# Circulating MicroRNA-122 and Fibrosis Stage Predict Mortality of Japanese Patients With Histopathologically Confirmed NAFLD

Norio Akuta,[1] Yusuke Kawamura,[1] Yasuji Arase,[1] Satoshi Saitoh,[1] Shunichiro Fujiyama,[1] Hitomi Sezaki ,[1] Tetsuya Hosaka,[1] Masahiro Kobayashi,[1] Mariko Kobayashi,[2] Yoshiyuki Suzuki,[1] Fumitaka Suzuki,[1] Kenji Ikeda,[1] and Hiromitsu Kumada[1]

**The impact of circulating microRNA-122 (miR-122) on mortality in patients with histopathologically confirmed nonalcoholic fatty liver disease (NAFLD) remains unclear. We analyzed the overall survival rates in 441 Japanese patients with histopathologically confirmed NAFLD after a median follow-up period of 4.7 years. We also determined the clinicopathologic, genetic, and epigenetic parameters, including serum miR-122 levels, for prediction of mortality. Of the 441 study patients, 21 (4.8%) died during the follow-up period. The cumulative survival rates were 95.4% and 90.6% at the end of 5 and 10 years, respectively. Multivariate analysis identified history of liver cancer (presence; hazard ratio [HR], 4.94; 95% confidence interval [CI], 1.84-13.3), serum miR-122 (<1.00 fold change; HR, 4.35; 95% CI, 0.06-0.83), and fibrosis-4 index (FIB-4 index) (≥1.30; HR, 15.7; 95% CI, 2.01-122) as significant risk factors of mortality. Cumulative survival rates varied significantly among patients with none/one risk factor, two risk factors, and three risk factors; particularly, the survival rate of patients with three risk factors was significantly lower than those with two risk factors and none/one risk factor. Two or more risk factors were identified in 17 of 21 (81.0%) death cases. *Conclusion:* The importance of serum miR-122 and FIB-4 index as risk factors for mortality in Japanese patients with histopathologically confirmed NAFLD is shown. Early diagnosis based on the presence of more than one risk factor and early treatment might improve the prognosis. (*Hepatology Communications* 2020;4:66-76).**

The most common liver disease worldwide is nonalcoholic fatty liver disease (NAFLD).[1-6] Liver pathology ranges from the typically benign nonalcoholic fatty liver to nonalcoholic steatohepatitis (NASH), which may progress to liver cirrhosis, liver cancer, and liver failure.[7] Studies have suggested that the fibrosis stage is a more reliable predictor of liver-specific mortality than the NAFLD activity score (NAS).[8] The fibrosis stage, but not other histopathologic features of steatohepatitis, was reported to be an independent and significant predictor of overall mortality, liver transplantation, and liver-related events.[9]

*Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; FIB-4, fibrosis-4; HR, hazard ratio; miR-122, microRNA-122; NAFLD, nonalcoholic fatty liver disease; NAS, NAFLD activity score; NASH, nonalcoholic steatohepatitis; PNPLA3, patatin-like phospholipase domain containing protein 3; SNP, single-nucleotide polymorphism; TM6SF2, transmembrane 6 superfamily member 2.*

*Received August 20, 2019; accepted October 11, 2019.*

*Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1445/suppinfo.*

*Supported in part by the Japan Agency for Medical Research and Development (Grant-in-Aid #JP19fk0210040 to N.A.) and the Ministry of Health, Labor, and Welfare of Japan (Grant-in-Aid for Scientific Research and Development #19HC1001 to N.A.).*



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*DOI 10.1002/hep4.1445*

*Potential conflict of interest: Dr. Akuta and Dr. Arase are on the speakers' bureau for AbbVie. Dr. Kobayashi is on the speakers' bureau for Eisai Co. Ltd. Dr. Kumada is on the speakers' bureau for MSD, Gilead, AbbVie, Eisai Co. Ltd., and Sumitomo Dainippon Pharma. The other authors have nothing to report.*

Various environmental, genetic, and epigenetic factors are known to influence the development and progression of NAFLD. For example, the level of circulating microRNA-122 (miR-122), an epigenetic factor, is reported to be associated with the histopathologic severity of liver disease,[10-12] and measurement of miR-22 can help in early prediction of histologic improvement following treatment with glucose-lowering agents.[13] We recently reported that serum miR-122 levels vary with the stage of liver cancer and/or NASH-related histopathologic changes. In this regard, longitudinal follow-up studies of patients with liver cancer demonstrated a tendency for serum miR-122 levels to decrease before progression to the fibrosis stage.[12] However, the relationship between serum miR-122 levels and mortality of patients with NAFLD remains unclear.

The purpose of the present retrospective study was to investigate the overall survival rates and determine the clinicopathologic, genetic, and epigenetic predictors of mortality in patients with NAFLD. For this purpose, we analyzed the clinical outcome of 441 Japanese patients with histopathologically confirmed NAFLD.

# Patients and Methods

## PATIENTS

This is a retrospective cohort study of patients with histopathologically confirmed NAFLD. Between 1976 and 2019, liver biopsy material was obtained at our hospital from patients with liver dysfunction and/or fatty liver diagnosed by abdominal ultrasonography. Of those patients, the diagnosis of NAFLD was confirmed in 441 patients by histopathology. The median duration of follow-up from diagnosis to death or last visit was 4.7 years (range, 0.0-42.9 years). The clinical features of the patients at the time of histopathologic diagnosis of NAFLD are summarized in Table 1. Patients with histopathologic changes of steatosis in at least 5% of hepatocytes and those with a history of alcohol intake of <20 g/day were included in the analysis. We excluded patients with (1) underlying liver disease (e.g., viral hepatitis, autoimmune hepatitis, drug-induced liver disease, or primary biliary cholangitis); (2) systemic autoimmune diseases (e.g., systemic lupus erythematosus or rheumatoid arthritis); and (3) metabolic diseases (e.g., hemochromatosis, α-1-antitrypsin deficiency, or Wilson's disease).

The study was conducted in compliance with the International Conference on Harmonization Guideline for Good Clinical Practice (E6) and the 2013 Declaration of Helsinki. The study protocol was approved by the Toranomon Hospital Institutional Review Board (#953). Written informed consent for a liver biopsy was provided by all patients.

## DIAGNOSIS AND FOLLOW-UP

In this study, we selected the following liver-related events for study outcome: liver cancer, hepatic encephalopathy, esophagogastric varices with bleeding, ascites, and jaundice. Other outcomes included cardiovascular events (e.g., coronary artery disease, heart valve disease, arrhythmia, heart failure, hypertension, orthostatic hypotension, shock, endocarditis, diseases of the aorta and its branches, disorders of the peripheral vascular system, and stroke) and type 2 diabetes mellitus (defined as high fasting blood glucose level ≥126 mg/dL, high hemoglobin A1c ≥6.5%, use of glucose-lowering agents, or self-reported history of clinical diagnosis).

Mortality was evaluated in all patients. Hematologic and biochemical data were collected at least twice yearly after the diagnosis of NAFLD. Ultrasonography,

**ARTICLE INFORMATION:**

From the [1]Department of Hepatology, Toranomon Hospital and Okinaka Memorial Institute for Medical Research, Tokyo, Japan; [2]Liver Research Laboratory, Toranomon Hospital, Tokyo, Japan.

**ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:**

Norio Akuta, M.D.
Department of Hepatology, Toranomon Hospital
2-2-2 Toranomon, Minato-ku
Tokyo 105-0001, Japan
E-mail: akuta-gi@umin.ac.jp
Tel.: +81-3-3588-1111

**TABLE 1. PATIENT CHARACTERISTICS AT THE TIME OF HISTOLOGIC DIAGNOSIS OF NAFLD**

| Characteristic | Value |
|---|---|
| Demographic data | |
| Number of patients | 441 |
| Sex, male/female, n | 266/175 |
| Age, year* | 52 (20-87) |
| Body mass index, kg/m$^2$* | 26.2 (18.1-42.4) |
| History of cardiovascular events, no/yes, n | 430/11 |
| Presence of previous malignancy (none/liver cancer/other malignancies), n | 385/28/33 |
| Type 2 diabetes mellitus, no/yes, n | 298/144 |
| Hypertension, no/yes, n | 244/198 |
| Hyperlipidemia, no/yes, n | 292/150 |
| Hyperuricemia, no/yes, n | 397/45 |
| Smoking, no/yes, n | 357/84 |
| Histopathologic findings | |
| Steatosis, 5%-33%/>33%-66%/>66%, n | 162/163/113 |
| Lobular inflammation (no foci/<2 foci/2-4 foci/>4 foci per 200× field), n | 28/254/142/14 |
| Ballooning (none/few cells/many cells), n | 40/281/117 |
| Stage, 0/1/2/3/4, n | 50/182/68/108/33 |
| NAFLD activity score, ≤2/3, 4/≥5, n | 38/190/213 |
| Diagnosis according to FLIP algorithm, NASH/non-NASH, n | 398/43 |
| Genetic variation | |
| *PNPLA3* rs738409 (CC/CG/GG/not determined) | 53/128/129/131 |
| *TM6SF2* rs58542926 (CC/CT/TT/not determined) | 234/69/7/131 |
| Circulating microRNA | |
| Serum miR-122, fold change* | 0.99 (0.01-27.42) |
| Laboratory data* | |
| Serum AST, U/L | 43 (3-378) |
| Serum ALT, U/L | 69 (14-783) |
| Gamma-glutamyl transpeptidase, U/L | 71 (11-990) |
| Platelet count, ×10$^3$/mm$^3$ | 213 (40-471) |
| Fasting plasma glucose, mg/dL | 102 (65-287) |
| Hemoglobin A1c, % | 5.9 (4.3-12.6) |
| Uric acid, mg/dL | 5.9 (1.9-11.1) |
| Total cholesterol, mg/dL | 204 (101-370) |
| Triglycerides, mg/dL | 139 (31-1,088) |
| High-density lipoprotein cholesterol, mg/dL | 45 (14-86) |
| Low-density lipoprotein cholesterol, mg/dL | 120 (27-243) |
| Serum ferritin, μg/L | 227 (<10-2,067) |
| High sensitive C-reactive protein, mg/dL | 0.095 (0.004-2.240) |
| FIB-4 index | 1.31 (0.19-14.8) |

*Data are median (range) values.
Abbreviations: FLIP, fatty liver: inhibition of progression; n, number.

computed tomography, or magnetic resonance imaging studies were conducted at least once annually during the follow-up.

## LIVER HISTOPATHOLOGY

Liver biopsy specimens were obtained using a 14-gauge modified Vim Silverman needle (Tohoku University style; Kakinuma Factory, Tokyo, Japan), a 16-gauge core tissue biopsy needle (Bard Peripheral Vascular, Inc., Tempe, AZ), or surgical resection. Liver biopsy samples >1.5 cm and/or containing more than 11 portal tracts were considered adequate for examination and diagnosis. The specimen was fixed in 10% formalin and cut into sections, which were then stained with hematoxylin and eosin, Masson trichrome, silver impregnation, or periodic acid–Schiff after diastase digestion. Four pathologists (Dr. Keiichi Kinowaki, Dr. Fukuo Kondo, Dr. Toshio Fukusato, and Dr. Takeshi Fujii), who were blinded to the clinical findings evaluated each of the specimens, and the final assessment was reached by consensus.

Steatosis grades 0, 1, 2, and 3 corresponded to steatosis of <5%, ≥5% to <33%, ≥33% to <66%, and ≥66% of hepatocytes, respectively. Lobular inflammation with no foci, <2 foci, 2-4 foci, and ≥4 foci per 200× field was scored 0, 1, 2, and 3, respectively. Hepatocyte ballooning of none, few, and many cells was scored as 0, 1, and 2, respectively. NAS represents the sum of scores of steatosis, lobular inflammation, and hepatocyte ballooning (range, 0-8 points).[14] Fibrosis stage was defined as 0, 1, 2, 3, and 4 using the defined criteria.[14,15] NASH was defined according to the fatty liver: inhibition of progression (FLIP) algorithm.[16]

## CLINICAL PARAMETERS

We analyzed the clinicopathologic and genetic parameters that could affect NAFLD prognosis. The fibrosis-4 (FIB-4) index, calculated as (age [year] × aspartate aminotransferase [AST] [IU/L])/(platelet count [10$^9$/L] × √alanine aminotransferase [ALT] [IU/L]), has been used as a parameter for progression of fibrosis.[17] The FIB-4 index is useful for excluding NASH with advanced fibrosis, with values <1.30 considered to represent nonadvanced fibrosis.[5] Obesity was defined as body mass index ≥30.0 kg/m$^2$. Patatin-like phospholipase domain containing protein 3 (*PNPLA3*) rs738409 and transmembrane 6 superfamily member 2 (*TM6SF2*) rs58542926 were genotyped by the TaqMan single-nucleotide polymorphism (SNP) genotyping assay (Applied Biosystems, Foster City, CA).

## MEASUREMENT OF SERUM miR-122

We selected serum miR-122 to represent the epigenetic parameters that could modulate NAFLD prognosis. The serum sample was frozen at −80°C within 4 hours of collection and thawed just before analysis. Circulating microRNA was extracted from 200 μL of serum samples using the QIAGEN miRNeasy Serum/Plasma Kit according to the manufacturer's instructions (QIAGEN K.K., Tokyo, Japan). RNA was reverse transcribed using TaqMan microRNA Reverse Transcription Kit (Life Technologies Japan, Tokyo, Japan). *Caenorhabditis elegans* miR-39 (cel-miR-39) was spiked in each sample as the control for extraction and amplification steps. The protocol used for measurement of serum miR-122 was based on the method provided by the manufacturer (TaqMan Small RNA Assays; Applied Biosystems). Serum miR-122 was amplified using primers and probes provided by Applied Biosystems in the TaqMan microRNA assay according to the instructions provided by the manufacturer. The relative expression of serum miR-122 was calculated using the comparative cycle threshold (CT) method ($2^{-\Delta\Delta CT}$),[18,19] with spiked cel-miR-39 as normalized internal control. The microRNA expression levels were reported relative to the levels of serum miR-122 measured in 286 clinical samples.[12] In the reproducibility of the serum miR-122 measurement, triplicate assay was performed and their mean values were adopted. Samples that indicated wide variations were re-examined.[20]

## STATISTICAL ANALYSIS

Parameters shown in Table 1 were used for analysis of survival/mortality rates. Parameters that indicated a strong correlation with others were considered confounding factors and excluded from statistical analysis. The incidence of each event was analyzed during the period from the time of histopathologic diagnosis of NAFLD until the last visit. Stepwise Cox regression analysis based on the backward selection method was used to determine independent predictive factors associated with mortality. The hazard ratio (HR) and 95% confidence interval (CI) were also calculated. Variables that were statistically significant on univariate analysis were entered into multivariate analysis to identify significant independent factors after conversion to categorical data of two simple ordinal numbers. Multiple comparisons were examined by the Bonferroni test. Significance was set at $P < 0.05$ by the two-tailed test. All statistical tests were performed with the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL).

# Results

## OVERALL SURVIVAL/MORTALITY RATES IN NAFLD

Data of the whole population sample were used for analysis of the overall survival/mortality rates. During the follow-up period of 4.7 years (range, 0.0-42.9 years), 21 (4.8%) deaths were recorded. In these cases, the median interval between histopathologic diagnosis of NAFLD and death was 4.1 years (range, 0.6-31.8 years). Of the cases, 76 (17.2%) stopped going to the hospital and 344 (78.0%) regularly went to the hospital. The cumulative survival rates were 95.4% and 90.6% at the end of 5 and 10 years, respectively.

## FACTORS ASSOCIATED WITH MORTALITY

Clinicopathologic, genetic, and epigenetic parameters shown in Table 1 were used for analysis of mortality. Parameters (e.g., age, AST, ALT, platelet count, NAS, and low-density lipoprotein cholesterol) that indicated a strong correlation with others were considered confounding factors and excluded from statistical analysis. Of these, seven parameters showed a significant relationship with mortality by univariate analysis: history of liver cancer, type 2 diabetes mellitus, stage, serum miR-122, FIB-4 index, fasting plasma glucose, and total cholesterol. These factors were entered into multivariate analysis, which identified three risk factors that significantly and independently influenced mortality: history of liver cancer (presence; HR, 4.94; 95% CI, 1.84-13.3; $P = 0.002$), serum miR-122 (<1.00 fold change; HR, 4.35; 95% CI, 0.06-0.83; $P = 0.025$), and FIB-4 index (≥1.30; HR, 15.7; 95% CI, 2.01-122; $P = 0.009$) (Table 2).

## CUMULATIVE SURVIVAL RATES ACCORDING TO FIB-4 INDEX LEVELS

Cumulative survival rates were significantly different among the four FIB-4 index subgroups (<1.30

**TABLE 2. FACTORS ASSOCIATED WITH SURVIVAL OF PATIENTS WITH NAFLD**

| | | Univariate* | | | Multivariate | | |
|---|---|---|---|---|---|---|---|
| Factor | Category | HR | (95% CI) | *P* Value† | HR | (95% CI) | *P* Value† |
| History of liver cancer | No | 1 | | | 1 | | |
| | Yes | 17.1 | (6.85-42.5) | <0.001 | 4.94 | (1.84-13.3) | 0.002 |
| Type 2 diabetes mellitus | No | 1 | | | | | |
| | Yes | 3.88 | (1.62-9.32) | 0.002 | | | |
| Stage | 0-2 | 1 | | | | | |
| | 3, 4 | 8.52 | (3.11-23.3) | <0.001 | | | |
| Serum miR-122 | ≥1.00 fold change | 1 | | | 1 | | |
| | <1.00 fold change | 4.55 | (1.67-12.3) | 0.007 | 4.35 | (1.20-16.7) | 0.025 |
| FIB-4 index | <1.30 | 1 | | | 1 | | |
| | ≥1.30 | 21.6 | (2.89-161) | 0.003 | 15.7 | (2.01-122) | 0.009 |
| Fasting plasma glucose | <110 mg/dL | 1 | | | | | |
| | ≥110 mg/dL | 2.59 | (1.05-6.43) | 0.040 | | | |
| Total cholesterol | ≥200 mg/dL | 1 | | | | | |
| | <200 mg/dL | 2.86 | (1.14-7.14) | 0.025 | | | |

*Seven variables that were statistically significant on univariate analysis were entered into multivariate analysis to identify significant independent factors.
†Significance was determined using the Cox proportional hazard model.

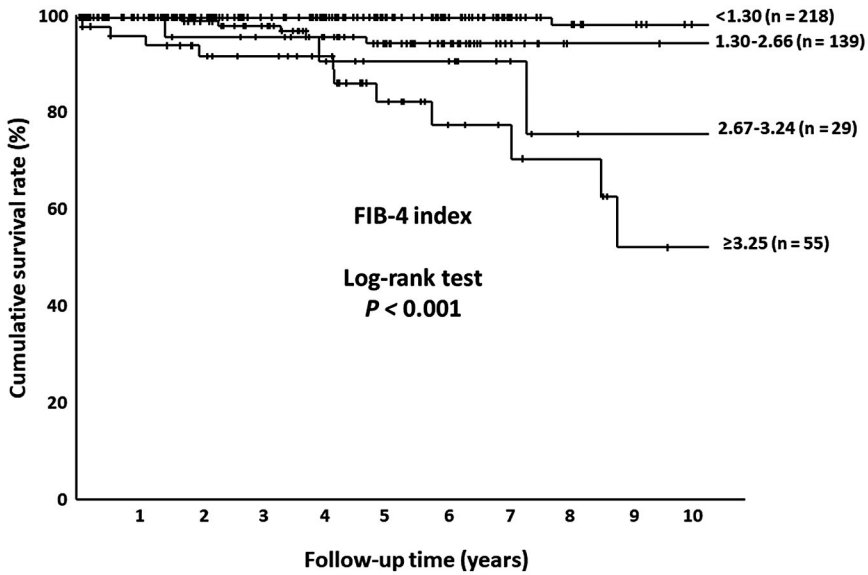


**FIG. 1.** Cumulative survival rates according to the FIB-4 index level. Rates were significantly different among the four FIB-4 index subgroups ($P < 0.001$; log-rank test).

[n = 218], 1.30-2.66 [n = 139], 2.67-3.24 [n = 29], and ≥3.25 [n = 55]; $P < 0.001$; log-rank test) (Fig. 1). Particularly, the rate with a FIB-4 index <1.30 was significantly higher than that of 1.30-2.66 ($P = 0.015$; log-rank test), 2.67-3.24 ($P < 0.001$; log-rank test), and ≥3.25 ($P < 0.001$; log-rank test). Furthermore, the rates with a FIB-4 index of 1.30-2.66 were significantly higher than that of ≥3.25 ($P = 0.015$; log-rank

test). The rate with a FIB-4 index of <1.30 was significantly higher than that of 2.67-3.24 ($P$ < 0.001) and ≥3.25 ($P$ < 0.001) by multiple comparisons.

As a reference, cumulative survival rates according to stage are shown in Supporting Fig. S1. Rates were significantly different among the four subgroups of stage (0 [n = 50], 1 [n = 182], 2 [n = 68], 3 [n = 108], and 4 [n = 33]; $P$ < 0.001; log-rank test).

## CUMULATIVE SURVIVAL RATES ACCORDING TO SERUM miR-122 LEVEL

A total of 392 cases had a saved serum sample ≥200 μL and were able to have a serum miR-122 level quantified. The cumulative survival rate varied significantly according to the serum level of miR-122 (<0.50 [n = 104], 0.50 to <1.00 [n = 94], and ≥1.00 fold change [n = 194]; $P$ = 0.003; log-rank test) (Fig. 2). Particularly, the survival rate of patients with serum miR-122 level ≥1.0 was significantly higher than that of patients with serum miR-122 level <0.50 ($P$ < 0.001; log-rank test) and tended to be higher than that of patients with serum miR-122 level 0.50 to <1.00 ($P$ = 0.064; log-rank test). The survival rate of patients with serum miR-122 level ≥1.00 was significantly higher than that of patients with serum miR-122 level <0.50 ($P$ = 0.001) by multiple comparisons.

In cases with severe fibrosis of stage 3 or 4, cumulative survival rates were significantly different among the three miR-122 subgroups (<0.50 [n = 36], 0.50 to <1.00 [n = 27], and ≥1.00 fold change [n = 62]; $P$ = 0.017; log-rank test) (Supporting Fig. S2).

In cases with a history of liver cancer, cumulative survival rates were not different among the three miR-122 subgroups (<0.50 [n = 15], 0.50 to <1.00 [n = 7], and ≥1.00 fold change [n = 2]; $P$ = 0.532; log-rank test).

## CUMULATIVE SURVIVAL RATES ACCORDING TO GENETIC VARIATION

Cumulative survival rates were not significantly different among the three genotypes of *PNPLA3* (CC, CG, and GG) ($P$ = 0.420; log-rank test) (Fig. 3). The rates were also not significantly different among the three genotypes of *TM6SF2* (CC, CT, and TT) ($P$ = 0.574; log-rank test).

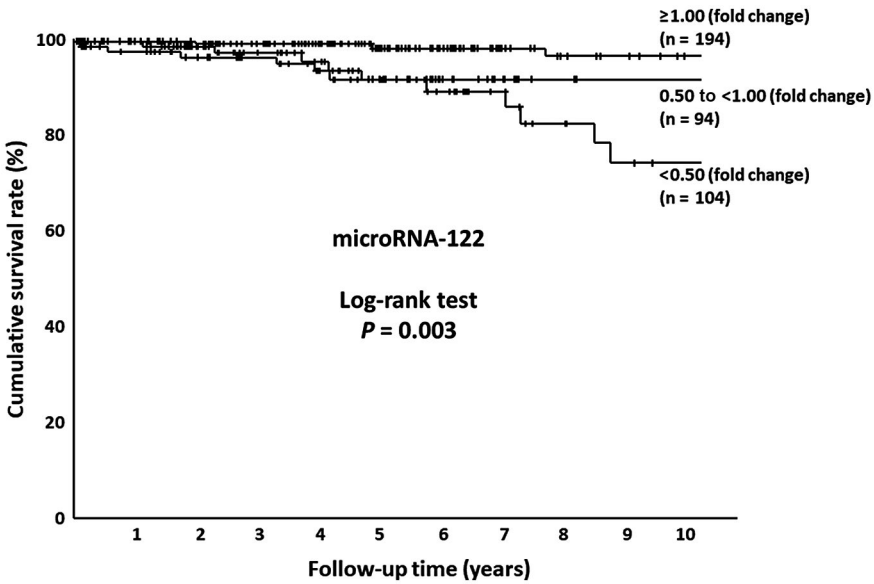


**FIG. 2.** Cumulative survival rates according to the level of serum miR-122. Rates were significantly different among the three serum miR-122 subgroups (<0.50, 0.50 to <1.00, and ≥1.00 fold change) ($P$ = 0.003; log-rank test).

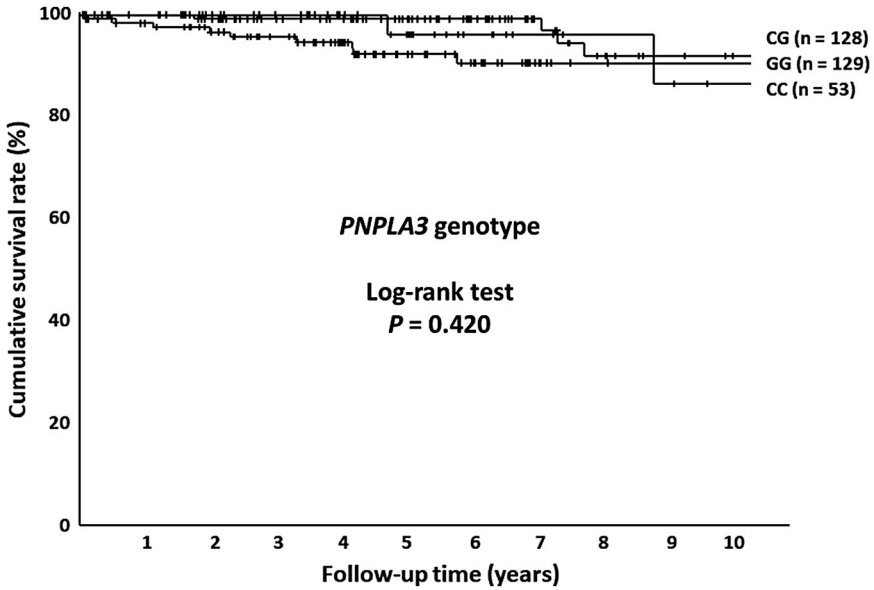


**FIG. 3.** Cumulative survival rates according to *PNPLA3* genotype. Rates were not significantly different among the three genotypes (CC, CG, and GG) ($P = 0.420$; log-rank test).

## CUMULATIVE SURVIVAL RATES ACCORDING TO A COMBINATION OF RISK FACTORS

Data of 392 patients who fulfilled the inclusion and exclusion criteria specified in this study were analyzed to determine the cumulative survival rates according to a combination of mortality risk factors. The cumulative survival rate was significantly different among the three subgroups based on the number of risk factors (none/one risk factor, two risk factors, and three risk factors; $P < 0.001$; log-rank test) (Fig. 4). Particularly, the survival rate for patients with three risk factors was significantly lower than that for patients with two risk factors and none/one risk factor ($P < 0.001$, each; log-rank test). Furthermore, the survival rate for patients with two risk factors was also significantly lower than that for patients with none/one risk factor ($P < 0.001$; log-rank test).

## CLINICAL CHARACTERISTICS OF PATIENTS WITH NAFLD WHO DIED DURING THE STUDY PERIOD

Clinical features of the 21 patients who died during the study period are listed in Table 3. Of these patients, 13 (61.9%) died of liver-related causes, 4 (19.0%) of malignancy excluding liver cancer, 2 (9.5%) of cardiovascular events, 1 (4.8%) of interstitial pneumonitis, and 1 (4.8%) of sepsis. Of the 13 patients who died from liver-related causes, 9 (69.2%) and 4 (30.8%) died from liver cancer and liver failure, respectively.

None/one risk factor, two risk factors, and three risk factors of mortality were identified in 4, 9, and 8 of 21 of the death cases, respectively. Thus, 17 of the 21 (81.0%) death cases exhibited two or more risk factors before death.

# Discussion

In patients with histopathologically confirmed NAFLD, liver fibrosis stage is the most important predictor of overall mortality and liver-related events, including liver cancer.[5,6,8,9] Our results confirmed this conclusion; multivariate analysis identified the FIB-4 index of fibrosis (HR, 15.7) as a significant and independent predictor of mortality. In addition, our study also identified the serum miR-122 level, representing an epigenetic factor (HR, 4.35), and history of liver cancer (HR, 4.94) as significant risk factors for poor prognosis in patients with histopathologically confirmed NAFLD. We reported in a longitudinal

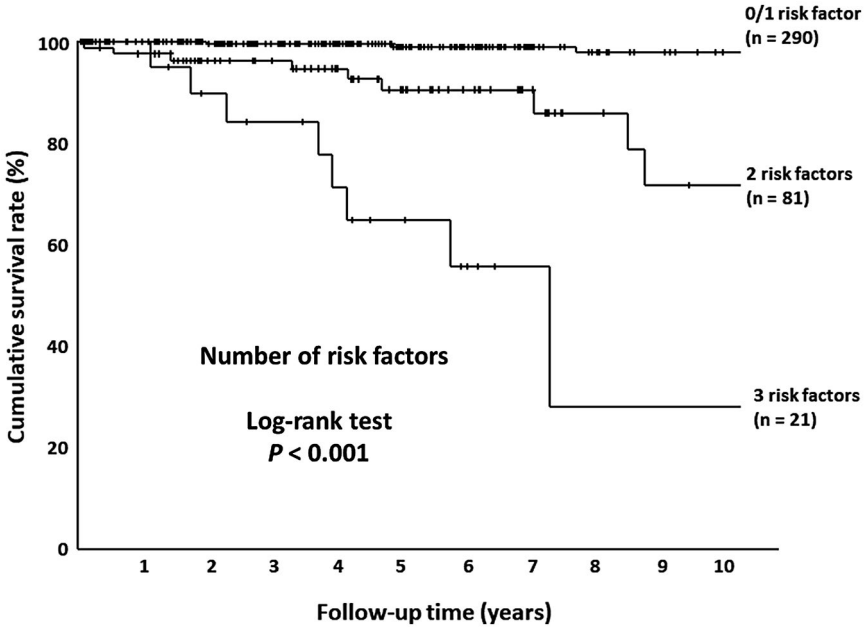


**FIG. 4.** Cumulative survival rates according to the number of risk factors for mortality (positive history of liver cancer, serum miR-122 <1.00 fold change, and FIB-4 index ≥1.30). Rates were significantly different among the three subgroups (0/1 risk factor, 2 risk factors, and 3 risk factors) ($P < 0.001$; log-rank test).

evaluation study of a patient with liver cancer that liver cancer and fibrosis stage independently modulated the serum miR-122 level and that the level tended to decrease before progression of the fibrosis stage.[12] The present results add support to that study, confirming that serum miR-122 and history of liver cancer are independent poor prognostic factors. Our results highlight the importance of assessment of the three risk factors (history of liver cancer, serum miR-122, and FIB-4 index) for early diagnosis and treatment to improve the prognosis of patients with NAFLD.

The impact of epigenetic factors on mortality of patients with NAFLD remains unclear. In particular, there is no information on the long-term survival rates associated with serum miR-122 levels in patients with histopathologically confirmed NAFLD. Reports have indicated that serum miR-122 is an independent predictor of overall survival of patients with liver cirrhosis[21] and liver cancer.[22,23] However, these studies could not investigate the mortality risk factors properly due to the small number of patients with NAFLD and short follow-up period. To our knowledge, the present study is the first to identify low serum level of miR-122 as a significant and independent risk factor of mortality in patients with NAFLD. The present findings indicated that cumulative survival rates were significantly different among the three serum miR-122 subgroups in cases of stage 3 or 4 but that the rates were not different in cases with a history of liver cancer. Hence, it is unclear whether the addition of serum miR-122 might improve the prognosis prediction based on two risk factors (FIB-4 index and history of liver cancer). Further study based on a large number of patients should be performed to investigate the impact of the serum miR-122 measurement. A recent meta-analysis of 11 studies involving 1,124 patients reported that low miR-122 expression in liver cancer tissues correlated significantly with unfavorable overall survival in patients with liver cancer who underwent curative resection; however, miR-122 expression level in the blood could not predict overall survival.[24] Thus, tissue microRNA and circulating microRNA expression profiles are not always consistent.[25] This discrepancy between the previous reports and the present study could be due to differences in the methods used to measure miR-122 level or differences in the etiology of liver cancer. One of the limitations of the present study is the lack of analysis of miR-122 expression in tissues. Further

**TABLE 3. CHARACTERISTICS OF 21 PATIENTS WHO DIED DURING THE STUDY PERIOD**

| | | History | | | | At Diagnosis of NAFLD | | | | At Time of Death | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Case | Sex | Cardiovascular Event | Liver Cancer | Malignancy Except for Liver Cancer | Type 2 Diabetes Mellitus | Age (Years) | Fibrosis Stage | FIB-4 Index | Serum miR-122 (Fold Change) | Age (Years) | Cause of Death |
| 1 | Male | Absence | Absence | Hemangiopericytoma | Absence | 29 | 3 | 0.77 | 2.92 | 37 | Hemangiopericytoma |
| 2 | Female | Absence | Absence | Absence | Absence | 40 | 1 | 4.85 | 0.67 | 41 | Interstitial pneumonitis |
| 3 | Male | Absence | Presence | Absence | Presence | 41 | 4 | 2.78 | 0.29 | 48 | Liver failure |
| 4 | Male | Absence | Absence | Absence | Presence | 45 | 4 | 4.97 | 0.06 | 54 | Liver cancer |
| 5 | Male | Angina pectoris | Presence | Absence | Presence | 57 | 3 | 2.85 | Not determined* | 58 | Liver cancer |
| 6 | Female | Absence | Absence | Colon cancer, lung cancer | Absence | 57 | 1 | 1.36 | 0.88 | 62 | Colon cancer |
| 7 | Male | Absence | Absence | Absence | Absence | 55 | 4 | 4.56 | 0.31 | 64 | Liver cancer |
| 8 | Male | Absence | Absence | Absence | Presence | 62 | 3 | 4.19 | 2.43 | 64 | Pancreas cancer |
| 9 | Female | Absence | Absence | Absence | Presence | 64 | 4 | 12.32 | 0.72 | 65 | Sepsis |
| 10 | Male | Absence | Absence | Absence | Absence | 64 | 3 | 1.82 | 0.80 | 67 | Lung cancer |
| 11 | Male | Absence | Presence | Absence | Presence | 67 | 1 | 1.72 | 0.19 | 71 | Liver failure |
| 12 | Female | Absence | Presence | Absence | Absence | 70 | 4 | 4.72 | 0.42 | 71 | Liver cancer |
| 13 | Male | Absence | Absence | Absence | Presence | 69 | 3 | 5.00 | 1.04 | 74 | Liver failure |
| 14 | Female | Absence | Absence | Absence | Absence | 68 | 4 | 14.83 | 0.25 | 75 | Infectious endocarditis |
| 15 | Female | Absence | Absence | Absence | Presence | 73 | 4 | 8.29 | 0.37 | 77 | Liver cancer |
| 16 | Male | Absence | Presence | Absence | Presence | 72 | 3 | 4.15 | 0.44 | 78 | Liver cancer |
| 17 | Female | Absence | Presence | Absence | Presence | 76 | 2 | 1.97 | 0.46 | 78 | Liver cancer |
| 18 | Female | Absence | Presence | Absence | Presence | 75 | 4 | 2.88 | 0.83 | 79 | Liver cancer |
| 19 | Female | Absence | Presence | Colon cancer | Presence | 79 | 3 | 2.22 | 0.88 | 81 | Aortic aneurysm |
| 20 | Male | Absence | Presence | Absence | Absence | 80 | 4 | 5.19 | 0.49 | 84 | Liver failure |
| 21 | Male | Absence | Absence | Absence | Absence | 57 | 2 | 1.50 | 1.10 | 89 | Liver cancer |

*Serum miR-122 was not measured due to an inadequate serum sample.

studies with a larger number of patients with NAFLD should be performed to determine the effects of miR-122 expression in both the serum and liver tissue on prognosis.

The impact of genetic factors on mortality in patients with NAFLD also remains unclear. Previous cross-sectional and genome-wide studies identified a close correlation between several SNPs and the pathology of NAFLD; among these SNPs, the genetic variant of *PNPLA3* rs738409 showed a strong relationship with the development and progression of NAFLD, NASH, and NAFLD-related liver cancer.[26,27] The genetic variant of *TM6SF2* rs58542926 was also associated with NAFLD, and its presence correlated with cardiovascular events.[27,28] However, genetic variations (e.g., *PNPLA3* and *TM6SF2*) failed to predict mortality based on the longitudinal study. The discrepancy among the studies could be due to racial differences, study design, and the small number of deaths registered in the present study (21 patients). Further studies with a large number of patients with NAFLD of different racial backgrounds or ethnic groups (non-Japanese) should be performed to investigate the impact of genetic factors on prognosis.

The impact of the FIB-4 index and cut-off value for the long-term prognosis of NAFLD is still unclear. In the present study, univariate analysis identified severe fibrosis stage, but multivariate analysis did not identified severe fibrosis stage as an independent predictor of overall mortality. One reason for this could be that the FIB-4 index might be the more powerful predictor, including platelet count (as one surrogate marker of fibrosis stage), AST/ALT (as surrogate markers of inflammation), and age, compared to the fibrosis stage. One report showed that a FIB-4 index <1.30 might be useful for excluding NAFLD with advanced fibrosis[5], and therefore it might not be practical to use a FIB-4 index ≥1.30 from the point of view of discriminating patients with NAFLD with a poor prognosis. On the other hand, the FIB-4 index value was low in approximately 80% of patients diagnosed with NAFLD during a health checkup, whereas a high value was noted in only approximately 1% of patients.[5] In the present study, multivariate analysis identified a FIB-4 index ≥1.30 as a significant and independent predictor of mortality. A further prospective study should be performed to investigate the suitable cut-off value for the prediction of prognosis.

In conclusion, the present results highlight the importance of serum miR-122 and the FIB-4 index on mortality in Japanese patients with histopathologically confirmed NAFLD. Early diagnosis based on the presence of more than one risk factor and early treatment might improve the prognosis.

*Acknowledgment:* We thank the following individuals for their assistance in histopathologic diagnosis: Keiichi Kinowaki, M.D. and Takeshi Fujii, M.D., Department of Pathology, Toranomon Hospital; Fukuo Kondo, M.D. and Toshio Fukusato, M.D., Department of Pathology, Teikyo University School of Medicine.

## REFERENCES

1) Angulo P. Nonalcoholic fatty liver disease. N Engl J Med 2002; 346:1221-1231.
2) Williams R. Global challenges in liver disease. Hepatology 2006; 44:521-526.
3) Torres DM, Harrison SA. Diagnosis and therapy of nonalcoholic steatohepatitis. Gastroenterology 2008;134:1682-1698.
4) Vuppalanchi R, Chalasani N. Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis: selected practical issues in their evaluation and management. Hepatology 2009;49:306-317.
5) Sumida Y, Nakajima A, Itoh Y. Limitations of liver biopsy and non-invasive diagnostic tests for the diagnosis of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. World J Gastroenterol 2014;20:475-485.
6) Akuta N, Kawamura Y, Arase Y, Saitoh S, Fujiyama S, Sezaki H, et al. Hepatocellular carcinoma is the most common liver-related complication in patients with histopathologically-confirmed NAFLD in Japan. BMC Gastroenterol 2018;18:165.
7) Kleiner DE, Brunt EM. Nonalcoholic fatty liver disease: pathologic patterns and biopsy evaluation in clinical research. Semin Liver Dis 2012;32:3-13.
8) Younossi ZM, Stepanova M, Rafiq N, Makhlouf H, Younoszai Z, Agrawal R, et al. Pathologic criteria for nonalcoholic steatohepatitis: interprotocol agreement and ability to predict liver-related mortality. Hepatology 2011;53:1874-1882.
9) Angulo P, Kleiner DE, Dam-Larsen S, Adams LA, Bjornsson ES, Charatcharoenwitthaya P, et al. Liver fibrosis, but no other histologic features, is associated with long-term outcomes of patients with nonalcoholic fatty liver disease. Gastroenterology 2015; 149:389-397.
10) Cermelli S, Ruggieri A, Marrero JA, Ioannou GN, Beretta L. Circulating microRNAs in patients with chronic hepatitis C and non-alcoholic fatty liver disease. PLoS One 2011;6:e23937.
11) Pirola CJ, Fernández Gianotti T, Castaño GO, Mallardi P, San Martino J, Mora Gonzalez Lopez Ledesma M, et al. Circulating microRNA signature in non-alcoholic fatty liver disease: from serum non-coding RNAs to liver histology and disease pathogenesis. Gut 2015;64:800-812.
12) Akuta N, Kawamura Y, Suzuki F, Saitoh S, Arase Y, Kunimoto H, et al. Impact of circulating miR-122 for histological features and hepatocellular carcinoma of nonalcoholic fatty liver disease in Japan. Hepatol Int 2016;10:647-656.
13) Akuta N, Watanabe C, Kawamura Y, Arase Y, Saitoh S, Fujiyama S, et al. Effects of a sodium-glucose cotransporter 2 inhibitor in nonalcoholic fatty liver disease complicated by diabetes mellitus:

preliminary prospective study based on serial liver biopsies. Hepatol Commun 2017;1:46-52.
14) Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Nonalcoholic Steatohepatitis Clinical Research Network. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 2005;41:1313-1321.
15) Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. Am J Gastroenterol 1999;94:2467-2474.
16) Bedossa P; FLIP Pathology Consortium. Utility and appropriateness of the fatty liver inhibition of progression (FLIP) algorithm and steatosis, activity, and fibrosis (SAF) score in the evaluation of biopsies of nonalcoholic fatty liver disease. Hepatology 2014;60:565-575.
17) Sterling RK, Lissen E, Clumeck N, Sola R, Correa MC, Montaner J, et al.; APRICOT Clinical Investigators. Development of a simple noninvasive index to predict significant fibrosis in patients with HIV/HCV coinfection. Hepatology 2006;43:1317-1325.
18) Kroh EM, Parkin RK, Mitchell PS, Tewari M. Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-PCR (qRT-PCR). Methods 2010;50:298-301. Erratum. In: Methods 2010;52:268.
19) Yu S, Liu Y, Wang J, Guo Z, Zhang Q, Yu F, et al. Circulating microRNA profiles as potential biomarkers for diagnosis of papillary thyroid carcinoma. J Clin Endocrinol Metab 2012;97:2084-2092.
20) Gong Y, Xu W, Chen Y, Liu Y, Yang Y, Wang B, et al. miR-20a-5p regulates pulmonary surfactant gene expression in alveolar type II cells. J Cell Mol Med 2019; https://doi.org/10.1111/jcmm.14639.
21) Waidmann O, Köberle V, Brunner F, Zeuzem S, Piiper A, Kronenberger B. Serum microRNA-122 predicts survival in patients with liver cirrhosis. PLoS One 2012;7:e45652.
22) Köberle V, Kronenberger B, Pleli T, Trojan J, Imelmann E, Peveling-Oberhag J, et al. Serum microRNA-1 and microRNA-122 are prognostic markers in patients with hepatocellular carcinoma. Eur J Cancer 2013;49:3442-3449.
23) Xu Y, Bu X, Dai C, Shang C. High serum microRNA-122 level is independently associated with higher overall survival rate in hepatocellular carcinoma patients. Tumour Biol 2015;36:4773-4776.
24) Zhang Y, Li Y, Jiang W, Li Q, Lan Y. The clinical significance of microRNA-122 in predicting the prognosis of patients with hepatocellular carcinoma: a meta-analysis validated by the Cancer Genome Atlas dataset. Medicine (Baltimore) 2019;98:e14810.
25) Morishita A, Masaki T. microRNAs as possible biomarkers for hepatocellular carcinoma. Hepatol Res 2018;48:499-501.
26) Romeo S, Kozlitina J, Xing C, Pertsemlidis A, Cox D, Pennacchio LA, et al. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. Nat Genet 2008;40:1461-1465.
27) Seko Y, Yamaguchi K, Itoh Y. The genetic backgrounds in nonalcoholic fatty liver disease. Clin J Gastroenterol 2018;11:97-102.
28) Kozlitina J, Smagris E, Stender S, Nordestgaard BG, Zhou HH, Tybjærg-Hansen A, et al. Exome-wide association study identifies a TM6SF2 variant that confers susceptibility to nonalcoholic fatty liver disease. Nat Genet 2014;46:352-356.

# Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1445/suppinfo.