lower cumulative survival rates than group that received a TACE repeat only when tumor growth was detected.

Systemic therapy

Recommendations
Sorafenib is recommended for the treatment of advanced stage patients (portal vein invasion or extrahepatic spread) who are not suitable for locoregional therapy and who have C-P class A liver function (1b, A).
Sorafenib may be used with caution in patients with C-P class B liver function (C).
Cytotoxic drugs are not routinely recommended but may be considered in highly selected patients whose general and hepatic conditions are adequate (3, C).

Recent advances in elucidating the molecular mechanisms of hepatocarcinogenesis have provided opportunities to develop molecular targeted therapy (MTT) for advanced HCC [344]. Sorafenib, an oral multikinase inhibitor, has shown survival benefit in two randomized, placebo-controlled trials [345, 346]. Several agents targeting tumor angiogenesis have also shown antitumor activity in patients with advanced HCC. Selected clinical trials of MTT for advanced HCC are summarized in Table 2.

Sorafenib

Sorafenib inhibits the kinase activity of both wild-type B-raf (IC_{50} = 6 nM) and mutant Raf^{V600E} (IC_{50} = 38 nM). In addition, sorafenib inhibits vascular endothelial growth factor receptors (VEGFR), platelet-derived growth factor receptors (PDGFR), c-kit, Flt-3, and RET (IC_{50} < 100 nM) [347]. Therefore, both antiproliferative and antiangiogenic mechanisms may account for the antitumor effects of sorafenib.

Two randomized, placebo-controlled trials of sorafenib for the treatment of advanced HCC have been reported [345, 346]. The first trial (SHARP trial) was conducted primarily in Europe and the United States with the primary end point of overall survival. The second trial was designed originally as a bridging study to evaluate the overall efficacy and safety of sorafenib in the Asia-Pacific population. Both trials recruited HCC patients whose tumors were not eligible for or had progressed after surgery or locoregional therapy, and patients with C-P class A liver function and Eastern Cooperative Oncology Group (ECOG) performance score was 2 or less. The treatment regimen was the same (sorafenib 400 mg twice daily). Both trials were stopped early because per-protocol interim analysis indicated significant survival benefit of sorafenib over placebo.

Patients in the Asia-Pacific trial were younger, had more symptomatic disease (ECOG score = 1 or 2), and extrahepatic metastases. Despite these differences in the baseline prognostic features, the overall treatment efficacy of sorafenib was similar between these two trials. The hazard ratios of overall survival and time to progression were 0.69 and 0.58 in the SHARP trial and 0.68 and 0.57 in the Asia-Pacific trial. Exploratory subgroup analyses of the two trials indicated that sorafenib treatment prolonged survival regardless of patients' age, performance status, and tumor burden (vascular invasion or extrahepatic spread). Time to symptomatic progression was not significantly different between patients who received sorafenib and patients who received placebo in either trial. Sorafenib is generally well tolerated. The most common drug-related adverse events included diarrhea, fatigue, hand-foot skin reaction, and rash/desquamation. These events occurred in 20–40% of patients, most of which were grade 1 or 2. The most common causes of treatment interruption or dose reduction were hand-foot skin reaction, rash, and diarrhea.

The efficacy and safety issues in patients with C-P class B cirrhosis need further clarification. A pharmacokinetic study suggested that patients with elevated bilirubin levels had lower tolerance to sorafenib treatment [348]. In the phase II trial of sorafenib for HCC, stable disease for 4 or more months was noted in 49% of patients with C-P class A cirrhosis (n = 98) and 26% of patients with C-P class B cirrhosis (n = 38). Patients with C-P class B cirrhosis had higher rate of elevated bilirubin (18 vs. 40%), encephalopathy (2 vs. 11%), and worsening ascites (11 vs. 18%) than patients with C-P class A cirrhosis, despite a similar incidence of all other adverse events and serious adverse events between these two groups of patients [349]. In the phase III SHARP trial, the incidence of serious hepatobiliary events was similar between the sorafenib group (11%) and the placebo group (9%). There are no clinical data for patients with C-P class C cirrhosis.

Antiangiogenic MTT

Hepatocellular carcinoma is typically a hypervascular tumor. Many antiangiogenic MTT have been tested for the treatment of HCC. The monoclonal anti-VEGF antibody bevacizumab has been tested at a dosing schedule of 5 or 10 mg/kg every 14 days in patients with advanced HCC [350]. The objective response rate was 13% (1 complete and 5 partial response in 46 patients). The median overall survival and progression-free survival were 12.4 and 6.9 months, respectively. The results suggest that bevacizumab may have a role in the treatment of patients with advanced HCC. The most common grade 3 or 4 toxicities included hypertension (15%), bleeding (11%), and thrombosis (6%). Careful evaluation of bleeding risk, such as esophageal and gastric varices, is recommended before the use of bevacizumab or similar agents.

Sunitinib is a multitarget tyrosine kinase inhibitor that inhibits tumor angiogenesis through its inhibition of

Table 2 Selected clinical trials of molecular targeted therapy for advanced HCC

	Treatment	Patient no.	Objective response	Median survival (months)				Level of evidence
				OS		TTP		
Phase III trials								
Llovet et al. [345]	Sorafenib 400 mg bid	299	RR: 2.3% (7 PR) SD: 71%	10.7	$P < 0.001$	5.5	$P < 0.001$	1b
	Placebo	303	RR: 0.7% (2 PR) SD: 67%	7.9		2.8		
Cheng et al. [346]	Sorafenib 400 mg bid	150	RR: 2.7% (4 PR) SD: 55%	6.5	$P = 0.014$	2.8	$P < 0.001$	1b
	Placebo	76	RR: 1.32% (1 PR) SD: 29%	4.2		1.4		
Phase II trials								
Siegel et al. [350]	Bevacizumab 5–10 mg/kg every 2 weeks	46	RR: 13% (1 CR and 5 PR) SD: 65% (progression free at 6 months)	12.4		6.9 (PFS)		4
Zhu et al. [352]	Sunitinib 37.5 mg qd for 4 weeks, followed by 2-week rest	34	RR: 2.9% (1 PR) SD: 47%	9.9		4.0		4
Faivre et al. [353]	Sunitinib 50 mg qd for 4 weeks, followed by 2-week rest	37	RR: 2.7% (1 PR) SD: 35.1%	10.3		4.8		4
Hsu et al. [356]	Thalidomide 100 mg bid	63	RR: 6.3% (1 CR, 3 PR in 63 evaluable patients)	4.3		NA		4
Patt et al. [357]	Thalidomide 400 mg qd	32	RR: 3.2% (1 PR in 32 evaluable patients) SD: 31%	6.8		NA		4
Philip et al. [368]	Erlotinib 150 mg qd	38	RR: 9% (3 PR in 34 evaluable patients) SD: 50%	13		3.2		4
Thomas et al. [369]	Erlotinib 150 mg qd	40	RR: 0 SD: 42.5%	10.8		6.5		4
O'Dwyer et al. [370]	Gefitinib 250 mg qd	31	RR: 3.2% (1 PR) SD: 22.6%	6.5		2.8 (PFS)		4
Zhu et al. [371]	Cetuximab 400 mg/m^2 loading, then 250 mg/m^2/week	30	RR: 0 SD: 16.7%	9.6		1.4 (PFS)		4

OS overall survival, *TTP* time to progression, *RR* response rate, *SD* stable disease, *PR* partial response, *PFS* progression-free survival

VEGFR and PDGFR activity [351]. Other targets of sunitinib include stem-cell factor receptor, colony-stimulating factor 1 (CSF-1), RET, and Flt-3. Two phase II trials of sunitinib for patients with advanced HCC reported a tumor stabilization rate of about 40% [352, 353]. Decreased tumor perfusion after sunitinib was demonstrated by dynamic computed tomography and magnetic resonance imaging, suggesting angiogenesis inhibition an important mechanism of its antitumor activity. Sunitinib at a daily dose of 50 mg was associated with a higher incidence of grade 3–5 toxicity, including ascites, edema, bleeding, and hepatic encephalopathy. At a daily dose of 37.5 mg, the most common toxicities included neutropenia, lymphopenia, thrombocytopenia, elevation of transaminases, fatigue, and skin rash. A phase III, randomized trial comparing the antitumor activity of sunitinib and sorafenib is under way.

Thalidomide showed antiangiogenic properties in the early 1990s and has been tested for the treatment of various cancers [354, 355]. Several phase II studies have explored the efficacy of thalidomide as a treatment of advanced HCC [21, 356–358]. Objective response, defined as complete and partial responses, was found in approximately 5% of the patients. In addition, about 10–30% of patients had disease stabilization for more than 2–4 months after thalidomide treatment. Disease stabilization after thalidomide treatment was associated with decreased tumor vascularity [359] and decreased blood perfusion [360], suggesting that the disease-controlling effect of thalidomide is mediated at

least, in part, by its antiangiogenic effect. The most common drug-related toxicities in all the series were somnolence, constipation, dizziness, and skin rash. These adverse effects were generally manageable.

Anti-EGFR MTT

The EGFR signaling pathway may play a role in hepatocarcinogenesis [361]. Expression of transforming growth factor-α, an EGFR ligand, can be induced by hepatitis viral proteins and may act synergistically with viral infection in hepatocarcinogenesis [362–364]. Results of EGFR expression in HCC tumor tissues, mainly by immunohistochemistry, varied in different studies [365], and activating mutation of EGFR, the major determinant of efficacy of EGFR inhibitors in lung cancer [366], was rarely found in HCC tumor tissue [367]. Both small-molecule EGFR inhibitors and monoclonal anti-EGFR antibodies have been tested in small-scale trials for the treatment of advanced HCC, and the response rates and patient survival were not consistent among the studies [368–371]. Correlation of tumor response with expression of EGFR yielded inconclusive results [368, 369]. The most common toxicities of these inhibitors were similar, including skin rash, diarrhea, and fatigue. The therapeutic potential of EGFR inhibitors remains unclear and needs more clinical data to support their role.

Cytotoxic therapy: single agent and combination

The role of conventional cytotoxic chemotherapy is limited by its myelosuppressive toxicity, which is particularly threatening in patients with cirrhosis, hypersplenism, and cytopenia. Objective tumor response rate to single-agent cytotoxic therapies is usually less than 10%, and no survival benefit has been observed [372–376]. An earlier randomized trial comparing doxorubicin, 60–75 mg/m^2 every 3 weeks, with no treatment indicated a borderline improvement in overall survival (10.6 vs. 7.5 weeks) for patients who received doxorubicin [377]. However, 25% of patients died of doxorubicin-related complications, including infection and cardiotoxicity. The antitumor activity of newer cytotoxic agents, such as gemcitabine [378, 379], oxaliplatin [380], and capecitabine [381], has been modest, with single-agent tumor response rate of 10% or less (Table 2). The most common grade 3–4 toxicity was myelosuppression, which occurred in 10–40% of the patients. Combination regimens, such as cisplatin/IFN/doxorubicin/fluorouracil (PIAF), gemcitabine/oxaliplatin (GEMOX), or capecitabine/oxaliplatin (XELOX), can increase the objective response rate to approximately 20% but at the expense of increased treatment-related toxicities [382, 383]. Therefore, cytotoxic chemotherapy can be used with caution only in selected patients with advanced HCC.

Future directions

Combination therapy with MTT has been continually investigated. A randomized phase II trial of sorafenib plus doxorubicin versus doxorubicin alone reported superior median overall survival (13.7 vs. 6.5 months) and time to progression (8.6 vs. 4.8 months) in patients receiving sorafenib plus doxorubicin versus doxorubicin alone [384]. These results should be interpreted with caution because a sorafenib-alone arm was not included and high incidence of adverse events related to doxorubicin was noted. Many small-scale trials of combining MTT with cytotoxic chemotherapy have been reported [385–389]. However, the treatment efficacy in terms of tumor response rate and patient survival were similar to those reported for the cytotoxic regimens alone (Table 3) [372, 375–382]. A second approach is to combine MTT targeting different molecular pathways. Preliminary results of a phase II trial combining bevacizumab with erlotinib showed a response rate of 20% and a median overall survival of 15.5 months [390]. However, these preliminary results must be validated by larger randomized trials.

Treatment algorithm

In general, treatment choice for a solid tumor should be decided taking into account the probability of cure and invasiveness of the treatments. Selecting treatment options for HCC is rather complicated because one should consider the background hepatic function that significantly affects the overall survival. In addition, probability of local cure is not a good surrogate for survival in HCC because intrahepatic recurrence occurs frequently even after curative resection. Therefore, a treatment algorithm should include both tumor- and hepatic reserve-related factors and should be based on results of studies that adopted survival as the primary end point. We propose a treatment algorithm for HCC as shown in Fig. 3.

Tertiary prevention

Recommendations
Interferon may be effective in reducing the recurrent HBV-related HCC after curative ablation of HCC (1b, B).
Lamivudine may be effective in reducing the recurrent HBV-related HCC after curative ablation of HCC (2c, C).
Interferon-based antiviral treatments after complete removal or ablation of HCV-related HCC may reduce HCC recurrence and improve survival (1b, B).

Tertiary prevention for HBV-related HCC

Interferon The short-term outcome of liver resection has dramatically improved over the last decade. The long-term

Table 3 Selected clinical trials of cytotoxic therapy for advanced HCC

Treatment	Patient no.	Objective response	Median survival (months)		Level of evidence
			OS	TTP	
Yeo et al. [375]					
Doxorubicin 60 mg/m^2 on day 1, every 3 weeks	94	RR: 10.5% (9 PR) SD: 39.4%	6.8	NA	1b
Doxorubicin 40 mg/m^2 on day 1, every 3 weeks Cisplatin 20 mg/m^2 Interferon α-2b 5 MU/m^2 5-FU 400 mg/m^2, days 1–4, every 3 weeks	94	RR: 20.9% (19 PR) SD: 37.2%	8.7	NA	
Gish et al. [376]					
Doxorubicin 60 mg/m^2 on day 1, every 3 weeks	222	RR: 2.7% (6 PR) SD: NA	7.4	2.3	1b
Nolatrexed 800 mg/m^2/ day, days 1–3, every 3 weeks	222	RR: 0.9% (1 PR) SD: NA	5.1	2.8	
Yang et al. [378]					
Gemcitabine 1,250 mg/m^2, days 1, 8, 15, every 4 weeks	28	RR: 17.8% (5PR) SD: 25%	4.3	2.8	4
Guan et al. [379]					
Gemcitabine 1,250 mg/m^2, days 1, 8, every 3 weeks	48	RR: 2.1% (2 PR) SD: 43.9%	3.2	1.5	4
Yen et al. [380]					
Oxaliplatin 100 mg/m^2 every 2 weeks	36	RR: 2.8% (1 PR) SD: 47%	6	2	4
Patt et al. [381]					
Capecitabine 2,000 mg/m^2/day, days 1–14, every 3 weeks	37	RR: 11% (1 CR, 3 PR) SD: 11%	10.1	NA	4

OS overall survival, *TTP* time to progression, *RR* response rate, *SD* stable disease, *PR* partial response

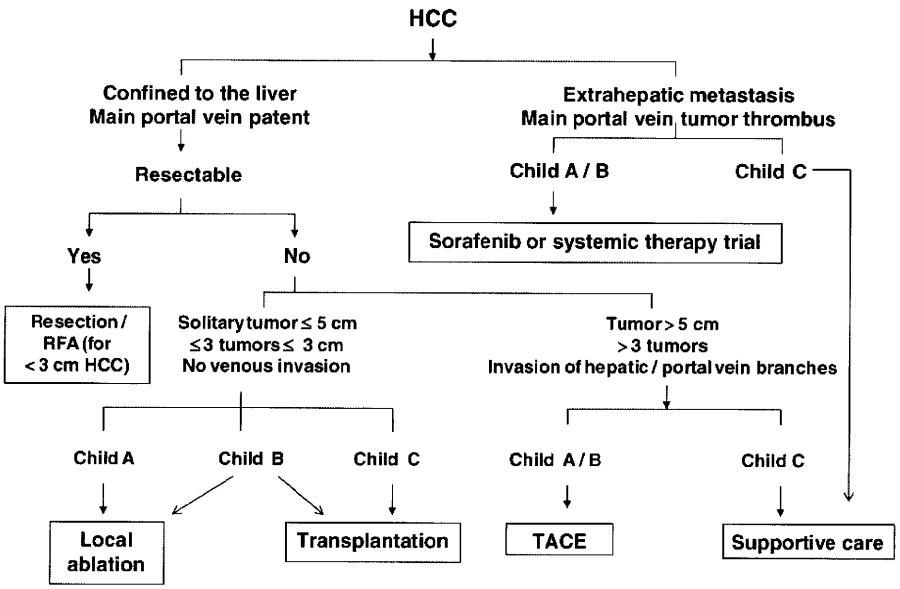

Fig. 3 Treatment algorithm of HCC

prognosis of HCC treated by hepatectomy remains a concern because of frequent development of tumor recurrence, which is the main cause of death in addition to concomitant hepatic decompensation. IFN has tumoricidal effect against a number of tumors including HCC. An RCT was performed to evaluate the safety and efficacy of adjuvant IFN

therapy after hepatic resection in a group of patients with predominantly HBV-related HCC [391]. The relative risk of death for IFN treatment was 0.42 (95% CI 0.17–1.05, $P = 0.063$). Subset analysis showed that adjuvant IFN had no survival benefit for pTNM stage I/II tumor (5-year survival 90% in both groups, $P = 0.917$) but prevented early recurrence and improved the 5-year survival of patients with stage III/IVA tumor from 24 to 68% ($P = 0.038$). HCC recurrence after locoablative treatment modalities is also common. Although candidates for medical ablation usually exhibit compensated hepatic functional status, the frequent recurrence of HCC after successful ablation contributes to short-term survival. A randomized controlled study with small sample size was conducted to evaluate the effectiveness of IFN therapy in preventing HCC recurrence after successful medical ablation therapy for primary tumors [392]. The cumulative HCC recurrence rate of the patients treated with IFN-alfa and the control group was 25 and 40% at the end of 1 year and 47 and 90% at the end of 4 years, respectively ($P = 0.0135$). Furthermore, this study also showed that the prevention of HCC recurrence using IFN-alfa was effective in HBV-related HCC [392]

Lamivudine A retrospective study was conducted to evaluate the efficacy with or without using LAM in patients following curative ablation of HBV-related HCC [393]. Cumulative recurrence rates of HCC were not significantly different between two groups ($P = 0.622$). However, median C-P score at the time of HCC recurrence was significantly different in the control group ($P = 0.005$). The cumulative survival rates of patients in the LAM group tended to be higher than those of patients in the control group ($P = 0.063$) [393]. The outcome of LAM treatment of patients with controlled HCC in terms C-P score and survival compared with a matched, LAM-untreated cohort showed no significant difference in the cumulative incidence of HCC recurrence and survival between the two groups [394]. However, there was a significant difference in the cumulative incidence of death due to liver failure ($P = 0.043$). A significant improvement in liver function was achieved by LAM treatment, even in patients with HCC. These results suggest that LAM treatment of patients with HCC may prevent death due to liver failure [394].

Recent randomized, placebo-controlled trial by Jang et al. [395] also showed that preemptive LAM therapy in patients receiving TACE significantly reduced the incidence of HBV reactivation ($P = 0.002$), overall hepatitis ($P = 0.021$), and severe hepatitis ($P = 0.035$) due to HBV reactivation after repeat TACE. However, the prevention of HCC by preemptive LAM therapy was not shown because of advanced stage of HCC in patients receiving TACE in that trial [395] Further prospective, randomized studies using a larger number of patients are required to assess its role in the tertiary prevention of HCC.

Tertiary prevention of HCV-related HCC

Hepatocellular carcinoma is characterized by very frequent recurrence even after successful initial treatments, either surgical resection or medical ablation, and the risk of recurrence remains high for many years. Recurrence is particularly frequent with HCV-related HCC, and a substantial proportion of recurrence, especially in late phase, is thought to represent de novo, or multicentric, hepatocarcinogenesis [396–398]. Therefore, it could be reasonably assumed that antiviral therapy would reduce the overall incidence of recurrence by preventing de novo carcinogenesis. Indeed, several small-sized RCTs, performed in Japan or Taiwan, showed that the incidence of recurrence was reduced in HCV-related HCC by IFN therapy subsequent to initial HCC treatment [392, 399, 400].

Other RCTs, also performed in Japan and Taiwan, failed to find a significant delay in the first recurrence with IFN therapy, but the second or third recurrence was significantly reduced especially in sustained responders and the overall survival was improved [401, 402]. Another RCT in Italy did not detect effects of IFN therapy on early recurrence but late recurrence, with more than 2 years of interval, seemed to be reduced among IFN responders [403]. These data are compatible with the hypothesis that de novo carcinogenesis was prevented by successful antiviral therapy. On the other hand, two reports on long-term observation of recurrence after IFN therapy following HCC treatment [404, 405] showed that recurrence rate in IFN-treated patients increased over time, suggesting that the growth of residual microscopic tumors had been delayed by IFN (in fact, the two presumed mechanisms are not necessarily mutually exclusive). Most of these studies used IFN monotherapy and suffered from low sustained response rates because most patients had advanced fibrosis or cirrhosis. Preventive effects of IFN on HCC recurrence are yet to be reevaluated using current more efficient protocols.

Microscopic, intrahepatic residual tumors, including intrahepatic metastases, are a possible cause of HCC recurrence. Theoretically, adjuvant chemotherapy may reduce or delay such recurrence, but few chemotherapeutic agents have been shown to be effective against HCC and not a few of them may be hepatotoxic. Hasegawa et al. [406] reported an RCT using oral administration of uracil-tegafur after curative hepatic resection but found no beneficial effects on recurrence and a possible adverse effect on overall survival. In 1966, Muto et al. [407] reported that administration of polyprenoic acid, an acyclic retinoid, reduced recurrence of HCC in an RCT. Updated, long-term

data were subsequently published [408], postulating that the eradication of premalignant or latent malignant clones is the mechanism of action. The effect is, however, yet to be confirmed in a large-scale RCT. Vitamin K_2 was reported to inhibit HCC development among female patients with cirrhosis, who had received the vitamin for the prevention of osteoporosis [122]. A small RCT suggested that vitamin K_2 was effective in suppressing HCC recurrence and may improve survival [124]. However, subsequent, large-scale RCT met with an early termination because of lacking evidence of effects.

Viral-unrelated tertiary prevention of HCC

Vitamin K_2 Apart from its use as a primary preventive agent for HCC, the use of vitamin K_2 as secondary preventive agent has also been investigated. Otsuka et al. [123] examined the biological effects of extrinsic supplementation of vitamin K_2 in HCC cells in vitro and in vivo. Administration of vitamin K_2 to nude mice inoculated with liver tumor cells reduced both tumor growth and weight loss. It was concluded that, similar to an acyclic retinoid, vitamin K_2 may be a promising therapeutic means for the management of HCC.

A pilot study by Mizuta et al. [124] on HCC patients who had undergone either percutaneous local ablation or surgery suggested that menatetrenone, a vitamin K_2 analogue, may have a suppressive effect on the recurrence of HCC and a beneficial effect on survival.

However, a later study (albeit smaller) by Hotta et al. [125] demonstrated that vitamin K_2 may not be as useful for the prevention of HCC recurrence as for primary prevention.

Sho-saiko-to Sho-saiko-to (SST or TJ9), a traditional (Chinese) herbal medicine, was demonstrated to improve liver function tests in patients with chronic active hepatitis in a multicenter, cross-over RCT by Hirayama et al. [409]. A later prospective, randomized (albeit nonblind) study by Oka et al. [410] could elucidate the use of TJ-9 in preventing the development of HCC in patients with cirrhosis, particularly in patients without HBsAg. Successive studies continued to confirm that TJ-9 could protect experimental liver injury caused by D-galactosamine and liver fibrosis by inhibition of lipid peroxide formation in liver cells [411, 412].

Juzen-taiho-to (TJ-48) A recently published study on Juzen-taiho-to, a traditional (Japanese) herbal formulation similar to Sho-saiko-to, presented new information on its anticancer effect in humans [413]. In this study, the administration of TJ-48 improved intrahepatic, recurrence-free survival after surgical treatment of HCC and its protective effects were probably due to reduction in oxidant and cytokine production by Kupffer cells.

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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-