Table 2 Univariate and multivariate analyses of overall survival

	Univariate analysis	Multivariate analysis		
	P	HR	95% CI	*P*
Age (<65/≥65 years)	0.8349			
Sex (male/female)	0.7584			
EOCG PS (0/1)	0.0895			
Child–Pugh score (5, 6/7)	0.1075			
Rate of tumor occupation in the liver (≤50%/>50%)	0.1042			
Vp (3/4)	0.6216			
Vv (0/2,3)	0.5705			
Platelet count ($<15 \times 10^4/\geq 15 \times 10^4$ μL)	<0.001	–	–	0.119
AFP (<1000/≥1000 ng/mL)	0.2462			
DCP (<1000/≥1000 mAU/mL)	0.2042			
3D-CRT (with/without combination)	0.3087			
Regimen (5-FU/IFN/low-dose FP)	0.4233			
Treatment response of intrahepatic HCC	<0.001	3.3	1.801–6.114	<0.001
Treatment response of PVTT only	<0.001	2.6	1.425–4.562	0.002

3D-CRT, 3-D conformal radiotherapy; 5-FU, 5-fluorouracil; AFP, α-fetoprotein; CI, confidence interval; DCP, des-c-carboxy prothrombin; EOCG PS, Eastern Cooperative Oncology Group performance status; FP, cisplatin plus 5-fluorouracil therapy; HCC, hepatocellular carcinoma; HR, hazard ratio; PVTT, portal vein tumor thrombosis; Vp3, tumor thrombus in the first branch of the portal vein; Vp4, tumor thrombus in the trunk of the portal vein; Vv2, tumor thrombus in the right, middle or left hepatic vein trunk, posterior inferior hepatic vein trunk or short hepatic vein; Vv3, tumor thrombus in inferior vena cava.

Table 3 Comparison of clinical profile between the RT group and non-RT group in intrahepatic HCC non-responders to HAIC

Variables	RT group ($n = 29$)	Non-RT group ($n = 25$)	*P*
Age (years)	67 (35–84)	64 (42–76)	0.430
Sex (male/female)	28/1	22/3	0.232
EOCG PS (0/1)	25/4	20/5	0.542
Etiology (HBV/HCV/NBNC/alcohol)	9/12/5/3	7/14/3/1	0.584
Child–Pugh score (5/6/7)	15/9/5	10/9/6	0.671
Rate of tumor occupation in the liver (≤50%/>50%)	19/10	14/11	0.474
Vp (3/4)	13/16	15/10	0.266
Vv (0/2/3)	27/1/1	22/1/2	0.759
Alb (g/dL)	3.6 (3.1–4.6)	3.8 (2.6–4.6)	0.709
Total bilirubin (mg/dL)	1.0 (0.4–3.0)	1.0 (0.5–2.9)	0.721
Prothrombin time activity (%)	86 (58–107)	86 (57–117)	0.584
Platelet count ($\times 10^4$/μL)	12.4 (3.9–25.1)	15.4 (4.6–88.8)	0.096
AFP (ng/mL)	514 (5–200000)	1421 (13–1895000)	0.440
DCP (mAU/mL)	9363 (36–287180)	6392 (15–722140)	0.538
Regimen (5-FU/IFN/low-dose FP)	19/10	14/11	0.474

Categorical data are represented as numbers of patients, and continuous data is represented as median and range.
5-FU, 5-fluorouracil; AFP, α-fetoprotein; Alb, albumin; DCP, des-c-carboxy prothrombin; EOCG PS, Eastern Cooperative Oncology Group performance status; FP, cisplatin plus 5-fluorouracil therapy; HAIC, hepatic arterial infusion chemotherapy; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; NBNC, non-B, non-C; RT, radiotherapy; Vp3, tumor thrombus in the first branch of the portal vein; Vp4, tumor thrombus in the trunk of the portal vein; Vv2, tumor thrombus in the right, middle or left hepatic vein trunk, posterior inferior hepatic vein trunk or short hepatic vein; Vv3, tumor thrombus in inferior vena cava.

Table 4 Univariate and multivariate analyses of overall survival in intrahepatic HCC non-responders to HAIC

	Univariate analysis	Multivariate analysis		
	P	HR	95% CI	*P*
Age (<65/≥65 years)	0.3083			
Sex (male/female)	0.0382	–	–	0.326
EOCG PS (0/1)	0.6603			
Child–Pugh score (5,6/7)	0.0116	2.1	1.061–4.265	0.033
Rate of tumor occupation in the liver (≤50%/>50%)	0.0940	–	–	0.064
Vp (3/4)	0.3633			
Vv (0/2, 3)	0.1575			
Platelet count ($<15 \times 10^4/\geq 15 \times 10^4$ μL)	0.0366	–	–	0.074
AFP (<1000/≥1000 ng/mL)	0.0678	–	–	0.232
DCP (<1000/≥1000 mAU/mL)	0.2420			
3D-CRT (combination with/without)	0.0002	3.2	1.692–6.021	<0.001
Regimen (5-FU/IFN/low-dose FP)	0.0583	–	–	0.053
Treatment response of PVTT only	0.0164	–	–	0.315

3D-CRT, 3-D conformal radiotherapy; 5-FU, 5-fluorouracil; AFP, α-fetoprotein; CI, confidence interval; DCP, des-c-carboxy prothrombin; EOCG PS, Eastern Cooperative Oncology Group performance status; FP, cisplatin plus 5-fluorouracil therapy; HCC, hepatocellular carcinoma; HR, hazard ratio; PVTT, portal vein tumor thrombosis; Vp3, tumor thrombus in the first branch of the portal vein; Vp4, tumor thrombus in the trunk of the portal vein; Vv2, tumor thrombus in the right, middle or left hepatic vein trunk, posterior inferior hepatic vein trunk or short hepatic vein; Vv3, tumor thrombus in inferior vena cava.

non-RT group (5.0 and 2.7 months, respectively; $P = 0.0024$) among intrahepatic HCC non-responders (Fig. 2c).

PPS

Seventy-one patients (85.5%) of all patients showed disease progression in their treatment courses. Among them, 21 patients (10 and 11 patients in the RT and non-RT groups, respectively) had achieved the objective response of intrahepatic HCC or responders, and 50 patients (28 and 22 patients in the RT and non-RT groups, respectively) were non-responders. The median PPS was 5.8 and 3.0 months in the RT and non-RT groups, respectively, with no significant difference ($P = 0.1488$, Fig. 3a). While median PPS was not significantly different between the RT and non-RT groups (12.1 and 14.6 months, respectively, $P = 0.9303$) among intrahepatic HCC responders, that was significantly longer in the RT group than in the non-RT group (5.3 and 1.5 months, respectively; $P = 0.0001$) among intrahepatic HCC non-responders (Fig. 3b,c).

Adverse reactions and complications

Treatment-related toxicities were observed in 72 patients (86.7%). Common adverse events were fever, fatigue, nausea and anorexia and these were mostly of CTCAE grade 1 or 2. CTCAE grade 3 or 4 adverse reactions included leukopenia, thrombocytopenia, increased alanine aminotransferase or aspartate aminotransferase, and increased blood bilirubin and these were observed in five (12.2%) and six (14.3%), five (12.2%) and six (14.3%), one (2.4%) and two (4.8%), and one (2.4%) and one (2.4%) patient among the RT and non-RT groups, respectively. No statistically significant differences were observed in adverse reactions between the RT and non-RT groups. None of the patients who received HAIC combined with 3D-CRT for PVTT developed hepatic failure that fulfilled the criteria of classic and non-classic RILD. One patient in the RT group developed hepatic failure with increasing alanine aminotransferase or aspartate aminotransferase, and hyperbilirubinemia; the cause of hepatic failure was considered to be the rapid progression of intrahepatic HCC.

DISCUSSION

IN THIS STUDY, we assessed tumor control, survival benefit and safety of the combination therapy of 3D-CRT for major PVTT (Vp3 or 4) with HAIC for advanced HCC by comparison with the group receiving HAIC alone. The reduction rate of PVTT was significantly higher in the RT group than in the non-RT group without any severe adverse reaction. However, there

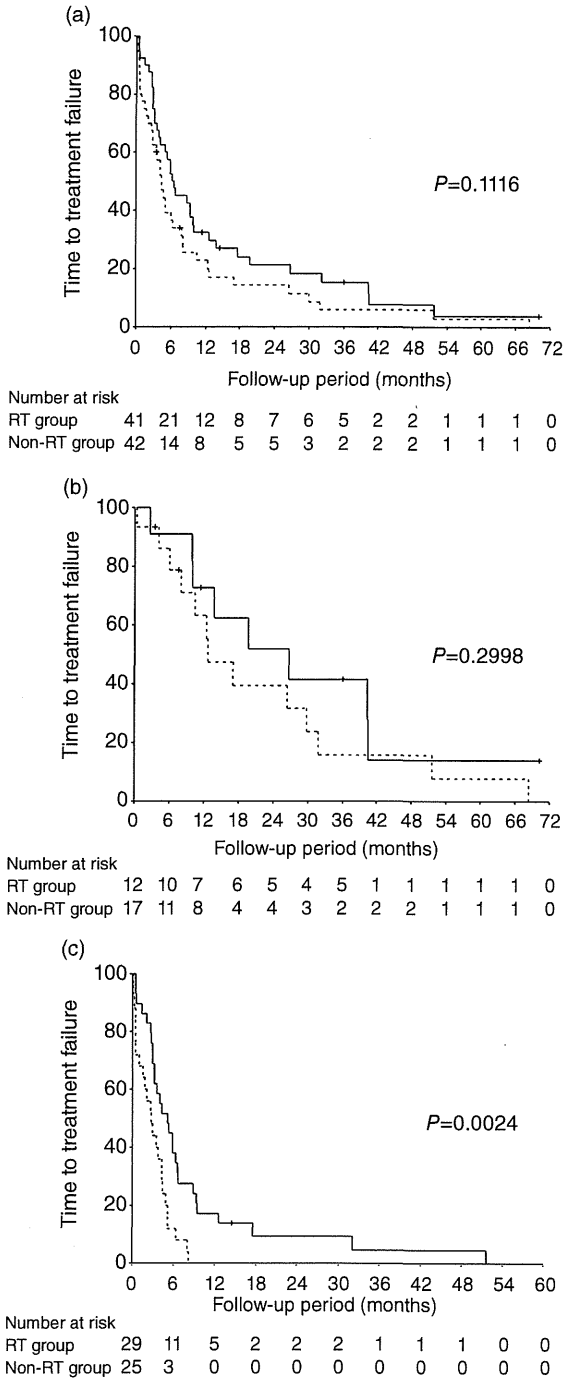

Figure 2 Time to treatment failure. (a) Comparison of time to treatment failure between the radiotherapy (RT) group and non-RT group. (b) Comparison of time to treatment failure between the RT group and non-RT group among intrahepatic tumor responders to hepatic arterial infusion chemotherapy (HAIC). (c) Comparison of time to treatment failure between the RT group and non-RT group among intrahepatic tumor non-responders to HAIC.

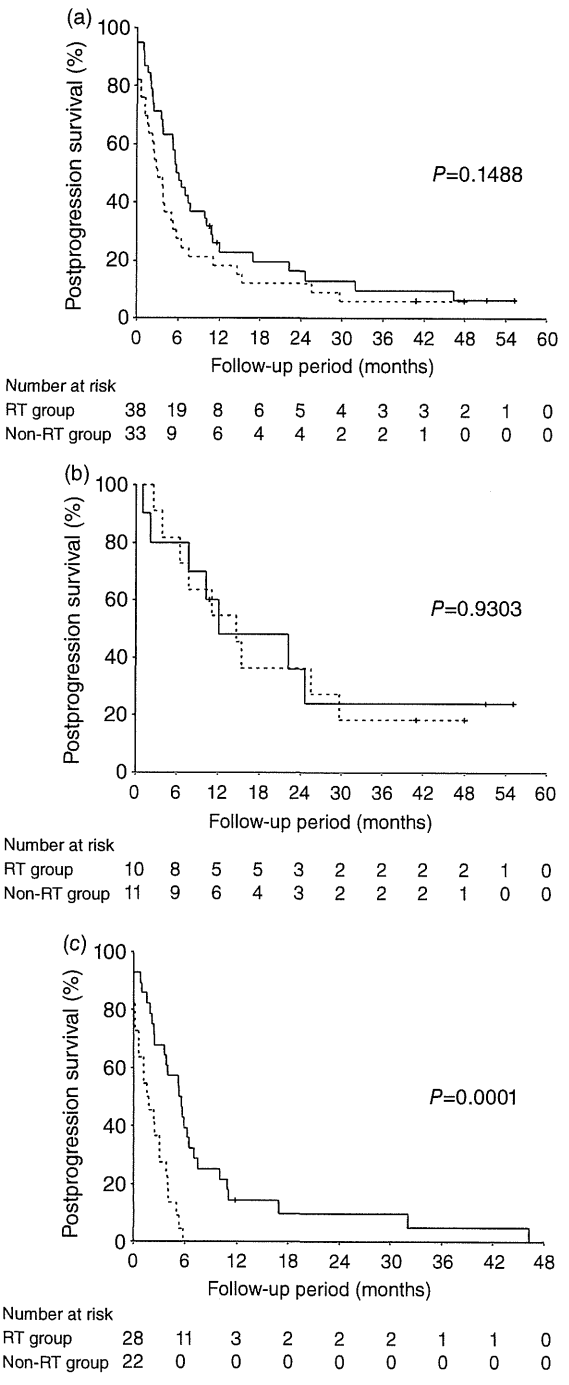

Figure 3 Post-progression survival rate. (a) Comparison of post-progression survival between the radiotherapy (RT) group and non-RT group. (b) Comparison of post-progression survival between the RT group and non-RT group among intrahepatic tumor responders to hepatic arterial infusion chemotherapy (HAIC). (c) Comparison of post-progression survival between the RT group and non-RT group among intrahepatic tumor non-responders to HAIC.

were no significant differences between these two groups in OS. By multivariate analysis, objective response of intrahepatic HCC and objective response of PVTT were independent factors for OS. Among non-responders to HAIC, MST was significantly longer in the RT group than in the non-RT group, and the combination therapy with HAIC and 3D-CRT was a significant and independent factor of OS by multivariate analysis. We consider that patients who obtained the reduction of PVTT with RT could receive more additional therapies due to the avoidance of hepatic functional deterioration even if patients could not respond to HAIC and showed disease progression in their treatment courses. In fact, among HAIC non-responders, we also showed that median TTF and median PPS were significantly longer in the RT group than in the non-RT group. Taken together, we considered that 3D-CRT for PVTT could potentially provide survival benefit to advanced HCC patients with HAIC who are refractory by prolongation TTF and PPS. However, most of patients enrolled in this study had advanced intrahepatic tumor with Vp3/4, and it was difficult to assess whether the cause of death was HCC spread or hepatic failure.

For patients with HCC and major vascular invasion, surgical resection is one of the treatments of choice. Pawlik *et al.* showed that surgical resection of HCC with major vascular invasion could be performed relatively safely and lead to long-term survival rate after resection.[24] However, it should be restricted to patients who have a good hepatic functional reserve and a relatively small primary tumor with distal branch occlusion by PVTT because of the potential for perioperative mortality and the high recurrence rate after operation.

Currently, sorafenib has become the first-line therapy for advanced HCC such as Barcelona Clinic Liver Cancer stage C, including that complicated with MVI. However, among patients treated with sorafenib, MST (8.1 months) in patients with MVI was shorter than that in patients without MVI (10.2 months).[25] In addition, Jeong *et al.* showed the extremely poor MST (3.1 months) in patients with advanced HCC with PVTT (Vp3/4) by sorafenib treatment.[26] Sorafenib is likely to delay disease progression and has been demonstrated to be effective in improving survival despite the low incidence of objective responses. Recently, it has been reported that administrating sorafenib potentially results in reduction of hepatic perfusion following deterioration of hepatic functional reserve.[27] In HCC patients with MVI, sorafenib may have a much greater impact on the reduction of hepatic perfusion and deterioration of liver function, and this may be one of the causes why OS in HCC patients with MVI treated with sorafenib is worse than that in patients without MVI.

Previous studies have demonstrated that treatment efficacy of HAIC for advanced HCC with MVI, using an intra-arterial 5-FU-based regimen (FP or 5-FU/IFN) or CDDP, shows a response rate ranging 12.2–52% which is higher than sorafenib.[15–18,28–31] Although these studies had several limitations, such as being retrospective, non-randomized controlled studies and with different regimens, survival of HAIC responders (MST, 21.7–31.6 months) generally improved when compared with non-responders (MST, 5.4–6.7 months). According to the Japanese HCC management guideline,[32] for HCC with MVI (Vp3/4), in addition to sorafenib, HAIC is regarded as one of the treatment options. On the other hand, survival of HAIC non-responders is generally poor and it may be similar to that of patients with MVI among placebo groups in the SHARP and Asia–Pacific studies.

Although RT for the treatment of HCC has been limited because of low radiation tolerance of the whole liver, local RT has been investigated for the treatment of HCC with the development of CT-based 3D-CRT. The efficacy of local RT in HCC patients with PVTT has been improved and MST and response rate were reported to be 6–13 months and 40–57%, respectively.[8,19,33–37] Furthermore, the efficacy of combination therapy of HAIC and 3D-CRT for PVTT have been reported. Han *et al.* reported a response rate of 45% and MST of 13.1 months in HCC patients with PVTT treated by HAIC with 5-FU/CDDP plus external beam RT.[20] We also reported that 5-FU/IFN in combination with 3D-CRT for PVTT improved the response rate of PVTT and reduced the incidence of portal hypertension-related events.[21] However, survival benefit has yet to be elucidated in this combination therapy of 3D-CRT for PVTT with HAIC.

The present study demonstrated a 56.1% objective response rate to 3D-CRT for PVTT without differences in adverse reactions. The RT group had significantly better survival than the non-RT group in intrahepatic HCC non-responders to HAIC, but no significance was seen in responders. Non-responders did not obtain the response of PVTT when only HAIC was performed. However, the RT group could have a high reduction of PVTT even if without intrahepatic HCC response. In this study, the RT group showed better TTF and PPS than the non-RT group despite the non-response of intrahepatic tumor to HAIC. These results show that 3D-CRT for PVTT combined with HAIC could provide survival benefit to non-responders to HAIC.

Although the present study has several limitations such as small sample size, a non-randomized and retrospective study design, and data generated from a single institution, it demonstrates that responders to HAIC could have better survival than non-responders similar to previous reports,[16,29] and that 3D-CRT for PVTT improves survival in non-responders to HAIC. It is the important issue to decide the indication of RT combined with HAIC for HCC patients with Vp3/4. In HAIC responders, little benefit was obtained by the combination therapy of 3D-CRT and HAIC. Unfortunately, no reliable predictors of prognosis and response to HAIC using pretreatment factors are currently available as well as sorafenib, although several studies have tested the utility of various biomarkers for advanced HCC.[31,38–41] Considering the progression and life-threatening condition of Vp3/4 HCC and low rate of severe adverse events on the combination of RT with HAIC, we consider that it may be reasonable to apply RT for PVTT at the first course of HAIC. Currently, several clinical studies regarding the efficacy of HAIC and sorafenib are being performed in Japan as follows. The combination of sorafenib and HAIC, including the randomized controlled trial comparing efficacy of sorafenib versus sorafenib in combination with low-dose cisplatin/5-FU HAIC in patients with advanced HCC (SILIUS, UMIN no. 000004315); the randomized phase II study of HCC_Sor_CDDP_rP2 of sorafenib combined with HAIC-cisplatin versus sorafenib for advanced HCC (UMIN no. 000005703); and sequential therapy of HAIC followed by sorafenib in the SCOOP-II trial (HAIC using cisplatin followed by sorafenib vs sorafenib alone for patients with advanced HCC, randomized phase II study, UMIN no. 000006147) and HICS55 (pilot study of HAIC using low-dose FP followed by sorafenib for advanced HCC, UMIN no. 000009094). Those results could help establish treatment strategies based on sorafenib and HAIC for advanced HCC in the future. Also, further studies are needed to identify predictors of response to these therapies that could allow the selection of HAIC or sorafenib for advanced HCC, and the combination with 3D-CRT for PVTT.

In conclusion, based on this study, we suggest that 3D-CRT for major PVTT combined with HAIC for advanced HCC could provide survival benefit to non-responders to HAIC for prolonged OS.

REFERENCES

1 Jemal A, Bray F, Center MM *et al.* Global cancer statistics. *CA Cancer J Clin* 2011; **61**: 69–90.

2 Kamangar F, Dores GM, Anderson WF. Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world. *J Clin Oncol* 2006; **24**: 2137–50.

3 Okita K. Management of hepatocellular carcinoma in Japan. *J Gastroenterol* 2006; **41**: 100–6.

4 Hasegawa K, Kokudo N, Makuuchi M *et al.* Comparison of resection and ablation for hepatocellular carcinoma: a cohort study based on a Japanese nationwide survey. *J Hepatol* 2013; **58**: 724–9.

5 Rossi S, Di Stasi M, Buscarini E *et al.* Percutaneous RF interstitial thermal ablation in the treatment of hepatic cancer. *AJR Am J Roentgenol* 1996; **167**: 759–68.

6 Llovet JM, Real MI, Montaña X *et al.* Arterial embolisation or chemoembolisation versus symptomatic treatment in patients with unresectable hepatocellular carcinoma: a randomised controlled trial. *Lancet* 2002; **359**: 1734–9.

7 Takayasu K, Arii S, Kudo M *et al.* Superselective transarterial chemoembolization for hepatocellular carcinoma. Validation of treatment algorithm proposed by Japanese guidelines. *J Hepatol* 2012; **56**: 886–92.

8 Kim DY, Park W, Lim DH *et al.* Three-dimensional conformal radiotherapy for portal vein thrombosis of hepatocellular carcinoma. *Cancer* 2005; **103**: 2419–26.

9 Ando E, Yamashita F, Tanaka M *et al.* A novel chemotherapy for advanced hepatocellular carcinoma with tumor thrombosis of the main trunk of the portal vein. *Cancer* 1997; **79**: 1890–96.

10 Llovet JM, Ricci S, Mazzaferro V *et al.* Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 378–90.

11 Cheng AL, Kong YK, Chen Z *et al.* Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomized, double-blind, placebo-controlled trial. *Lancet Oncol* 2009; **10**: 25–34.

12 Abou-Alfa GK, Amadori D, Santoro A *et al.* Safety and efficacy of sorafenib in patients with hepatocellular carcinoma (HCC) and Child-Pugh A versus B cirrhosis. *Gastrointest Cancer Res* 2011; **4** (2): 40–4.

13 Hollebecque A, Cattan S, Romano O *et al.* Safety and efficacy of sorafenib in hepatocellular carcinoma: the impact of the Child-Pugh score. *Aliment Pharmacol Ther* 2011; **34**: 1193–201.

14 Itamoto T, Nakahara H, Tashiro H *et al.* Hepatic arterial infusion of 5-fluorouracil and cisplatin for unresectable or recurrent hepatocellular carcinoma with tumor thrombus of the portal vein. *J Surg Oncol* 2002; **80**: 143–8.

15 Sakon M, Nagano H, Dono K *et al.* Combined intraarterial 5-fluorouracil and subcutaneous interferon-alpha therapy for advanced hepatocellular carcinoma with tumor thrombi in the major portal branches. *Cancer* 2002; **94**: 435–42.

16 Obi S, Yoshida H, Toune R *et al.* Combination therapy of intraarterial 5-fluorouracil and systemic interferon-alpha

for advanced hepatocellular carcinoma with portal venous invasion. *Cancer* 2006; **106**: 1990–97.
17 Ota H, Nagano H, Sakon M *et al.* Treatment of hepatocellular carcinoma with major portal vein thrombosis by combined therapy with subcutaneous interferon-alpha and intra-arterial 5-fluorouracil; role of type 1 interferon receptor expression. *Br J Cancer* 2005; **93**: 557–64.
18 Uka K, Aikata H, Takaki S *et al.* Similar side effects of recombinant interferon-alpha-2b and natural interferon-alpha when combined with intra-arterial 5-fluorouracil for the treatment of advanced hepatocellular carcinoma. *Liver Int* 2007; **27**: 1209–16.
19 Toya R, Murakami R, Baba Y *et al.* Conformal radiation therapy for portal vein tumor thrombosis of hepatocellular carcinoma. *Radiother Oncol* 2007; **84**: 266–71.
20 Han KH, Seong J, Kim JK *et al.* Pilot clinical trial of localized concurrent chemoradiation therapy for locally advanced hepatocellular carcinoma with portal vein thrombosis. *Cancer* 2008; **113**: 995–1003.
21 Katamura Y, Aikata H, Takaki S *et al.* Intra-arterial 5-fluorouracil/interferon combination therapy for advanced hepatocellular carcinoma with or without three-dimensional conformal radiotherapy for portal vein tumor thrombosis. *J Gastroenterol* 2009; **44**: 492–502.
22 Eisenhauer EA, Therasse P, Bogaerts J *et al.* New response evaluation criteria in solid tumors: revised RECIST guideline (version 1.1). *Eur J Cancer* 2009; **45**: 228–47.
23 Pan CC, Kavanagh BD, Dawson LA *et al.* Radiation-associated liver injury. *Int J Radiat Oncol Biol Phys* 2010; **76**: S94–S100.
24 Pawlik TM, Poon RT, Abdalla EK *et al.* Hepatectomy for hepatocellular carcinoma with major portal or hepatic vein invasion: results of a multicenter study. *Surgery* 2005; **137**: 403–10.
25 Bruix J, Raoul JL, Sherman M *et al.* Efficacy and safety of sorafenib in patients with advanced hepatocellular carcinoma: subanalyses of a phase III trial. *J Hepatol* 2012; **57**: 821–9.
26 Jeong SW, Jang JY, Shim KY *et al.* Practical effect of sorafenib monotherapy on advanced hepatocellular carcinoma and portal vein tumor thrombosis. *Gut Liver* 2013; **7**: 696–703.
27 Sugimoto K, Moriyasu F, Saito K *et al.* Hepatocellular carcinoma treated with sorafenib: early detection treatment response and major adverse events by contrast-enhanced US. *Liver Int* 2013; **33**: 605–15.
28 Yamasaki T, Sakaida I. Hepatic arterial infusion chemotherapy for advanced hepatocellular carcinoma and future treatments for the poor responders. *Hepatol Res* 2011; **42**: 340–8.
29 Ueshima K, Kudo M, Takita M *et al.* Hepatic arterial infusion chemotherapy using low-dose 5-fluorouracil and cisplatin for advanced hepatocellular carcinoma. *Oncology* 2010; **78**: 148–53.
30 Yamashita T, Arai K, Sunagozaka H *et al.* Randomized, phase II study comparing interferon combined with hepatic arterial infusion of fluorouracil plus cisplatin and fluorouracil alone in patients with advanced hepatocellular carcinoma. *Oncology* 2011; **81**: 281–90.
31 Niizeki T, Sumie S, Torimura T *et al.* Serum vascular endothelial growth factor as a predictor of response and survival in patients with advanced hepatocellular carcinoma undergoing hepatic arterial infusion chemotherapy. *J Gastroenterol* 2012; **47**: 686–95.
32 Liver Cancer study Group of Japan. *Guidelines for Evidence-Based Clinical Practice for the Treatment of Liver Cancer. Clinical Practice Guidelines for Hepatocellular Carcinoma.* Tokyo: Kanehara & Co., Ltd., 2013. (In Japanese.)
33 Yoon SM, Lim YS, Won HJ *et al.* Radiotherapy plus transarterial chemoembolization for hepatocellular carcinoma invading the portal vein: long-term patient outcomes. *Int J Radiat Oncol Biol Phys* 2012; **82**: 2004–11.
34 Huang YJ, Hsu HC, Wang CY *et al.* The treatment responses in cases of radiation therapy to portal vein thrombosis in advanced hepatocellular carcinoma. *Int J Radiat Oncol Biol Phys* 2009; **73**: 1155–63.
35 Kim TH, Kim DY, Park JW *et al.* Three-dimensional conformal radiotherapy of unresectable hepatocellular carcinoma patients for whom transcatheter arterial chemoembolization was ineffective or unsuitable. *Am J Clin Oncol* 2006; **29**: 568–75.
36 Zeng ZC, Fan J, Tang ZY *et al.* Prognostic factors for patients with hepatocellular carcinoma with macroscopic portal vein or inferior vena cava tumor thrombi receiving external-beam radiation therapy. *Cancer Sci* 2008; **99**: 2510–7.
37 Tanaka Y, Nakazawa T, Komori S *et al.* Radiotherapy for patients with unresectable advanced hepatocellular carcinoma with invasion to intrahepatic large vessels: efficacy and outcomes. *J Gastroenterol Hepatol* 2014; **29**: 352–7.
38 Tsuchiya K, Asahina Y, Matsuda S *et al.* Changes in plasma vascular endothelial growth factor at 8 weeks after sorafenib administration as predictors of survival for advanced hepatocellular carcinoma. *Cancer* 2014; **120**: 229–37.
39 Kawaoka T, Aikata H, Murakami E *et al.* Evaluation of the mRECIST and α-fetoprotein ratio for stratification of the prognosis of advanced-hepatocellular-carcinoma patients treated with sorafenib. *Oncology* 2012; **83**: 192–200.
40 Llovet JM, Peña CE, Lathia CD *et al.* Plasma biomarkers as predictors of outcome in patients with advanced hepatocellular carcinoma. *Clin Cancer Res* 2012; **18**: 2290–300.
41 Miyahara K, Nouso K, Tomoda T *et al.* Predicting the treatment effect of sorafenib using serum angiogenesis markers in patients with hepatocellular carcinoma. *J Gastroenterol Hepatol* 2011; **26**: 1604–11.

Journal of Gastroenterology and Hepatology

JGHF

doi:10.1111/jgh.12508

HEPATOLOGY

Long-term outcome of patients with gastric varices treated by balloon-occluded retrograde transvenous obliteration

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Key words
B-RTO, gastric varices, portal hypertension, prognosis esophageal varices.

Accepted for publication 3 December 2013.

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Conflict of interest: There are no potential conflicts of interest in this manuscript.

Abstract

Background and Aim: To assess the short- and long-term outcome of patients with gastric varices (GV) after balloon-occluded retrograde transvenous obliteration (B-RTO) by comparing bleeding cases with prophylactic cases.

Methods: Consecutive 100 patients with GV treated by B-RTO were enrolled in this retrospective cohort study. We compared the technical success, complications, and survival rates between bleeding and prophylactic cases.

Results: Of 100 patients, 61 patients were bleeding cases and 39 patients were prophylactic cases. Technical success was achieved in 95% of bleeding case and in 100% of prophylactic case, with no significant difference between these groups (overall technical success rate, 97%). The survival rates at 5 and 10 years were 50% and 22% in bleeding case, and 49% and 36% in prophylactic case, respectively. There was also no significant difference ($P = 0.420$). By multivariate analysis, survival rates correlated significantly with liver function (hazard ratio 2.371, 95% CI 1.457–3.860, $P = 0.001$) and hepatocellular carcinoma development (HR 4.782, 95% CI 2.331–9.810, $P < 0.001$). The aggravating rates of esophageal varices (EV) were 21%, 50%, and 54% at 12, 60, and 120 months after B-RTO. By multivariate analysis, aggravating rates significantly correlated with EV existing before B-RTO (HR 18.114, 95% CI 2.463–133.219, $P = 0.004$).

Conclusion: B-RTO for GV could provide the high rate of complete obliteration and favorable long-term prognosis even in bleeding cases as well as prophylactic cases. Management of EV after B-RTO, especially in coexisting case of GV and EV, would be warranted.

Introduction

Gastric variceal bleeding is associated with higher mortality and more difficulty in obtaining hemostasis rather than esophageal variceal bleeding[1,2] because gastric varices (GV) without passing through palisade vein has more increased blood flow and higher portal vein pressure. Therefore, optimal management of GV requires multidisciplinary approaches. Although various treatment modalities, such as pharmacotherapy, endoscopic procedures, interventional radiologic treatment, and surgery, have been widely performed at present, the standard treatments for GV have not yet been established. With endoscopic and pharmacological treatment for GV with abundant blood volume, large size, and high portal vein pressure, complete hemostasis and eradication of GV are often difficult to obtain. Surgeries such as Hassab's devascularization and transection, and splenectomy are among the useful treatment options; however, these are invasive and have a limitation of indication due to unfavorable hepatic functional reserve. In uncontrolled hemorrhage or rebleeding from GV, a transjugular intrahepatic portosystemic shunt (TIPS) is one of the important tools.[3–10] However, because of relatively high rates of rebleeding and the development of hepatic encephalopathy, this procedure seems not satisfactory. Balloon-occluded retrograde transvenous obliteration (B-RTO) involves the occlusion of blood flow by inflation of a balloon catheter into an outflow shunt and injection of 5% ethanolamine oleate into GV in a retrograde manner. Although it is reported that B-RTO has been safely performed for GV with almost complete eradication,[11–15] the long-term outcome and prognostic factors after B-RTO have yet to be elucidated. In the present study, we evaluated the clinical outcome and prognostic factors after B-RTO for GV by comparing bleeding cases with prophylactic cases.[16,17]

Table 1 Clinical characteristics of patients

	All cases (n = 100)	Bleeding cases (n = 61)	Prophylactic cases (n = 39)	P
Gender (male/female)	59/41	41/20	18/21	0.041¶
Age (years)†	72	70	72	n.s.††
Etiology (HBV/HCV/alcohol/others)	4/54/24/18	2/29/17/13	2/25/7/5	n.s.¶
Child-Pugh (A/B/C)	39/48/13	19/31/11	20/16/3	n.s.¶
Gastric variceal size (F1/F2/F3)‡	0/50/50	0/34/27	0/16/23	n.s.¶
Hirota's grade (1/2/3/4/5)§	11/27/36/23/3	5/14/22/17/3	6/13/14/6/0	n.s.¶
HCC development (no/yes)	39/61	42/19	20/19	n.s.¶
Portal systemic shunt (GR shunt/GC shunt)	96/4	57/4	39/0	n.s.¶
Esophageal varices (nonexisting/existing)	32/68	19/42	13/26	n.s.¶
Esophageal variceal size (F1/F2/F3)	37/29/2	24/16/2	13/13/0	n.s.¶
HVPG (mm Hg)†	7	5	8	n.s.††

†Data are median values. ‡Endoscopic findings for gastric varices were evaluated according to the general rules for recording endoscopic findings of esophagogastric varices. §Criteria for difficulty of retrograde transvenous obliteration according to retrograde venography under balloon occlusion.[12] ¶Chi-square test. ††Mann–Whitney *U*-test.

F, form; GC, gastrocaval; GR, gastrorenal; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HVPG, hepatic venous pressure gradient; n.s., not significant.

Methods

Patients. In this study, consecutive 100 patients of GV treated by B-RTO between December 1994 and April 2013 were enrolled in this retrospective cohort study. Patients were classified into two groups based on the presence or absence of bleeding from GV: the prophylactic cases and the bleeding cases. In prophylactic cases, appearances of red color sign or F3, or rapidly growing varices with the high risk of rupture, were indications for B-RTO. Also, in bleeding cases, those of portal systemic shunts by contrast-enhanced computed tomography (CT) after temporary hemostasis with balloon tamponade or endoscopically were indication for B-RTO. Patients' characteristics are shown in Table 1. Patients comprised 61 bleeding cases and 39 prophylactic cases. There was no difference of clinical background between bleeding and prophylactic cases except gender (male/female) ($P = 0.041$). The study was approved by the institutional review board of the participating clinical sites before study initiation, and the study was conducted according to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all patients at the time of enrollment.

B-RTO. Selective angiography from the celiac- and superior mesenteric arteries, and CT during arterial portography via superior mesenteric- or/and splenic arteries using an angio-CT system (Miyabi, Siemens, Erlangen, Germany; or INFX-8000C + Aquilion, Toshiba, Otawara, Japan) were performed before B-RTO in order to evaluate portal-systemic collaterals via unilateral femoral artery.

A 5-Fr catheter with a 1- or 2-cm-diameter balloon (Selecon balloon catheter; Terumo Clinical Supply, Gifu, Japan) was inserted into the draining vein of the portal systemic shunt through inferior vena cava via a right femoral or right jugular vein under local anesthesia. During balloon occlusion of outflow vessels, retrograde venography was performed to determine the hemodynamics of the GV and collateral veins. During balloon occlusion, the degree of progression of the GV and collateral veins was graded according to the classification reported by Hirota *et al.* (grade 1, GV were well opacified without evidence of collateral veins; grade 2, collateral veins were small and few in number, and the contrast medium remained in the GV for 3 min or more; grade 3, collateral veins were medium to large, there were few veins, and the contrast medium filled the GV only partially and disappeared within 3 min; grade 4, there were many large collateral veins, and the GV were not opacified; and grade 5, the left adrenal vein could not be occluded with the balloon catheter because of a very large gastrorenal shunt with rapid blood flow).[12] B-RTO was commonly performed using 5% ethanolamine oleate (Oldamin; Takeda Pharmaceutical, Osaka, Japan) mixed with iopamidol (Iopamiron 300; Bayer Health Care, Osaka, Japan) (5% ethanolamine oleate mixed with iopamidol [EOI]) under balloon occlusion. When necessary, minor collateral vessels of the shunts were embolized by 50% glucose solution[18] and microcoils[19] before EOI injection. Especially in the case of Hirota's grade 3 or 4, additional specialized techniques, such as stepwise injection of EOI, to treat minor collaterals were utilized.[19,20] The amount of EOI was defined as below 0.4 mL/kg body weight in one session to reduce the side effect. To avoid incomplete therapeutic efficacy and pulmonary infarction due to an unstable thrombus, the balloon catheter was left in the draining vein with balloon inflation for overnight and removed after confirmation of complete obliteration by retrograde venography. When obliteration of shunt was insufficient on retrograde venography, additional B-RTO was subsequently performed until disappearance of inflow vessels. To prevent renal dysfunction related to hemolysis that occurs as an adverse effect of EOI, 2000–4000 units of haptoglobin were administered to all patients before B-RTO. All patients underwent gastrointestinal endoscopy and intravenous contrast-enhanced CT approximately 1 week after B-RTO.

Technical success. When the contrast-enhanced CT scan showed GV with low attenuation, including the afferent veins or the draining veins of the GV, we considered obliteration to be complete. On the other hand, when contrast-enhanced CT showed GV with partial enhancement, we considered obliteration to be

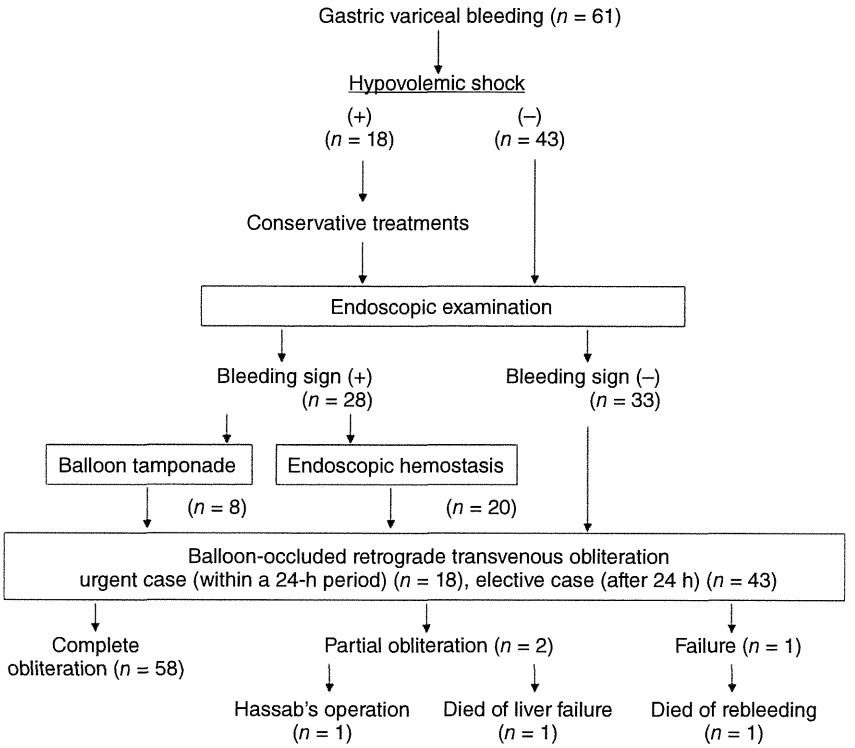

Figure 1 Clinical course of bleeding cases from gastric varices.

partial. And when contrast-enhanced CT showed GV with whole enhancement, we considered obliteration to be failure.

Complications. We adequately assessed complications related to the procedure of B-RTO according to the Society of Interventional Radiology.[21] Minor complication means not to require medical attention. Major complication means therapy is required, permanent adverse sequelae, and death.

Follow-up. We estimated technical success and overall survival in bleeding cases and prophylactic cases. Follow-up diagnostic imaging, such as gastrointestinal endoscopy or contrast-enhanced CT, was performed consecutively at 1, 3, 6, and 12 months, and then every 6 months or 1 year after B-RTO.

Statistical analysis. While technical success and complications were examined as the short-term outcome, survival rates and aggravation of esophageal varices (EV) were examined as the long-term outcome. Categorical variables were compared using chi-square test or the Fisher exact test when appropriate. Quantitative variables or ordinal variables were compared with the Mann–Whitney *U*-test. The cumulative survival rates and aggravation rates of EV were determined using the Kaplan–Meier method, and comparison of survivals between groups was done with the log–rank test or the log–rank trend test in case of ordered groups. The Cox's proportional hazards model was used to estimate the significance of independent variables. A value of $P < 0.05$ was regarded as statistically significant. The Statistical Package for Social Sciences (SPSS, Chicago, IL, USA) was used for statistical analysis.

Results

The clinical course of GV bleeding cases. The clinical course of GV bleeding cases is shown in Figure 1. The 18 patients had sustained hypovolemic shock on admission. They were initially treated to stabilize the hemodynamic circulation by conventional treatment, such as intravenous fluid administration, vasopressor drugs, and blood transfusion. After improvement of their general condition, endoscopic examination was done. Twenty-eight patients with bleeding signs were treated for temporary hemostasis with balloon tamponade ($n = 8$) and/or endoscopically ($n = 20$; 12 patients were treated with injection of cyanoacrylate[22] and 8 with endoscopic variceal ligation). Then, all patients underwent contrast-enhanced CT to evaluate the presence of portal systemic shunts and to determine whether they can be treated with B-RTO.[23] Eighteen patients underwent urgent B-RTO (within a 24-h period), and 43 patients underwent elective B-RTO (after 24 h).

B-RTO procedure. Hirota's grade, which was evaluated by balloon-occluded retrograde transvenous venography, is listed in Table 2. The 11, 27, 37, and 25 patients were diagnosed as Hirota's grade 1, 2, 3, and 4, respectively. Three patients were evaluated as grade 5 because balloon catheter could not be inserted into the shunt. By reevaluation from the internal carotid vein approach, these patients were graded and listed as grade 3 and grade 4 in Tables 2 and 3. The median amounts of 5% EOI were between 18 mL and 22.5 mL in each grade, as shown in Table 2. In two, one and one patient in Hirota grade 2, 3, and 4, GV was completely obliteration with 50% glucose and coiling for main vessel without

Table 2 B-RTO procedure according to Hirota's grade

Hirota's grade	All cases				Bleeding cases				Prophylactic cases			
	Number of patients	5% EOI (mL)†	50% glucose (mL)†	Coil (%)‡	Number of patients	5% EOI (mL)†	50% glucose (mL)†	Coil (%)‡	Number of patients	5% EOI (mL)†	50% glucose (mL)†	Coil (%)‡
1	11	20 (7–38)	40 (0–90)	0	5	28 (7–38)	40 (0–90)	0	6	16.5 (10–30)	20 (20–60)	0
2	27	18 (0–42)	20 (0–100)	12	14	19 (0–42)	20 (0–80)	21	13	17 (0–35)	40 (0–130)	0
3	37	18 (0–56)	60 (0–300)	43	23	18 (4–56)	60 (0–102)	30	14	18 (0–55)	55 (40–300)	64
4	25	22.5 (0–82)	102 (0–200)	44	19	23 (7–82)	102 (0–200)	37	6	20 (0–60)	70 (0–130)	67

†Data are median values (range). ‡The number is population of patients used with micro-coil for microvessels.
B-RTO, balloon-occluded retrograde transvenous obliteration, EOI, 5% ethanolamine oleate mixed with iopamidol.

EOI administration. The median amounts of EOI used were not significantly different between Hirota's grade 1/2 and 3/4 (18.5 mL and 19.5 mL, respectively, $P = 0.651$). In contrast, the median amounts of 50% glucose administered were 40 mL and 60 mL in grade 1/2 and 3/4, respectively. The proportion of patients embolized by coils for microvessels was 8% and 44% in grade 1/2 and 3/4, respectively. The amount of 50% glucose administered ($P = 0.009$) and the proportion of patients embolized by coils for microvessels ($P < 0.001$) were significantly different between Hirota's grade 1/2 and 3/4. When compared between bleeding cases and prophylactic cases, there were no differences in the amount of 5% EOI administered (bleeding cases and prophylactic cases: 22 mL and 18 mL, respectively, $P = 0.748$), 50% glucose administered (bleeding cases and prophylactic cases: 60 mL and 50 mL, respectively, $P = 0.128$), and proportion embolized by coils (bleeding cases and prophylactic cases: 28% and 33%, respectively, $P = 0.386$).

Technical success. Of 100 patients, overall complete obliteration was achieved in 97 of 100 (97%) patients, partial was in 2 (2%), and failure was in 1 (1%). Thus, technical success was 97%. In bleeding cases, complete obliteration was achieved in 58 of 61 (95%) patients, partial obliteration was in 2 of 61 (3.3%), and failure was in 1 of 61 (1.6%). In prophylactic cases, complete obliteration was achieved in all 39 of 39 (100%) patients. There was no difference between bleeding and prophylactic cases in complete obliteration. Concerning Hirota's grade, the success rates were 100% ($n = 11$), 100% ($n = 27$), 97% ($n = 37$), and 92% ($n = 25$) in grade 1, 2, 3, and 4, respectively (Table 3). One patient with Hirota's grade 3 and one patient with Hirota's grade 4 resulted in partial obliteration, and one patient with Hirota's grade 4 resulted in failure. All these three patients were bleeding cases. One of the partial underwent Hassab's devascularization 9 days after B-RTO, and survived. The other of the partial died shortly of liver failure. The patient with failure obtained spontaneous hemostasis but died of gastric variceal rebleeding 1 year later.

Complications. Major complications were not observed by this treatment. Minor complications were pain (13%), fever (35%), gross hematuria (32%), transaminase elevation (29%), jaundice (9%), renal dysfunction (6%), and ascites (18%). Among minor complications, significant differences were observed in transaminase elevation between bleeding and prophylactic cases (bleeding cases: $n = 22$; prophylactic cases: $n = 6$, $P = 0.035$).

Survival. We examined overall survival of 97 patients with complete obliteration. The median value of observation periods after B-RTO was 60 (range: 0–191) months. Thirty-one patients in bleeding cases and 15 in prophylactic cases died during the follow-up period. The causes of death were hepatocellular carcinoma (HCC) in 8, hepatic failure in 20, esophageal variceal bleeding in 3, and extrahepatic diseases in 15. Overall cumulative survival rates were 50% and 26% at 60 and 120 months after B-RTO, respectively. The cumulative survival rates at 60 and 120 months were 50% and 22% in patients with bleeding, and 49% and 36% in patients with prophylactic cases, respectively (Fig. 2a). No significance were seen between the two groups ($P = 0.420$).

Factors affecting overall survival. Overall survival correlated significantly with hepatic functional reserve assessed by

Table 3 Technical success according to Hirota's grade

Hirota's grade	All cases		Bleeding cases		Prophylactic cases	
	Number of patients	Success rates (%)	Number of patients	Success rates (%)	Number of patients	Success rates (%)
1	11	100	5	100	6	100
2	27	100	14	100	13	100
3	37	97	23	96	14	100
4	25	92	19	89	6	100

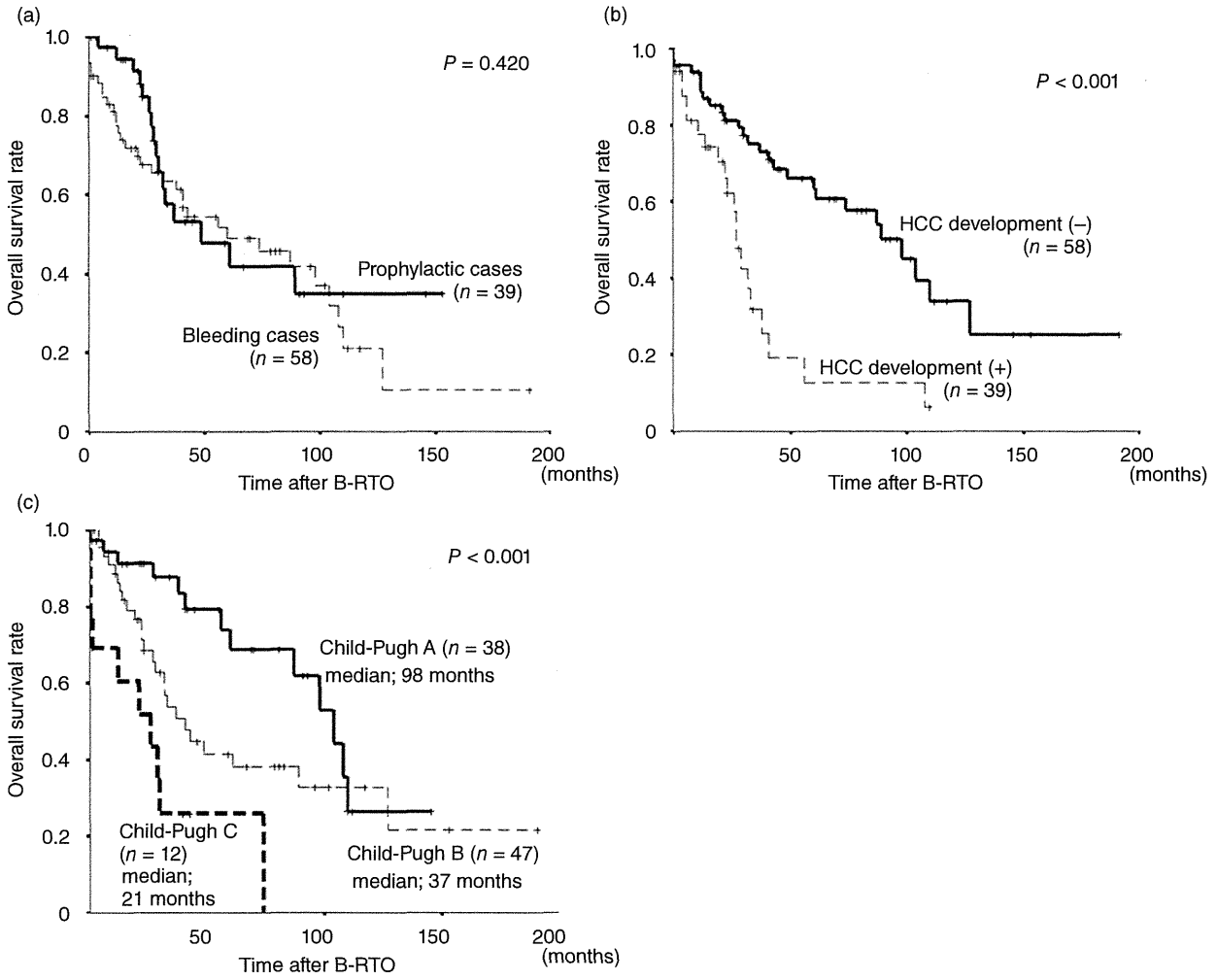

Figure 2 (a) Cumulative overall survival rate in bleeding or prophylactic cases from gastric varices. No significance was seen between the two groups (log–rank test, $P = 0.420$). (b) Cumulative overall survival rate between patients with and without development of hepatocellular carcinoma. There was a significant difference between the two groups (log–rank test, $P < 0.001$). (c) Cumulative overall survival rate according to Child-Pugh classification (A/B/C). There was a significant trend in survival with respect to Child-Pugh classification (log–rank trend test, $P < 0.001$). B-RTO, balloon-occluded retrograde transvenous obliteration; HCC, hepatocellular carcinoma.

Child-Pugh grading ($P = 0.004$), HCC development ($P < 0.001$), and existing EV before B-RTO ($P = 0.035$) by univariate analysis. Multivariate analysis revealed that hepatic functional reserve (hazard ratio 2.371, 95% CI 1.457–3.860, $P = 0.001$) and HCC development (hazard ratio 4.782, 95% CI 2.331–9.810, $P < 0.001$) were significant independent factors for overall survival (Table 4).

The cumulative survival rates in patients with HCC development were 16% and 0% at 60 and 120 months, whereas those

Table 4 Univariate and multivariate analysis for survival rate

	Univariate analysis	Multivariate analysis		
	P	Hazard ratio	95% CI	*P*
Gender (male/female)	0.258	—	—	—
Age (year)	0.222	—	—	—
Etiology				
HBV	0.693	—	—	—
HCV	0.245	—	—	—
NBNC	(reference)			
Child-Pugh classification (A/B/C)	0.004	2.371	1.457–3.860	0.001
HCC development (no/yes)	< 0.001	4.782	2.331–9.810	< 0.001
PSS (GR shunt/GC shunt)	0.759	—	—	—
Gastric varices (bleeding/prophylactic)	0.420	—	—	—
Esophageal varices before B-RTO (nonexistence/existence)	0.035	—	—	—
HVPG before B-RTO (under 10/over 10 mm Hg)	0.887	—	—	—

B-RTO, balloon-occluded retrograde transvenous obliteration; GC, gastrocaval; GR, gastrorenal; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HVPG, hepatic venous pressure gradient; NBNC, non-HBV and non-HCV; PSS, portal systemic shunt.

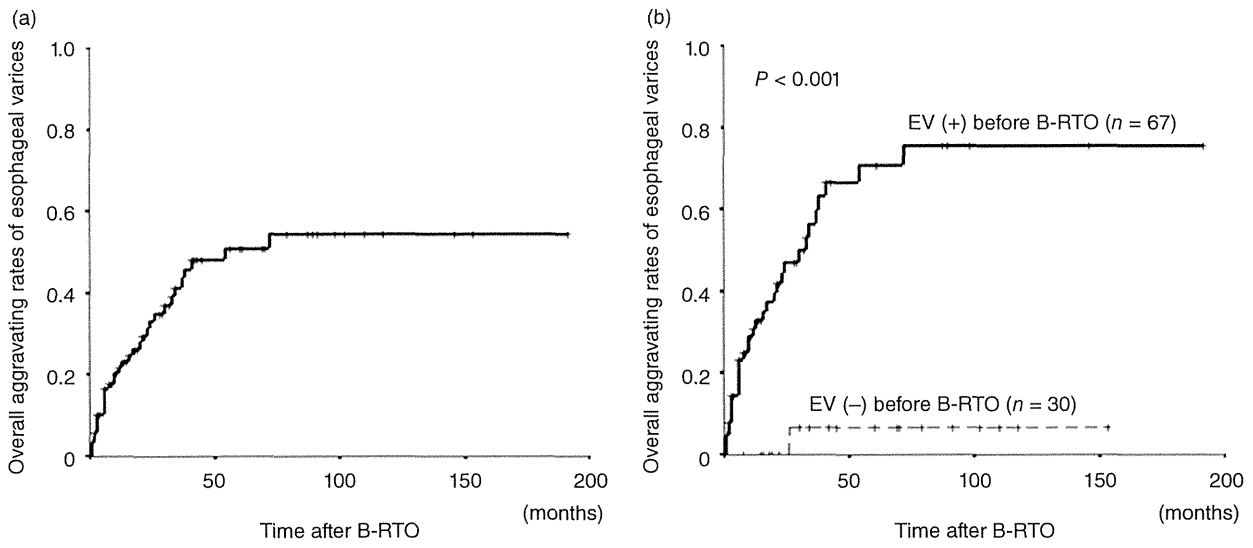

Figure 3 (a) Cumulative esophageal variceal aggravation. (b) Cumulative esophageal variceal aggravation classified by the existence of esophageal varices (EV) before balloon-occluded retrograde transvenous obliteration (B-RTO). There was a significant difference between the two groups (log–rank test, $P < 0.001$).

without HCC development were 69% and 34% at 60 and 120 months, respectively. There was a significant difference between the two groups ($P < 0.001$) (Fig. 2b). The cumulative survival rates of Child A/B/C were 69%/42%/26% at 60 months, and 27%/33%/0% at 120 months, respectively. A significant trend in survival with respect to Child-Pugh grading was demonstrated, indicating that better prognosis was associated with hepatic functional reserve (log–rank trend test, $P < 0.001$) (Fig. 2c). Four patients with Child C and one patient with Child A, those were all bleeding cases, died within 1 month. This patient of Child-Pugh A died of pneumonia 20 days after B-RTO despite of successful complete obliteration.

Aggravation of EV after B-RTO. We analyzed the aggravation of EV after B-RTO. Aggravating EV was recognized in 36 patients. Overall cumulative aggravation rates of EV were 21%, 41%, 50%, and 54% at 12, 36, 60, and 120 months after B-RTO, respectively (Fig. 3a). By univariate analysis, aggravating rates correlated significantly with existence of EV before B-RTO ($P < 0.001$) (Fig. 3b) and HCC development (yes/no) ($P = 0.071$). By multivariate analysis, overall aggravating rates correlated significantly and independently with existence of EV before B-RTO (hazard ratio 18.114, 95% CI 2.463–133.219, $P = 0.004$). The cumulative aggravating rates in patients with existence of EV before B-RTO were 28%, 50%, and 68% at 12, 36, and 120

months, respectively. The cumulative aggravating rates in patients with nonexistence of EV before B-RTO were 0%, 7%, and 7% at 12, 36, and 120 months, respectively.

Discussion

In the present study, we examined the short- and the long-term outcome of patients with GV after B-RTO by comparing bleeding cases with prophylactic cases. There were no differences in complete obliteration rates of B-RTO between bleeding cases and prophylactic cases. Also, no differences were found in survival rates between bleeding cases and prophylactic cases ($P = 0.420$) (Fig. 2a). We identified hepatic functional reserve and HCC development as significant and independent factors for overall survival (Table 4). That is, hepatic functional reserve and HCC development influenced the long-term outcome after B-RTO. The complete obliteration rates of overall cases, bleeding cases, and prophylactic cases were 97%, 95%, and 100%, respectively. According to the classification of Hirota's grade, all cases of partial or failure of B-RTO were Hirota's grade 3 or 4. All complications were minor complications. Aggravating rates of EV after B-RTO is reported to be high.[16,17,24–26] Similarly, those in our study were 50% and 54% at 60 and 120 months after B-RTO, respectively.

In bleeding cases, initial hemostasis of GV is mandatory before B-RTO. After obtaining initial hemostasis, it is important to undergo contrast-enhanced CT to evaluate the presence of portal systemic shunts and to determine whether they can be treated with B-RTO.[23] Considering that survival rates were similar between bleeding cases and prophylactic cases ($P = 0.420$), even in bleeding cases, successful treatment by initial hemostasis with endoscopy or balloon tamponade, and successive complete B-RTO, would provide a good prognosis comparable to prophylactic cases.

Hassab's devascularization and transection, percutaneous transhepatic obliteration, TIPS, and shunt-occluded endoscopic injection sclerotherapy are performed as other modalities for treatment of GV. Hassab's devascularization and transection have a limited role in the treatment of GV from the aspect of hepatic dysfunction.[27,28] Percutaneous transhepatic obliteration could obtain hemostasis, but rebleeding may occur.[29] It is likely that the antegrade procedure could obliterate inflow vessels but not easy to obliterate varices completely. After the treatment of TIPS for gastric variceal hemorrhage, the rates of hemostasis are greater than 90%, but the incidence of rebleeding is approximately 30%.[30,31] TIPS may achieve hemostasis, but rebleeding rates are not satisfactory depressed. Moreover, this procedure may induce hepatic encephalopathy or chronic hepatic failure.[31] On the other hand, B-RTO shows high rates of hemostasis and low rates of rebleeding. These findings would indicate that the retrograde procedure could easily obliterate whole varices in comparison with antegrade obliteration. Of course, there were unavoidable cases. Five patients, all were bleeding cases, died of pneumonia (one patient with Child A) and liver dysfunction (four patients with Child C) within 1 month after B-RTO.

There were no differences in the amounts of EOI ($P = 0.748$) and 50% glucose ($P = 0.128$), and the proportion of patients treated with micro coil ($P = 0.386$) between bleeding and prophylactic cases. There were significant differences in glucose ($P = 0.009$) administered and usage of coil ($P < 0.001$) between Hirota's grade 1/2 and 3/4. On the other hand, the amount of EOI administered has no difference between them ($P = 0.651$). As the higher grade of Hirota's classification, it means that more micro vessels would exist. It is very important to decrease the amount of EOI that is used to embolize these microvessels, and it is key to achieve high successful rates of B-RTO. Even in a higher Hirota's grade, a good success rate would be kept by the use of glucose and coil to reduce the amount of EOI. Recently, foam embolization has been reported in B-RTO. This could also reduce the amounts of EOI.[32,33] This procedure also needs to treat microvessels according to Hirota's grade.

All complications were minor, and there were no difference between bleeding cases and prophylactic cases. Aggravating rates of EV after B-RTO were high, especially in coexisting case of GV and EV before B-RTO. This showed the need for more delicate follow-up or additive endoscopic treatment for EV after B-RTO in coexisting case of GV and EV. When these treatments were not applicable, Hassab's operation might be done. In HCC patients with GV, B-RTO effectively prevented the rupture of GV, and this would enable the continued treatment for HCC. Prospective large-scale study concerning long-term prognosis and complications after B-RTO in the future would be needed.

In conclusion, B-RTO for GV could provide high rates of complete obliteration and favorable long-term prognosis even in bleeding cases as well as prophylactic cases.

References

1 Kim T, Shijo H, Kokawa H *et al.* Risk factors for hemorrhage from gastric fundal varices. *Hepatology* 1997; **25**: 307–12.
2 Ryan BM, Stockbrugger RW, Ryan JM. A pathophysiologic gastroenterologic, and radiologic approach to the management of gastric varices. *Gastroenterology* 2004; **126**: 1175–89.
3 Rossle M, Haag K, Ochs A *et al.* The transjugular intrahepatic portosystemic stent-shunt procedure for variceal bleeding. *N. Engl. J. Med.* 1994; **330**: 165–71.
4 Sanyal AJ, Freedman AM, Luketic VA *et al.* The natural history of portal hypertension after transjugular intrahepatic portosystemic shunts. *Gastroenterology* 1997; **112**: 889–98.
5 Rossle M, Siegerstetter V, Olschewski M, Ochs A, Berger E, Haag K. How much reduction in portal pressure is necessary to prevent variceal rebleeding? A longitudinal study in 225 patients with transjugular intrahepatic portosystemic shunts. *Am. J. Gastroenterol.* 2001; **96**: 3379–83.
6 Tripathi D, Therapondos G, Jackson E, Redhead DN, Hayes PC. The role of the transjugular intrahepatic portosystemic stent shunt (TIPSS) in the management of bleeding gastric varices: clinical and haemodynamic correlations. *Gut* 2002; **51**: 270–4.
7 Zhuaug ZW, Teng GJ, Jeffery RF, Gemery JM, Janne d'Othee B, Bettmann MA. Long-term results and quality of life in patients treated with transjugular intrahepatic portosystemic shunts. *AJR Am. J. Roentgenol.* 2002; **179**: 1597–603.
8 Boyer TD. Transjugular intrahepatic portosystemic shunt: current status. *Gastroenterology* 2003; **124**: 1700–10.
9 Boyer TD, Haskal ZJ, American Association for the Study of Liver Diseases. The role of transjugular intrahepatic portosystemic shunt in the management of portal hypertension. *Hepatology* 2005; **41**: 386–400.
10 Tesdal IK, Filser T, Weiss C, Holm E, Dueber C, Jaschke W. Transjugular intrahepatic portosystemic shunts: adjunctive embolotherapy of gastroesophageal collateral vessels in the prevention of variceal rebleeding. *Radiology* 2005; **236**: 360–7.

11 Koito K, Namieno T, Nagakawa T, Morita K. Balloon-occluded retrograde transvenous obliteration for gastric varices with gastrorenal or gastrocaval collaterals. *AJR Am. J. Roentgenol.* 1996; **167**: 1317–20.
12 Hirota S, Matsumoto S, Tomita M, Sako M, Kono M. Retrograde transvenous obliteration of gastric varices. *Radiology* 1999; **211**: 349–56.
13 Matsumoto A, Hamamoto N, Nomura T *et al.* Balloon-occluded retrograde transvenous obliteration of high risk gastric fundal varices. *Am. J. Gastroenterol.* 1999; **94**: 643–9.
14 Sonomura T, Sato M, Kishi K *et al.* Balloon-occluded retrograde transvenous obliteration for gastric varices: a feasibility study. *Cardiovasc. Intervent. Radiol.* 1998; **21**: 27–30.
15 Katoh K, Sone M, Hirose A, Inoue Y, Fujino Y, Onodera M. Balloon-occluded retrograde transvenous obliteration for gastric varices: the relationship between the clinical outcome and gastrorenal shunt occlusion. *BMC Med. Imaging* 2010; **10**: 2.
16 Hiraga N, Aikata H, Takaki S *et al.* The long-term outcome of patients with bleeding gastric varices after balloon-occluded retrograde transvenous obliteration. *J. Gastroenterol.* 2007; **42**: 663–72.
17 Kitamoto M, Imamura M, Kamada K *et al.* Balloon-occluded retrograde transvenous obliteration of gastric fundal varices with hemorrhage. *AJR Am. J. Roentgenol.* 2002; **178**: 1167–74.
18 Yamagami T, Kato T, Hirota T, Yoshimatsu R, Matsumoto T, Nishimura T. Infusion of 50% glucose solution before injection of ethanolamine oleate during balloon-occluded retrograde transvenous obliteration. *Australas. Radiol.* 2007; **51**: 334–8.
19 Takahashi K, Yamada T, Hyodoh H *et al.* Selective balloon-occluded retrograde sclerosis of gastric varices using a coaxial microcatheter system. *AJR Am. J. Roentgenol.* 2001; **177**: 1091–3.
20 Kiyosue H, Mori H, Matsumoto S, Yamada Y, Hori Y, Okino Y. Transcatheter obliteration of gastric varices: part 2. Strategy and techniques based on hemodynamic features. *Radiographics* 2003; **23**: 921–37.
21 Sacks D, Cardella J, Lewis C. Society of Interventional Radiology clinical practice guidelines. *J. Vasc. Interv. Radiol.* 2003; **14**: 199–202.
22 Hong CH, Kim HJ, Park JH *et al.* Treatment of patients with gastric variceal hemorrhage: endoscopic N-butyl-2-cyanoacrylate injection versus balloon-occluded retrograde transvenous obliteration. *J. Gastroenterol. Hepatol.* 2009; **24**: 372–8.
23 Matsumoto A, Kitamoto M, Imamura M *et al.* Three-dimensional portography using multislice helical CT is clinically useful for management of gastric fundic varices. *AJR Am. J. Roentgenol.* 2001; **176**: 899–905.
24 Tanihata H, Minamiguchi H, Sato M *et al.* Changes in portal systemic pressure gradient after balloon-occluded retrograde transvenous obliteration of gastric varices and aggravation of esophageal varices. *Cardiovasc. Intervent. Radiol.* 2009; **32**: 1209–16.
25 Elsamman MK, Fujiwara Y, Kameda N *et al.* Predictive factors of worsening of esophageal varices after balloon-occluded retrograde transvenous obliteration in patients with gastric varices. *Am. J. Gastroenterol.* 2009; **104**: 2214–21.
26 Nakamura S, Torii N, Yatsuji S *et al.* Long-term follow up of esophageal varices after balloon-occluded retrograde transvenous obliteration for gastric varices. *Hepatol. Res.* 2008; **38**: 340–7.
27 Hassab MA. Gastroesophageal decongestion and splenectomy in the treatment of esophageal varices in bilharzial cirrhosis: further studies with a report on 355 operations. *Surgery* 1967; **61**: 169–76.
28 George P, Brown C, Ridgway G, Crofts B, Sherlock S. Emergency oesophageal transection in uncontrolled variceal haemorrhage. *Br. J. Surg.* 1973; **60**: 635–40.
29 L'Hermine C, Chastanet P, Delemazure O, Bonniere PL, Durieu JP, Paris JC. Percutaneous transhepatic embolization of gastroesophageal varices: results in 400 patients. *AJR Am. J. Roentgenol.* 1989; **152**: 755–60.
30 Chau TN, Patch D, Chan YW, Nagral A, Dick R, Burroughs AK. "Salvage" transjugular intrahepatic portosystemic shunts: gastric fundal compared with esophageal variceal bleeding. *Gastroenterology* 1998; **114**: 981–7.
31 Saad WE, Darcy MD. Transjugular Intrahepatic Portosystemic Shunt (TIPS) versus Balloon-occluded Retrograde Transvenous Obliteration (BRTO) for the management of gastric varices. *Semin. Intervent. Radiol.* 2011; **28**: 339–49.
32 Choi SY, Won JY, Kim KA, Lee do Y, Lee KH. Foam sclerotherapy using polidocanol for balloon-occluded retrograde transvenous obliteration (BRTO). *Eur. Radiol.* 2011; **21**: 122–9.
33 Clements W, Cavanagh K, Ali F *et al.* Variant treatment for gastric varices with polidocanol foam using balloon-occluded retrograde transvenous obliteration: a pilot study. *J. Med. Imaging Radiat. Oncol.* 2012; **56**: 599–605.

JOURNAL OF VIRAL HEPATITIS
JVH

Journal of Viral Hepatitis, 2014, **21**, e89–e97 doi:10.1111/jvh.12240

Human microRNA hsa-miR-1231 suppresses hepatitis B virus replication by targeting core mRNA

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Received November 2013; accepted for publication February 2014

SUMMARY. Pathogen-specific miRNA profiles might reveal potential new avenues for therapy. To identify miRNAs directly associated with hepatitis B virus (HBV) in hepatocytes, we performed a miRNA array analysis using urokinase-type plasminogen activator (uPA)–severe combined immunodeficiency (SCID) mice where the livers were highly repopulated with human hepatocytes and human immune cells are absent. Mice were inoculated with HBV-infected patient serum samples. Eight weeks after HBV infection, human hepatocytes were collected from liver tissues, and miRNAs were analysed using the Toray 3D array system. The effect of miRNAs on HBV replication was analysed using HBV-transfected HepG2 cells. Four miRNAs, hsa-miR-486-3p, hsa-miR-1908, hsa-miR-675 and hsa-miR-1231 were upregulated in mouse and human livers with HBV infection. These miRNAs were associated with immune response pathways such as inflammation mediated by chemokine and cytokine signalling. Of these miRNAs, hsa-miR-1231, which showed high homology with HBV core and HBx sequences, was most highly upregulated. In HBV-transfected HepG2 cells, overexpression of hsa-miR-1231 resulted in suppression of HBV replication with HBV core reduction. In conclusion, a novel interaction between hsa-miR-1231 and HBV replication was identified. This interaction might be useful in developing new therapeutic strategies against HBV.

Keywords: HB core, hepatitis B virus, hsa-miR-1231, human hepatocyte chimeric mouse, microRNA.

INTRODUCTION

Hepatitis B virus (HBV) is a member of the *Hepadnaviridae* family, which contains a group of hepatotropic small DNA viruses that infect their respective animal hosts [1–3]. Once HBV infects human hepatocytes, the HBV genome translocates into the nucleus. Some genome copies are converted into a covalently closed circular DNA (cccDNA) form and organized into a minichromosome with histone and nonhistone proteins [4–8]. HBV cccDNA utilizes the cellular transcriptional machinery to produce all viral RNAs including the pregenomic RNA [9], and these gene products regulate viral replication and pathogenesis by regulating host gene expression [10,11].

MicroRNAs (miRNAs) are small noncoding RNAs of 21-25 nucleotides in length, processed from hairpin-shaped transcripts [12]. MiRNAs can bind the 3′-untranslated regions (UTRs) of messenger RNAs and downregulate gene expression by cleaving messenger RNA or inhibiting translation. Several miRNAs associated with HBV infection, HBV replication and hepatocarcinogenesis have recently been identified [13–19]. However, the direct influence of HBV infection on miRNA expression is still unclear.

MicroRNAs are currently being investigated for their therapeutic potential in antiviral therapy. As several studies have demonstrated that hsa-miR-122, which is specifically and abundantly expressed in hepatocytes, supported hepatitis C virus (HCV) replication by improving RNA

Abbreviations: HBc, hepatitis B core; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; miRNA, microRNA; RI, replication intermediates.

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*These authors contributed equally to this work.
This study was supported in part by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Labor and Health and Welfare.

stability [20–24], small molecules or siRNAs which are able to knock down miR-122 expression have been explored as a new therapeutic agent for HCV eradication.

A similar microRNA-based antiviral approach is also sought for the treatment of chronic hepatitis B, as it is difficult to eradicate HBV genomes converted into cccDNA or minichromosomes under present antiviral therapies. To develop new strategies for complete eradication of the viral genome from hepatocytes, it is important to clarify the direct associations between hepatic miRNAs and HBV infection.

In this study, miRNA microarray analysis was performed using human hepatocyte chimeric mouse livers to assess the direct impact of HBV infection on miRNA expression. We successfully demonstrated that HBV infection attenuated the expression of miRNAs under immunodeficient conditions to protect early viral propagation. A novel interaction between hsa-miR-1231 and HBV replication was identified.

MATERIALS AND METHODS

Human serum inoculum

Serum samples were obtained from a carrier infected with HBV genotype C after obtaining written informed consent for the donation and evaluation of blood samples. Inoculum was positive for HBs and HBe antigens with high-level viremia (HBV DNA: 7.1 log copies/mL). The experimental protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the Hiroshima University Hospital ethical committee (Approval ID: D08-9).

Human hepatocyte chimeric mice experiments

Human hepatocyte chimeric mice (PXB mice), in which human hepatocytes were transplanted into $uPA^{+/+}/SCID^{+/+}$ mice, were purchased from Phoenix Bio (Hiroshima, Japan). Mouse experiments were performed in accordance with the guidelines of the local committee for animal experiments at Hiroshima University.

Six chimeric mice, in which more than 90% of the liver tissue was replaced with human hepatocytes, were divided into two experimental groups. Group A contained three uninfected mice. Group B consisted of three mice that were inoculated via the mouse tail vein with human serum containing 6×10^6 copies of HBV. Serum HBV DNA titres were quantified every 2 weeks by real-time PCR, and human albumin levels were measured using the Human Albumin ELISA Quantitation kit (Bethyl Laboratories Inc., Montgomery, TX, USA) as described previously [25]. Eight weeks after inoculation, all three infected mice were sacrificed. Infection, extraction of serum samples and sacrifice were performed under ether anaesthesia as described previously [26].

miRNA microarray analysis

Human hepatocytes were finely dissected from the mouse livers and stored in liquid nitrogen after submerging in RNA*later*® solution (Applied Biosystems, Foster City, CA, USA). Experimental sample RNAs were isolated using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and analysed using TORAY 3-D Gene Chip human miRNA ver. 12.1 (TORAY, Chiba, Japan).

Data analysis

Gene expression profiles were analysed using GeneSpring GX 10.0.2 software (Tomy Digital Biology, Tokyo, Japan). Expression ratios were normalized per chip to the 50th percentile. To determine whether there were miRNAs differentially expressed among samples, we performed two Welch's *t*-tests ($P < 0.01$) on this prescreened list of miRNAs with Benjamini and Hochberg's correction. Complete linkage hierarchical clustering analysis was applied using Euclidean distance.

Pathway analysis

The miRNA target genes were predicted by the online database miRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/index.html). Target prediction was performed using 3′-UTR sequences of mRNAs, and the probability distributions were calculated using the Poisson distribution [27]. The mRNAs with *P* values <0.01 were considered significant. To improve the accuracy of target gene selection, the predicted genes were screened using other prediction programs, including miRanda (August 2010 release), miRDB (April 2009 release) and TargetScan version 5.1 (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). Genes that were predicted by at least two alternate programs were selected. Pathway analysis was performed by PANTHER version 8.1 (http://www.pantherdb.org/) to determine the effects of the predicted target genes on pathways.

Quantification of miRNAs

Small RNAs were extracted from liver tissues or HepG2 cells with mirVana™ miRNA Isolation Kit (Applied Biosystems) and reverse-transcribed according to the manufacturer's instructions. The selected miRNAs were quantified with TaqMan® MicroRNA Assays (Applied Biosystems) using the 7300 Real-Time PCR System (Applied Biosystems), and the expression of RNU6B served as a control.

Quantification of mRNAs

Total RNA was extracted from HepG2 cells transfected with control miRNA or miR-1231 expression plasmid using

RNeasy Mini Kit and reverse-transcribed (RT) using ReverTra Ace (TOYOBO, Osaka, Japan) with random primer according to the manufacturer's instructions. The selected cDNAs were quantified by real-time PCR. Differences between groups were examined for statistical significance using Student's *t*-test. The primer sequences were as follows: GAPDH forward 5′-ACAACAGCCTCAAGATCATCAG-3′ and reverse 5′-GGTCCACCACTGACACGTTG-3′; Mx1 forward 5′- TTCGGCTGTTTACCAGACTCC-3′ and reverse 5′- CAAAGCCTGGCAGCTCTCTAC-3′; 2′–5′ oligoadenylate synthetase 1 (OAS1) forward 5′- ACCTGGTTGTCTTCCTCA GTCC-3′ and reverse 5′- GAGCCTGGACCTCAAACTTCAC-3′; double stranded RNA dependent protein kinase (PKR) forward 5′- TGGCCGCTAAACTTGCATATC-3′ and reverse 5′- AGTTGCTTTGGGACTCACACG-3′; and SOCS1 forward 5′-ACGAGCATCCGCGTGCACTT-3′ and reverse 5′-AAGAGG CAGTCGAAGCTCTC-3′.

Plasmid construction

The construction of wild-type HBV 1.4 genome length, pTRE-HB-wt, was described previously [25]. The nucleotide sequence of the cloned HBV genome was deposited into GenBank AB206817. The HBc and HBx genes, amplified from pTRE-HB-wt, were cloned into pcDNA3 and p3xFLAG-CMV10 vectors and designated pcDNA-HBc and p3FLAG-HBx, respectively. The human miR-1231 precursor expression plasmid (HmiR0554-MR04) and the control miRNA plasmid (CmiR0001-MR01), which was a miRNA-scrambled control clone, were commercially produced (GeneCopoeia™, Rockville, MD, USA).

Transfection of HepG2 cell lines with the plasmids

The HBV expression plasmid was transfected into HepG2 cells with control miRNA or miR-1231 expression plasmid using *Trans*IT-LT1 (Mirus, Madison, WI, USA) reagent according to the manufacturer's instructions. 24–48 h after transfection, core-associated HBV DNA and HBV RNA were extracted and quantified by real-time PCR or RT real-time PCR, respectively [28]. For identifying targets within the HBV genome, HBc or HBx expression plasmids were transiently transfected with miR-1231 expression plasmid into HepG2 cells. Twenty-four hours after transfection, the cells were harvested to perform Western blot analysis.

Analysis of HBV replication intermediates

Quantitative analysis of HBV replication intermediates was performed as described previously [29]. The HBV-specific primers used for amplification were 5′-TTTGGGCATGGAC-ATTGAC-3′ and 5′-GGTGAACAATGTTCCGGAGAC-3′. The lower detection limit of this assay was 300 copies.

Western blot analysis

Cell lysates, prepared with RIPA like buffer [50 mM Tris-HCl (pH 8.0), 0.1% SDS, 1% NP-40, 150 mM sodium chloride, and 0.5% sodium deoxycholate] containing protease inhibitor cocktail (Sigma-Aldrich, Tokyo, Japan), were separated on 5–20% (wt/v) SDS-polyacrylamide gels (Bio-Rad Laboratories, Inc., Tokyo, Japan). Immunoblotting was performed with anti-FLAG M2 monoclonal antibody (Sigma-Aldrich) or anti-HBV core monoclonal antibody HB91 (Advanced Life Science Institute Inc., Saitama, Japan) or anti β-actin monoclonal antibody (Sigma-Aldrich) followed by incubation with horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin (GE Healthcare, Buckinghamshire, UK). Expression of HBc protein was quantified based on the densities of the immunoblot signals by Quantity One® software (Bio-Rad Laboratories, Inc.).

RESULTS

miRNA expression alterations associated with HBV infection

To analyse the influence of HBV infection on human hepatocytes, miRNA microarray expression profiles were compared between groups A (mice without HBV infection) and B (mice with HBV infection). Among the 900 miRNAs on the microarray, 10 miRNAs showed a more than 2.0-fold change with HBV infection. Five of the 10 miRNAs were upregulated, and the remaining five were downregulated (Fig. S1). Because immunity was severely suppressed in the chimeric mice, changes in miRNA expression are thought to be closely associated with HBV infection, and the upregulated miRNAs might play a protective role against HBV infection. Thus, we focused on these 5 upregulated miRNAs.

Comparison of expression of the 5 upregulated miRNAs in human liver tissues

To verify the microarray results, quantitative analysis of miRNAs was performed using liver tissues from the chimeric mice. Three of the 5 miRNAs were significantly upregulated by HBV infection (Fig. 1). Expression changes in the other 2 miRNAs (hsa-miR-675 and hsa-miR-1908) showed a similar trend but were not significant due to individual variation. Therefore, further quantitative analysis was performed using human liver tissues. Nine liver tissue samples were obtained from patients with chronic hepatitis B ($N = 3$), chronic hepatitis C ($N = 2$) or alcoholic liver dysfunction ($N = 4$), and miRNA expression levels were compared. Expressions of all miRNAs except for miR-886-5p were significantly higher in liver tissues with chronic hepatitis B than in those with other liver diseases (Fig. 2).

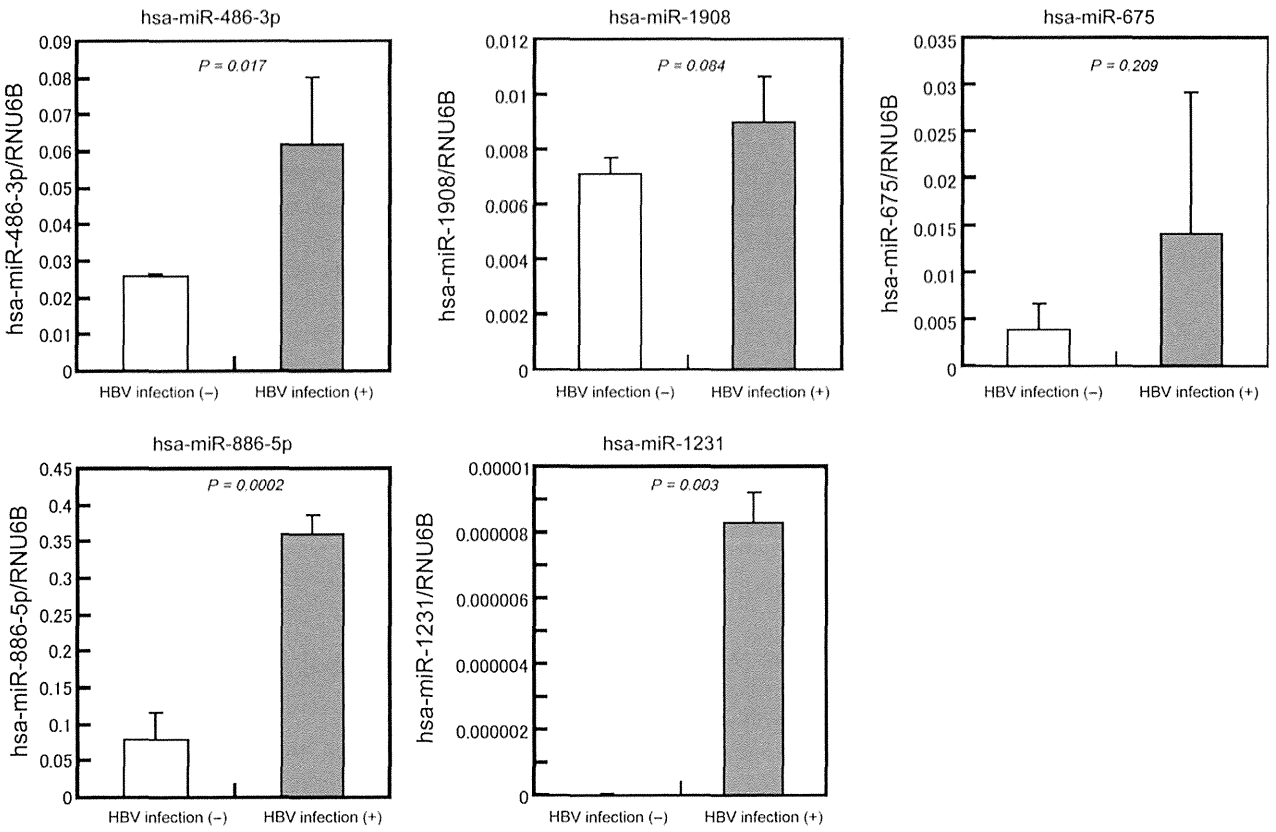

Fig. 1 Upregulation of microRNA by HBV infection. Signal intensities of five upregulated miRNAs were compared between HBV-infected and noninfected mouse livers. All 5 miRNAs were significantly upregulated by HBV infection. *P* values were calculated by the Mann–Whitney *U*-test.

Associations between signalling pathways and the upregulated miRNAs

To analyse the influence of miRNA upregulation on signalling pathways, pathway analysis was performed. However, there are several obstacles in analysing the association between miRNAs and pathways, such as the lack of reliable miRNA target prediction algorithms, differences in the results among target prediction systems, and the small number of validated target genes. To improve the reliability of the targets, we performed the pathway analysis in combination with four prediction tools (miRWalk, TargetScan, miRanda and miRDB). After this operation, 482 targets were predicted (hsa-miR-1231: 203 targets, hsa-miR-1908: 3 targets, hsa-miR-486-3p: 251 targets, hsa-miR-675: 25 targets), and these 482 targets were submitted to the PANTHER classification system for pathway analysis. As shown in Table 1, several immunological pathways such as inflammation mediated by chemokine and cytokine signalling pathway, and the interleukin signalling pathway were identified, but it was difficult to identify characteristic pathways.

Suppression of HBV replication with miR-1231 overexpression

Because hsa-miR-1231 was most the highly upregulated among these four miRNAs and had a high homology with the HBV genome, we focused on hsa-miR-1231. Using GENETYX ver. 8.2.1 (GENETYX, Tokyo, Japan), the hsa-miR-1231 sequence was predicted to hybridize at the HB core and X regions of the HBV genome (Fig. 3). To analyse the influence of hsa-miR-1231 on HBV replication, changes in HBV replication intermediates were evaluated using an *in vitro* HBV replication model. As shown in Fig. 4a, HBV replication intermediates were significantly reduced by hsa-miR-1231 overexpression, and the suppression of HBV RNA and HBc proteins were also observed by hsa-miR-1231 overexpression (Figs 4b,c). Thus, HBV replication was concluded to be inhibited by hsa-miR-1231 at the post-transcriptional level.

Specific regulation of HBV-related protein levels with hsa-miR-1231 overexpression

As the preceding results indicated an association between the production of HBV-related protein or HBV particles and

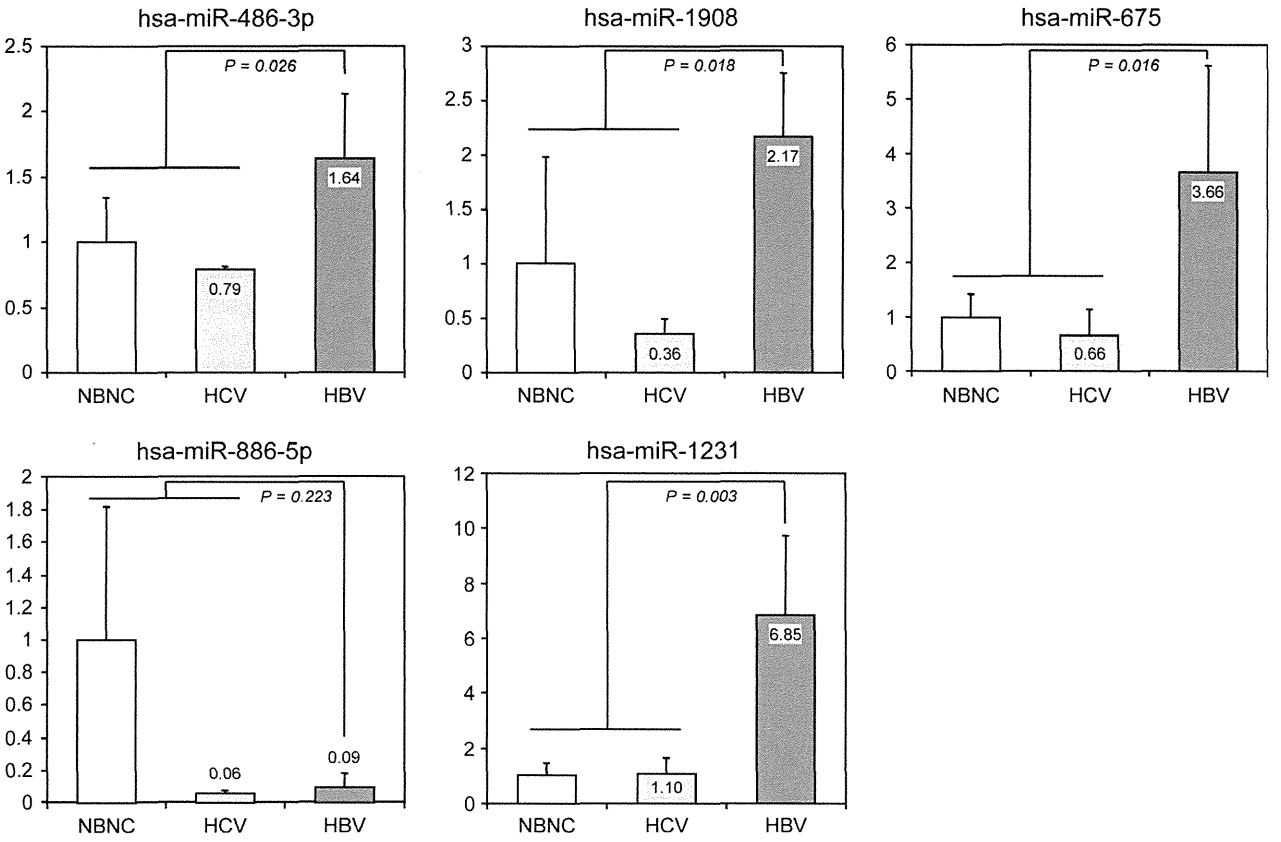

Fig. 2 Comparison of microRNA expression in clinical liver tissues. Quantification of miRNAs was performed by real-time PCR using nine human liver tissues obtained from the patients who had chronic hepatitis B (N = 3), C (N = 2) or alcoholic liver dysfunction (N = 4). Expression levels of four miRNA were significantly higher in the chronic hepatitis B patients than in those of other liver diseases. The results of miR-886-5p levels were not statistically significant. P values were assessed by Mann–Whitney U-test.

hsa-miR-1231 expression, further analysis was performed to identify the region hybridized by hsa-miR-1231. As shown in Fig. 5, HBc protein expression was remarkably reduced by hsa-miR-1231 expression, but no reduction in HBx protein was observed. These results indicate that hsa-miR-1231 might interact with HBV core mRNA and suppress HBV replication by inhibiting HBV core protein production.

The effects of hsa-miR-1231 on the expression of interferon-stimulated genes

Alternatively, hsa-miR-1231 might suppress HBV replication through activation of the interferon signalling pathway. We thus evaluated mRNA expression of interferon-stimulated genes (ISGs) with or without hsa-miR-1231 overexpression. None of the examined ISGs (MxA, PKR, OAS-1 and SOCS1) were regulated by hsa-miR-1231 expression (Fig. S3). These results suggest that hsa-miR-1231 suppresses HBV replication at the post-transcriptional level but not through the activation of interferon signalling.

DISCUSSION

Previously, we have demonstrated that human hepatocyte chimeric mice can be chronically infected with hepatitis B and C viruses [25,30,31]. This mouse model facilitates analysis of the effect of viral infection under immunodeficient conditions. In the present study, we performed miRNA array analysis using this mouse model and obtained miRNA expression profiles reflecting the direct influence of HBV infection on human hepatocytes. Furthermore, we found a novel mechanism for HBV replication mediated by hsa-miR-1231.

To avoid contamination with mouse tissue, human hepatocyte chimeric mice were used in which liver tissue was largely (>90%) replaced by human hepatocytes. Although it is feasible to use microarray analysis in this chimeric mouse model [32], signals from miRNA array analysis may be influenced by cross-hybridization with mouse miRNA from a small amount of contaminated mouse-derived cells because of the high homology between the human and mouse genomes. To compensate

Table 1 Pathways associated with the 4 miRNAs upregulated by HBV infection

Pathway	Number of gene hits	Ratio of genes %
Inflammation mediated by chemokine and cytokine signalling pathway (P00031)	11	2.60
Angiogenesis (P00005)	10	2.30
Integrin signalling pathway (P00034)	9	2.10
Gonadotropin releasing hormone receptor pathway (P06664)	7	1.60
Wnt signalling pathway (P00057)	7	1.60
Parkinson disease (P00049)	7	1.60
EGF receptor signalling pathway (P00018)	7	1.60
Alzheimer's disease–presenilin pathway (P00004)	6	1.40
PDGF signalling pathway (P00047)	6	1.40
B-cell activation (P00010)	6	1.40
Interleukin signalling pathway (P00036)	5	1.20
Huntington disease (P00029)	5	1.20
FGF signalling pathway (P00021)	5	1.20
Cadherin signalling pathway (P00012)	5	1.20
VEGF signalling pathway (P00056)	4	0.90
Toll receptor signalling pathway (P00054)	4	0.90
T-cell activation (P00053)	4	0.90
Ras pathway (P04393)	4	0.90
Heterotrimeric G-protein signalling pathway-Gi alpha and Gs alpha-mediated pathway (P00026)	4	0.90
Endothelin signalling pathway (P00019)	4	0.90

for contamination, mice that were negative for HBV infection were set up as negative controls.

Only 5 miRNAs showed more than 2.0-fold upregulation with HBV infection under miRNA array analysis using chimeric mouse livers (Fig. S1). Comparing these results with our previous study using patient sera, only hsa-miR-486-3p showed a similar change in sera from chronic hepatitis B patients, but no upregulation of the other 4 miRNAs was observed [15]. These results suggest that miRNA expression in sera from chronic hepatitis B patients might be regulated not only by HBV infection but also by human immune responses. In addition, it might be difficult to analyse changes in expression of miRNAs that are expressed at low levels in human hepatocytes, including hsa-miR-1231, using human serum.

To identify targets of miR-1231, we searched using four prediction systems. Although 632 target genes were identified (data not shown), and involvement of a number of pathways was indicated (Table S1), critical targets associated with human immunity or HBV replication could not be identified. Interferon signalling was also a potential mechanism of HBV suppression, but several ISG mRNAs were not induced by hsa-miR-1231 overexpression *in vitro* (Fig. S2). Therefore, we concluded that hsa-miR-1231 does not suppress HBV replication via interferon signalling.

To examine the possibility that miR-1231 directly regulates HBV replication by interacting with HBV-related mRNAs, we searched for hsa-miR-1231-binding motifs and found two candidate sequences in the HBV core and X genes (Fig. 3). As shown in Fig. 5, one target in the HBV core region could hybridize with hsa-miR-1231, and HBc expression was found to be suppressed by hsa-miR-1231 overexpression. The hsa-miR-1231-binding motif in the HBV core region was conserved in more than 90% of the HBV sequences in GenBank, regardless of HBV genotype (data not shown). Thus, we speculate that hsa-miR-1231 binds to the HBc target region and suppresses HBc production to inhibit HBV replication.

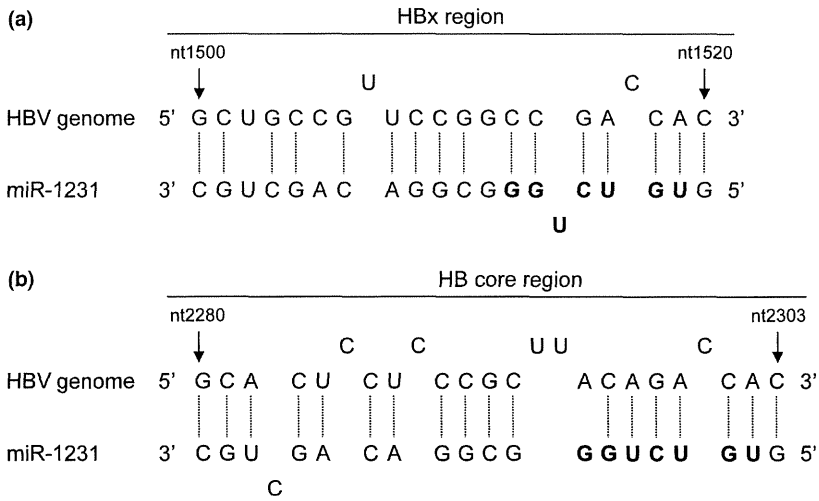

Fig. 3 Alignment of hsa-miR-1231 to HBV genome. Alignment of hsa-miR-1231 to the HBV genome was performed. MiR-1231 sequence was predicted to hybridize at the HBV core (**a**) and HBV X region (**b**).