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Original Article

Follow up of the 987 blood donors found with hepatitis C virus infection over 9–18 years

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Aim: To follow up blood donors found with hepatitis C virus (HCV) infection, to improve the outcome by antiviral treatments.

Methods: Between 1991 and 2001, 3377 of the 1 925 860 donors (0.18%) were found to have HCV infection at the Hiroshima Red Cross Blood Center in Japan. Of them, 987 were able to be followed regularly over 9–18 years until 2009, and received antiviral treatments as required.

Results: At the start, chronic hepatitis was diagnosed in 541 (54.8%), cirrhosis in five (0.5%) and hepatocellular carcinoma (HCC) in one (0.1%), whereas the remaining 439 (44.5%) had persistently normal aminotransferase levels (PNAL). Hospital visits were terminated voluntarily in 24.3% within the first year, 46.8% by 10 years and 50.9% by 17 years. Liver disease improved in 178 (18.0%), remained stable in 606 (61.4%) and aggravated in 170 (17.2%). Of the 541 donors with chronic hepatitis, HCC developed in 28 (5.2%) and cirrhosis in 11 (2.0%), whereas HCV infection was cleared in 107 (19.8%) by antiviral treatments. In addition, HCV infection resolved in 54 of the 439 donors (12.3%) with PNAL after they had developed chronic hepatitis and received treatments. In donors with chronic hepatitis, the cumulative incidence of HCC was 4.1% at 10 years. By multivariate analysis, age and diagnosis of chronic hepatitis at the entry were found to be independent risk factors for the development of HCC.

Conclusion: Individuals with undiagnosed HCV infection need to be identified and receive medical care. They have to be motivated to merit from this health-care program.

Key words: alanine aminotransferase, chronic hepatitis C, hepatitis C virus, hepatocellular carcinoma, interferon, natural history

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INTRODUCTION

OVER THE WORLD, an estimated 130–170 million people are infected with hepatitis C virus (HCV),[1] and most of them are unaware of their HCV infection. Because HCV infection evolves insidiously, it takes decades before overt liver disease develops, such as decompensated cirrhosis and hepatocellular carcinoma (HCC).[2] Hence, it is necessary to identify individuals with undiagnosed HCV infection, and provide them with medical care for clearing HCV and preventing severe liver disease developing in them.

To make plans for management of individuals with undiagnosed HCV infection, it needs to be established to what extent liver disease has progressed in them, and how it advances over the long run, with or without medical interventions. Blood donation offers a unique opportunity to pursue such an undertaking, because it can identify HCV infections that have not been diagnosed previously.[3–9]

In 1991, when screening for antibody to HCV (anti-HCV) was introduced, a program was launched by the Hiroshima Hepatitis Study Group in Japan to identify HCV carriers at the blood donation. Liver disease was

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diagnosed in the donors infected with HCV, so as to provide them with medical care to prevent the development of serious complications. Initially, 1019 blood donors with HCV infection were enrolled in the program, and the outcome was reported for 408 (40.0%) of them who had been followed for 5 years or longer by the year 2003.[10]

Here, we report the outcome of liver disease in 987 (96.9%) of them over 18 years of follow up. Various issues came up during the study, and they would need to be taken into consideration for improving health-care of the population with undiagnosed HCV infection hidden in the society. Further, the obtained results are hoped to lessen the national burden for management of hepatitis C that is expected to increase substantially in the foreseeable future.

METHODS

Study population

BETWEEN AUGUST 1991 and November 2001, 1 925 860 individuals wished to donate blood at the Japanese Red Cross (JRC) Hiroshima Blood Center, and 3377 (0.18%) were found to have HCV infection with high-titered anti-HCV and HCV RNA in the serum. They were informed of their HCV infection, and recommended to consult hepatology specialists in the 20 institutions constituting the Hiroshima Hepatitis Study Group: Hiroshima City Asa Hospital, Akitsu Prefectural Hospital, Hiroshima Prefectural Hospital, Kure City Medical Association Hospital, Kure Kyosai Hospital, Hiroshima University Hospital, KKR Hiroshima Kinen Hospital, Hiroshima City Hospital, Hiroshima Red Cross Hospital, Hiroshima General Hospital, Hiroshima JR Hospital, National Hospital Organization Kure Medical Center, National Hospital Organization Fukuyama Medical Center, Mitsubishi Mihara Hospital, Shobara Red Cross Hospital, Chugoku Rousai Hospital, Chuden Hospital, Nippon Kokan Fukuyama Hospital, Onomichi General Hospital and National Hospital Organization West Medical Center. Serving there as hepatology specialists were: Keiji Tsuji, M.D., Toshio Miura, M.D., Mikiya Kitamoto, M.D., Norihiko Katayama, M.D., Shuji Yamaguchi, M.D., Shoichi Tanakahshi, M.D., Hideaki Kodama, M.D., Yasuyuki Araki, M.D., Yasuyuki Aisaka, M.D., Kunio Ishida, M.D., Keitaro Yamashina, M.D., Hiroshi Kouno, M.D., Toshihiko Kaneyoshi, M.D., Kazushi Teramen, M.D., Kouji Kamada, M.D., Takashi Moriya, M.D., Hiroto Ishihara, M.D., Tomoo Yoshida, M.D., and Makoto Obayashi, M.D.

Of the 3377 donors infected with HCV, who were advised to undergo medical examination, 1097 (32.5%) visited liver clinics. The initial diagnosis was established in 1019 of them (30.2% of the total), and the date of initial visit, the date of birth and the baseline liver disease were filed in the computer. They were recommended to take regular check-ups, and receive antiviral treatment as required. The results of 408 carriers (40.0%), who had been followed for 5 years or longer, were reported in 2007.[10] Since then, they had been followed for an additional 6 years. The present study compiled all the data that had been accumulated on them over 18 years from 1991 to 2009, in an attempt to portray the outcome of undiagnosed HCV infection, and evaluate the efficacy of preventing liver disease by antiviral treatments. The 32 donors with normal aminotransferase levels at the initial diagnosis were excluded, because they visited clinics only once, and therefore the diagnosis of liver disease was not established in them. The remaining 987 donors entered the present study.

The study design conformed to the Declaration of Helsinki, and was approved by the Ethic Committees of Hiroshima University. Informed consent was obtained from each blood donor who was infected with HCV.

Data collection

A questionnaire form was distributed among hepatology specialists in the 20 institutions of the Hiroshima Hepatitis Study Group. They were asked to log the following: (i) initial diagnosis; (ii) compliance to regular visits; (iii) changes in liver disease over time; (iv) treatments with interferon (IFN); and (v) development of HCC. These data were made anonymous for the personal identification of any participant, and analyzed collectively.

Diagnosis of liver disease

Four clinical states were classified. They were: (i) persistently normal aminotransferase levels (PNAL); (ii) chronic hepatitis; (iii) cirrhosis; and (iv) HCC. PNAL was judged by: (i) values of alanine aminotransferase (ALT) within normal limits (≤40 IU/L) twice or more within 6 months at least 2 months apart; (ii) normal platelet counts ($\geq 150 \times 10^3/\text{mm}^3$); (iii) lack of abnormal findings in those examined by imaging modalities; and (iv) no pathological findings in the liver biopsy for those who received it. Each attending specialist was asked his/her comprehensive opinion on the absence of liver disease in the HCV-infected donor with PNAL. The diagnosis of chronic hepatitis, cirrhosis and HCC was left to the judgment of the attending hepatology specialist, who took into consideration the results of biochemical, imaging and other tests. The specialist made the decision as to whether his/her patient should receive IFN-based treatment or would better be followed regularly without treatment.

Markers of HCV infection

Hepatitis C virus RNA was determined by reverse-transcription polymerase chain reaction (RT–PCR) with primers deduced from conserved sequences in the 5′-non-coding region of the genome, irrespective of genotypes.[11] Genotypes of HCV were determined by RT–PCR with type-specific primers[12]

Genotypes of the interleukin 28B (*IL28B*) gene

The three donors who cleared HCV spontaneously were examined for *IL28B* genotypes.[13–15] The genomic DNA was extracted from the serum by RT–PCR, and polymorphisms of the *IL28B* gene at rs12979860 and rs8099917 were determined by the direct sequencing.

IFN-based treatments

Interferon was administrated according to the regular protocol at the discretion of the attending doctor and with the agreement of his/her patient. Types of IFN were natural IFN-α, recombinant IFN-α2 and -α2b until 2002. Natural IFN or recombinant IFN-α2a or -α2b at a daily dose of 6–9 million units (MU) was administrated during the initial 2 weeks, followed by 3 MU three times per week until 24 weeks after the start of IFN (total dose, 300–342 MU). After 2002, ribavirin was combined with recombinant IFN. After 2005, the standard-of-care therapy was implemented with pegylated-IFN-α2b (PEG-Intron; Shering Plough, Kenilworth, NJ) s.c. at a median dose of 1.5 μg/kg [range, 1.3–2.0 μg/kg]) once a week until 48 weeks, together with a daily dose of 600–1000 mg ribavirin (Rebetol [600–1000 mg]; Shering Plough) that was adjusted by the bodyweight. Sustained virological response (SVR) to IFN was diagnosed by the clearance of HCV RNA from serum 24 weeks after the treatment completion and thereafter.

Statistical analyses

Means and proportions were compared between groups by Student's *t*-test and χ^2-test or Fisher's exact test, respectively. For comparison of the frequency of the response to IFN (SVR and non-SVR) and the lack of IFN treatments in blood donors in whom liver disease was improved, unchanged and aggravated, post-hoc pairwise comparisons were carried out using the χ^2-test, and the *P*-value was adjusted by the method of Bonferroni. The Kaplan–Meier's life-table analysis and Cox proportional hazards models were employed in assessing the risk of developing chronic hepatitis or HCC with reference to sex, age and diagnosis at the entry, as well as the response to IFN-based treatments, utilizing JMP ver. 9 software (SAS Institute, Cary, NC, USA). All *P*-values were two-tailed, and those less than 0.05 were considered statistically significant.

RESULTS

Compliance with the follow up of blood donors who had HCV infection

FIGURE 1 ILLUSTRATES the compliance with follow up and shifting status of blood donors with HCV over 18 years. Notably, 24.3% of them were lost to follow up within the first year. Dropouts increased to 46.8% at 10 years and reached 51.6% at 15 years. Exclusive of the 52 blood donors, who entered the study in the first year and were followed for 18 years, only approximately 20% of donors with HCV infection complied with observation between 14 and 17 years. HCV was cleared in 17.4% of them over 17 years, including 8.3% who complied with observation and 9.1% who were lost to follow up.

Liver disease in the 987 blood donors with HCV infection

Clinical states of the 987 blood donors with HCV infection, at the time of donation, are summarized in Table 1. Cirrhosis had developed in five (0.5%) and HCC in one (0.1%) already. Chronic hepatitis was diagnosed in 541 (54.8%) of them, leaving only 439 donors (44.5%) who had PNAL. Acute hepatitis was diagnosed in a single donor infected with HCV-2a, who had been negative for HCV RNA at the previous donation. He developed chronic hepatitis 9 months later, and achieved SVR to antiviral treatment 1 year thereafter. Chronic hepatitis was more frequent in men than women (65.7% vs 45.2%, $P < 0.001$). Conversely, women possessed PNAL more often than men (54.4% vs 33.3%, $P < 0.001$). Liver biopsy was performed in

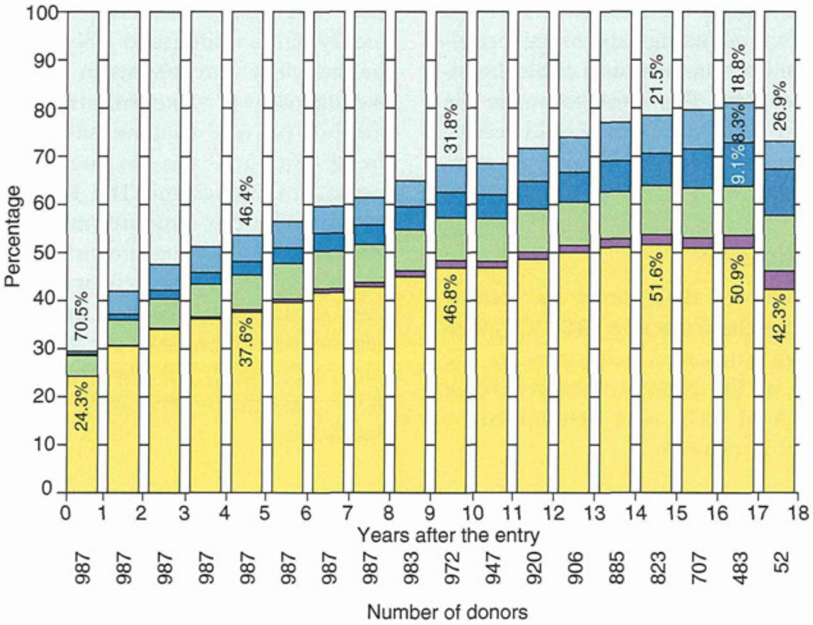


Figure 1 Follow up of the 987 donors with hepatitis C virus (HCV) infection over 18 years. □, on observation (HCV kept); 🟦, on observation (HCV cleared); 🟦, lost to follow up (HCV cleared); 🟩, transferred (HCV kept); 🟪, deceased (HCV kept); 🟨, lost to follow up (HCV kept).

356 (36.1%) of the 987 donors, and 393 (39.8%) received IFN-based treatments. Among the 709 donors, for whom genotyping was feasible, genotype 1b was the most prevalent both in men (67.6%) and women (65.7%).

Evolution of liver disease in the 987 donors with HCV during the follow up

Figure 2 depicts the outcome of 987 blood donors with HCV infection during an average follow-up period

Table 1 Clinical states of the 987 blood donors found with HCV infection at the donation

Features	Total ($n = 987$)	Men ($n = 463$)	Women ($n = 524$)	Differences Men vs. Women
Persistently normal aminotransferase levels	439 (44.5%)	154 (33.3%)	285 (54.4%)	$P < 0.001$
Chronic hepatitis	541 (54.8%)	304 (65.7%)	237 (45.2%)	$P < 0.001$
Cirrhosis	5 (0.5%)	3 (0.7%)	2 (0.4%)	$P = 0.670$
Hepatocellular carcinoma	1 (0.1%)	1 (0.2%)	0	$P = 0.470$
Acute hepatitis	1 (0.1%)	1 (0.2%)	0	$P = 0.470$
Age at the entry	45.1 ± 11.3	43.2 ± 11.1	46.8 ± 11.2	$P < 0.001$
Observation period (years)	7.3 ± 6.6	6.8 ± 6.4	7.7 ± 6.8	$P = 0.024$
Biopsy	356 (36.1%)	181 (39.1%)	177 (33.8%)	$P = 0.096$
IFN-based therapy	393 (39.8%)	190 (41.0%)	203 (38.7%)	$P = 0.503$
SVR	166 (42.2%)	84 (44.2%)	82 (40.4%)	$P = 0.507$
Genotypes identified	709 (71.8%)	339 (73.2%)	370 (70.6%)	$P = 0.402$
Genotype 1b	472 (66.6%)	229 (67.6%)	243 (65.7%)	$P = 0.366$
Genotype 2a	153 (21.6%)	71 (20.9%)	82 (22.2%)	$P = 0.962$
Genotype 2b	68 (9.6%)	35 (10.3%)	33 (8.9%)	$P = 0.512$
Mixed Genotype	16 (2.3%)	4 (1.2%)	12 (3.2%)	$P = 0.129$

SVR, sustained virological response.

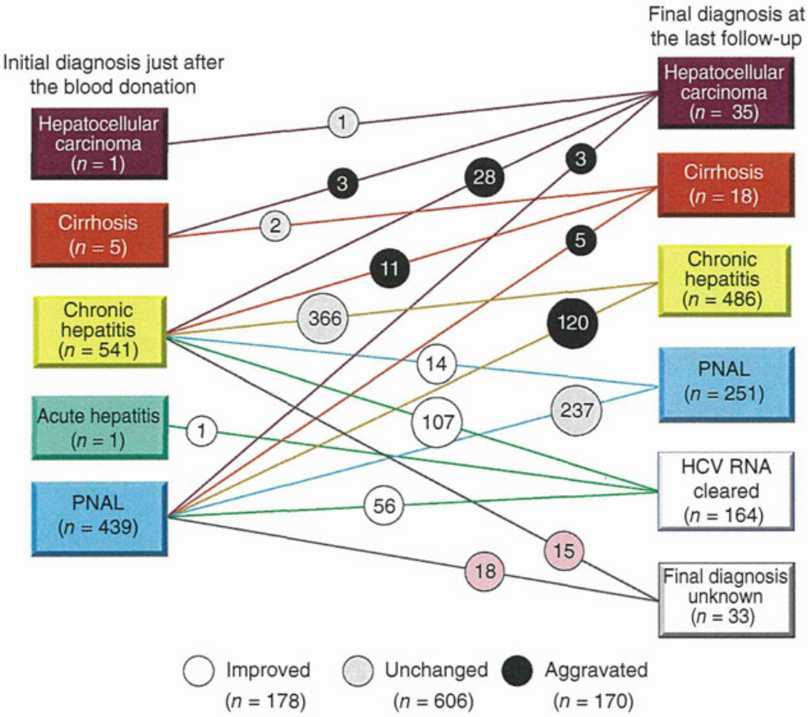

Initial diagnosis just after the blood donation
Final diagnosis at the last follow-up
Hepatocellular carcinoma (n = 1)
Cirrhosis (n = 5)
Chronic hepatitis (n = 541)
Acute hepatitis (n = 1)
PNAL (n = 439)
Hepatocellular carcinoma (n = 35)
Cirrhosis (n = 18)
Chronic hepatitis (n = 486)
PNAL (n = 251)
HCV RNA cleared (n = 164)
Final diagnosis unknown (n = 33)
1
3
2
28
3
11
5
366
120
14
107
237
1
56
15
18
Improved (n = 178)
Unchanged (n = 606)
Aggravated (n = 170)

Figure 2 Outcome of hepatitis C virus (HCV) donors with HCV infection. ○, improved ($n = 178$); ○, unchanged ($n = 606$); ●, aggravated ($n = 170$). PNAL, persistently normal aminotransferase levels.

of 7.3 ± 6.6 years; they were stratified by the initial diagnosis. Liver disease improved in 178 (18.0%), and remained stable in 606 (61.4%), whereas it worsened in the remaining 170 (17.2%). HCV infection was cleared by antiviral treatments in 107 of the 541 donors (19.8%) with chronic hepatitis. Chronic hepatitis developed in 120 of the 439 donors (27.3%) with PNAL. Of them, HCV infection was cleared in 54 by treatments given after they had developed chronic hepatitis, whereas it resolved spontaneously in two. Cirrhosis occurred in 16 donors (1.6%), including 11 with chronic hepatitis and five with PNAL at entry. HCC developed in 34 donors (3.4%), and in three of them, it was detected at 1, 4 and 7 years, respectively, after they had cleared HCV infection by antiviral treatments.

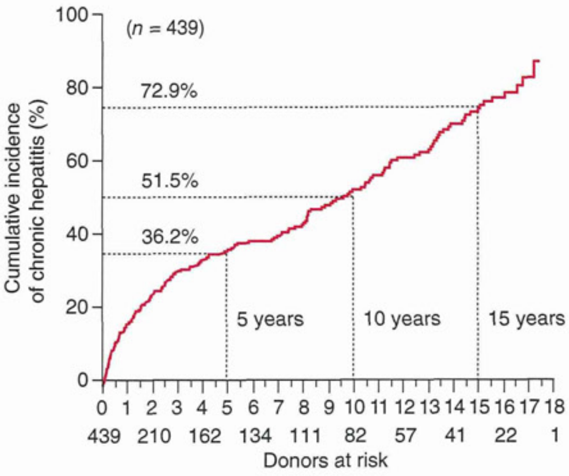
Cumulative incidence of chronic hepatitis in donors with PNAL

Figure 3 shows the cumulative incidence of chronic hepatitis in the 439 donors with PNAL. Chronic hepatitis developed in 36.2% by 5 years, 51.5% by 10 years and 72.9% by 15 years, with an annual incidence of 4.9–7.2% (Fig. 3a). Men tended to develop chronic hepatitis more frequently than women (Fig. 3b). Figure 3c compares the development of chronic hepatitis in donors with PNAL classified into four age groups. Chronic hepatitis developed in the group aged 40–49 years at entry, more frequently than in those aged 39 years or younger or 50 years or older, although the difference fell short of being significant ($P = 0.089$). The cumulative incidence of chronic hepatitis reached 66.3% by 10 years in the group aged 40–49 years at entry.

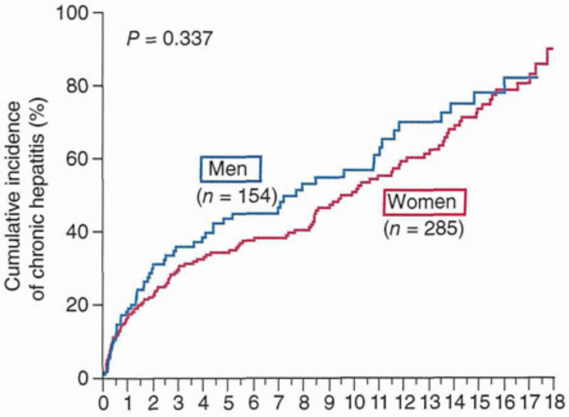
Development of HCC in donors with chronic hepatitis

Overall, 680 donors (68.9%) were diagnosed with chronic hepatitis either at entry or during this study, and they were followed for the development HCC. The cumulative incidence of HCC was 9.0% by 15 years after they had been diagnosed with chronic hepatitis (Fig. 4a). HCC developed comparably frequently in men and women (Fig. 4b). It developed more frequently ($P < 0.001$) in the donors in whom chronic hepatitis had been diagnosed at ages 60 years or older than 59 years or younger (Fig. 4c). HCC occurred less frequently ($P = 0.037$) in responders than non-responders to IFN, or the donors who did not receive IFN (Fig. 4d).

(a) Total



(b) Stratified by the gender



(c) Stratified by the age

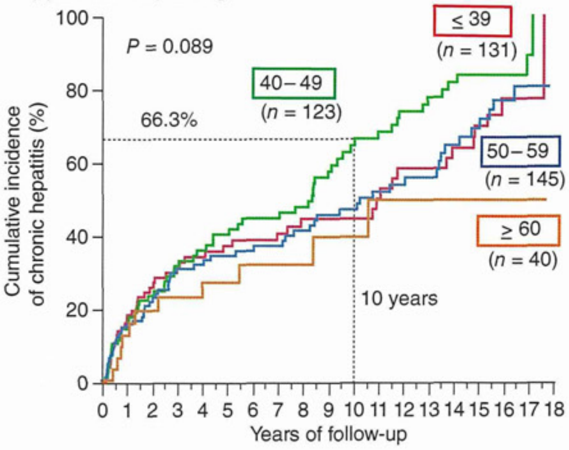


Figure 3 Cumulative incidence of chronic hepatitis in the 439 donors with persistently normal aminotransferase levels (PNAL). Development of chronic hepatitis is depicted in the total donors with PNAL (a), those stratified by sex (b), and those in different age groups (c). (b) Men, ($n = 154$); Women, ($n = 285$); (c) ≤ 39, ($n = 131$); 40–49, ($n = 123$); 50–59, ($n = 145$); ≥ 60, ($n = 40$).

Independent risk factors for the development of HCC were sorted by multivariate analysis (Table 2). Age at the diagnosis of chronic hepatitis was the highest risk (Odds Ratio [OR], 22.70 for ≥60 years), followed by the initial diagnosis of chronic hepatitis (OR, 6.52) and the male sex (OR, 1.78). Failure to gain SVR to IFN (OR, 2.11), or the lack of IFN-based treatment (OR, 2.06), increased the risk of HCC, as well.

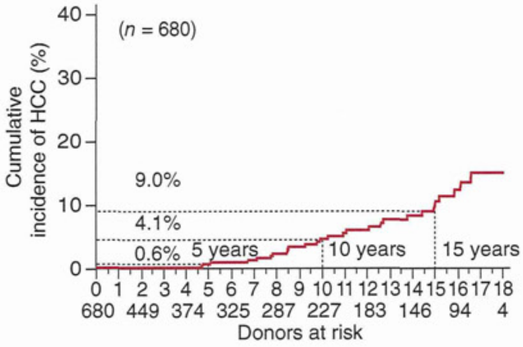
Influence of the response to IFN on the outcome of donors

Of the 476 donors with PNAL or chronic hepatitis, who had been followed for 5 years or longer, 280 (58.8%) received IFN-based treatments, and the outcome of liver disease was evaluated with reference to the response in three combinations, namely, SVR versus non-SVR, SVR versus without IFN, and non-SVR versus without IFN (Table 3). Of the 50 patients with improvement in liver disease, SVR was more frequent than non-SVR and without IFN ($P < 0.001$ for both). Of the 281 patients in whom liver disease did not change, SVR was more frequent than without IFN ($P < 0.001$), and non-SVR was more frequent than without IFN ($P < 0.01$). There were no differences among the frequency of SVR, non-SVR and without IFN in the 145 patients in whom liver disease aggravated.

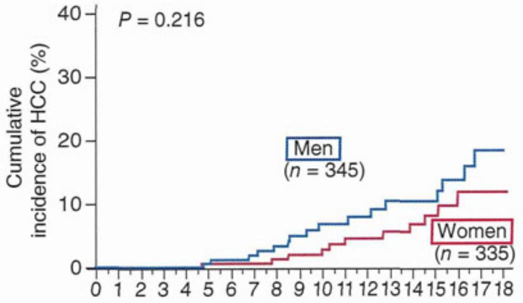
Spontaneous clearance of HCV infection

Hepatitis C virus RNA was cleared from the serum in three donors who had not received antiviral treatments (Table 4). HCV RNA disappeared 6, 15 and 15 years, respectively, after they had been found with HCV infection at the blood donation. Two of them had PNAL and the remaining one had chronic hepatitis at entry. HCV genotypes were able to be determined in two, and they were 2a and 2b, respectively; neither of them was infected with the genotype 1b that is most prevalent and detected in 82% of Japanese blood donors.[12] All three donors possessed CC at rs12979860 and TT at rs8099917 in the *IL28B* gene, which increase the response to IFN in hepatitis C patients,[13–15] and promote the spontaneous clearance of HCV infection.[16] Among

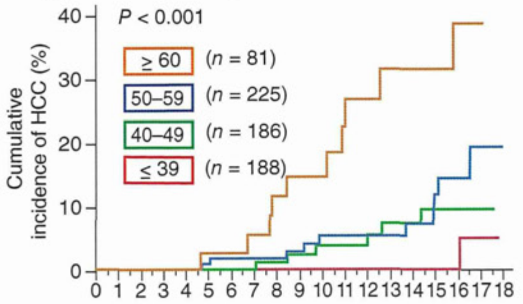
(a) Total



(b) Stratified by the gender



(c) Stratified by the age



(d) Stratified by IFN-based therapy and the response to it

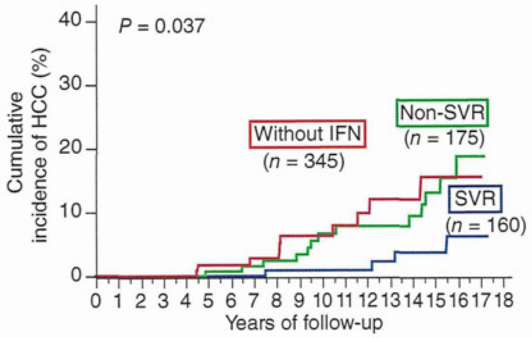


Figure 4 Cumulative incidence of hepatocellular carcinoma (HCC) in donors with chronic hepatitis. Development of HCC is portrayed in the 680 donors since the day of diagnosis with chronic hepatitis (start point) till the day of diagnosis with HCC (end-point) (a). Influence of sex (b), age (c) and interferon (IFN)-based treatments (d) is evaluated, also. (b) Men, (*n* = 345); Women, (*n* = 335); (c) ≥ 60, (*n* = 81); 50–59, (*n* = 225); 40–49, (*n* = 186); ≤ 39, (*n* = 188); (d) Non-SVR, (*n* = 175); Without IFN, (*n* = 345) SVR, (*n* = 160). SVR, sustained virological response.

the 316 donors with PNAL who did not receive IFN treatment, HCV was cleared spontaneously in only two, at the rate of 13.0/10^4 person-years (95% confidence interval, 1.6–47.1/10^4 person-years).

DISCUSSION

A LONG-TERM prospective study was performed on blood donors who were found to have HCV infection. For 18 years (1991–2009), 987 donors with HCV infection were followed up for an average of 7.3 years. The entry to this study was started in 1991 when an anti-HCV test was introduced to blood screening, and terminated in 2000 when merely a few donors were found to have HCV infection at the Hiroshima Red Cross Blood Center annually.[17] Despite our initial expectation, only one-third (32.5%) of blood donors with HCV infection visited liver clinics, even though they were advised to do so strongly. Another distressing issue was the low compliance with the study (Fig. 1). Within the first year after the entry, 24.3% of donors ceased to visit liver clinics. The rate of dropouts increased gradually to 46.8% over 10 years; it plateaued thereafter and stayed at 50.9% over 17 years. Thus, approximately one-half of blood donors with HCV infection were unable to receive any possible benefit of this study. It is not known whether these dropouts differed from the followed donors in the outcome of HCV infection and response to IFN-based treatments. Therefore, there remain possible biases in the results obtained only in the followed donors in the present study. We can say that the symptoms at the onset were almost the same among donors because they were well enough to give someone their blood regardless of whether they consulted later or not. It is of utmost importance to motivate blood donors with HCV infection to visit liver clinics, and encourage early dropouts to stay in follow up, to gain the full effect of screening blood donors.

Over 17 years of follow up, IFN-based treatments cleared HCV infection in 17.4%. Restricted to the

Table 2 Independent risk factors for the development of hepatocellular carcinoma in the 680 donors with chronic hepatitis

Factors	*n*	Odds ratio	95% CI	Differences
Sex				
Female	335	1.00		
Male	345	1.78	0.81–4.04	$P = 0.148$
Age (years) at the diagnosis of CH				
≤39	188	1.00		
40–49	186	5.32	0.90–100.94	$P = 0.068$
50–59	225	8.63	1.59–160.31	$P = 0.009$
≥60	81	22.70	4.14–424.53	$P < 0.001$
Initial diagnosis				
PNAL	161	1.00	7.94	
CH	519	6.52	1.36–116.95	$P = 0.014$
IFN treatment				
SVR	160	1.00		
Non-SVR	176	2.11	0.71–7.73	$P = 0.187$
Without	344	2.06	0.62–7.94	$P = 0.237$

CH, chronic hepatitis; CI, confidence interval; IFN, interferon; PNAL, persistently normal aminotransferase levels; SVR, sustained virological response.

donors who stayed on the surveillance (49.1%), 39.8% were helped by antiviral treatments. A high SVR of 42.2% was gained by blood donors who received IFN-based treatment. A similarly high SVR (42%) has been reported in blood donors with minimal to moderate liver disease who had received IFN monotherapy.[18] This would underscore the need for identifying people with undiagnosed HCV infection, and provide them with treatments as required. In this study, IFN-based treatments were offered to blood donors who presented with chronic hepatitis or those with PNAL who had developed chronic hepatitis. Since the standard-of-care therapy with pegylated IFN and ribavirin was initiated in the early 2000s, it has been indicated to HCV-infected individuals with PNAL and achieved an excellent efficacy.[19,20] If the indication of the combination therapy would have been extended to blood donors with PNAL in this study, a further gain may have been brought about in the clearance of HCV.

Sustained virological response was accomplished by the 166 donors with chronic hepatitis or PNAL who received IFN-based treatments. Spontaneous clearance of HCV without IFN treatment occurred in two of the 316 donors with PNAL at entry, at a rate of $13.0/10^4$ (95% confidence interval, $1.6–47.1/10^4$) person-years. Notably, they all possessed CC at rs12979860 and TT at rs8099917 in the *IL28B* gene, which improve the response to IFN,[13-15] and accelerate spontaneous clearance of HCV.[16]

Table 3 Clinical outcomes of the 280 donors with and the 196 without interferon-based treatment who had been followed for longer than 5 years

	Liver disease		
	Improved ($n = 50$)	Unchanged ($n = 281$)	Aggravated ($n = 145$)
Treatments			
With IFN			
SVR	42 (84.0%)[a]	62 (22.1%)[d]	34 (23.4%)
Non-SVR	1 (2.0%)[b]	85 (30.2%)[e]	56 (38.6%)
Without IFN	7 (14.0%)[c]	134 (47.7%)[f]	55 (37.9%)

Improved: a vs b, $P < 0.001$; a vs c, $P < 0.001$ (comparison was made by the post-hoc test with Bonferroni's adjustment).
Unchanged: d vs f, $P < 0.001$; e vs f, $P < 0.01$.
IFN, interferon; SVR, sustained virological response.

Table 4 Blood donors who resolved HCV infection spontaneously

Case No.	Sex	Age (years) at		Diagnosis at		Genotype	*IL28B* genotypes	
		Entry	HCV loss	Entry	HCV loss		rs12979860	rs8099917
1	Male	64	70	PNAL	Normal	2a	CC	TT
2	Female	43	58	PNAL	Normal	ND	CC	TT
3	Male	54	69	CH	LC/HCC	2b	CC	TT

CH, chronic hepatitis; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; LC, cirrhosis; ND, not determined due to low HCV RNA titers; PNAL, persistently normal aminotransferase levels.

Although blood donors with HCV infection were healthy, both subjectively and objectively, many had developed chronic hepatitis with the prevalence higher in men than women (65.7% vs 45.2%, $P < 0.001$). This would reflect the progression of hepatitis C that is more rapid in men than women.[21] Cirrhosis had developed in five of the 987 donors (0.5%) with HCV, and HCC accompanied by chronic hepatitis in one (0.1%) at entry. Donors with PNAL, defined by ALT below the upper limit of normal (≤40 U/L) at least twice during 6 months, accounted only for 44.5%. Development of significant liver disease in donors found to have HCV infection has been reported from various countries,[3–9] but a long-term follow up on a large scale was rarely performed. During a follow up for 7 years of 118 blood donors with HCV infection, 52.5% dropped out.[22]

Such a failure is ascribable to the lack of motivation of individuals with asymptomatic HCV infection who are forced to bear substantial economic and psychological burdens.[23] In Japan, 807 903 individuals are estimated to have undiagnosed HCV infection, corresponding to 0.63% of the total population.[24] The national campaign for screening undiagnosed HCV infections in the Japanese aged 40 years or older was started in 2002.[25] Although approximately one half of those infected with HCV visited hepatology specialists, only a minor portion of them (12–15% of those with HCV) have received IFN-based treatments (unpubl. obss.).

During the follow up of 7.3 ± 6.6 years, liver disease improved in 178 donors (18.0%), stayed unchanged in 606 (61.4%) and aggravated in 170 (17.2%). Although 237 of the 439 donors (54.0%) with PNAL kept normal ALT levels during a follow up of 7.3 years, the cumulative incidence of chronic hepatitis increased almost linearly over 15 years, from 36.2% at 5 years, to 51.5% at 10 years, and to 72.9% at 15 years (Fig. 3). Thus, chronic hepatitis developed in 7–8% of blood donors with PNAL yearly, at a rate comparable with that estimated by the Markov model based on the transition probability.[26]

Among 680 donors with chronic hepatitis at entry or in whom it was diagnosed during the follow up, HCC developed in 27 (4.0%). It increased steadily over time, and reached 4.1% and 9.0% at 10 and 15 years, respectively (Fig. 4). As expected, HCC developed more rapidly in the donors aged 60 years or older than 40–59 years; the development was the least frequent in the donors aged 39 years or younger. HCC developed less frequently in the donors with SVR to IFN-based treatments than those with no treatments or the lack of SVR. Independent factors for the development of HCC were age (OR, 22.70 for ≥60 years), initial diagnosis of chronic hepatitis (OR, 6.52), male sex (OR, 1.78) and response to IFN-based treatments (OR, 2.11 for non-responders and 2.06 for donors without IFN). These risk factors for HCC are in accord with those in previous reports.[27,28] The response to IFN-based treatments did not influence the risk for HCC (OR, 2.11 for non-responders [$P = 0.187$] and 2.06 for donors without IFN [$P = 0.237$]). In view of the influence of SVR on the risk of HCC observed in the Kaplan–Maier analysis (Fig. 4d), it would be expected to gain significant difference in the risk for HCC in the multivariate analysis by studying patients in larger scales. It has to be noted that HCC developed in three donors 1, 4 and 7 years, respectively, after they had achieved SVR to antiviral treatments. This underscores the need for continuing the follow up of donors with chronic hepatitis who have achieved SVR.

In conclusion, this study has demonstrated a wide range of liver disease, low compliance with follow up and high efficacy to IFN-based treatments in blood donors found with HCV infection. The results reported herein are hoped to help in coping with many undiagnosed HCV infections over the world, which are predicted to increase in the foreseeable future.[29] Efforts along this line would improve the health-care of people with undiagnosed HCV infection, and decrease social and economic burdens on the nation.

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REFERENCES

1 World Health Organization Media Center. *Fact sheet Hepatitis C.* [Cited August 2011.] Available from URL: http://www.who.int/mediacentre/factsheets/fs164/en/
2 Seeff LB. Natural history of chronic hepatitis C. *Hepatology* 2002; **36**: S35–46.
3 Alberti A, Chemello L, Cavalletto D *et al.* Antibody to hepatitis C virus and liver disease in volunteer blood donors. *Ann Intern Med* 1991; **114**: 1010–12.
4 Conry-Cantilena C, VanRaden M, Gibble J *et al.* Routes of infection, viremia, and liver disease in blood donors found to have hepatitis C virus infection. *N Engl J Med* 1996; **334**: 1691–6.
5 Esteban JI, Lopez-Talavera JC, Genesca J *et al.* High rate of infectivity and liver disease in blood donors with antibodies to hepatitis C virus. *Ann Intern Med* 1991; **115**: 443–9.
6 Irving WL, Neal KR, Underwood JC, Simmonds PN, James V. Chronic hepatitis in United Kingdom blood donors infected with hepatitis C virus. *BMJ* 1994; **12** (308): 695–6.
7 Salmeron FJ, Palacios A, Perez-Ruiz M *et al.* Epidemiology, serological markers, and hepatic disease of anti-HCV ELISA-2-positive blood donors. *Dig Dis Sci* 1996; **41**: 1933–8.
8 Serfaty L, Nousbaum JB, Elghouzzi MH, Giral P, Legendre C, Poupon R. Prevalence, severity, and risk factors of liver disease in blood donors positive in a second-generation anti-hepatitis C virus screening test. *Hepatology* 1995; **21**: 725–9.
9 Yuki N, Hayashi N, Takehara T *et al.* Serum hepatitis C virus RNA levels and liver injury in volunteer blood donors. *Am J Gastroenterol* 1994; **89**: 1462–6.
10 Mizui M, Tanaka J, Katayama K *et al.* Liver disease in hepatitis C virus carriers identified at blood donation and their outcomes with or without interferon treatment: Study on 1019 carriers followed for 5–10 years. *Hepatol Res* 2007; **37**: 994–1001.
11 Okamoto H, Okada S, Sugiyama Y *et al.* Detection of hepatitis C virus RNA by a two-stage polymerase chain reaction with two pairs of primers deduced from the 5′-noncoding region. *Jpn J Exp Med* 1990; **60**: 215–22.
12 Okamoto H, Sugiyama Y, Okada S *et al.* Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. *J Gen Virol* 1992; **73**: 673–9.
13 Ge D, Fellay J, Thompson AJ *et al.* Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 2009; **461**: 399–401.
14 Suppiah V, Moldovan M, Ahlenstiel G *et al.* IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat Genet* 2009; **41**: 1100–4.
15 Tanaka Y, Nishida N, Sugiyama M *et al.* Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet* 2009; **41**: 1105–9.
16 Thomas DL, Thio CL, Martin MP *et al.* Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* 2009; **461**: 798–801.
17 Tanaka J, Mizui M, Nagakami H *et al.* Incidence rates of hepatitis B and C virus infections among blood donors in Hiroshima, Japan, during 10 years from 1994 to 2004. *Intervirology* 2008; **51**: 33–41.
18 Prati D, Zanella A, Zanuso F *et al.* Sustained response to interferon-alpha2a monotherapy of young blood donors with minimal-to-mild chronic hepatitis C. *J Viral Hepat* 2000; **7**: 352–60.
19 Zeuzem S, Diago M, Gane E *et al.* Peginterferon alfa-2a (40 kilodaltons) and ribavirin in patients with chronic hepatitis C and normal aminotransferase levels. *Gastroenterology* 2004; **127**: 1724–32.
20 Puoti C, Pellicelli AM, Romano M *et al.* Treatment of hepatitis C virus carriers with persistently normal alanine aminotransferase levels with peginterferon alpha-2a and ribavirin: a multicentric study. *Liver Int* 2009; **29**: 1479–84.
21 Poynard T, Ratziu V, Charlotte F, Goodman Z, McHutchison J, Albrecht J. Rates and risk factors of liver fibrosis progression in patients with chronic hepatitis C. *J Hepatol* 2001; **34**: 730–9.
22 Cividini A, Rebucci C, Silini E, Mondelli MU. Is the natural history of hepatitis C virus carriers with normal aminotransferase really benign? *Gastroenterology* 2001; **121**: 1526–7.
23 Ryan KE, MacLennan S, Barbara JA, Hewitt PE. Follow up of blood donors positive for antibodies to hepatitis C virus. *BMJ* 1994; **308**: 696–7.
24 Tanaka J, Koyama T, Mizui M *et al.* Total numbers of undiagnosed carriers of hepatitis C and B viruses in Japan estimated by age- and area-specific prevalence on the national scale. *Intervirology* 2011; **54**: 185–95.
25 Yoshizawa H, Tanaka J, Miyakawa Y. National prevention of hepatocellular carcinoma in Japan based on epidemiology of hepatitis C virus infection in the general population. *Intervirology* 2006; **49**: 7–17.
26 Tanaka J, Kumada H, Ikeda K *et al.* Natural histories of hepatitis C virus infection in men and women simulated by the Markov model. *J Med Virol* 2003; **70**: 378–86.

27 El-Serag HB. Hepatocellular carcinoma and hepatitis C in the United States. *Hepatology* 2002; **36:** S74–83.

28 Ikeda K, Saitoh S, Arase Y *et al.* Effect of interferon therapy on hepatocellular carcinogenesis in patients with chronic hepatitis type C: A long-term observation study of 1,643 patients using statistical bias correction with proportional hazard analysis. *Hepatology* 1999; **29:** 1124–30.

29 Alter MJ. Epidemiology of hepatitis C virus infection. *World J Gastroenterol* 2007; **13:** 2436–41.

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Comprehensive miRNA Expression Analysis in Peripheral Blood Can Diagnose Liver Disease

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Abstract

Background: miRNAs circulating in the blood in a cell-free form have been acknowledged for their potential as readily accessible disease markers. Presently, histological examination is the golden standard for diagnosing and grading liver disease, therefore non-invasive options are desirable. Here, we investigated if miRNA expression profile in exosome rich fractionated serum could be useful for determining the disease parameters in patients with chronic hepatitis C (CHC).

Methodology: Exosome rich fractionated RNA was extracted from the serum of 64 CHC and 24 controls with normal liver (NL). Extracted RNA was subjected to miRNA profiling by microarray and real-time qPCR analysis. The miRNA expression profiles from 4 chronic hepatitis B (CHB) and 12 non alcoholic steatohepatitis (NASH) patients were also established. The resulting miRNA expression was compared to the stage or grade of CHC determined by blood examination and histological inspection.

Principal Findings: miRNAs implicated in chronic liver disease and inflammation showed expression profiles that differed from those in NL and varied among the types and grades of liver diseases. Using the expression patterns of nine miRNAs, we classified CHC and NL with 96.59% accuracy. Additionally, we could link miRNA expression pattern with liver fibrosis stage and grade of liver inflammation in CHC. In particular, the miRNA expression pattern for early fibrotic stage differed greatly from that observed in high inflammation grades.

Conclusions: We demonstrated that miRNA expression pattern in exosome rich fractionated serum shows a high potential as a biomarker for diagnosing the grade and stage of liver diseases.

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Introduction

MicroRNAs (miRNAs) are a gene family that is evolutionarily conserved and have important roles in the control of many biological processes, such as cellular development, differentiation, proliferation, apoptosis, and metabolism [1]. Aberrant expression of miRNAs in liver tissue has been implicated in the progression of liver fibrosis, and hepatocarcinogenesis [2,3,4]. Recently, two independent groups showed that miR-122 plays a critical role in the maintenance of liver homeostasis and anti-tumor formation [5,6].

Exosome in one of the endoplasmic reticulum carries mRNAs and miRNAs [7]. Recently, it has become clear that exosome perform intercellular signaling through miRNA. miRNAs are released through a ceramide-dependent secretory machinery and are then transferred and become functional in the recipient cells [8]. In a prior study using human blood and cultured cells, several miRNAs were selectively packaged into microvesicle (MV) and actively secreted [9]. In another study, miRNAs originating from EBV was transported by exosome and then participated in the immune response of host cells [10]. In HCC cells as well, this type of exosome-mediated miRNA transfer is an important mechanism of intercellular communication [11].

It has also become clear that exosome can adjust to immune function, control infection or carry the virus itself. Exosomes of T, B and dendritic immune cells contain a repertoire of miRNAs that differ from that of their parent cells [12,13]. Exosomes released from nasopharyngeal carcinoma cells harboring latent EBV were shown to contain LMP1, signal transduction molecules, and virus-encoded miRNAs [14]. Retroviruses evade adaptive immune responses by using nonviral or host exosome biogenesis pathways to form infectious particles and as a mode of infection [15].

Recent evidence has shown that the expression patterns of serum or plasma miRNAs are altered in several diseases, in particular heart disease, sepsis, malignancies, and autoimmune diseases (reviewed in [16]). Discoveries such as this is encouraging and has propelled further research leading to the hypothesis that circulating miRNAs are detectable in serum and plasma in a form sufficiently stable to serve as biomarkers [17,18]. One such example is that tumour-associated miRNAs were found in the serum of diffuse large B-cell lymphoma patients [19]. In other examples, serum levels of miR-34a and miR-122 were associated with histological disease severity in patients with CHC or non-alcoholic fatty-liver disease (NAFLD) [20]. In fact, the serum level of miR-122 strongly correlates with serum ALT activity and with necro-inflammatory activity in patients with CHC and elevated ALT levels. However, there seems to be no significant correlation between fibrosis stage and functional capacity of the liver [21]. The expression levels of miR-122 and miR-194 correlated negatively with age in patients with CHB and HBV associated acute-on-chronic liver failure [22]. The expression level of miR-122 in serum was found to be closely related to non drug-induced acute liver injury [23]. Based on the above, it comes as no surprise that recently, the expression profile from extracellular miRNA is being used clinically to diagnose various diseases.

Here, in order to obtain data with high resolution that is reproducible, we extracted MVs from serum using exoquick and then performed a comprehensive microarray analysis. We attempted to diagnose HCV infection, and ascertain the degree of liver inflammation and fibrosis stage using exosome-rich fractioned miRNA. In short, we investigate if serum-derived miRNAs had the potential to serve as non-invasive bio-markers for various liver diseases.

Results

Reproducible Gene-analysis Using Microarray

In microarray experiments, serum analysis is comparatively easy; however, the downside is that the accuracy and reproducibility of the results are usually not satisfactory. To circumvent this drawback, we devised a procedure that would give us higher accuracy and reproducibility. Serum samples from NL subjects were prepared and divided into two groups; for the first, RNA was extracted using exoquick treated serum, and in the second, RNA was extracted from total serum. Next, miRNA expression was analyzed using Agilent miRNA microarray. The above procedure was performed independently twice (**Fig. 1A**). We compared the miRNA expression pattern among the four microarray results (**Fig. 1B**) and found that miRNA expression analysis using exoquick was the more reliable and reproducible (**Fig. 1C**).

Exosome from normal human prostatic cell lines PNT-2, was yielded by the conventional ultra-centrifugation method [8]. We prepared serums with and without exoquick treatment and performed immunoblot analysis with anti-CD63 (**Fig. 1D**). Bands of the expected relative sizes were detected in serum treated with exoquick. We designated RNA extracted using exoquick treated serum as exosome-rich fractionated RNA.

Unique Expression Pattern of miRNA in CHC

We attempted to diagnose CHC using the miRNA expression pattern found in the peripheral blood samples from 64 CHC and 24 NL. The expression of nine miRNAs (miR-1225-5p, miR-1275, miR-638, miR-762, miR-320c, miR-451, miR-1974, miR-1207-5p, and miR-1246) allowed us to categorize patients as CHC or NL with 96.59% accuracy (**Fig. 2, 3** Table 1 and Table S1). As shown in **Fig. 2C**, CHC and NL were well differentiated due to their distinct miRNA expression patterns. The expression pattern of 12 miRNAs led to the distinction of CHC, CHB, NASH, and NL with 87.50% accuracy (**Fig. 4, S1A,** and Table S1). The accuracy of determining whether samples were CHC or CHB, CHC or NASH, CHB or NASH was 98.35%, 97.37%, and 87.50%, respectively. The accuracy of judging whether samples were NL or CHB, NL or NASH, was 89.29% and 88.89%, respectively (**Fig. 3, S1B** and Table S1). Unlike CHC and NL, there were relatively fewer analyses done of CHB and NASH (due to a small sample size), therefore, we used *"in silico"* resampling to overcome any possible bias. With *"in silico"* we found that it was highly reproducible to determine with high accuracy whether samples were CHC, CHB, NASH, or NL, CHC or CHB, CHC or NASH, CHC or NL, CHB or NASH, CHB or NL, or finally NASH or NL (**Fig. S2 to S8** and Supporting Information).

In order to validate our above-mentioned classifications, we prepared a separate independent sample consisting of 31 CHC, 16 CHB, and 8 NASH. We established miRNA expression patterns using microarray for each of these chronic liver disease groups. We tried to discriminate among the classifications in the independent cohort using the semi-supervised learning method [24] based only on the labels in the original sample group and the selected miRNAs shown in Table S1. The accuracy of judging whether samples were CHB or CHC, CHC or NASH, CHB or NASH, was 74.47%, 87.18%, and 79.19%, respectively (**Fig. S9**, Table 1, and Supporting Information). During the process of obtaining these results, we noticed that different versions of the Feature Extraction (FE) Software provided slightly different results, however it was not possible to fully unify these versions of FE. This may explain the relatively lower performance of the independent group compared with the original samples that mostly used the same FE Software versions.

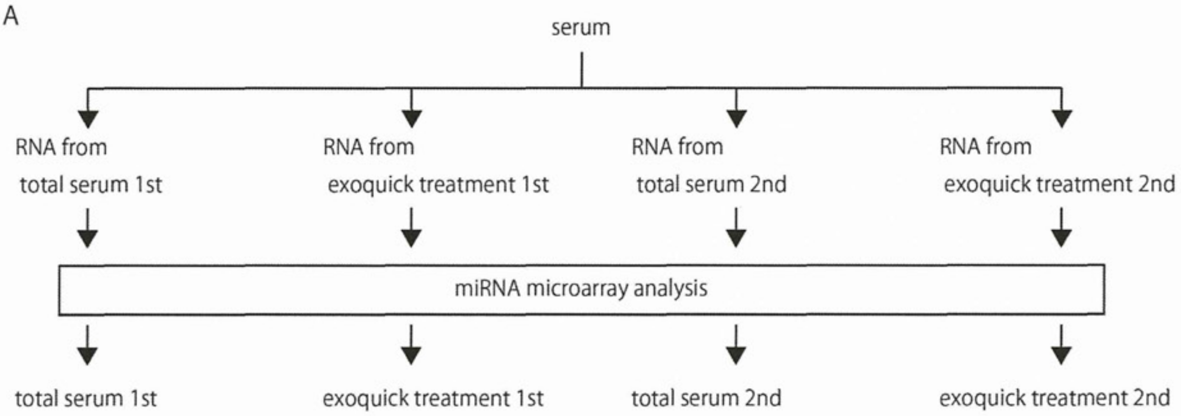
miRNA Expression Correlates with the Grade of Liver Inflammation

The grade of inflammation for CHC patients was ascertained by liver histological examination, and then samples were divided into four groups A0, A1, A2, and A3 based on their fibrosis stage. miRNA expression profiles were then established for CHC according to each of their inflammation grade. From the four groups (A0 to A3), a combination of six arbitrary pairs is possible. miRNAs which had significant differential expression in five or more of the six pairs were extracted ($p<0.05$). Five miRNAs (miR-1914*, miR-193a-5p, miR-22, miR-659, and miR-711) had expression levels that increased as the severity of liver inflammation progressed. On the other hand, the expression levels of nine miRNAs (miR-1274b, miR-197, miR-1974, miR-21, miR-34a, miR-451, miR-548d-5p, miR-760, and miR-767-3p) significantly decreased with the progression of liver inflammation (**Fig. 5, S10** and Table S2).

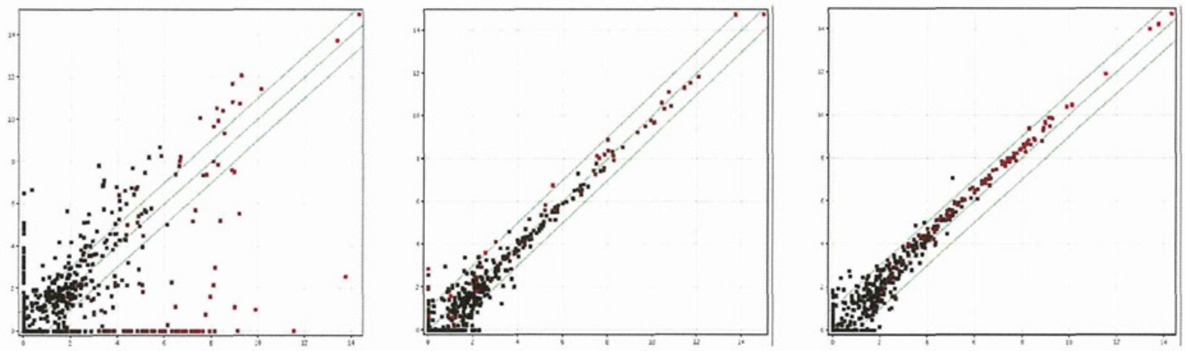
The Grade of Liver Fibrosis Corresponded with the Expression Level of miRNAs

As previously noted, CHC samples were divided into F0, F1, F2, and F3 based on patients' fibrotic stage. From these four fibrotic groups, a combination of six arbitrary pairs were possible. miRNAs that had significant differential expression in all six pairs were extracted ($p<0.05$). The expression levels of two miRNAs (miR-483-5p and miR-671-5p) significantly increased the higher the fibrotic stage and the expression level of 14 miRNAs (let-7a, miR-106b, miR-1274a, miR-130b, miR-140-3p, miR-151-3p, miR-181a, miR-19b, miR-21, miR-24, miR-375, miR-548l, miR-93, and miR-941) became progres-

A


serum
RNA from
total serum 1st
RNA from
exoquick treatment 1st
RNA from
total serum 2nd
RNA from
exoquick treatment 2nd
miRNA microarray analysis
total serum 1st
exoquick treatment 1st
total serum 2nd
exoquick treatment 2nd

B



C

	exoquick 1st	total serum 1st	exoquick 2nd	total serum 2nd
exoquick 1st	1	0.836	0.998	0.809
total serum 1st	–	1	0.831	0.930
exoquick 2nd	–	–	1	0.821
total serum 2nd	–	–	–	1

D

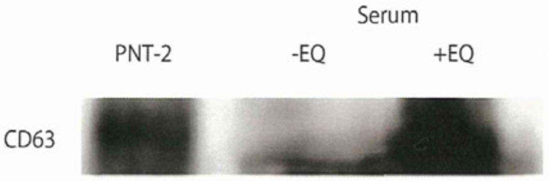
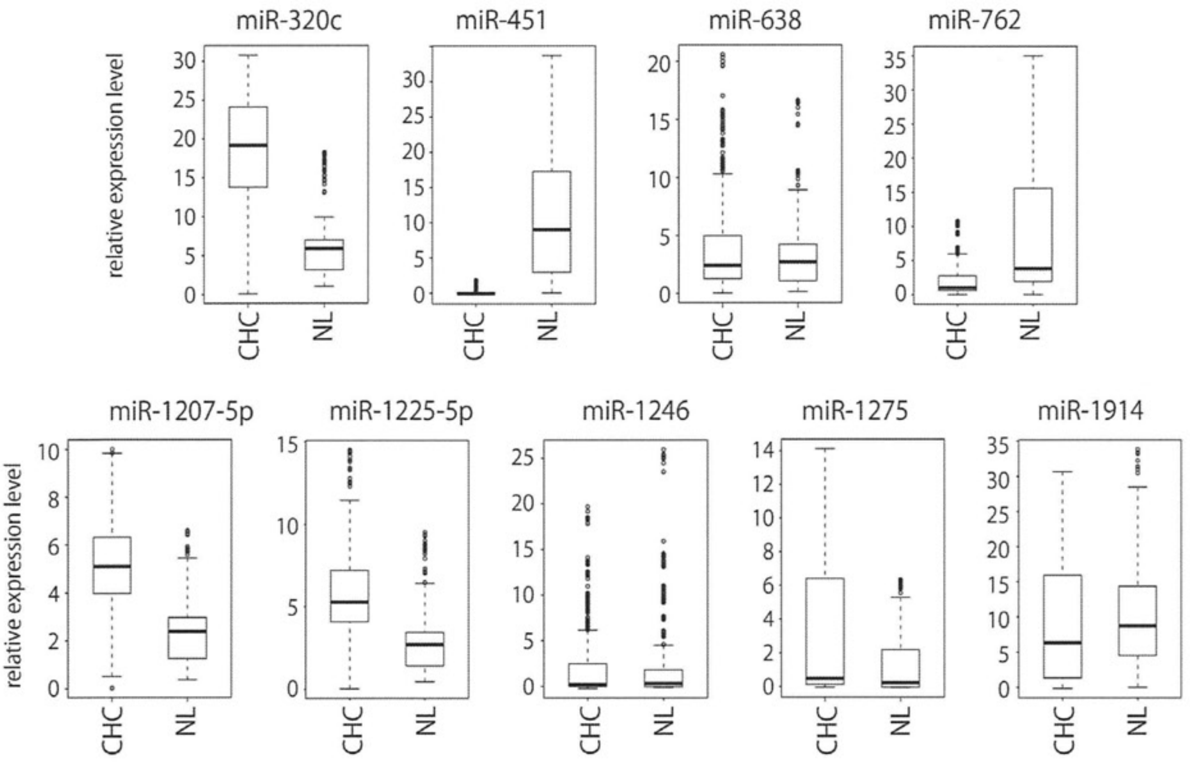

Serum
PNT-2
-EQ
+EQ
CD63

Figure 1. The method used to obtain reproducible data for microarray analysis conducted on serum-extracted samples. A. NL patients' serum were sampled twice. In the first, RNA was extracted first from untreated serum, and then extracted again from serum treated with exoquick. In the second serum sample, RNA was also extracted from both untreated serum and serum treated with exoquick. Microarray analysis was conducted for RNA in a total of four samples. B. Reproducibility test of microarray data. Scatter plots comparing non- normalized signal intensities of miRNAs in two independent experiments from human total serum and exosome rich fraction. Red and black denotes high and low miRNA expressions respectively. Total serum extracted first, versus exosome rich fraction first (left), total serum extracted first versus second (middle), and exosome rich fraction extracted first versus second (right). C. Pearson's pairwise correlations of signal intensities of miRNAs from human total serum and exosome rich fraction. D. Western blot was performed for untreated serum, serum extracted by exoquick and exosome fraction from PNT-2, using anti-CD63.

doi:10.1371/journal.pone.0048366.g001

A



B

	result	
	CHC	NL
prediction CHC	🟩 64	3
NL	0	🟩 21

accuracy 96.6%

C

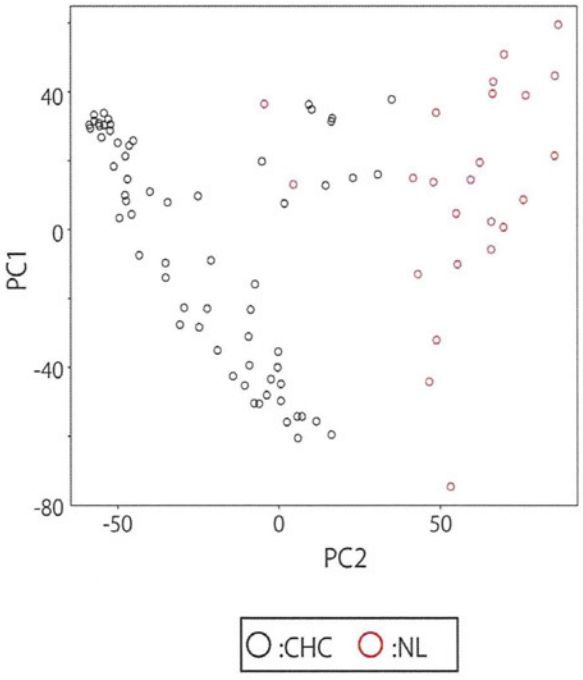


Figure 2. Expression patterns of miRNA used for discriminating between CHC and NL. A. Box plots of expression patterns of the nine miRNAs used for discriminating between CHC and NL. B. Classification of CHC and NL using LOOCV from miRNA expression profile. C. PCA in CHC and NL. The two dimensional embedding of CHC and NL by PCA. The first and second principal component scores computed (not selected for discrimination) of normalized miRNA expression were employed for this plot. Computation was done with ALL.
doi:10.1371/journal.pone.0048366.g002

sively downregulated as liver fibrotic stage increased (**Fig. 6, S11** and Table S2).

Classification of Liver Inflammation Grade and Fibrotic Stage Using miRNA Expression Pattern

We attempted to classify liver inflammation grade and fibrosis stage using miRNA expression pattern. Liver inflammation was diagnosed by Leave One Out Cross-Validation (LOOCV); the accuracy of determining A1 from other inflammation grade was 71.88% and its odds ratio was 7.08. The accuracy of determining A2 and A3 was 75.00% and 82.81%, and their odds ratios were 9.50 and 11.08, respectively. In our study, we were unable to accurately classify A0 because we were limited to only one sample for that grade (**Fig. 7A**). Diagnosis of liver fibrosis by LOOCV showed that determining F0 from the other fibrotic stages had an accuracy of 87.50% and an odds ratio of 14.25. The classification of F1, F2, and F3 had accuracy rates of 65.63%, 70.31%, and 73.44% and odds ratio of 3.16, 6.39 and 5.80, respectively (**Fig. 7B**).

miRNA Expression Level Detected by Real-time qPCR Validated the Microarray Result

Four miRNAs (miR-1207-5p, miR-134, miR-1249, and miR-1183) with expression levels that differed among liver inflammation grades and liver fibrotic stages were chosen in order to confirm the microarray results using stem-loop based real-time qPCR. miRNAs that correlated with other clinical characteristics besides liver fibrosis and inflammation were listed using the Wilcoxon test. We performed two Wilcoxon tests and ranked miRNAs based on their p-value from smallest to largest and selected the miRNAs with the four smallest p-values that were common among the two Wilcoxon tests.

The real-time qPCR result was consistent with the microarray analysis (**Fig. 8**). Here also, we applied *"in silico"* resampling to compensate for the small number of patients used in the real-time qPCR analysis. The results of the "*in silico*" resampling conferred with the results of the real-time qPCR (**Fig. S12**).

miRNA Expression Pattern was Closely Related to Several Clinical Parameters in CHC

Although we observed that miRNA expression correlated with ALT value, we were unable to identify miRNAs that displayed a strong correlation. 12 miRNAs were chosen sequentially from miRNAs with a high absolute correlation coefficient. One to 12 of these selected miRNAs were used to compare the canonical correlation coefficient of the above. When the expression patterns of six of the 12 miRNAs were compared with serum ALT value, the correlation coefficient and p-value were 0.44 and 4.91E−02, respectively. Similarly, when serum Albumin value was compared with the expression pattern of all 12 miRNAs, the correlation coefficient and p-value were 0.59 and 2.04E−02, respectively. Finally when the amount of serum HCVRNA was compared with the expression pattern of 12 miRNAs, the resulting correlation coefficient and p-value were 0.59 and 1.89E−02, respectively (**Fig. 9, S13** and Table S3).

Expression Pattern of a Several miRNAs Correlated to Serum and Hepatic Tissue

In a previous report, we described the miRNA expression pattern found in liver tissues obtained from 105 CHC [2]. From this group, we analyzed the miRNA expression of hepatic tissue and serum in 60 samples. We observed that the expression pattern of three miRNAs (miR-134, miR-200b, miR-324-5p) in hepatic tissue negatively correlated with that in serum, and the expression pattern of miR-370 in hepatic tissue positively correlated with that in serum ($p<0.05$) (Table S4). However, there was no significant correlation between the expression pattern of miR-122 in the hepatic tissue and serum (**Fig. S14** and Table S4).

Discussion

In this comprehensive miRNA analysis in various chronic liver diseases, we observed that aberrant expression of miRNAs was closely related to disease progression. Based on this, we believe that these miRNAs are potential readily accessible biomarkers, useful for diagnosing hepatic viral infection and for grading or staging liver diseases.

Many investigators have elected to use miRNA from serum instead of miRNA from exosome as the candidate for diagnosing diseases [18,20,22,25,26]. In our study, when exoquick was used, exosome could not be isolated therefore other MVs similar in size to exosome were also extracted. In other words, exoquick not only collected miRNAs contained in exosome, but also miRNA that were or were not combined with protein. Despite this, we found that exoquick delivered results that were superior to those obtained without exoquick. Therefore, although the process of analyzing miRNA from serum is simple, we chose to analyze miRNA from exosome rich fraction since it has a higher rate of reproducibility. Moreover, since exosome is closely related to intercellular signaling [14,27], it is expected that data obtained by exosome analysis can clarify the mechanism of chronic infection and inflammation [28].

When we extended our analysis from miR-122 to all miRNAs, it became clear that the expression level of several miRNAs correlated with the progression of liver fibrosis. In fact, recent studies have stated that when the expression levels of adequate numbers of miRNAs is used to identify disease, diagnostic ability is significantly higher than using a single miRNA [29]. In this study, when liver fibrosis was diagnosed using miRNA expression, distinguishing between F0 and F1-3 was done with 87.50% accuracy. Since F0 cannot be distinguished from other stages of chronic liver disease using blood examination, we propose that using miRNA expression pattern may be useful for diagnosing chronic liver disease that is in the early stage.

Previous studies have shown that the level of miR-122 in blood plasma increased earlier than in ALT in the presence of toxic liver injury in rodents [30]. Serum levels of miR-122 in patients with CHC are frequently elevated compared with healthy individuals [21]. Bihrer et al. mentioned that variations in the concentration of miR-122 in serum or plasma tend to be more specific for liver diseases than ALT and AST. This is because miR-122 is almost exclusively expressed in the liver, whereas ALT and AST originate from skeletal muscles and other tissues, therefore their diagnostic value is low [31]. In our study, the expression level of miR-122 had

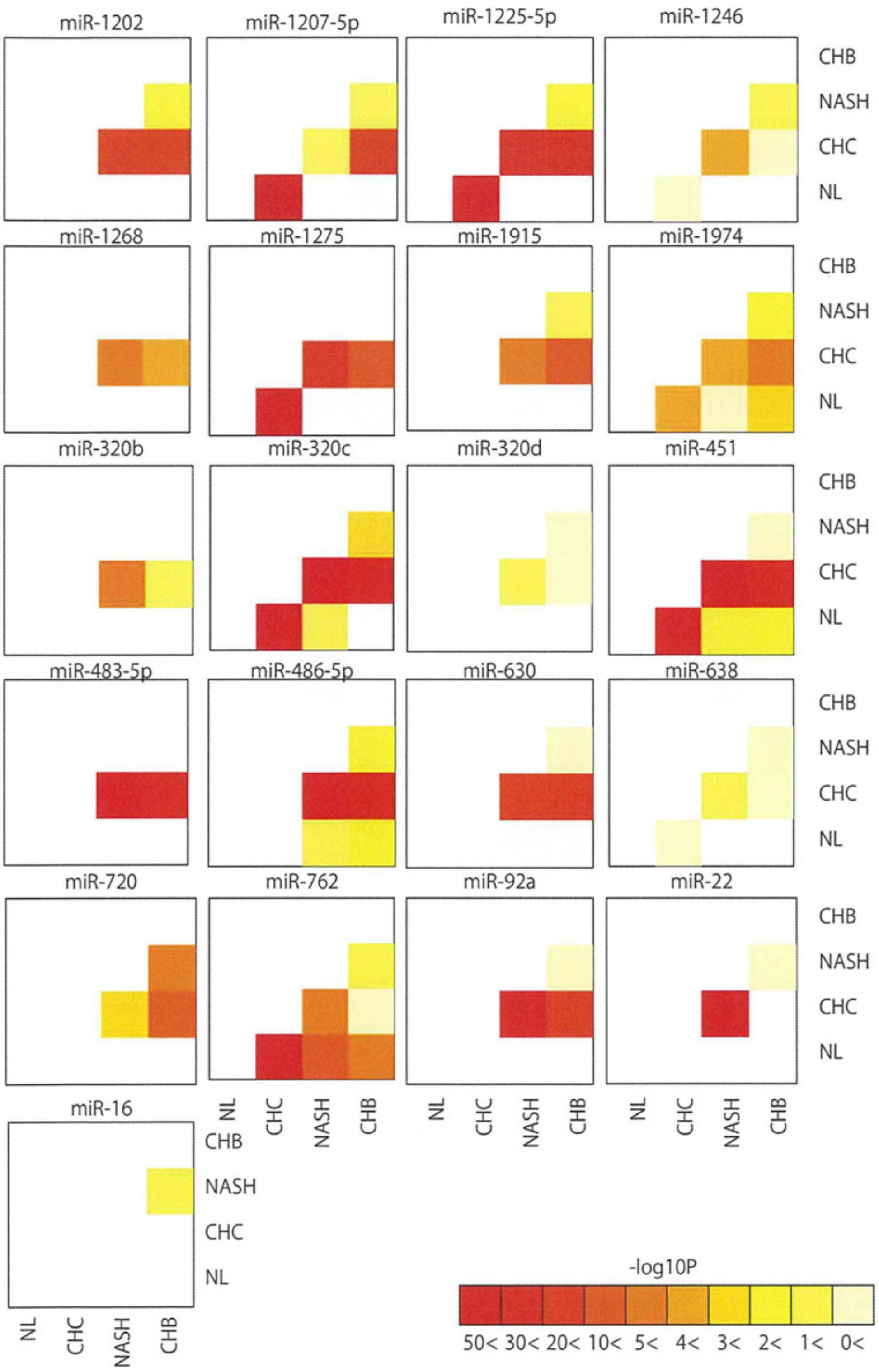


Figure 3. Pairwise heatmap of the miRNAs used for classifying two arbitrary groups. Pairwise heatmap showed the miRNAs and their p-value of two arbitrary groups.
doi:10.1371/journal.pone.0048366.g003

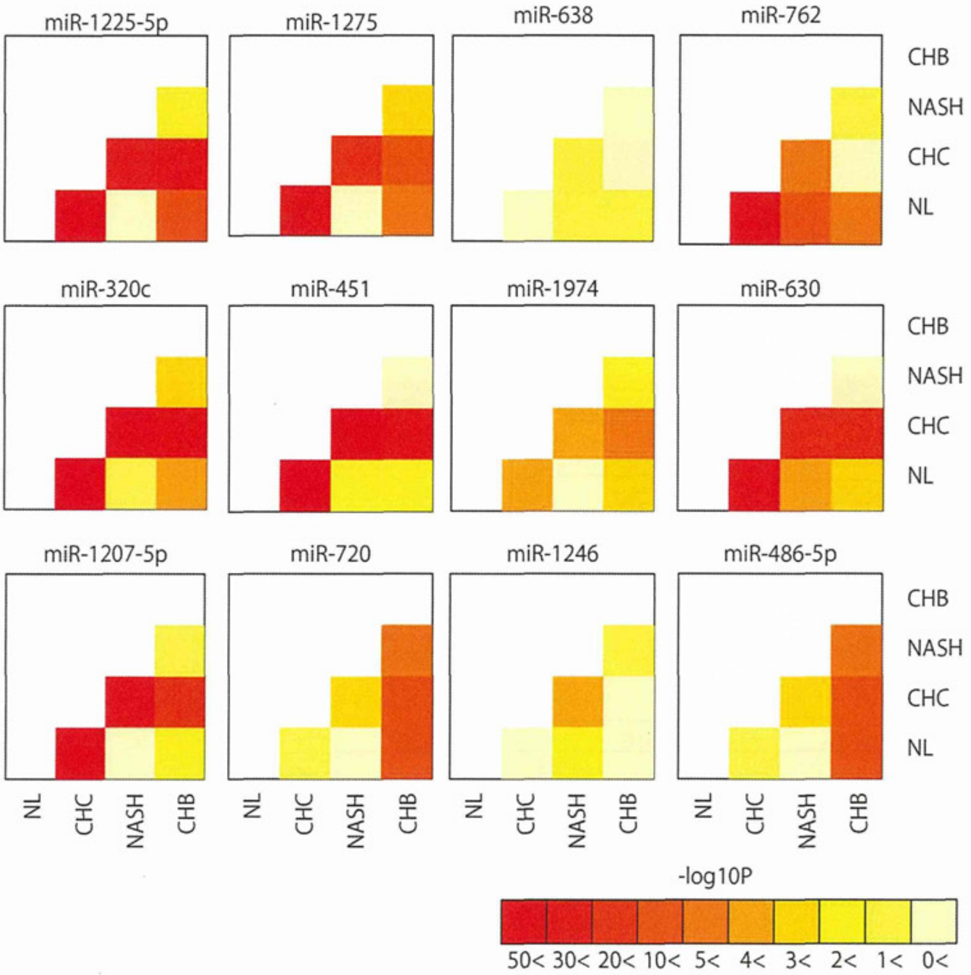


Figure 4. Pairwise heatmap of the miRNAs used for classifying among four groups.
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a significant positive correlation with the grade of liver inflammation, serum albumin value, or serum HCVRNA value. However, miR-122 expression did not significantly correlate with liver fibrosis stage. Moreover, there was no correlation between the expression level of miR-122 in liver tissue, and that in serum in the same 60 samples (**Fig. S14**). The expression pattern of only four miRNAs out of total liver tissue miRNAs correlated with the expression patterns of miRNA found in the serum (Table S4). Most serum miRNA had expression patterns that differed from those observed in hepatic tissue samples. Moreover, we observed differences in miRNAs expression between various tissues [32]. These differences were observed even in tissues taken from the same subject; at present we are unclear as to the reason for this phenomenon.

In regards to the progression of liver fibrosis and the expression pattern of miR-21, previous studies concur with our result that miR-21 expression level significantly decreased in response to the progression of liver fibrosis [20]. Taken together, this suggests that any miRNAs that may have been emitted from liver tissue cannot be detected in serum after hepatic cell injury.

The expression pattern of many miRNAs in serum positively correlated with serum ALT, albumin, and HCVRNA levels in this study (**Fig. 9, S13** and Table S3). This result contradicts prior assumptions that no correlation exists between serum miR-122 and HCVRNA serum levels [21]. Three likely reasons for this difference in results are: 1) the detection method used (real-time qPCR versus microarray), 2) the difference in the subjects' ages (the subjects in this study were older), and 3) the difference in the amount of miRNAs (multiple miRNAs vs. a single miRNA) used to identify the clinical parameters of the disease.

CHC and NL were classified with a high level of accuracy using the expression pattern of miRNA. In order to elucidate if the miRNA expression in CHC is common to other chronic liver diseases including CHB, we compared the miRNA expression pattern of CHC with those of NASH and CHB. The result of this analysis was that CHC could be clearly distinguished from both CHB and NASH. These results demonstrate that the varying forms of chronic liver disease have their own unique miRNA expression pattern. NASH is a histological diagnosis that rests on a combination of features and can only be confirmed by liver biopsy. Recently, NASH was diagnosed by first determining the existence of NAFLD from blood samples and then performing an ultrasound tomography. Finally, liver fibrosis stage was determined by Fibroscan

Table 1. Characteristics of subjects in this study of original samples and independent samples.

Original samples

Characteristics	CHC	CHB	NASH	NL
Gender	F: 34/M: 30	F: 2/M: 2	F: 3/M: 9	F:11/M: 13
Age (years)	59.5±8.3	46.8±14.5	52.3±13.1	50.8±12.0
AST (IU/L)	50.1±29.8	83.3±53.7	46.2±16.0	N.D
ALT (IU/L)	57.6±40.6	167.8±170.3	74.5±34.9	N.D
WBC (x10^{3}/mm^{3})	5.1±1.5	4.7±1.5	6.2±1.6	N.D
Platelet (x10^{4}/mm^{3})	16.6±5.9	14.8±6.3	24.7±8.0	N.D
Total Bilirubin (mg/dl)	0.65±0.22	0.83±0.40	0.76±0.25	N.D
Weight (kg)	57.9±9.18	58.8±4.3	74.9±24.8	59.6±9.6
ALP (IU/L)	267.0±88.4	223.3±25.0	232.7±36.2	N.D
γGTP (IU/L)	46.9±42.3	77.3±82.2	58.4±20.9	N.D
Hemoglobin (g/dl)	13.8±1.2	14.5±0.59	14.7±1.6	N.D
Albumin (g/dl)	4.1±0.4	4.2±0.5	4.4±0.3	N.D

Independent samples

Characteristics	CHC	CHB	NASH
Gender	F: 18/M: 13	F: 10/M: 6	F: 6/M: 2
Age (years)	59.5±8.3	46.8±14.5	54.8±12.7
AST (IU/L)	50.1±29.8	83.3±53.7	80.9±50.0
ALT (IU/L)	57.6±40.6	167.8±170.3	108.9±76.2
WBC (x10^{3}/mm^{3})	5.1±1.5	4.7±1.5	5.5±1.8
Platelet (x10^{4}/mm^{3})	16.6±5.9	14.8±6.3	19.3±7.6
Total Bilirubin (mg/dl)	0.65±0.22	0.83±0.40	0.73±0.25
Weight (kg)	57.9±9.18	58.8±4.3	66.4±9.9
ALP (IU/L)	267.0±88.4	223.3±25.0	278.6±100.6
γGTP (IU/L)	46.9±42.3	77.3±82.2	130.1±81.23
Hemoglobin (g/dl)	13.8±1.2	14.5±0.59	13.6±1.4
Albumin (g/dl)	4.1±0.4	4.2±0.5	3.8±0.3

Abbreviations; CHC, chronic hepatitis C; CHB, chronic hepatitis B; NASH, non alcoholic steatohepatitis; NL, normal liver (healthy control); N.D, no data.

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(reviewed in [33]). However, when the results of these and other measures fail to yield a diagnosis then a pathology evaluation is necessary. Using "*in silico*" resampling to increase the reliability of our data, has led us to believe that NASH diagnosis may be possibly through blood examination.

We tested the reliability of our analysis in two ways and obtained reproducible results in both cases. First we enrolled an independent sample group, and second, we created virtual cohorts using in silico resampling to overcome our small sample size.

In this study we concluded that miRNA profiling is a promising alternative to diagnosing liver disease. This is based on our demonstration that the following evaluations could be performed using suitable miRNA expression profiles (1) determining the stage or grade of chronic liver disease, (2) ascertaining the clinical status of chronic liver diseases, and (3) distinguishing among various forms of chronic liver diseases. While these results suggest there is great potential and benefit of miRNA profiling, future studies in a larger population of CHC patients are warranted to fully elucidate the diagnostic potential of serum miRNA expression.

Materials and Methods

Patient Selection

A cohort of 64 CHC, 4 CHB, and 12 NASH patients who had undergone liver biopsy, as well as 24 healthy control subjects was enrolled. We also prepared independent samples consisting of 31 CHC, 12 CHB, and 8 NASH to validate our results. Patient characteristics are summarized in Table 1 and detailed clinical data is depicted in Table S5. The criteria for exclusion for CHC, CHB, and NASH were: co-infection with human immunodeficiency virus (HIV) types 1 and 2, decompensated liver disease, organ transplantation, immune suppression, autoimmune disorders, consumption of >20 g/day alcohol, and past history of intravenous drug abuse. Healthy controls were selected if they were not infected with HBV, HCV, nor HIV, had normal liver function tests, and had no history of liver disease.

All patients or their guardians provided written informed consent, and Ogaki Municipal Hospital and Kyoto University Graduate School and Faculty of Medicine's Ethics Committee approved all aspects of this study in accordance with the Helsinki Declaration.

Liver Histology and Blood Examination

A liver biopsy specimen was collected from each patient before anti-viral treatment. Histological grading and staging of CHC liver biopsy specimens were performed according to the Metavir classification system [34]. NASH was diagnosed histologically [35].

Serum HCV RNA was quantified before IFN treatment using Amplicor-HCV Monitor Assay (Roche Molecular Diagnostics Co., Tokyo, Japan), while serum HBV DNA was quantified before treatment using Amplicor HBV Monitor Assay (Roche). Pretreatment blood tests were conducted to determine each patient's level of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, alkaline phosphatase, gamma-glutamyl transpeptidase, white blood cell (WBC), platelets, and hemoglobin.

Blood Sampling

Peripheral blood was collected from all subjects directly into serum tubes before anti-viral treatment. The tubes were centrifuged at 1,500 *g* for 10 min at 4°C, sera were aliquoted and additionally centrifuged at 2,000 *g* to completely remove any remaining cells. Sera were stored at −80°C until use.

RNA Preparation

Total RNA from 200 ul of serum was prepared using miRNeasy mini kit (Qiagen, Hilden Germany) according to the manufacturer's instruction. Exosome rich fractionated RNA was prepared using Exoquick (System Biosciences, CA, USA). Briefly, 900 ul of serum was mixed with 250 ul of Exoquick and incubated for 12 hr at 4°C. The tubes were centrifuged at 1500 g for 30 min at room temperature and then supernatant was discarded. The pellet was dissolved with 200 ul of PBS with vigorous vortex. RNA was extracted using miRNeasy mini kit (Qiagen).

Immunoblot Analysis and Exosome Preparation

The procedure for exosome preparation has been previously described [8]. SDS-PAGE gels, SuperSep Ace 5–20% (194–15021) (Wako, Osaka, Japan), were calibrated with Precision Plus Protein Standards (161–0375) (Bio-Rad), and anti-CD63 (1:200)

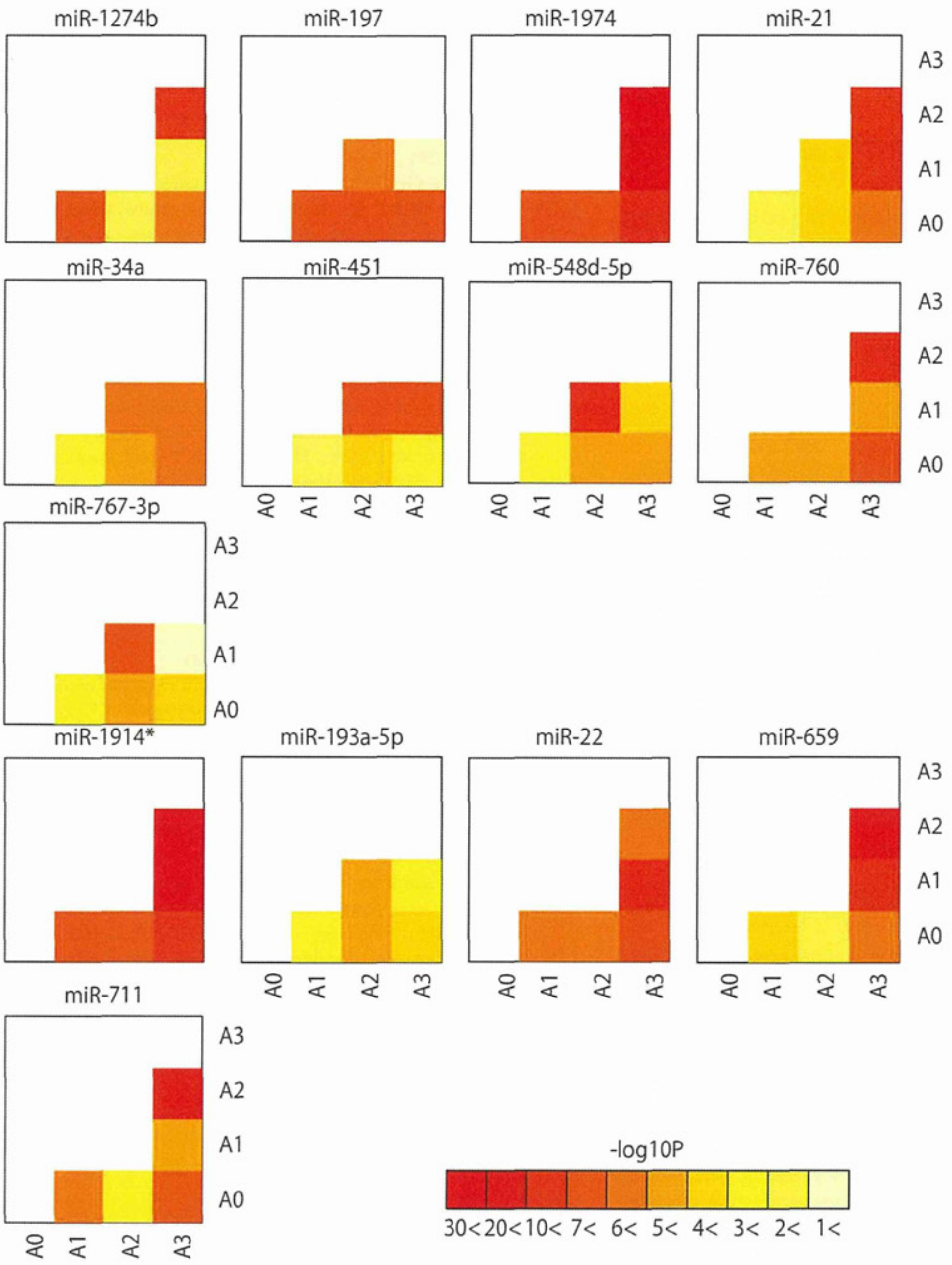

Figure 5. Significantly differentially expressed miRNAs according to liver inflammation grade. Pairwise heatmap showing the miRNAs and p-value of two arbitrary grades.
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was used as primary antibodies. The dilution ratio of each antibody is indicated in parentheses. Two secondary antibodies (peroxidase-labeled anti-mouse and anti-rabbit antibodies) were used at a dilution of 1:5000. Bound antibodies were visualized by chemiluminescence using the ImmunoStar LD (Wako) and luminescent images were analyzed by a LuminoImager (LAS-3000; Fuji Film, Inc.). Only gels for CD63 (BD, NJ, USA) detection were run under non-reducing conditions. To exclude the albumin and IgG in serum, Albumin & IgG Depletion SpinTrap kit was used (GE health care, WI, USA). After aliquots isolation, exosome-contained fraction was isolated by Exoquick according to standard instructions.

miRNA Microarray

To detect serum miRNA, 60 ng of RNA was labeled and hybridized using the Human microRNA Microarray Kit (Rel