ribosomal RNA using $2^{-\Delta\Delta Ct}$ method (Applied Biosystems,, Foster City, CA, User Bulletin No. 2).

Immunoblot analysis

Liver tissue extracts were prepared by using M-PER® Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Rockford, IL) plus Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific, Rockford, IL). Immunoblot analysis was performed with specific antibodies against uMtCK (dilution, 1:1,000; Abcam, Cambridge, United Kingdom) and beta-actin (dilution, 1:2,000; Sigma-Aldrich, St. Louis, MO) as described-previously.[9] Immunoreactive proteins were visualized using a chemiluminescence kit (GE Healthcare, Buckinghamshire, United Kingdom), and recorded using a LAS-4000 image analyzer (Fuji Film, Tokyo, Japan). The intensities of immunodetected bands were quantified with NIH Image J software.

Immunohistochemical analysis

Excised liver specimens were fixed immediately in 10% formalin and embedded in paraffin. Serial 4-μm-thick liver tissue sections were deparaffinized, and incubated in citrate buffer at 95°C for 40 min for antigen retrieval, and then incubated overnight at 4°C with anti-uMtCK antibody (Proteintech, Chicago, IL). Biotinylated secondary antibodies (Pharmingen, San Diego, CA) were added and incubated for 20 min at room temperature. Streptavidin–horseradish peroxidase (Pharmingen, San Diego, CA) was added and after 30 min the sections were developed with 3,3′-diaminobenzidine substrate and counterstained hematoxylin.

Patient follow-up and diagnosis of HCC

Patients were followed up at the outpatient clinic with blood tests including tumor markers every 1–3 months, and ultrasonography every 4–6 months. Contrast-enhanced CT was performed when serum tumor markers showed an abnormal rise and/or tumor(s) was detected as possible HCC on ultrasonography. The diagnosis of HCC was based on the typical findings on CT, that is, hyperattenuation in the arterial phase and hypoattenuation in the equilibrium phase.[19,20]

The end points consisted of the interval between the first measurement of serum MtCK activity and the detection of HCC development, death without HCC development or the last examination until May 30, 2013, whichever came first. Death without HCC development was treated as censored data.

Statistical analysis

Categorical data were compared by χ^2-test or Fisher's exact test. Distributions of continuous variables were analyzed with Student's *t*-test for two groups. All tests of significance were two-tailed, and $p < 0.05$ was considered statistically significant. The potential associations between the MtCK and the following factors were assessed using Spearman's rank correlation coefficient: age, serum albumin, AST, ALT, GGT, total bilirubin, AFP, DCP, platelet count, prothrombin time and liver stiffness measured by Fibroscan. Cumulative incidence of hepatocarcinogenesis was calculated by the Kaplan–Meier method, and differences among groups were assessed using the log-rank test. The following factors were assessed as candidate risk factors for hepatocarcinogenesis by time-fixed Cox proportional hazard regression: age, sex, hepatitis virus, serum albumin, AST, ALT, GGT, total bilirubin, AFP, DCP, platelet count, prothrombin time, liver stiffness and MtCK. We used univariate and multivariate time-fixed Cox proportional hazard models and stepwise variable selection based on Akaike Information Criteria. Data processing and analysis were performed using SPSS software version 17.0 or 19.0 (SPSS, Chicago, IL).

Table 1. Characteristics of the enrolled chronic hepatitis C patients

Parameter	*N* = 171
Age (year)[1]	68 (60–75.5)
Female[2]	75 (43.9)
MtCK (U/L)[1]	4.50 (3.20–7.19)
Albumin (g/dL)[1]	4.0 (3.7–4.3)
AST (U/L)[1]	40 (29–63)
ALT (U/L)[1]	35 (23–55.5)
GGT (U/L)[1]	28 (20–49.5)
Total bilirubin (mg/dL)[1]	0.8 (0.6–1.2)
AFP (ng/dL)[1]	5.0 (3.0–10.1)
DCP (mAU/mL)[1]	16 (12–22.5)
Platelet ($\times 10^4/\mu L$)[1]	12.1 (8.8–17.5)
Prothrombin time (sec)[1]	11.7 (11.2–12.5)
LSV measured by Fibroscan (kPa)[1]	10.5 (5.7–17.0)

[1]Data were expressed as mean (1st–3rd. quartile).
[2]Data were expressed as number (%).

Results

Increased serum MtCK activity in patients with chronic hepatitis C

Clinical and laboratory variables of the enrolled patients are listed in Table 1. The mean level of serum albumin and total bilirubin and the mean platelet count in the enrolled patients were 4.0 g/dL, 0.8 mg/dL and 12.1 × $10^4/\mu L$, suggesting that the patients would have developed various stages of liver fibrosis, not exclusively liver cirrhosis. In agreement with this fact, the mean liver stiffness value in the enrolled patients was 10.5 kPa, suggesting the fibrosis stage of F3.[17] In these patients, serum MtCK activity was higher than the previously reported values in healthy subjects ($p < 0.001$): the mean serum MtCK activity was 4.5 U/L in patients with chronic hepatitis C, whereas 3.4 U/L in healthy subjects as described previously.[8]

Relationships between serum MtCK activity and various parameters

Relationships between serum MtCK activity and various clinical parameters are summarized in Table 2. Serum MtCK activity was significantly correlated with serum albumin levels, platelet counts and liver stiffness values ($p < 0.001$, 0.026

Table 2. Relation between serum MtCK activity and various parameters

Parameter	Spearman's ρ	*p*-Value
Age (year)	0.1829	0.016
Albumin (g/dL)	−0.4041	<0.001
AST (U/L)	0.2419	0.0014
ALT (U/L)	0.1556	0.042
GGT (U/L)	0.0427	0.58
Total bilirubin (mg/dL)	−0.0044	0.96
AFP (ng/dL)	0.2207	0.0037
DCP (mAU/mL)	0.0667	0.39
Platelet ($\times 10^4/\mu L$)	−0.1703	0.026
Prothrombin time (sec)	0.1482	0.086
LSV measured by Fibroscan (kPa)	0.2843	<0.001

and <0.001), suggesting that the increase in serum MtCK activity may be associated with the stage of liver fibrosis. On the other hand, the significant correlations between serum MtCK activity and serum levels of AST ($p = 0.0014$) and ALT ($p = 0.042$) were observed, which may suggest that serum MtCK activity is increased in association with hepatocellular damage. Furthermore, serum MtCK activity was significantly correlated with serum AFP levels ($p = 0.0037$).

Increased uMtCK mRNA and protein expressions and immunoreactivity for uMtCK in fibrotic livers in mice

As described earlier, among two tissue-specific isozymes of MtCK, that is, uMtCK and sMtCK, we have found that the increase in serum MtCK activity in HCC patients was mostly owing to that in serum uMtCK activity but not in serum sMtCK activity.[8] As the current evidence suggests that serum MtCK activity may be increased in association with the stage of liver fibrosis, we wondered whether uMtCK expression might be enhanced in fibrotic livers. To test this hypothesis, we first measured uMtCK mRNA levels in the livers of mice treated with bile duct ligation for 4 weeks. As shown in Figure 1*a*, uMtCK mRNA levels in the livers were significantly enhanced in bile duct-ligated mice at 4 weeks after the operation compared to sham-operated mice ($p = 0.02$; Fig. 1*a*). An increased immunoreactivity for uMtCK was detected in bile duct-ligated mouse livers, predominantly in hepatocytes at the periductular area, as compared to sham-operated livers, where immunoreactivity was very low or absent (Fig. 1*b*). This increased immunoreactivity was confirmed to be owing to uMtCK protein expression by immunoblot analysis (Fig. 1*c*). These results suggest that uMtCK expression may be increased in fibrotic livers predominantly in hepatocytes, possibly leading to enhanced serum MtCK activity.

Increased serum MtCK activity as an independent risk for hepatocarcinogenesis

The enrolled patients were then followed up to detect HCC occurrence. During the mean follow-up period of 2.7 years (1st–3rd quartile: 2.4–3.1 years), HCC developed in 21 patients. To carefully exclude MtCK production by HCC, HCC was ruled out at the enrollment by ultrasonography, dynamic CT and/or magnetic resonance imaging. The cumulative incidence rates of HCC at 1, 2 and 3 years estimated by the Kaplan–Meier method were 3.5, 8.8 and 12.3%, respectively, as shown in Figure 2*a*. In these patients who developed HCC, serum MtCK activity was significantly higher than that in patients who did not develop HCC ($p < 0.001$) as shown in Figure 2*b*; serum MtCK activity was 10.6 U/L (interquartile range, 4.4–20.7) in patients who developed HCC and 4.3 U/L (interquartile range, 3.1–6.6) in patients who did not develop HCC. Then, significant risk factors for HCC occurrence by univariate Cox regression analysis were as follows (Table 3): older age ($p = 0.018$), lower albumin ($p < 0.001$), higher AST ($p = 0.017$), higher AFP ($p < 0.001$), lower platelet count ($p = 0.0025$), longer prothrombin time ($p = 0.0013$), elevated liver stiffness value ($p < 0.001$) and higher serum MtCK activity ($p < 0.001$). Multivariate analysis using stepwise variable selection based on Akaike Information Criteria identified higher serum MtCK activity (HR: 1.09/year, $p < 0.001$), higher AFP (HR: 1.01/year, $p = 0.002$) and longer prothrombin time (HR: 1.48/year, $p = 0.002$) as the significant risk factors.

As our multivariate analysis identified serum MtCK activity as an independent factor associated with a risk for HCC development, we determined a cutoff value of serum MtCK activity for the prediction of HCC development by receiver operating characteristics (ROC) analysis. From this analysis, serum MtCK activity of 9.0 U/L was identified as a cutoff value (Fig. 3*a*), and with this cutoff value, area under receiver operating characteristics curve for serum MtCK activity was 0.754 (95% confidence interval [CI]: 0.613–0.894), with a sensitivity of 61.9%, a specificity of 92.8%, a positive predictive value of 56.5% and a negative predictive value of 94.2%. As this negative predictive value was high, the patients with serum MtCK activity of ≤9.0 U/L are suggested to be at a lower risk for HCC development. In fact, as shown in Figure 3*b*, patients with serum MtCK activity of >9.0 U/L were at a significantly higher risk for HCC development compared to those with serum MtCK activity of ≤9.0 U/L ($p < 0.001$). As serum MtCK activity seemed to be correlated with liver fibrosis as observed above, a relationship between serum MtCK activity and HCC development was analyzed in stratified patients by liver stiffness values. As shown in Figures 3*c* and 3*d*, in both patient groups with liver stiffness values of >15 and ≤15 kPa, serum MtCK activity of >9.0 U/L was a significantly higher risk for HCC development compared to those with serum MtCK activity of ≤9.0 U/L ($p < 0.001$). Notably, the cumulative incidence of HCC at 1,100 days of follow-up period in patients with serum MtCK activity of >9.0 U/L was comparable, approximately 0.5, irrespective of their liver stiffness values, that is ≤15 or >15 kPa. Collectively, the higher serum MtCK activity may be an independent risk for HCC development in chronic hepatitis C patients.

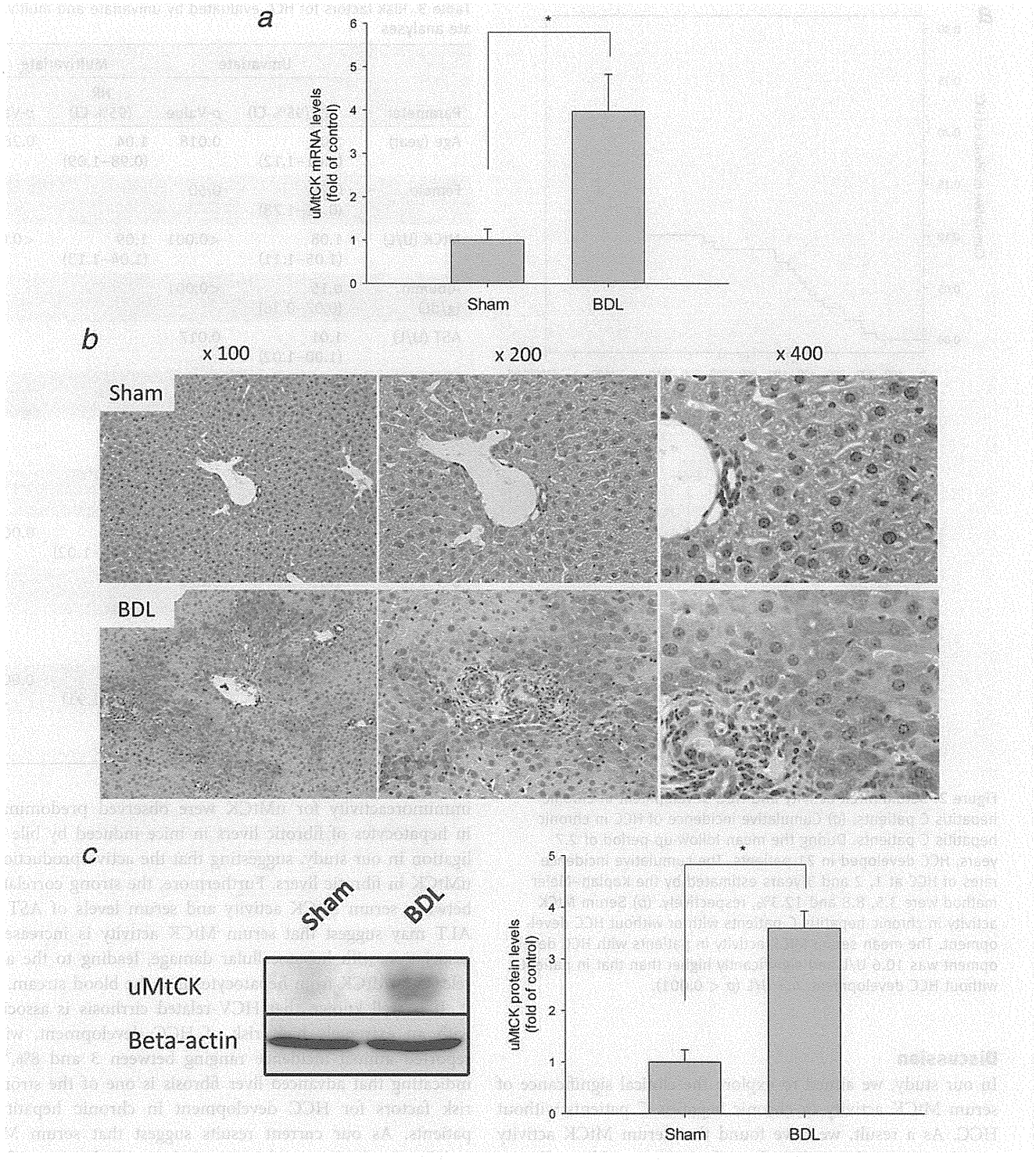

Figure 1. uMtCK mRNA and protein expressions in fibrotic livers induced by bile duct ligation in mice. (*a*) uMtCK mRNA expressions were evaluated by quantitative real-time PCR in the livers of bile duct-ligated and sham-operated mice at 4 weeks after the operation. Results represent a fold of control mice (means ± SEM, $n = 4$). uMtCK mRNA expressions were significantly enhanced in fibrotic livers induced by bile duct ligation in mice ($p = 0.02$) compared to control livers; an asterisk indicates a significant difference. (*b*) uMtCK protein expressions were evaluated immunohistochemically in fibrotic livers induced by bile duct ligation in mice in comparison with control livers. Increased immunoreactivity for uMtCK was observed predominantly in hepatocytes in fibrotic livers compared to control livers. (*c*) uMtCK protein expressions, evaluated by immmunoblot analysis, were significantly enhanced in fibrotic livers induced by bile duct ligation in mice ($p = 0.03$) compared to control livers; an asterisk indicates a significant difference.

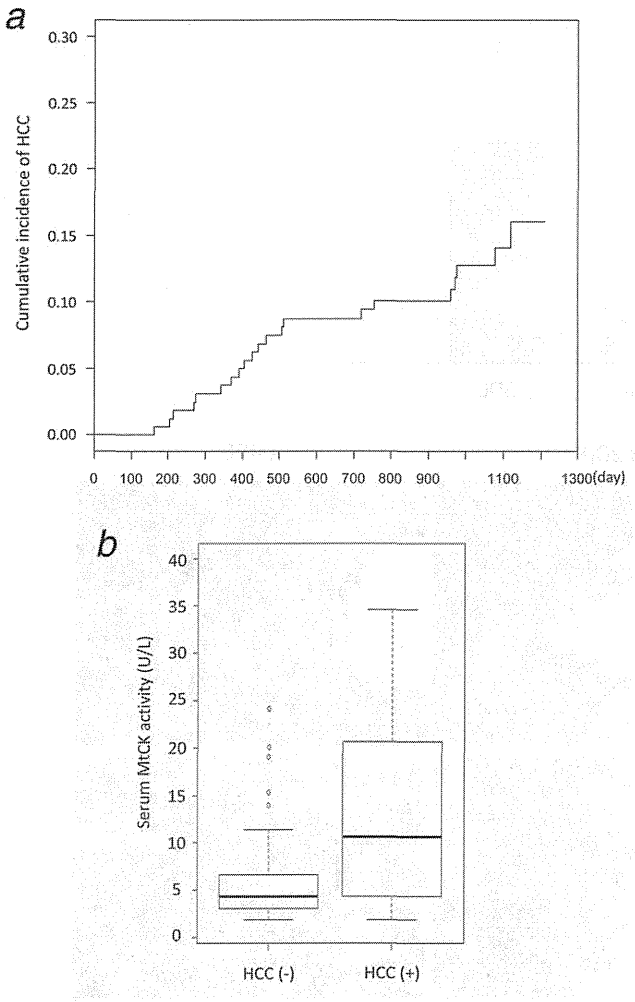

Figure 2. Serum MtCK activity and HCC development in chronic hepatitis C patients. (*a*) Cumulative incidence of HCC in chronic hepatitis C patients. During the mean follow-up period of 2.7 years, HCC developed in 21 patients. The cumulative incidence rates of HCC at 1, 2 and 3 years estimated by the Kaplan–Meier method were 3.5, 8.8 and 12.3%, respectively. (*b*) Serum MtCK activity in chronic hepatitis C patients with or without HCC development. The mean serum MtCK activity in patients with HCC development was 10.6 U/L and significantly higher than that in patients without HCC development, 4.3 U/L ($p < 0.001$).

Table 3. Risk factors for HCC evaluated by univariate and multivariate analyses

	Univariate		Multivariate	
Parameter	HR (95% CI)	*p*-Value	HR (95% CI)	*p*-Value
Age (year)	1.06 (1.01–1.12)	0.018	1.04 (0.98–1.09)	0.28
Female	0.74 (0.31–1.78)	0.50		
MtCK (U/L)	1.08 (1.05–1.11)	<0.001	1.09 (1.04–1.13)	<0.001
Albumin (g/dL)	0.15 (0.07–0.36)	<0.001		
AST (U/L)	1.01 (1.00–1.02)	0.017		
ALT (U/L)	1.002 (0.998–1.010)	0.66		
GGT (U/L)	1.001 (0.997–1.006)	0.54		
Total bilirubin (mg/dL)	2.36 (0.99–5.61)	0.053		
AFP (ng/dL)	1.02 (0.98–1.02)	<0.001	1.01 (1.004–1.02)	0.002
DCP (mAU/mL)	1.02 (0.98–1.04)	0.020		
Platelet ($\times 10^4/\mu L$)	0.87 (0.80–0.95)	0.0025		
Prothrombin time (sec)	1.53 (1.18–1.98)	0.0013	1.48 (1.28–1.91)	0.002
LSV (kPa)	1.06 (1.04–1.08)	<0.001		

Discussion

In our study, we aimed to explore the clinical significance of serum MtCK activity in chronic hepatitis C patients without HCC. As a result, we have found that serum MtCK activity may be increased correlatively with the stage of liver fibrosis and hepatocellular damage, and that the increased serum MtCK activity is an independent risk for hepatocarcinogenesis, which could be the important information for physicians.

As MtCK is not naturally secreted from the cells, the active production of MtCK in a certain tissue or organ and its active release into the blood stream are assumed to be necessary for the increase in serum MtCK activity. Indeed, the increased uMtCK mRNA expression and the increased immunoreactivity for uMtCK were observed predominantly in hepatocytes of fibrotic livers in mice induced by bile duct ligation in our study, suggesting that the active production of uMtCK in fibrotic livers. Furthermore, the strong correlations between serum MtCK activity and serum levels of AST and ALT may suggest that serum MtCK activity is increased in association with hepatocellular damage, leading to the active release of MtCK from hepatocytes into the blood stream.

It is well known that HCV-related cirrhosis is associated with an extremely high risk of HCC development, with a reported annual incidence ranging between 3 and 8%,[4,21,22] indicating that advanced liver fibrosis is one of the strongest risk factors for HCC development in chronic hepatitis C patients. As our current results suggest that serum MtCK activity may be increased in association with the stage of liver fibrosis, the increased serum MtCK activity as a risk factor for hepatocarcinogenesis in chronic hepatitis C patients could be explained, at least in part, by the association between serum MtCK activity and liver fibrosis. In our study, higher serum MtCK activity but not elevated liver stiffness value was determined as a risk for HCC development on multivariate analysis. This finding may be explained by that liver stiffness value, being strongly correlated with serum MtCK

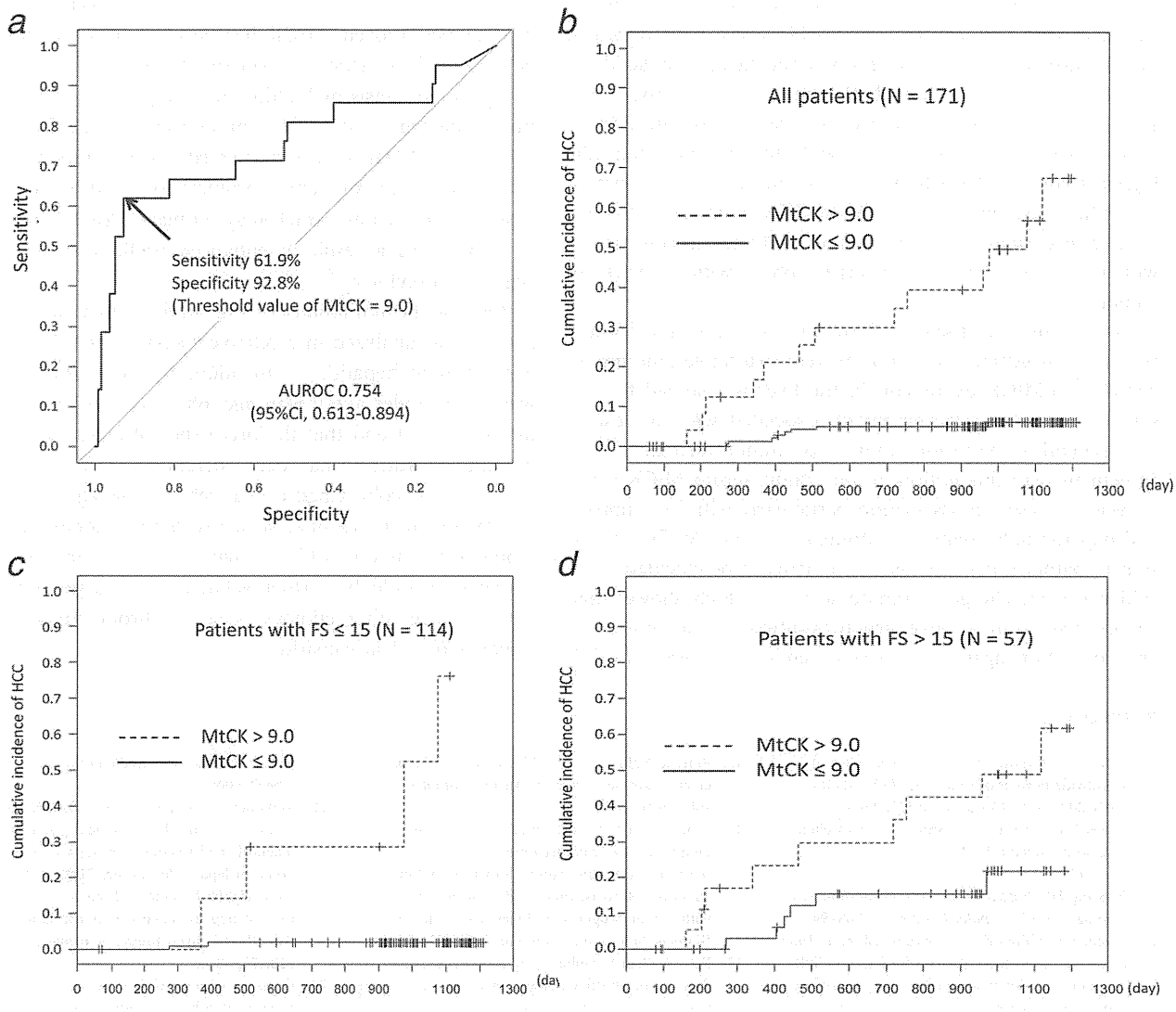

Figure 3. ROC curve showing the overall accuracy of serum MtCK activity for the prediction of HCC development and cumulative incidence of HCC subdivided according to serum MtCK activity in chronic hepatitis C patients. (*a*) ROC curve showing the overall accuracy of serum MtCK activity for the prediction of HCC development in chronic hepatitis C patients. The arrow identifies the best cutoff value (*i.e.*, 9.0 U/L) of serum MtCK activity. Then, cumulative incidence rates of HCC were estimated by the Kaplan–Meier method in all patients (*b*), in patients with liver stiffness value (LSV) of ≤15 kPa (*c*), and in patients with LSV of >15 kPa (*d*) subdivided according to their serum MtCK activity of 9.0 U/L. Serum MtCK activity of >9.0 U/L was a significantly higher risk for HCC development compared to those with serum MtCK of <9.0 U/L ($p < 0.001$) in all patient groups. Solid line, MtCK ≤ 9.0 U/L; dashed line, MtCK > 9.0 U/L.

activity as a predicting factor for liver fibrosis, was not retained as an independent risk for HCC development as a confounding factor. When evaluating this result, we should also bear in mind that another factor other than liver fibrosis may be responsible for the strong association between serum MtCK activity and HCC development. In this context, of interest is the evidence that the higher serum ALT levels were associated with the higher rate of HCC development[23] and HCC recurrence after the surgical treatment[24] in HCV-related cirrhosis, suggesting that the active hepatocellular damage may also be a risk for HCC development. Thus, the association between serum MtCK activity and hepatocellular damage, in addition to liver fibrosis, may explain the reason why serum MtCK activity was retained as an independent risk for hepatocarcinogenesis on multivariate analysis.

In our study, a significant association between serum MtCK activity and serum AFP levels was observed. As it is well known, serum AFP levels have been widely used as a serological marker for HCC[25] although the combination with other serological markers and imaging techniques is recommended to increase diagnostic accuracy.[26] However, elevated serum AFP levels are often observed in patients with chronic hepatitis C without HCC.[27–29] Although the mechanism(s) underlying this finding has not been fully understood yet, it was reported that serum AFP levels were independently associated with liver fibrosis and serum AST levels.[28,30] Thus, it

may be reasonable to assume that serum MtCK activity would behave similarly to serum AFP levels, both of which may be associated with liver fibrosis and hepatocellular damage. Indeed, in our study, both serum MtCK activity and serum AFP levels were retained as a risk for hepatocarcinogenesis, which may be in line with the evidence that the higher serum AFP levels were a risk for HCC development in cirrhotic patients.[31,32] Serum MtCK activity as a risk for HCC development should be further evaluated in comparison with serum AFP levels in a larger cohort with a variety of etiology.

As healthy liver tissue is known to be one of the few tissues that, in general, does not express detectable amounts of uMtCK,[33] uMtCK expression in the liver is assumed to be a sign of pathological development associated with, for example, ischemic–reperfusion injury[34] or tumor formation.[35] In agreement with this notion, in our study, serum MtCK activity was increased in association particularly with liver fibrosis and hepatocellular damage. Although a role of MtCK expression in pathological liver tissues remains to be elucidated, the evidence from CK gene transgenic mice, which showed that CK expression in the liver led to inhibition of apoptosis[36,37] and protection against hypoxia or endotoxin perfusion,[38–40] may suggest a protective role of MtCK expression in injured liver tissues. Indeed, MtCK has been assumed to be important for the energetics of oxidative tissues to control cellular energy homeostasis by building up a large pool of rapidly diffusing phosphocreatine for temporal and spatial buffering of ATP levels.[33] Hence, it is speculated that the increased MtCK activity may support active proliferation of the injured liver tissues to regenerate, which may ultimately lead to hepatocarcinogenesis as a result of enhanced proliferative activity as suggested previously.[32]

One of the limitations of our study is that serum MtCK activity was analyzed in a relatively small number of patients with chronic hepatitis C. In addition, the enrolled patients were at an older age (mean age, 68 years), which may be in line with the trend that the prevalence of older patients with chronic hepatitis C has been increasing in Japan.[41] In our study, as our cohort had a relatively narrow age distribution, age might not be retained as a risk for hepatocarcinogenesis. Nonetheless, serum MtCK activity as a risk for hepatocarcinogenesis should be further validated in a larger number of patients with other etiology, such as chronic hepatitis B or nonalcoholic steatohepatitis.

References

1. Ferlay J, Shin HR, Bray F, et al. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010;127:2893–917.
2. Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. *Lancet* 2003;362: 1907–17.
3. El-Serag HB. Epidemiology of hepatocellular carcinoma in USA. *Hepatol Res* 2007;37:S88–94.
4. Yoshida H, Shiratori Y, Moriyama M, et al. Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. IHIT Study Group. Inhibition of Hepatocarcinogenesis by Interferon Therapy. *Ann Intern Med* 1999;131:174–81.
5. Bertino G, Di Carlo I, Ardiri A, et al. Systemic therapies in hepatocellular carcinoma: present and future. *Future Oncol* 2013;9:1533–48.
6. Biondi A, Malaguarnera G, Vacante M, et al. Elevated serum levels of Chromogranin A in hepatocellular carcinoma. *BMC Surg* 2012;12:S7.
7. Bertino G, Ardiri A, Malaguarnera M, et al. Hepatocellualar carcinoma serum markers. *Semin Oncol* 2012;39:410–33.
8. Soroida Y, Ohkawa R, Nakagawa H, et al. Increased activity of serum mitochondrial isoenzyme of creatine kinase in hepatocellular carcinoma patients predominantly with recurrence. *J Hepatol* 2012;57:330–36.
9. Uranbileg B, Enooku K, Soroida Y, et al. High ubiquitous mitochondrial creatine kinase expression in hepatocellular carcinoma denotes a poor prognosis with highly malignant potential. *Int J Cancer* 2013 Oct 15. doi: 10.1002/ijc.28547. [Epub ahead of print]
10. Kanemitsu F, Kawanishi I, Mizushima J, et al. Mitochondrial creatine kinase as a tumor-associated marker. *Clin Chim Acta* 1984;138:175–83.
11. Pratt R, Vallis LM, Lim CW, et al. Mitochondrial creatine kinase in cancer patients. *Pathology* 1987;19:162–65.
12. Qian XL, Li YQ, Gu F, et al. Overexpression of ubiquitous mitochondrial creatine kinase (uMtCK) accelerates tumor growth by inhibiting apoptosis of breast cancer cells and is associated with a poor prognosis in breast cancer patients. *Biochem Biophys Res Commun* 2012;427:60–66.
13. Patra S, Bera S, SinhaRoy S, et al. Progressive decrease of phosphocreatine, creatine and creatine kinase in skeletal muscle upon transformation to sarcoma. *FEBS J* 2008;275:3236–47.
14. Stein W, Bohner J, Renn W, et al. Macro creatine kinase type 2: results of a prospective study in hospitalized patients. *Clin Chem* 1985;31:1959–64.
15. Lee KN, Csako G, Bernhardt P, et al. Relevance of macro creatine kinase type 1 and type 2 isoenzymes to laboratory and clinical data. *Clin Chem* 1994;40:1278–83.
16. Hoshino T, Sakai Y, Yamashita K, et al. Development and performance of an enzyme immunoassay to detect creatine kinase isoenzyme MB activity using anti-mitochondrial creatine kinase monoclonal antibodies. *Scand J Clin Lab Invest* 2009;69:687–95.
17. Castera L, Vergniol J, Foucher J, et al. Prospective comparison of transient elastography, Fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. *Gastroenterology* 2005;128:343–50.
18. Kageyama Y, Ikeda H, Watanabe N, et al. Antagonism of sphingosine 1-phosphate receptor 2 causes a selective reduction of portal vein pressure in bile duct-ligated rodents. *Hepatology* 2012;56:1427–38.
19. Torzilli G, Minagawa M, Takayama T, et al. Accurate preoperative evaluation of liver mass lesions without fine-needle biopsy. *Hepatology* 1999;30:889–93.
20. Makuuchi M, Kokudo N, Arii S, et al. Development of evidence-based clinical guidelines for the diagnosis and treatment of hepatocellular carcinoma in Japan. *Hepatol Res* 2008;38:37–51.
21. Tsukuma H, Hiyama T, Tanaka S, et al. Risk factors for hepatocellular carcinoma among patients with chronic liver disease. *N Engl J Med* 1993; 328:1797–801.
22. Ikeda K, Saitoh S, Koida I, et al. A multivariate analysis of risk factors for hepatocellular carcinogenesis: a prospective observation of 795 patients with viral and alcoholic cirrhosis. *Hepatology* 1993;18:47–53.
23. Tarao K, Rino Y, Ohkawa S, et al. Association between high serum alanine aminotransferase levels and more rapid development and higher rate of incidence of hepatocellular carcinoma in patients with hepatitis C virus-associated cirrhosis. *Cancer* 1999;86:589–95.
24. Tarao K, Takemiya S, Tamai S, et al. Relationship between the recurrence of hepatocellular carcinoma (HCC) and serum alanine aminotransferase levels in hepatectomized patients with hepatitis C virus-associated cirrhosis and HCC. *Cancer* 1997; 79:688–94.
25. El-Serag HB, Marrero JA, Rudolph L, et al. Diagnosis and treatment of hepatocellular carcinoma. *Gastroenterology* 2008;134:1752–63.
26. Bertino G, Neri S, Bruno CM, et al. Diagnostic and prognostic value of alpha-fetoprotein, des-gamma-carboxy prothrombin and squamous cell carcinoma antigen immunoglobulin M complexes in hepatocellular carcinoma. *Minerva Med* 2011; 102:363–71.
27. Bayati N, Silverman AL, Gordon SC. Serum alpha-fetoprotein levels and liver histology in

patients with chronic hepatitis C. *Am J Gastroenterol* 1998;93:2452–56.
28. Goldstein NS, Blue DE, Hankin R, et al. Serum alpha-fetoprotein levels in patients with chronic hepatitis C. Relationships with serum alanine aminotransferase values, histologic activity index, and hepatocyte MIB-1 scores. *Am J Clin Pathol* 1999;111:811–16.
29. Chu CW, Hwang SJ, Luo JC, et al. Clinical, virologic, and pathologic significance of elevated serum alpha-fetoprotein levels in patients with chronic hepatitis C. *J Clin Gastroenterol* 2001;32:240–44.
30. Hu KQ, Kyulo NL, Lim N, et al. Clinical significance of elevated alpha-fetoprotein (AFP) in patients with chronic hepatitis C, but not hepatocellular carcinoma. *Am J Gastroenterol* 2004;99:860–65.
31. Oka H, Tamori A, Kuroki T, et al. Prospective study of alpha-fetoprotein in cirrhotic patients monitored for development of hepatocellular carcinoma. *Hepatology* 1994;19:61–66.
32. Sangiovanni A, Colombo E, Radaelli F, et al. Hepatocyte proliferation and risk of hepatocellular carcinoma in cirrhotic patients. *Am J Gastroenterol* 2001;96:1575–80.
33. Schlattner U, Tokarska-Schlattner M, Wallimann T. Mitochondrial creatine kinase in human health and disease. *Biochim Biophys Acta* 2006;1762:164–80.
34. Vaubourdolle M, Chazouilleres O, Poupon R, et al. Creatine kinase-BB: a marker of liver sinusoidal damage in ischemia-reperfusion. *Hepatology* 1993;17:423–28.
35. Kanemitsu F, Kawanishi I, Mizushima J. A new creatine kinase found in mitochondrial extracts from malignant liver tissue. *Clin Chim Acta* 1983;128:233–40.
36. Dolder M, Walzel B, Speer O, et al. Inhibition of the mitochondrial permeability transition by creatine kinase substrates. Requirement for microcompartmentation. *J Biol Chem* 2003;278:17760–66.
37. Hatano E, Tanaka A, Kanazawa A, et al. Inhibition of tumor necrosis factor-induced apoptosis in transgenic mouse liver expressing creatine kinase. *Liver Int* 2004;24:384–93.
38. Miller K, Halow J, Koretsky AP. Phosphocreatine protects transgenic mouse liver expressing creatine kinase from hypoxia and ischemia. *Am J Physiol* 1993;265:C1544–51.
39. Hatano E, Tanaka A, Iwata S, et al. Induction of endotoxin tolerance in transgenic mouse liver expressing creatine kinase. *Hepatology* 1996;24:663–69.
40. Miller K, Sharer K, Suhan J, et al. Expression of functional mitochondrial creatine kinase in liver of transgenic mice. *Am J Physiol* 1997;272:C1193–202.
41. Tanaka Y, Hanada K, Mizokami M, et al. A comparison of the molecular clock of hepatitis C virus in the United States and Japan predicts that hepatocellular carcinoma incidence in the United States will increase over the next two decades. *Proc Natl Acad Sci USA* 2002;99:15584–89.

J Gastroenterol
DOI 10.1007/s00535-013-0909-8

REVIEW

The role of microRNAs in hepatocarcinogenesis: current knowledge and future prospects

Motoyuki Otsuka · Takahiro Kishikawa · Takeshi Yoshikawa · Motoko Ohno · Akemi Takata · Chikako Shibata · Kazuhiko Koike

Received: 17 October 2013 / Accepted: 4 November 2013

Abstract MicroRNAs (miRNAs) are small, noncoding RNA molecules that regulate gene expression post-transcriptionally through complementary base pairing with thousands of messenger RNAs. Although the precise biological functions of individual miRNAs are still unknown, miRNAs are speculated to play important roles in diverse biological processes through fine regulation of their target gene expression. A growing body of data indicates the deregulation of miRNAs during hepatocarcinogenesis. In this review, we summarize recent findings regarding deregulated miRNA expression and their possible target genes in hepatocarcinogenesis, with emphasis on inflammation-related hepatocarcinogenesis. Because miRNA-based strategies are being applied to clinical therapeutics, precise knowledge of miRNA functions is crucial both scientifically and clinically. We discuss the current open questions from these points of view, which must be clarified in the near future.

Keywords MicroRNA · Hepatocarcinogenesis · Inflammation

M. Otsuka (✉) · T. Kishikawa · T. Yoshikawa · M. Ohno · A. Takata · C. Shibata · K. Koike
Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo, 5-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
e-mail: otsukamo-tky@umin.ac.jp

M. Otsuka
Japan Science and Technology Agency, PRESTO, Kawaguchi, Saitama 332-0012, Japan

Published online: 21 November 2013

Introduction

MicroRNAs (miRNAs) are short, single-stranded, noncoding RNAs, which are expressed in most organisms, from plants to vertebrates [1]. Since the discovery of the miRNA lin-4 in *Caenorhabditis elegans* [2, 3], 1,872 miRNA precursors and 2,578 mature miRNA sequences in humans have been deposited in miRBase, a public repository hosted by the Sanger Institute, as of November 2013 [4]. Bioinformatic predictions suggest that miRNAs regulate more than 30 % of human protein-coding genes [5–7]. Through the regulation of gene expression, miRNAs are involved in various physiological and pathological processes, including cell proliferation, apoptosis, differentiation, metabolism, oncogenesis and oncogenic suppression [8, 9]. Thus, it is not surprising that deregulation of miRNAs is linked closely to various human pathological conditions. In this review, we will describe the crucial role of miRNAs in liver carcinogenesis, especially inflammation-related hepatocarcinogenesis.

Biogenesis and functions of miRNAs

Transcription is the first step in miRNA expression (Fig. 1). Similar to most protein-coding genes, transcriptional factors, enhancers and silencers are involved in miRNA transcription [10–12]. Epigenetic mechanisms, such as promoter methylation or histone modification, also regulate miRNA transcription, and it was shown that histone deacetylase (HDAC) inhibition results in transcriptional changes in ~40 % of miRNAs [13].

Primary miRNAs, which possess stem-loop structures, are transcribed by RNA polymerase II [8]. These pri-miRNAs are processed by a microprocessor complex

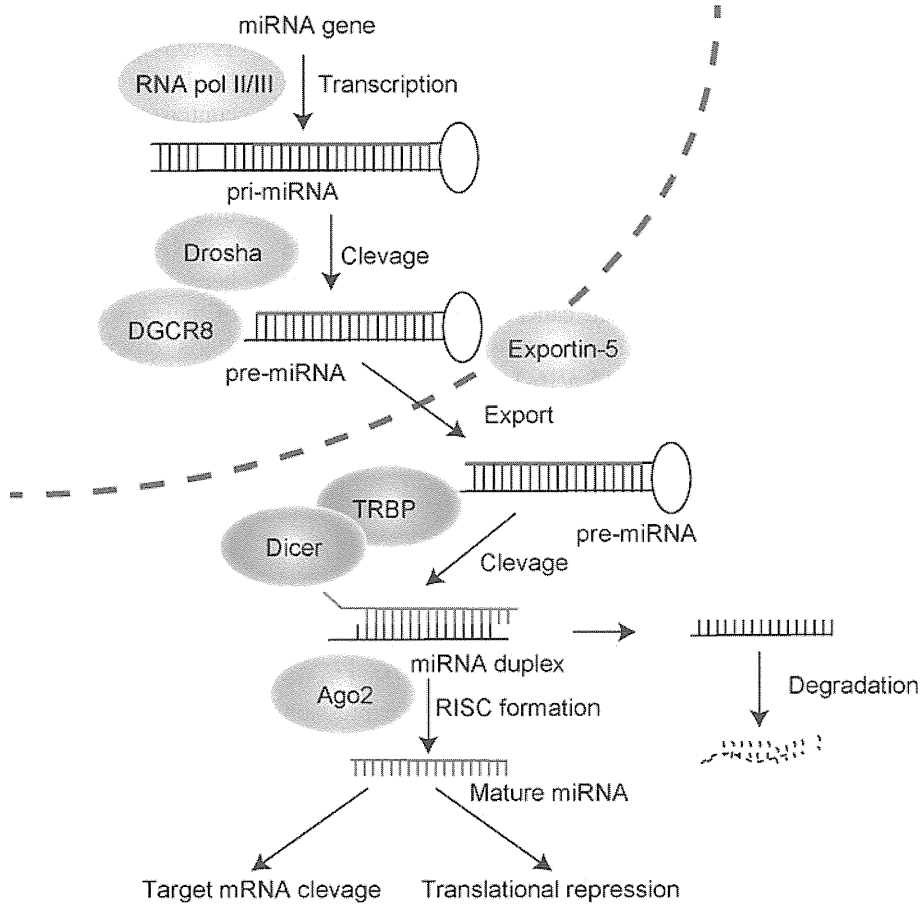

Fig. 1 Biogenesis of miRNAs. The primary miRNA transcript (pri-miRNA) is transcribed from the genome by RNA polymerase II or III. The microprocessor complex Drosha–DGCR8 cleaves the pri-miRNA into the precursor hairpin, pre-miRNA in the nucleus. The pre-miRNA is exported from the nucleus by exportin-5–Ran-GTP. In the cytoplasm, the RNase Dicer in complex with the double-stranded RNA-binding protein, TRBP, cleaves the pre-miRNA hairpin to its mature length. The functional strand of the mature miRNA is loaded together with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC), where it guides RISC to silence target mRNAs through mRNA cleavage or translational repression. The passenger strand (*black*) is degraded

comprising Drosha (RNAase III) [14] and DGCR8/Pasha [15] in the nucleus [16]. The processed products are approximately 65-nucleotide hairpin-shaped precursors (pre-miRNAs) that are transported to the cytoplasm via exportin-5 [17, 18]. Pre-miRNAs are further cleaved into mature miRNAs by Drosha and Dicer RNA polymerase III. Mature miRNA duplexes are loaded onto an RNA-induced silencing complex (RISC) and are unwound into the single-stranded mature form [19–21]. The resulting co-complex directly targets the 3′-untranslated regions (3′-UTRs) of target mRNAs, depending on the sequence similarities, to negatively regulate their expression by enhancing mRNA cleavage or inhibiting translation (Fig. 1) [8, 22]. Because most miRNAs guide the recognition of imperfect matches of target mRNAs, individual miRNAs have multiple (probably hundreds) of mRNA targets. In addition, multiple miRNAs can cooperate to regulate the expression of the same transcript [6]. Thus, depending upon the identity of the target mRNAs, miRNAs play roles as "fine-tuners of gene expression" in the control of various biological functions.

Identifying functionally important miRNA target genes is crucial for understanding the impact of specific miRNAs on cellular function. However, this is challenging because miRNAs usually have imperfect complementarity with their targets [22]. In mammals, the most consistent requirement for miRNA-target interaction, although not always essential, is a contiguous and perfect pairing of the miRNA (nt 2–8), representing the "seed" sequence [22]. In many cases, the seed sequences determine this recognition, but in other cases, additional determinants are required, such as reasonable complementarity to the miRNA 3′ half to stabilize the interaction. In addition, target pairing to the center of some miRNAs has also been reported [23]. Although public miRNA target prediction algorithms, such as TargetScan [24] and PicTar [25], have facilitated the rapid identification of miRNA target genes [22], candidates should be validated experimentally.

miRNAs and cancer

The involvement of miRNAs in cancer pathogenesis is well established. miRNAs can affect six hallmarks of malignant cells, which are (1) self-sufficiency in growth signals, (2) insensitivity to anti-growth signals, (3) evasion of apoptosis, (4) limitless replicative potential, (5) angiogenesis, and (6) invasion and metastasis [26]. miRNAs are frequently

up- or downregulated in malignant tissues and can be considered oncogenes or tumor suppressors, respectively. However, it is essential to test experimentally whether the deregulated miRNAs are actually causative to carcinogenesis, since miRNAs have a very restricted tissue-specific expression and the apparent miRNA modulation in cancer tissues may only reflect the different constituents of a cell population as compared to normal tissues. Extensive analyses have confirmed the causative roles of miRNAs in cancer by using either human cancer cells or genetically engineered animal models, such as transgenic expression of miR-155, miR-21 and miR-15-a/16-1, which are sufficient to initiate lymphomagenesis in mice [27–29]. These results suggest the potential role of miRNAs in the pathogenesis of carcinogenesis and as therapeutic targets.

miRNAs and hepatocarcinogenesis

Numerous reports regarding the deregulated expression of miRNAs in human hepatocellular carcinoma (HCC) are extant. Most studies compared the miRNA expression levels between cancer tissues and background non-tumorous tissues, selected candidate miRNA(s) and revealed their target genes, which may be involved in carcinogenesis. As shown in Tables 1 and 2, many miRNAs have been identified as downregulated or upregulated in recent studies (Tables 1, 2). However, these numerous results are not always superimposable due to the large variances in the results. These significant differences may be due to several reasons, such as the use of different techniques or different samples as controls, normal liver tissues versus peritumoral non-neoplastic tissues. In addition, one may need to take into consideration the fact that HCCs arise in background livers with different etiologies, such as hepatitis B, hepatitis C or steatohepatitis, and also the age or sex of the tissue-derived patients and background liver condition, such as fibrosis staging or inflammation activity, which may result in differences in the expression status of miRNAs. Despite these considerable limitations, the list suggests that diverse miRNAs play crucial roles in hepatocarcinogenesis. We will briefly describe some of them below.

The expression levels of miRNAs have restricted tissue specificities. In the liver, miR-122, miR-192 and miR-199a/b-3p are the three most expressed miRNAs, accounting for 52, 17 and 5 % of all mRNAs in the tissues, respectively [30]. The tumorigenic role of the loss of miR-122 was confirmed in gene-knockout mice [31, 32] and its expression is indeed decreased in half of the HCCs, especially non-viral HCCs [30]. We also reported that decreased expression of miR-122 is linked with poor prognosis of HCC [33]. While miR-192 does not appear to be deregulated in HCC samples in previous studies, miR-199a/b-3p is decreased with high frequency in HCC, which is closely linked to a poor prognosis of HCC [30]. In contrast, miR-21, whose expression is increased following rat hepatectomy [34], is upregulated as a known oncomiRNA and represses PTEN signaling, resulting in promotion of HCC development [35]. Although individual miRNAs may be involved in hepatocarcinogenesis, because miRNAs often function co-operatively, the extent of their involvement remains to be determined.

As described above, miRNAs usually have multiple mRNA targets. Thus, it is not practical to describe only a few genes as being responsible for the phenotypes by deregulation of specific miRNAs, while many studies identify specific genes as targets of specific miRNAs. Nonetheless, the identified targeted genes are generally related to at least one of the hallmarks of cancer, such as cell growth, apoptosis, invasion, and so on. These results suggest that the deregulation of miRNA expression might mediate hepatocarcinogenesis through deregulating the expression of their target genes.

The miRNAs identified as deregulated in hepatocarcinogenesis may be useful as diagnostic and prognostic markers [36], because miRNAs in the circulation are reported to be relatively stable [37]. Also, deregulated miRNAs may be candidate therapeutic and preventive targets against HCC. However, to include the obtained results in clinical interventional applications, it is necessary to confirm if the deregulated miRNAs are truly drivers or are simply passive in hepatocarcinogenesis. To this end, genetically modified mice may provide some information. In addition, to correctly interpret the data, a standard method of normalizing the microRNAome data between studies may also be crucial. Since there are multiple target genes of miRNAs and, conversely, one transcript can be targeted by multiple miRNAs, a more systematic comparison using miRNA data, transcriptome data and proteome data would increase our understanding of the consequences of the deregulation of miRNAs during hepatocarcinogenesis. From this point of view, systematic and comprehensive target gene analyses for in silico systems biology models may be one option to resolve these issues.

miRNAs linked to inflammation-mediated hepatocarcinogenesis

Inflammation is considered to be a major cause of cancer [38, 39]. In the liver, hepatocarcinogenesis frequently occurs in persistently inflamed liver tissues caused by chronic hepatitis viral infection or non-alcoholic steatohepatitis. However, the molecular linkage between chronic inflammation and carcinogenesis is not well characterized.

Table 1 Upregulated miRNAs in hepatocarcinogenesis

miRNA	Expression levels	Targets	Main tested samples	References
miR-17-5p	Upregulated	p38 pathway	Cultured cells, human tissues	[52]
miR-18a	Upregulated	ER1a	Human tissues, cultured cells	[53]
miR-21	Upregulated	C/EBPb	Mouse CDAA model	[54]
	Upregulated	PTEN	Human tissues, cultured cells	[35]
miR-22	Upregulated	ERa, IL-1a	Human tissues, cultured cells, DEN model	[55]
miR-23a	Upregulated	PGC-1a,G6PC	Human tissues, cultured cells	[56]
miR-26a	Upregulated	Lin28B, Zcchc11	Human tissues, xenograft model	[57]
	Upregulated	NF-κB, IL-6 pathways	Human tissues	[58]
miR-30d	Upregulated	GNAI2	Human tissues, cultured cells	[59]
miR-100	Upregulated		Human tissues	[60]
miR-106b	Upregulated	APC	Human tissues, cultured cells	[61]
miR-122	Upregulated		Human tissues	[60]
miR-130b	Upregulated	TP53INP1	Human tissues, xenograft model	[62]
miR-135a	Upregulated	FOXM1, MTSS1	Human tissues, cultured cells, xenograft	[63]
miR-143	Upregulated	FNDC3B	Human tissues, HBX transgenic mouse	[64]
miR-146a	Upregulated in endothelial cells	BRCA, PDGFRA	Cultured cells	[65]
miR-151	Upregulated	FAK	Human tissues, cultured cells	[66]
	Upregulated	FAK, RhoGDIA	Human tissues, cultured cells	[67]
miR-155	Upregulated	SOCS1	Orthotropic transplant model	[68]
	Upregulated	DKK1, APC	Human tissues, cultured cells	[69]
	Upregulated	PTEN	Mouse CDAA model	[54]
miR-181	Upregulated	TIMP3	Mouse CDAA model	[70]
	Upregulated	CDX2, GATA6, NLK	Cultured cells	[71]
miR-183	Upregulated	AKAP12	Human tissues	[72]
miR-186	Upregulated	AKAP12	Human tissues	[72]
miR-200	Upregulated	NRF2 pathway	Rat HCC model,	[73]
miR-210	Upregulated	VMP1	Human tissues, cultured cells	[74]
miR-216a	Upregulated	TSLC1	Human tissues, cultured cells	[75]
miR-216a/217	Upregulated	PTEN, SMAD7	Cultured cells, Human tissues	[76]
miR-221	Upregulated	CDK inhibitors	Transgenic mouse	[77]
	Upregulated	p27, p57, Arnt	Primary hepatocytes	[78]
	Upregulated	Bmf	Cultured cells, human tissues	[79]
	Upregulated	p27, p57	Cultured cells, human tissues	[80]
miR-221/222	Upregulated	p27, DDIT4	Human tissues, mouse model	[81]
miR-224	Upregulated		Human tissues	[82]
	Upregulated	Atg5, Smad4, autophagy	Human tissues, HBV X transgenic mice	[83]
	Upregulated	API-5	Cultured cells, human tissues	[84]
	Upregulated		Human tissues	[85]
	Upregulated	API-5	Human tissues	[86]
miR-423	Upregulated	p21/waf1	Human tissues, cultured cells	[87]
miR-485-3p	Upregulated	MAT1, LIN28B	Human tissues, xenograft model	[88]
miR-490-3p	Upregulated	ERCIC3	Human tissues, cultured cells	[89]
miR-494	Upregulated	MCC	Human tissue, mouse liver cancer model	[90]
miR-495	Upregulated	MAT1, LIN28B	Human tissues, xenograft model	[88]
miR-517a	Upregulated		Human tissues, cultured cells	[91]
miR-657	Upregulated	TLE1, NF-κB	Human tissues, cultured cells	[92]
miR-664	Upregulated	MAT1, LIN28B	Human tissues, xenograft model	[88]
miR-1323	Upregulated		Human tissues	[93]

Table 2 Downregulated miRNAs in hepatocarcinogenesis

miRNA	Expression levels	Targets	Main tested samples	References
let-7a	Downregulated	STAT3	Cultured cells	[94]
let-7c	Downregulated		Human tissues, cultured cells	[95]
let-7g	Downregulated	COL12A	Cultured cells, human tissues	[96]
miR-7	Downregulated	PIK3CD	Cultured cells, human tissues	[97]
miR-10a	Downregulated	EphA4	Cultured cells	[98]
miR-10b	Downregulated		Human tissues	[99]
miR-15a/16	Downregulated		Cultured cells	[100]
miR-21	Downregulated		Human tissues	[82]
miR-26a	Downregulated	IL-6	Human tissues, xenograft model	[101]
	Downregulated	CyclinD2, E2	Cultured cells, mouse model	[102]
miR-29	Downregulated	Bcl2, Mcl1	Human tissues, cultured cells	[103]
miR-29b	Downregulated	MMP-2	Human tissues, cultured cell	[104]
miR-29c	Downregulated	SIRT1	Cultured cells	[105]
miR-34a	Downregulated	CCL22	Human tissues, cultured cells	[106]
miR-99a	Downregulated	PLK1	Human tissues, cultured cells	[107]
	Downregulated	IGF-1R	Human tissues, cultured cells	[108]
miR-100	Downregulated	PLK1	Human tissues, cultured cells	[107]
miR-101	Downregulated	EZH2, EED	Human tissues, cultured cells	[109]
	Downregulated		Human tissues, cultured cells	[95]
	Downregulated	Mcl1	Cultured cells, human tissues	[110]
	Downregulated	Fos	Human tissues, cultured cells	[111]
miR-122	Downregulated	c-Myc	Human tissues, cultured cells	[112]
	Downregulated		Cultured cells	[113]
	Downregulated	MTTP	Knockout mice	[32]
	Downregulated	IL6, TNF	Knockout mice	[31]
	Downregulated	IGF-1R	Human tissues	[114]
	Downregulated	Cyclin G1	Human tissues, cultured cells	[115]
miR-124	Downregulated	ROCK2, EZH2	Human tissues, cultured cells	[116]
	Downregulated	CDK6, VIM, SMYD3, IQGAP1	Human tissues, cultured cells	[117]
miR-125a/125b	Downregulated		Human tissues, cultured cells	[118]
miR-125b	Downregulated	SUV39H	Human tissues, cultured cells	[119]
	Downregulated	Mcl1, Bclw, IL6R	Human tissues, cultured cells	[120]
	Downregulated		Human tissues, cultured cells	[95]
	Downregulated	PIGF, MMP-2, MMP-9	Human tissues, cultured cells	[121]
	Downregulated	Lin28B	Human tissues, cultured cells	[122]
miR-139	Downregulated	ROCK2	Human tissues, cultured cells	[123]
miR-139-5p	Downregulated		Human tissues, cultured cells	[95]
miR-140-5p	Downregulated	TGFBR1, FGF9	Human tissues, cultured cells	[124]
		DNMT1	Knockout mice	[125]
miR-141	Downregulated	DLC-1	Human tissues	[126]
miR-145	Downregulated		Human tissues	[60]
	Downregulated	IRS1, IRS2, IGF-1R, b-catenin	Human tissues, cultured cells	[127]
	Downregulated		Human tissues	[85]
miR-148a	Downregulated	c-Met	Human tissues, cultured cells	[128]
	Downregulated	HRIP	Mouse xenograft model, cultured cells	[129]
	Downregulated	e-cadherin	Human tissues, cultured cells	[130]
	Downregulated	c-Myc	Cultured cells	[131]
miR-152	Downregulated	DNMT1, GSTP1, CDH1	Human tissues	[132]

Table 2 continued

miRNA	Expression levels	Targets	Main tested samples	References
miR-195	Downregulated	NF-κB pathway	Cultured cells	[133]
	Downregulated	VEGF, VAV2, CDC42	Cultured cells, human tissues	[134]
	Downregulated	Cyclin D1, CDK6, E2F3	Cultured cells, human tissues	[135]
miR-198	Downregulated		Human tissues	[60]
miR-199a/b-3p	Downregulated	PAK4	Human tissues, cultured cells	[30]
miR-199b	Downregulated		Human tissues	[85]
miR-200a	Downregulated	H3 acetylation	Human tissues, cultured cells	[136]
miR-200b	Downregulated		Human tissues, cultured cells	[95]
miR-200c	Downregulated		Human tissues	[82]
miR-200	Downregulated		Human tissues	[82]
miR-203	Downregulated	ABCE1	Human tissues, cultured cells	[117]
miR-214	Downregulated	HDGF	Human tissues, cultured cells	[137]
miR-222	Downregulated		Human tissues	[82]
miR-223	Downregulated	STMN1	Human tissues	[138]
miR-224	Downregulated		Human tissues	[139]
miR-363-3p	Downregulated	c-Myc	Cultured cells	[131]
miR-375	Downregulated	ATG7	Human tissues, cultured cells	[140]
	Downregulated	AEG-1	Human tissues, cultured cells	[141]
miR-429	Downregulated	Rab18	Cultured cells	[142]
miR-449	Downregulated	c-MET	Xenograft, cultured cells	[143]
miR-520e	Downregulated	NIK	Human tissues, cultured cells	[69]
miR-612	Downregulated	AKT2	Cultured cells, human tissues	[144]
miR-637	Downregulated	STAT3 activation	Human tissues, cultured cells	[145]
miR-1271	Downregulated	GLP3	Human tissues, cultured cells	[99]

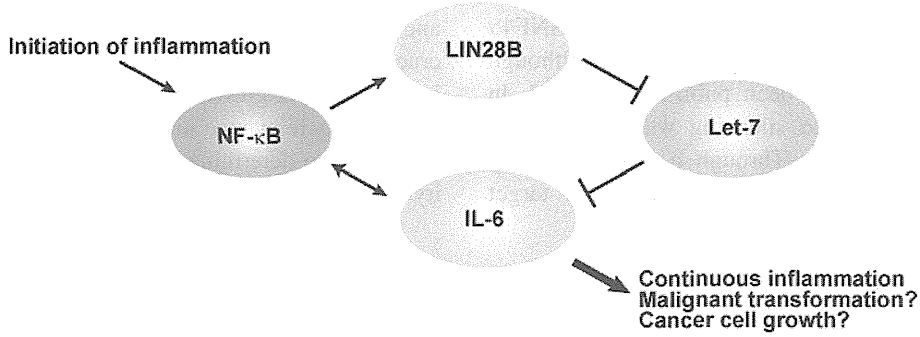

Fig. 2 A model bridging chronic inflammation and transformation by miRNA. Inflammation triggers activation of NF-κB, which leads to transcription of LIN28B. LIN28B inhibits the production of Let-7. Let-7 normally inhibits IL-6 expression, resulting in higher levels of IL-6 than are achieved by NF-κB activation. IL-6 mediated STAT3 activation is necessary for transformation and IL-6 activates NF-κB, completing a positive feedback loop

miRNAs, as a new class of gene expression regulators, may be involved in chronic inflammation-induced carcinogenesis and, in fact, several studies have clarified one such linkage, in which miRNAs may serve as a bridge between continuous inflammation and carcinogenesis.

A flagship report addresses a positive feedback loop of an inflammatory response mediated by NF-κB that activates Lin28B transcription (Fig. 2) [40]. LIN28B, which is an inhibitor of miRNA processing, reduces let-7 levels. Let-7 inhibits IL-6 expression, resulting in higher levels of IL-6 than achieved by NF-κB activation. IL-6-mediated STAT3 activation is necessary for transformation and IL-6 activates NF-κB, completing a positive feedback loop. Although the experiments mainly used MCF10A cells (breast cancer cells), a similar feedback loop was observed in HCC tissues. The authors termed these mechanisms an

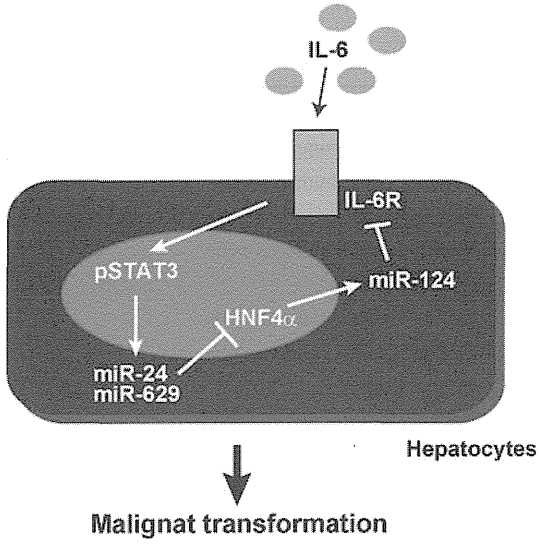

Fig. 3 A model describing a positive feedback loop mediated by miRNAs from transient HNF4α inhibition to transformation. Transient silencing of HNF4α is mediated by miR-24 and miR-629, both of which are induced by STAT3 activation following IL-6 stimulation. miR-124, whose promoter region contains HNF4α-binding sites, targets IL-6R and, thus, HNF4α silencing results in reduced expression of miR-124 and enhanced expression of IL-6R and activation of STAT3, which induces miR-24 and miR-629. This microRNA feedback-inflammatory loop is thought to be crucial in IL-6-mediated liver cancer

"epigenetic switch" because the loop maintains the epigenetic transformed state even in the absence of induction by inflammation (Fig. 2).

Another report addressed hepatocarcinogenesis induced by transient inhibition of HNF4α (Fig. 3) [41]. HNF4α was reported to be involved in liver oncogenesis, although discrepant reports have also been published [42–44]. In that report, transient HNF4α silencing was sufficient to maintain cell transformation. Through a miRNA library screen, miR-24 and miR-629 were identified to target HNF4α. Interestingly, both miRNAs were induced following HNF4α silencing, supporting their involvement in the HNF4α-dependent feedback loop. miR-24 and miR-629 contain the STAT3-binding motif in their promoter region. The authors showed that in response to IL-6, STAT3 binding to their promoters increased, resulting in miRNA expression. They also identified miR-124, whose promoter region contains HNF4α binding sites. miR-124 targets IL-6R and, thus, HNF4α silencing results in reduced expression of miR-124 and enhanced expression of IL-6R and activation of STAT3. The importance of these feedback loops was confirmed in vivo using a mouse HCC model induced by diethylnitrosamine. miR-124 delivery by cationic liposomes prevented tumor development. Thus, these microRNA feedback-inflammatory loops are important and can be a therapeutic target for liver cancer (Fig. 3) [41].

A recent paper reported a similar but distinct observation (Fig. 4). The authors found that when using DEN-induced foci of altered hepatocytes (FAH), LIN28-expressing cells are present in FAH, in which let-7 is down-regulated, resulting in the enhanced expression of IL-6, mediating the progression of malignancies from progenitors. An important difference between the cells in FAH and those in early hepatocarcinogenesis is that IL-6 signaling is autocrine, being mediated by reduced let-7 due to upregulation of LIN28B in FAH cells. This mechanism may contribute to malignant progression from HCC progenitor cells (Fig. 4) [45].

These three reports are from related research groups, and rely on the hypothesis that the IL-6-STAT3 pathway is crucial for hepatocarcinogenesis. Although IL-6 has been implicated as a growth factor in various epithelial cancers [46, 47], its relevance in hepatocarcinogenesis needs to be confirmed to determine the applicability and reproducibility of these findings to the clinical setting.

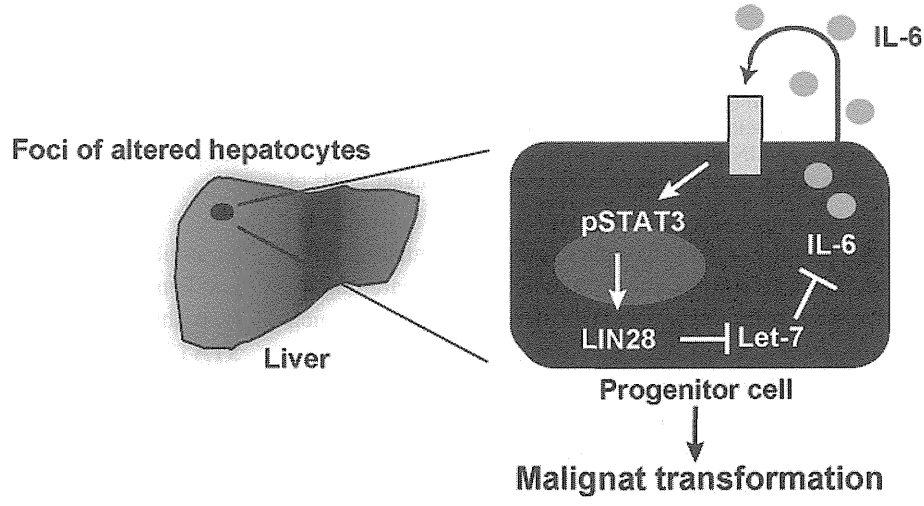

Fig. 4 A model bridging the malignant transformation of precursor cells and autocrine-mediated inflammation by microRNA. LIN28-expressing cells exist in the foci of altered hepatocytes, in which let-7 is downregulated, resulting in enhanced IL-6 expression, which mediates the progression of malignancies from progenitor cells

miRNAs as therapeutic targets in the liver

Recently, miravirsen, a LNA-modified DNA phosphorothioate antisense oligonucleotide against miR-122, became the first miRNA-targeting drug for clinical use [48]. It was developed to target HCV, as the stability and propagation of this virus is dependent on a functional interaction between the HCV genome and miR-122 [49, 50]. No harmful events were observed in Phase I studies in healthy volunteers, and Phase II studies proceeded to evaluate the safety and efficacy of miravirsen in 36 patients with chronic HCV genotype 1 infection. The patients were randomly assigned to receive 5 weeks of subcutaneous miravirsen injections at 3, 5 or 7 mg per kg body weight or a placebo over a 29-day period. Miravirsen resulted in a dose-dependent reduction in HCV levels, without major adverse events and with no escape mutations in the miR-122 binding sites of the HCV genome [48]. The success of miravirsen is promising, not only as a novel anti-HCV drug, but also as the first trial of miRNA-targeting therapy.

In addition to miravirsen, a clinical trial of MRX34 as a mimic of miR-34 is underway. MRX34 is a liposome-formulated mimic of the tumor suppressor miR-34 (Mirna Therapeutics, Austin, TX, USA). Further study of MRX34 is being conducted by Mirna Therapeutics, which initiated a Phase I study in May 2013 to examine the effects of MRX34 on unresectable primary liver cancer or advanced or metastatic cancer with liver involvement (ClinicalTrials.gov Identifier: NCT01829971). If these oligonucleotide therapies are successful, therapeutic options based on the numerous miRNAs deregulated during hepatocarcinogenesis appear promising [51].

Issues to be resolved in miRNA involvement in hepatocarcinogenesis

As described above, along with recent discoveries of the diverse effects of miRNAs in hepatocarcinogenesis, miRNA-mediated intervention is promising for the development of new diagnostic, preventive and therapeutic tools. However, the data obtained to date are far from complete. The following are some of the critical issues that we believe need to be resolved.

1. The reason for the non-reproducible results among studies should be determined to utilize the available data more reasonably and efficiently.
2. Identification of crucial driver miRNAs among the diverse deregulated miRNAs is critical to develop useful therapeutics in clinics, although even passive miRNAs may be utilized as markers for diagnosis or prediction of prognosis.
3. Comprehensive target gene analyses using in silico systems biology models should be applied.
4. For effective interventions using miRNA, the delivery method, improved oligonucleotide modification and safety must be further considered. Since miRNAs generally have diverse effects due to targeting multiple mRNAs, undesired outcomes, so called off-target effects, may be encountered, even when a specific miRNA is targeted.

Finding solutions to these issues should be considered as critically important for the near future in order to understand more fully the physiological function of miRNAs in hepatocarcinogenesis and utilize this knowledge in translational research.

Conclusions

The discovery of miRNA has, without doubt, opened up new possibilities for understanding the molecular mechanisms of gene regulation. As numerous findings regarding miRNA, from diverse perspectives, have been reported, the speed of discovery in this field is astonishing. In fact, novel therapeutics targeting miRNAs have already been successfully applied in clinical trials. Some miRNAs may be useful as novel biomarkers. Additionally, the discovery of novel concepts in the pathogenesis of hepatocarcinogenesis frequently involves miRNA. On the other hand, several important issues remain to be resolved in this field. Thus, continuous research in this field is still necessary to develop truly innovative concepts in our understanding of pathogenesis related to miRNA and to transform the obtained knowledge into real clinical applications.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Carrington J, Ambros V. Role of microRNAs in plant and animal development. Science. 2003;301:336–8.
2. Lee RC, Feinbaum RL, Ambros V. The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell. 1993;75:843–54.
3. Wightman B, Ha I, Ruvkun G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*. Cell. 1993;75:855–62.
4. Kozomara A, Griffiths-Jones S. miRBase: integrating microRNA annotation and deep-sequencing data. Nucleic Acids Res. 2011;39:D152–7.
5. John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS. Human MicroRNA targets. PLoS Biol. 2004;2:e363.
6. Krek A, Grün D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, et al. Combinatorial microRNA target predictions. Nat Genet. 2005;37:495–500.

7. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell. 2005;120:15–20.
8. Ambros V. The functions of animal microRNAs. Nature. 2004;431:350–5.
9. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116:281–97.
10. Cai X, Hagedorn CH, Cullen BR. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. RNA. 2004;10:1957–66.
11. Lee Y, Kim M, Han J, Yeom KH, Lee S, Baek SH, et al. MicroRNA genes are transcribed by RNA polymerase II. EMBO J. 2004;23:4051–60.
12. Borchert GM, Lanier W, Davidson BL. RNA polymerase III transcribes human microRNAs. Nat Struct Mol Biol. 2006;13:1097–101.
13. Scott GK, Mattie MD, Berger CE, Benz SC, Benz CC. Rapid alteration of microRNA levels by histone deacetylase inhibition. Cancer Res. 2006;66:1277–81.
14. Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, et al. The nuclear RNase III Drosha initiates microRNA processing. Nature. 2003;425:415–9.
15. Han J, Lee Y, Yeom KH, Kim YK, Jin H, Kim VN. The Drosha-DGCR8 complex in primary microRNA processing. Genes Dev. 2004;18:3016–27.
16. Denli AM, Tops BB, Plasterk RH, Ketting RF, Hannon GJ. Processing of primary microRNAs by the microprocessor complex. Nature. 2004;432:231–5.
17. Yi R, Qin Y, Macara IG, Cullen BR. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev. 2003;17:3011–6.
18. Lund E, Güttinger S, Calado A, Dahlberg JE, Kutay U. Nuclear export of microRNA precursors. Science. 2004;303:95–8.
19. Maniataki E, Mourelatos Z. A human, ATP-independent, RISC assembly machine fueled by pre-miRNA. Genes Dev. 2005;19:2979–90.
20. Mourelatos Z, Dostie J, Paushkin S, Sharma A, Charroux B, Abel L, et al. miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. Genes Dev. 2002;16:720–8.
21. Gregory RI, Chendrimada TP, Cooch N, Shiekhattar R. Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. Cell. 2005;123:631–40.
22. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009;136:215–33.
23. Shin C, Nam JW, Farh KK, Chiang HR, Shkumatava A, Bartel DP. Expanding the microRNA targeting code: functional sites with centered pairing. Mol Cell. 2010;38:789–802.
24. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res. 2009;19:92–105.
25. Lall S, Grün D, Krek A, Chen K, Wang YL, Dewey CN, et al. A genome-wide map of conserved microRNA targets in *C. elegans*. Curr Biol. 2006;16:460–71.
26. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144:646–74.
27. Costinean S, Sandhu SK, Pedersen IM, Tili E, Trotta R, Perrotti D, et al. Src homology 2 domain-containing inositol-5-phosphatase and CCAAT enhancer-binding protein beta are targeted by miR-155 in B cells of Emicro-MiR-155 transgenic mice. Blood. 2009;114:1374–82.
28. Medina PP, Nolde M, Slack FJ. OncomiR addiction in an in vivo model of microRNA-21-induced pre-B-cell lymphoma. Nature. 2010;467:86–90.
29. Klein U, Lia M, Crespo M, Siegel R, Shen Q, Mo T, et al. The DLEU2/miR-15a/16-1 cluster controls B cell proliferation and its deletion leads to chronic lymphocytic leukemia. Cancer Cell. 2010;17:28–40.
30. Hou J, Lin L, Zhou W, Wang Z, Ding G, Dong Q, et al. Identification of miRNomes in human liver and hepatocellular carcinoma reveals miR-199a/b-3p as therapeutic target for hepatocellular carcinoma. Cancer Cell. 2011;19:232–43.
31. Hsu SH, Wang B, Kota J, Yu J, Costinean S, Kutay H, et al. Essential metabolic, anti-inflammatory, and anti-tumorigenic functions of miR-122 in liver. J Clin Invest. 2012;122:2871–83.
32. Tsai WC, Hsu SD, Hsu CS, Lai TC, Chen SJ, Shen R, et al. MicroRNA-122 plays a critical role in liver homeostasis and hepatocarcinogenesis. J Clin Invest. 2012;122:2884–97.
33. Kojima K, Takata A, Vadnais C, Otsuka M, Yoshikawa T, Akanuma M, et al. MicroRNA122 is a key regulator of α-fetoprotein expression and influences the aggressiveness of hepatocellular carcinoma. Nat Commun. 2011;2:338.
34. Castro RE, Ferreira DM, Zhang X, Borralho PM, Sarver AL, Zeng Y, et al. Identification of microRNAs during rat liver regeneration after partial hepatectomy and modulation by ursodeoxycholic acid. Am J Physiol Gastrointest Liver Physiol. 2010;299:G887–97.
35. Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, Patel T. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. Gastroenterology. 2007;133:647–58.
36. Chiu LY, Kishnani PS, Chuang TP, Tang CY, Liu CY, Bali D, et al. Identification of differentially expressed microRNAs in human hepatocellular adenoma associated with type I glycogen storage disease: a potential utility as biomarkers. J Gastroenterol. 2013 (in press).
37. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci USA. 2008;105:10513–8.
38. Clevers H. At the crossroads of inflammation and cancer. Cell. 2004;118:671–4.
39. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell. 2010;140:883–99.
40. Iliopoulos D, Hirsch HA, Struhl K. An epigenetic switch involving NF-kappaB, Lin28, Let-7 MicroRNA, and IL6 links inflammation to cell transformation. Cell. 2009;139:693–706.
41. Hatziapostolou M, Polytarchou C, Aggelidou E, Drakaki A, Poultsides GA, Jaeger SA, et al. An HNF4α-miRNA inflammatory feedback circuit regulates hepatocellular oncogenesis. Cell. 2011;147:1233–47.
42. Horiguchi N, Takayama H, Toyoda M, Otsuka T, Fukusato T, Merlino G, et al. Hepatocyte growth factor promotes hepatocarcinogenesis through c-Met autocrine activation and enhanced angiogenesis in transgenic mice treated with diethylnitrosamine. Oncogene. 2002;21:1791–9.
43. Ning BF, Ding J, Yin C, Zhong W, Wu K, Zeng X, et al. Hepatocyte nuclear factor 4 alpha suppresses the development of hepatocellular carcinoma. Cancer Res. 2010;70:7640–51.
44. Walesky C, Edwards G, Borude P, Gunewardena S, O'Neil M, Yoo B, et al. Hepatocyte nuclear factor 4 alpha deletion promotes diethylnitrosamine-induced hepatocellular carcinoma in rodents. Hepatology. 2013;57:2480–90.
45. He G, Dhar D, Nakagawa H, Font-Burgada J, Ogata H, Jiang Y, et al. Identification of liver cancer progenitors whose malignant progression depends on autocrine IL-6 signaling. Cell. 2013;155:384–96.
46. Akira S, Kishimoto T. The evidence for interleukin-6 as an autocrine growth factor in malignancy. Semin Cancer Biol. 1992;3:17–26.
47. He G, Karin M. NF-κB and STAT3—key players in liver inflammation and cancer. Cell Res. 2011;21:159–68.

48. Janssen HL, Reesink HW, Lawitz EJ, Zeuzem S, Rodriguez-Torres M, Patel K, et al. Treatment of HCV infection by targeting microRNA. N Engl J Med. 2013;368:1685–94.
49. Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P. Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. Science. 2005;309:1577–81.
50. Fukuhara T, Matsuura Y. Role of miR-122 and lipid metabolism in HCV infection. J Gastroenterol. 2013;48:169–76.
51. Bouchie A. First microRNA mimic enters clinic. Nat Biotechnol. 2013;31:577.
52. Yang F, Yin Y, Wang F, Wang Y, Zhang L, Tang Y, et al. miR-17-5p Promotes migration of human hepatocellular carcinoma cells through the p38 mitogen-activated protein kinase-heat shock protein 27 pathway. Hepatology. 2010;51:1614–23.
53. Liu WH, Yeh SH, Lu CC, Yu SL, Chen HY, Lin CY, et al. MicroRNA-18a prevents estrogen receptor-alpha expression, promoting proliferation of hepatocellular carcinoma cells. Gastroenterology. 2009;136:683–93.
54. Wang B, Majumder S, Nuovo G, Kutay H, Volinia S, Patel T, et al. Role of microRNA-155 at early stages of hepatocarcinogenesis induced by choline-deficient and amino acid-defined diet in C57BL/6 mice. Hepatology. 2009;50:1152–61.
55. Jiang R, Deng L, Zhao L, Li X, Zhang F, Xia Y, et al. miR-22 promotes HBV-related hepatocellular carcinoma development in males. Clin Cancer Res. 2011;17:5593–603.
56. Wang B, Hsu SH, Frankel W, Ghoshal K, Jacob ST. Stat3-mediated activation of microRNA-23a suppresses gluconeogenesis in hepatocellular carcinoma by down-regulating glucose-6-phosphatase and peroxisome proliferator-activated receptor gamma, coactivator 1 alpha. Hepatology. 2012;56:186–97.
57. Fu X, Meng Z, Liang W, Tian Y, Wang X, Han W, et al. miR-26a enhances miRNA biogenesis by targeting Lin28B and Zcchc11 to suppress tumor growth and metastasis. Oncogene. 2013 (in press).
58. Ji J, Shi J, Budhu A, Yu Z, Forgues M, Roessler S, et al. MicroRNA expression, survival, and response to interferon in liver cancer. N Engl J Med. 2009;361:1437–47.
59. Yao J, Liang L, Huang S, Ding J, Tan N, Zhao Y, et al. MicroRNA-30d promotes tumor invasion and metastasis by targeting Galphai2 in hepatocellular carcinoma. Hepatology. 2010;51:846–56.
60. Varnholt H, Drebber U, Schulze F, Wedemeyer I, Schirmacher P, Dienes HP, et al. MicroRNA gene expression profile of hepatitis C virus-associated hepatocellular carcinoma. Hepatology. 2008;47:1223–32.
61. Shen G, Jia H, Tai Q, Li Y, Chen D. miR-106b downregulates adenomatous polyposis coli and promotes cell proliferation in human hepatocellular carcinoma. Carcinogenesis. 2013;34:211–9.
62. Ma S, Tang KH, Chan YP, Lee TK, Kwan PS, Castilho A, et al. miR-130b Promotes CD133(+) liver tumor-initiating cell growth and self-renewal via tumor protein 53-induced nuclear protein 1. Cell Stem Cell. 2010;7:694–707.
63. Liu S, Guo W, Shi J, Li N, Yu X, Xue J, et al. MicroRNA-135a contributes to the development of portal vein tumor thrombus by promoting metastasis in hepatocellular carcinoma. J Hepatol. 2012;56:389–96.
64. Zhang X, Liu S, Hu T, He Y, Sun S. Up-regulated microRNA-143 transcribed by nuclear factor kappa B enhances hepatocarcinoma metastasis by repressing fibronectin expression. Hepatology. 2009;50:490–9.
65. Zhu K, Pan Q, Zhang X, Kong LQ, Fan J, Dai Z, et al. MiR-146a enhances angiogenic activity of endothelial cells in hepatocellular carcinoma by promoting PDGFRA expression. Carcinogenesis. 2013;34:2071–9.
66. Luedde T. MicroRNA-151 and its hosting gene FAK (focal adhesion kinase) regulate tumor cell migration and spreading of hepatocellular carcinoma. Hepatology. 2010;52:1164–6.
67. Ding J, Huang S, Wu S, Zhao Y, Liang L, Yan M, et al. Gain of miR-151 on chromosome 8q24.3 facilitates tumour cell migration and spreading through downregulating RhoGDIA. Nat Cell Biol. 2010;12:390–9.
68. Yan XL, Jia YL, Chen L, Zeng Q, Zhou JN, Fu CJ, et al. Hepatocellular carcinoma-associated mesenchymal stem cells promote hepatocarcinoma progression: role of the S100A4-miR155-SOCS1-MMP9 axis. Hepatology. 2013;57:2274–86.
69. Zhang S, Shan C, Kong G, Du Y, Ye L, Zhang X. MicroRNA-520e suppresses growth of hepatoma cells by targeting the NF-κB-inducing kinase (NIK). Oncogene. 2012;31:3607–20.
70. Wang B, Hsu SH, Majumder S, Kutay H, Huang W, Jacob ST, et al. TGFbeta-mediated upregulation of hepatic miR-181b promotes hepatocarcinogenesis by targeting TIMP3. Oncogene. 2010;29:1787–97.
71. Ji J, Yamashita T, Budhu A, Forgues M, Jia HL, Li C, et al. Identification of microRNA-181 by genome-wide screening as a critical player in EpCAM-positive hepatic cancer stem cells. Hepatology. 2009;50:472–80.
72. Goeppert B, Schmezer P, Dutruel C, Oakes C, Renner M, Breinig M, et al. Down-regulation of tumor suppressor A kinase anchor protein 12 in human hepatocarcinogenesis by epigenetic mechanisms. Hepatology. 2010;52:2023–33.
73. Petrelli A, Perra A, Cora D, Sulas P, Menegon S, Manca C, et al. MiRNA/gene profiling unveils early molecular changes and NRF2 activation in a rat model recapitulating human HCC. Hepatology. 2013 (in press).
74. Ying Q, Liang L, Guo W, Zha R, Tian Q, Huang S, et al. Hypoxia-inducible microRNA-210 augments the metastatic potential of tumor cells by targeting vacuole membrane protein 1 in hepatocellular carcinoma. Hepatology. 2011;54:2064–75.
75. Chen PJ, Yeh SH, Liu WH, Lin CC, Huang HC, Chen CL, et al. Androgen pathway stimulates microRNA-216a transcription to suppress the tumor suppressor in lung cancer-1 gene in early hepatocarcinogenesis. Hepatology. 2012;56:632–43.
76. Xia H, Ooi LL, Hui KM. MicroRNA-216a/217-induced epithelial–mesenchymal transition targets PTEN and SMAD7 to promote drug resistance and recurrence of liver cancer. Hepatology. 2013;58:629–41.
77. Callegari E, Elamin BK, Giannone F, Milazzo M, Altavilla G, Fornari F, et al. Liver tumorigenicity promoted by microRNA-221 in a mouse transgenic model. Hepatology. 2012;56:1025–33.
78. Yuan Q, Loya K, Rani B, Möbus S, Balakrishnan A, Lamle J, et al. MicroRNA-221 overexpression accelerates hepatocyte proliferation during liver regeneration. Hepatology. 2013;57:299–310.
79. Gramantieri L, Fornari F, Ferracin M, Veronese A, Sabbioni S, Calin GA, et al. MicroRNA-221 targets Bmf in hepatocellular carcinoma and correlates with tumor multifocality. Clin Cancer Res. 2009;15:5073–81.
80. Fornari F, Gramantieri L, Ferracin M, Veronese A, Sabbioni S, Calin GA, et al. MiR-221 controls CDKN1C/p57 and CDKN1B/p27 expression in human hepatocellular carcinoma. Oncogene. 2008;27:5651–61.
81. Pineau P, Volinia S, McJunkin K, Marchio A, Battiston C, Terris B, et al. miR-221 overexpression contributes to liver tumorigenesis. Proc Natl Acad Sci USA. 2010;107:264–9.
82. Ladeiro Y, Couchy G, Balabaud C, Bioulac-Sage P, Pelletier L, Rebouissou S, et al. MicroRNA profiling in hepatocellular tumors is associated with clinical features and oncogene/tumor suppressor gene mutations. Hepatology. 2008;47:1955–63.
83. Lan SH, Wu SY, Zuchini R, Lin XZ, Su IJ, Tsai TF, et al. Autophagy suppresses tumorigenesis of hepatitis B virus-

associated hepatocellular carcinoma through degradation of miR-224. Hepatology. 2013 (in press).
84. Scisciani C, Vossio S, Guerrieri F, Schinzari V, De Iaco R, D'Onorio de Meo P, et al. Transcriptional regulation of miR-224 upregulated in human HCCs by NFκB inflammatory pathways. J Hepatol. 2012;56:855–61.
85. Gao P, Wong CC, Tung EK, Lee JM, Wong CM, Ng IO. Deregulation of microRNA expression occurs early and accumulates in early stages of HBV-associated multistep hepatocarcinogenesis. J Hepatol. 2011;54:1177–84.
86. Wang Y, Lee AT, Ma JZ, Wang J, Ren J, Yang Y, et al. Profiling microRNA expression in hepatocellular carcinoma reveals microRNA-224 up-regulation and apoptosis inhibitor-5 as a microRNA-224-specific target. J Biol Chem. 2008;283:13205–15.
87. Lin J, Huang S, Wu S, Ding J, Zhao Y, Liang L, et al. MicroRNA-423 promotes cell growth and regulates G(1)/S transition by targeting p21Cip1/Waf1 in hepatocellular carcinoma. Carcinogenesis. 2011;32:1641–7.
88. Yang H, Cho ME, Li TW, Peng H, Ko KS, Mato JM, et al. MicroRNAs regulate methionine adenosyltransferase 1A expression in hepatocellular carcinoma. J Clin Invest. 2013;123:285–98.
89. Zhang LY, Liu M, Li X, Tang H. miR-490-3p modulates cell growth and epithelial to mesenchymal transition of hepatocellular carcinoma cells by targeting endoplasmic reticulum-Golgi intermediate compartment protein 3 (ERGIC3). J Biol Chem. 2013;288:4035–47.
90. Lim L, Balakrishnan A, Huskey N, Jones KD, Jodari M, Ng R, et al. MiR-494 within an oncogenic MicroRNA megacluster regulates G1/S transition in liver tumorigenesis through suppression of MCC. Hepatology. 2013 (in press).
91. Toffanin S, Hoshida Y, Lachenmayer A, Villanueva A, Cabellos L, Minguez B, et al. MicroRNA-based classification of hepatocellular carcinoma and oncogenic role of miR-517a. Gastroenterology. 2011;140(1618–1628):e1616.
92. Zhang L, Yang L, Liu X, Chen W, Chang L, Chen L, et al. MicroRNA-657 promotes tumorigenesis in hepatocellular carcinoma by targeting transducin-like enhancer protein 1 through nuclear factor kappa B pathways. Hepatology. 2013;57:1919–30.
93. Law PT, Qin H, Ching AK, Lai KP, Co NN, He M, et al. Deep sequencing of small RNA transcriptome reveals novel non-coding RNAs in hepatocellular carcinoma. J Hepatol. 2013;58:1165–73.
94. Wang Y, Lu Y, Toh ST, Sung WK, Tan P, Chow P, et al. Lethal-7 is down-regulated by the hepatitis B virus x protein and targets signal transducer and activator of transcription 3. J Hepatol. 2010;53:57–66.
95. Au SL, Wong CC, Lee JM, Fan DN, Tsang FH, Ng IO, et al. Enhancer of zeste homolog 2 epigenetically silences multiple tumor suppressor microRNAs to promote liver cancer metastasis. Hepatology. 2012;56:622–31.
96. Ji J, Zhao L, Budhu A, Forgues M, Jia HL, Qin LX, et al. Let-7g targets collagen type I alpha2 and inhibits cell migration in hepatocellular carcinoma. J Hepatol. 2010;52:690–7.
97. Fang Y, Xue JL, Shen Q, Chen J, Tian L. MicroRNA-7 inhibits tumor growth and metastasis by targeting the phosphoinositide 3-kinase/Akt pathway in hepatocellular carcinoma. Hepatology. 2012;55:1852–62.
98. Yan Y, Luo YC, Wan HY, Wang J, Zhang PP, Liu M, et al. MicroRNA-10a is involved in the metastatic process by regulating Eph tyrosine kinase receptor A4-mediated epithelial–mesenchymal transition and adhesion in hepatoma cells. Hepatology. 2013;57:667–77.
99. Maurel M, Jalvy S, Ladeiro Y, Combe C, Vachet L, Sagliocco F, et al. A functional screening identifies five microRNAs controlling glypican-3: role of miR-1271 down-regulation in hepatocellular carcinoma. Hepatology. 2013;57:195–204.
100. Wang Y, Jiang L, Ji X, Yang B, Zhang Y, Fu XD. Hepatitis B viral RNA directly mediates down-regulation of the tumor suppressor microRNA miR-15a/miR-16-1 in hepatocytes. J Biol Chem. 2013;288:18484–93.
101. Yang X, Liang L, Zhang XF, Jia HL, Qin Y, Zhu XC, et al. MicroRNA-26a suppresses tumor growth and metastasis of human hepatocellular carcinoma by targeting interleukin-6-Stat3 pathway. Hepatology. 2013;58:158–70.
102. Kota J, Chivukula RR, O'Donnell KA, Wentzel EA, Montgomery CL, Hwang HW, et al. Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. Cell. 2009;137:1005–17.
103. Xiong Y, Fang JH, Yun JP, Yang J, Zhang Y, Jia WH, et al. Effects of microRNA-29 on apoptosis, tumorigenicity, and prognosis of hepatocellular carcinoma. Hepatology. 2010;51:836–45.
104. Fang JH, Zhou HC, Zeng C, Yang J, Liu Y, Huang X, et al. MicroRNA-29b suppresses tumor angiogenesis, invasion, and metastasis by regulating matrix metalloproteinase 2 expression. Hepatology. 2011;54:1729–40.
105. Bae HJ, Noh JH, Kim JK, Eun JW, Jung KH, Kim MG, et al. MicroRNA-29c functions as a tumor suppressor by direct targeting oncogenic SIRT1 in hepatocellular carcinoma. Oncogene. 2013 (in press).
106. Yang P, Li QJ, Feng Y, Zhang Y, Markowitz GJ, Ning S, et al. TGF-β-miR-34a-CCL22 signaling-induced Treg cell recruitment promotes venous metastases of HBV-positive hepatocellular carcinoma. Cancer Cell. 2012;22:291–303.
107. Petrelli A, Perra A, Schernhuber K, Cargnelutti M, Salvi A, Migliore C, et al. Sequential analysis of multistage hepatocarcinogenesis reveals that miR-100 and PLK1 dysregulation is an early event maintained along tumor progression. Oncogene. 2012;31:4517–26.
108. Li D, Liu X, Lin L, Hou J, Li N, Wang C, et al. MicroRNA-99a inhibits hepatocellular carcinoma growth and correlates with prognosis of patients with hepatocellular carcinoma. J Biol Chem. 2011;286:36677–85.
109. Wang L, Zhang X, Jia LT, Hu SJ, Zhao J, Yang JD, et al. c-Myc-mediated epigenetic silencing of microRNA-101 contributes to dysregulation of multiple pathways in hepatocellular carcinoma. Hepatology. 2013 (in press).
110. Su H, Yang JR, Xu T, Huang J, Xu L, Yuan Y, et al. MicroRNA-101, down-regulated in hepatocellular carcinoma, promotes apoptosis and suppresses tumorigenicity. Cancer Res. 2009;69:1135–42.
111. Li S, Fu H, Wang Y, Tie Y, Xing R, Zhu J, et al. MicroRNA-101 regulates expression of the v-fos FBJ murine osteosarcoma viral oncogene homolog (FOS) oncogene in human hepatocellular carcinoma. Hepatology. 2009;49:1194–202.
112. Wang B, Hsu SH, Wang X, Kutay H, Bid HK, Yu J, et al. Reciprocal regulation of miR-122 and c-Myc in hepatocellular cancer: role of E2F1 and TFDP2. Hepatology. 2013 (in press).
113. Song K, Han C, Zhang J, Lu D, Dash S, Feitelson M, et al. Epigenetic regulation of MicroRNA-122 by peroxisome proliferator activated receptor-gamma and hepatitis b virus X protein in hepatocellular carcinoma cells. Hepatology. 2013 (in press).
114. Zeng C, Wang R, Li D, Lin XJ, Wei QK, Yuan Y, et al. A novel GSK-3 beta-C/EBP alpha-miR-122-insulin-like growth factor 1 receptor regulatory circuitry in human hepatocellular carcinoma. Hepatology. 2010;52:1702–12.
115. Gramantieri L, Ferracin M, Fornari F, Veronese A, Sabbioni S, Liu CG, et al. Cyclin G1 is a target of miR-122a, a microRNA frequently down-regulated in human hepatocellular carcinoma. Cancer Res. 2007;67:6092–9.

116. Zheng F, Liao YJ, Cai MY, Liu YH, Liu TH, Chen SP, et al. The putative tumour suppressor microRNA-124 modulates hepatocellular carcinoma cell aggressiveness by repressing ROCK2 and EZH2. Gut. 2012;61:278–89.
117. Furuta M, Kozaki KI, Tanaka S, Arii S, Imoto I, Inazawa J. miR-124 and miR-203 are epigenetically silenced tumor-suppressive microRNAs in hepatocellular carcinoma. Carcinogenesis. 2010;31:766–76.
118. Kim JK, Noh JH, Jung KH, Eun JW, Bae HJ, Kim MG, et al. Sirtuin7 oncogenic potential in human hepatocellular carcinoma and its regulation by the tumor suppressors MiR-125a-5p and MiR-125b. Hepatology. 2013;57:1055–67.
119. Fan DN, Tsang FH, Tam AH, Au SL, Wong CC, Wei L, et al. Histone lysine methyltransferase, suppressor of variegation 3–9 homolog 1, promotes hepatocellular carcinoma progression and is negatively regulated by microRNA-125b. Hepatology. 2013;57:637–47.
120. Gong J, Zhang JP, Li B, Zeng C, You K, Chen MX, et al. MicroRNA-125b promotes apoptosis by regulating the expression of Mcl-1, Bcl-w and IL-6R. Oncogene. 2013;32:3071–9.
121. Alpini G, Glaser SS, Zhang JP, Francis H, Han Y, Gong J, et al. Regulation of placenta growth factor by microRNA-125b in hepatocellular cancer. J Hepatol. 2011;55:1339–45.
122. Liang L, Wong CM, Ying Q, Fan DN, Huang S, Ding J, et al. MicroRNA-125b suppressesed human liver cancer cell proliferation and metastasis by directly targeting oncogene LIN28B2. Hepatology. 2010;52:1731–40.
123. Wong CC, Wong CM, Tung EK, Au SL, Lee JM, Poon RT, et al. The microRNA miR-139 suppresses metastasis and progression of hepatocellular carcinoma by down-regulating Rho-kinase 2. Gastroenterology. 2011;140:322–31.
124. Yang H, Fang F, Chang R, Yang L. MicroRNA-140-5p suppresses tumor growth and metastasis by targeting transforming growth factor β receptor 1 and fibroblast growth factor 9 in hepatocellular carcinoma. Hepatology. 2013;58:205–17.
125. Takata A, Otsuka M, Yoshikawa T, Kishikawa T, Hikiba Y, Obi S, et al. MicroRNA-140 acts as a liver tumor suppressor by controlling NF-κB activity by directly targeting DNA methyltransferase 1 (Dnmt1) expression. Hepatology. 2013;57:162–70.
126. Banaudha K, Kaliszewski M, Korolnek T, Florea L, Yeung ML, Jeang KT, et al. MicroRNA silencing of tumor suppressor DLC-1 promotes efficient hepatitis C virus replication in primary human hepatocytes. Hepatology. 2011;53:53–61.
127. Law PT, Ching AK, Chan AW, Wong QW, Wong CK, To KF, et al. MiR-145 modulates multiple components of the insulin-like growth factor pathway in hepatocellular carcinoma. Carcinogenesis. 2012;33:1134–41.
128. Gailhouste L, Gomez-Santos L, Hagiwara K, Hatada I, Kitagawa N, Kawaharada K, et al. miR-148a plays a pivotal role in the liver by promoting the hepatospecific phenotype and suppressing the invasiveness of transformed cells. Hepatology. 2013;58:1153–65.
129. Xu X, Fan Z, Kang L, Han J, Jiang C, Zheng X, et al. Hepatitis B virus X protein represses miRNA-148a to enhance tumorigenesis. J Clin Invest. 2013;123:630–45.
130. Zhang JP, Zeng C, Xu L, Gong J, Fang JH, Zhuang SM. MicroRNA-148a suppresses the epithelial–mesenchymal transition and metastasis of hepatoma cells by targeting Met/Snail signaling. Oncogene. 2013 (in press).
131. Han H, Sun D, Li W, Shen H, Zhu Y, Li C, et al. A c-Myc-MicroRNA functional feedback loop affects hepatocarcinogenesis. Hepatology. 2013;57:2378–89.
132. Huang J, Wang Y, Guo Y, Sun S. Down-regulated microRNA-152 induces aberrant DNA methylation in hepatitis B virus-related hepatocellular carcinoma by targeting DNA methyltransferase 1. Hepatology. 2010;52:60–70.
133. Ding J, Huang S, Wang Y, Tian Q, Zha R, Shi H, et al. Genome-wide screening reveals that miR-195 targets the TNF-α/NF-κB pathway by down-regulating IκB kinase alpha and TAB 3 in hepatocellular carcinoma. Hepatology. 2013;58:654–66.
134. Wang R, Zhao N, Li S, Fang JH, Chen MX, Yang J, et al. MicroRNA-195 suppresses angiogenesis and metastasis of hepatocellular carcinoma by inhibiting the expression of VEGF, VAV2, and CDC42. Hepatology. 2013;58:642–53.
135. Xu T, Zhu Y, Xiong Y, Ge YY, Yun JP, Zhuang SM. MicroRNA-195 suppresses tumorigenicity and regulates G1/S transition of human hepatocellular carcinoma cells. Hepatology. 2009;50:113–21.
136. Yuan JH, Yang F, Chen BF, Lu Z, Huo XS, Zhou WP, et al. The histone deacetylase 4/SP1/microrna-200a regulatory network contributes to aberrant histone acetylation in hepatocellular carcinoma. Hepatology. 2011;54:2025–35.
137. Shih TC, Tien YJ, Wen CJ, Yeh TS, Yu MC, Huang CH, et al. MicroRNA-214 downregulation contributes to tumor angiogenesis by inducing secretion of the hepatoma-derived growth factor in human hepatoma. J Hepatol. 2012;57:584–91.
138. Wong QW, Lung RW, Law PT, Lai PB, Chan KY, To KF, et al. MicroRNA-223 is commonly repressed in hepatocellular carcinoma and potentiates expression of Stathmin1. Gastroenterology. 2008;135:257–69.
139. Guichard C, Amaddeo G, Imbeaud S, Ladeiro Y, Pelletier L, Maad IB, et al. Integrated analysis of somatic mutations and focal copy-number changes identifies key genes and pathways in hepatocellular carcinoma. Nat Genet. 2012;44:694–8.
140. Chang Y, Yan W, He X, Zhang L, Li C, Huang H, et al. miR-375 inhibits autophagy and reduces viability of hepatocellular carcinoma cells under hypoxic conditions. Gastroenterology. 2012;143(177–187):e178.
141. He XX, Chang Y, Meng FY, Wang MY, Xie QH, Tang F, et al. MicroRNA-375 targets AEG-1 in hepatocellular carcinoma and suppresses liver cancer cell growth in vitro and in vivo. Oncogene. 2012;31:3357–69.
142. You X, Liu F, Zhang T, Li Y, Ye L, Zhang X. Hepatitis B virus X protein upregulates oncogene Rab18 to result in the dysregulation of lipogenesis and proliferation of hepatoma cells. Carcinogenesis. 2013;34:1644–52.
143. Buurman R, Gürlevik E, Schäffer V, Eilers M, Sandbothe M, Kreipe H, et al. Histone deacetylases activate hepatocyte growth factor signaling by repressing microRNA-449 in hepatocellular carcinoma cells. Gastroenterology. 2012;143:811–20. e811–15.
144. Tao ZH, Wan JL, Zeng LY, Xie L, Sun HC, Qin LX, et al. miR-612 suppresses the invasive-metastatic cascade in hepatocellular carcinoma. J Exp Med. 2013;210:789–803.
145. Zhang JF, He ML, Fu WM, Wang H, Chen LZ, Zhu X, et al. Primate-specific microRNA-637 inhibits tumorigenesis in hepatocellular carcinoma by disrupting signal transducer and activator of transcription 3 signaling. Hepatology. 2011;54:2137–48.

Hepatology Research

Hepatology Research 2013 doi: 10.1111/hepr.12221

Original Article

Discrimination of fibrotic staging of chronic hepatitis C using multiple fibrotic markers

Kenji Ikeda,[1,2] Namiki Izumi,[3] Eiji Tanaka,[8] Hiroshi Yotsuyanagi,[4] Yoshihisa Takahashi,[6] Junichi Fukushima,[7] Fukuo Kondo,[6] Toshio Fukusato,[6] Kazuhiko Koike,[5] Norio Hayashi,[9] Hirohito Tsubouchi[10] and Hiromitsu Kumada[1,2]

[1]*Department of Hepatology, Toranomon Hospital,* [2]*Okinaka Memorial Institute for Medical Research,* [3]*Department of Gastroenterology, Musashino Red Cross Hospital,* [4]*Department of Infectious Disease,* [5]*Department of Gastroenterology, Graduate School of Medicine, Tokyo University,* [6]*Department of Pathology, Teikyo University School of Medicine,* [7]*Department of Pathology, NTT Medical Center Tokyo, Tokyo,* [8]*Department of Gastroenterology, Shinshu University of Medicine, Matsumoto,* [9]*Department of Gastroenterology, Kansai-Rosai Hospital, Hyogo, and* [10]*Department of Gastroenterology, Kagoshima University of Medicine, Kagoshima, Japan*

Aim: In order to evaluate and judge a fibrotic stage of patients with chronic hepatitis C, multivariate regression analysis was performed using multiple fibrotic markers.

Methods: A total of 581 patients from eight hepatology units and institutes were diagnosed by needle biopsy as having chronic liver disease caused by hepatitis C virus. Twenty-three variables and their natural logarithmic transformation were employed in the multivariate analysis.

Results: Multivariate regression analysis finally obtained the following function: $z = 2.89 \times ln$ (type IV collagen 7S) (ng/mL) $- 0.011 \times$ (platelet count) ($\times 10^3/mm^3$) $+ 0.79 \times ln$ (total bilirubin) (mg/dL) $+ 0.39 \times ln$ (hyaluronic acid) (μg/L) $- 1.87$. Median values of the fibrotic score of F1 ($n = 172$), F2 ($n = 80$), F3 ($n = 37$) and F4 ($n = 16$) were calculated as 1.00, 1.45, 2.82 and 3.83, respectively. Multiple regression coefficient and coefficient of determination were 0.56 and 0.320, respectively. Validation with patient data from other institutions demonstrated good reproducibility of the fibrotic score for hepatitis C (FSC), showing 1.10 in F1 ($n = 156$), 2.35 in F2 ($n = 73$), 3.16 in F3 ($n = 36$) and 3.58 in F4 ($n = 11$).

Conclusion: A concise multiple regression function using four laboratory parameters successfully predicted pathological fibrotic stage of patients with hepatitis C virus infection.

Key words: chronic hepatitis, hepatitis C virus, liver cirrhosis, liver fibrosis, multiple regression analysis, stage

INTRODUCTION

WHEN HEPATITIS C virus (HCV)-related chronic liver disease was found by biochemical and virological examination, peritoneoscopy and/or liver biopsy can establish the definitive diagnosis of chronic hepatitis and liver cirrhosis. Although these pathological procedures are reliable and informative both in diagnosis and treatment, they sometimes require medical invasion and financial costs, including the risk of bleeding from needle puncture, some pain experienced during the examination, medical expenses and hospitalization for a few days. The pathological examination is, therefore, rarely performed repeatedly in a short period of time, even when disease activity is severe and progression of liver disease is highly suspected. Recently, many authors described the usefulness of ultrasonographic elastography and magnetic resonance imaging technology in the estimation of staging of chronic hepatitis and cirrhosis.[1–4] These ways of estimation using the imaging apparatuses seem truly useful for current patients, but it cannot evaluate and compare with past fibrotic states of patients retrospectively. Moreover, the same apparatus for elastometry will not be available for repeated measurement for a follow-up examination, several years later for example.

In spite of the accuracy of biopsy and of convenience of elastography in chronic liver disease, clinical diagnosis based on biochemistry and hematology is still indispensable for the daily practice of many patients with

Correspondence: Dr Kenji Ikeda, *Department of Hepatology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105-8470, Japan. Email: ikedakenji@tora.email.ne.jp*

Received 13 May 2013; revision 4 August 2013; accepted 8 August 2013.