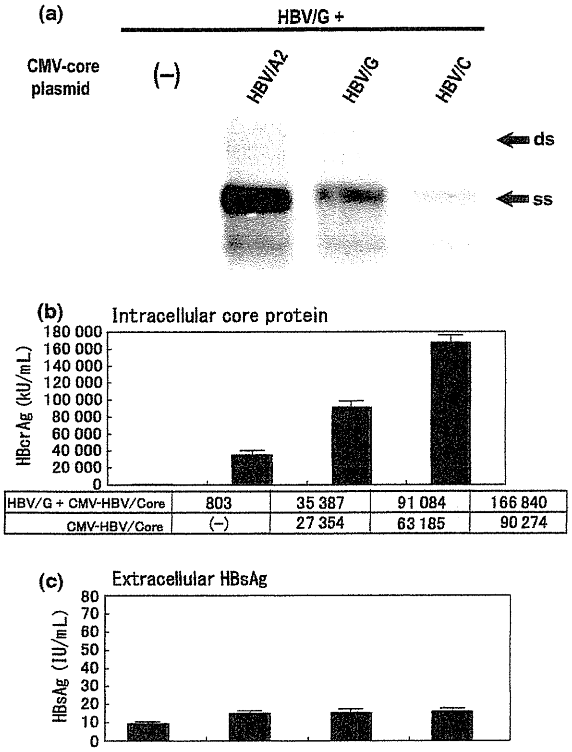

HBV/G + CMV-HBV/Core	803	35 387	91 084	166 840
CMV-HBV/Core	(–)	27 354	63 185	90 274

Fig. 4 (a) Southern blot analysis for the expression of intracellular HBV/G DNA during co-transfection with the three core protein expression constructs for each genotype (HBV/G, HBV/A2 and HBV/C) driven by the CMV promoter, which produced core protein in the absence of a preceding ε signal. (b) Intracellular expression of core protein. (c) The expression of HBsAg in the culture supernatant.

levels of core protein (HBcrAg) expression were observed for the HBV/G/A2-CP and HBV/G/A2-CP/core-transfected cultures, which was in sharp contrast with the low levels observed in the HBV/G/A2-core and the wild-type HBV/G cultures. The discrepancy between viral replication and core production of the HBV/G/A2-CP clone might indicate insufficient virion assembly. Figure 5c shows the HBeAg levels measured in culture supernatants. The expression of HBeAg was the highest in the HBV/G/A2-CP/core culture distantly followed by that in the HBV/G/A2-core culture. The HBV/G/A2-CP and wild-type HBV/G clones expressed HBeAg protein at levels close to or below the level of detection. Nevertheless, a high HBcrAg titre was detected in the cell lysate of the HBV/G/A2-CP clone, although its DNA level was as low as that of the wild-type HBV/G clone (Fig. 5a). These results indicated that low replication of HBV/G might be explained by low synthesis of HBV/G core protein due to weak core promoter activity or dysfunction, as well as insufficient virion assembly due to the larger core protein of HBV/G (12-aa unique insertion).

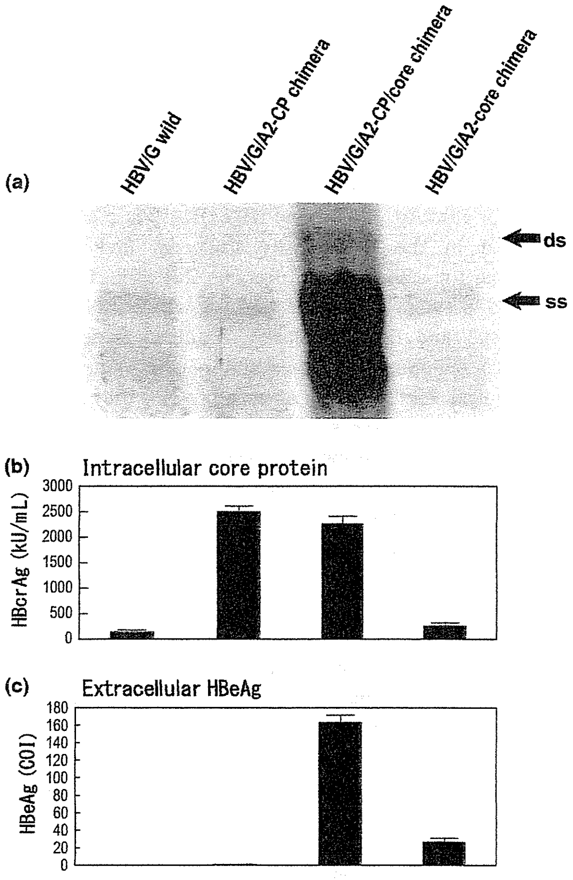

Fig. 5 (a) Southern blot analysis for HBV replication among HBV/G and three chimeric replicating constructs created by recombination of different genomic sections of HBV/G and HBV/A2 (see Materials and methods). The 'HBV/G/A2-CP' clone was a HBV/G-based construct in which the fragment containing the core promoter (CP) region but not the precore or core was replaced by the corresponding sequence from HBV/A2. The 'HBV/G/A2-Core' clone was an HBV/G-based construct in which the section of the precore and core region was replaced with that of HBV/A2. For the 'HBV/G/A2-CP/core' clone, the CP, precore and core region of HBV/G were replaced with that of HBV/A2. (b) Intracellular expression of core protein. (c) Extracellular expression of HBeAg levels detected by a commercial chemiluminescent enzyme immunoassay (mean and standard deviation, *n* = 3).

Dane particles produced by HBV/G during co-transfection with HBV/A2 were packed in HBV/A2 protein

To investigate the effects of HBV/A2 core protein during HBV/G viral assembly, we tried to define whether the Dane particles in B Huh7 cells that had been co-transfected with wild-type HBV/G and the CMV-HBV/A2-core plasmid (source of the Fig. 4 lane 2) contained HBV/A2 or HBV/G core protein. To extract the Dane particles, we employed ultracentrifugation of the culture media through a 10–60% (w/w) sucrose density gradient followed by immunoprecipitation using anti-HBs-coated magnetic beads. We thereby extracted Dane particles, which were then analysed using Western blotting. The obtained fractions were tested for HBcrAg, HBsAg and HBV DNA (Fig. 6a). HBcrAg appeared in the high-density fractions, and its levels peaked in the same fraction (fraction 22) as HBV DNA. As reported previously, the fraction in which the levels of HBV DNA and HBcrAg peaked contained Dane particles [22]. To eliminate contamination of the Dane particles with 'naked' core particles or core protein, they were specifically retrieved from sucrose high-density fraction 22 by means of immunoprecipitation using anti-HBs-coated magnetic beads. The media supernatant obtained from the culture of cells that had been subjected to CMV-HBV/A2/core clone monotransfection was also subjected to sucrose gradient ultracentrifugation using the same protocol. Sucrose high-density fraction 22, in which the HBcrAg concentration peaked, presumably contained 'naked' core particles or core protein (Fig. 6b). This fraction was collected and processed in the same manner via anti-HBs-coated magnetic bead separation and was used as negative control for this procedure (Fig. 6c, lane 4). To discriminate between HBV/G and HBV/A2 core proteins on Western blot analysis probed with anti-HBc antibody, we employed cell lysates produced from cells that had been transfected with the wild-type HBV/G clone and those produced with the HBV/A2 clone as controls. As can be seen on the Western blotting image (Fig. 6c), HBV/G-transfected cells (lane 1) produced larger proteins than the HBV/A2-transfected cells (lane 2), which can be explained by the 12-aa insertion in the core protein of HBV/G coded by its 36-nt unique insertion. Interestingly, the most saturated band associated with the Dane particles produced by HBV/G that had been co-transfected with CMV-HBV/A2/core (lane 3) was the same size as that for HBV/A2, suggesting that HBV/G competitively produces Dane particles consisting of HBV/A2 core protein during virion assembly.

DISCUSSION

HBV/G was first isolated in 2000 in France and the USA and was later found in Thailand, Japan and Mexico, indicating its global dissemination and association with specific risk groups, such as injection drug users (IDU) and men who had sex with men (MSM) [25]. Studies have also demonstrated that throughout the world, HBV/G strains possess unprecedented genetic homology and are mainly detected during co-infection with another genotype that is endemic in the area. Further studies have suggested that genotype G represents a 'replication-defective' variant of HBV that requires co-infection with another genotype to establish a persistent infection. We and others have reported *in vitro* and *in vivo* experimental evidence of this HBV/G dependence [13–15]. The unique 36-nt insertion within core coding region increases core protein level and genome replication in genotype G but impairs replication, not core protein expression, in other genotypes [14]. These results strongly suggest the 36-nt/12-aa insertion has pleiotropic effects on core protein expression, genome replication and virion secretion [14]. To obtain clues about the mechanism by which genotype G works in combination with genotype A to effect its replication, we performed co-transfection experiments using Huh7 cells.

Using HBV/A2 viral proteins expressing plasmids, we determined that a HBV/A2 plasmid that selectively expressed core protein was capable of increasing the replication of the wild-type HBV/G (Fig. 3a). The replication of HBV/G during co-transfection was not affected by other viral elements of HBV/A2 because of the presence of the 'packaging-negative mutation' in the epsilon-coding region and stop codons preventing the translation of the other three viral proteins (the polymerase, surface and X proteins). The specific role of the core protein was further confirmed in experiments with CMV promoter–driven core expressing constructs, in which the core protein alone enhanced HBV/G replication in the absence of HBV pregenomic RNA. Interestingly, co-transfection of HBV/G with the CMV-HBV/A2/core expression construct produced the highest levels of intracellular DNA, even though this combination produced the lowest intracellular core protein level, compared with the CMV-core constructs of the other two genotypes (HBV/G and HBV/C) (Figs 4a,b). The replication of HBV/G was the highest during co-transfection with the CMV-HBV/A2/core expression construct, which agreed with the results of experiments using other genotype (HBV/D, HBV/B1) CMV-core constructs (data not shown). Thus, the core protein of HBV/A2 was confirmed to play an important role in upregulating HBV/G replication and performed this task more efficiently than the other genotypes. These experimental results might explain why HBV/A is the genotype that is most frequently found in co-infections with HBV/G [12,26].

Moreover, HBV/G core protein overexpression achieved by the co-transfection of HBV/G with CMV-HBV/G/core did not enhance replication, suggesting that HBV/G core protein is functionally defective; that is, results in insufficient viral packaging. To investigate the functional defect in the HBV/G core protein, we exchanged the core gene of the wild-type HBV/G for the corresponding gene of HBV/A2 (HBV/G/A2-core); the introduction of the HBV/A2 core promoter together with core coding region into the HBV/G genome (HBV/G/A2-CP/core) significantly enhanced replication. However, the replication of the recombinant construct (HBV/A2 core coding region; HBV/G/A2-core) did not differ from that of the wild-type HBV/G, suggesting that the replacement of HBV/G/A2-core alone was not

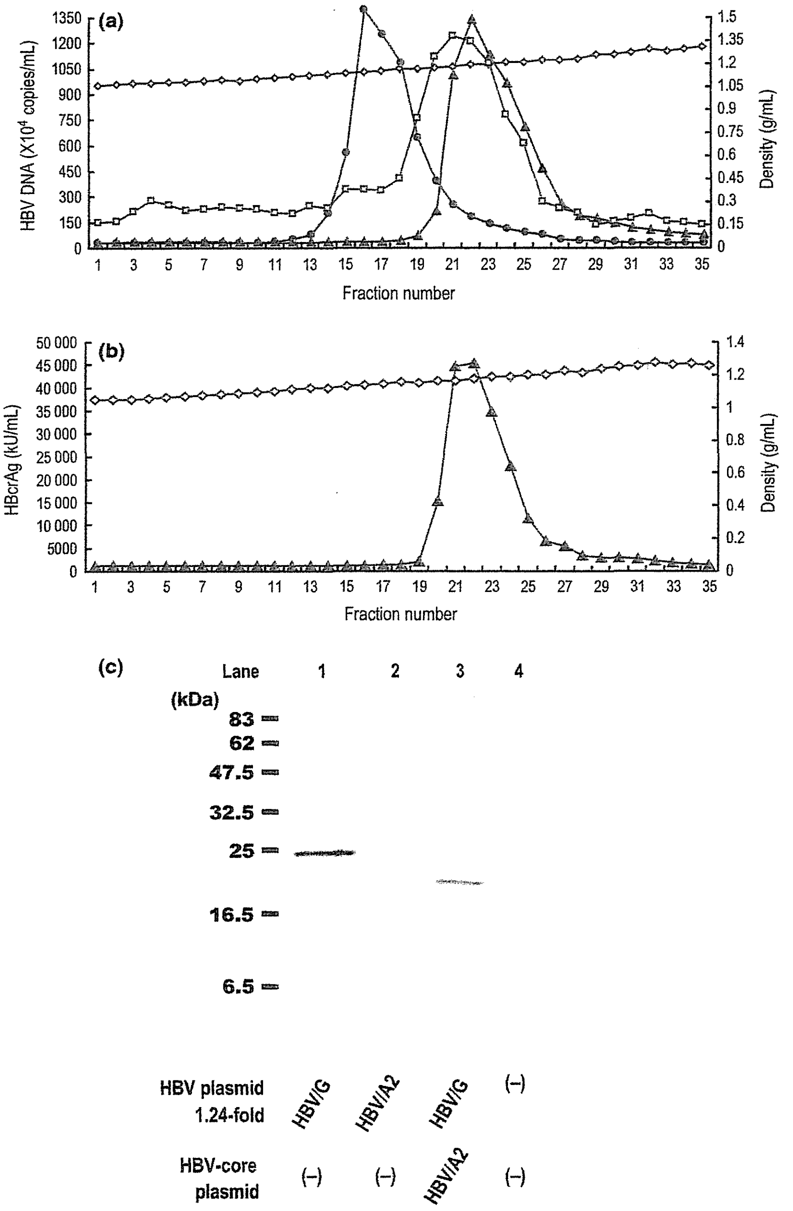

Fig. 6 (a) Sucrose gradient analysis of the culture media of Huh7 cells that had been co-transfected with wild-type HBV/G and the CMV-HBV/A2-core plasmid. It was subjected to ultracentrifugation through a 10–60% (w/w) sucrose density gradient. Density of each fraction is shown as a line with diamond symbols. Fractions were diluted 10-fold and tested for HBsAg (●) (IU/mL), HBcrAg (▲) (KU/mL) and HBV DNA (□) (10^4 copies/mL). (b) Sucrose gradient analysis of culture supernatant obtained from the cells that were subjected to CMV-HBV/A2/core monotransfection using the same protocol. (c) Western blot analysis for HBV core protein was probed by anti-HBc antibody. HBV/G/core and HBV/A2/core were obtained from cell lysates that were transfected with the wild-type HBV/G clone and the wild-type HBV/A2 clone, respectively. The 'HBV/G + CMV-HBV/A2/core' was obtained from sucrose high-density fraction 22 (Fig. 6a) that had been co-transfected with wild-type HBV/G and the CMV-HBV/A2-core plasmid by means of immunoprecipitation using anti-HBs-coated magnetic beads.

enough for viral replication because the core promoter of HBV/G was not capable of generating sufficient amounts of core protein to enhance HBV replication. As well, an HBV/G/A2-CP construct containing the HBV/A core promoter region in the context of the wild-type HBV/G genome did not enhance replication, even though its core protein production was significantly increased (Figs 5a,b). Although it was previously reported that the 36-nt insertion of the HBV/G core gene was required for both efficient core protein expression and HBV/G replication [13], in this study, the discrepancy between viral replication and core production of the HBV/G/A2-CP clone might indicate insufficient virion assembly due to the larger core protein of HBV/G (12-aa unique insertion). *Trans*-complementation experiments carried out by Gutelius *et al.* [14] demonstrated an association between enhanced core protein level and reduced replication capacity only when the core and polymerase proteins are expressed from the same RNA template. Thus, it was indicated that HBV/G itself could not replicate sufficiently due to a defect in its core protein and/or the core promoter of HBV/G.

Finally, we investigated whether HBV/G utilises the core protein of HBV/A2 for virion packaging. Dane particles obtained from the culture supernatants of cells that had been co-transfected with HBV/G and CMV-HBV/A2/core were assessed by Western blotting, and it was found that the Dane particles of HBV/G contained HBV/A core proteins. Thus, it was implied that HBV/G replication is enhanced by the core protein of HBV/A because it is more suitable for virion packaging than its own core protein, suggesting that the core protein of HBV/A is a key element enhancing the replication of HBV/G during co-infection. Interestingly, our experiments demonstrated that there were large differences in core protein expression among the CMV-core constructs of different genotypes, despite the fact that all of the CMV-core constructs had the same CMV promoter (Fig. 4b). In a previous report, it was speculated that the core protein binds to its own mRNA to influence protein translation [13]. For example, dihydrofolate reductase protein has been found to downregulate its own translation by binding to cognate mRNA [27,28]. Therefore, we predict that the core protein of HBV/A2 enhances HBV/G replication by affecting viral promoters or transcription in addition to its role in virion packaging.

In conclusion, enhanced replication of HBV/G requires the HBV/A2 core protein during co-infection with HBV/A2. Our findings provide a possible explanation that the core protein of HBV/A2 is more suitable for virion packaging rather than that of HBV/G, and the replication of HBV/G occurs at a very low level, which may be due to defects in its core protein functions and/or core promoter activity. Further experiments are warranted to clarify the detailed roles of the enhanced HBV/G replication by co-infection with the other genotype and the clinical manifestation of HBV/G infection.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST STATEMENT

None declared.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Data S1. Plasmid construct of HBV/G.

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ORIGINAL ARTICLE

RASSF1A methylation indicates a poor prognosis in hepatoblastoma patients

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Abstract
Purpose The RAS association domain family protein 1 (RASSF1A) is known to be frequently inactivated by promoter hypermethylation in cancers. This study investigated the association of *RASSF1A* methylation with clinical outcomes in hepatoblastoma patients and whether it is correlated with the histological phenotype of hepatoblastoma tumors.
Methods Seventy-four hepatoblastoma tumors were obtained from patients enrolled in the Japanese study group for pediatric liver tumor protocol-2. From nine formalin-fixed, paraffin-embedded specimens, we extracted DNA by dissection under a light microscope. We examined the methylation status of the *RASSF1A* promoter region by bisulfite pyrosequencing.
Results Twenty-five (33.8 %) hepatoblastoma tumors were classified as having methylated *RASSF1A*. The *RASSF1A* methylation was significantly associated with metastatic tumors and a poor prognosis. Despite the complete resection, five pretreatment extent of disease II tumors showed recurrence or distant metastasis postoperatively. Among these cases, four tumors were found to show *RASSF1A* methylation. When compared to histologically different types of cell, *RASSF1A* methylation values in samples of the normal liver, fetal type, and embryonal type, were significantly elevated in ascending order.
Conclusions We confirmed that *RASSF1A* methylation is a significant prognostic indicator in hepatoblastomas, and it may become a promising molecular marker to stratify patients into appropriate risk groups.

Keywords Hepatoblastoma · *RASSF1A* methylation · Prognostic marker

Introduction

Hepatoblastoma is the most common malignant neoplasm of the liver in children. Despite the progress of therapy, the mortality rate remains at 35–50 % in high-risk patients, such as those with extrahepatic tumors, macroscopic invasion of large vessels, or distant or lymph node metastases [1]. Complete surgical resection or liver transplantation and mainstream treatment with cytotoxic drugs are essential for achieving a favorable long-term outcome. To

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improve the mortality of hepatoblastoma patients in advanced stages, innovative treatment and potent prognostic markers for better therapy planning are needed.

Histologically, hepatoblastoma tumors are classified as wholly epithelial, or mixed epithelial and mesenchymal types. In the wholly epithelial type, there are two major subtypes, the fetal subtype and the mixed fetal and embryonal subtype [2]. Fetal and embryonal components often develop in combination, that is, heterogeneity is present. The RAS association domain family protein 1 (RASSF1A) is known to be frequently inactivated by promoter hypermethylation in many adult and childhood cancers [3]. We previously reported that *RASSF1A* methylation was correlated with a poor outcome by multivariate analysis, and suggested that *RASSF1A* may be a promising molecular–genetic marker predicting the treatment outcome in hepatoblastoma patients [4]. The association between the histological type and *RASSF1A* methylation is ambiguous despite the fact that the histologic features are associated with different prognoses; a pure fetal histology is favorable and small cell undifferentiated and macrotrabecular histologies are unfavorable [1]. Therefore, the current study was undertaken to determine the association with histological types by examining each type of hepatoblastoma cell dissected separately.

In this study, we investigated the methylation status of *RASSF1A* in hepatoblastoma tumors by bisulfite pyrosequencing, which is a rapid and accurate method to quantify DNA methylation. We analyzed the results with regard to patients' clinicopathological characteristics and prognosis, and evaluated its association with the histological phenotype on the basis of the epigenetic alteration of hepatoblastomas.

Methods

Patients and samples

Seventy-four hepatoblastoma patients with a median age of 18 months underwent tumor resection and partial hepatectomy between December 1999 and December 2008 at the institutions of the Japanese Study Group for Pediatric liver Tumors (JPLT). All patients were treated in the JPLT-2 study [5]. The extent of disease was determined at the time of initial biopsy or resection according to the classification of the pretreatment extent of disease (PRETEXT) staging system [6]. Metastatic tumors were found in 15 % of the patients (Table 1). The 5-year overall survival and event-free survival rates were 86.7 and 73.4 % for the 74 patients, respectively.

The DNA samples of the 74 hepatoblastoma tumors were supplied by JPLT, and they were extracted from fresh-frozen specimens. Furthermore, formalin-fixed, paraffin-embedded (FFPE) specimens were obtained from nine patients referred to our institution for surgical treatment between 1995 and 2011. We extracted DNA from different types of cell: fetal type, embryonal type, and normal liver, by dissection under a light microscope in order to avoid contamination with normal tissues and mesenchymal components. The ethics committee of our institution approved the study protocol, and signed

Table 1 Clinicopathological factors and *RASSF1A* methylation status in 74 patients with hepatoblastoma

Clinicopathological factors		No. of patients	*RASSF1A*		p value[1]
			Methylated	Unmethylated	
Sex	Male	45	14	31	0.360
	Female	29	11	18	
Age at diagnosis	<365 days	22	0	22	0.000064
	≥365 days	52	25	27	
PRETEXT	I	5	1	4	0.319
	II	27	7	20	
	III	29	10	19	
	IV	13	7	6	
Metastasis	No	63	15	48	0.000039
	Yes	11	10	1	
Histological type	Fetal	28	9	19	0.508
	Mixed fetal and embryonal	40	14	26	
	Unknown	6			
Outcome	Alive	63	15	48	0.000039
	Dead	11	10	1	

[1] Fisher's exact test

informed consent was obtained in all cases by local physicians of the participating institutions.

Evaluation of *RASSF1A* methylation level

We examined the methylation status of the *RASSF1A* promoter region by bisulfite pyrosequencing, which can calculate the level of methylation at each CpG site in samples after bisulfite treatment. Genomic DNA (500 ng) was modified with sodium bisulfite using an EpiTect bisulfite kit (Qiagen, Netherlands). Bisulfite pyrosequencing was carried out as described previously [7]. After PCR, the biotinylated PCR product was purified, made single-stranded, and used as a template in the pyrosequencing reaction. Briefly, the PCR products were bound to streptavidin sepharose beads HP (Amersham Biosciences, USA), after which beads containing the immobilized PCR product were purified, washed, and denatured using a 0.2 mol/L NaOH solution. After the addition of 0.3 μmol/L sequencing primer to the purified PCR product, pyrosequencing was carried out using a PSQ96MA system (Biotaga) and Pyro Q-CpG software (Biotage). The mean value of the methylation levels at two CpG sites in the *RASSF1A* promoter region was calculated. Primer sequences used in this study were as follows: forward, GAAGGAGGGAAGGAAGGGTAAG; reverse, GCCTCC CCCAAAATCCAA; sequencing primer, TTGTATTTAG GTTTTTATTG.

Statistical analysis

Correlations between the *RASSF1A* methylation status and clinicopathological factors were analyzed using the Fisher's exact test. Survival curves were constructed according to the methods of Kaplan and Meier, and comparisons of survival curves were performed with a log-rank test. One-way ANOVA followed by Student's *t* test with Bonferroni correction was used to compare methylation values of histologically different types of cell. A *P* value <0.05 was considered statistically significant.

Results

RASSF1A methylation status in 74 hepatoblastomas

The average of the *RASSF1A* methylation values in 74 hepatoblastoma tumors was 25.8 % (2.0–74.8 %). We performed the ROC analysis to determine the cutoff value of the *RASSF1A* methylation and adopted a cutoff value of 36.2 % in this study (Fig. 1). On the basis of this cutoff value, 25 (33.8 %) tumors were classified as having methylated *RASSF1A*, and the sensitivity and specificity for the patients having an event postoperatively were 77.8 and 80.4 %, respectively. There was only one patient who died in those with tumors with *RASSF1A* unmethylated (Fig. 2).

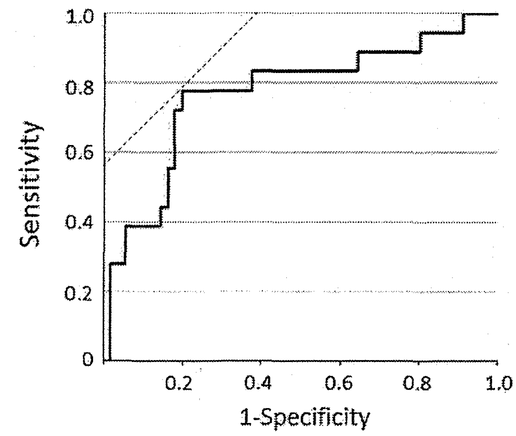

Fig. 1 ROC analysis to determine the cutoff value of the *RASSF1A* methylation

Associations between clinicopathological factors and *RASSF1A* methylation status

We evaluated the associations between the clinicopathological factors and *RASSF1A* methylation status in 74 patients. As Table 1 shows, there were no patients aged under 1 year who had a tumor with *RASSF1A* methylated; however, about half of the patients aged over 1 year were found to have a tumor with *RASSF1A* methylated. 10 of 25 patients (40 %) with a tumor with *RASSF1A* methylated suffered from metastatic tumors, although there was only one patient with metastasis in those with a tumor with *RASSF1A* unmethylated. This demonstrated that age at diagnosis and metastatic tumors were significantly associated with *RASSF1A* methylation. In Kaplan–Meier analyses, the patients with a tumor with methylated *RASSF1A* were significantly associated with a poor outcome: the 5-year overall survival and event-free survival rates were 63.6 and 35.5 %, respectively (Fig. 3).

The *RASSF1A* methylation was detected in 1 of 5 PRETEXT I tumors and 7 of 27 PRETEXT II tumors (Table 1). Despite complete resection, five PRETEXT II tumors showed recurrence or distant metastasis postoperatively, and three patients died. Among these cases, four tumors were found to have *RASSF1A* methylated.

Associations between histological types and *RASSF1A* methylation status

Next, we evaluated whether *RASSF1A* methylation is associated with the histopathological subtypes. Four of the

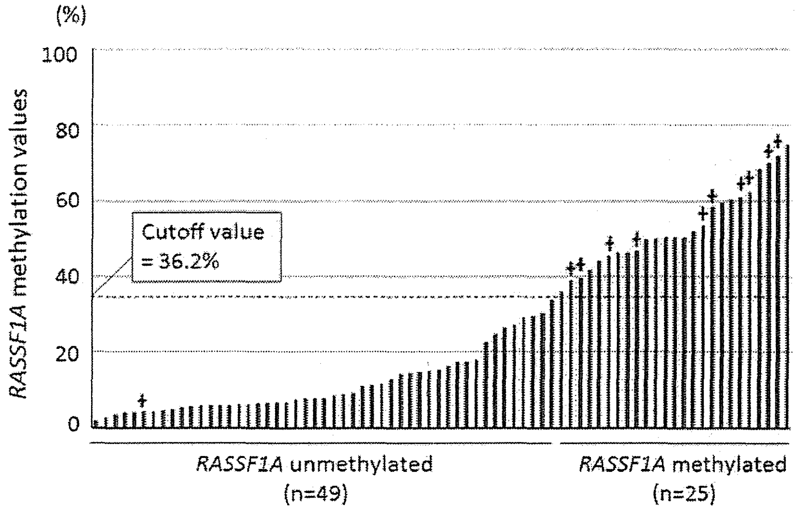

Fig. 2 *RASSF1A* methylation values in 74 patients with hepatoblastoma. *Plus* indicates the patient who died of the disease

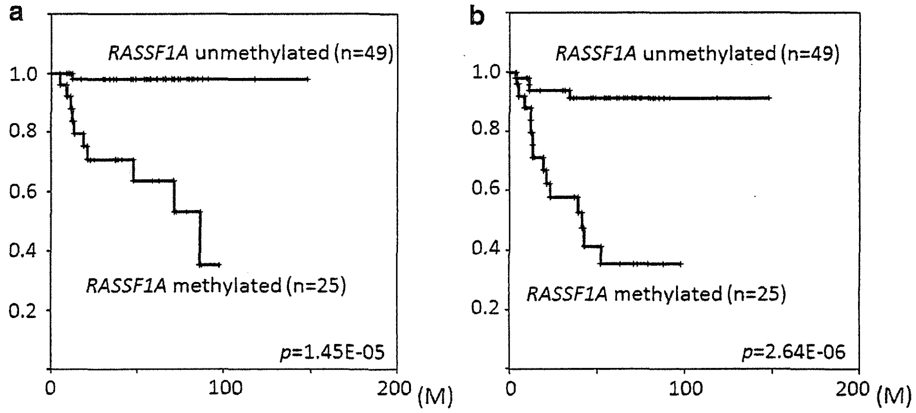

Fig. 3 **a** Overall survival curves and **b** event-free survival curves for hepatoblastoma patients classified by the methylation status of *RASSF1A*

nine tumors in FFPE specimens were classified pathologically into the mixed fetal and embryonal subtype, and DNA was extracted from the tumor cells of each subtype and the normal liver. The other five tumors were the pure fetal subtype, so DNA was extracted from fetal hepatoblastoma and normal liver cells.

The mean methylation values of *RASSF1A* in nine normal liver, nine fetal type, and four embryonal type samples were 8.6, 19.7, and 42.2 %, respectively (Fig. 4). This showed that fetal and embryonal types were significantly associated with *RASSF1A* methylation. Moreover, *RASSF1A* methylation values in samples of the normal liver, fetal type, and embryonal type were elevated in ascending order, when compared to each type of cell taken from the same patient.

Discussion

Complete surgical resection and chemotherapy including cisplatin remains the mainstay of hepatoblastoma treatment. In contrast to standard-risk patients, of who over 90 % achieve long-term survival, the treatment of patients with unrespectable and metastatic disease remains a challenge. Furthermore, there seems to exist a group of patients with high-risk tumors in PRETEXT II, which have a poorer prognosis despite the high-level resectability [5]. First, this study demonstrated that *RASSF1A* methylation was significantly associated with metastatic tumors and a poor prognosis, and that *RASSF1A* methylation may be useful to identify high-risk tumors in PRETEXT II. Secondly, *RASSF1A* methylation was also shown to be histologically correlated with different types of tumor. These findings suggest that *RASSF1A* may be a promising molecular marker to stratify the patients into appropriate risk groups in order to develop better therapeutic approaches.

The present factors predicting the outcome in hepatoblastoma patients include the age at diagnosis, histology, local growth pattern of the tumor, presence of metastasis, and the level of alpha–feto protein [1]. Chromosomal gains of 2q, 8q, and 20, high-level expression of *TERT* or *PLK1*, *CTNNB1* mutation, and *RASSF1A* methylation were shown

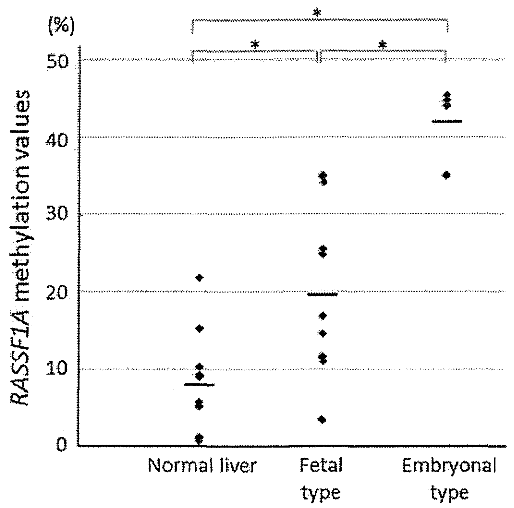

Fig. 4 *RASSF1A* methylation value for each sample is plotted by histological type. *Horizontal bars* indicate the mean value of each type. The *p* values were calculated by one-way ANOVA followed by Student's *t* test with Bonferroni correction (*$p < 0.017$)

to be molecular–genetic markers predicting a poor outcome [4, 8, 9]. We have been focusing on *RASSF1A* methylation in hepatoblastomas, since it has been proven to be an independent prognostic factor by multivariate analysis [4]. RASSF1A inhibits tumor formation by apoptosis, and regulates microtubule dynamics and mitotic arrest via multiple effectors. By dysregulation of the Ras-signaling pathway, *RASSF1A* methylation is correlated with poor differentiation and vascular invasion of cancer cells, and an unfavorable outcome [10]. In child cancers, *RASSF1A* methylation was shown to be associated with a poor outcome in neuroblastoma and Wilms tumor [11, 12]. In this study, we newly adopted bisulfite pyrosequencing as a tool for methylation analysis because it is a highly effective and practical method and offers higher throughput compared to quantitative methylation-specific PCR used in the previous study [4]. We believe that bisulfite pyrosequencing can be a reliable tool when used in a clinical setting.

Cairo et al. [13] identified a 16-gene signature discriminating tumors with a fairly well-differentiated histology and a favorable prognosis against advanced and poorly differentiated tumors with a dismal outcome. In this study, *RASSF1A* methylation was also shown to be correlated with different types of histological phenotype by examining FFPE samples dissected separately. As shown in Table 1, there was no apparent difference in *RASSF1A* methylation values between the fetal subtype and the mixed fetal and embryonal subtype, probably because contamination with different types of tumor cell, normal tissues, and mesenchymal components could not be avoided using fresh-frozen specimens. With these gene signatures based on different phenotypes, the molecular classification of hepatoblastoma tumors may become possible after thorough clinical testing. Although the number of cases in this study is too small to draw definite conclusions, we expect that these molecular markers can be used as prognostic markers predicting the treatment outcome when larger clinical trials are carried out.

In conclusion, *RASSF1A* methylation was significantly associated with metastatic tumors and a poor prognosis in hepatoblastoma patients, and it may be especially useful to identify high-risk tumors in PRETEXT II. The *RASSF1A* methylation was also shown to be correlated with different histological phenotypes. We hope that this work will contribute to establishing a useful molecular marker to predict the outcome of hepatoblastoma patients, stratify the patients efficiently, and develop better therapeutic strategies.

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Conflict of interest The authors who have taken part in this study declare that they do not have anything to disclose regarding funding or any conflict of interest with respect to this manuscript. The first and the corresponding authors are JSPS members, and this abstract was selected for presentation at the 50th Annual Meeting of the Japanese Society of Pediatric Surgeons.

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Wakayama *et al. World Journal of Surgical Oncology* 2013, **11**:259
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RESEARCH

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Surgical management of hepatocellular carcinoma with tumor thrombi in the inferior vena cava or right atrium

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Abstract

Background: The prognosis for advanced hepatocellular carcinoma (HCC) with tumor thrombi in the inferior vena cava (IVC) or right atrium (RA) is poor, and there is no established effective treatment for this condition. Thus study aimed to evaluate the efficacy of surgical resection and prognosis after surgery for such cases.

Methods: Between January 1990 and December 2012, 891 patients underwent hepatectomy for HCC at our institution. Of these, 13 patients (1.5%) diagnosed with advanced HCC with tumor thrombi in the IVC or RA underwent hepatectomy and thrombectomy. Data detailing the surgical outcome were evaluated and recurrence-free and overall survival rates were calculated using the Kaplan-Meier method.

Results: Seven patients had an IVC thrombus and six had an RA thrombus. Extra-hepatic metastasis was diagnosed in 8 of 13 patients. Surgical procedures included three extended right lobectomies, three extended left lobectomies, five right lobectomies, and two sectionectomies. Right adrenal gland metastases were excised simultaneously in two patients. All IVC thrombi were removed under hepatic vascular exclusion and all RA thrombi were removed under cardiopulmonary bypass (CPB). Four patients (30.8%) experienced controllable postoperative complications, and there was no surgical mortality. The mean postoperative hospital stay for patients with IVC and RA thrombi was 23.6 ± 12.5 days and 21.2 ± 4.6 days, respectively. Curative resection was performed in 5 of 13 cases. The 1- and 3-year overall survival rates were 50.4%, and 21.0%, respectively, and the median survival duration was 15.3 months. The 1- and 3-year overall survival rates for patients who underwent curative surgical resection were 80.0% and 30.0%, respectively, with a median survival duration of 30.8 months. All patients who underwent curative resection developed postoperative recurrences, with a median recurrence-free survival duration of 3.8 months. The 1-year survival rate for patients who underwent noncurative surgery and had residual tumors was 29.2%, with a median survival duration of 10.5 months.

Conclusions: Aggressive surgical resection for HCC with tumor thrombi in the IVC or RA can be performed safely and may improve the prognoses of these patients. However, early recurrence and treatment for recurrent or metastatic tumors remain unresolved issues.

Keywords: Hepatocellular carcinoma, Inferior vena cava, Right atrium, Tumor thrombus, Surgery

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Background

Hepatocellular carcinoma (HCC) is a highly malignant tumor with a propensity for invading intrahepatic blood vessels such as the portal vein (PV) or hepatic vein in advanced stages [1]. Further extension of tumor thrombi from any of the three main hepatic veins or the right inferior hepatic vein can give rise to thrombi in the inferior vena cava (IVC) or right atrium (RA) [1-3]. Commonly, the prognosis of HCC patients presenting with IVC or RA thrombosis is extremely poor [4-6], and there is no established management for such cases [4,5,7-17]. Surgical removal of IVC and RA thrombi combined with hepatectomy is the only radical treatment to decrease the risk of systemic metastasis and sudden death due to pulmonary embolism or occlusion of the tricuspid valve with a tumor thrombus [18-20]. However, aggressive surgical resection is not common because the surgical approach to IVC and RA thrombi is considered complicated and hazardous and is applicable only in limited cases with good hepatic reserve [4,6,9,16,17]. Therefore, the efficacy of surgical treatment for HCC with IVC or RA thrombi remains unclear. In this study, we retrospectively investigated the surgical outcomes and prognoses of patients who underwent surgery for HCC with IVC or RA tumor thrombi in a single institution to clarify the safety and efficacy of surgical resection.

Methods

Patients and diagnoses

Between January 1990 and December 2012, 891 patients underwent hepatectomy for HCC at the Department of Gastroenterological Surgery, Hokkaido University, Japan. The diagnosis of HCC was determined by enhanced computed tomography (CT) and magnetic resonance imaging (MRI). IVC or RA thrombi were evaluated by CT (Figure 1). Among those studied, 13 patients (1.5%) diagnosed with advanced HCC and tumor thrombi in the IVC or RA underwent hepatectomy. This study was approved by the Institutional Review Board of the Hokkaido University School of Advanced Medicine.

The mean age at diagnosis was 63.4 years. The most common cause of HCC was hepatitis B virus (HBV) infection (53.8%), followed by hepatitis C virus (HCV) infection (15.4%). A total of 12 (92.3%) patients were male, and according to the Child-Pugh classification, all cases had Child-Pugh class-A disease. Six (46.2%) patients had a single tumor and seven (53.8%) had multiple tumors. The mean main tumor size was 11.8 cm, with nine tumors (69.2%) located in the right lobe and four (30.8%) in the left lobe. Extra-hepatic metastases were detected in eight of thirteen (61.5%) patients (five with lung, two with right adrenal gland and one with mediastinal lymph node metastases). Seven (53.8%) patients had an IVC thrombus and five (46.2%) had an RA thrombus (Table 1).

The tumor thrombus arose from the right hepatic vein in four patients (30.8%), middle hepatic vein in three (23.1%), left hepatic vein in one (7.7%), inferior right hepatic vein in two (15.4%), right hepatic vein combined with the middle hepatic vein in one (7.7%), and right hepatic vein combined with the inferior right hepatic vein in one (7.7%). In one patient with right adrenal gland metastasis, the tumor thrombus arose from the right adrenal vein (7.7%). Two patients had a mural thrombus and eleven had a massive thrombus. The massive thrombi in 10 patients did not completely occlude the IVC because circinate or arc-like luminal flow in the IVC around the tumor thrombi was present and the outflow canals of the intact hepatic veins were maintained. The tumor thrombus of one patient completely occluded the IVC inferior to the influx of hepatic veins accompanied by an aggregating blood thrombus. The outflow canals of the intact hepatic veins were severely narrowed but not completely occluded. One patient with a thrombus completely occluding the IVC and two patients with a massive IVC thrombus suffered preoperative renal insufficiency, and two had evident leg edema (Table 2).

Surgical procedures

A lobectomy was performed for patients with an indocyanine green retention rate at 15 minutes after injection (ICG R_{15}) of <15% and total bilirubin levels of <1.5 mg/dl without ascites. Patients with an ICG R_{15} of 15 to 20% and total bilirubin levels of 1.5 to 2.0 mg/dl were eligible for sectionectomy according to our criteria [21]. The type of surgical procedure was selected based on Couinaud's classification [22] and included three extended right hepatectomies, three extended left hepatectomies, five right hepatectomies, and two sectionectomies accompanied by thrombectomies. The right adrenal gland was resected simultaneously in two patients with right adrenal gland metastases (Table 3).

Hepatic resections were performed with an ultrasonic dissector using the Pringle maneuver in all cases. All IVC thrombi were removed under hepatic vascular exclusion (HVE). Before thrombectomy, hepatic transection was performed and the IVC was clamped below and above the liver. The IVC thrombus was excised en-bloc from the incised IVC, with satisfactory visualization of the intraluminal space under HVE. The IVC incision was closed by a simple continuous suture without a patch. All RA thrombi were removed under cardiopulmonary bypass (CPB). Following a laparotomy, a median sternotomy was performed to prepare for prompt CPB in anticipation of an undesirable pulmonary tumor embolism from a dislodged thrombus. Hepatic transection was then performed. The liver was handled gently, particularly if the thrombus had a long, thin neck, to

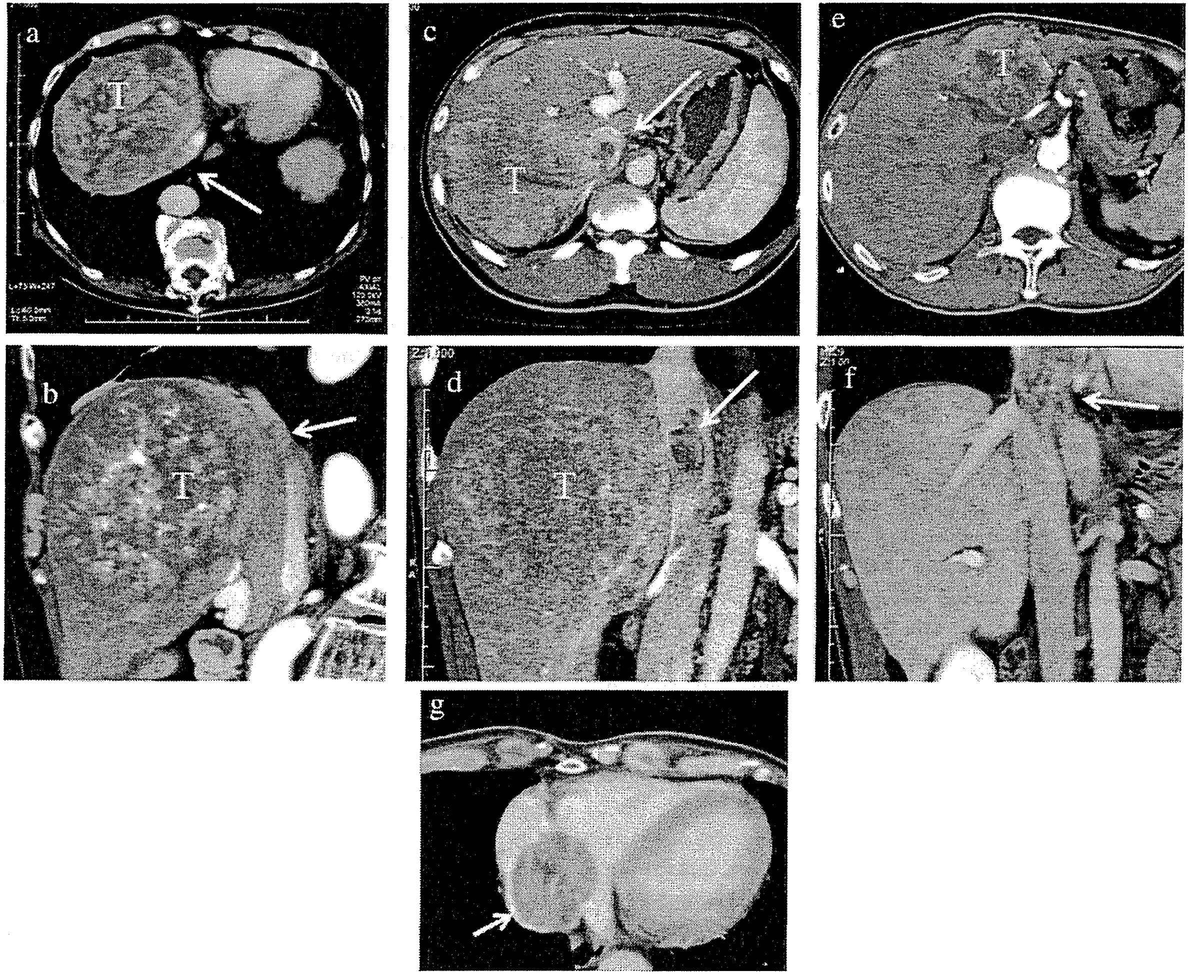

Figure 1 Representative computed tomography findings of hepatocellular carcinoma (HCC) with inferior vena cava (IVC) and right atrium (RA) thrombi. (**a** and **b**) A large HCC lesion in the right lobe (T), with a tumor thrombus arising from the right hepatic vein into the IVC (arrow). (**c** and **d**) HCC in the right lobe (T), with a tumor thrombus arising from the inferior right hepatic vein into the IVC (arrow). (**e**, **f**, and **g**) HCC in the left lobe (T), with a tumor thrombus arising from the left hepatic vein into the RA (arrow).

prevent dissemination of tumor thrombi. Then, the superior vena cava (SVC) and IVC below the liver were clamped and blood flow was bypassed to the ascending aorta via an oxygenator. The RA was incised and the thrombus was excised en-bloc under direct vision. In most cases, the RA was reconstructed by simple sutures, but in two cases, an invaded RA wall was partially excised and reconstructed using an artificial graft or pericardial patch. In addition to CPB, one patient with complete IVC occlusion accompanied by severe obstruction of intact hepatic outflow and one patient with tumor thrombi that arose from two major hepatic veins showed gross hepatic congestion due to outflow block at surgery. These cases needed extracorporeal bypass from the portal vein (PV) and IVC to SVC (Table 3). In all cases, thrombi were intraoperatively monitored by transesophageal echocardiography. In this study, we defined curative resection as macroscopic complete excision of the tumors, including metastatic lesions.

Follow up

The median duration of follow up was 11.2 (range, 1.8 to 51.8) months. Hospital death was defined as death occurring within 30 days of the first hospitalization. After surgery, CT or MRI was performed at 1- to 3-month intervals to determine recurrence. Data on surgical outcomes, postoperative management, recurrence, treatment of recurrence, and survival was analyzed for all cases.

Statistical analysis

Survival rates were analyzed by the Kaplan-Meier method and statistical significance was determined by

Table 1 Characteristics of patients and tumors

Characteristic	Value
Total number of patients	13
Age, years	
Mean ± SD (range)	63.4 ± 11.8 (37 to 86)
Sex	
Male/female	12/1
Hepatitis B virus	
Positive/negative	7/6
Hepatitis C virus	
Positive/negative	2/11
Child-Pugh classification	
A/B/C	13/0/0
Main tumor location	
Anterior/posterior/median/lateral section	5/4/3/1
Tumor size, cm	
Mean ± SD (range)	11.8 ± 4.3 (3.5–19)
Number of tumors	
Single/multiple	6/7
Extension of thrombus	
Inferior vena cava/right atrium	7/6
Preoperative extrahepatic metastases	
None/lung/adrenal gland/lymph nodes	5/5/2/1
Status of metastases after surgery	
Resected/regressed or stationary/progressed	3/1/4
Postoperative metastatic recurrence	
Liver/lung/lymph node/adrenal gland/inferior vena cava/brain	8/7/4/2/2/2

the log-rank test using JMP Pro 10.0.0 software (SAS, Cary, NC, USA). Significance was defined as $P < 0.05$.

Results

Surgical outcomes and postoperative complications

With regard to patients with an IVC thrombus, the mean surgical duration was 349 ± 30 minutes, the median blood loss was 950 ± 100 ml, and the mean HVE duration was 8.8 ± 3.1 minutes. Two of seven (28.6%) patients needed blood transfusions. No patient required an ICU stay, and the mean postoperative hospital stay was 23.6 ± 12.5 days. After surgery, one patient experienced biloma and one experienced controllable ascites. With regard to patients with an RA thrombus, the mean surgical duration was 608 ± 169 minutes, the median blood loss was 6540 ± 5404 ml, and the mean CPB duration was 32.2 ± 18.3 minutes. Five of six (83.3%) patients needed blood transfusions. The mean postoperative ICU stay was 1.7 ± 0.8 days and the mean postoperative hospital stay was 21.2 ± 4.6 days. After surgery, one patient experienced acute renal failure and one experienced atrial fibrillation, but these patients recovered with medical therapy. There was no postoperative mortality. All IVC and RA thrombi were excised completely. Curative resection was performed in five of thirteen (38.5%) cases (Table 4).

Postoperative management

Among the five patients (38.5%) who underwent curative resection, adjuvant systemic chemotherapy was administered to four. The chemotherapeutic agents used in combination included intravenous 5-fluorouracil (5-FU; 500 mg weekly) and peroral tegafur uracil (UFT; 300 mg daily) in three patients and peroral UFT (300 mg daily) in one. One patient was followed up without adjuvant chemotherapy.

Tumors remained after surgery in eight (61.5%) patients, including lung metastases in four, intrahepatic metastases in two, both intrahepatic and lung metastases in one, and mediastinal lymph node metastases in one. Residual lung metastases were treated with oral administration of UFT in two patients, 5-FU + UFT in one patient, and oral administration of tegafur gimeracil oteracil potassium (S-1) followed by surgical resection in one patient. Unresectable intrahepatic metastases were treated with UFT in two patients and transarterial chemoembolization (TACE) in one patient. A patient with residual mediastinal lymph node metastasis received radiation after surgery.

Recurrence and survival

All five patients who underwent curative resection experienced postoperative recurrences. Intrahepatic recurrences appeared in all five patients, lung metastases in four, intra-IVC metastases in one, and left adrenal gland metastases in one patient. The median recurrence-free survival duration of the patients who underwent curative resection was 3.8 months. Intrahepatic recurrences were treated with TACE in three patients, radiofrequency ablation (RFA) in two, and radiotherapy in one patient. Lung metastases were treated with systemic chemotherapy in three patients (5-FU + UFT in two, cisplatin (CDDP) + S-1 followed by oral administration of sorafenib in one patient), and surgical resection in one patient. Left adrenal gland metastases were surgically excised.

Among the eight patients who underwent noncurative resection, four of five with lung metastases exhibited progression of the metastases. In one patient, lung metastasis was resected but recurred after resection. Intrahepatic residual tumors in three patients progressed after surgery; however, mediastinal lymph node metastases treated by irradiation remained unchanged. Among these eight patients, seven experienced further dissemination of the tumor to new locations, including the lung in three, lymph

Table 2 Characteristics of tumor thrombi

Patients age/sex	Involved veins	Extent of thrombus	Advance of thrombus	Symptoms associated with the thrombus
68/M	RHV	Massive	RA	Renal insufficiency/lower limb edema
57/M	RAdV	Massive	IVC	Renal insufficiency
70/M	RHV	IVC occlusive	RA	Renal insufficiency/lower limb edema
86/F	RHV	Massive	IVC	(–)
68/M	IRHV	Mural	IVC	(–)
66/M	RHV	Massive	RA	(–)
37/M	IRHV	Massive	IVC	(–)
56/M	RHV/IRHV	Massive	IVC	(–)
51/M	LHV	Massive	RA	(–)
72/M	MHV	Mural	IVC	(–)
59/M	LHV/MHV	Massive	RA	(–)
69/M	MHV	Massive	IVC	(–)
65/M	MHV	Massive	RA	(–)

M male, *RHV* Right hepatic vein, *RAdV* right adrenal vein, *IRHV* inferior right hepatic vein, *LHV* left hepatic vein, *MHV*, middle hepatic vein, *IVC* inferior vena cava, *RA* right atrium. (–), no symptom.

nodes in three, brain in two, IVC in one, and adrenal gland in 1 (Tables 1 and 5).

The 1-, and 3-year overall survival rates for all 13 patients were 50.4% and 21.0%, respectively, and the overall median survival duration was 15.3 months. The cause of postoperative death in all patients was cancer, which remained at surgery or recurred after surgery (Table 5). The overall survival rate for patients with IVC thrombi was 57.1% at 1 year and 42.9% at 3 years, with median survival duration of 15.3 months. The 1-year overall survival rate for patients with RA thrombi was 40.0%, with median survival duration of 11.2 months. There was no significant difference between the IVC thrombi and RA thrombi groups (Figure 2a). The survival rates for patients who underwent curative surgical resection were 80.0% at 1 year and 30.0% at 3 years, with a median survival time of 30.8 months. Meanwhile, the 1-year survival rate for patients who underwent noncurative surgery and had residual tumors was 29.2%, with a median survival time of 10.5 months (Figure 2b). The longest survival time was 51.8 months for patients who underwent complete resection and 29.3 months (to date) for those who underwent incomplete resection, and they are still alive (Table 5).

Table 3 Surgical procedure

	Inferior vena cava thrombus (n = 7)	Right atrium thrombus (n = 6)
Surgical procedure		
Extended right hepatectomy	1	1
Extended right hepatectomy + right adrenectomy	0	1
Right hepatectomy	3	1
Right hepatectomy + right adrenectomy	1	0
Extended left hepatectomy	1	2
Sectionectomy	1	1
Inflow vascular control		
Hepatic vascular exclusion	7	0
Cardiopulmonary bypass	0	4
CPB + portal vein/inferior vena cava to superior vena cava bypass	0	2
Vascular wall reconstruction		
Simple closure	7	4
Patch reconstruction	0	2

Discussion

IVC and RA tumor thrombi arising from HCC are uncommon and are found in approximately 3 to 4% of HCC patients [2,23]. It is recognized that tumor invasion into intrahepatic vessels, such as the portal or hepatic veins, is an important prognostic factor for patients with HCC [24]. In particular, the prognosis of patients presenting with ICV or RA thrombi is extremely dismal [6]. Although surgical treatments as well as nonsurgical treatments such as TACE, radiotherapy, and chemotherapy are reported, optimal therapeutic management of IVC and RA thrombi has not been established because of the paucity of data [5,7,8,10-16]. Some reports demonstrate the potential benefit of surgical resection, but there are few reports that consolidate the efficacy of a surgical approach because IVC and RA thrombi are rare and because these reports are typically case reports or descriptions of a small number of patients [4,9,16,17,20,25,26]. Reports detailing the surgical treatment of RA thrombi

Table 4 Surgical outcomes

	Inferior vena cava thrombus	Right atrium thrombus
Surgical duration (minutes)		
Mean ± SD (range)	349 ± 30 (288 to 377)	608 ± 169 (449 to 911)
Blood loss (ml)		
Median ± SE (range)	950 ± 100 (750 to 1,520)	6540 ± 5404 (1,050 to 35,820)
Blood transfusion		
Yes/no	2/5	5/1
HVE time (minutes)		
Mean ± SD (range)	8.8 ± 3.1 (8 to 13)	-
CPB time (minutes)		
Mean ± SD (range)	-	32.2 ± 18.3 (4 to 54)
Curative resection		
Yes/no	3/4	2/4
ICU stay (days)		
Mean ± SD (range)	-	1.7 ± 0.8 (0–2)
Hospital stay (days)		
Mean ± SD (range)	23.6 ± 12.5 (14 to 48)	21.2 ± 4.6 (16 to 28)
Complications		
Yes/no	2 (ascites, 1; biloma, 1)/5	2 (ARF, 1; Af, 1)/4

HVE hepatic vascular exclusion, *CPB* cardiopulmonary bypass, *ARF* acute renal failure, *Af* atrial fibrillation.

are particularly rare, and to our knowledge this is the first report on the surgical treatment of IVC and RA thrombi, including six cases of RA thrombectomy, from a single institute.

It is generally assumed that liver resection combined with IVC or RA thrombectomy is a challenging and hazardous procedure that involves a high surgical risk. According to past reports, hepatectomy together with IVC or RA thrombectomy was associated with a high morbidity of 40% and a high mortality of 15% [4,15]. However, recent surgical innovations such as the inflow vascular control method together with refinement of the assessment of preoperative hepatic reserve have improved the safety of hepatectomy and thrombectomy procedures [26,27]. This progress has encouraged us to accept the challenge of aggressive surgical treatment for IVC and RA thrombi.

Effective control of intraoperative hemorrhage plays a crucial role in hepatectomy procedures combined with

Table 5 Characteristics and prognosis of patients

Patients age/sex	Tumor thrombus	Residual tumor		Metastatic recurrence		Outcome (cause of death)
		Hepatic	Distant	Hepatic	Distant	
68/M	RA	(−)	(−)	(+)	(+) (lung, Ad)	30.8 months; dead (cancer)
57/M	IVC	(−)	(−)	(+)	(−)	10.1 months; dead (cancer)
70/M	RA	(+)	(−)		(+) (lung)	9.1 months; dead (cancer)
86/F	IVC	(−)	(−)	(+)	(+) (lung, IVC)	15.3 months; dead (cancer)
68/M	IVC	(−)	(−)	(+)	(+) (lung, LN)	51.8 months; alive
66/M	RA	(−)	(+) (lung)	(+)	(+) (LN)	11.2 months; dead (cancer)
37/M	IVC	(−)	(+) (lung)	(−)	(+) (Ad, LN, Brain)	10.5 months; dead (cancer)
56/M	IVC	(−)	(+) (lung)	(−)	(−)	29.3 months; alive
51/M	RA	(−)	(+) (lung)	(+)	(+) (Brain)	8.5 months; dead (cancer)
72/M	IVC	(+)	(+) (lung)		(+) (LN)	1.9 months; dead (cancer)
59/M	RA	(−)	(−)	(+)	(+) (lung)	16.5 months; alive
69/M	IVC	(−)	(+) (LN)	(+)	(+) (lung, IVC)	16.0 months; alive
65/M	RA	(+)	(−)		(+) (lung)	7.6 months; alive

Ad adrenal gland, *IVC* inferior vena cava, *LN* lymph node.

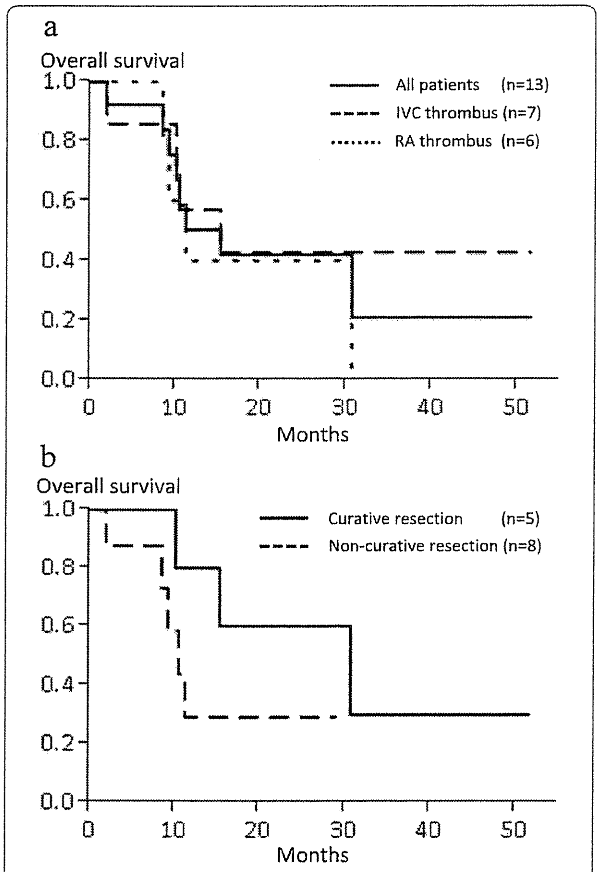

Figure 2 Survival curve for patients with hepatocellular carcinoma (HCC) and inferior vena cava (IVC) and right atrium (RA) thrombi. (a) Overall survival curve for all patients (n = 13), patients with IVC thrombus, and patients with RA thrombus. $P = 0.6056$ by log-rank test, IVC thrombus versus RA thrombus. **(b)** Overall survival curve for patients who underwent curative surgical resection and noncurative resection. $P = 0.2103$ by log-rank test, curative resection versus noncurative resection.

IVC or RA thrombectomy, because the degree of bleeding is a major predictive factor for operative morbidity and mortality [21]. In this study, hepatic parenchymal transection was routinely performed prior to thrombectomy using the Pringle maneuver. IVC occlusion at the suprahepatic portion with bulky tumor thrombi evokes Budd-Chiari syndrome and massive hepatic congestion [28]. In this study, we observed hepatic congestion in patients with outflow obstruction of spared hepatic veins by a massive tumor thrombus. Furthermore, occlusion of two of three major hepatic veins by venous invasion induced hepatic congestion, even though the spared hepatic vein was not obstructed. We used extracorporeal bypass from the PV and IVC to SVC to decompress the liver parenchyma and decrease bleeding during hepatic transection in two patients with an RA thrombus [29]. We performed IVC thrombectomy under a favorable field with good bleeding control by HVE. The duration of HVE, which could trigger hemodynamic deterioration, was short enough. Although CPB was mandatory for RA thrombectomy and was accompanied with a larger amount of blood loss and a higher rate of blood transfusion, patients with RA thrombi required minimal ICU stays and shorter postoperative hospitalization. These procedures contributed to a low incidence of postoperative non-serious complications that were medically manageable. Furthermore, we did not observe any operative mortality in this study. Therefore, hepatectomy with IVC or RA thrombectomy, although technically challenging, can be performed safely with appropriate inflow vascular control for patients with good hepatic reserve. Because almost all thrombi had capsules and did not adhere to the wall of the IVC or RA, they were simply removed by thrombectomy without wall resection. Although some authors indicate the efficacy of IVC resection, the benefits are controversial [30]. We experienced two cases of intra-IVC recurrence after surgery (Table 5); therefore, the management of such tumor thrombi should be reconsidered.

The prognosis of HCC patients with IVC or RA tumor thrombi is extremely poor. Earlier observations revealed a median survival duration after diagnosis of 1 to 5 months for untreated patients [4,15,31]. Although there is no consensus on the therapeutic options for HCC with IVC or RA thrombi, nonsurgical treatments such as TACE, as well as radiotherapy and chemotherapy, have been attempted. Previous reports concerning the therapeutic benefits of TACE with or without radiotherapy revealed insufficient results, with a median survival duration of 9.2 months (range, 4.2 to 18.4 months) [4,8,11-13,32]. Currently, the outcome of systemic chemotherapy for HCC has been disappointing, although sorafenib, which is the only effective agent against HCC, demonstrated a slightly better prognosis of 10.7 months in patients with unresectable HCC [33]. Some case reports have suggested the efficacy of surgical resection, and, recently, Wang *et al.* reported the significant superiority of a surgical approach to HCC with IVC or RA thrombi, with a median survival duration of 19 months, compared with TACE with or without chemoradiotherapy or no treatment [4,6,9,16,17,20,26,27,34]. This study included 56 patients, of whom 25 underwent surgery, although 7 had an RA thrombus and only 3 underwent surgical resection for the same [4]. Therefore, the therapeutic benefit of surgical resection for HCC with an RA thrombus remains unclear. In the present study, the median overall survival duration of patients with an IVC or RA thrombus was comparable at 15.3 months and 11.2 months, respectively (Figure 2a). This finding indicates the equivalent therapeutic efficacy of surgical resection for RA or IVC thrombi. These results for patient survival are slightly worse than those of the previous study [4] because our study included patients who underwent non-curative resection. The median survival duration of patients who underwent curative resection was 30.8 months, which is longer than that in the previous report [4] (Figure 2b). This result also surpasses nonsurgical treatment with sorafenib, which resulted in median survival duration of 8.1 months in patients with macrovascular invasion [35].

All patients who underwent curative surgical resection experienced local recurrence or distant metastasis in the early postoperative phase, despite the fact that almost all patients received adjuvant chemotherapy (Table 5). It has been recognized that the poor prognosis of HCC with tumor thrombi in the IVC or RA is strongly related to a high incidence of postoperative recurrence at a relatively early stage, even after curative surgery. In this study, most patients who underwent curative resection developed postoperative lung metastases and intrahepatic recurrence at an equal rate. Preoperative or intraoperative dissemination of tumor cells to the lung can contribute to postoperative metastatic recurrence. To prevent potential intraoperative dissemination by intraoperative handling, some authors indicated the benefit of separated thrombectomy before hepatic transection [36]. However, it could be technically difficult to remove a thrombus en-bloc without up-front hepatic transection; therefore, further improvements in surgical techniques are required. To date, there is no clear modality established for preventing HCC recurrence [4,16,37]. The efficacy of preoperative radiotherapy is indicated for PV tumor thrombi [38]; however, the benefit for IVC or RA thrombi is unclear and there remains a risk of thrombi dislodgment during radiation. In this study, locally recurrent tumors were controlled by TACE or RFA and distant metastatic tumors were treated by chemotherapy with or without radiotherapy or surgical excision if resectable. These vigorous repetitive treatments contribute to improvement in survival, even after recurrence.

Surgical resection is commonly contraindicated for patients with unresectable metastatic tumors because incomplete resection is a crucial factor for poor prognosis [6]. However, hepatic lesions, but not distant metastasis, are the major factors influencing poor prognosis for death in the early postoperative phase [39]. On the basis of the fact that the survival duration of patients with IVC or RA thrombi is extremely short with nonsurgical treatment, distant metastasis itself should not be considered a contraindication for surgery. In this study, eight patients underwent noncurative surgery, and the median survival duration of 10.5 months was relatively better than that for patients who underwent nonsurgical treatment or no treatment in previous studies, including patients who received sorafenib, which is the only highly evidenced agent for advanced HCC treatment and results in median survival duration of 8.9 months in patients with extrahepatic metastases [4,8,11-13,15, 30-32,35]. Recent reports indicate the efficacy of aggressive treatment for HCC metastases, including those to the lung, adrenal gland, and lymph nodes, by surgery or radiotherapy [40-42]. In this study, a patient with lung metastases at primary surgery underwent resection of the metastases and survived for 29.3 months. These findings indicate that reductive surgical resection can be justified in patients with IVC or RA thrombi accompanied by distant metastases or unresectable intrahepatic metastases. Control of the life-threatening progression of intrahepatic HCC and prevention of unexpected death by pulmonary embolism would give these patients a chance to undergo multidisciplinary treatments for improving survival. Therefore, if intrahepatic HCC and IVC or RA thrombi can be totally or partially resected, surgical resection may be beneficial.

The limitations of this study include its retrospective design, its single-center design, the small sample size, and patient heterogeneity. Because IVC and RA thrombi associated with HCC are rare, a multicenter prospective study with a large patients sample is necessary to definitively establish the benefits of surgical management.

Conclusions

In conclusion, surgical resection of HCC with IVC or RA thrombosis can be performed safely with appropriate inflow vascular control in patients with good hepatic reserve. We suggest that aggressive surgical resection may be more beneficial than existing therapeutic modalities; however, early recurrence and treatment of recurrent or metastatic tumors remain unresolved issues. Further studies on adjuvant therapies and establishment of therapeutic strategies for recurrent and metastatic tumors are important challenges to improve survival.

Abbreviations

Af: Atrial fibrillation; ARF: Acute renal failure; HBV: Hepatitis B virus; CDDP: Cisplatin; CPB: Cardiopulmonary bypass; CT: Computed tomography; 5-FU: 5-fluorouracil; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HVE: Hepatic vascular exclusion; ICG R_{15}: Indocyanine green retention rate at 15 minutes after injection; IRHV: Inferior right hepatic vein; IVC: Inferior vena cava; LHV: Left hepatic vein; MHV: Middle hepatic vein; MRI: Magnetic resonance imaging; PV: Portal vein; RA: Right atrium; RAdV: Right adrenal vein; RFA: Radio frequency ablation; RHV: Right hepatic vein; S-1: Tegafur gimeracil oteracil potassium; SVC: Superior vena cava; TACE: Transarterial chemoembolization; UFT: Tegafur uracil.

Competing interests

The authors have no conflicts of interest to declare.

Authors' contributions

KW and TK designed the research, KW, TK, HY, TK, HK, YT, KN, TS, ST, and AT contributed to acquisition of data, and KW and TK analyzed and interpreted data. All authors read and approved the final manuscript.

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