Identification of microRNA profiles associated with refractory primary biliary cirrhosis

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Abstract. MicroRNAs (miRNAs) are small, endogenous, non-coding RNAs that control the target gene translation by RNA interference; miRNAs are associated with cellular processes, including proliferation, differentiation, apoptosis, and cell survival. Primary biliary cirrhosis (PBC) is a chronic cholestatic liver disease of unknown etiology. One third of patients with PBC demonstrate suboptimal responses, which result in worse outcomes. It has been previously reported that miRNAs are involved in drug resistance, however, the association between miRNA expression levels and refractory PBC remains to be fully elucidated. In the present study, among the 20 patients with PBC treated with ursodeoxycholic acid or bezafibrate, 15 patients were classed as treatment-effective, and 5 were classed as being treatment-resistant. Using the miRNA array technique, miRNA profiles were identified for each group. A total of 35 miRNAs were significantly upregulated, and 23 were significantly downregulated in the treatment-resistant group compared with the treatment-effective group. In order to examine the association between the highly altered miRNAs and clinical features of the two groups, numerous parameters were analyzed. Elevated levels of direct bilirubin, aspartate transaminase (AST), and alanine transaminase (ALT) were identified to be associated with miRNA-122 upregulation. AST, ALT, and γ guanosine triphosphate were additionally associated with miRNA-378f upregulation. However, the reduction of miRNA-4311 was associated with reduced levels of AST and ALT. miRNA-4714-3p was also negatively correlated with total bilirubin and lactate dehydrogenase. Therefore, identifying the miRNA profile was demonstrated to be a useful approach in the characterization of PBC development. It is suggested that highly altered miRNAs may be potential biomarkers for use in the development of treatment of patients with refractory PBC.

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Abbreviations: UDCA, ursodeoxycholic acid

Key words: primary biliary cirrhosis, microRNA, treatment resistancy, ursodeoxycholic acid, bezafibrate

Introduction

MicroRNAs (miRNAs) are small, non-coding RNAs that are 21-30 nucleotides in length and that interfere with their target mRNAs, and there are approximately 2,000 of these sequences in the human genome (1). Each miRNA negatively regulates protein translation through the degradation of mRNA cleaved by a miRNA-associated RNA-induced silencing complex (2). It is now apparent that an individual miRNA can modulate more than 200 miRNAs (3,4), and greater than 2,000 miRNAs have been identified at present (5).

Previous studies have demonstrated that miRNAs can serve an important role in autoimmune diseases (AID) (6), including systemic lupus erythematosus (7) and rheumatoid arthritis (8). Several miRNAs have been associated with the maturation of various immune cells and with the regulation of their functions (9-11), suggesting that certain miRNAs may affect the development and etiology of AID. In addition, although it has been demonstrated that miRNAs are involved in numerous types of liver disease, the association between miRNA profiles and autoimmune liver diseases, including the clinical relevance of miRNA in treating these diseases, remains unclear.

Primary biliary cirrhosis (PBC) is a slow, progressive, chronic cholestatic disease that is characterized by the destruction of the intrahepatic bile ducts and fibrosis, and with portal inflammation, it may develop into liver cirrhosis (12). Several processes are observed during the development of PBC: i) A specific immune response including antimitochondrial antibodies (AMA), which are directed towards the E2 component of the 2-oxo-dehydrogenase pathway, particularly towards the pyruvate dehydrogenase-E2 complex; ii) abnormal innate immunity, in which the ligands of the Toll-like receptors 3 and 4 stimulate type 1 T helper cell responses, resulting in biliary cell destruction; and iii) a higher frequency autoreactive T cell

Table I. Characteristics of the study groups.

Characteristic	Treatment-effective group (n=15)	Treatment-resistant group (n=5)
Gender (F/M)	15/0	4/1
Age (years)	59.3±9.0	40±14.6
TP (mg/dl)	7.79±0.62	7.82±0.37
Alb (mg/dl)	4.01±0.35	3.9±0.44
T-bil (mg/dl)	0.61±0.24	0.88±0.48
D-bil (mg/dl)	0.21±0.15	0.32±0.11
AST (IU/l)	75±66.8	87.6±50.8
ALT (IU/l)	94.9±131.7	101.6±52.7
ALP (IU/l)	602.8±282.2	1080.4±504.4
LDH (IU/l)	246.1±56.5	246.1±65.7
γ-GTP (IU/l)	215.5±140.3	399±277.1
IgA (mg/dl)	272.4±124.6	341.2±140.5
IgG (mg/dl)	1908.3±598.2	1955.4±810.8
IgM (mg/dl)	381.9±258.4	404.4±126.1
AMA (positive/negative)	9/6	3/2
AMA-M2 (positive/negative)	11/4	3/2

F, female; M, male; TP, total protein; Alb, albumin; T-bil, total bilirubin; D-bil, direct bilirubin; AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; γ-GTP, γ-guanosine triphosphate; Ig, immunoglobulin; AMA, antimitochondrial antibody; AMA-M2, AMA M2 subtype.

precursor in the liver (13,14). During PBC treatment, it has been demonstrated that patients with efficient biochemical responses to ursodeoxycholic acid (UDCA) have a low risk of developing liver failure or cirrhosis in the long term (15). In addition, the normalization of alanine transaminase (ALT) with additional bezafibrate treatment reduces the risk of occurrence of liver-associated symptoms in patients with PBC with insufficient responses to UDCA (16). At present, no biomarker has been discovered for the prediction of refractory PBC. Notably, alterations in hepatic miRNAs, including miRNA-122a, miRNA-26a, miRNA-328 and miRNA-299-5p, have been previously associated with PBC (17). However, the association between these alterations and refractory PBC remains to be fully elucidated.

The aim of the current study was to identify the miRNA profiles associated with drug resistance to UDCA, bezafibrate and prednisolone, in addition to the various clinical parameters in patients with PBC.

Materials and methods

Patients. The current study involved 20 patients with PBC treated at Kagawa University Hospital (Miki-Cho, Japan) between 2001 and 2013. The PBC diagnosis was established when two of the following three criteria were met: i) Biochemical evidence of cholestasis, primarily based on elevated alkaline phosphatase levels (ALP), ii) the presence of AMA, and iii) histological evidence of nonsuppurative destructive cholangitis and destruction of the interlobular bile ducts (12).

Clinical presentation. The treatment-effective group was defined by reductions in ALP (less than 600 IU/l) and γ-guanosine triphosphate (γ-GTP) (less than 100 IU/l) within a year of being treated with UDCA at a maximum dose of 900 mg/day. Bezafibrate administration was decided upon within a year subsequent to initiation of the UDCA medication, in accordance with the response to UDCA monotherapy (n=8). The treatment-resistant group included the patients who did not meet a condition of the treatment-effective group. Subsequent to undergoing continuous treatment more than a year, serum samples were collected from those patients. The baseline characteristics of PBC at the time of miRNA sampling are presented in Table I. The present study was approved by the ethics committee of Kagawa University Faculty of Medicine, and informed consent was obtained from all patients.

Analysis of the microRNA array. Total RNA was extracted from the samples derived from the serum samples using a miRNeasy Mini kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. RNA samples typically exhibited $A_{260/280}$ ratios between 1.9 and 2.1, which were measured using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA).

Subsequent to measuring the RNA with an RNA 6000 Nano kit (Agilent Technologies, Inc.), the samples were labeled using a miRCURY Hy3/Hy5 Power Labeling kit (Takara Bio, Inc., Otsu, Japan) and were hybridized onto a human miRNA Oligo chip (version 19.0; Toray Industries, Inc., Tokyo, Japan). Scanning was performed with the 3D-Gene Scanner 3000 (Toray Industries, Inc.), and 3D-Gene Extraction software (version 1.2; Toray Industries, Inc.) was used to read the raw intensity of the image. To determine the alterations in miRNA expression between the treatment-resistant and treatment-effective groups, the raw data were analyzed with

Table II. Statistical results of the miRNAs that were upregulated in the serum of patients with primary biliary cirrhosis.

Upregulated miRNA	P-value	Fold (resistant/effective group)
hsa-let-7b[a]	0.026804	1.567847
hsa-let-7f-1[*]	0.047298	1.215035
hsa-miR-122[a]	0.026881	1.561976
hsa-miR-1233	0.038244	1.208806
hsa-miR-1260	0.041029	1.227792
hsa-miR-136[*]	0.037889	1.249572
hsa-miR-150	0.039072	1.253923
hsa-miR-2053	0.039291	1.182613
hsa-miR-210[a]	0.003102	1.564477
hsa-miR-218-1[*]	0.045877	1.240002
hsa-miR-2467-3p	0.035871	1.246314
hsa-miR-3065-3p	0.003962	1.417027
hsa-miR-3123	0.043136	1.298624
hsa-miR-3173-5p	0.025762	1.276267
hsa-miR-3616-3p	0.028351	1.273818
hsa-miR-378f[a]	0.013278	1.527677
hsa-miR-378g	0.022622	1.330441
hsa-miR-3976	0.014323	1.320455
hsa-miR-409-5p	0.030094	1.220102
hsa-miR-4424	0.041228	1.236549
hsa-miR-4535	0.007167	1.394004
hsa-miR-4655-3p	0.005221	1.25409
hsa-miR-4670-3p	0.007172	1.489381
hsa-miR-4781-3p	0.012059	1.290433
hsa-miR-504	0.026989	1.349747
hsa-miR-509-3p	0.037295	1.333371
hsa-miR-511	0.01903	1.348944
hsa-miR-542-5p	0.03619	1.344654
hsa-miR-548ac	0.035305	1.230733
hsa-miR-548f	0.049632	1.239292
hsa-miR-610	0.026332	1.228792
hsa-miR-612	0.048399	1.251725
hsa-miR-650	0.02361	1.182034
hsa-miR-659[a]	0.001558	1.500267
hsa-miR-802	0.040635	1.305961

[a]Five miRNAs were significantly upregulated greater than 1.5 times in the treatment-resistant group as compared with the treatment-effective group. miRNA/miR, microRNA.

GeneSpring GX software, version 10.0 (Agilent Technologies, Inc.). Samples were first normalized relative to the 28S RNA, and then the baseline was corrected to the median of all samples.

Replicate data were consolidated into two groups: The treatment-resistant and treatment-effective groups. Hierarchical clustering was performed with the farthest neighbor method using the absolute Pearson's correlation coefficient as a metric. The base-2 log-transformed intensities were median-centered for each row (miRNA probe) and were color-coded, as presented on the heat map. The P-value cutoff was set to 0.05. Only alterations greater than 50% in a minimum of one of the time points for each sample were considered to be significant. All analyzed data were scaled by global normalization. The statistical significance of the differentially expressed miRNAs was analyzed with Student's t-test.

Statistical analysis. Statistical analyses were performed using the computer-assisted StatFlex software, version 6.0 (Artec Co., Ltd., Osaka, Japan). A paired analysis between the groups was conducted using Student's t-test and Pearson's correlation coefficient. $P<0.05$ was considered to indicate a statistically significant difference.

Table III. Statistical results of the miRNAs that were downregulated in the serum of the patients with primary biliary cirrhosis.

Downregulated miRNA	P-value	Fold (resistant/effective group)
hsa-miR-125b-2*	0.014229	0.7001
hsa-miR-1301	0.031384	0.77713
hsa-miR-2277-3p	0.02819	0.772271
hsa-miR-3136-3p[a]	0.003916	0.662795
hsa-miR-33b*	0.013788	0.815009
hsa-miR-340*	0.008022	0.78203
hsa-miR-3675-5p	0.015949	0.685806
hsa-miR-3689a-3p[a]	0.015287	0.659541
hsa-miR-3690[a]	0.017489	0.614312
hsa-miR-380*	0.018128	0.755665
hsa-miR-4260	0.046847	0.753042
hsa-miR-4311[a]	0.044072	0.659274
hsa-miR-4423-5p	0.035972	0.773386
hsa-miR-4504	0.030226	0.696011
hsa-miR-4655-5p	0.038655	0.752697
hsa-miR-4714-3p[a]	0.007293	0.655707
hsa-miR-4720-3p	0.027112	0.719039
hsa-miR-520a-5p	0.012847	0.732509
hsa-miR-548z	0.046249	0.746099
hsa-miR-602	0.003259	0.722861
hsa-miR-607	0.043153	0.796838
hsa-miR-770-5p	0.012182	0.719448
hsa-miR-873	0.040941	0.769026

[a]A total of 5 miRNAs were significantly downregulated less than 0.67 times in the treatment-resistant group compared with the treatment-effective group. miRNA/miR, microRNA.

Table IV. Association between representative upregulated miRNAs and clinical parameters in patients with primary biliary cirrhosis.

Parameter	let-7b	miR-122	miR-210	miR-378f	miR-659
TP	0.1038	0.4242	0.095	0.281	0.0973
Alb	0.1084	0.2757	-0.0793	-0.1152	-0.1483
T-Bil	0.0636	0.1186	-0.0581	-0.1196	-0.1074
D-Bil	0.3542	0.4811[a]	0.1879	0.0569	-0.0729
AST	0.3694	0.5539[a]	0.2774	0.4814[a]	0.3036
ALT	0.4883[a]	0.7018[a]	0.3105	0.5642[a]	0.3424
ALP	0.3182	0.3793	0.2231	0.2928	0.0445
LDH	-0.3468	-0.2167	-0.16	-0.1538	-0.3315
γ-GTP	0.2733	0.5706	0.296	0.5359[a]	0.4495[a]
IgG	-0.1361	0.0219	-0.0543	0.2627	0.1534
IgM	0.0957	0.1882	0.0436	-0.0893	-0.0768

[a]P<0.05. miRNA/miR, microRNA; TP, total protein; Alb, albumin; T-bil, total bilirubin; D-bil, direct bilirubin; AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; γ-GTP, γ-guanosine triphosphate; Ig, immunoglobulin.

Results

miRNA expression in the serum of patients with PBC. miRNAs are present in human serum and are highly stable due to the resistance to RNase digestion (18). An miRNA array was performed in the current study using the serum

Table V. Association between representative downregulated miRNAs and clinical parameters in patients with primary biliary cirrhosis.

Parameter	miR-3136-3p	miR-3689a-3p	miR-3690	miR-4311	miR-4714-3p
TP	-0.1468	-0.1996	-0.2365	-0.3657	-0.1489
Alb	0.0189	-0.1204	-0.2857	0.2015	-0.0633
T-Bil	0.3531	0.0764	-0.2528	0.0557	0.4763[a]
D-Bil	0.1435	-0.2297	-0.3576	-0.0678	0.3744
AST	-0.0728	-0.3675	-0.1569	-0.5766[a]	0.1138
ALT	-0.1218	-0.458[a]	-0.2569	-0.559[a]	0.0184
ALP	0.1701	-0.1837	-0.1857	-0.3857	0.2868
LDH	0.3869	0.3149	-0.0115	-0.1767	0.5841[a]
γ-GTP	-0.3012	-0.3801	-0.3301	-0.4519	-0.0204
IgG	-0.2189	-0.0718	0.2479	-0.2368	-0.3262
IgM	-0.0193	0.0584	-0.2388	-0.2487	-0.0262

[a]P<0.05. miRNA/miR, microRNA; TP, total protein; Alb, albumin; T-bil, total bilirubin; D-bil, direct bilirubin; AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; γ-GTP, γ-guanosine triphosphate; Ig, immunoglobulin.

of patients with PBC. The expression patterns of 1,769 miRNAs were examined using extracted serum miRNAs. As presented in Table II, 35 miRNAs were significantly upregulated in the treatment-resistant group compared with the treatment-effective group. However, 23 miRNAs in the treatment-resistant group were significantly downregulated compared with those in the treatment-effective group (Table III). In addition, unsupervised hierarchical clustering analysis, using Pearson's correlation, demonstrated that the treatment-effective group was clustered separately from the treatment-resistant group (Fig. 1). Furthermore, highly altered miRNAs (greater than 1.5-fold) were analyzed, and the two groups were clustered separately using highly altered miRNAs (Fig. 2 and Tables I and II).

miRNA expression and clinical parameters. To examine the association between highly altered miRNAs and clinical features, various parameters were analyzed. As presented in Table IV, elevated levels of direct bilirubin (D-bil), aspartate transaminase (AST), and ALT were associated with miRNA-122 upregulation (Table IV). AST, ALT, and γ-GTP were also related to miRNA-378f upregulation (Table IV). However, the reduction of miRNA-4311 was related to the decreases of AST and ALT (Table V). miRNA-4714-3p was also negatively correlated to total bilirubin and lactate dehydrogenase (Table V). These results suggest that these miRNAs may be new biomarkers for the development of PBC.

Discussion

It has been demonstrated that patients with PBC with efficient biochemical responses to UDCA or bezafibrate have low risks of developing liver failure or cirrhosis in the long term (15,16). However, one third of patients with PBC exhibited suboptimal responses to these treatment strategies (19), and these patients demonstrated poorer outcomes (20-22). At present, no useful biomarker has been reported for the development of PBC. In the present study, miRNA expression patterns in the treatment-resistant group markedly differed compared with those in the treatment-effective group. The expression levels of highly altered miRNAs in the resistant group were also associated with the clinical parameters of PBC. Notably, miRNA-122 was significantly upregulated and was associated with D-bil, AST, and ALT in Tables II and IV. Roderburg *et al* (23) demonstrated that elevated miRNA-122 serum levels are a potent and independent marker of liver injury and are not seen in patients with liver cirrhosis without ongoing liver damage. In addition, it has also been reported that miRNA-122 is upregulated in acute and chronic liver injury (24), myocardial infarction (25) and cancer (26). These results support those indicating that miRNA-122 was enhanced in the serum of the treatment-resistant group, due to the fact that clinical parameters, including AST and ALT, were raised sufficiently to indicate ongoing inflammation in the liver.

In addition, several miRNAs were identified to be associated with cell cycle arrest, including let-7 and miR-210 among the upregulated miRNAs in the treatment-resistant group. It has been previously demonstrated that let-7 and miR-210 targeted cell cycle regulatory molecules (27,28). Han *et al* (29) reported that miR-194 suppresses cell proliferation through the inhibition of insulin-like growth factor receptor 1. These reports suggest that various miRNAs, including let-7, miR-210 and miR-194, may inhibit hepatocyte regeneration by regulating cell cycle and cell proliferation in the treatment control group.

Downregulated miRNAs, including miR-125b, miR-380 and miR-602, were detected in the treatment-resistant group. Notably, it has been previously reported that miR-125b and miR-380 are negative regulators of p53 (30,31) and miR-602 regulates the tumor-suppressive gene Ras-association domain family 1, isoform A (32). This suggests that downregulation of miR-125b, miR-380 and miR-602 may suppress cell proliferation and cell cycle acceleration, and induce apoptosis in the hepatocytes of treatment-resistant patients.

It has been previously reported that miRNAs are able to regulate the efficacy of drugs interacting with the miRNA

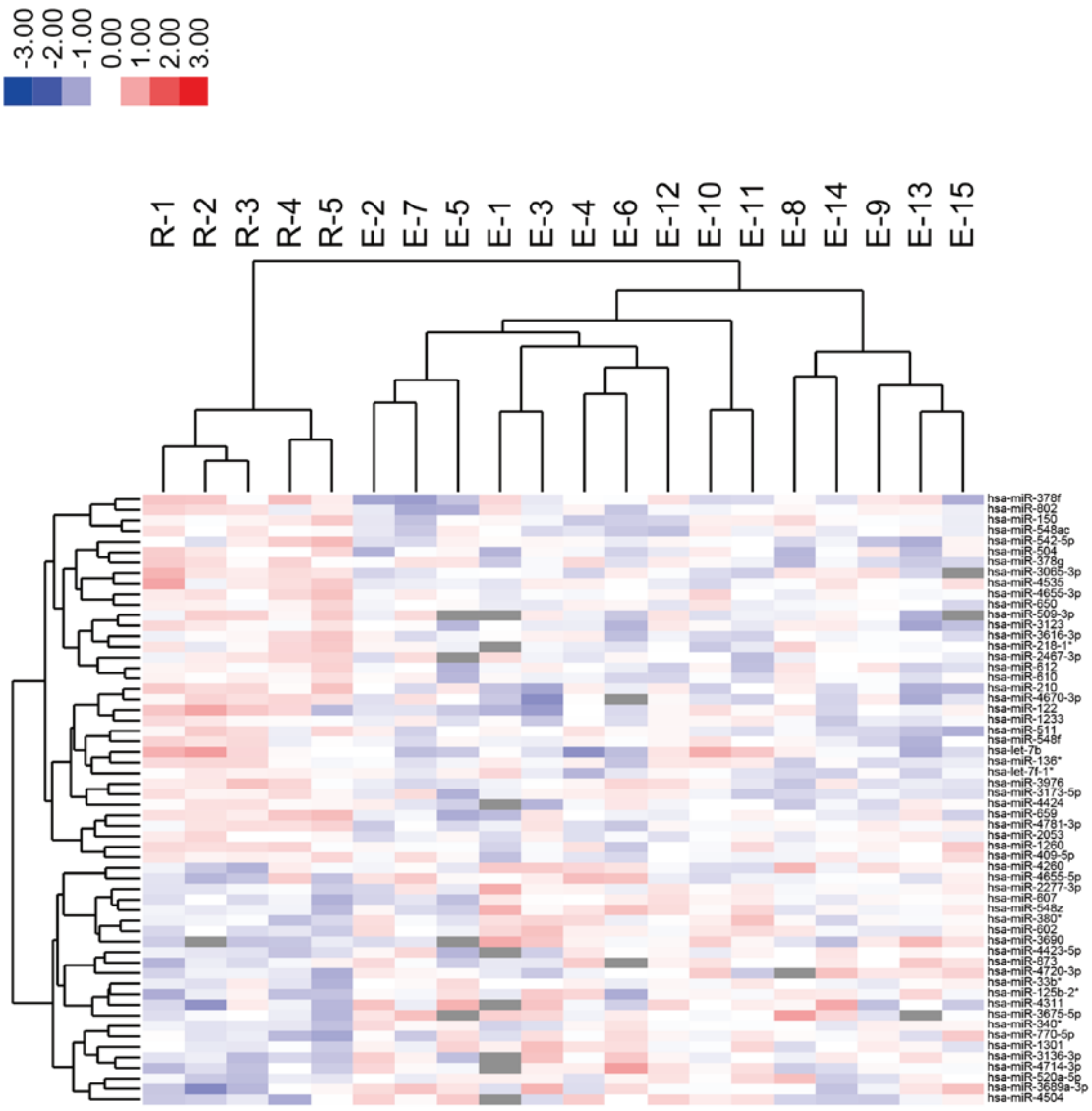

Figure 1. Hierarchical clustering of the treatment-effective and treatment-resistant groups. The patients with primary biliary cirrhosis were clustered according to their expression profiles of the 35 upregulated miRNAs and 23 downregulated miRNAs in the treatment-resistant group compared with the treatment-effective group. The miRNA clustering tree is presented on the left, with the sample-clustering tree at the top. The color scale presented illustrates the relative expression level of the miRNAs; red, high expression levels; blue, low expression levels. miRNA/miR, microRNA.

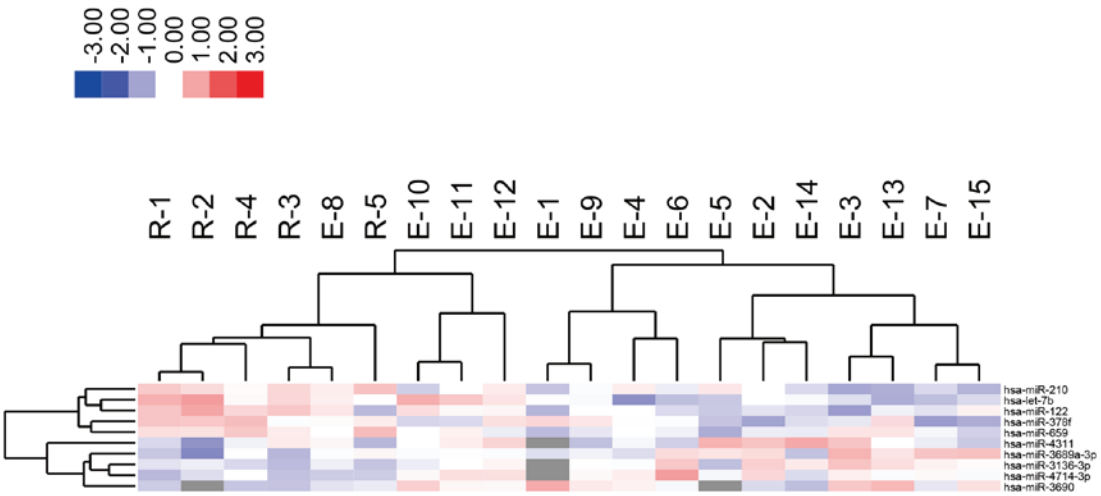

Figure 2. miRNAs that were markedly altered (greater than 1.5-fold) were analyzed and clustered between the treatment-effective and treatment-resistant groups. The miRNA clustering tree is presented on the left, with the sample-clustering tree at the top. The color scale presented illustrates the relative expression level of the miRNAs; red, high expression levels; blue, low expression levels. miRNA/miR, microRNA.

target and protein (33). Several miRNAs, including miR-125b, interact with the cytochrome P450, family 1, member A1 gene and induce drug resistance (33). In the current study, miR-125b was downregulated in the treatment-resistant group. The results of the current study are in agreement with a previous study that indicated that miRNAs are associated with drug resistance to UDCA or bezafibrate in patients with PBC (33).

In conclusion, identification of miRNA profiles is useful in characterizing the development of PBC, and representative miRNAs, including miR-125b, let-7b and miR-520a-5p are suggested to be potential biomarkers for refractory PBC.

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