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Differential Expression of MicroRNAs in Hepatitis C Virus-Mediated Liver Disease Between African Americans and Caucasians: Implications for Racial Health Disparities

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African Americans (AAs) have higher hepatocellular carcinoma (HCC) mortality rates than Caucasian Americans (CAs). Chronic hepatitis C virus (HCV) infection leads to cirrhosis and HCC. HCV infection is highly prevalent in the AA population compared to other racial groups. AAs are also less likely to naturally clear HCV, potentially contributing to higher prevalence of HCV. However, the explanation for this disparity is currently unknown. Circulating microRNAs (miRNAs) in the blood are emerging as biomarkers for pathological conditions. Expression analysis of miRNAs in major racial groups would be important for optimizing personalized treatment strategies. Here we assessed the differential expression of circulatory miRNAs from HCV-infected AA and CA patients. We identified increased expression of miR-146a, miR-150, and miR-155 in HCV-infected AA patient sera compared to that of CA. Further analysis demonstrated that these miRNAs were significantly elevated in AA patients diagnosed with HCV-mediated HCC. Higher expression of miR-150 was also noted in cirrhosis and HCC in AA patients, which may serve as a predictor of liver disease progression in this population. The differential expression of miRNAs suggests that these miRNAs and their target genes could be useful to gain further mechanistic insight of racial disparity associated with HCV-mediated pathogenesis.

Key words: Hepatitis C virus (HCV); Hepatocellular carcinoma (HCC); MicroRNAs (miRNAs); African Americans; Caucasian Americans

INTRODUCTION

Hepatitis C infection is disproportionately prevalent in African American (AA) populations[1], and AAs with hepatitis C virus (HCV) exhibit higher liver cancer rates than seen in other ethnic groups. Higher frequency of the TT interleukin-28B (IL-28B) minor polymorphism (rs12979860) in AAs partially explains the higher prevalence due to the negative association of this polymorphism with rates of spontaneous viral clearance or sustained virological response following antiviral treatment. AAs have a twofold higher rate of death than expected from chronic HCV infection, largely due to a two- to threefold higher hepatocellular carcinoma incidence rate[1–3]. In 2013, the rate of HCV-related deaths among AAs was nearly double that of Caucasian Americans (CAs) (http://www.cdc.gov/hepatitis/statistics2013surveillance/index.htm#tabs-801919-5). The explanation for this disparity is currently unknown. Attempts to understand the underlying pathogenic mechanisms are limited by the absence of convenient cellular and animal models of HCV infection[4,5]. Genetic parameters like microRNAs (miRNAs) could explain some of the disparate risk of HCC incidence and mortality in AAs.

Dysregulation of miRNA expression occurs frequently in a variety of diseases including viral hepatitis, alcoholic and nonalcoholic steatohepatitis, drug-induced liver injury, and liver cancer[6,7]. Evidence is also emerging that miRNA expression profiles are distinct between liver diseases with different etiologies[6,8,9]. miRNAs may be modulated differentially during cancer progression in different racial populations. For instance, differences in miRNA expression levels of miR-182 have been reported to contribute to decreased survival in AA colon cancer patients compared to CA patients[10]. Potential racial differences in circulating miRNA expression have also been reported in breast cancer and papillary thyroid carcinoma[11,12].

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Considering the emerging utility of miRNAs as potential biomarkers in diseases and promising aspects of miRNAs in therapeutic interventions, we hypothesized that certain miRNAs might be endowed with a capacity to function as crucial modulators of HCV-mediated HCC and could be potential biomarkers to monitor disease progression among AA patients. We identified an increased expression of circulatory miRNAs—miR-146a, miR-150, and miR-155—in HCV-infected AA patients compared with CAs. Our results demonstrate that these miRNAs were significantly elevated in AA patients diagnosed with HCV-mediated HCC. Higher expression of miR-150 during cirrhosis and HCC in AA patients may serve as a predictor of liver disease progression. To our knowledge, this is the first report describing ethnicity-based modulation of a group of miRNAs during HCV infection, which could be used as potential predictive biomarkers and will help in understanding the mechanisms associated with racial disparity during HCV pathogenesis.

MATERIALS AND METHODS

Study Design and Patient Samples

Our study was approved by the Saint Louis University Hospital and Corporal Michael J. Crescenz VA Medical Center Institutional Review Board (IRB), and written informed consent was obtained from all subjects. HCV diagnosis was performed by screening the blood samples for the detection of HCV RNA in respective medical centers. We have received the viral load data from the clinical chart. A total of 90 sera samples including HCV-infected and HCC-associated AA patients ($n=30$) and CA patients ($n=16$) were included in this study. We also included serum samples from chronic hepatitis C (CHC) patients (AA, $n=9$; CA, $n=9$) and liver cirrhosis patients (AA, $n=12$; CA, $n=14$). Table 1 shows the characteristics of patients infected with HCV. Liver disease stages of most of the patients were diagnosed by biopsy and some (especially for cirrhosis patients) by Fibrosure. The diagnosis of HCC was made as described previously[13].

Serum miRNA Expression Profiling and miRNA-Specific Quantitative Real-Time RT-PCR

To generate a comprehensive set of miRNA expression profiles in HCV-infected AA and CA patients, we analyzed the expression of 84 miRNAs from four pooled serum samples of each group as described previously[14]. Briefly, total RNA was isolated from 200 µl of serum or plasma by miRVana PARIS kit (Invitrogen) according to the manufacturer's instructions. RNA was reverse transcribed to complementary DNA (cDNA) using the miScript Reverse Transcription kit (Qiagen) according to the manufacturer's instructions. Real-time polymerase chain reaction (PCR) was performed using the miScript SYBR Green PCR kit (Qiagen) with the manufacturer-provided miScript Universal primer. Array data were analyzed using free Web-based software (http://pcrdataanalysis.sabiosciences.com/mirna/arrayanalysis.php) and automatically performed all $^{\Delta\Delta}$Ct fold change calculations. For validation and subsequent work, miRNA-specific quantitative real-time RT-PCR was performed using TaqMan assays (Invitrogen). Synthetic spiked-in *Caenorhabditis elegans* miR-39 was added to the serum/plasma samples prior to RNA extraction as an internal control as described previously[14].

Liver biopsy specimens from HCC adult patients (CA and AA, $n=4$ from each group) for our study were used from the Saint Louis University Liver Center repository and were approved by IRB, and miRNA was analyzed as described previously[14]. The relative expression levels of miRNA were normalized to U6 (as internal control), and fold changes in gene expression were calculated using the $2^{-\Delta\Delta}$Ct method.

Statistical Analysis

Data were analyzed by Mann–Whitney *U* test for two nonparametric groups. Receiver operating characteristic (ROC) curves were constructed, and the area under the curve (AUC) was calculated to evaluate specificity and sensitivity of predictive value or feasibility of using

Table 1. Patient Characteristics

	Caucasian Americans (CAs)			African Americans (AAs)		
Characteristics	CHC	Cirrhosis	HCC	CHC	Cirrhosis	HCC
No. of samples	9	14	16	9	12	30
Age (years)*	54±4.5	59±8	61±5	59±5.5	56±3.4	60±4.5
Gender (male/female)	4/5	14/0	14/3	7/2	12/0	26/4
ALT (IU/L)†	49 (12–118)	ND	49 (5–132)	37 (18–139)	ND	55 (14–272)
AFP (ng/ml)†	NA	4.2 (0.8–77)	12.1 (3.4–58,700)	NA	6.5 (0.9–67.4)	58 (4.7–12,600)
HCV viral load (log10 copies/ml)‡	6.6±2.5	UD	4.8±1.6	6.2±2.2	UD	6.1±1.4

N/A denotes not applicable; ND, not done; UD, undetermined; CHC, chronic hepatitis C; HCC, hepatocellular carcinoma.
*Data given as mean ± SD.
†Data given as median (ranges).
‡HCV viral load in log10 ± SEM.

serum/plasma miRNA as a marker for liver disease progression. A value of $p<0.05$ was considered statistically significant. All statistical analyses were performed, and graphs were generated using GraphPad Prism 6.0 (GraphPad Software, CA, USA).

RESULTS

Profiling of Serum miRNA Levels in HCV-Infected AA and CA Patients

The pathway-specific circulatory miRNA expression profiling by miScript miRNA PCR Array allowed us to focus on the comprehensive set of miRNA expression profiles in HCV-infected AA/CA HCC patient sera. Clustering analysis revealed that miR-146a, miR-150, and miR-155 were significantly upregulated, and miR-125b and miR-143 were downregulated in HCV-infected AA sera compared to sera from CA patients (data not shown). We chose these five miRNAs for subsequent studies.

Candidate miRNAs Are Highly Modulated in Serum of HCV-Infected AA Patients

We next determined the five miRNAs individually in a cohort of sera from HCV-infected AA ($n=41$) and CA ($n=30$) patients. We observed significant upregulation of miR-150 and miR-155 in AA patient sera compared to that of CA patients (Fig. 1A and B). We also evaluated the predictive value of these miRNAs in identifying the HCV-mediated liver disease in AA patients. The levels of two miRNAs in the serum samples were measured, and ROC analysis was performed on individual miRNAs. Between the two miRNAs, miR-150 showed the highest AUC of 0.719 ± 0.06 (95% CI = 0.599–0.834) with sensitivity of 86% and specificity of 53%, and miR-155 showed an AUC of 0.657 ± 0.065 (95% CI = 0.530–0.785) with sensitivity of 80% and specificity of 56% in separating the AA patients from CA patients (Fig. 1). However, miR-125b, miR-143, and miR-146a were not significantly altered between sera/plasma of the two ethnic groups (Fig. 2A–C).

Candidate miRNAs Are Highly Modulated in Serum of HCV-Infected AA Patients With HCC

We further evaluated the five miRNAs individually in a small cohort of sera from HCV-infected AA and CA HCC patients. We observed a significant upregulation of miR-146a, miR-150, and miR-155 in AA patients compared to CA patients ($p=0.04$, $p=0.008$, and $p=0.03$, respectively) (Fig. 3A–C). However, we did not observe a significant modulation of miR-125b and miR-143 between the two ethnic groups (data not shown).

We also determined the predictive value of these miRNAs in identifying the HCV-mediated HCC in AA patients. The levels of three miRNAs in the serum samples were measured, and ROC analysis was performed on individual miRNAs. Among the three miRNAs, miR-150 showed the highest AUC of 0.731 ± 0.074 (95% CI = 0.585–0.878) with sensitivity of 59% and specificity of 84%, miR-155 showed an AUC of 0.702 ± 0.077 (95% CI = 0.545–0.854) with sensitivity of 87.5% and specificity of 57%, and miR-146a showed an AUC of 0.689 ± 0.087 (95% CI = 0.517–0.861) with sensitivity of 50% and specificity of 93.3% in separating the AA from CA HCC patients (Fig. 3).

Expression Level of miR-146a, miR-150, and miR-155 in HCV-Infected AA and CA Patients With Different Clinical Status

To further stratify the disease progression-specific modulation of these miRNAs in two different ethnic populations, we examined the expression level of these circulatory miRNAs in HCV-infected AA and CA patients with cirrhosis stages. Our data demonstrated that only miR-150 level was significantly elevated in AA sera compared with CA sera ($p=0.02$) (Fig. 4). We also performed ROC analysis to determine the predictive value of this miRNA. Our analysis showed that miR-150 had an AUC of 0.769 ± 0.09 (95% CI = 0.58–0.958) with sensitivity of 85% and specificity of 67% in separating the AA cirrhosis from CA cirrhosis patients. The expression of other miRNAs remained unaltered (data not shown). We also examined the expression level of these circulatory miRNAs in HCV-infected AA and CA patients with chronic HCV infection. Interestingly, we did not observe a significant alteration of these miRNAs between AA and CA CHC patients (Fig. 5A–E).

Correlations Between miRNAs and Clinicopathological Characteristics

We analyzed if there are any correlations that exist among upregulated miRNAs with clinical characteristics such as ALT, AFP levels, and viral load. We did not find any correlation with upregulation of miR-146a, miR150, or miR155 and liver enzymes or viral load. However, a correlation between serum miR-150 and miR-155 levels was found in AA patients with HCV-mediated HCC (Fig. 6A).

miR-150 and miR-155 Are Highly Expressed in Liver Biopsy Samples of AA HCC Patients

We further determined the expression level all five miRNAs in liver biopsy specimens of CA and AA HCC patients. miR-150 and miR-155 were upregulated in HCV-infected HCC AA liver biopsy specimens compared to that of CA samples (Fig. 6B). However, no significant difference in expression level of miR-125b, miR-143, and miR-146a was observed between the two ethnic groups (data not shown). These data suggested that modulation

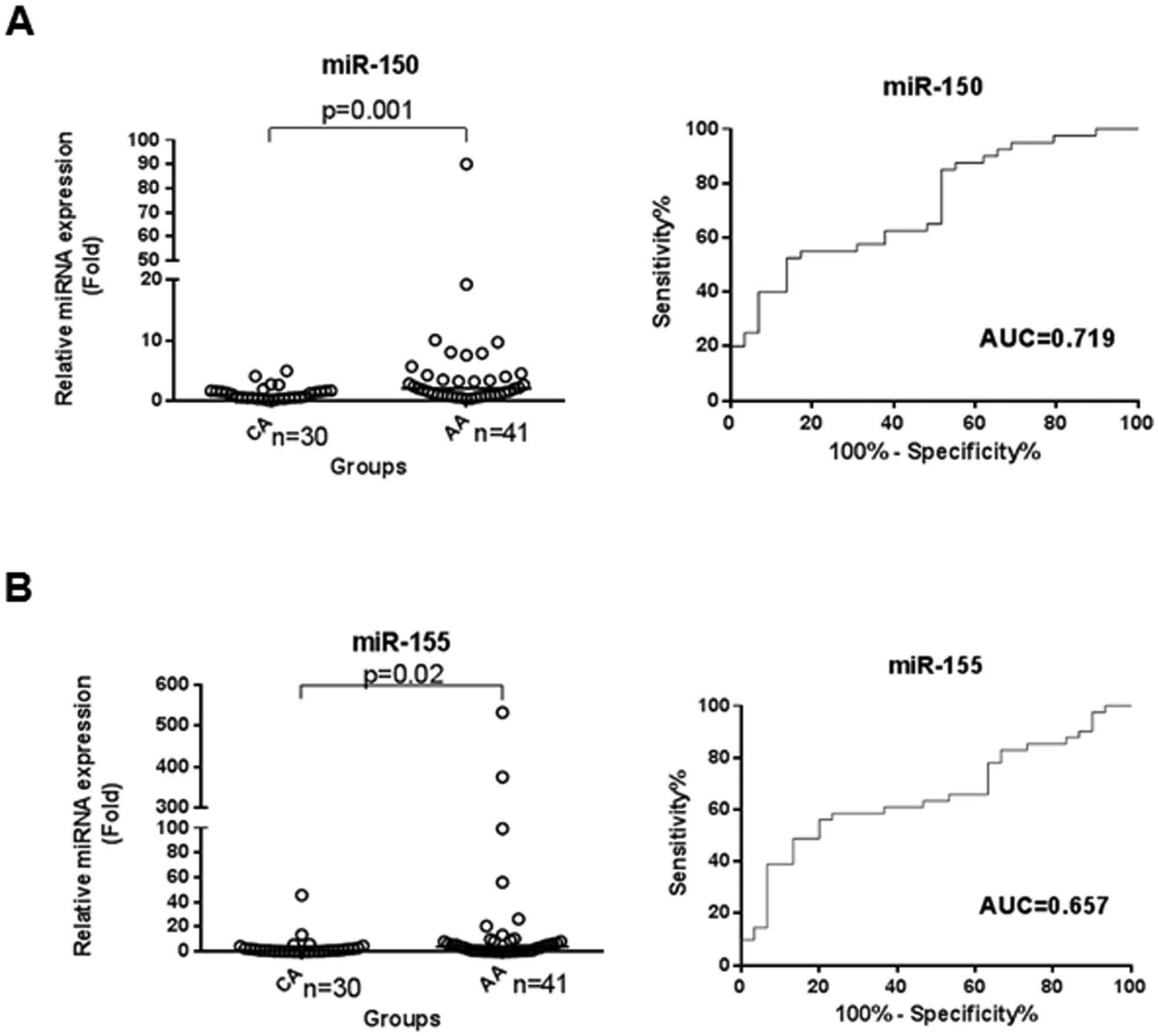


Figure 1. Differential expression of serum miRNAs in HCV-infected African American (AA) patients compared to Caucasian American (CA) patients. Scatter plots of serum levels of (A) miR-150 and (B) miR-155 from HCV-infected CA ($n=30$) and AA ($n=41$) patients as assessed by qRT-PCR. The line indicates the median value per group. Fold regulation is expressed as RQ based on the $2^{-\Delta\Delta Ct}$ method. Mann–Whitney *U* test was used to determine statistical significance. Receiver operating characteristic (ROC) curve analysis of two miRNAs (right), miR-150 and miR-155, was used to differentiate AA individuals from CA individuals. The area under the ROC curve (AUC) for each miRNA conveys its accuracy for differentiation of AA patients from CA patients in terms of sensitivity and specificity.

of these miRNAs might reflect disease severity in the AA population.

DISCUSSION

The rationale for differences in HCV natural history and disease progression based on racial and ethnicity is not completely understood. In this study, we demonstrated that upregulation of selected miRNAs—miR-146a, miR-150, and miR-155—was associated with liver disease in HCV-infected patients and significantly modulated in AA patients compared to CA patients. Circulating miRNA levels differ according to physiological and pathological states at different diseases, suggesting that miRNAs may be useful biomarkers for the diagnosis and clinical progression of various diseases[15]. We also observed a significant upregulation of miR-146a, miR-150, and miR-155 in the HCV-infected HCC patients' sera from both ethnic groups when compared with the respective healthy volunteers' sera (data not shown).

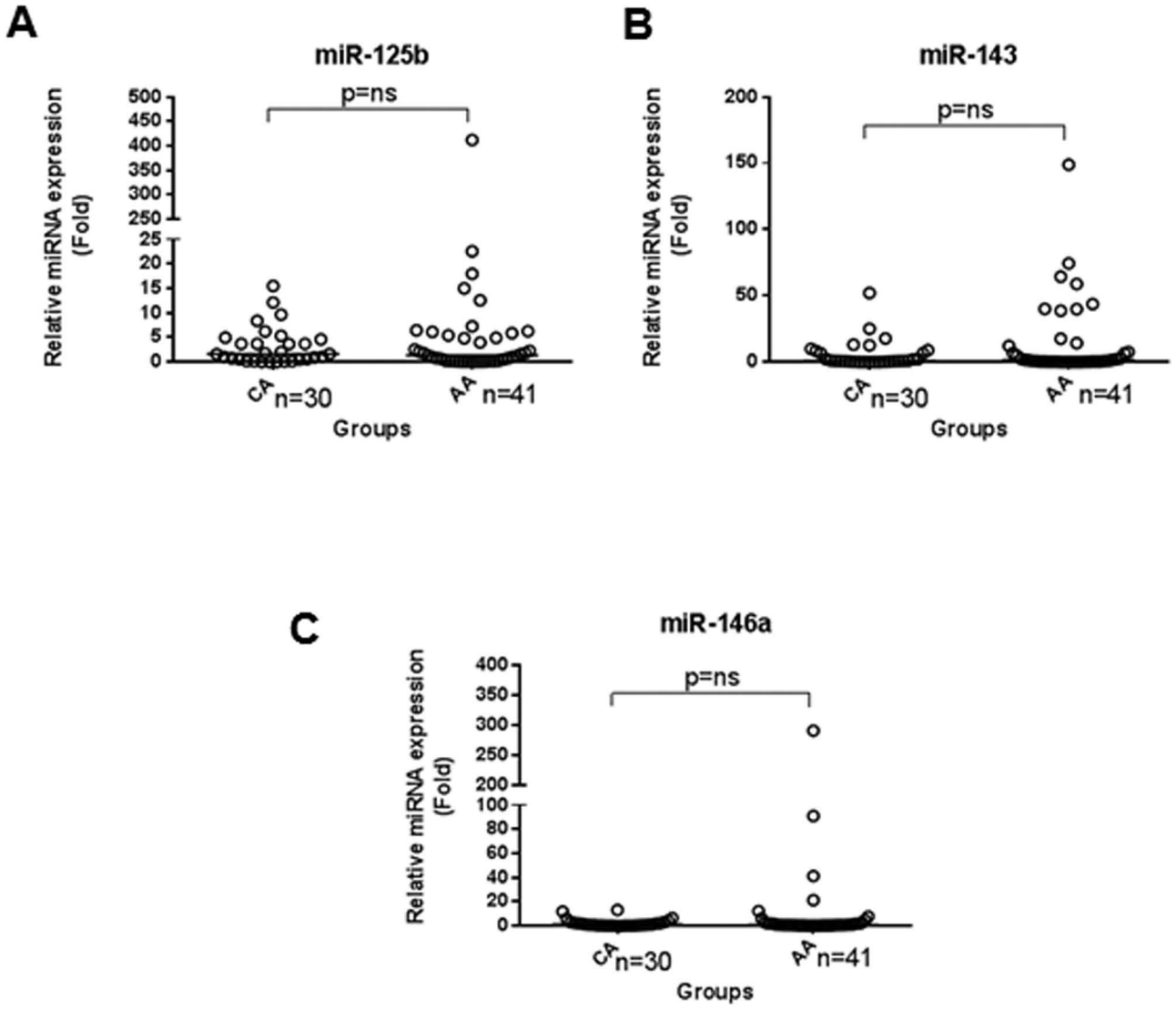
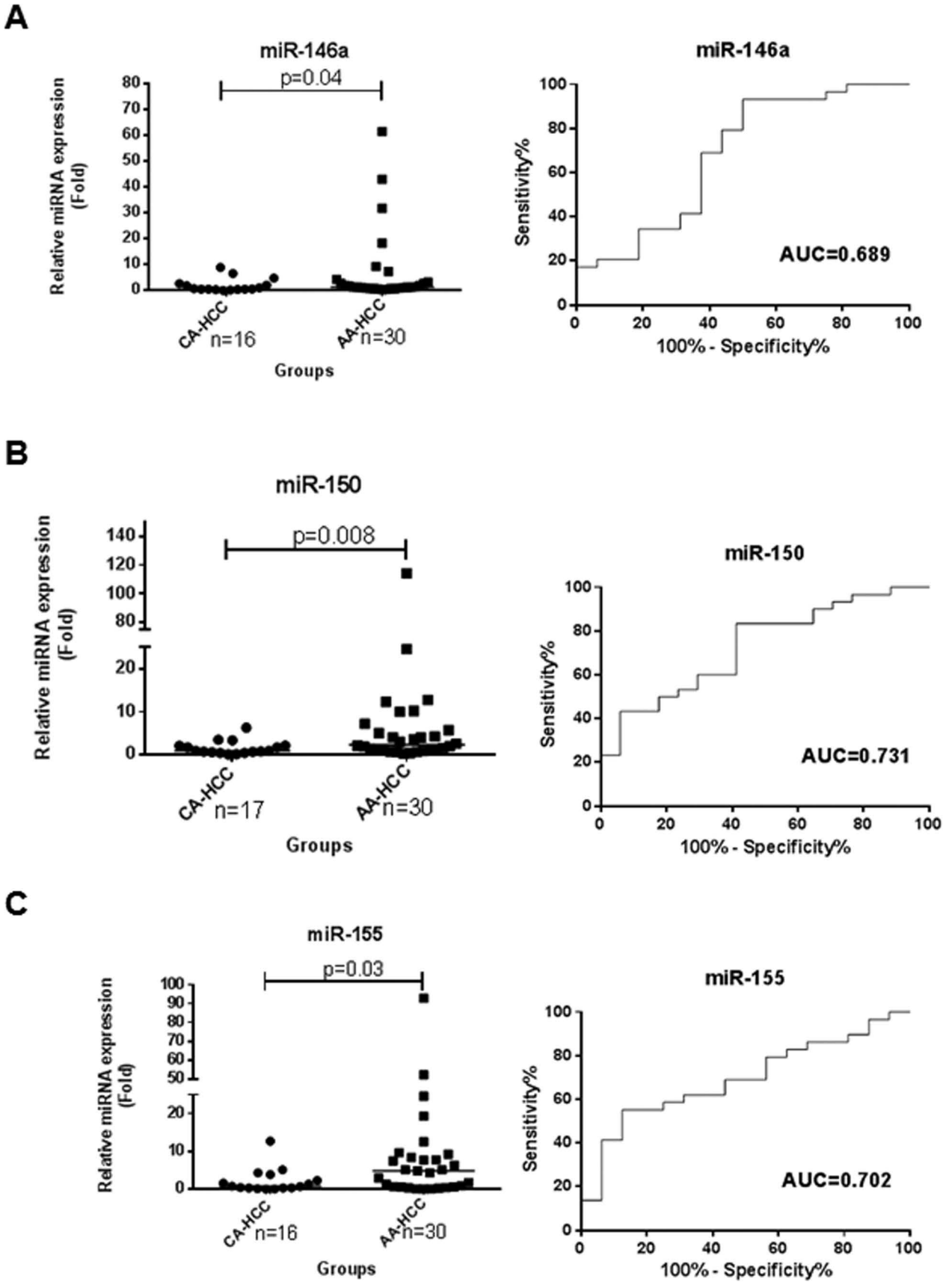


Figure 2. Differential expression of serum miRNAs in HCV-infected AA patients compared to CA patients. Scatter plots of serum levels of (A) miR-125b, (B) miR-143, and (C) miR-146a from HCV-infected CA ($n=30$) and AA ($n=41$) patients as assessed by qRT-PCR. The line indicates the median value per group. Fold regulation is expressed as RQ based on the $2^{-\Delta\Delta Ct}$ method. Mann–Whitney *U* test was used to determine statistical significance.

miR-155, miR-146a, and miR-125b are reported to be relevant to monocyte inflammatory activation as they regulate inflammation at multiple levels[16,17]. Serum levels of these miRNAs were increased in chronic HCV patients. We also observed significant upregulation of these miRNAs in both CA and AA chronic HCV patients compared to the respective healthy controls (data not shown), although there was no difference between the ethnic groups. Further, miR-150 expression was higher not only in liver cirrhosis but also in HCV-mediated HCC in AA patients' sera, suggesting its role as a useful biomarker for the diagnosis and clinical progression of liver disease among the AA population. Abnormal expression of miR-150 has been found in other solid tumor tissues, such as lung cancer, breast cancer, esophagus cancer, colorectal cancer, and pancreatic cancer, and associated with tumorigenesis[18]. miR-150 plays an important role in T-cell development through targeting the Notch pathway, a main regulator of T-cell differentiation, resulting in decreased T-cell proliferation and survival[16]. The higher expression of miR-150 may be associated with the induction of immune tolerance in AA patients infected with HCV. High circulating levels of miR-150 was associated with severe A/H1N1 illness and correlated with different


A
miR-146a
p=0.04
Relative miRNA expression (Fold)
80
70
60
50
40
30
20
10
0
CA-HCC n=16
AA-HCC n=30
Groups
miR-146a
Sensitivity%
100
80
60
40
20
0
AUC=0.689
0
20
40
60
80
100
100% - Specificity%
B
miR-150
p=0.008
Relative miRNA expression (Fold)
140
120
100
80
20
10
0
CA-HCC n=17
AA-HCC n=30
Groups
miR-150
Sensitivity%
100
80
60
40
20
0
AUC=0.731
0
20
40
60
80
100
100% - Specificity%
C
miR-155
p=0.03
Relative miRNA expression (Fold)
100
90
80
70
60
50
20
10
0
CA-HCC n=16
AA-HCC n=30
Groups
miR-155
Sensitivity%
100
80
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AUC=0.702
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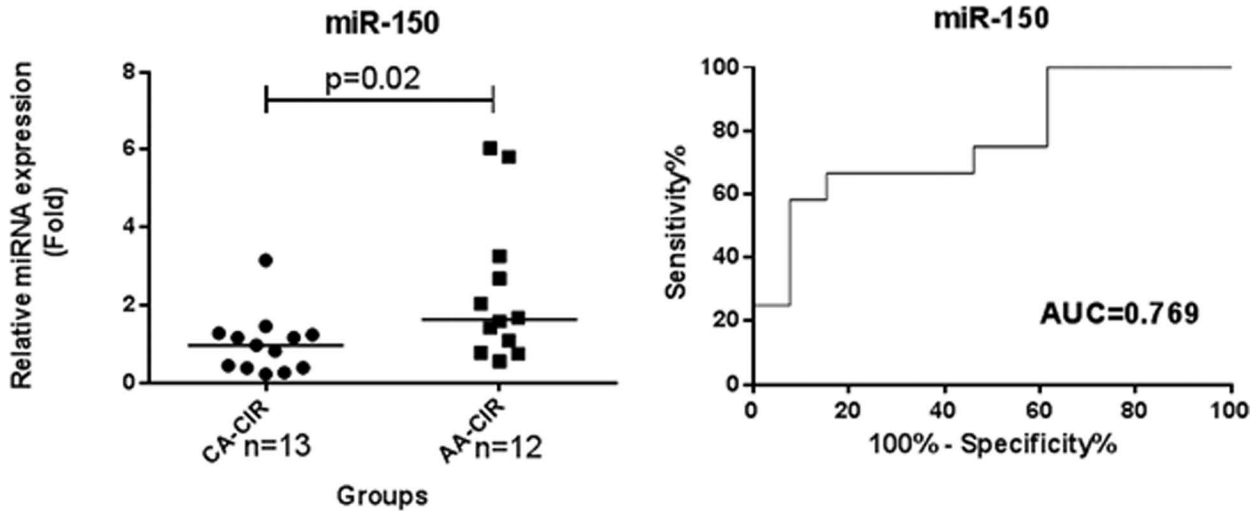

Figure 4. miRNA expression from CA and AA patients affected with HCV-mediated liver cirrhosis. Upregulation of miR-150 in AA patient sera compared to CA patients affected with liver cirrhosis (CIR). ROC curve analysis of miR-150 conveys its accuracy for differentiation of AA patients from CA patients with liver cirrhosis in terms of sensitivity and specificity.

proinflammatory mediators and growth factors including IL-1β, interferon-γ (IFN-γ), CXCL8, and G-CSF, particularly in critically ill A/H1N1 patients[19]. This implicates one of the plausible mechanisms of inflammation-mediated liver damage in AA patients infected with HCV.

miR-155 enhances T-cell proliferation by targeting SOCS1 and promotes T-cell activation by targeting CTLA-4, a negative regulator of T-cell activation[20]. miR-155 also plays an important role in regulating both inflammation and tumorigenesis. In limited liver biopsy specimens, we observed a higher expression of miR-150 and miR-155 in the AA group. miR-150 targets the 3′-UTR of p53, and the p53 protein promotes the expression of miRNAs, including miR-34a, miR-184, miR-181a, and miR-148, which affect the cell cycle progression of non-small cell lung cancer[21]. miR-150 enhances the proliferation and migration of lung cancer through specifically targeting the 3′-UTR of SRCIN1 and BAK1[22]. On the other hand, miR-155 promotes hepatocyte proliferation and tumorigenesis by activating Wnt signaling[23]. miR-155 and miR-150 are highly expressed in $EpCAM^+$ HCC cells compared to $EpCAM^-$ HCC cells, normal hepatic stem cells (HpSCs), hepatocytes, as well as normal fetal and adult livers[24]. However, we do not know whether circulating miR-150 and/or miR155 is coming from HCV-infected hepatocytes to the serum, and further study is necessary to elucidate this issue. Higher expression of these miRNAs in AA HCC patients might be one of the contributing factors for higher rates of HCC and mortality in AAs.

miR-146a modulates both the innate and adaptive immune responses via negative feedback loops involving downregulation of its target genes[16]. HBx upregulates miR-146a in HBV-infected hepatocytes, which subsequently targets complement factor H (CFH), an important negative regulator of the alternative pathway of complement activation[25]. Higher miR-146a expression leads to a more severe suppression of CFH in hepatocytes, resulting in enhanced chronic complement-mediated cytotoxicity and subsequent liver fibrosis, cirrhosis, and HCC. HCV-induced increase in miR-146a-5p expression in different cell-based models of HCV infection and HCV patient-derived liver tissue implicated its role in promoting both viral infection and is relevant for pathogenesis of liver disease[26]. We observed a significant upregulation of miR-146a in AA HCC patients compared to CA HCC patients, suggesting a possible role of miR-146a in the lower rate of spontaneous viral clearance in AAs and in

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Figure 3. miRNA expression from CA and AA patients affected with HCV-mediated hepatocellular carcinoma (HCC). Scatter plots of serum levels of (A) miR-146a, (B) miR-150, and (C) miR-155 from HCV-infected CA ($n=17$) and AA ($n=30$) patients with HCC as assessed by qRT-PCR. miR-146a, miR-150, and miR-155 were significantly upregulated in AA patient sera compared to that of CA patients. The line indicates the median value per group. Fold regulation is expressed as RQ based on the $2^{-\Delta\Delta Ct}$ method. Mann–Whitney *U* test was used to determine statistical significance. ROC analysis with corresponding AUC for miR-146a, miR-150, and miR-155 in discriminating AA patients from CA patients is shown on the right, respectively.

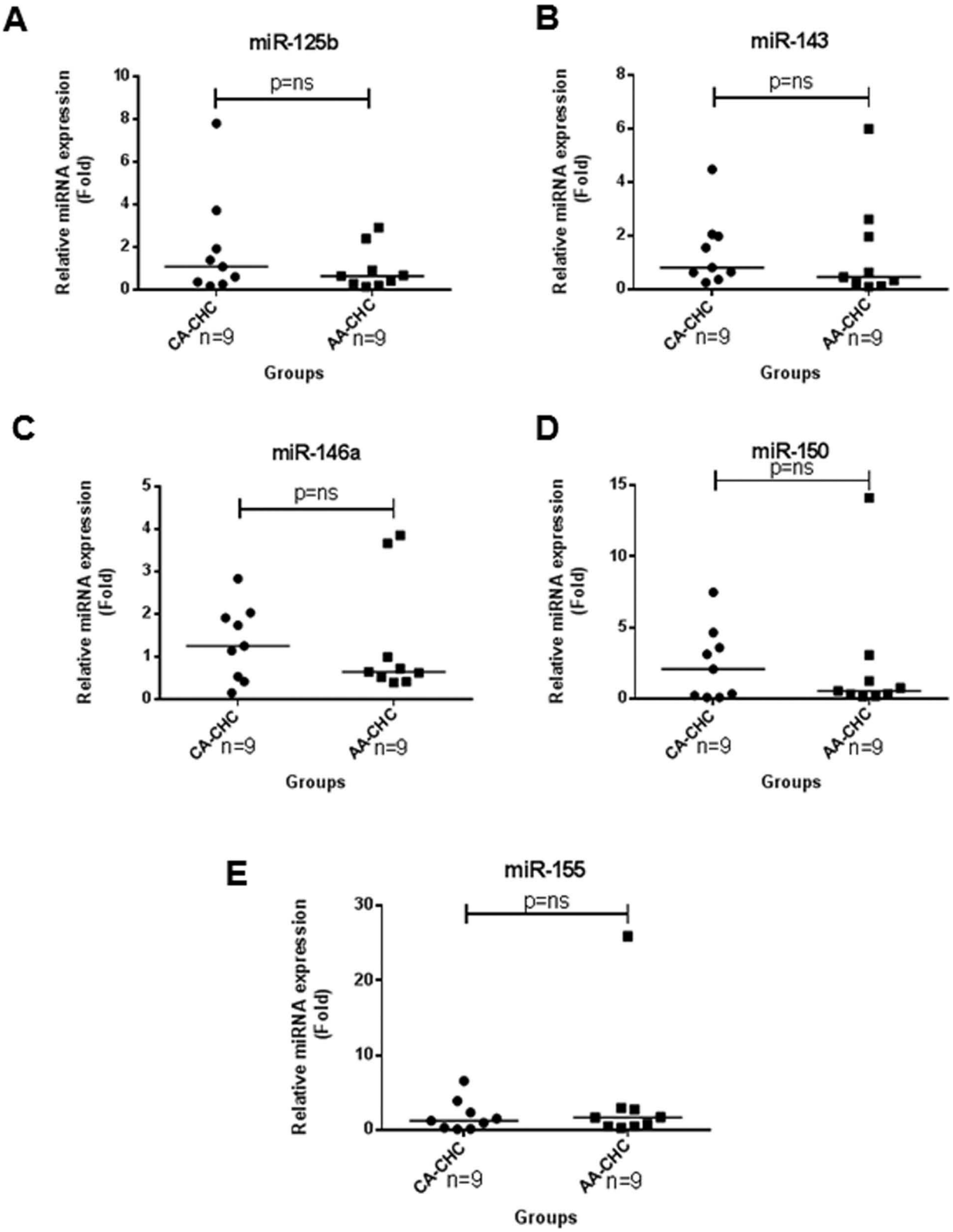

Figure 5. miRNA expression from CA and AA patients affected with chronic hepatitis C (CHC). Scatter plots of serum levels of (A) miR-125b, (B) miR-143, (C) miR-146a, (D) miR-150, and (E) miR-155 from CA ($n=9$) and AA ($n=9$) patients as assessed by qRT-PCR. The line indicates the median value per group. Fold regulation is expressed as RQ based on the $2^{-\Delta\Delta Ct}$ method. Mann–Whitney *U* test was used to determine statistical significance.

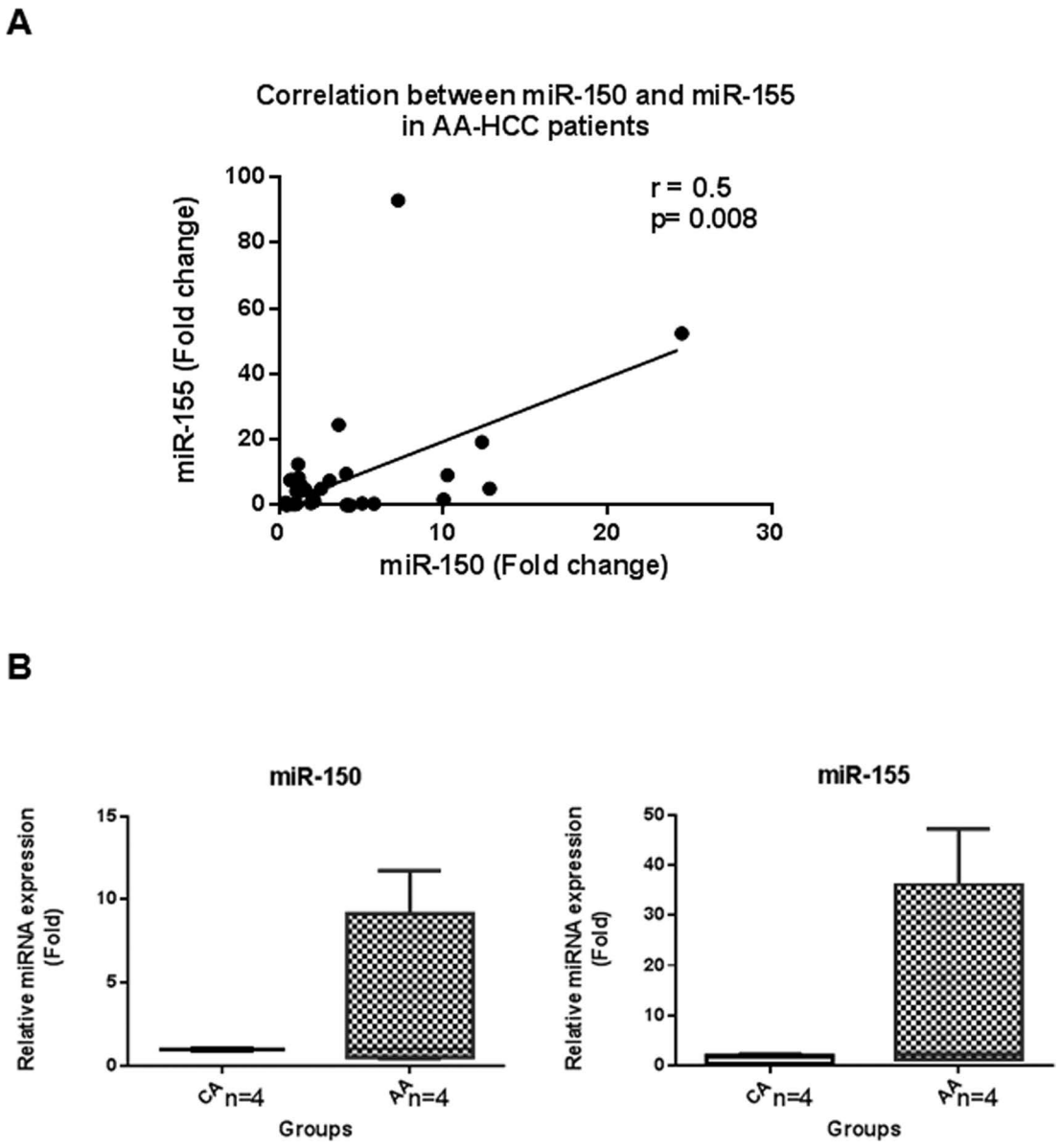

Figure 6. Correlations between miR-150 and miR-155. (A) Increased circulating miR-150 levels correlate with miR-155 levels in AA patients with HCV-mediated HCC. (B) Higher expression of miR-150 and miR-155 in liver biopsy samples from AA compared to CA patients infected with HCV. Relative expression of miR-150 and miR-155 is shown. Data are presented as means ± SEM.

modulating pathways that are related to liver disease and HCC development.

To the best of our knowledge, this is the first report demonstrating differential miRNA expression between CA and AA patients during HCV pathogenesis, suggesting modulation of different pathways for the final outcome of liver disease in AA patients. We acknowledge that our sample size is limited, which is a limitation of our study. Our study demonstrated significant upregulation of miR-146a, miR-150, and miR-155 in HCV-infected AA patients compared to CA patients. Interestingly, circulating miR-150 expression was high not only in liver cirrhosis but also in HCV-mediated HCC in AA patients, suggesting its role as a useful biomarker for the diagnosis and clinical progression of liver disease among the AA population. Further studies are needed to understand the mechanistic details behind the modulation of these miRNAs in HCV pathogenesis with respect to racial disparity.

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