Figure 3. Diagnostic algorithm for hypovascular nodule (APASL Guideline). ※1: When the nodule is hypovascular on dynamic CT or dynamic MRI, Sonazoid-enhanced contrast US is recommended to confirm whether it is truly a hypovascular nodule.

are in use. Use of imaging diagnosis is increasing, whereas the use of biopsy is decreasing. New imaging techniques are continuously being developed. Practice guidelines should be updated to reflect the development of new imaging techniques.

PATHOLOGICAL DIAGNOSIS OF EARLY HCC

In 2009, pathologists from all over the world made great progress by reaching a consensus on the pathological diagnosis of early HCC. A consensus paper was published in the journal, *Hepatology* (21). The main topic of the consensus paper was histopathological definition of early HCC, together with premalignant lesions, dysplastic nodules and progressed HCC. Representative early HCC is a small, well-differentiated tumor, of vaguely nodular type. Microscopically, the border is unclear, and very well-differentiated cancer cells show a replacing growth pattern. They also frequently show stromal invasion, which is quite useful for making a diagnosis of cancer. However, histological atypia or histological alteration is usually very slight in early HCC, which is quite similar to the case of early cancers in other organs. Biopsy diagnosis of early HCC is especially difficult. In an example case, a slight increase in chromatin staining with substantial increase in the nuclear density is seen. Several standard techniques reveal slight changes or alterations in the tumor portion, such as a decrease in reticulin and a slight increase in proliferative activity. However, the use of some new markers, such as heat shock protein (HSP) 70, clearly highlights the tumor portion, making it more easily recognized. Greater use of tumor markers, including glypican 3 and HSP70, is likely and will increase the accuracy of diagnosis of early HCC.

Much has been learned about early HCC, but various problems remain. We know that cancer development is a multi-step process, especially when there are cirrhotic changes. Early HCC grows very slowly and has a favorable outcome, whereas progressed, small HCC has a greater likelihood of showing intrahepatic spread and a worse prognosis. It is necessary to recognize that there is a gray zone between pre-cancerous lesion and early HCC. Liver biopsy is recommended for small, equivocal lesions. Also, molecular markers are expected to raise the diagnostic accuracy, especially in the case of biopsy diagnosis of HCC. At the same time, controversy remains regarding which lesions should be examined by biopsy, and there is a risk of over-diagnosis of early cancer.

CURRENT TREATMENT STRATEGIES

Since 2001, when the Barcelona group published their consensus guideline, at least eight other guidelines have been released worldwide regarding the diagnosis and/or treatment of HCC. In 2003, the Korean guidelines were published, and in 2005, the Japanese guidelines for evidence-based clinical practice (Fig. 4) (16) were released. Clinical practice guidelines should be evidence-based, and they should represent the consensus of expert committees. Sometimes, it is very difficult to reach a consensus in the field of HCC. Guidelines must also take into consideration the socioeconomic status and current daily practice in the country or region. The socioeconomic background and daily practice regarding HCC were compared among Europe and the USA, Asia (Korea) and Japan. The major etiology of HCC is HCV in Europe, the USA and Japan, but HBV in Asia (Korea). A surveillance system has been established in Japan, is being
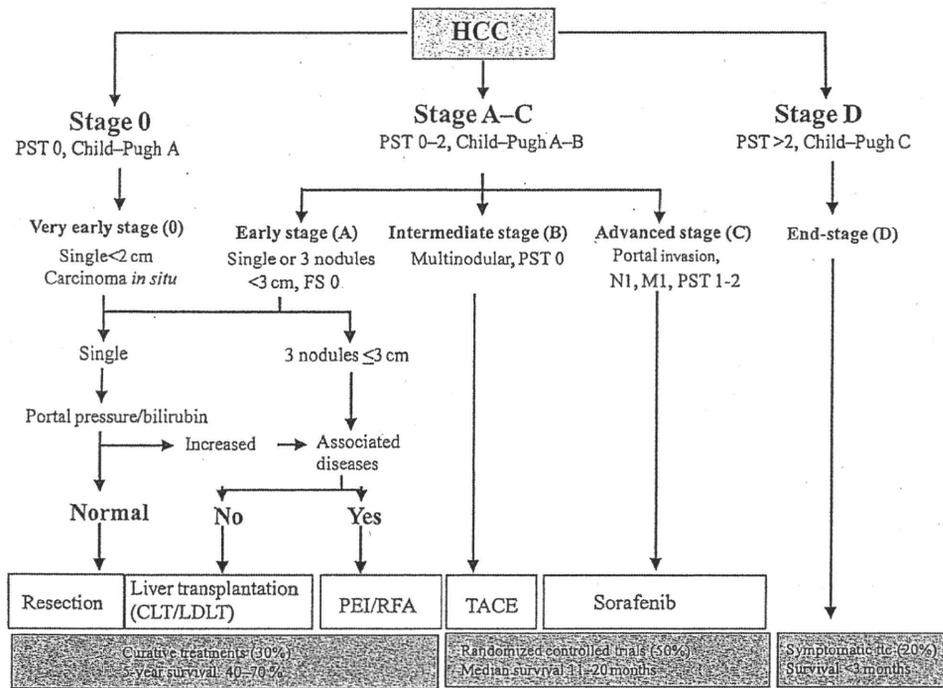
Figure 4. BCLC staging [Llovet et al. (10)]. BCLC, Barcelona Clinic of Liver Cancer; PST, performance status; CLT, cadaveric liver transplantation; LDLT, living donor liver transplantation; PEI, percutaneous ethanol injection; RFA, radiofrequency ablation; TACE, transcatheter arterial chemoembolization.
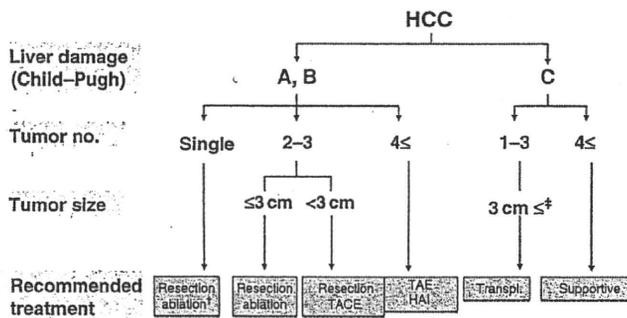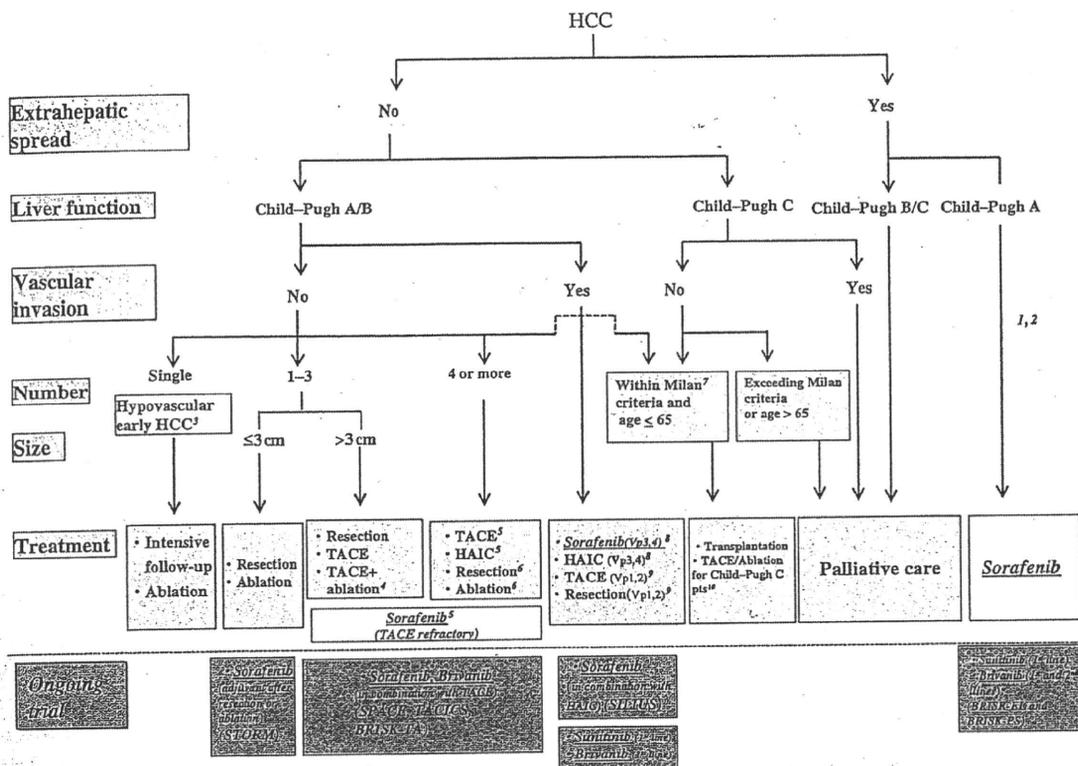

Figure 5. EBM-based algorithm for HCC treatment (J-HCC Guidelines 2009). Resection or transarterial chemoembolization (TACE) may be selected for liver damage A patients with vascular invasion. Chemotherapy may be selected for extrahepatic HCC. LT is only for ≤65 years old. [†]Recommended for Child B; [‡]<2 cm for solitary lesion. HAI, hepatic arterial infusion.

developed in Asia (Korea), but does not exist in the Western countries. As a result, most HCC patients are diagnosed in an early stage in Japan, but at a very advanced stage in Western countries. As tumor markers, only AFP is measured in Western countries, whereas three tumor markers are measured in Japan. The risk of treatment of HCC must also be considered. The mortality of liver resection is as high as 4–5% in Western countries, but only 0.7% in Japan. Brain-dead donors for liver transplantation are very rare in Japan, but common in Western countries (22). These factors must be considered for development of treatment strategies for HCC.

The BCLC guidelines to staging and treatment of HCC are probably the most popular treatment algorithm in Western countries, but not in Asia. The Japanese guidelines were just revised in 2009, are very simple and cover a majority of early- and intermediate-stage HCC patients (Fig. 5). A Japanese consensus-based algorithm for HCC covers even very advanced-stage HCC, including patients with extrahepatic spread and vascular invasion (Fig. 6) (17,19). Sorafenib is recommended for such advanced disease with good liver function, and an ongoing trial is evaluating its use as an adjuvant therapy. The Korean guideline for management of HCC was initially published in 2003, after which they accumulated evidence, held a nation-wide forum for revision of the guidelines and created a revision committee. As a result, their updated guidelines were published in 2009 (23). The algorithm for the Korean HCC treatment plan lists hepatic resection, liver transplantation, radiofrequency ablation and ethanol injection as curative treatments. There is no evidence showing which treatment is superior for cure of HCC in each patient, so the guideline recommends that the physician decide which treatment will be used. The APASL Consensus on Treatment of HCC (24) was published in 2010 and may be utilized in the Asian region.

In conclusion, several practice guidelines presenting treatment strategies for HCC in Asia have been developed. They were created based on evidence-based medicine methodology and consensus among experts in the region. They also reflect the socioeconomic status and current daily practice in the region. A number of ongoing clinical trials aim to

Figure 6. Consensus-based treatment algorithm for HCC proposed by Japan Society of Hepatology (JSH) 2009 revised in 2010. *1*, Treatment should be performed as if extrahepatic spread is negative, when extrahepatic spread is not regarded as a prognostic factor. *2*, Sorafenib is the first choice of treatment in this setting as a standard of care. *3*, Intensive follow-up observation is recommended for hypovascular nodules by the Japanese Evidence-Based Clinical Practice Guidelines. However, local ablation therapy is frequently performed in the following case: (i) when the nodule is diagnosed pathologically as early HCC, (ii) when the nodules show decreased uptake on gadolinium-ethoxybenzyl-diethylene triamine pentaacetic acid or (iii) when the nodules show decreased portal flow by CTAP, since these nodules are known to frequently progress to the typical advanced HCC. *4*, Even for HCC nodules exceeding 3 cm in diameter, combination therapy of TACE and ablation is frequently performed when resection is not indicated. *5*, TACE is the first choice of treatment in this setting. Hepatic arterial infusion chemotherapy (HAIC) using an implanted port is also recommended for TACE refractory patients. The regimen for this treatment is usually low-dose FP (5FU + CDDP) or intra-arterial 5FU infusion combined with systemic interferon therapy. Sorafenib is also recommended for TACE refractory patients. *6*, Resection is sometimes performed even when numbers of nodules are over 4. Furthermore, ablation is sometimes performed in combination with TACE. *7*, Milan criteria: Tumor size ≤3 cm and tumor numbers ≤3; or solitary tumor ≤5 cm. Even when liver function is good (Child–Pugh A/B), transplantation is sometimes considered for frequently recurring HCC patients. *8*, Sorafenib and HAIC are recommended for HCC patients with Vp3 (portal invasion at the first portal branch) or Vp4 (portal invasion at the main portal branch). *9*, Resection and TACE are frequently performed when portal invasion is minimum such as Vp1 (portal invasion at the third or more peripheral portal branch) or Vp2 (portal invasion at the second portal branch). *10*, Local ablation therapy or subsegmental TACE is performed even for Child–Pugh C patients when transplantation is not indicated when there is no hepatic encephalopathy, no uncontrollable ascites and a low bilirubin level (<3.0 mg/dl). However, it is regarded as an experimental treatment since there is no evidence of its survival benefit in Child–Pugh C patients. A prospective study is necessary to clarify this issue.

generate evidence for a better treatment algorithm. Guidelines should be updated every 3 or 4 years, incorporating new evidence.

FUTURE PERSPECTIVES, ESPECIALLY IN REGARD TO SORAFENIB

There was no established systemic chemotherapy for HCC. However, sorafenib has become a standard systemic treatment for advanced HCC. This section addresses the future perspectives for sorafenib and beyond sorafenib. Two randomized control studies have shown the survival benefit of sorafenib in advanced HCC patients with good liver function of Child–Pugh A. The SHARP trial (25), carried out mainly in European countries, and an Asia-Pacific trial (26) both showed that sorafenib provides a survival benefit in advanced HCC patients. Both trials yielded similar hazard ratio of 0.69 and 0.68, respectively, in favor of sorafenib over placebo. Other published reports on sorafenib for HCC include a Phase II trial conducted in Western countries (27), a Phase I Japanese study (28), a Korean study (29) and a Phase 2 Hong Kong study (30). The studies had various differences in patient background, such as involvement of HBV, HCV or others, liver function of Child–Pugh A and B, and the ECOG performance status. Those differences affected the survival outcomes in the four studies like outcomes after other treatment modalities.

Although sorafenib has become a standard systemic treatment for advanced HCC, there are still issues to be investigated with regard to this agent, including its efficacy and safety in patients with Child–Pugh B moderate liver

function, combination therapy with other treatment methods, and the need to identify predictive factors and markers for sorafenib. Various studies are currently attempting to elucidate those issues. The Phase III STORM global trial will evaluate sorafenib as an adjuvant therapy after surgery or radiofrequency ablation. A Japanese Phase II study will evaluate the efficacy and safety of sorafenib in patients with Child–Pugh A and B, with investigation of biomarkers. A global trial of combination of sorafenib with TACE is ongoing, while two Japanese Phase I studies of combination of sorafenib with hepatic arterial infusion are in progress (19). Arterial infusion chemotherapy is a very common and useful treatment in Japan (31), and one of these studies combines sorafenib with cisplatin, whereas the other combines sorafenib with 5-FU and cisplatin. It is anticipated that these trials will lead to Phase III studies.

OTHER MOLECULARLY TARGETED AGENTS

Sorafenib is the first systemic therapy approved for advanced-stage HCC, and widely used. Sorafenib prolongs time to progression and overall survival in patients with advanced HCC; however, predictive factors are unknown at the present. Good responders show a good response, but how can they be identified in advance? Researchers are currently looking for biomarkers that will identify good responders and lead to modification of the treatment algorithm. Also, a 'good response' has limitations. How can a 'complete response' be attained? Combination therapy and some adjuvant treatment, after palliative or curative treatment, will be needed. There are also many poor responders. How can a poor response be overcome? Second-line agents are necessary, as is combination therapy. Various targeted agents in addition to sorafenib are under development for HCC. They include brivanib, bevacizumab, cediranib, erlotinib, gefitinib, lapatinib, RAD001, sunitinib, thalidomide and TSU-68. These agents have similar yet slightly different mechanisms of action. The results of various clinical studies of these molecular targeted therapy agents were summarized in *Hepatology* (32). The results look good, and many Phase II and Phase III trials are ongoing. The trials can be categorized into three types: first-line or combination studies, second-line studies and adjuvant studies.

First-line or combination studies are being carried out as Phase III trials of sunitinib vs. sorafenib (terminated in 2010 because of severe adverse effect); brivanib vs. sorafenib; lilifanib vs. sorafenib; erlotinib plus sorafenib vs. sorafenib; and erlotinib plus bevacizumab vs. sorafenib. The results of these trials should be available in 2 or 3 years. There are also many first-line Phase II studies. There are two second-line Phase II studies, of brivanib vs. the placebo and RAD001 vs. the placebo, for patients who failed to respond to sorafenib. There are three Phase III adjuvant studies. The STORM study investigates sorafenib vs. placebo after resection or ablation. A second adjuvant study investigated sorafenib vs. placebo after TACE; this is already finished and the

results were presented at ASCO-GI in 2010 (33). The third Phase III adjuvant study compares brivanib vs. placebo after TACE. In a first-line Phase II study of brivanib, 46% of the patients showed stable disease, and in the second-line Phase II study, 43% showed stable disease (34,35). These results were promising, and at least three trials are now ongoing for brivanib.

In conclusion, molecularly targeted therapy (MTT) has emerged as a promising approach for advanced HCC. Sorafenib impacted on MTT agents in HCC, but the benefits of sorafenib were reported to be relatively modest. Several MTT agents for first- and second-line treatments are undergoing clinical trials. The advantages of MTT agents are being explored in combination treatments as well as adjuvant therapy with resection, local ablation, radiation, hepatic arterial infusion chemotherapy and TACE.

CONCLUSION

HCC is a highly prevalent disease in many Asian countries and incidence of HCC varies enormously across Asia, but tends to follow incidences of hepatitis B infection and liver cirrhosis. Incidence and etiology of HCC in Japan is different from the rest of Asia, but similar to Western countries since hepatitis C infection is the main etiological factor. Screening program improves detection of early HCC and has some positive impact on survival, but the majority of HCC patients in Asia still present with advanced HCC. Long-term outcomes following treatment of early, intermediate or advanced disease are still unsatisfactory because of lack of effective adjuvant or systemic therapies. Sorafenib is the first systemic therapy to demonstrate prolonged survival vs. placebo in patients with advanced HCC. New molecular targeting therapies hold great promise. Many new agents are under investigation and their results are awaited.

Conflict of interest statement

The author, Joong-Won Park, participated in phase II and phase III clinical studies sponsored by Bristol-Myers Squibb, Pfizer Inc., Bayer Healthcare and Bukwang Pharmaceutical Co. He is also a member of BMS Brivanib study steering committee, Pfizer Sunitinib advisory committee, and Bukwang Pharmacetutical Co. advisory committee.

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BMC
Gastroenterology

A novel biomarker TERTmRNA is applicable for early detection of hepatoma

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Abstract

Backgrounds: We previously reported a highly sensitive method for serum human telomerase reverse transcriptase (hTERT) mRNA for hepatocellular carcinoma (HCC). α-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP) are good markers for HCC. In this study, we verified the significance of hTERTmRNA in a large scale multi-centered trial, collating quantified values with clinical course.

Methods: In 638 subjects including 303 patients with HCC, 89 with chronic hepatitis (CH), 45 with liver cirrhosis (LC) and 201 healthy individuals, we quantified serum hTERTmRNA using the real-time RT-PCR. We examined its sensitivity and specificity in HCC diagnosis, clinical significance, ROC curve analysis in comparison with other tumor markers, and its correlations with the clinical parameters using Pearson relative test and multivariate analyses. Furthermore, we performed a prospective and comparative study to observe the change of biomarkers, including hTERTmRNA in HCC patients receiving anti-cancer therapies.

Results: hTERTmRNA was demonstrated to be independently correlated with clinical parameters; tumor size and tumor differentiation (P < 0.001, each). The sensitivity/specificity of hTERTmRNA in HCC diagnosis showed 90.2%/85.4% for hTERT. hTERTmRNA proved to be superior to AFP, AFP-L3, and DCP in the diagnosis and underwent an indisputable change in response to therapy. The detection rate of small HCC by hTERTmRNA was superior to the other markers.

Conclusions: hTERTmRNA is superior to conventional tumor markers in the diagnosis and recurrence of HCC at an early stage.

Background

Since the discovery of circulating nucleic acids (CNAs) in plasma in 1948, many diagnostic applications have emerged. Recently, CNAs instead of a protein has appeared on this scene of practical diagnostic assay, suggesting that cell-free CNAs in the plasma/serum of cancer patients have characteristics of tumor-derived nucleic acids. In addition to DNA-derived from tumor cells [1-4], a recent development in this new field is the finding of tumor-related RNA in the plasma/serum of cancer patients [5]. These features include tyrosine kinase

mRNA [6], telomerase components [7,8], the mRNAs that are encoded by different tumor-related genes [9-13], and viral mRNA [14]. In one study, two telomerase markers in breast cancer yielded 44% of positive rates [7]. Nevertheless, telomerase RNA seems to be a promising marker by the reason that it can be found even in the serum of patients with small, undifferentiated breast cancers without any metastatic lesions. Dasi et al. showed that circulating telomerase RNA is a sensitive marker, using real-time reverse transcription-PCR (RT-PCR) [8].

The telomerase catalytic subunit (hTERT) exerts important cellular functions, including telomere homeostasis, genetic stability, cell survival and perhaps differentiation [15-20]. hTERTmRNA in serum was detected in

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breast cancer but not in benign diseases, suggesting that hTERT is available for cancer diagnosis [4].

HCC ranks high among the most common and fatal malignancies associated with hepatitis B virus (HBV) and hepatitis C virus (HCV) infection [5]. Although HCC patients receive possible medical treatments such as transcatheter arterial chemoembolization/embolization (TACE/TAE), radiofrequency ablation (RFA), and surgery for primary tumors, intrahepatic and extrahepatic recurrence frequently limit patient's survival [6]. Although the modalities such as ultrasonography (US) and conventional tumor markers such as α-fetoprotein-L3 (AFP-L3) and DCP are widely used and important for HCC detection in clinical scenes [7], they still do not provide an entirely satisfactory solution to detect HCC at the early stage. Since HCC has been recently classified as a complex disease with a wide range of risk factors and many cellular signaling pathways have been reported to be involved in hepatocarcinogenesis, a novel biomarker for HCC is required [21]. We previously reported that measurement of serum hTERTmRNA by real-time RT-PCR method was sensitive in detection of tumor-derived hTERTmRNA even in the HCC patients whose AFP levels were low [9], and was also useful even for other malignancies such as non-small cell lung cancer, ovarian cancer, and gastric cancer [22-24]. In this large-scale study that includes follow-up cases, we focused on HCC of all malignancies and assessed the clinical significance of hTERTmRNA measurement in HCC diagnosis and monitored the clinical course.

Methods

Patients and Sample Collection

Four hundred-thirty seven consecutive patients (303 patients with HCC, 89 with CH, and 45 with LC), who were admitted at Tottori University related Hospitals, Osaka Red Cross Hospital, and Fukuoka University Chikushi Hospital between November, 2002 and December, 2006, were enrolled in this study. All the HCC patients had LC or CH as the underlying liver disease. The mean ages of patients with HCC, LC, and CH were 65, 66, and 61 years, respectively. One hundred-sixty seven patients were infected with HCV, 97 with HBV, 24 with both viruses and 15 with no viral markers. The patients were diagnosed by blood chemistry, US, computed tomography (CT), AFP and/or biopsy under US. The clinicopathological findings (age, gender, etiology, underlying liver disease (adjacent lesion), Pugh score, Child classification, total bilirubin (TB), albumin (Alb), alanine aminotransferase (ALT), AFP, AFP-L3, DCP, HCV titer, HCV subtype, tumor number, tumor size, differentiation degree of tumor, and presence of metastasis) were evaluated (Figure 1). HCC was diagnosed according to the AASLD guidelines and the differentiation of HCC was

diagnosed by liver biopsy. Two hundred one healthy individuals including 144 females (from 24-87 years old: mean age 57 years) served as controls. Informed consent was obtained from each patient and the study protocols followed the ethical guidelines of the 1975 Declaration of Helsinki and were approved by the human research committee of Tottori University. The therapies for HCC include TAE, transcatheter arterial infusion (TAI), percutaneus ethanol injection therapy (PEIT), and RFA. Regarding follow-up patients, blood samples were taken basically every two months.

RNA extraction and Real-time quantitative RT-PCR

Harvesting serum samples were performed as previously described [9]. RNA was extracted with DNase treatment from serum as reported previously [4,9]. The quantitative RT-PCR was performed as described previously [5,10]. (a) for hTERT. The RT-PCR condition was an initial incubation at 50 for 30 min followed by a 12-min incubation at 95, then 50 cycles at 95 (0 s), 55 (10 s), and 72 (15 s), and a 20 second melting at 40°C. The dynamic ranges of real-time PCR analysis for hTERTmRNA were more than approximately 5 copies in this assay and we were able to exclude the possibility of false negativity in serum samples from patients with CH, LC and controls. The PCR yielded products of 143 bp for hTERT (data not shown). The RT-PCR assay was repeated twice and the quantification was confirmed by using LightCycler (Roche, Basel, Switzerland) with reproducibility.

hTERTmRNA during the treatment and detection of small HCC

We examined the therapeutic effectiveness of hTERTmRNA during the clinical course. Serum hTERTmRNA was measured before and 7 days after TAE in 16 HCC patients. In comparison with AFPmRNA, the half-life of hTERTmRNA was examined. By monitoring gene expression in serum up to 6 months after the beginning of therapy such as TAE, TAI, RFA, PEIT, surgical treatment, the effect of therapies were estimated in 20 patients. Furthermore, we examined hTERTmRNA expression and level of other conventional tumor markers after they were categorized by the tumor size (less than 10 mm, 11-20 mm, 21-30 mm, more than 30 mm).

Immunohistochemistry

For immunohistochemical analysis, of 303 patients, 50 HCC patients (24 patients with HCV, 9 with HBV, 10 with both viruses, and 7 with unknown etiology; 5 patients with well-, 3 with well~moderately-, 32 with moderately-, 3 with moderately~poorly-, and 7 with poorly-differentiated HCC) with 35 positive and 15 negative conventional tumor markers, who underwent surgical treatment, were chosen. The immunohistochemical procedures were done as reported previously [25]. The sections were incu-

Clinical parameters		No. of HCC patient 303	hTERT mRNA p	AFP p	AFP-L3 p	DCP p
Age mean:65 years old (range:22 to 101)			NS	NS	NS	NS
Gender	M	196	NS	NS	NS	NS
	F	107				
Etiology	HBV	97	NS	NS	NS	NS
	HCV	167				
	HBV+HCV	24				
	NBNC	15				
Background lesion	CH	113	NS	NS	NS	NS
	LC	190				
Numbers of tumors	1	199	NS	NS	0.003	0.029
	2	55				
	>3	49				
Tumor size (cm)	<1	25	<0.001	0.008	NS	NS
	1~2	79				
	2~3	100				
	>3	99				
Differentiation of tumor (Edmondson grade)	I	24	<0.001	0.019	0.001	NS
	II	43				
	III	33				
	IV	1				
	unknown	101				

hTERT mRNA was independently correlated with tumor size eand differentiation degree. NS: not significant.

Figure 1 Multivariate analysis of tumor markers with clinical parameters in patients with HCC.

bated with the following monoclonal antibodies: anti-hTERT (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Ki67 (Santa Cruz Biotechnology), anti-TUNEL (Sigma Chemical, MO, USA), HBsAg (Sigma Chemical), and HCV core antibody (Sigma Chemical). Expression degree was confirmed and estimated of hTERT, Ki-67, and TUNEL by the percentage of positively-stained cell number [26-28].

Statistical analysis

Multivariate analysis was performed using SPSS 13.0 (SPSS Corp., Tokyo, Japan). Stratified categories in each clinical parameter were evaluated by One Way ANOVA and multivariate analysis using a logistic regression analysis model. To assess the accuracy of the diagnostic tests, the matched data sets (chronic liver diseases patients and HCC patients) regarding AFP, AFP-L3, DCP, and hTERT-mRNA were analyzed by using receiver operator characteristic (ROC) curve analysis. The correlation of hTERTmRNA between HCC tissue and serum was analyzed using both Paired t test and Spearman's test. The detection rates of HCC in comparison with tumor size were evaluated by Friedman test.

Results

RNA extraction and Real-time quantitative RT-PCR

In each quantitative assay, a strong linear relation was demonstrated between copy number and PCR cycles using RNA controls for concentration ($r^2 > 0.99$; data not shown). hTERTmRNA expression showed stepwise up-regulation with disease progression and the quantification was significantly higher in HCC than in LC, CH and healthy individuals ($P < 0.001$, $P < 0.01$ and $P < 0.001$, respectively, Figure 2A). ROC curve analyses showed that the sensitivity/specificity of hTERTmRNA for HCC were 90.2%/85.4% (Figure 2B). Optimal cut-off values for hTERTmRNA expressions were predicted as 9,332 copies/0.2 ml by stressing the higher specificity. Forty six (15%) of HCC patients, whose AFP, AFP-L3, and DCP were within normal limits, had 4.23 ± 0.32 logarithmic values of hTERTmRNA, and 20 patients of 46 patients were positive for this assay.

Multivariate analysis showed that hTERTmRNA was associated with tumor size and differentiation degree of tumor ($P < 0.001$, each, Figure 1 &3). However, hTERT-mRNA was not associated with age, gender, etiology, background lesion or number of tumor. On the other hand, AFP was related to tumor size and differentiation
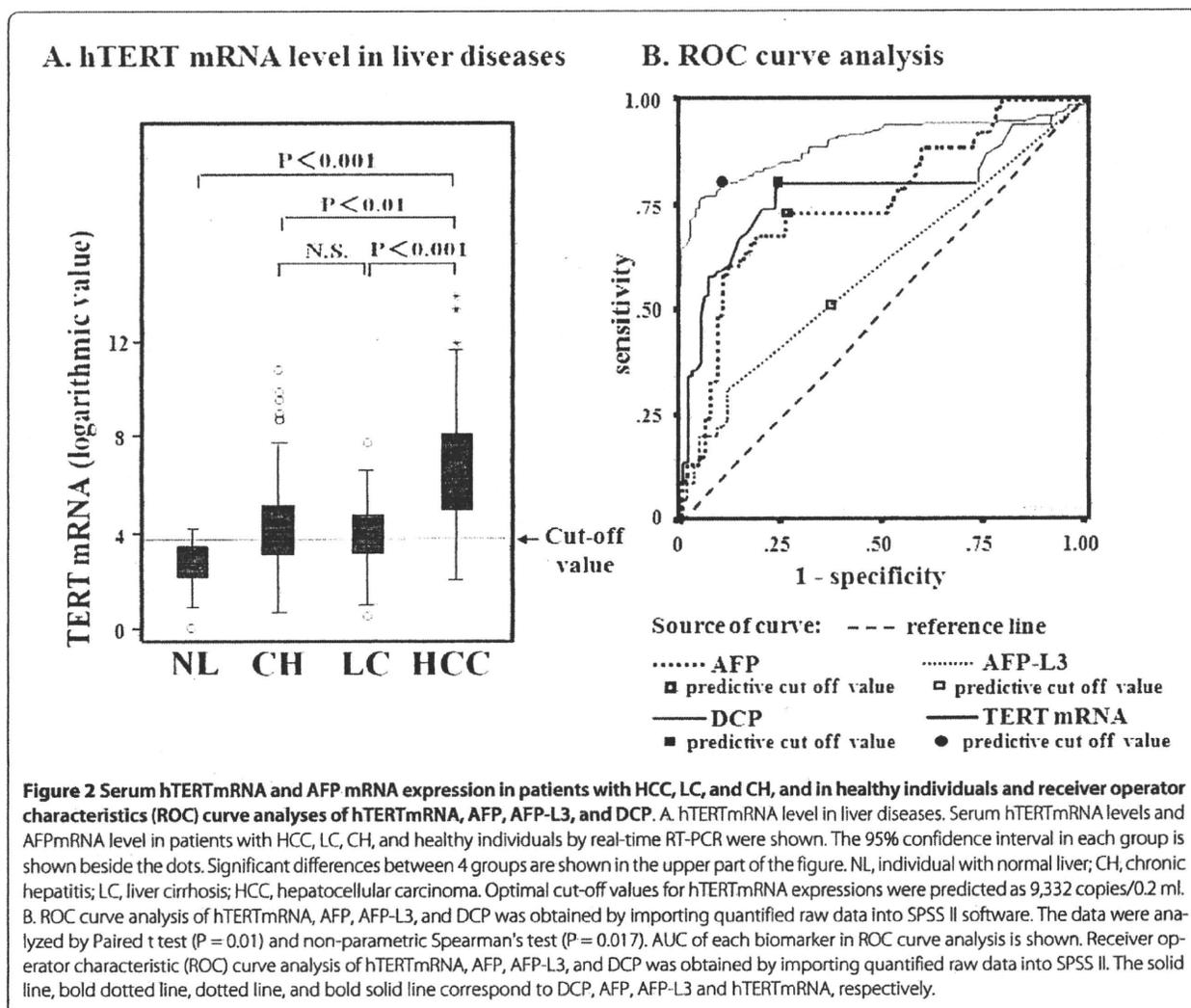
Figure 2 Serum hTERTmRNA and AFP mRNA expression in patients with HCC, LC, and CH, and in healthy individuals and receiver operator characteristics (ROC) curve analyses of hTERTmRNA, AFP, AFP-L3, and DCP. A. hTERTmRNA level in liver diseases. Serum hTERTmRNA levels and AFPmRNA level in patients with HCC, LC, CH, and healthy individuals by real-time RT-PCR were shown. The 95% confidence interval in each group is shown beside the dots. Significant differences between 4 groups are shown in the upper part of the figure. NL, individual with normal liver; CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma. Optimal cut-off values for hTERTmRNA expressions were predicted as 9,332 copies/0.2 ml. B. ROC curve analysis of hTERTmRNA, AFP, AFP-L3, and DCP was obtained by importing quantified raw data into SPSS II software. The data were analyzed by Paired t test (P = 0.01) and non-parametric Spearman's test (P = 0.017). AUC of each biomarker in ROC curve analysis is shown. Receiver operator characteristic (ROC) curve analysis of hTERTmRNA, AFP, AFP-L3, and DCP was obtained by importing quantified raw data into SPSS II. The solid line, bold dotted line, dotted line, and bold solid line correspond to DCP, AFP, AFP-L3 and hTERTmRNA, respectively.

(P = 0.008 and P = 0.0199), AFP-L3 was related to number of tumor and differentiation degree (P = 0.003 and P = 0.001), and DCP was associated with only number of tumor (P = 0.029). By Pearson relative test, serum hTERTmRNA significantly associated with tumor size and number of tumors (P < 0.033 and P < 0.003, respectively, Table 1). Importantly, hTERTmRNA was related only to DCP (P = 0.03).

ROC curve analyses showed that the sensitivity/specificity of hTERTmRNA for HCC were 90.2%/85.4% (Table 2). The sensitivity/specificity of AFP, AFP-L3, and DCP were 76.6/66.2, 60.5/88.7, and 83.4/80.3, respectively. Thus, hTERTmRNA was superior to other markers especially in sensitivity. The positive predictive value (PPV)/negative predictive value (NPV) of hTERTmRNA were 83.0/85.9. On the other hand, the PPV/NPV for AFP, AFP-L3, and DCP were 74.6/67.7, 59.6/92.2, and 78.4/73.5, respectively. Consequently, hTERTmRNA was superior to other markers in the diagnosis of HCC. Combina-

tions of hTERTmRNA with AFP level improved the sensitivity/specificity up to 96.0%/87.2%. ROC curve analysis categorized by viruses was examined and sensitivity/specificity in HBV-infected cases was similar to that of HCV-infected cases (additional file 1). hTERT and other markers in LC was not statistically and significantly different in comparison with that in CH.

Estimation of therapeutic effect and the possibility of early HCC detection of hTERTmRNA in comparison with other biomarkers

To examine the significance of hTERTmRNA before and after TAE, serum hTERTmRNA was measured before and 7 days after TAE in 16 HCC patients (Figure 4A). As a result, hTERTmRNA significantly decreased after TAE (P = 0.018), suggesting that changes in hTERTmRNA are indicative of therapeutic effects on HCC. Comparing the follow-up data of hTERTmRNA and AFP (Figure 4B, C),
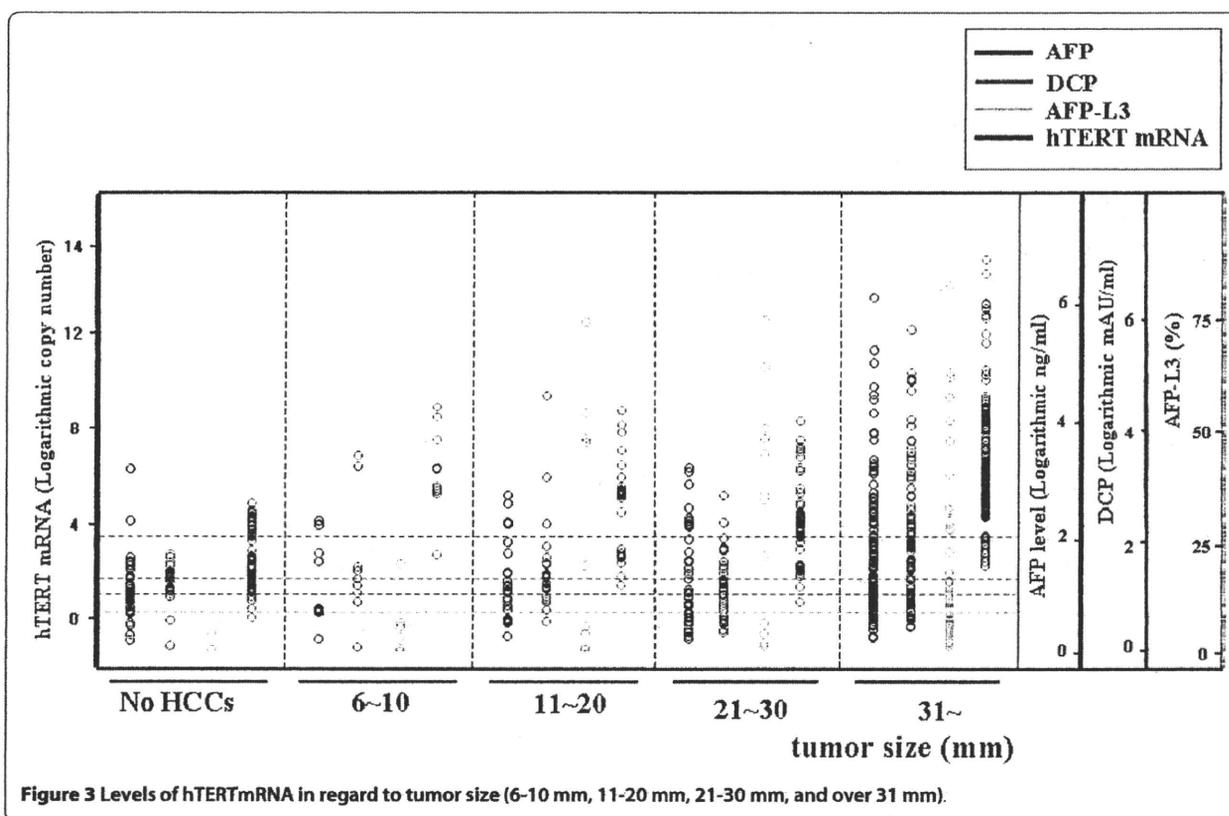
Figure 3 Levels of hTERTmRNA in regard to tumor size (6-10 mm, 11-20 mm, 21-30 mm, and over 31 mm).

the half-life of hTERTmRNA was shorter than that of AFP.

To clarify the significance of hTERTmRNA in monitoring the effect of therapies in comparison with other biomarkers, two representative cases were depicted in Figure 5. The quantification of hTERTmRNA was performed before, 2 and 5 months after RFA in a 73-year-old male patient whose HCC was a single 21 mm-sized (Figure 5A). hTERTmRNA changed similar to AFP, AFP-L3, and DCP, suggesting that hTERTmRNA is useful for monitoring the clinical course of HCC. In a 78-year-old female patient whose HCC was a single 38 mm-sized, a surgical operation was performed (Figure 5B). The values of AFP, DCP, and hTERTmRNA were measured before, 2 and 7 months after the operation. The operation was performed successfully in this patient, however recurrence was found by dynamic CT at 7 months after the operation. Although neither AFP nor DCP detected the recurrence, only hTERTmRNA did. In all the cases that hTERT detected recurrence in the earlier stage, no other imaging modality could detect it at the same time, but when we could find HCC in images such as US, CT, or MR, other markers began to arise.

Finally, we examined the relationship between the positive rates of biomarkers and tumor size. Positive rate of hTERTmRNA was higher than that of the other markers

in each category of tumor size; 6-10 mm, 11-20 mm, 21-30 mm, over 31 mm by Friedman test (P = 0.017) (Figure 3). However, the positivity of hTERTmRNA expression tended to reduce slightly in tumors with diameters that exceeded than 51 mm (5.2 ± 1.9 for 56 patients with 31-50 mm of HCC, 5.0 ± 1.8 for 43 patients with HCC over 51 mm; mean ± S.D.) (additional file 2). Dot blot regarding the correlation of hTERT mRNA quantification with tumor differentiation is shown in additional file 3. In a 6 mm HCC case, no marker other than hTERTmRNA was elevated and only abdominal US caught the evidence of HCC (Figure 6(a) A, B).

Immunohistochemistry

Immunohistochemical analysis showed that Ki-67 positivity was observed in the nuclei of cancer cells (Figure 6(b) A). hTERT was observed in both the nuclei and cytoplasm of cancer cells (Figure 6(b) B). Some TUNEL-positive cells were present in cancerous lesions, however the prevalence was low (Figure 6(b) C). hTERT expression was significantly associated with the labeling index of Ki-67 (P = 0.023). When the labeling indices of Ki-67, hTERT and TUNEL were compared with the differentiation degree of HCC, both hTERT and Ki-67 were higher in poorly differentiated HCC than in well and moderately differentiated HCC (Figure 6(b) D).

Table 1: The sensitivity/specificity of each tumor marker for hepatocellular carcinoma and a statistic evaluation of hTERTmRNA level to clinical parameter were shown.

clinical parameter	average ± S.E.	Pearson test P value	Multivariate analysis P value
tumor size (mm)	21.2 ± 0.1	0.033	<0.001
(range: 6-90)			
tumor number	1.8 ± 0.1	0.003	N.S.
tumor differentiation		N.S.	<0.001
AFP (ng/ml)	6146 ± 4554	N.S.	N.S.
(n = 353)			
AFP-L3 (%)	6.7 ± 1.0	N.S.	N.S.
(n = 213)			
DCP (mAU/ml)	18780 ± 1044	0.03	N.S.
	n = 346)		

Discussion

Since HCC has been recently classified as a complex disease with a wide range of risk factors and many cellular signaling pathways have been reported to be involved in hepatocarcinogenesis, a novel biomarker for HCC is required [21]. Since an epoch-making assay to detect telomerase activity was established [11], telomerase has been examined in many kinds of cancers, precancerous lesions and normal tissues using the telomeric repeat amplification protocol and investigated the correlation with telomere length [29,30]. Notwithstanding that telomerase was definitely an unprecedented candidate tumor marker due to its specificity to cancer, it has clinically remained inapplicable because telomerase expres-

Table 2: The sensitivity/specificity of each tumor marker for HCC was depicted.

|	Sensitivity	Specificity	OR	PPV/NPV	Cut-off point
hTERTmRNA	90.2	85.4	19.0	83.0/85.9	3. 97 (logarithmic copy number)
AFP	76.6	66.2	11.1	74.6/67.7	<10 (ng/ml)
AFP-L3	60.5	88.7	2.2	59.6/92.2	<10 (%)
DCP	83.4	80.3	7.6	78.4/73.5	<40 (mAU/ml)

The sensitivity/specificity values are 90.2%/85.4% for hTERTmRNA, 76.6%/66.2% for AFP, 60.5%/88.7% for AFP-L3, and 83.4%/80.3% for DCP. Regarding a diagnostic assessment in sensitivity and specificity, hTERTmRNA is identified as the most excellent tumor marker. OR: odds ratio, PPV: positive predictive value (%), NPV: negative predictive value (%).

A. The change in hTERT mRNA before and after TAE treatment
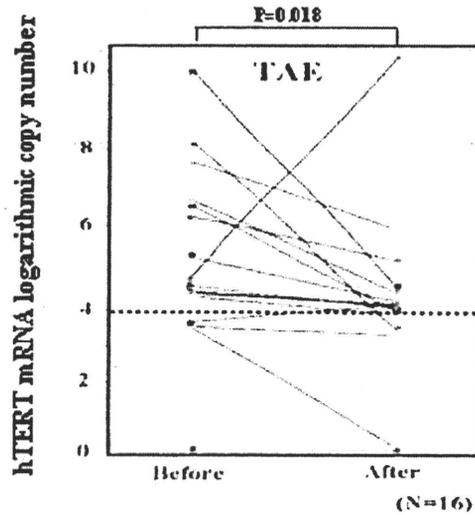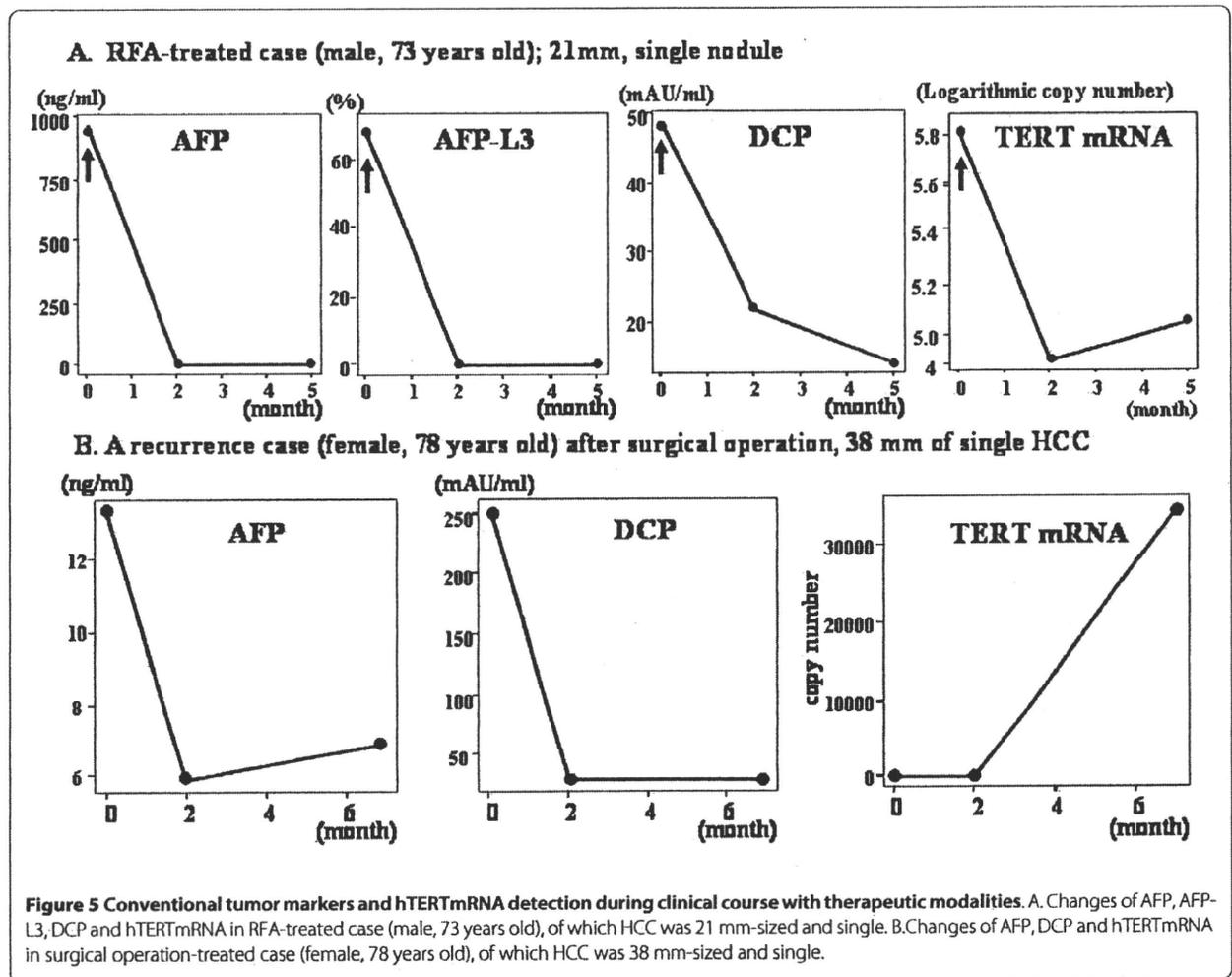

B. Follow-up of hTERT mRNA after TAE

C. Follow-up of AFP after TAE
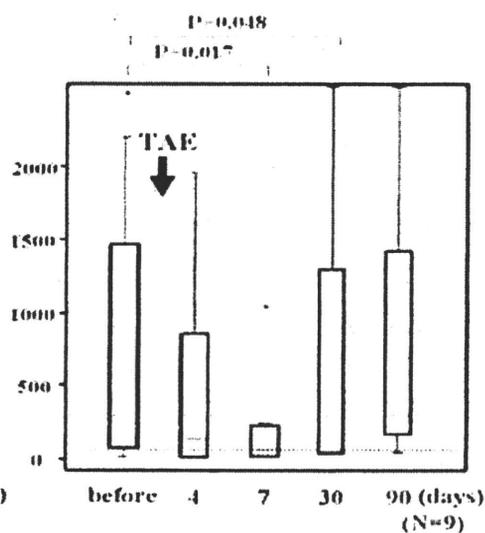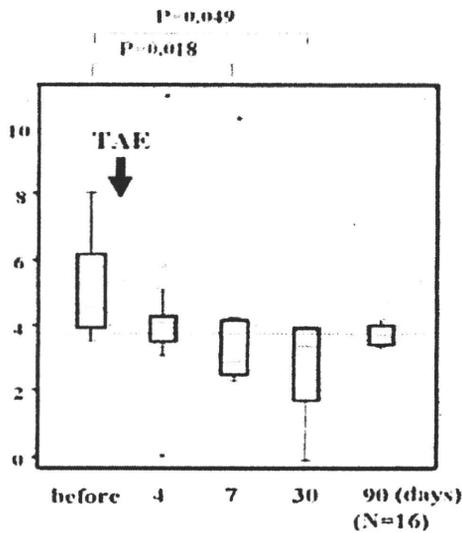
Figure 4 The change of hTERTmRNA before and 7days after TAE. A. Follow-up of serum hTERTmRNA before, 4, 7, 30 and 90 days after TAE. B. Follow-up of serum AFP before, 4, 7, 30 and 90 days after TAE.

sion has not been detected stably in body fluid [12]. In serum, the hTERTmRNA derived from cancer cells seemed to be undetectable because it becomes instable by RNase in blood. Since RNAs in serum are unexpectedly stable within 24 hrs after drawing blood due to particle-associated complex in structure [13,14], it has been suggested that they can be generally detected even in RNase-rich blood. Actually, hTERTmRNA can be detected in serum from breast cancer patients and its maximum sensitivity and specificity are at most 40% and 100%, respectively [4]. The sensitivity in patients with HCC rose to 89.7% in the semi-quantitative assay, and

thus compared favorably with the previous findings in which the sensitivity and specificity of AFPmRNA were 69% and 50% for HCC, respectively [31]. Besides, with respect to HCC detection, AFPmRNA was superior to AFP level used routinely in clinic [32]. Recently, in the present study, we reported the sensitivity to detect the nucleotides in blood in the process of RNA extraction, including centrifugation steps less than 1500 × g to remove cellular proteins in serum and a primer set that can detect hTERTmRNA more efficiently than primers in the previous reports (data not shown). We previously reported that hTERT expression was very faint in the

A. RFA-treated case (male, 73 years old); 21mm, single nodule

B. A recurrence case (female, 78 years old) after surgical operation, 38 mm of single HCC

Figure 5 Conventional tumor markers and hTERTmRNA detection during clinical course with therapeutic modalities. A. Changes of AFP, AFP-L3, DCP and hTERTmRNA in RFA-treated case (male, 73 years old), of which HCC was 21 mm-sized and single. B.Changes of AFP, DCP and hTERTmRNA in surgical operation-treated case (female, 78 years old), of which HCC was 38 mm-sized and single.
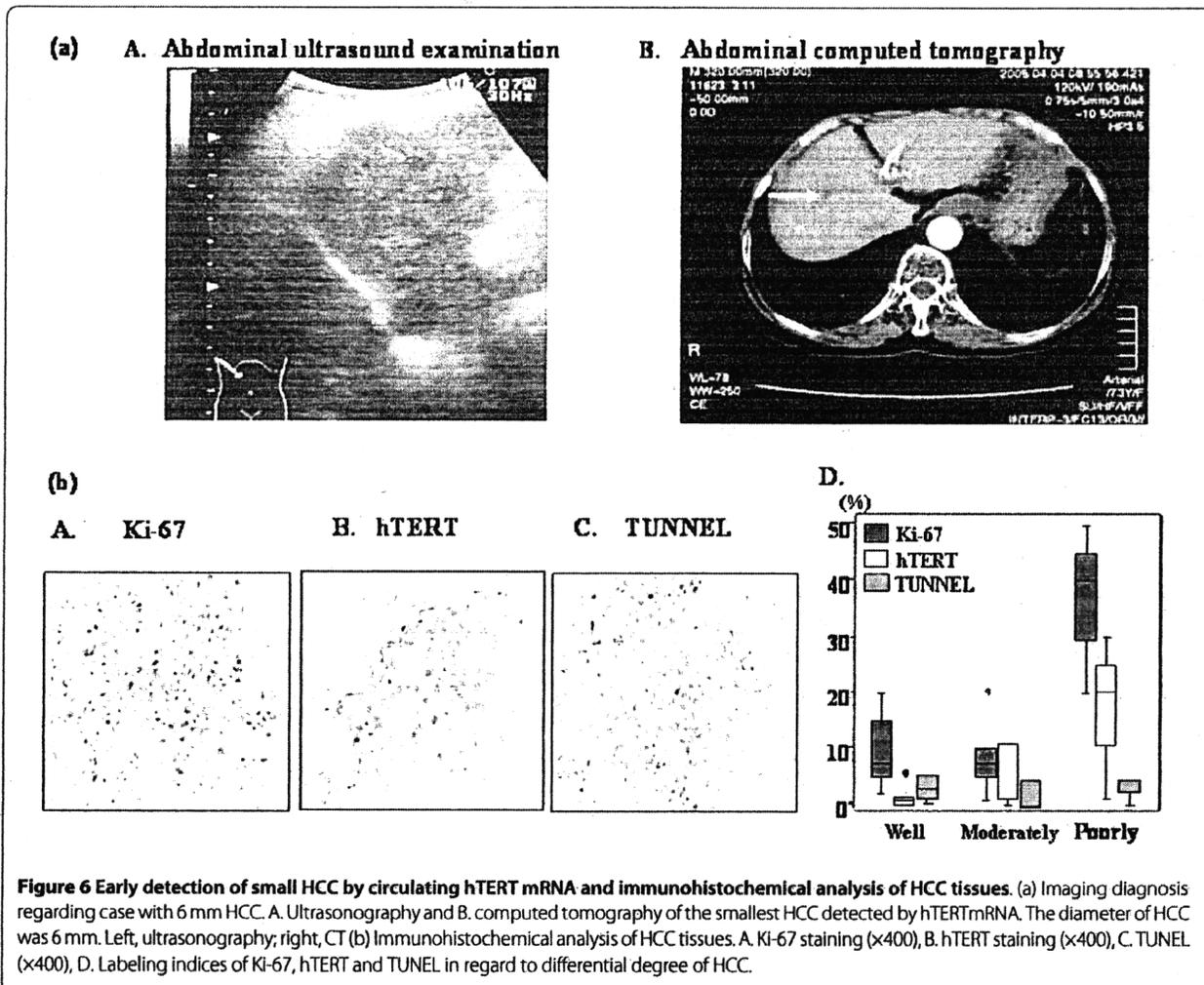
serum from normal individuals indicating that lymphocytes and circulating normal cells express very low levels of hTERTmRNA [9]. Because hTERTmRNA in lymphocytes is very low, elevated hTERTmRNA levels in serum may mean that hTERTmRNA is derived from cancer cells. Since we could detect negligible amounts of lymphocyte markers after three steps of centrifugation of blood samples, the RNA extraction procedure seemed to remove lymphocytes effectively. In addition, normal or damaged hepatocytes express negligible amounts of hTERT [33,34]. Furthermore, we previously showed the significant correlation of hTERTmRNA expression between tumor tissue and serum [32]. These data suggest that hTERTmRNA detected in serum is derived from tumor cells.

Previously, we reported that qualitative analysis of serum hTERTmRNA was superior to AFP for the purpose of the early detection of HCC, because hTERTmRNA was detectable in HCC patients with normal AFP levels [9]. AFP is being widely used as a reliable marker of HCC not in earlier stage but in the advanced stage [35]. However,

in this study, neither AFP was able to distinguish HCC from non-cancerous liver diseases, nor hTERTmRNA was correlated with AFP level $(P = 0.201)$, suggesting that quantitative analysis of serum hTERTmRNA was much more sensitive for HCC diagnosis even in the early stage. Because the induction of the abdominal (enhanced-)US, CT, and MRI into the clinical scene enabled us to detect smaller-sized HCC [36], the sensitivity of AFP in the early detection of HCC became less than 70%. Unlike AFP level, AFPmRNA was significantly correlated with hTERTmRNA $(P < 0.001)$ and more sensitive than AFP. In the present study, we measured AFP-L3, since AFP-L3 has been reported to be a more HCC-specific marker than AFP [37]. Indeed, the level of AFP-L3 correlated significantly with differentiation and number of HCC although that of AFP was correlated with tumor size and differentiation.

In the present study, of 303 HCC patients, 24 patients were negative below the calculated cut-off value (9,332; 3.97 as logarithmic number) for serum hTERTmRNA. Although the reason why hTERTmRNA was negative in

Figure 6 Early detection of small HCC by circulating hTERT mRNA and immunohistochemical analysis of HCC tissues. (a) Imaging diagnosis regarding case with 6 mm HCC. A. Ultrasonography and B. computed tomography of the smallest HCC detected by hTERTmRNA. The diameter of HCC was 6 mm. Left, ultrasonography; right, CT (b) Immunohistochemical analysis of HCC tissues. A. Ki-67 staining (×400), B. hTERT staining (×400), C. TUNEL (×400), D. Labeling indices of Ki-67, hTERT and TUNEL in regard to differential degree of HCC.

these patients is not clear, eleven of 24 hTERTmRNA-negative HCC patients had decompensated liver cirrhosis as the underlying disease. It has been reported that decompensated liver cirrhosis had higher levels of serum TGF-β that promotes apoptosis of immortalized hepatocytes and, in these cases, elevated TGF-β may stimulate apoptosis, resulting in reduction of hTERTmRNA [34,38,39]. hTERT-negative cases had no other common characteristics with age, gender, etiology, child classification etc. than tumor size, ALT, and surrounding lesion. In 23 cases (95.8%), ALT was within 1.5 fold normal limits. In 17 cases (70.8%), surrounding lesion was LC including decompensated situation. Tumor size in 12 cases (50%) was over 30 mm, reflecting on the biological features of cancer itself, as referred in Norton-Simon models regard tumor growth [40]. AFP and DCP were positive in 16 (66.7%) and 11 (45.8%) cases, respectively, suggesting that combinative use of these markers contributes to improve the diagnostic specificity.

Thus, hTERTmRNA is not only improved in both sensitivity and specificity but has a close correlation with tumor size and number in an early stage of HCC. Since HCC repeatedly recurs polyclonally after any treatment as a biological characteristic, the measurement of serum hTERTmRNA makes it possible to recognize recurrence or therapeutic effect in details as well as the usefulness for one-point diagnosis. In this respect, we have to undergo follow-up study after the treatment of HCC [24]. hTERTmRNA expression was closely associated with well to moderate differentiation degree of HCC and was enhanced with the proliferation. We should clarify that serum hTERTmRNA can be detected by what alterations of other molecules during the cancer progression [41-43]. In lower differentiated HCC, tumor cells are proliferating and hTERTmRNA has a tendency to correlate with the differentiation degree and an apoptotic event never reflect on the serum detection of cancer cell-derived mRNAs (Figure 6). Nakashio et al. previously reported the significant correlation of HCC differentiation with

telomerase expression [44]. The results in the present study confirmed their findings. hTERTmRNA showed more sensitivity and specificity compared with AFPmRNA in HCC patients. However, in liver diseases other than HCC, hTERTmRNA was not correlated with AFPmRNA. The higher specificity of hTERTmRNA in HCC may be related to fact that AFPmRNA is produced in HCC cells and injured hepatocytes and hTERT is produced mainly in HCC cells. Furthermore, we could detect serum hTERTmRNA expression even in HCC patients with less than 10 mm moderate-differentiated tumor, indicating that hTERT are upregulated during rapid proliferation of tumor at the early phase of oncogenesis, dedifferentiation.

Waguri et al. proved that there exist circulating cancer cells derived from original HCC tissues in blood and they can detect hTERTmRNA in blood [45]. The present study suggests that quantification of hTERTmRNAs in serum has diagnostic implications for HCC. Unless apoptosis of cancer cells contributes to the early detection of HCC using serum mRNA, the essence may be immunoreactions [46]. The development of micro vessels may be also involved in the step [47]. We will evaluate the correlation of prognosis with hTERTmRNA and the availability of hTERTmRNA in other cancers by comparison of hTERTmRNA with other tumor markers [48], and will study its usefulness for inflammatory diseases in which cellular reactions are active [49]. This method depends on RNA stability in each process of RNA purification, storage, and quantification. In the light of its superior positivity to other markers, the assay will be applied for clinical use in the strict condition because it is required to keep the serum RNA as it is in blood and avoid the degradation of RNA quality. Now we are improving RNA stability and PCR condition to better cost/benefit of this assay. In the future, another large-scale study will be required to confirm our results for monitoring HCC and the feasibility for its detection even on a primary care level.

Conclusions

In sum, our results support the suggestion that quantification of circulating hTERTmRNA expression is clinically useful for the early detection of HCC. Furthermore, hTERTmRNA is superior to conventional tumor markers in the diagnosis and recurrence of HCC at the early stage.

Additional material

Additional file 1 TIF ROC curve analysis and AUC in measurement categorized by viruses. ROC curve analysis and AUC in measurement categorized by viruses are demonstrated. Sensitivity/specificity of hTERTmRNA expression in HBV-infected cases is similar to that in HCV-infected cases.

Additional file 2 MS word Positivity of each marker for HCC. Positivity of each marker for HCC was shown, categorized by tumor size.

Additional file 3 TIF Dot blot regarding the correlation of hTERTmRNA quantification with tumor differentiation. Serum hTERTmRNA quantification in HCC patients (n = 101) diagnosed by liver biopsy was shown, categorized by tumor differentiation. The quantification in serum of HCC patients with well-/moderately-/poorly-/un-differentiation was 4.4 ± 1.4/5.4 ± 2.0/6.3 ± 3.3/5.9 ± 1.8 (mean ± SD).

Abbreviations
(h)TERT: (human) telomerase reverse transcriptase protein; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; HBV: hepatitis B virus; LC: liver cirrhosis; CH: chronic hepatitis; AFP: α-fetoprotein; DCP: des-γ-carboxy prothrombin; ALT: alanine aminotransferase; Alb: albumin; CNA: Circulating nucleic acids.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
YO analyzed biomedical data and provided blood sample as main researcher in Osaka Red Cross Hospital. MN analyzed biomedical data in Kinki University. MK analyzed biomedical data and provided blood sample as main researcher in Kinki University. KY analyzed biomedical data and provided blood sample in San-in Labor Welfare Hospital. TK analyzed clinical data and the practical analysis in San-in Labor Welfare Hospital. KO analyzed HCC imaging data and the analysis in Saiseikai Gotsu General Hospital. YK was in charge for case study in Saiseikai Gotsu General Hospital. SM analyzed biomedical data and provided blood sample as main researcher in Saiseikai Gotsu General Hospital EN was in charge for case study and biomedical analysis in Saiseikai Gotsu General Hospital. YH analyzed clinical and biomedical data as main researcher in Saiseikai Gotsu General Hospital comprehensively. MK analyzed biomedical data and provided blood sample as main researcher in Matsue City Hospital. SS analyzed biomedical data and provided blood sample as main researcher in Fukuoka University Chikushi Hospital. YH performed biomedical and clinical analysis in surgical case in Tottori University. HK analyzed biomedical data and provided blood sample as chief researcher in San-in Labor Welfare Hospital. JH provided the environment to analyze the data comprehensively. All authors read and approved the final manuscript.

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Pre-publication history
The pre-publication history for this paper can be accessed here:
http://www.biomedcentral.com/1471-230X/10/46/prepub

A multi-institution phase II study of gemcitabine/S-1 combination chemotherapy for patients with advanced biliary tract cancer

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Abstract

Purpose We aimed to evaluate the efficacy and safety of gemcitabine/S-1 combination chemotherapy for the treatment of patients with advanced biliary tract cancer.

Methods Patients with histologically or cytologically confirmed unresectable or recurrent biliary tract cancer were eligible for inclusion. The primary endpoint was overall survival. Gemcitabine was administered intravenously at a dose of 1,000 mg/m^2 over 30 min on days 1 and 8, and oral S-1 was administered daily at a dose of 60 mg/m^2 on days

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1–14. This schedule was repeated every 3 weeks until disease progression or patient refusal.

Results Twenty-five patients were enrolled between October 2007 and January 2009. Eleven patients (44%) had extrahepatic bile duct cancer, 5 (20%) had intrahepatic bile duct cancer, 8 had gallbladder cancer (32%), and 1 (4%) had ampulla of Vater cancer. The median overall survival time was 12.7 months (95% CI, 8.4–23.5 months), and the 1-year survival rate was 52.0% (95% CI, 31.2–69.2%). Of the 23 patients with evaluable target regions, seven patients experienced a partial response, and an overall response rate was 30.4%. The following grade 3–4 hematological toxicities occurred: neutropenia (56%), leukopenia (24%), anemia (8%) and thrombocytopenia (4%). In spite of the high incidence of grade 3–4 neutropenia, no patients developed febrile neutropenia in the present study. The major grade 3–4 non-hematological toxicities were fatigue (8%), anorexia (8%) and diarrhea (4%).

Conclusions Gemcitabine/S-1 combination chemotherapy offered a promising survival benefit with acceptable toxicity in patients with advanced biliary tract cancer.

Keywords Biliary tract cancer · Gemcitabine · S-1 · Chemotherapy

Introduction

Biliary tract cancer is one of the most lethal malignancies worldwide, with surgery representing the only potentially curative treatment for this disease. However, many patients are diagnosed too late for curative resection, and even if surgery can be performed, the likelihood of relapse is very high [7, 13]. Over the past decade, gemcitabine has been widely used to treat unresectable or recurrent biliary tract

cancer [3, 4, 9, 17, 18, 23, 27], although no phase III trials have established this drug as a standard treatment for advanced biliary tract cancer. We have previously evaluated the outcome of consecutive 22 patients with advanced biliary tract cancer who received gemcitabine monotherapy as first line and reported that median survival time (MST) was 8.3 months (95% CI 6.4–11.2 months) [9].

In the ABC-02 study, the first prospective multicenter phase III study in this field, gemcitabine/cisplatin combination chemotherapy was compared with gemcitabine monotherapy. The study found that the combination regimen significantly prolonged MST (from 8.1 to 11.7 months; $P < 0.001$) [26]. The superiority of gemcitabine/cisplatin combination chemotherapy over gemcitabine monotherapy was also demonstrated in a randomized phase II study conducted in Japan (the BT-22 study) [6]. Given these findings, gemcitabine/cisplatin combination chemotherapy is now becoming accepted as a new standard regimen for advanced biliary tract cancer.

S-1 is an oral fluoropyrimidine prodrug that has confirmed efficacy against various solid tumors, both alone and in combination with other cytotoxic drugs [1, 12, 14, 19, 29]. S-1 monotherapy has yielded good results against advanced biliary tract cancer [5, 24], and gemcitabine/S-1 combination therapy has yielded promising results with acceptable toxicity levels for patients with advanced pancreatic cancer [15, 16, 28]. At the time of planning this clinical trial in 2007, there had been no reports on gemcitabine/S-1 combination chemotherapy for patients with advanced biliary tract cancer, so we designed this clinical trial to determine its efficacy and safety in this context.

Patients and methods

Eligibility criteria

Patients with advanced biliary tract cancer that was not amenable to potentially curative surgery or that had recurred after surgery were eligible for inclusion if they met the following criteria: histologically or cytologically confirmed biliary tract cancer; Eastern Cooperative Oncology Group performance status of 0–2; age ≥ 20 years; adequate bone marrow function (neutrophil count $\geq 1,500/mm^3$, and platelet count $\geq 100,000/mm^3$), liver function (total bilirubin ≤ 3 times the upper limit of normal (ULN) and aspartate aminotransferase [AST]/alanine aminotransferase [ALT] ≤ 5 times ULN) and renal function (creatinine ≤ 1.5 mg/dL); adequate oral intake; life expectancy ≥ 3 months. All patients provided written informed consent. Exclusion criteria included a history of chemotherapy or radiotherapy (patients who had undergone adjuvant chemotherapy were not excluded if at least 6 months had passed since the last

administration), pregnancy or lactation, a history of severe drug allergy and other severe comorbid diseases. This phase II study (UMIN ID 000000792) was conducted in five institutions in Japan. The protocol was approved by the institutional review board at each institution, and patient registration and data management were conducted at an independent data center at Translational Research Center, Kyoto University Hospital. All procedures were performed in accordance with the 1964 Declaration of Helsinki.

Treatment

Gemcitabine was infused at a dose of 1,000 mg/m^2 over 30 min on days 1 and 8. S-1 was given orally twice a day for 14 consecutive days. Doses of S-1 were calculated according to body surface area (BSA) as follows: BSA < 1.25 m^2, 60 mg/day; 1.25 m$^2 \leq$ BSA < 1.5 m^2, 80 mg/day; BSA ≥ 1.5 m^2, 100 mg/day. The gemcitabine and S-1 treatment regimen was repeated every 3 weeks. Doses were reduced in response to adverse effects (graded according to the Common Terminology criteria for Adverse Events v 3.0) [22].

Chemotherapy was started if on day 1 the neutrophil count was $\geq 1,500/mm^3$, platelet count was $\geq 100,000/mm^3$, total bilirubin was ≤ 3 times the ULN, AST/ALT was ≤ 5 times ULN, and there were no non-hematological toxicities of grade 3 or higher (except for abnormal blood test results not relevant to the chemotherapy regimen). Chemotherapy was continued if on day 8 the neutrophil count was $\geq 1,000/mm^3$, platelet count was $\geq 75,000/mm^3$, total bilirubin was ≤ 3 times the ULN, AST/ALT was ≤ 5 times the ULN, and there were no non-hematological toxicities of grade 3 or higher. If the patient did not meet the above criteria, chemotherapy was delayed by 1 week. If neutropenia (grade 3–4), thrombocytopenia (grade 3–4), febrile neutropenia or non-hematological toxicity associated with gemcitabine (grade 3) occurred, the subsequent gemcitabine dose was reduced to 800 mg/m^2. If further toxicity occurred with the reduced dose, it was further reduced to 600 mg/m^2. If a further dose reduction was necessary, the subsequent gemcitabine dose was reduced by 20%. If diarrhea or stomatitis (grade 3–4) associated with S-1 occurred, S-1 was discontinued, and patients were withdrawn from the study. No dose reescalation was allowed. The treatment regimen was continued until disease progression, unacceptable toxicity including non-hematological toxicity of grade 4 or patient refusal occurred.

Pretreatment and follow-up evaluation

Pretreatment evaluation included obtaining the patient's medical history and performing a physical examination, imaging using contrast-enhanced computed tomography

(CT) or magnetic resonance imaging (MRI), a complete blood cell count, serum biochemical tests, an electrocardiogram and chest X-rays. During the treatment cycles, physical examinations and blood tests were scheduled on days 1 and 8. Carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) were measured at the time patients were enrolled in the study and every month thereafter. Toxicity was evaluated using the Common Terminology criteria for Adverse Events v3.0.

Statistical analysis

The primary endpoint was overall survival. The secondary endpoints were toxicity and response rate. Twenty-five patients were enrolled, a sample size that would allow rejection of a null hypothesis of a 30% 1-year survival rate and acceptance of an alternative hypothesis of a 50% 1-year survival rate, with a significance level of 0.05 and a power of 80%. Overall survival was calculated using the Kaplan–Meier method and was defined as the time from initiation of therapy to death from any cause or the final follow-up. Among patients with measurable target lesions, the objective response rate was evaluated according to Response Evaluation Criteria in Solid Tumors (RECIST) version 1.0 [21]. Patients were enrolled between October 2007 and January 2009, and the final analysis was conducted in January 2010 after a 1-year follow-up period. All analyses were conducted on an intention-to-treat basis and were performed using SAS version 9.2 (SAS Institute, Cary, NC, USA).

Results

Patient characteristics

Twenty-five patients were enrolled between October 2007 and January 2009. The patient characteristics are shown in Table 1. The median age was 63 years (range 32–78 years), and 18 patients (72%) were men. Four of the 25 patients (16%) experienced recurrent disease after undergoing curative surgery. Out of the 21 patients with unresectable disease, distant metastasis was reported in 13 patients at the time of enrollment. Eleven patients (44%) had extrahepatic bile duct cancer, 8 had gallbladder cancer (32%), 5 (20%) had intrahepatic bile duct cancer, and 1 (4%) had ampulla of Vater cancer.

Efficacy

Seventeen patients (68%) died during the study period. The median overall survival time was 12.7 months (95% CI, 8.4–23.5 months), and the 1-year overall survival rate was

Table 1 Characteristics of patients with advanced biliary tract cancer ($n = 25$)

Sex	
Male	18 (72.0%)
Female	7 (28.0%)
Median age (years)	63 (range 32–78)
Primary lesion	
Intrahepatic	5 (20.0%)
Extrahepatic	11 (44.0%)
Gallbladder	8 (32.0%)
Ampulla of Vater	1 (4.0%)
Disease status	
Unresectable	21 (84.0%)
Recurrent*	4 (16.0%)
Target lesion	
Primary	18
Liver	7
Lymph node	3
Peritoneum	2
Local recurrence	2
Lung	1
None	2
Median no. treatment cycles	7 (range 1–20)
Median CEA (ng/mL)	4.5 (range 0.3–468)
Median CA19-9 (U/mL)	167 (range 1–6,373)

CEA carcinoembryonic antigen, *CA 19-9* carbohydrate antigen

* One patient had a history of adjuvant chemotherapy using gemcitabine

52% (95% CI, 31.2–69.2%, $P = 0.02$ under a null hypothesis of 30%). Of the 23 patients with target regions that were evaluable according to RECIST, 7 (30.4%) experienced a partial response, and 13 (56.5%) had stable disease, with an overall disease control rate of 87.0%.

Toxicity

In total, 229 cycles of gemcitabine/S-1 combination chemotherapy were delivered, with a median of 7 cycles per patient (range 1–20 cycles; Table 1). The mean relative dose intensities of gemcitabine and S-1 were 75% and 84%, respectively. The incidence rates of hematological and non-hematological adverse events are summarized in Tables 2 and 3, respectively. The most common grade 3–4 hematological toxicity was neutropenia (56%); however, no instances of febrile neutropenia were observed in this study. The incidence rates of grade 3–4 anemia and thrombocytopenia were 8% and 4%, respectively. Grade 3–4 hyperbilirubinemia and ALT were observed in 16% and 8% of patients, respectively, mostly associated with obstructive jaundice caused by the primary disease. Other grade 3–4

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