specific forward primer (5′-CAATCAGCAAGTATACTGCC-3′), 0.3 μM of universal reverse primer (5′-GCAGGGTCCGAGG TATTC-3′), 1 μL of cDNA and nuclease-free water in a final volume of 12.5 μL. Thermal profiles were optimized for each miRNA as the following: initial denaturation at 95°C for 3 min and then 45 cycles of amplification including 95°C for 15 s and 60°C for 30 s.

The expression level (copies/μL) of miR-34a in serum was determined via absolute quantitation by comparing the Ct value of each sample with the standard curve. The standard curve was prepared by serial 10-fold dilutions of the standard in vitro transcribed miRNAs ranging from 10^8 to 10 copies/μL.

Sample size calculation

Sample size was calculated based on primary outcome. A recent study showed that a correlation coefficient between serum miR-34a and NAS divided into ≤4 and ≥5 was 0.46 [20]. We determined that α was to be 0.05 and the power of the study was to be 90%. Therefore, the calculated sample size was 46. Because the correlation coefficient between the level of serum miR-34 and liver inflammation was evaluated by NAS based on nonparametric data, we added 10% to the calculated sample size. Therefore, the total sample size was equal to 50. The control group was calculated to be 23.

Statistical analyses

We used SPSS version 16.0 for statistical analyses. The correlation coefficient is presented as Spearman's rho. The difference of the mean between two continuous data sets was calculated by the unpaired student *t* test or an analysis of variance. The difference of the percentage between two ordinal data points was calculated using a χ^2 test. A two-tailed $P < 0.05$ was considered significant for all analyses.

Results

The demographic data of the 50 patients and their basic laboratory test results are presented in **Table 1**. The mean age of control group was 52.6 ± 11.9 years, and 60% of the patients were female. These data did not significantly differ from NAFLD patients ($P = 0.84$, 0.34, respectively). Nevertheless, the control group had significantly lower BMI (21.6 ± 2.2 kg/m^2, $P < 0.001$).

The liver histopathology is presented in **Table 2**: 54% of patients had NAS ≥4 and the remaining 46% had NAS <4. Two-thirds of the patients (66%) had histopathology compatible with NASH, and 22% of patients were found to have significant fibrosis (≥F2). Other parameters of NAS are also shown in **Table 2**.

Serum levels of miR-34a were significantly correlated with the following liver histological parameters: NAS ($r = 0.39$, $P = 0.005$) (**Figure 1**), and degree of steatosis ($r = 0.28$, $P = 0.049$), ballooning ($r = 0.30$, $P = 0.034$), and fibrosis ($r = 0.39$, $P = 0.005$). However, miR-34a was not correlated with the degree of lobular inflammation ($r = 0.19$, $P = 0.188$). There was no significant correlation between serum miR-34a and other clinical variables measured including age, body weight, height, or BMI. Nevertheless, serum alanine aminotransferase (ALT) and high-density

lipoprotein (HDL) had a fair correlation (r = 0.42, P = 0.002 and r = 0.30, P = 0.03, respectively) with serum miR-34a.

We found that serum miR-34a in NASH was 28.12 ± 34.38 copies/μL and was significantly higher compared with that in the control group (P = 0.002), but not compared to simple steatosis (13.1 ± 21.0 copies/μL, P = 0.141) (**Figure 2**).

The patients with NAFLD and NAS ≥4 had significantly higher age, body weight, ALT, ALP, and HDL than those with NAS <4. Interestingly, the serum level of miR-34a in NAFLD patients with NAS ≥4 was 33.1 ± 37.5 copies/μL, which was significantly higher than those with NAS <4 (11. 6 ± 15.6 copies/μL, P = 0.011) and the control group (2.7 ± 3.7 copies/μL, P < 0.001) (**Figure 3**).

In addition, the serum level of miR-34a in the patients with NAS < 4 was not significantly different from that of the controls (P = 0.478).

With respect to the degree of fibrosis, those patients with significant fibrosis (≥ F2) had significantly higher serum miR-34a levels than those with F0-1 (41.9 ± 44.8 copies/μL vs. 17.9 ± 24.3 copies/μL, P = 0.022). However, there was no significant difference in serum miR-34a level between those patients with F0-1 and the control (P = 0.07).

In addition, there were no significant differences in serum miR-34a levels between NAFLD patients with and without obesity (18.6 ± 24.0 vs 28.1 ± 37.3 copies/μL, P = 0.30), those with and without metabolic syndrome (21.4 ± 30.3 vs 28.8 ± 34.4 copies/μL, P = 0.52), those with and without diabetes mellitus (21.2 ± 26.0 vs 24.8 ± 35.3 copies/μL, P = 0.68), and those with and without hypertriglyceridemia (15.9 ± 18.3 vs 26.3 ± 35.0 copies/μL, P = 0.17).

We performed receiver operating characteristic (ROC) curve analyses to evaluate whether serum miR-34a could be used to assess the severity of liver inflammation. The area under the ROC curve (AUC) values for serum miR-34a in the comparison between NAFLD patients with NAS ≥4 and with NAS <4 were 0.67 (95% CI 0.52, 0.82) (**Figure 4**). The optimal cut-off level of serum miR-34a for diagnosing NAS ≥4 was 18.3 copies/μL. It presented sensitivity was 56% and specificity was 83%.

Table 1. Baseline demographic and biochemical findings from patients with NAFLD

Characteristics, mean (SD) and number (%)	NAFLD patients (n = 50)
Demographics	
Age (year)	46.0 (13.7)
Female patients (%)	26 (52)
Weight (kg)	93.0 (43.6)
Height (m)	161.8 (9.6)
Body Mass Index (BMI) (kg/m^2)	35.8 (15.8)
Obesity classification (%)	
Normal (BMI <23.0 kg/m^2)	3 (6)
Overweight (BMI 23.0–24.9 kg/m^2)	8 (16)
Obesity grade 1(BMI 25.0–29.9 kg/m^2)	14 (28)
Obesity grade 2 (BMI 30.0–39.9 kg/m^2)	9 (18)
Morbid obesity (BMI ≥40 kg/m^2)	16 (32)
Comorbidities (%)	
Metabolic syndrome	38 (76)
Hypertension	30 (60)
Hypercholesterolemia	30 (60)
Diabetes mellitus and impaired fasting glucose	27 (54)
Hypertriglyceridemia	15 (30)
Biochemical profile	
Albumin (g/dL)	4.3 (0.4)
Total bilirubin (mg/dL)	0.91 (0.39)
Aspartate aminotransferase (AST) (U/L)	50 (32)
Alanine aminotransferase (ALT) (U/L)	71 (45)
Alkaline phosphatase (ALP) (U/L)	75 (22)
Fasting plasma glucose (mg/dL)	114 (36)
Hemoglobin A_{1c} (%)	6.3 (1.2)
Triglyceride (mg/dL)	143 (54)
Total cholesterol (mg/dL)	197 (38)
High-density lipoprotein (HDL) (mg/dL)	43 (10)
Low-density lipoprotein (LDL) (mg/dL)	124 (35)

Table 2. Liver histological parameters of NAFLD patients

Characteristics	NAFLD patients (n = 50)
NASH (%)	33 (66)
Simple steatosis (%)	17 (34)
NAFLD Activity Score (NAS) (%)	
1	7 (14)
2	5 (10)
3	11 (22)
4	15 (30)
5	9 (18)
6	3 (6)
Fibrosis score (%)	
F0	19 (38)
F1	20 (40)
F2	5 (10)
F3	6 (12)
Degree of steatosis (%)	
1 (5-33%)	24 (48)
2 (34-66%)	18 (36)
3 (>66%)	8 (16)
Degree of lobular inflammation	
0 (none)	14 (28)
1 (<2 foci/20 fields)	27 (54)
2 (2-4 foci/20 fields)	9 (18)
3 (>4 foci/20 fields)	0
Degree of hepatocyte ballooning (%)	
0 (none)	16 (32)
1 (few)	24 (48)
2 (many)	10 (20)

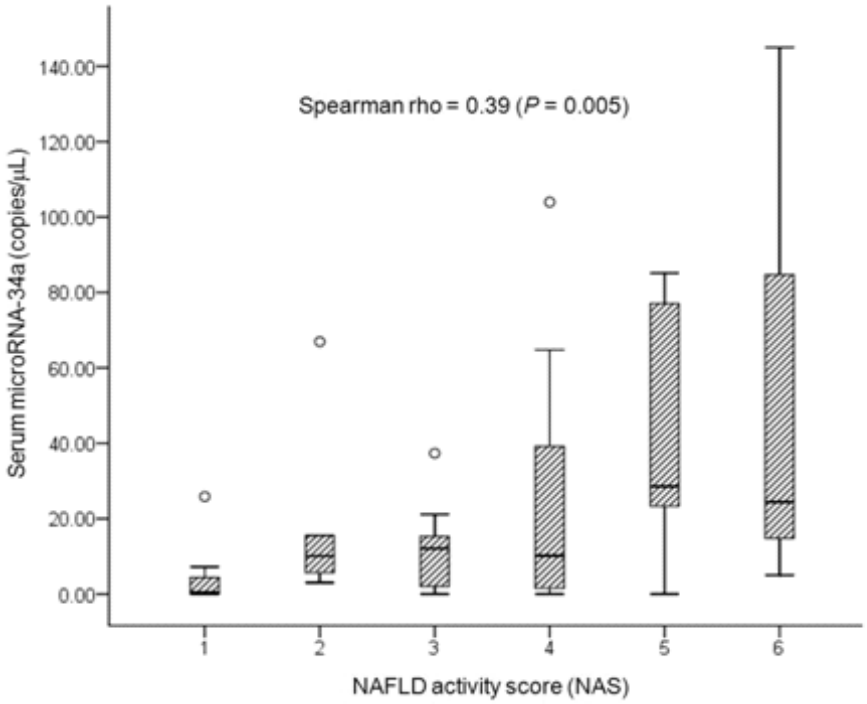

Figure 1. Correlation between serum microRNA-34a and nonalcoholic fatty liver disease activity score

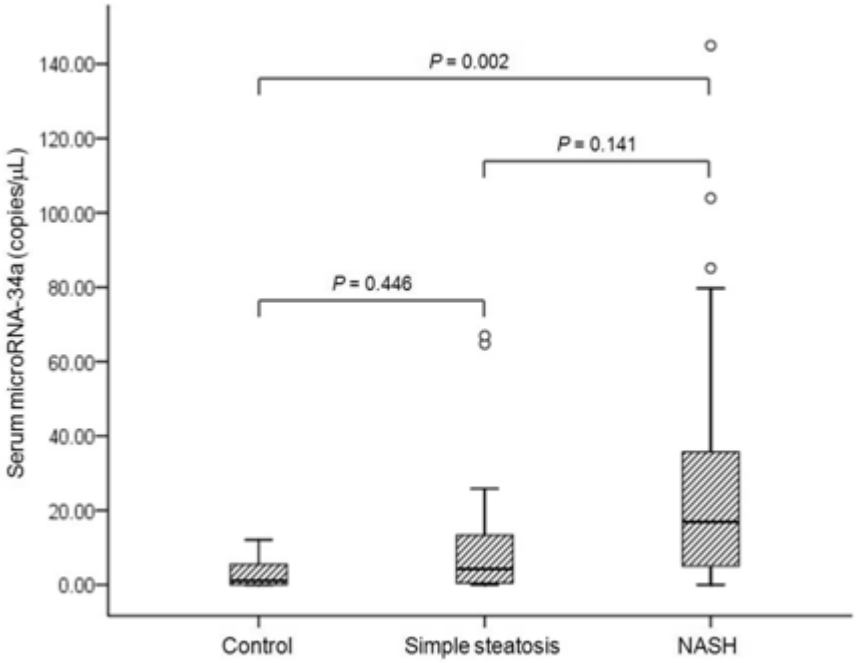

Figure 2. Level of serum microRNA-34a in control, simple steatosis, and nonalcoholic steatohepatitis (NASH)

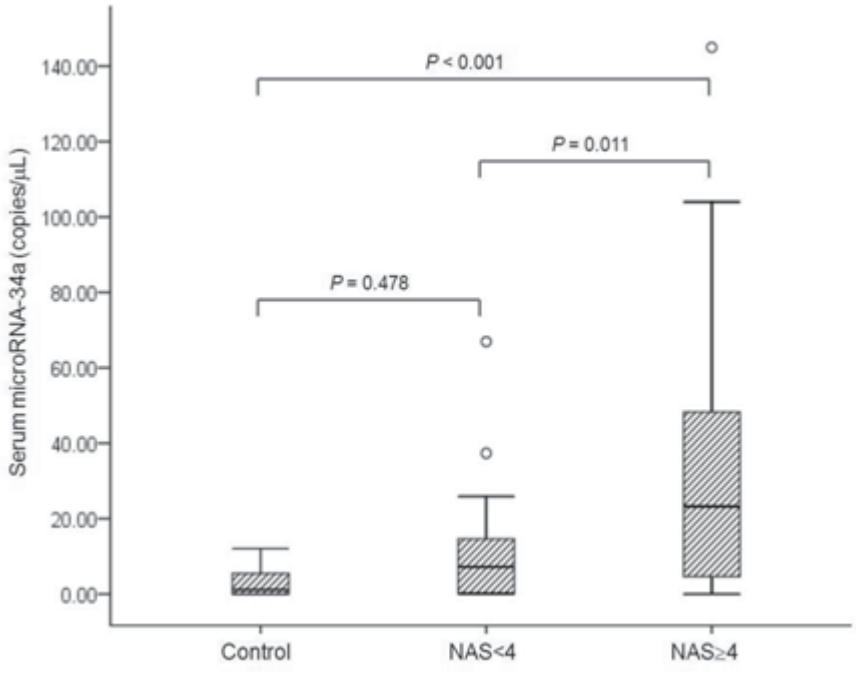

Figure 3. Level of serum microRNA-34a in patients with control, nonalcoholic fatty liver disease activity score (NAS) <4, and NAS ≥4

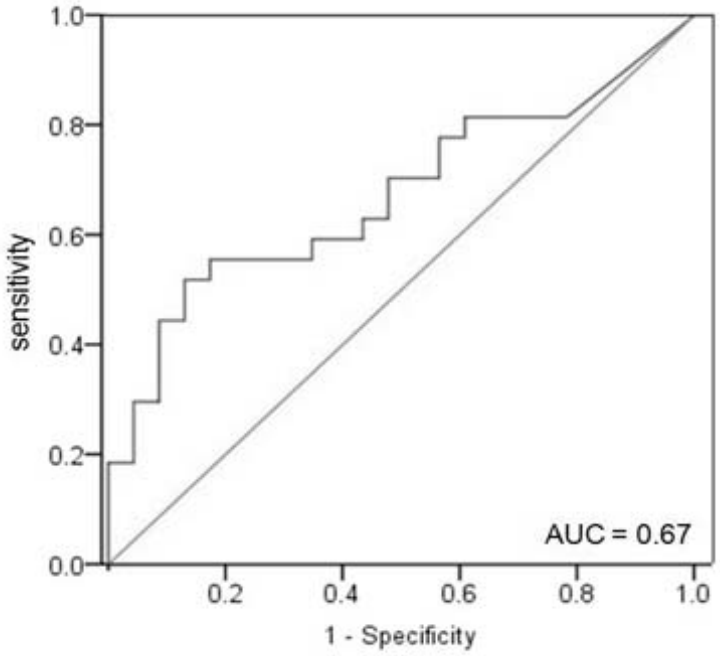

Figure 4. Receiver operating characteristic analysis of serum miR-34a from patients with nonalcoholic fatty liver disease (NAFLD) and NAFLD activity score (NAS) ≥4 versus with NAS <4

Discussion

Several microRNAs have been extensively proven and accepted to be potent regulators in the pathogenesis of NAFLD. The expression of miR-34a in human liver has clearly been confirmed to have a crucial role in regulating liver inflammation. Thus, it is associated with significantly increased severity in NAFLD. Moreover, serum miR-34a could distinguish NASH from simple steatosis and healthy individuals. However, the correlation between serum level of miR-34a and degree of liver inflammation has not been directly reported from past studies. The previous study from Cermelli et al. [20] showed the correlation coefficient between serum miR-34a and degree of liver inflammation was 0.46. However, our study differs from previous study in three aspects. First, the ethnic group is not similar. Second, we calculated the correlation coefficient from every NAS score while a previous study calculated correlations from only two NAS groups (NAS ≥5 and NAS <5) [21]. In this regard, our study presented more reliable and accurate correlation between serum miR-34a and liver inflammation as assessed histologically by NAS. Third, we determined NAS ≥4 to represent a subgroup with a high degree of liver inflammation because a study showed that NAS ≥4 was an optimal cut-off level for predicting steatohepatitis [8]. It was contrast to the previous study using NAS ≥5 [21]. Our meaningful data affirmed that serum miR-34 can be used as a biomarker for the degree of disease severity in patients with NAFLD. Moreover, a significant correlation between serum miR-34a and degree of hepatocyte ballooning supports a putative role for this microRNA because this histological feature represents degenerative changes, dilated endoplasmic reticulum and cytoskeletal injuries.

Patients with NASH tended to have a higher level of serum miR-34a compared to those with simple steatosis, but statistical significance was not obtained. This may be due to the fact that the patients who had steatosis levels >66% had significantly higher serum miR-34a as compared to other steatosis grades. Consequently, the proportion of the patients with steatosis of more than 66% was 22.3% in those with NAS ≥4 and was found more often 12.1% in those with NASH.

Data comparing serum miR-34a levels with each clinical feature of NAFLD patients such as diabetic mellitus and degree of obesity were not clearly presented in previous studies [19, 20]. The results from Cermelli et al. showed poor correlation between serum miR-34a and serum glucose and triglycerides. Additionally, serum miR-34a did not significantly change in morbid obesity cases [21]. In our study, obesity and metabolic syndrome were observed in approximately three-fourths of patients, and diabetes

mellitus or impaired fasting glucose was found in 54%. We found that there was no significant difference in level of serum miR-34a between patients with NAFLD and with and without these metabolic diseases. This implies that other clinical features of metabolic syndrome do not directly influence serum miR-34a.

With respect to clinical application, serum miR-34a may be used as a biomarker for diagnosing NASH instead of invasive liver biopsy. At this time, there is no standard biomarker for identifying patients with steatohepatitis, although serum cytokertin-18 (CK-18) is one of the promising candidates. However, a recent meta-analysis showed that CK-18 has moderate overall accuracy for diagnosing NASH (66% sensitivity, 82% specificity) [22]. Additionally, its optimal cut-off level is variable in each study. The optimal cut-off level of serum miR-34a from our study had high specificity for diagnosing NAS ≥4. Further studies should be conducted to calculate the optimal cut-off point of serum miR-34a. Drugs targeting microRNAs are novel therapeutic agents for liver diseases, such as miR-122-targeting by locked nucleic acid (LNA)-based antisense oligonucleotides in hepatitis C virus infection. Nevertheless, the results of a miRNA antagonist in NALFD were not impressive in animal studies [23-26]. The current miR-34a data may lead to the development of drugs for reducing liver inflammation in NASH.

Our study has certain limitations. First, we did not measure the level of hepatic miR-34a to compare with serum levels. Whereas previous studies have indicated that hepatic expression of miR-34a increases in NAFLD. This finding may support a concordant association between serum and liver tissue levels. Second, we could not perform liver biopsy to confirm normal liver histology in the control group because of ethical issues. Nevertheless, we applied new noninvasive diagnostic tools (transient elastography and controlled attenuation parameter) to choose the populations with the lowest risk of fatty liver disease. Finally, our study is cross-sectional, and long-term follow-up regarding natural history or prognosis cannot be assessed. Prospective studies are required.

Conclusion

Serum level of microRNA-34a has a significant fair to good correlation with NAS and degree of fibrosis, which represents the severity of inflammation. The serum miR-34a level in patients with NAS ≥4 was significantly higher than in those with NAS <4 and in the control group. The optimal cut-off level of serum miR-34a had high specificity for diagnosing NAS ≥4. Therefore, microRNA-34a serves as a potential biomarker of liver inflammation and fibrosis in NAFLD patients.

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Conflict of interest statement

The authors declare that there is no conflict of interest in this research.

Authors' contributions

The roles of each author are summarized as follows: Puth Muangpaisarn: proposal of the study concept and design and interpretation of data and editing the manuscript; Kanisa Jampoka and Sunchai Payungporn: data acquisition, microRNA extraction and measurement; Naruemon Wisedopas: pathological evaluation; Chalermrat Bunchorntavakul and Pisit Tangkijvanich: suggestion of the study concept and critical revision of the manuscript; Sombat Treeprasertsuk: suggestion of the study concept, critical revision and correspondence of the manuscript.

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