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abbreviations

miRNAs, microRNAs; ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase; T-Bil, total bilirubin; HB, hepatitis B; HC, hepatitis C; PBC, primary biliary cirrhosis; AIH, autoimmune hepatitis; NASH, non-alcoholic steatohepatitis, DILI, drug-induced liver injury; Ctrl, control subjects; PCR, polymerase chain reaction; Ct, cycle threshold; PCA, principal component analysis; ANOVA, analysis of variance; PC, principal component

Abstract

Purpose: Some blood biomarkers or histological examination by liver biopsy are used for the diagnosis of liver diseases in clinics. However, conventional blood biomarkers show poor specificity and sensitivity, and liver biopsy is highly invasiveness. Therefore, to overcome such disadvantages, specific/sensitive and noninvasive options are desirable. In recent years, circulating microRNAs (miRNAs) have been acknowledged for their potential as disease markers. Actually, several miRNAs have been reported to be biomarker candidates of liver diseases. However, these earlier studies were performed for one disease. Therefore, the specificity as biomarkers was not guaranteed, because they didn't study for the other types of liver injury. In this study, we examined if circulating miRNA could distinguish different types of liver diseases.

Methods: Serum miRNA profiles in 28 patients with chronic hepatitis B, chronic hepatitis C, primary biliary cirrhosis, autoimmune hepatitis, nonalcoholic steatohepatitis or drug-induced liver injury as well as 4 control subjects were determined by TaqMan MicroRNA array analysis. Principal component analysis (PCA) of selected miRNAs was performed.

Results: We identified 37 miRNAs whose levels were significantly different between any of the groups. Although individual miRNAs could not distinguish different types of liver diseases, probably because of similar liver pathology, their profiling by PCA could classify different liver disease groups.

Conclusions: The profiling of the selected miRNAs can be useful to distinguish different types of liver diseases.

1. Introduction

Liver diseases are caused by various factors, including viral infection, excessive alcohol intake, drugs, metabolic abnormalities, overnutrition or malnutrition, or other health problems. To select the best therapy, an appropriate diagnosis of the types of liver disease is required. At present, alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), or total bilirubin (T-Bil) levels in blood or histological examination by liver biopsy are used for the diagnosis of liver diseases [1]. However, these blood markers cannot distinguish different types of liver diseases, and may be increased by extrahepatic injury, including muscle damage or cardiac injury [2, 3]. Furthermore, ALT levels are not always correlated with histomorphological patterns of the liver [4]. The disadvantages of liver biopsy include invasiveness and pain, as well as potential side effects, which include tenderness, internal bleeding, pneumothorax, and, rarely, death. Under such situations, additional non-invasive biomarkers with improved specificity and sensitivity have been warranted.

MicroRNAs (miRNAs) are a class of endogenous, short non-coding RNAs that regulate the expression of target genes by binding to complementary regions of transcripts to repress their translation or cause mRNA degradation [5]. Gene regulations by miRNA are involved in various biological processes, including cell differentiation, proliferation, apoptosis, and development [6]. Dysregulation of miRNAs is relevant to onset or progression of diseases [6, 7]. More than 2,500 human miRNAs have been identified (miRBase version 21). Some miRNAs are expressed in a tissue-specific manner, and others are ubiquitously expressed [8]. The expression of intracellular miRNAs in tissues is known to be altered in various diseases (http://www.miR2disease.org/), and the dysregulation of miRNAs is closely linked with the incidence or progress of diseases.

In 2008, miRNAs were discovered to be stably present in plasma or serum [9].

Subsequently, the presence of miRNAs in saliva [10] and urine [11] was reported. Such extracellular miRNAs are stable, even in RNase-rich environments, because they are enveloped in vesicles such as exosomes, microvesicles, apoptotic bodies, and ectosomes [12], or are associated with RNA-binding proteins, including nucleophosmin 1 [13], or high-density lipoproteins [14]. Following the discovery of extracellular miRNA in body fluids, a significant number of papers have reported that the levels of some extracellular miRNAs are changed with different pathophysiological conditions. Examples include miR-141 and miR-375 in prostate cancer [15], miR-29a and miR-92a in colorectal cancer [16], miR-499 in myocardial infarction [17]. The most well-known example is liver-specific miR-122, which is a sensitive biomarker of various kinds of liver injuries [18-20]. In addition, several individual miRNAs have been raised as markers of various types of liver injuries: miR-25, miR-375, and let-7f in hepatitis B (HB) [21], miR-21 in hepatitis C (HC) [22], and miR-29 in hepatic fibrosis [23] and hepatocellular carcinoma [24]. However, these earlier studies were performed for one disease. Therefore, the specificity as biomarkers was not guaranteed, because they didn't study for the other types of liver injury. Previously, we compared plasma miRNA profiles in rat models with different types of liver injury (acute hepatocellular injury, cholestasis, steatosis, steatohepatitis, and fibrosis), and identified that miRNAs that can be specific and sensitive biomarkers of each type of liver injury [25].

In this study, expanding our previous animal study to humans, we sought to examine whether the circulating miRNAs in patients can be biomarkers to distinguish different types of liver injury. Profiles of serum miRNAs in patients with HB, HC, primary biliary cirrhosis (PBC), autoimmune hepatitis (AIH), nonalcoholic steatohepatitis (NASH), or drug-induced liver injury (DILI), and control subjects with no liver injury were also analyzed to determine biomarkers.

2. Materials and Methods

2.1. Chemicals and Reagents.

*mir*Vana PARIS Kit, Megaplex Primer Pools, TaqMan MicroRNA Reverse Transcription Kit, TaqMan Universal PCR Master Mix (No AmpErase UNG) and TaqMan Human MicroRNA Array A and B Card v2.0 were from Life Technologies (Carlsbad, CA). All other chemicals and solvents were of the highest grade commercially available.

2.2 Human serum samples.

Sera were collected from a total of 28 patients with HB (n = 6), HC (n = 4), PBC (n = 3), AIH (n = 3), NASH (n = 5), and DILI (n = 7) as well as age-matched 4 control subjects without liver injury. Age, sex, and serum ALT and ALP levels are shown in Table 1. Sera were stored at -80°C until use. This study was approved by the Ethics Committees of Kanazawa University (No. 211) and Toyama University (No. G21-1). Written informed consent was obtained from all subjects.

2.3 Total RNA isolation from serum and TaqMan MicroRNA Array Analysis.

Total RNA was isolated from 600 μl of serum using a mirVana PARIS Kit as described previously [25]. The profiles of miRNAs were assessed using TaqMan Human MicroRNA Array A+B Cards Sets v2.0 containing 377 (A array) or 287 (B array) primer-probe sets for individual miRNAs (total 664 miRNAs). All procedures were performed following the manufacturer's instruction. All primers and probes were designed by Life Technologies. Briefly, total RNA was reverse-transcribed to cDNA using Megaplex RT Primer Pool A or B and TaqMan MicroRNA Reverse Transcription Kits. Pre-amplification was carried out using Megaplex PreAmp Primers and TaqMan Preamp Master Mix. The profiles of miRNAs were determined by quantitative real-time polymerase chain reaction (PCR) using TaqMan Human

MicroRNA Arrays with 7900HT Fast Real-Time PCR System (Life Technologies) and the manufacturer's recommended cycling conditions. The PCR was run for 40 cycles. Cycle threshold (Ct) values were calculated using SDS software v.2.3, with a baseline of 3 to 15 and an assigned minimum threshold of 0.2. Data were expressed as 40-Ct, which were frequently used for the analysis of miRNA/RNA expression [26, 27] to show that the higher value means the higher expression level. We confirmed that the levels of cel-miR-39-3p which was spiked as an external control were almost equal between samples. The RT-qPCR protocol, and data analysis were followed by the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [28].

2.4 Principal component analysis.

The miRNAs presenting 40-Ct > 8 in at least one sample were subjected to data analysis, because the manufacturer has reported that the Ct values >32 might be non-specific signal with low reproducibility. To ignore the data of miRNAs whose levels are low, the levels of miRNAs with 40-Ct < 8 were regarded as 0. Among detected miRNAs, 37 miRNAs whose levels were significantly different between any groups by one-way analysis of variance (ANOVA) ($P < 0.05$) were subjected to principal component analysis (PCA) using Partek Genomics Suite version 6.12 (Partek, St. Louis, MO).

3 Result

3.1 Serum miRNA profiles in HB, HC, PBC, AIH, NASH, and DILI patients.

We determined serum miRNA levels in 28 patients with HB, HC, PBC, AIH, NASH, or DILI as well as 4 control subjects using the TaqMan MicroRNA Array. The numbers of miRNAs that were detected or over the cut-off value (40-Ct > 8) in each subject are shown in Table 1. The number of miRNAs whose levels were over the cut-off value in at least one sample was

210. Among them, 37 miRNAs were uncovered as miRNAs whose expression was significantly ($P < 0.05$) different between any of the groups by one-way ANOVA (Table 2 and supplemental Fig. 1). The explanation of the differences in the levels of each miRNA is as follows:

The miR-218, miR-363, miR-518f, miR-628-5p, miR-888, miR-523, miR-141, miR-302b, and miR-643 were substantially detected in sera from control subjects, but they were hardly detected at all in patients with any type of liver injury. The miR-29a levels in patients with all types of liver injury were significantly lower than those in control subjects. Additionally, a significant difference in the miR-29a levels between the DILI and AIH groups was observed. In comparison with control subjects, the miR-573 levels in patients with DILI were significantly lowered, and those in patients with PBC or AIH were undetectable.

The following miRNAs were the ones of whose levels in patients with some liver injuries were higher than those in control subjects. Although the miR-378 and let-7b were not detected in control subjects, they were highly detected in almost all patients with liver injury. Statistical significance was particularly observed between the DILI and control groups. The levels of miR-122 and miR-192 were significantly higher in almost all patients with liver injury than in control subjects, and statistical differences were observed in some liver injury groups.

Interestingly, the levels of miR-574-3p in the DILI group were significantly higher than those in the control group, whereas levels in the HC, PBC, and AIH groups were not increased at all. The levels of miR-193a-5p and miR-148a in the DILI group were also significantly higher than those in the control group, whereas the levels in the HC and AIH groups were not increased at all. The miR-520d-5p levels were only significantly increased in the DILI group. The miR-16, miR-222, and miR-320 levels were significantly higher in DILI patients than those in the control and HC groups.

The levels of miR-345, miR-483-5p, and miR-193b were significantly higher in the DILI, HB, and NASH groups than those in the control group, and significant differences were observed with the other liver injury groups (miR-345: DILI versus HC or AIH, and HB versus AIH; miR-483-5p: DILI versus AIH; miR-193b: AIH versus DILI or NASH).

The levels of miR-21, miR-20a, miR-92a, and miR-375 were significantly higher in the DILI group than those in the HC group. The miR-423-5p level was increased in some patients with DILI and PBC. The levels of miR-374a in the HC group were significantly lower than those in the DILI and HB groups. The levels of miR-19a, miR-19b, and miR-26b were significantly lower in the HC group than those in the DILI, HB, and NASH groups. The levels of miR-142-3p, miR-25, and miR-451 were significantly lower in the HC group than those in the DILI, HB, PBC, and NASH groups. Taken together, statistical differences were observed in the levels of these 37 miRNAs between any of groups, but since the expression profiles were similar between liver injury groups, it was suggested that individual miRNAs could not distinguish the different types of liver diseases. Accordingly, we sought to perform PCA to determine whether the expression profiles of 37 miRNAs can be used to distinguish different types of liver injury.

3.2 Principal component analysis.

PCA was performed using the levels of the selected 37 miRNAs. Firstly, the greatest variance of the data set, called the first principal component (PC1), was calculated. Sequentially, the second greatest variance, called the second principal component (PC2), followed by PC3, PC4 were calculated. In this study, PC1 encompassed 43.9% proportion of the total variance, followed by PC2 (22.6%), PC3 (6.99%), and PC4 (5.43%). As shown in Fig. 2A, a 3-D scatter plot of PC1, PC2, and PC3 (total ~95% of the variation), clearly demonstrates that the profiles of 37 miRNAs were different between patient groups and the control group. However,

patients in HB, PBC, NASH and DILI groups were nearly plotted. Fig. 2B, which shows a 3-D scatter plot of PC2, PC3, and PC4 (total ~56% of the variation), demonstrated that the profiles of 37 miRNAs can distinguish different types of liver injury, whereas plots of HB and NASH groups were close. These results suggest that the profiling of 37 miRNAs in sera can be utilized to diagnose liver diseases.

4 Discussion

Circulating miRNAs are currently receiving considerable attention as potential biomarkers of various diseases [29]. Studies reporting specific miRNAs as potential biomarkers have continuously been accumulated. However, the limitation of such studies is that miRNA levels in patients with the studied liver injury were compared with those in healthy controls, even though there are many different types of liver diseases. Therefore, it is uncertain whether the proposed miRNA candidates are specific markers that can diagnose different types of liver disease. To overcome the issue, we evaluated serum miRNA profile in patients with a series of liver injuries, including HB, HC, PBC, AIH, NASH, and DILI as well as control subjects.

Since a previous study [30] reported that miRNA profiles changed due to aging, we set age-matched control subjects with no liver injury. In addition, some reports have revealed that there is a sex difference in the levels of some miRNAs, such as miR-130b, miR-18b in serum [27], and miR-548-3p, miR-1323, miR-940, and miR-1292 in plasma [31]. In our study, the sample numbers in each group were not enough to examine whether there are sex differences in the expression of miRNAs. Since the comparison of miRNA expression in male and female subjects over groups (liver injury groups and control group) would be inappropriate, we sought to find miRNAs whose expression was different between groups irrelevant to sex.

It is well known that circulating miR-122 can be a biomarker of a wide variety of liver injuries, including HB [32], HC [33], NASH [32], alcoholic liver disease [20], DILI [19, 20], and hepatocarcinoma [32, 33]. In our study, the miR-122 levels in patients with DILI, HB, PBC, and NASH were higher than those in control subjects. Additionally, the miR-122 levels in patients with HC (40-Ct values = 8.8 ± 6.0) and AIH (40-Ct values = 7.9 ± 6.9) also tended to be higher than those in control subjects (40-Ct values = 4.2 ± 4.8). These data support previous reports. The increase in the miR-122 level is convincing because this is the most abundant and liver-specific miRNA, and it is released from the damaged hepatocytes into the bloodstream. Although the miR-122 is a good marker of a variety of liver diseases, the limitation is that it cannot distinguish different types of liver diseases.

The levels of miR-218, miR-363, miR-518f, miR-628-5p, miR-888, miR-523, miR-141, miR-302b, miR-643, and miR-29a were substantially detected in sera from control subjects, but their levels were significantly lowered in patients with all types of liver injury. Surprisingly, miR-218 has been reported to be elevated in plasma or serum from patients with acetaminophen-induced acute liver injury [19]. In that paper, the authors argued that this elevation might be due to two reasons: miR-218 is highly expressed in the brain, and acetaminophen-induced acute liver injury is associated with encephalopathy. This was evidenced in their study by the finding that the miR-218 levels were significantly higher in patients whose encephalopathy score was grade 4 compared to patients with a score of grade 0. Therefore, miR-218 is unlikely to act as a biomarker of liver injury. Rather, the decrease in a series of miRNAs could be useful in detecting any types of liver injury.

Circulating miR-29a has been reported to be decreased in patients with hepatic fibrosis in chronic liver disease, such as alcoholic cirrhosis [23]. We found that the miR-29a levels were lowered in patients with all types of liver injury (40-Ct values were approximately 10 or below). Although HB, HC, PBC, AIH and NASH are usually associated with fibrosis, DILI is

not, because DILI is usually found in the acute phase. Therefore, miR-29a might be a marker of general liver diseases, rather than liver fibrosis. Overall, 10 miRNAs mentioned in the previous paragraph, whose levels were dramatically lowered in patients with all types of liver injury, may act as general markers of liver diseases.

Although miR-192 has been reported to be elevated in DILI [19], our study revealed that the levels were elevated in patients with DILI and HB. Shwetha et al. have reported that the miR-483-5p level was significantly increased by HCV infection [34]. Our data showed that the miR-483-5p levels tended to increase in HC patients, whereas they were significantly increased in patients with DILI, HB, and NASH. Shrivastava et al. have reported that the increased levels of circulating miR-20a and miR-92a have potential as sensitive biomarkers for early detection of HCV infection [35]. However, in our data, the levels of miR-20a and miR-92a in patients with HC were similar to those in control subjects, rather than showing an increase in patients with DILI. Thus, miR-20a and miR-92a are unlikely to act as specific markers for HC infection.

The expression of intracellular miRNAs in tissues is altered in various diseases (http://www.miR2disease.org/), and the dysregulation of miRNAs is closely linked with the incidence or progress of diseases. For 37 miRNAs selected in this study, some reports are available on their relevance with liver diseases. For example, it has been reported that miR-29 down-regulates collagen which facilitates liver fibrosis [23], miR-21 down-regulates programmed cell death 4 and promotes anti-inflammatory responses [36], and miR-16 down-regulates Bcl2, anti-apoptotic gene and facilitates TNF-mediated apoptosis [36]. However, the functional relevance of major part of 37 miRNAs remains to be clarified. The present study is the first step to find biomarker miRNAs that can distinguish different types of liver injury. Next important challenge that needs to be addressed is to clarify their functional relevance with liver diseases.

As mentioned, single or limited numbers of miRNA(s) are unlikely to distinguish different types of liver diseases, probably because pathology of liver would be close beyond types of liver injury. Therefore, we sought to determine whether the profiles of a set of selected miRNAs, whose levels were different between any groups of liver injury or control, can be used for diagnosis of different types of liver injury by PCA. As shown in Fig. 2, the profiles of 37 miRNAs could clearly separate patients with liver injury from control subjects, and further, could distinguish different types of liver injury. Our finding has strength beyond that of a previous study [37] reporting that the profiles of 9 miRNAs could distinguish patients with HC from the other groups, because of the broad coverage of multiple types of liver injury.

In conclusion, the present study demonstrated that the profiling of 37 miRNAs could be used to distinguish different types of liver disease. This finding was accomplished by simultaneous comparison of miRNA profiles in multiple groups of different types of liver injury. Such comparative analysis for miRNA profiles should be applied to other diseases in the future, especially cancer, because the reported miRNA biomarkers, found by conventional studies for each type of cancer, overlapped between some kinds of cancers and cannot be used for diagnosis. It is important to perform comparative studies for miRNA profiles across various types of diseases by using the same experimental platform in order to identify specific biomarkers for concerned disease.

Conclusion

We identified 37 miRNAs whose levels in sera were significantly different between patients with various types of liver diseases and control subjects. PCA clearly demonstrated that the profile of 37 miRNAs can be useful to distinguish different types of liver diseases. Because of the relatively small number of patients in each group, our finding should be

confirmed in a larger sample of subjects.

Declaration of conflicting interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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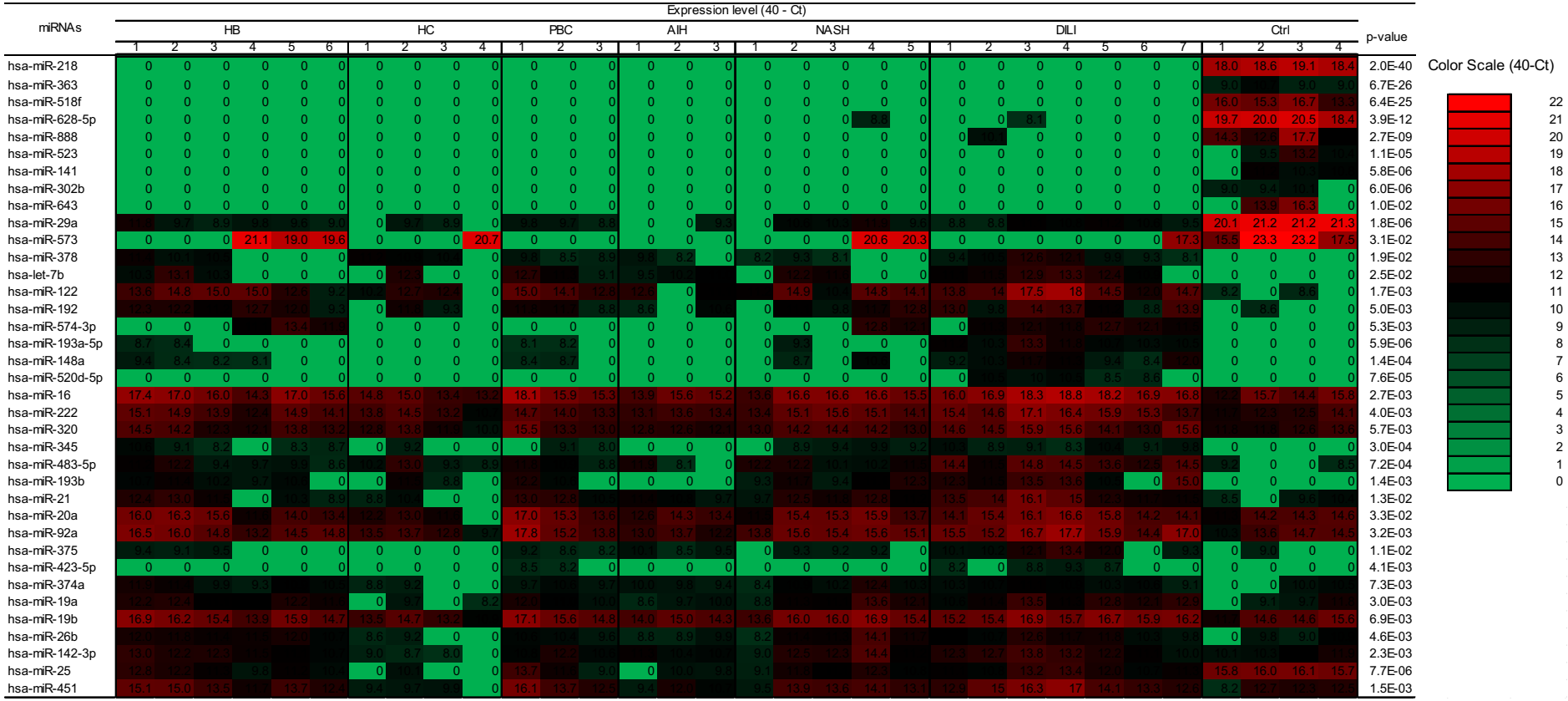


Figure 1. Levels of 37 miRNAs whose expression in serum was significantly different between the groups. The values are 40-Ct. The heat map was generated with Excel 2016 (Microsoft). The color scale of 40-Ct is shown at the right of the figure. Red and green color represents high and low expression of miRNA, respectively.

Fig. 2

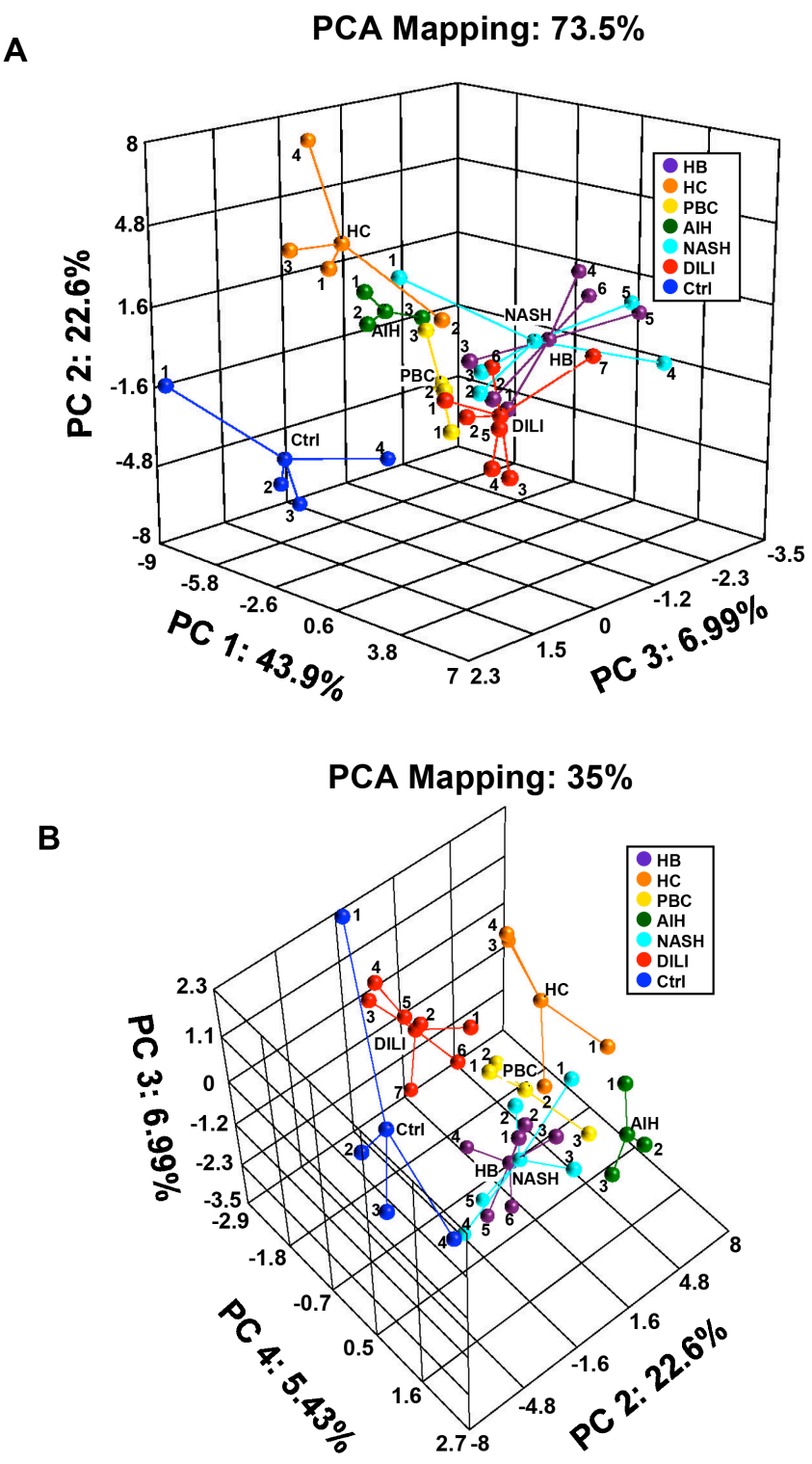


Figure 2. PCA of serum miRNA expression in 6 hepatitis B (HB), 4 hepatitis C (HC), 3 primary biliary cirrhosis (PBC), 3 autoimmune hepatitis (AIH), 5 non-alcoholic steatohepatitis (NASH), 7 drug-induced liver injury (DILI), and 4 control subjects (Ctrl). PCA was performed using 37 miRNAs that exceeded the cut-off value in all subjects (n = 32). (A)

A three-component model was developed that explained a total of 73.49% (PC1, 43.9%; PC2, 22.6%; PC3, 6.99%) of the variability of the data. (B) A three-component model was developed that explained a total of 35.02% (PC2, 22.6%; PC3, 6.99%; PC4, 5.43%) of the variability of the data. Each ball representing an individual is connected to the centroid of each group. The numbers near the balls represent the subject number. This plot illustrates the level of spread between individuals and groups using three principal components.

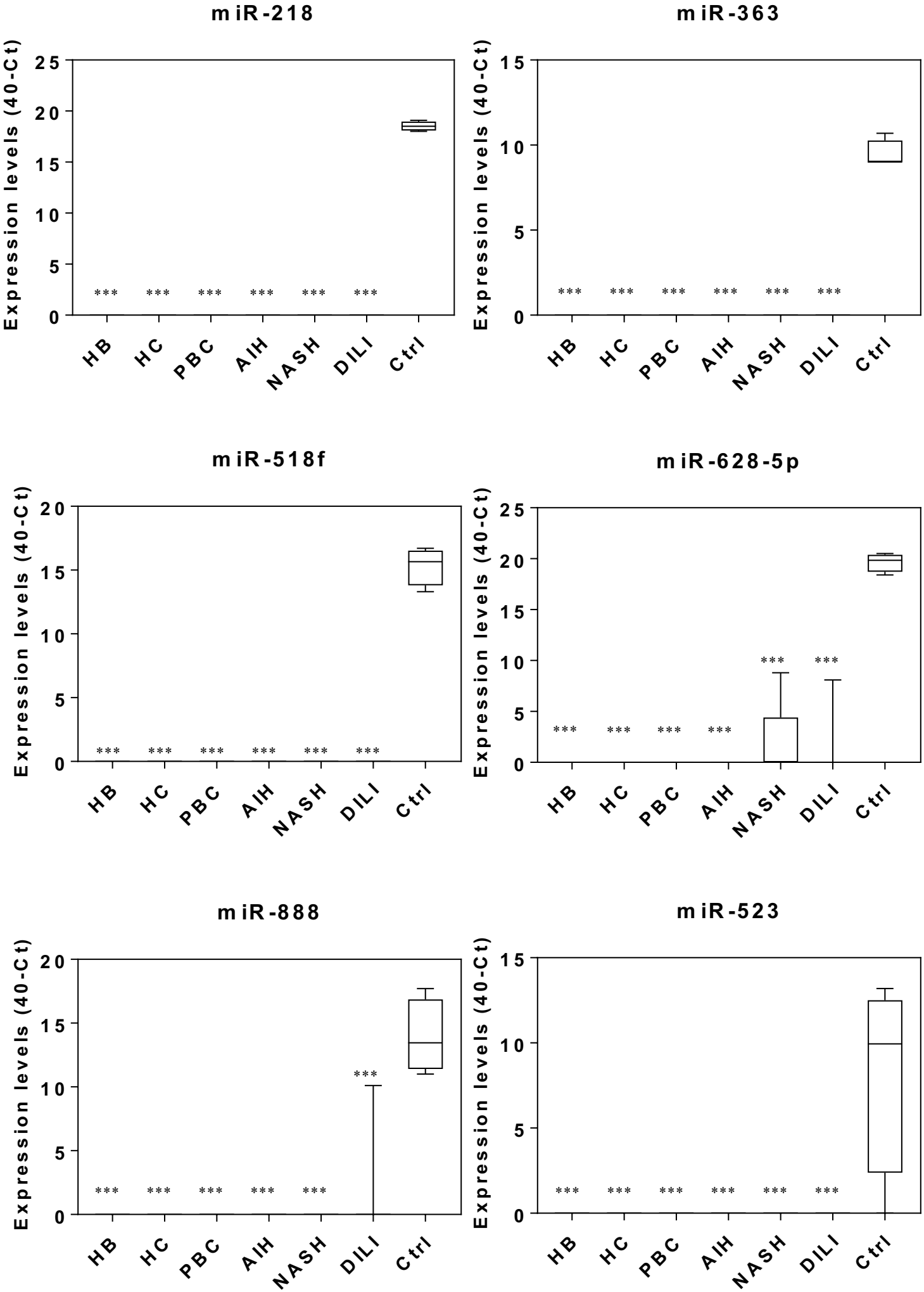
Table 1. Clinical data of patients with liver injury or control subjects and the number of miRNAs detected in serum or whose levels were over the cut-off value.

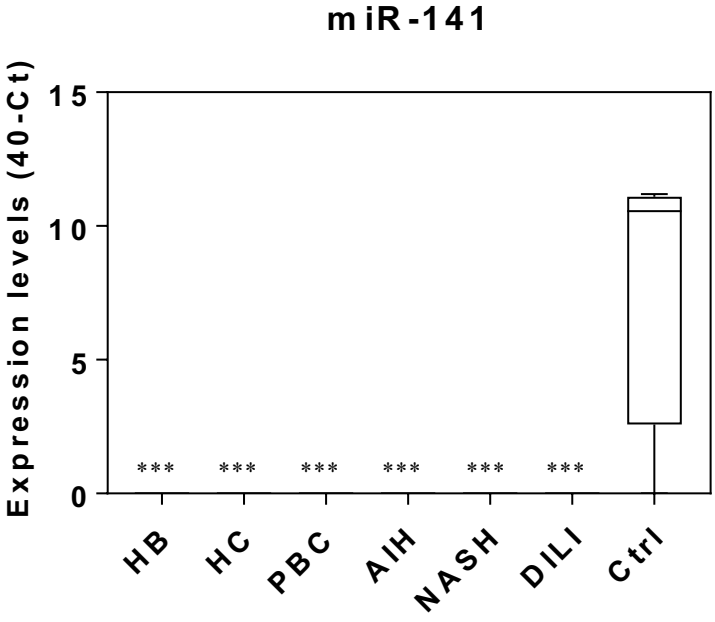
Control or diseases	No.	Age	Gender	ALT	ALP	Number of miRNA	
						Detectable	(40-Ct)>8
HB	1	60	M	43	317	257	127
	2	57	F	181	259	229	111
	3	59	M	61	222	206	91
	4	34	F	26	27	133	63
	5	54	M	27	192	212	97
	6	59	F	18	239	175	85
HC	1	52	M	47	251	185	76
	2	66	F	146	233	181	89
	3	50	F	52	230	172	67
	4	61	M	27	31	105	37
PBC	1	40	F	114	481	177	78
	2	54	F	66	837	162	71
	3	54	F	33	364	165	74
AIH	1	46	F	323	382	209	105
	2	67	F	22	260	206	100
	3	54	F	83	286	193	86
NASH	1	47	M	183	455	148	60
	2	52	M	110	129	204	98
	3	53	M	33	251	205	101
	4	41	F	218	138	236	130
	5	28	F	123	214	235	121
DILI	1	53	M	175	1473	223	95
	2	43	M	212	736	257	111
	3	69	M	1141	528	279	135
	4	70	M	843	661	241	127
	5	45	F	236	359	217	112
	6	50	M	684	487	208	87
	7	65	F	722	222	146	87

Ctrl	1	68	F	22	231	143	55
	2	68	F	18	348	146	65
	3	58	M	11	182	181	91
	4	52	M	15	171	199	101

Supplement Figure 1. Comparison of expression of serum miRNAs in patients with various types of liver injury, HB (n = 6), HC (n = 4), PBC (n = 3), AIH (n = 3), NASH (n = 5), and DILI (n = 7), as well as age-matched control subjects (n=4). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with control subjects. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared with other patient.

Supplement Figure 1

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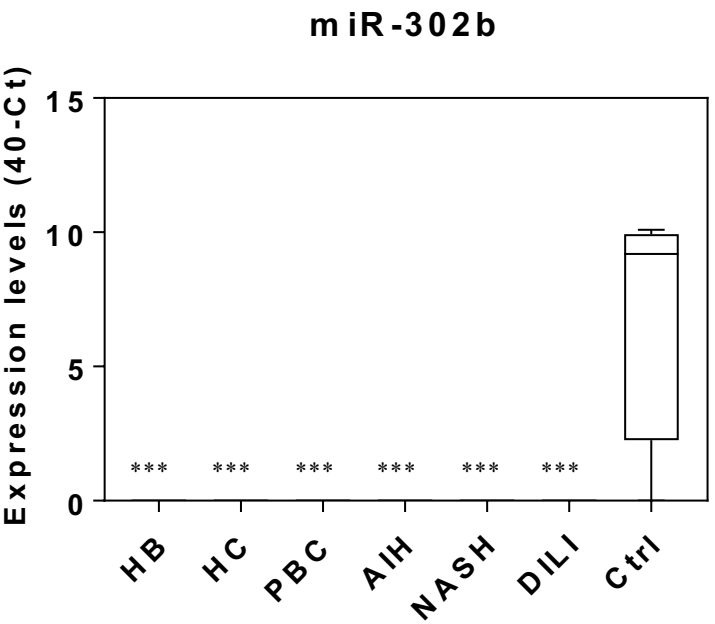
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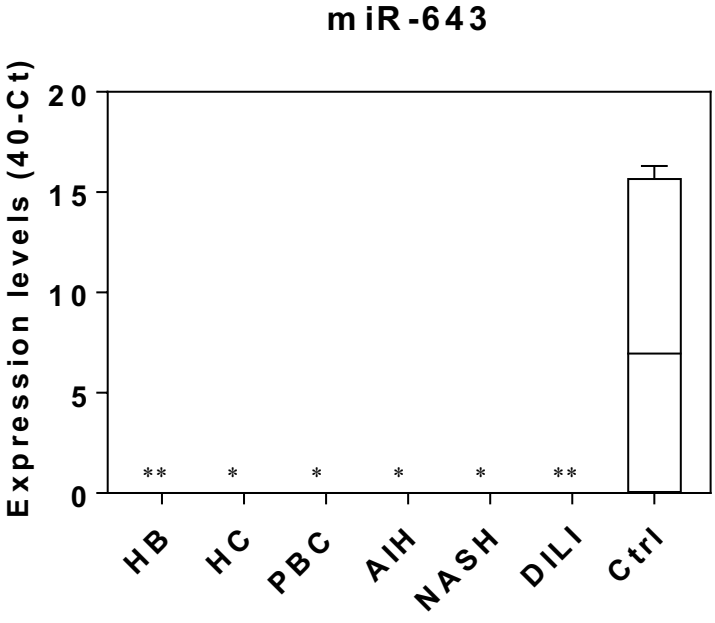
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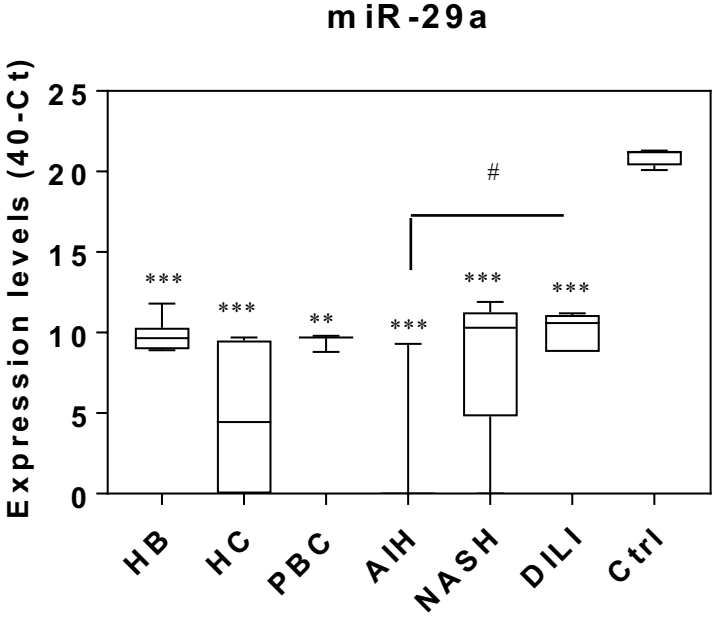
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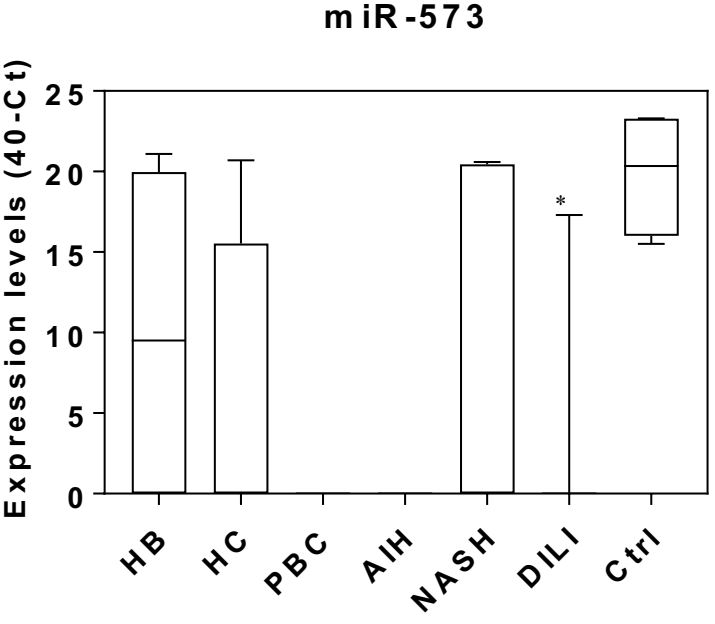
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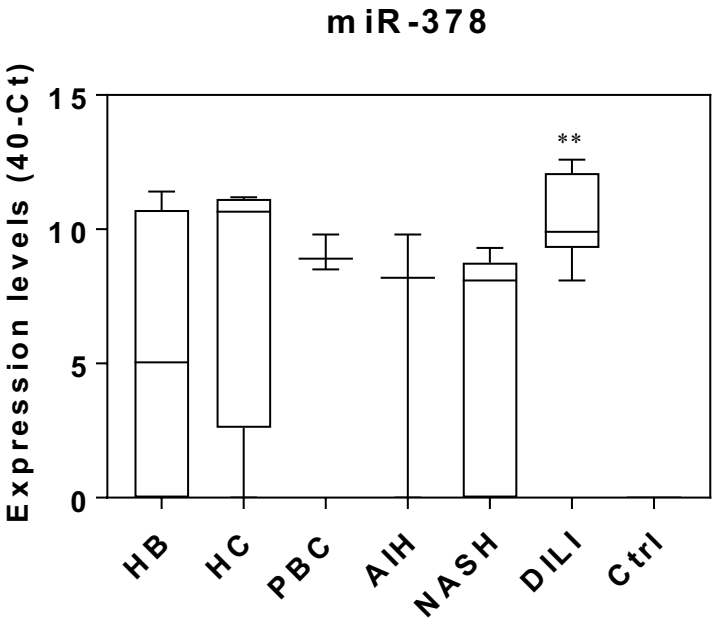
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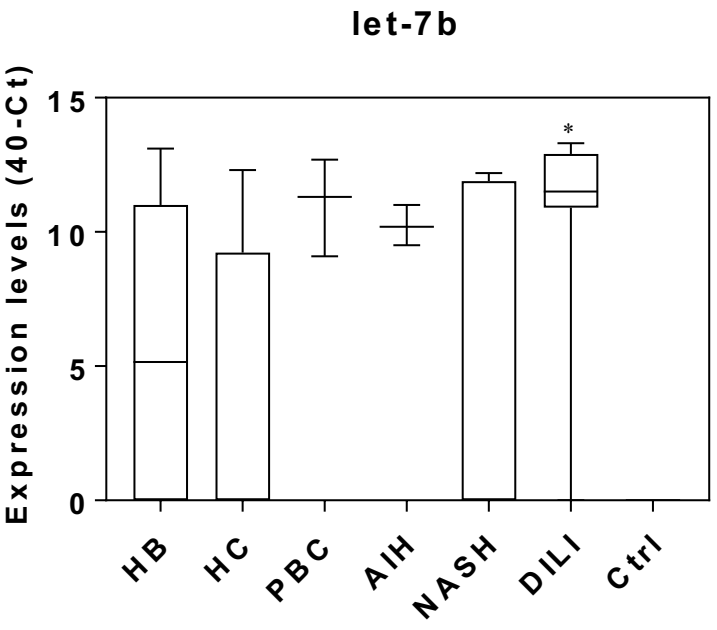
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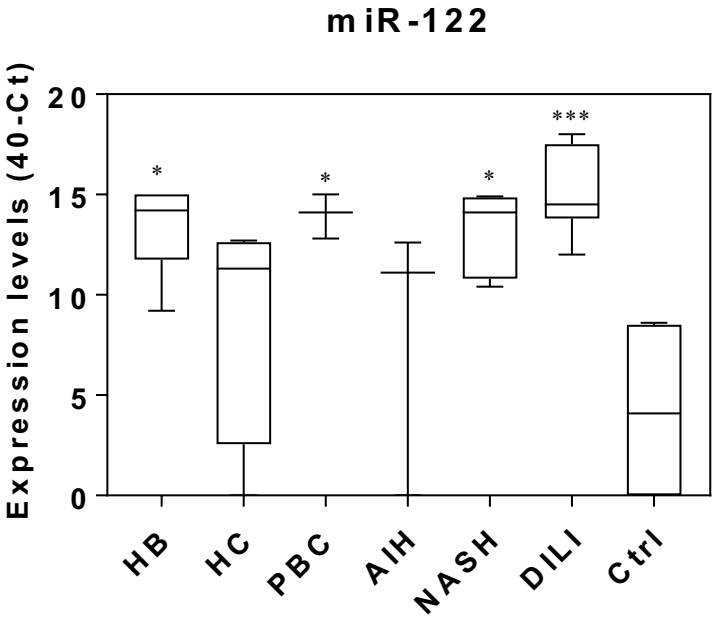
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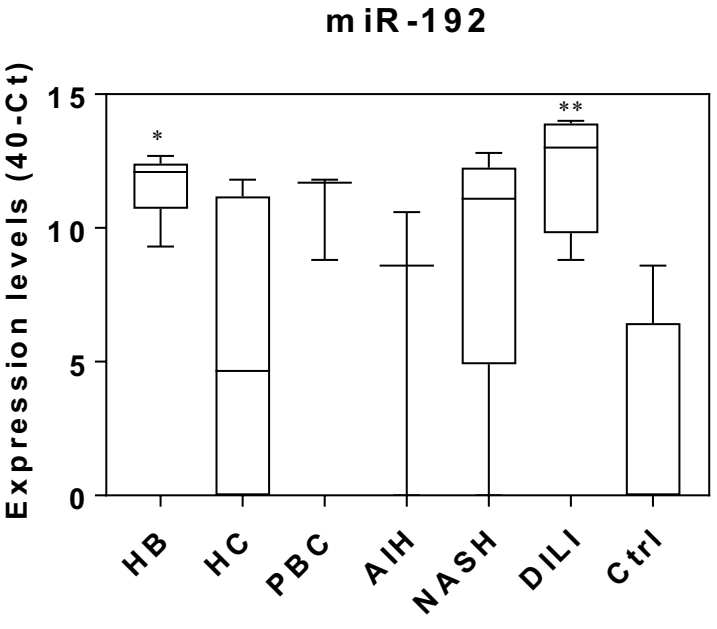
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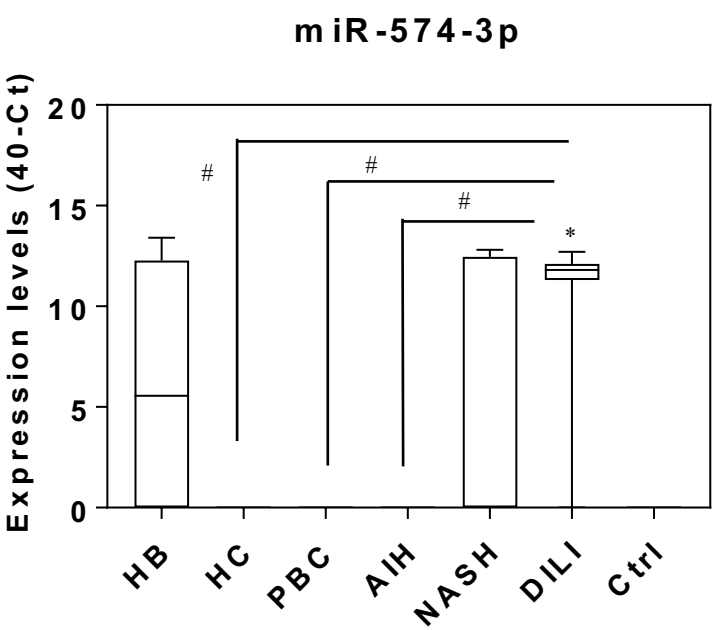
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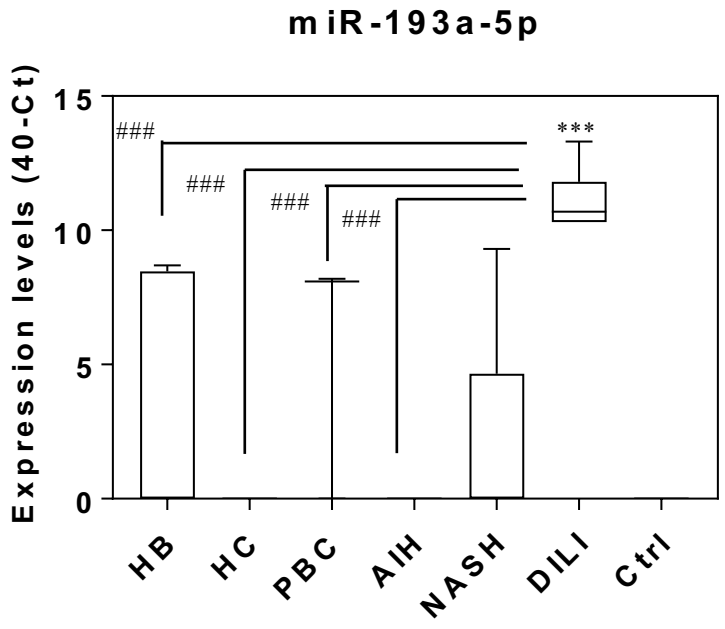
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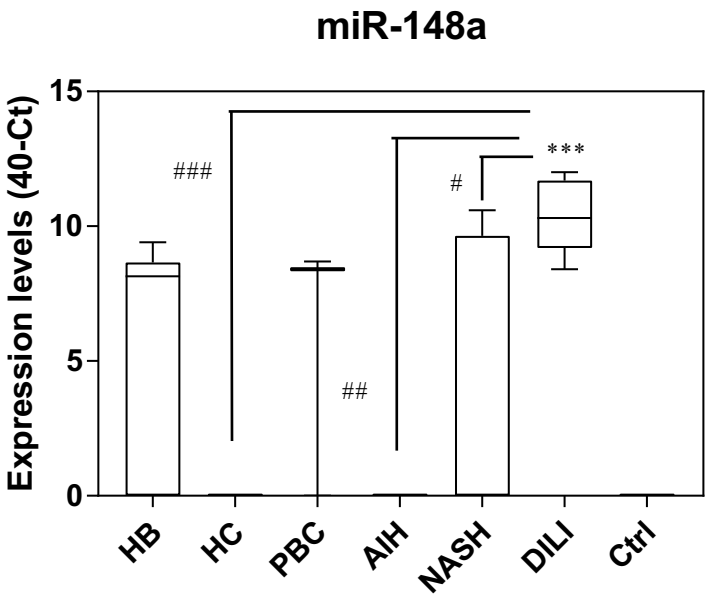
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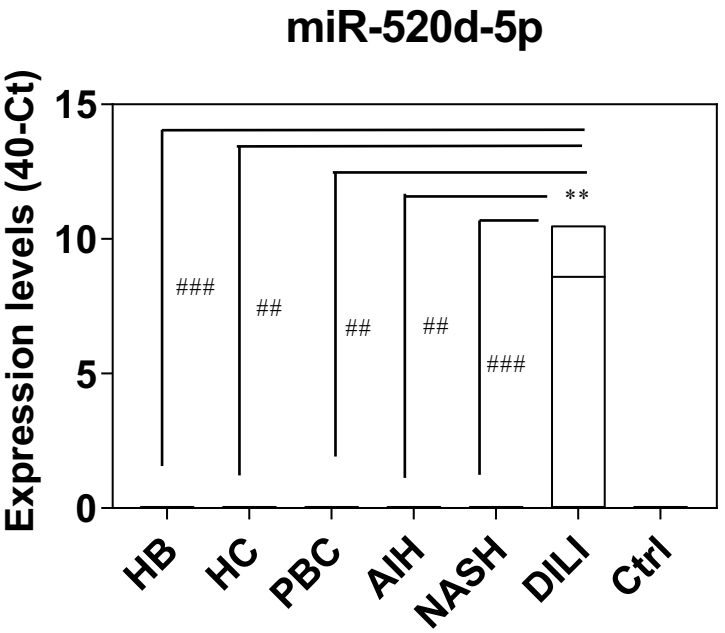
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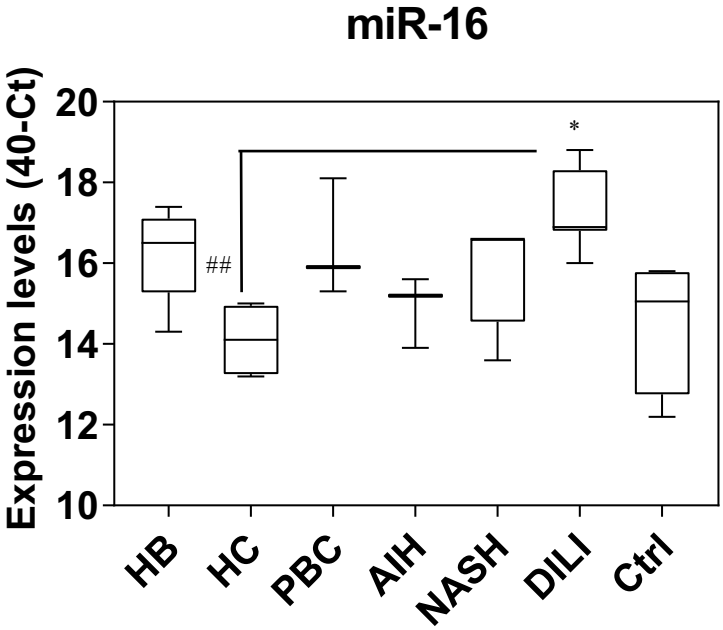
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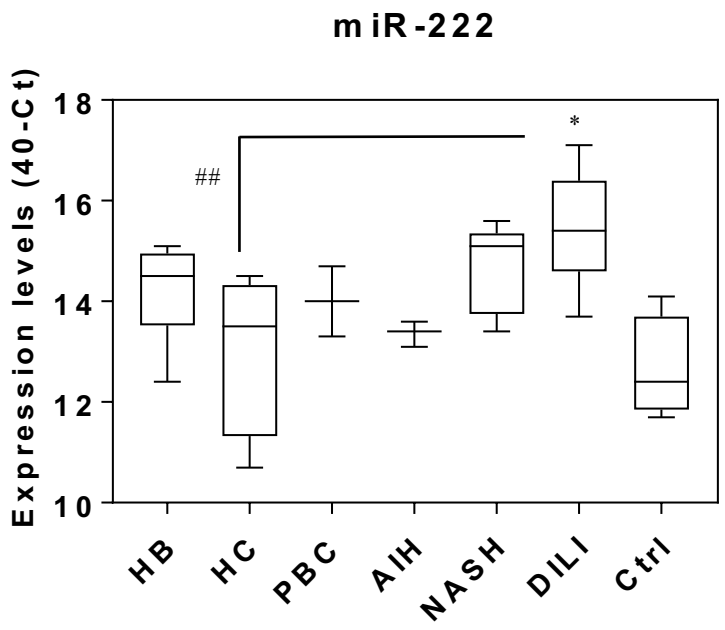
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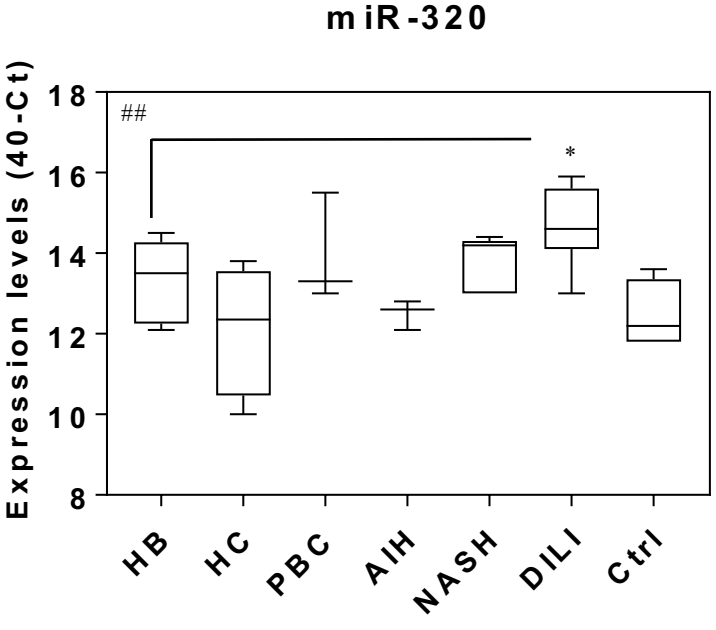
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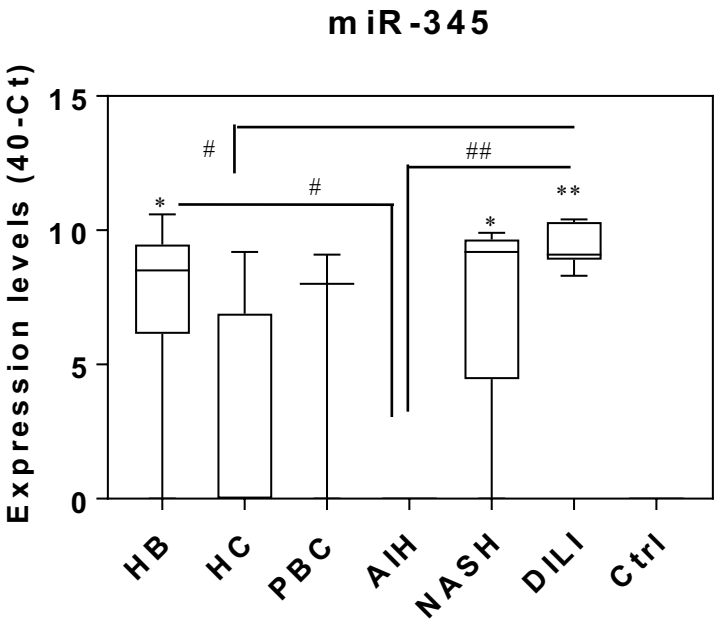
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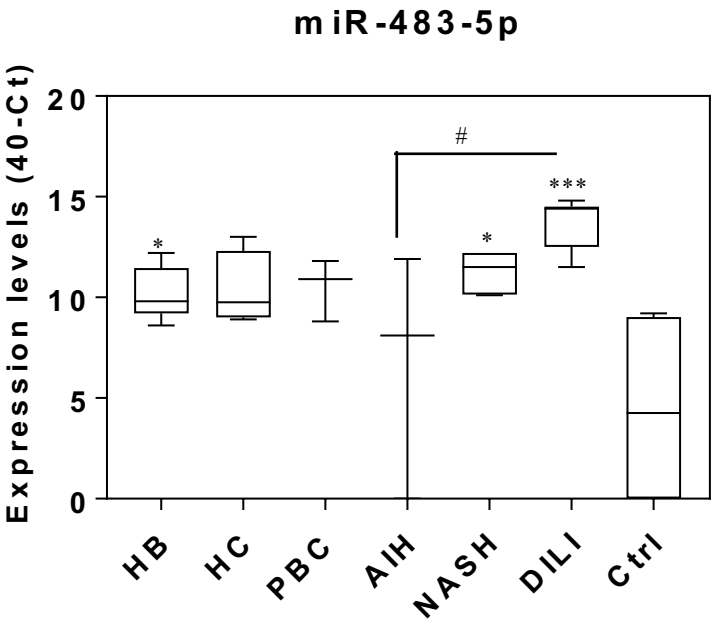
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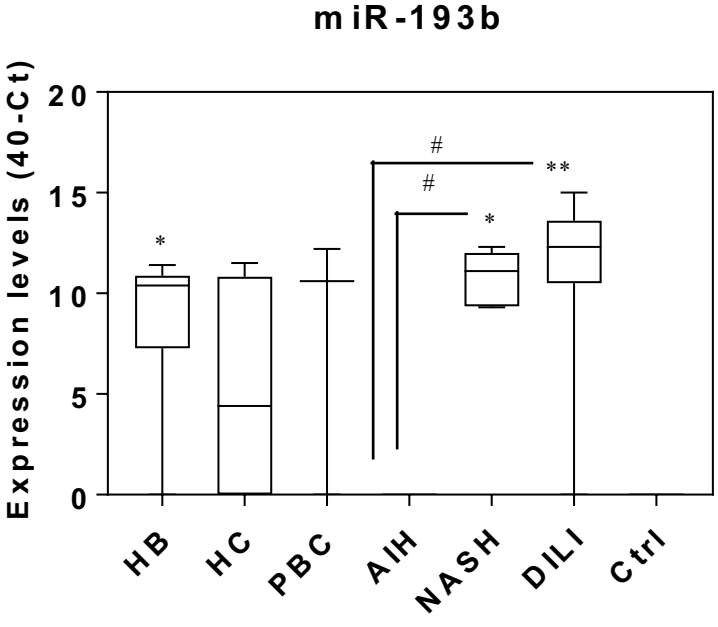
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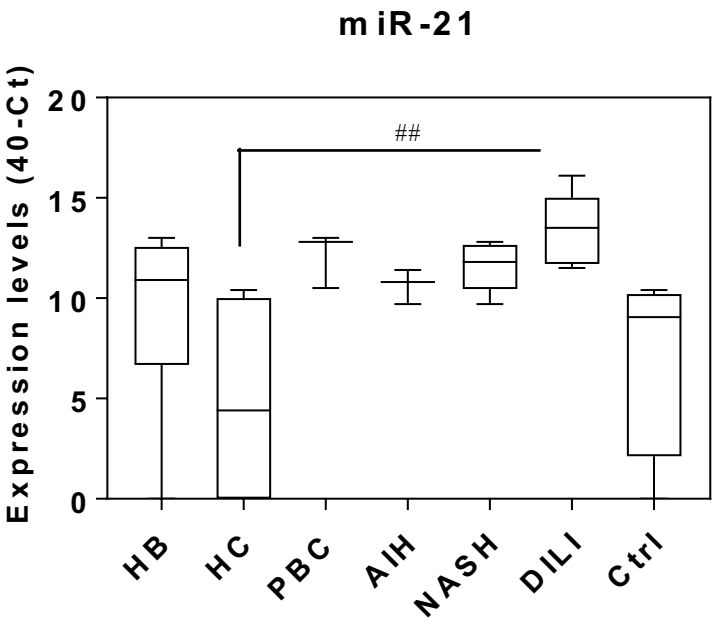
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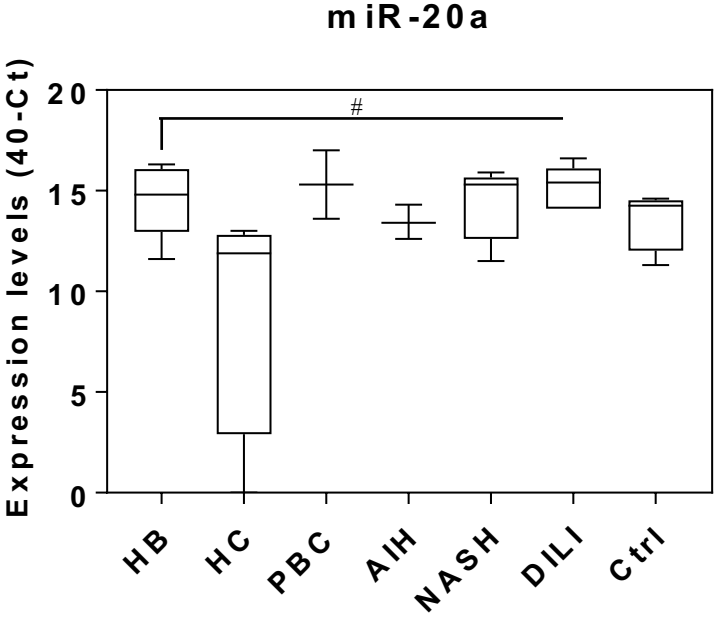
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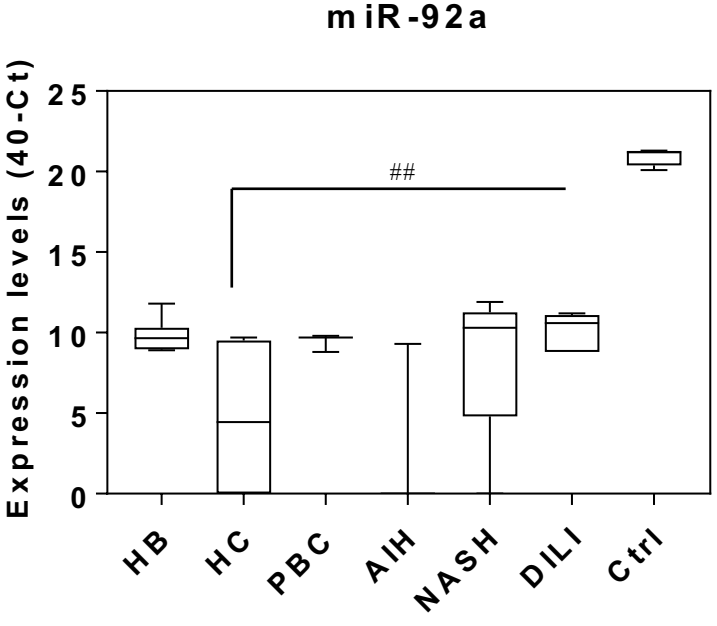
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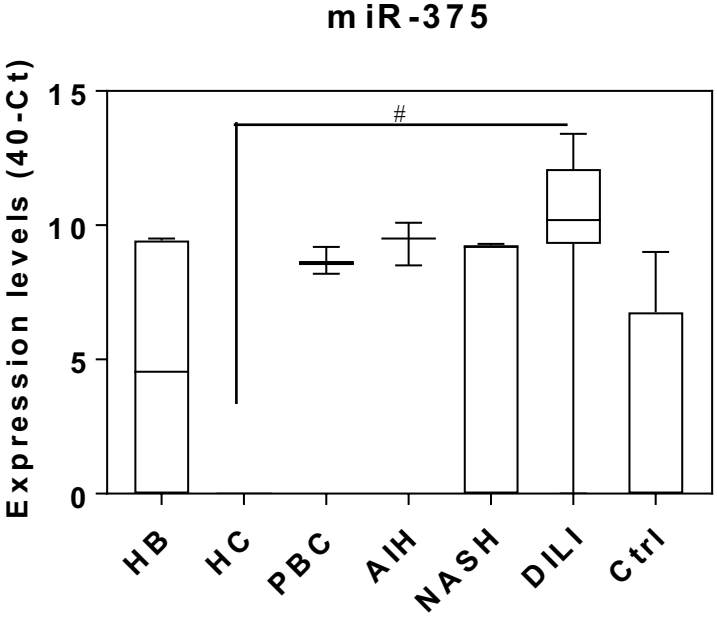
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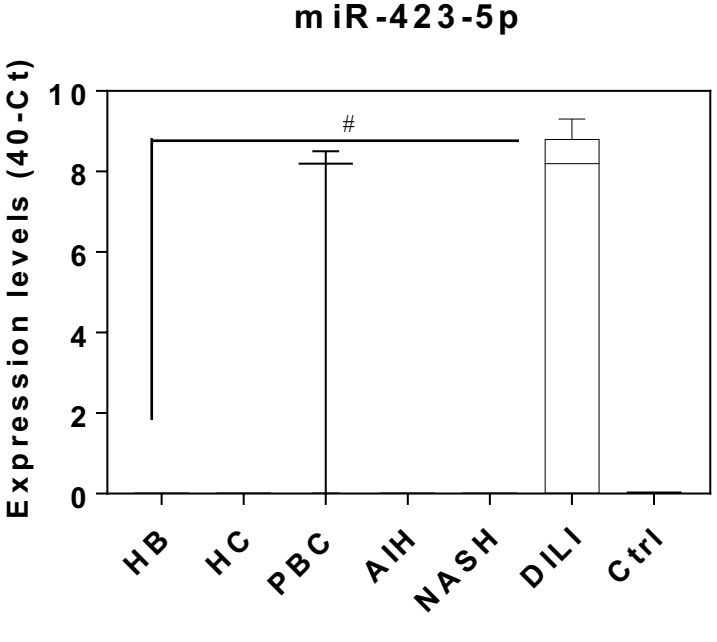
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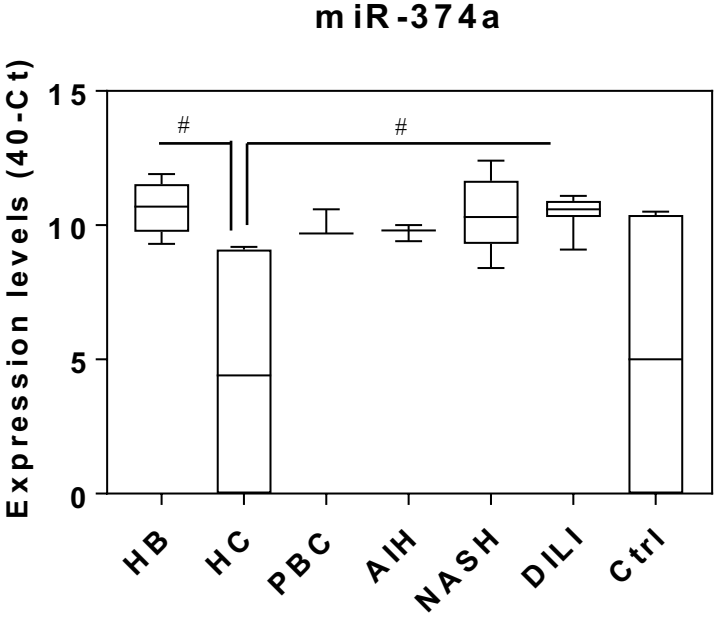
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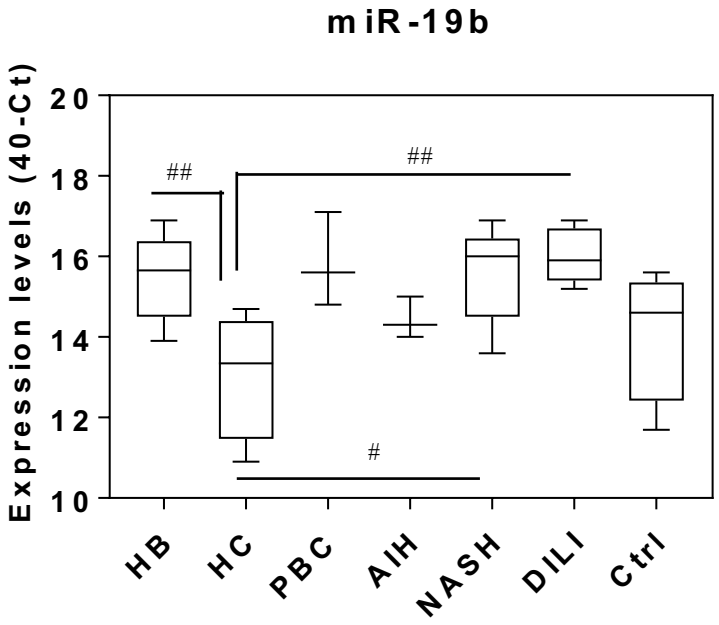
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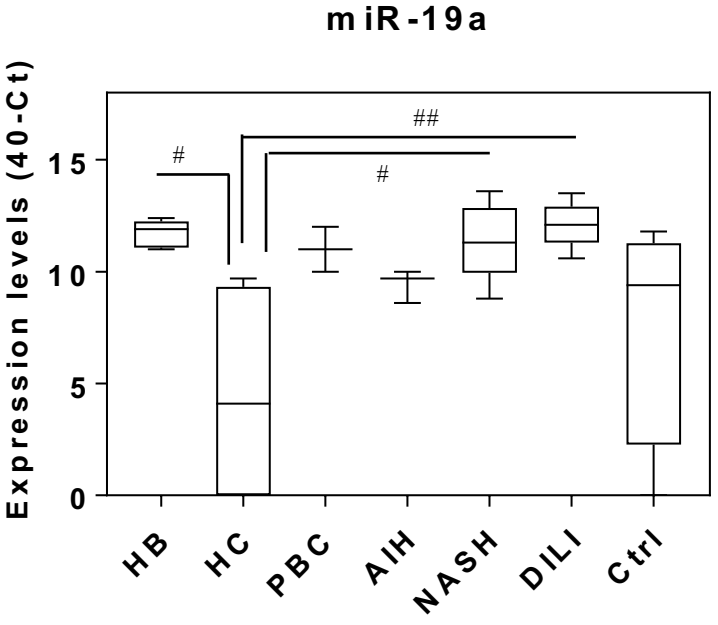
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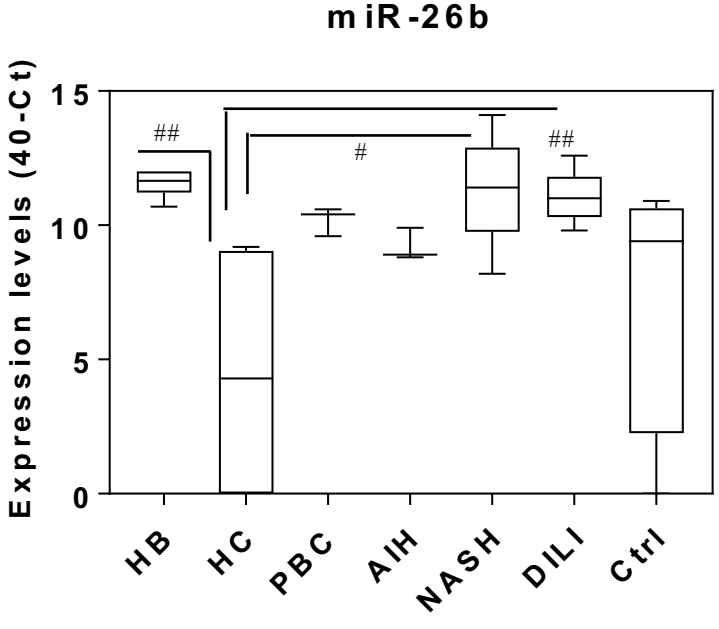
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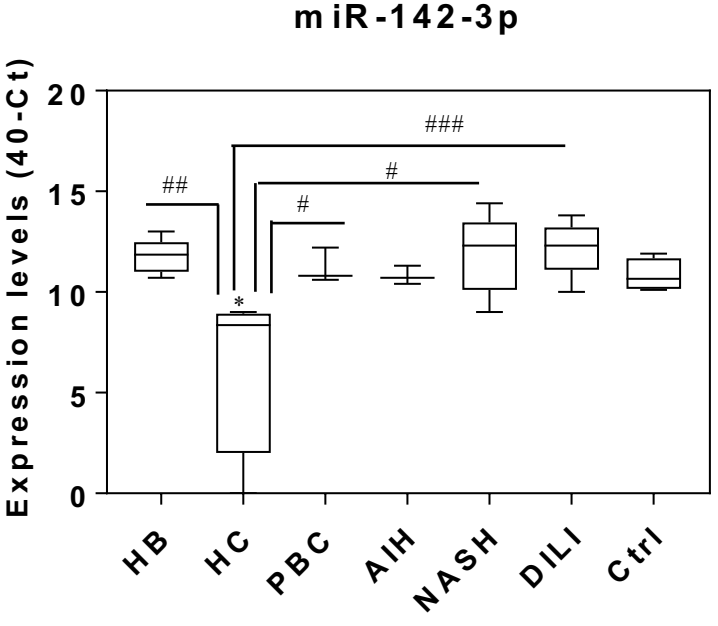
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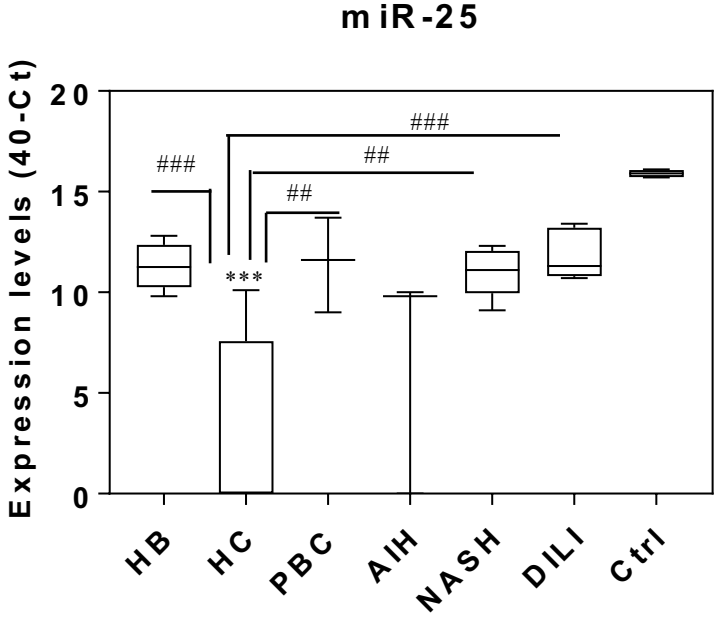
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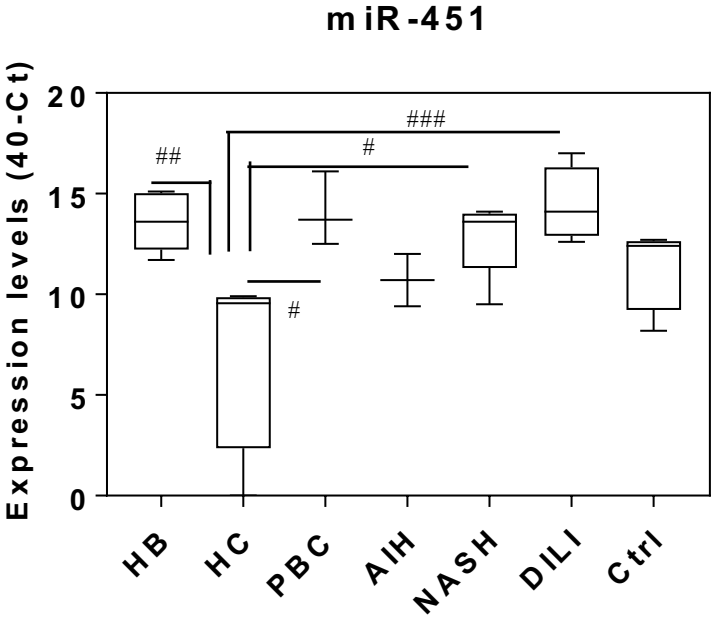
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