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Preoperative Plasma Transcript AA454543 Level Is an Independent Prognostic Factor for Hepatocellular Carcinoma after Partial Hepatectomy[1]*

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Abstract

BACKGROUND*: We have previously reported that tissue expression levels of transcript AA454543 in hepatocellular carcinoma (HCC) are significantly higher than those of normal livers, livers with cirrhosis, and livers with hepatitis. In addition, a higher level of transcript AA454543 in tumor tissues is associated with poor prognosis. We aim to examine whether quantitative measurement of preoperative plasma transcript AA454543 can provide similar prognostic information.** ***PATIENTS AND METHODS: **Blood samples were obtained from 84 HCC patients before surgery. Real-time quantitative reverse transcription–polymerase chain reaction, using *TaqMan* system, was employed to measure plasma transcript AA454543 and α-fetoprotein (AFP) RNA levels. We assessed their prediction power in prognosis using univariate and multivariate analyses.** ***RESULTS:*** **High plasma transcript AA454543 RNA levels were associated with poor overall survival (log-rank test, $P < .01$). Patients with different plasma AFP RNA levels revealed no difference in overall survival (log-rank test, $P = .88$). By multivariate Cox regression analysis, plasma transcript AA454543 RNA level (hazard ratio = 4.8, $P < .01$) and tumor stage (hazard ratio = 1.7, $P < .01$) were determined to be independent risk factors for the prediction of overall survival.** ***CONCLUSION:*** **Preoperative plasma transcript AA454543 RNA level can provide prognostic information for HCC patients receiving curative partial hepatectomy.**

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Keywords: Plasma RNA, circulating nucleic acid, liver cancer, prognosis, biomarker.

Introduction

Studies on the detection of cell-free nucleic acids in plasma and serum have revealed potential in the initial diagnosis and follow-up monitoring of a variety of cancers [1–3]. Different RNA targets have been used in diverse cancer studies, and cytokeratin-19 frequently serves as a universal marker for solid tumors of epithelial cell origin [4]. In addition, studies on RNA targets of viral and cellular origins have been reported, including studies on Epstein-Barr virus RNA in nasopharyngeal carcinoma, tyrosinase RNA in melanoma, catenin RNA in adenoma and colorectal cancer, hnRNP B1 RNA in lung cancer, and telomerase RNA in breast cancer, melanoma, and thyroid cancer [5–10].

We have previously reported that the tissue expression of transcript AA454543 (GenBank accession no. BC043195) was specific for hepatocellular carcinoma (HCC), with a significantly higher level in tumor tissues than in liver tissues adjacent to tumors (comprising hepatitis and cirrhotic livers) and in normal livers [11,12]. Furthermore, a higher level of transcript AA454543 in tumor tissues was associated with aggressive tumor features, including venous infiltration, microsatellite nodules, late tumor stage, and poor overall survival [12]. Such association could be explained by the biologic role of AA454543 in cell invasion. An increased level of transcript AA454543 might enhance the invasive ability of tumor cells, subsequently resulting in venous infiltration and formation of microsatellite nodules. Importantly, transcript AA45453 levels in tumor tissues and tumor stage were independent prognostic factors for overall survival [12]. The prognostic significance of transcript AA454543 was first identified in our earlier cDNA microarray dataset [11], in which data were validated by quantitative reverse transcription–polymerase chain reaction (RT-PCR) and then confirmed in a separate HCC sample set [12]. Thus, quantitative assay of the molecular marker transcript AA454543 in tumor tissues can provide general prognostic information (two independent cohorts of HCC patients, and prediction is independent of the assay method used).

Abbreviations: HCC, hepatocellular carcinoma; AFP, α-fetoprotein

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In the current study, we aim to investigate whether preoperative plasma RNA levels of transcript AA454543 can provide prognostic information. Plasma RNA levels of transcript AA454543 and α-fetoprotein (AFP) were examined. Plasma RNA data were compared with serum AFP protein data and tumor stage to determine their prediction power on overall survival. We report here that preoperative plasma transcript AA454543 level is one of the independent prognostic factors for HCC following partial hepatectomy.

Patients and Methods

Patients

The study protocol was approved by the Ethics Committee of The University of Hong Kong, and informed consent was obtained from the patients. Between October 1999 and May 2004, 84 patients undergoing partial hepatectomy for HCC at the Department of Surgery, The University of Hong Kong, Queen Mary Hospital, were recruited for the current study. The same team of surgeons performed all operations throughout this period. All patients had been diagnosed with primary HCC and have not received other treatments before surgery. Clinicopathological data were prospectively collected (Table 1). Overall survival was measured from the date of surgery to the date of death due to liver cancer as endpoint by the end of follow-up. Disease-free survival was calculated from the date of surgery to the date of recurrence and/or liver cancer death as endpoint by the end of follow-up. Diagnosis of recurrence was based on typical imaging findings in a contrast-enhanced computed tomography scan and an increased serum AFP level. In case of uncertainty, hepatic arteriography and post-Lipiodol computed tomography scan were performed; when necessary, fine-needle aspiration cytology was used for confirmation. Up to the date of analysis, the median follow-up time was 25.4 months.

Table 1. Clinicopathological Features of HCC.

HCC Features	Total (n = 84)
Age in years [median (range)]	52 (24–79)
Gender	
Male	72
Female	12
Tumor stage*	
I and II	46
III and IV	38
Tumor size (cm) [median (range)]	11 (3–22)
Serum AFP level (ng/ml) [median (range)]	249.5 (1–1,043,700)
HBsAg	
Positive	74
Negative	10
Underlying liver disease	
Noncirrhosis	59
Cirrhosis	25
Disease recurrence	
With	50
Without	34
Disease mortality	
Death	31
Living	53

*Tumor stage according to the *American Joint Committee on Cancer Staging Manual* [15].

Isolation of Plasma RNA

Blood samples (10 ml each) were collected from each patient before the operation. The blood samples collected were stored at 4°C, and the plasma separated within 24 hours. The blood samples were centrifuged at 3000 rpm for 20 minutes to separate the plasma. Parallel plasma samples that had been centrifuged once and twice were compared, and they did not reveal a significant difference in plasma RNA levels, including 18*S*, AFP, and AA454543 in our pilot study. Thereafter, including all plasma samples used in the current study, the blood samples were centrifuged once. Plasma specimens were stored at −70°C until use. Total RNA was extracted using TRIZOL LS reagent (Invitrogen, Carlsbad, CA). Briefly, 200 μl of plasma was added to 600 μl of TRIZOL LS, mixed, incubated at room temperature for 5 minutes, and added to 160 μl of chloroform. After thorough mixing, the mixture was centrifuged at 13,000 rpm for 30 minutes at 4°C. The aqueous phase (360 μl) on top was transferred to a clean tube, then added to 10 μg of glycogen and 400 μl of isopropanol. RNA was precipitated by centrifugation, and RNA pellet was washed once with 75% ethanol. The RNA pellet was then dissolved with 50 μl of diethylpyrocarbonate (DEPC)–water for subsequent quantitative assays.

Real-Time Quantitative RT-PCR

Real-time quantitative RT-PCR was performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), as described [12]. First-strand cDNA was synthesized with 4.5 μl of total RNA in 50 μl of reaction mixture using High-Capacity cDNA Achive kit (Applied Biosystems), following the manufacturer's instructions. The 5-μl first-strand cDNA was then used in each 25-μl quantitative assay with 1× PCR buffer II; 5.5 mM $MgCl_2$; 0.2 mM dATP, dCTP, and dGTP; 0.4 mM dUTP; and 0.625 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA). The primers and probe for transcript AA454543 were AA454543-F (5′-CAA TCA GAC AGG CTG CTT TTC TC-3′), AA454543-R (5′-CTT CCT AAT TAA TGT TTG CAC CAT ATG-3′), and AA454543-P (5′-6FAM AAC TCA TAG GTA ACA AAC ACA AA-MGBNFQ-3′). The primers and probe for AFP RNA were AFP-F (5′-ACT CCA GTA AAC CCT GGT GTT G-3′), AFP-R (5′-ACA TAT GTT TCA TCC ACC ACC AA-3′), and AFP-P (5′-6FAM CAC TTC TTC ATA TGC C-MGBNFQ-3′). The primers and probe reagents for control ribosomal 18*S* were ready-made reagents (Pre-Developed *TaqMan* Assay Reagents; Applied Biosystems) and served as quality control for all samples in all assays. Transcript quantification was performed at least in duplicate for every sample. The PCR profile for the quantitative assay included incubation at 95°C for 10 minutes and then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The amplification plots of PCR were used to determine the threshold cycle (C_T), and the threshold line was usually set at R_n = 0.1. The C_T value represented the PCR cycle at which an increase in reporter fluorescence above a baseline signal could first be detected. Calibrator

control was the sample at which the transcript amount was at the baseline level of the sample series. The relative amount of plasma RNA, after normalization with the calibrator and after adjustment for plate-to-plate variation, presented the fold difference (log 2 base scale) relative to baseline level. The relative amount of plasma transcript AA454543 is expressed as dCT(AA454543), where dCT(AA454543 test sample) = [C_T(AA454543 calibrator) − C_T(AA454543 test sample)]. Similarly, the relative amount of plasma AFP mRNA is expressed as dCT(AFP), where dCT(AFP test sample) = [C_T(AFP calibrator) − C_T(AFP test sample)].

Study Report

We followed the recommendation for tumor marker prognostic studies by the National Cancer Institute-European Organization for Research and Treatment of Cancer (NCI-EORTC) [13]. Plasma quantitative assays were performed by investigators blinded to the study endpoint. We have a designated staff member who was tasked to build up the prospective clinical database, including follow-up details, and who was not aware of assay data. Assays were performed by another research staff member who did not know the clinical details of the specimen. The assay data of the biomarker, together with the clinical information, were then combined and analyzed by an independent investigator.

Statistical Methods

The quantitative data of plasma transcript AA454543 RNA, AFP RNA, and serum AFP protein levels were continuous variables, and the data were modeled as categorical variables in Kaplan-Meier and Cox regression analyses. The Youden index (sensitivity + specificity −1) [14] was employed to determine the optimal cutoff point for prognosis prediction. Other cutoff values, including the mean and the median, have also been considered and examined. They were all able to segregate patients with similar clinical implications. The Youden index was employed to simultaneously maximize the sensitivity and the specificity of the prediction. The association of transcript AA454543 data, AFP data, and tumor stage [15] with overall survival and disease-free survival was examined by multivariate Cox proportional hazards regression with a forward stepwise selection procedure. All statistical analyses were performed by a statistical software (SPSS version 12.0 for Windows; SPSS, Inc., Chicago, IL). Differences were considered significant when $P < .05$.

Results

Ribosomal 18*S* RNA was used as an endogenous quality control for all assays, and 18*S* RNA was detectable in all plasma specimens from HCC patients [84 of 84 (100%); median = 29.92, range = 24.65–33.02]. 18*S* RNA data indicated the RNA quality and integrity of plasma specimens. Plasma transcript AA454543 RNA was detectable in the majority of specimens from HCC patients (78 of 84; 93%), with a wide data range (median = 14.84, range = 0–22.02). The positive detection rates of transcript AA454543 in plasma (current study) and liver cancer tissues [12] were similar, reflecting the significance of circulating tumor nucleic acids. Plasma AFP RNA was detectable only in a subset of HCC patients (65 of 84; 77%), with a diverse data range (median = 12.43, range = 0–18.75). There was no correlation between plasma RNA levels of transcript AA454543 and AFP (Pearson correlation = −0.035, $P = .752$). The positive detection rate of plasma AFP RNA (current study) was comparable to that of serum AFP in the literature, as AFP was only expressed in a subset of HCC tissues. The current data further highlighted the importance of identifying novel tumor markers with sensitivity and specificity comparable to or better than those of AFP for liver cancer management.

To evaluate if a high plasma transcript AA454543 RNA level in HCC patients is associated with poor prognosis, we analyzed its level for overall survival. Kaplan-Meier plot was used to examine the prediction power by the quantity of plasma transcript AA454543. The patients were segregated into low-plasma or high-plasma transcript AA454543 groups using the Youden index to determine the optimal cutoff value. There were 64 patients in the low-plasma transcript AA454543 group (range = 0–16.37) and 20 patients in the high-plasma transcript AA454543 group (range = 16.38–22.02). By the plasma transcript AA454543 level alone, patients with a high plasma transcript AA454543 level were found to have poor overall survival (log-rank test, $P < .01$) (Figure 1). The analysis was repeated based on the plasma transcript AA454543 levels and tumor stages [15] of patients. In early-stage (stages I and II) patients, a high plasma transcript AA454543 level was associated with poor overall survival (log-rank test, $P = .01$). In late-stage (stages III and IV) patients, a high plasma transcript AA454543 level was also associated with poor overall survival (log-rank test, $P < .01$).

AFP RNA levels were also examined to evaluate their prediction power. The patients were segregated into low-plasma or high-plasma AFP RNA groups using the Youden index to determine the optimal cutoff value. There were 27 patients in the low-plasma AFP RNA group (range = 0–5.56) and 57 patients in the high-plasma AFP RNA group (range = 5.57–18.75). By Kaplan-Meier analysis, patients with different plasma AFP RNA levels did not reveal significant differences in overall survival (log-rank test, $P = .88$) (Figure 2).

AFP protein levels were also evaluated for association with overall survival. Patients were segregated according to AFP protein levels, and the optimal cutoff value was 99 ng/ml, as determined by the Youden index. There were 36 patients in the low-AFP protein group (range = 0–99 ng/ml) and 48 patients in the high-AFP protein group (range = 100–1,043,700 ng/ml). By Kaplan-Meier analysis, patients with high AFP protein levels revealed poor overall survival (log-rank test, $P = .03$) (Figure 3). In early-stage patients, the difference in AFP protein levels revealed no difference in overall survival (log-rank test, $P = .43$). Nonetheless, high AFP protein levels showed a significant association with poor overall survival only in late-stage patients (log-rank test, $P < .01$).

Cox regression analysis was employed to compare gene data with tumor stage on the prediction for overall survival

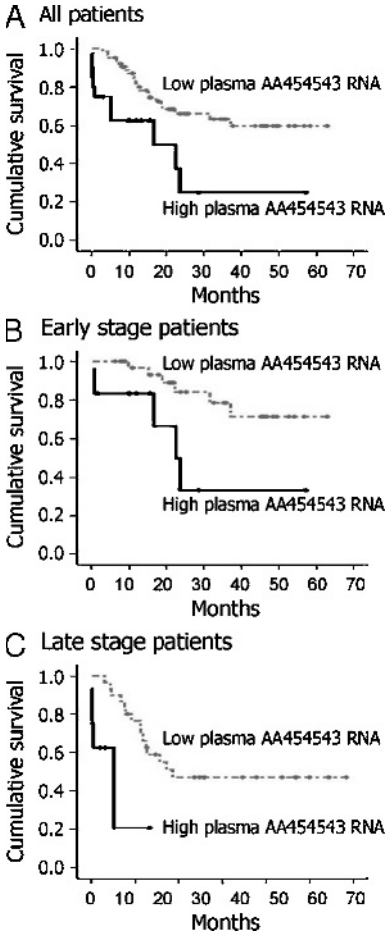

Figure 1. *Kaplan-Meier overall survival plot according to plasma transcript AA454543 RNA levels. (A) All patients (log-rank test, P < .01). (B) Early-stage (stages I and II) patients (log-rank test, P = .01). (C) Late-stage (stages III and IV) patients (log-rank test, P < .01).*

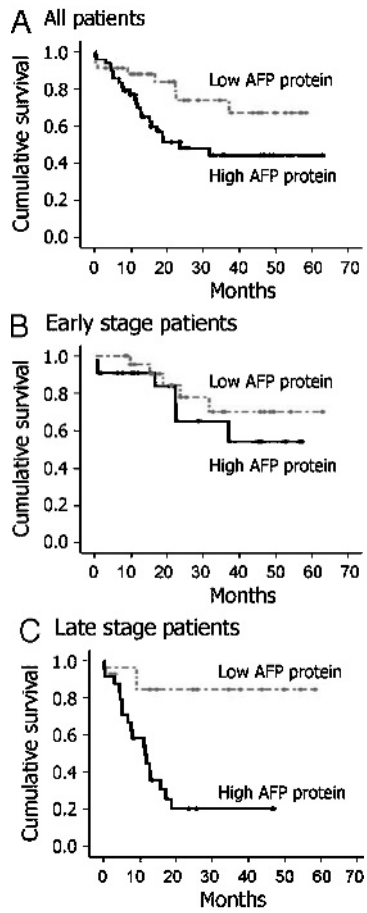

Figure 3. *Kaplan-Meier overall survival plot according to serum AFP protein levels. (A) All patients (log-rank test, P = .03). (B) Early-stage (stages I and II) patients (log-rank test, P = .43). (C) Late-stage (stages III and IV) patients (log-rank test, P < .01).*

(Table 2). By univariate Cox regression analysis, high plasma transcript AA454543 RNA level [hazard ratio (HR) = 2.9; 95% confidence interval (95% CI) = 1.4–6.3; $P < .01$], high AFP protein level (HR = 2.4; 95% CI = 1.1–5.3; $P = .04$), and late tumor stage (HR = 1.5; 95% CI = 1.1–2.2; $P = .02$) were significantly associated with overall survival (Table 2). The plasma AFP RNA level did not demonstrate an association with overall survival ($P = .88$). By multivariate Cox regression analysis, high plasma transcript AA454543 level (HR = 4.8; 95% CI = 2.1–10.6; $P < .01$), AFP protein level (HR = 3.0; 95% CI = 1.3–6.8; $P < .01$), and late tumor stage (HR = 1.7; 95% CI = 1.2–2.5; $P < .01$) were found to be independent prognostic factors for overall survival.

Other clinicopathological features that might influence prognosis were also examined together with plasma transcript

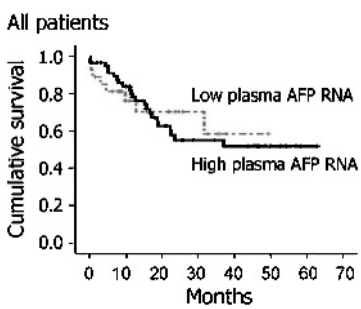

Figure 2. *Kaplan-Meier overall survival plot according to plasma AFP RNA levels in all patients (log-rank test, P = .88).*

Table 2. Cox Regression Analyses for Overall Survival on Plasma Transcript AA454543 RNA Level, Plasma AFP RNA Level, Serum AFP Protein Level, and Tumor Stage.

Variables*	Univariate Analysis		Multivariate Analysis	
	HR (95% CI)	*P*	Adjusted HR (95% CI)	*P*
Transcript AA454543†	2.9 (1.4–6.3)	< .01	4.8 (2.1–10.6)	< .01
AFP RNA†	–	.88	–	–
AFP protein†	2.4 (1.1–5.3)	.04	3.0 (1.3–6.8)	< .01
Tumor stage‡	1.5 (1.1–2.2)	.02	1.7 (1.2–2.5)	< .01

*All data were modeled as categorical variables.
†For plasma transcript AA454543 RNA, AFP RNA, and serum AFP protein levels, the optimal cutoff value used to segregate patients was determined by the Youden index.
‡Tumor stage according to the *American Joint Committee on Cancer Staging Manual* [15].

AA454543 data by multivariate Cox regression analysis (Tables W1 and W2). A similar analysis in relation to disease-free survival was performed (Figures W1–W3). Irrespective of the inclusion of other clinicopathological variables in multivariate Cox regression analyses, the same conclusion can be drawn: plasma transcript AA454543 RNA level is an independent risk factor for the prediction of overall survival and disease-free survival.

To further investigate whether the quantitative measurement of plasma RNA levels could serve as a diagnostic tool for HCC, we compared the data obtained from HCC patients to those obtained from healthy individuals (n = 10) and cirrhotic patients with no underlying HCC (n = 10) (Figures W4 and W5). Concerning the plasma RNA levels of ribosomal 18*S*, transcript AA454543, and AFP, the outliers were all HCC patients. However, control and patient groups revealed an overlap in data range. Thus, plasma RNA levels of transcript AA454543 could provide prognostic information on HCC patients, but preliminary analysis showed that it would not be applicable for disease diagnosis with the current experimental design.

Discussion

We have previously reported that a high level of transcript AA454543 in tumor tissues is predictive of poor prognosis in HCC patients [12]. In the current study, we showed that plasma transcript AA454543 level could provide similar prognostic information in both early-stage and late-stage HCC patients. Conversely, plasma AFP RNA level revealed no prognostic significance, and serum AFP protein level only demonstrated prognostic information for late-stage patients, but not for early-stage patients. Similar AFP protein data have been reported. In patients with large HCCs (> 5 cm), a high AFP protein level was significantly associated with a lower 10-year overall survival, but AFP protein levels revealed no association with overall survival in patients with small HCC [16]. Thus, AFP protein level can generally be referred to as a reliable marker only in the advanced stage of HCC, but not in the early stage, and plasma AFP RNA level revealed no prognostic information in the current study. The performance of transcript AA454543 and AFP as plasma RNA target was compared. Plasma AFP RNA was detectable only in a subset of HCC patients (77%)—a positive rate comparable to that of AFP proteins in the literature. However, plasma transcript AA454543 was detectable in the majority of HCC patients (93%)—a positive rate comparable to that of transcript AA454543 expression in HCC tissues (96 of 101; 95%) [12]. Thus, transcript AA454543 would be an applicable marker for the majority of HCC patients, whereas AFP (as plasma RNA target or serum protein) would be an appropriate marker only for a subgroup of HCC patients. Existing data demonstrated that the performance of transcript AA454543 was better than that of AFP as plasma RNA target.

The quantitation of plasma transcript AA454543 for prognosis prediction will encompass important clinical information to stratify patients into different risk groups. Stratification is even better than tumor staging because, among patients in early tumor stage, further stratification into a subgroup of patients whose survival rates are similar to late-tumor-stage patients is possible. This helps to identify high-risk patients who have a high plasma transcript AA454543 level in the early tumor stage, as they may benefit from adjuvant therapy after partial hepatectomy. Conversely, low-risk patients developing recurrence but with poor liver function may have good prognosis if they undergo salvage liver transplantation [17], even though they have large tumors or have been diagnosed at a late stage.

To further understand the significance of plasma RNA levels, we have analyzed the clinicopathological features of patients with different plasma RNA levels of transcript AA454543 and AFP (data not shown). Other than the outcomes of overall survival and disease-free survival, the plasma RNA levels of AA454543 and AFP were not significantly associated with tumor stage, tumor size, age, venous infiltration, microsatellite nodules, tumor encapsulation, liver cirrhosis, differentiation status (Edmondson-Steiner grade), hepatitis B surface antigen (HBsAg) status, and intrahepatic or extrahepatic recurrence. The exact mechanism of how tumor RNA entered the circulation is unknown [1–3]; thus, its clinicopathological significance is not clear. Nonetheless, the higher transcript AA454543 levels in HCC tissues were associated with aggressive tumor features [12]. As plasma transcript AA454543 levels were a reflection of tumor levels, they could provide prognostic information.

Earlier reports on circulating tumor contents were mostly assays on circulating tumor cells, and controversial data on diagnostic and prognostic values were presented [18–31]. Recently, extracellular-based assays (circulating DNA/RNA) were found to be as efficacious as, or better than, cell-based assays (circulating tumor cells) in the detection of preneoplastic lesions and micrometastases [32]. Extracellular-based assays demonstrated potential for broad clinical applications because they allow automation and high-throughput platforms, and are less prone to sampling errors [2,32]. Furthermore, the relative proportion of tumor-derived nucleic acids was reported to be higher in plasma than in serum [33,34]. Thus, in the current study, we employed quantitative measurements on plasma RNA rather than on serum RNA. Exactly how RNA entered the circulation was unknown [1–3]. RNA in apoptotic bodies was protected from degradation in the circulation [35], and apoptosis was a mechanism through which nucleic acids entered the circulation [36]. Accordingly, it was speculated that circulating nucleic acids might be released from the apoptotic cells of the tumor mass. Alternatively, tumor cells were detached from the tumor mass (process of metastasis), and some of these circulating tumor cells underwent apoptosis because of detachment stress in the bloodstream, and their contents were released as circulating nucleic acids.

The quantitative assay of plasma RNA is feasible for direct clinical application as the test is not expensive (< US$30), assays have automation platforms, and only 0.2 ml of plasma (equivalent to 0.5 ml of whole blood) is needed. The assay, in the form of a noninvasive blood test, can provide prognostic information and is more practical than assays involving tissue

specimens. The need for tissue specimens will make the test less useful for clinical applications, as tissue specimens may not be readily available: biopsies may have morbidity and mortality consequences, and surgical specimens are available only after the operation. A simple blood test is therefore more feasible.

The current data indicate, for the first time, that preoperative plasma transcript AA454543 RNA level can predict prognosis for HCC patients receiving curative partial hepatectomy. To consolidate the current findings, we have initiated a large-scale prospective study to examine, in parallel, other factors/biomarkers that have reported associations with cancer recurrence. This will enhance our understanding on the relative importance of each factor/biomarker and the formulation for accurate prognosis.

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Table W1. Multivariate Cox Regression Analyses for Overall Survival.

Variables*	Adjusted HR (95% CI)	*P*
Transcript AA454543†	8.9 (3.4–23.1)	< .01
AFP protein†	5.9 (2.3–15.3)	< .01
Albumin‡	0.2 (0.1–0.6)	< .01
Multiple tumor nodules	2.4 (12.9)	< .01

*All data were modeled as categorical variables. Insignificant variables with *P* > .05 were not listed in the table, including AFP RNA data, tumor stage, venous infiltration, histologic grade (Edmondson-Steiner grade), tumor size, age, sex, cirrhosis, HBsAg, Child-Pugh classification, and bilirubin level.
†For plasma transcript AA454543 RNA and serum AFP protein levels, the optimal cutoff value used to segregate patients was determined by the Youden index, as described in the text.
‡For serum albumin level, the optimal cutoff value (40.5 g/l) used to segregate patients was determined by the Youden index.

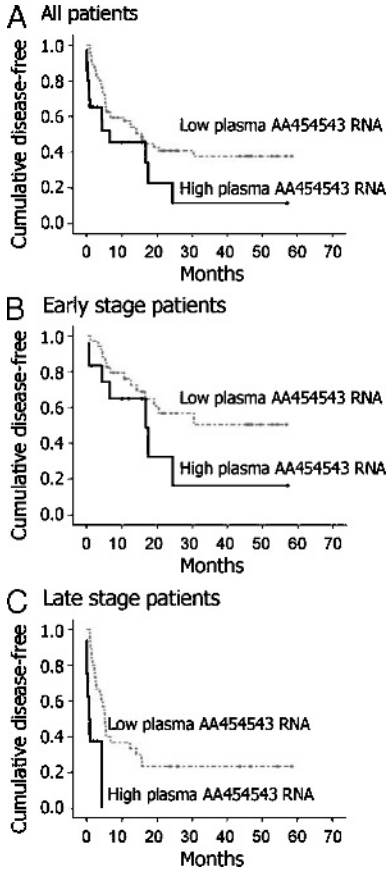

Figure W1. *Kaplan-Meier disease-free survival plot according to plasma transcript AA454543 RNA levels. (A) All patients (log-rank test, P = .05). (B) Early-stage (stages I and II) patients (log-rank test, P = .11). (C) Late-stage (stages III and IV) patients (log-rank test, P < .01).*

Table W2. Multivariate Cox Regression Analyses for Disease-Free Survival.

Variables*	Adjusted HR (95% CI)	*P*
Transcript AA454543†	4.7 (2.2–10.5)	< .01
Albumin‡	0.5 (0.3–0.9)	.03
Multiple tumor nodules	4.7 (2.5–8.9)	< .01
Venous infiltration	3.5 (1.5–8.2)	< .01

*All data were modeled as categorical variables. Insignificant variables with *P* > .05 were not listed in the table, including AFP RNA data, serum AFP, tumor stage, histologic grade (Edmondson-Steiner grade), tumor size, age, sex, cirrhosis, HBsAg, Child-Pugh classification, and bilirubin level.
†For plasma transcript AA454543 RNA level, the optimal cutoff value used to segregate patients was determined by the Youden index, as described in the text.
‡For serum albumin level, the optimal cutoff value (40.5 g/l) used to segregate patients was determined by the Youden index.

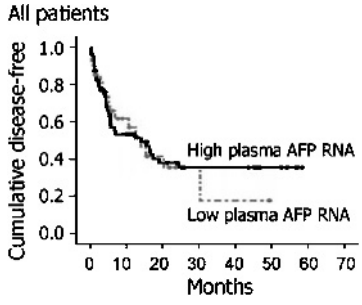

Figure W2. *Kaplan-Meier disease-free survival plot according to plasma AFP RNA levels in all patients (log-rank test, P = .86).*

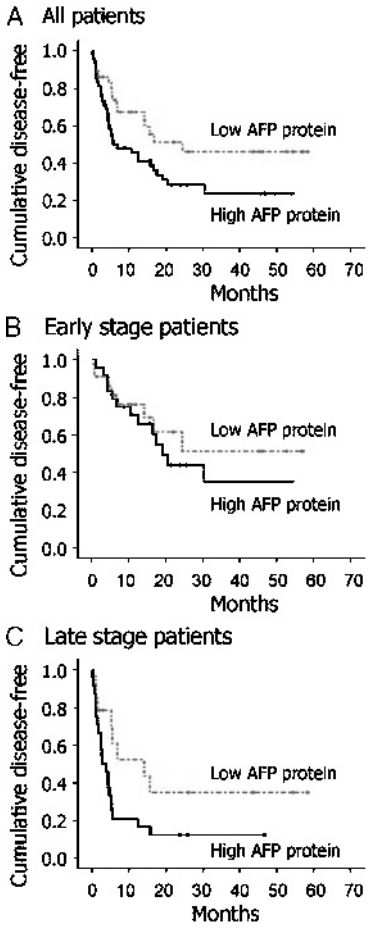

Figure W3. *Kaplan-Meier disease-free survival plot according to serum AFP protein levels. (A) All patients (log-rank test, P = .04). (B) Early-stage (stages I and II) patients (log-rank test, P = .48). (C) Late-stage (stages III and IV) patients (log-rank test, P = .05).*

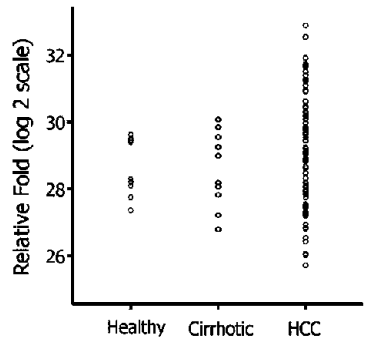

Figure W4. *Plasma RNA levels in healthy individuals (n = 10), cirrhotic patients without HCC (n = 10), and HCC patients (n = 84). The relative quantitative value of plasma ribosomal 18S RNA was shown. Plasma transcript AA454543 and AFP RNA showed similar distributions in controls and patients. As control and patient groups revealed an overlap in the date range, plasma transcript AA454543 could only provide prognostic information to HCC patients, but would not be applicable for disease diagnosis.*

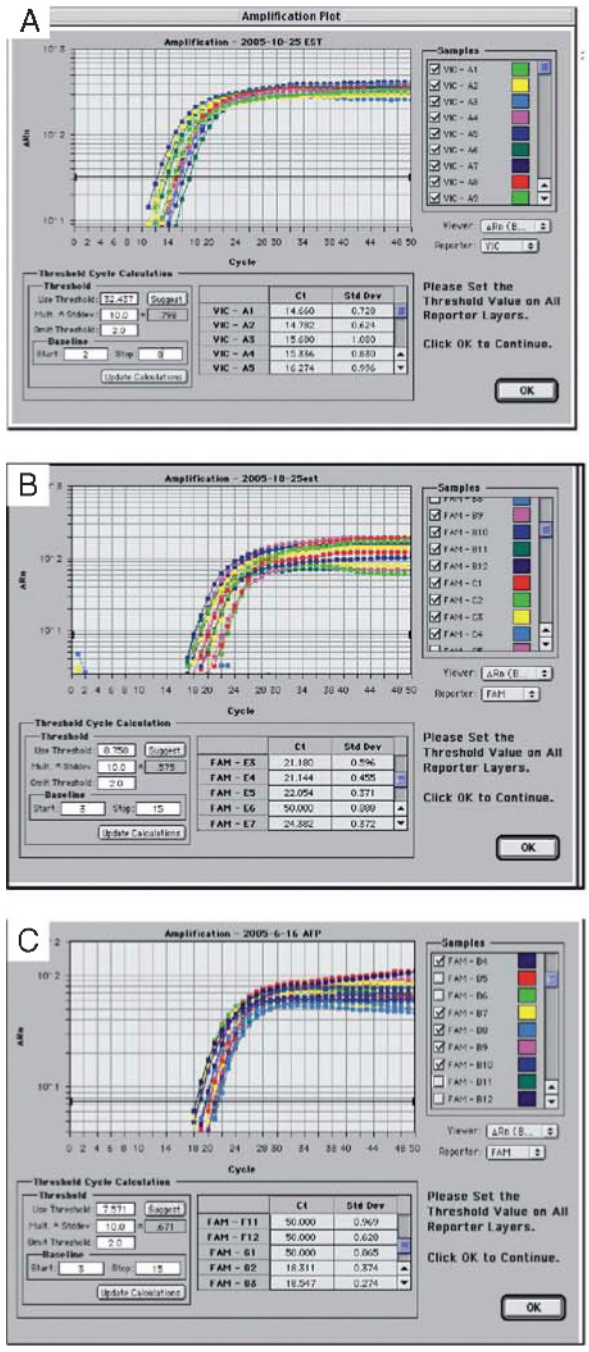

Figure W5. *Quantitative real-time RT-PCR curves. (A) Ribosomal 18S RNA. (B) Transcript AA454543. (C) AFP.*