

Figure 1. Changes in the percentages of patients with elevated tumor markers before and after hepatectomy ($n = 187$). (A) Percentage of patients with AFP \geq 20 ng/mL. The percentage decreased significantly after hepatectomy (35.8% before hepatectomy vs. 16.6% after hepatectomy, $P < 0.0001$). (B) Percentage of patients with DCP \geq 40 mAU/mL. The percentage decreased significantly after hepatectomy (50.3% before hepatectomy vs. 7.0% after hepatectomy, $P < 0.0001$). (C) Percentage of patients with conventional AFP-L3 \geq 10%. The percentage decreased significantly after hepatectomy (19.8% before hepatectomy vs. 7.0% after hepatectomy, $P = 0.0005$). (D) Percentage of patients with highly sensitive AFP-L3 \geq 5%. The percentage was similar before and after hepatectomy (45.5% before hepatectomy vs. 52.4% after hepatectomy, $P = 0.2145$).

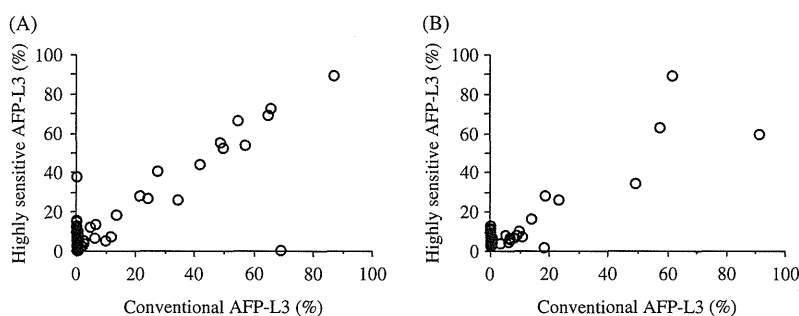


Figure 2. Correlation between conventional AFP-L3 and highly sensitive AFP-L3 levels (A) before and (B) after hepatectomy. The correlation of AFP-L3 measured with two different methods decreased after hepatectomy (r^2 , 0.76 before hepatectomy and 0.47 after hepatectomy).

A) and patients whose pretreatment tumor marker levels elevated but normalized after hepatectomy (group B). Recurrence-free and overall survival rates were significantly lower in patients with persistently elevated levels of hsAFP-L3 before and after hepatectomy (group C) than patients with normal levels of hsAFP-L3 before and after hepatectomy (group A). Recurrence-free survival rate was significantly lower in patients with persistently elevated

levels of hsAFP-L3 before and after hepatectomy (group C) than patients whose pretreatment tumor marker levels elevated but normalized after hepatectomy (group B). In contrast, both recurrence-free and overall survival rates of patients with normal hsAFP-L3 levels before hepatectomy but elevated levels after hepatectomy (group D) was significantly lower than those of patients with normal post-operative levels of hsAFP-L3 regardless of pretreatment

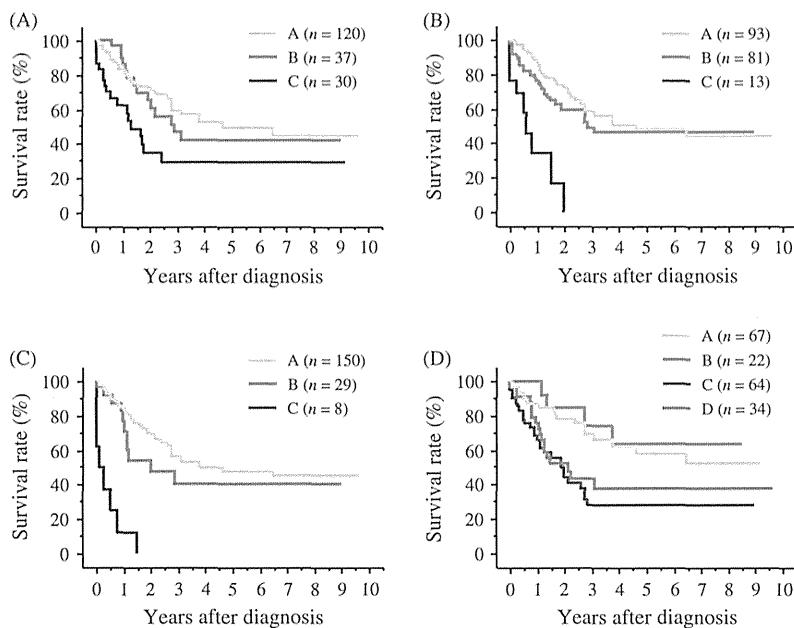


Figure 3. Recurrence-free survival rates of patients stratified based on the treatment response of tumor markers after hepatectomy. A: Tumor marker was normal before and after hepatectomy. B: Tumor marker was elevated before hepatectomy but normalized after hepatectomy. C: Tumor marker was elevated before and after hepatectomy. D: Tumor marker was normal before hepatectomy but elevated after hepatectomy. (A) Survival rates based on the treatment response of AFP. The survival rate of patients in group C was significantly lower than that of patients in group A ($P = 0.0016$) and in group B ($P = 0.0345$). (B) Survival rates based on the treatment response of DCP. The survival rate of patients in group C was significantly lower than that of patients in group A ($P < 0.0001$) and in group B ($P = 0.0003$). (C) Survival rates based on the treatment response of conventional AFP-L3. The survival rate of patients in group C was significantly lower than that of patients in groups A and B (both, $P < 0.0001$). (D) Survival rates based on the treatment response of highly sensitive AFP-L3. The survival rate of patients in group C was significantly lower than that of patients in group A ($P = 0.0005$) and patients in group B ($P = 0.0065$). The survival rate of patients in group D was significantly lower than that of patients in group A ($P = 0.0024$) and patients in group B ($P = 0.0345$).

levels (groups A and B), and the rate was similar to that of patients with persistent elevations of hsAFP-L3 before and after hepatectomy (group C).

HCC characteristics based on the elevations of conventional and highly sensitive AFP-L3 after hepatectomy

Table 2 lists the characteristics of HCC tumors according to elevations of conventional and hsAFP-L3 after hepatectomy. The size of HCC was significantly greater in patients with elevations in cAFP-L3 than in patients without the elevation ($P = 0.0143$). The percentages of moderately or poorly differentiated HCC and HCC with infiltrative growth was significantly higher in patients with elevations in cAFP-L3 than in patients without the elevation ($P = 0.0454$ and $P = 0.0203$, respectively). In contrast, no differences were found in the characteristics of HCC between patients with and without postoperative elevations of hsAFP-L3. Although postoperative total AFP was elevated (≥ 20 ng/mL) in 10 of 13 patients (76.9%) in

whom cAFP-L3 was elevated postoperatively, total AFP was elevated in only 22 of 98 patients (22.4%) in whom postoperative elevations of hsAFP-L3 were observed. Especially, the total AFP concentration after hepatectomy was within normal range (< 20 ng/mL) in all patients in whom hsAFP-L3 became elevated after hepatectomy despite the normal values before hepatectomy (data not shown).

Discussion

In this study, we investigated changes in newly developed hsAFP-L3 in patients treated with hepatectomy with curative intent. The analytical sensitivity of the conventional assay system for AFP-L3 is insufficient in patients with low total AFP levels; cAFP-L3 cannot be measured when total AFP is less than 10 ng/mL [23,24]. The new generation of assays for AFP-L3 (micro total analysis system; μ TAS), which used novel advanced microfluidics-based separation technology, has enabled the accurate measurement of AFP-L3 even at very low total AFP

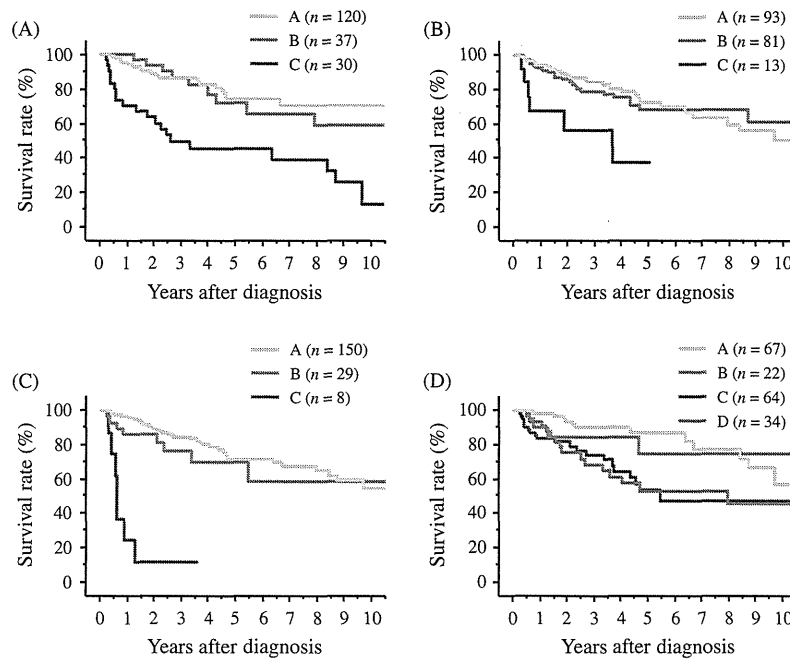


Figure 4. Overall survival rates of patients stratified based on the treatment response of tumor markers after hepatectomy. A: Tumor marker was normal before and after hepatectomy. B: Tumor marker was elevated before hepatectomy but normalized after hepatectomy. C: Tumor marker was elevated before and after hepatectomy. D: Tumor marker was normal before hepatectomy but elevated after hepatectomy. (A) Survival rates based on the treatment response of AFP. The survival rate of patients in group C was significantly lower than that of patients in group A ($P < 0.0001$) and in group B ($P = 0.0012$). (B) Survival rates based on the treatment response of DCP. The survival rate of patients in group C was significantly lower than that of patients in group A ($P = 0.0016$) and in group B ($P = 0.0091$). (C) Survival rates based on the treatment response of conventional AFP-L3. The survival rate of patients in group C was significantly lower than that of patients in groups A and B (both, $P < 0.0001$). (D) Survival rates based on the treatment response of highly sensitive AFP-L3. The survival rate of patients in group C was significantly lower than that of patients in group A ($P = 0.0026$). The survival rate of patients in group D was significantly lower than that of patients in group A ($P = 0.0024$).

Table 2. Characteristics of resected hepatocellular carcinoma specimens according to elevations of conventional or highly sensitive AFP-L3 after hepatectomy ($n = 187$).

	Conventional AFP-L3		Highly sensitive AFP-L3	
	Negative ($n = 174$)	Positive ($n = 13$)	Negative ($n = 89$)	Positive ($n = 98$)
Child-Pugh class (A/B)	166 (95.4)/8 (4.6)	13 (100)/0	87 (97.8)/2 (2.2)	92 (93.9)/6 (6.1)
Tumor size (cm)	3.09 ± 2.30^a	5.19 ± 4.21^a	3.28 ± 2.56	3.20 ± 2.50
Number of tumors (single/multiple)	143 (82.2)/31 (17.8)	10 (76.9)/3 (23.1)	73 (82.0)/16 (18.0)	80 (81.6)/18 (18.4)
Differentiation (well-/moderately or poorly)	52 (29.9)/122 (70.1) ²	0/13 (100) ^b	25 (29.1)/64 (71.9)	27 (27.6)/71 (72.4)
Growth pattern (expansive/infiltrative)	161 (92.5)/13 (7.5) ^c	9 (69.2)/4 (30.8) ^c	81 (91.0)/8 (9.0)	89 (90.8)/9 (9.2)
Capsular formation (absent/present) ¹	62 (38.5)/99 (61.5)	2 (22.2)/7 (77.8)	28 (34.6)/53 (65.4)	36 (40.4)/53 (59.6)
Capsular infiltration (absent/present) ²	38 (38.4)/61 (61.6)	1 (14.3)/6 (85.7)	23 (43.4)/30 (56.6)	16 (30.2)/37 (69.8)
Portal vein invasion (absent/present) ³	146 (83.9)/28 (16.1)	8 (61.5)/5 (38.5)	69 (77.5)/20 (22.5)	85 (86.7)/13 (13.3)

Unless otherwise indicated, values are means \pm SD and percentages are indicated in parentheses. ^a $P = 0.0143$, ^b $P = 0.0454$, ^c $P = 0.0203$.

¹Evaluated only in HCC with expansive growth.

²Evaluated only in HCC with capsular formation.

³On pathologic evaluation.

concentrations [16, 25, 30, 34–37]. With this method, the percentage of hsAFP-L3 is measurable when total AFP concentration is 2 ng/mL or higher.

The percentage of patient with elevated AFP, DCP, and cAFP-L3 decreased after hepatectomy with normalization of these values in patients with elevated pretreatment

value. Surprisingly, in contrast, the percentage of patients with elevated hsAFP-L3 did not decrease after hepatectomy, despite the high curativity. The percentage of patients in whom hsAFP-L3 was elevated after hepatectomy was greater than 50% despite its curative intent. The percentage of AFP-L3 is calculated as the serum AFP-L3 concentration divided by the total serum AFP concentration. Therefore, the analytical sensitivity for AFP-L3 depends on the concentration of total AFP. The improved sensitivity of AFP-L3 measurement might, therefore, account for this unexpected change in the percentage of patients with elevated hsAFP-L3 after hepatectomy. The decrease in the correlation between cAFP-L3 and hsAFP-L3 after hepatectomy will be due to the elevation of hsAFP-L3 in patients with normal total AFP after hepatectomy. Indeed, the total AFP concentration after hepatectomy was within normal range (<20 ng/mL) in all patients in whom hsAFP-L3 became elevated after hepatectomy despite its normal range before hepatectomy.

The postoperative elevation of respective tumor markers predicts patient recurrence-free and overall survival after hepatectomy regarding to all tumor markers. Both recurrence-free and overall survival rates of patients in whom tumor markers were persistently elevated before and after hepatectomy was lower than those of patients with normal tumor marker levels after hepatectomy that included patients whose tumor markers were not elevated before and after hepatectomy and patients whose elevated pretreatment values normalized after hepatectomy, for AFP, DCP, and cAFP-L3. This was similar for hsAFP-L3. In addition, the survival rate of patients whose normal pretreatment hsAFP-L3 levels became elevated after hepatectomy was lower than that of patients with normal posthepatectomy hsAFP-L3 levels and was comparable to patients with persistently elevated hsAFP-L3 before and after hepatectomy, showing that postoperative elevation of hsAFP-L3 levels predict unfavorable outcome regardless of its pretreatment status.

It is unknown why hsAFP-L3 levels increase after hepatectomy despite normal preoperative levels in some patients. We did not find any differences in the characteristics of HCC between patients with and without elevations of hsAFP-L3 after hepatectomy, whereas HCC had more advanced characteristics in patients with postoperative elevations of cAFP-L3. The elevation of cAFP-L3 after hepatectomy might partly be explained by potential residual HCC cells in the liver that were not detected by imaging studies postoperatively, due to the progressive nature of resected HCC. In contrast, postoperative elevation of hsAFP-L3 was not associated with morphologic and pathologic findings of resected HCC. Postoperative elevations of hsAFP-L3, therefore, cannot be predicted based on the

progression of resected HCC. The postoperative elevation of hsAFP-L3 may reflect undefined malignant potential of HCC tumor that was not shown by pathologic and imaging examinations.

In conclusion, this study showed that the percentage of patients with elevated hsAFP-L3 did not decrease after hepatectomy when it was measured with the highly sensitive method, although the percentage of patients with elevated AFP, DCP, and cAFP-L3 levels decreased markedly. Postoperative elevations of hsAFP-L3 were observed in more than half of the patients who underwent hepatectomy with curative intent, and suggest unfavorable outcome.

Conflict of Interest

There are no conflicts of interest. The employment status of C. Kagebayashi and S. Satomura by Wako Pure Chemical Ltd. did not influence the data and the interpretation of the study.

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Keywords: hepatocellular carcinoma; prognosis; prognostic models; biomarkers; AFP; DCP; AFP-L3; bilirubin; albumin

Biomarker-based prognosis in hepatocellular carcinoma: validation and extension of the BALAD model

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Background: The Japanese 'BALAD' model offers the first objective, biomarker-based, tool for assessment of prognosis in hepatocellular carcinoma, but relies on dichotomisation of the constituent data, has not been externally validated, and cannot be applied to the individual patients.

Methods: In this Japanese/UK collaboration, we replicated the original BALAD model on a UK cohort and then built a new model, BALAD-2, on the original raw Japanese data using variables in their continuous form. Regression analyses using flexible parametric models with fractional polynomials enabled fitting of appropriate baseline hazard functions and functional form of covariates. The resulting models were validated in the respective cohorts to measure the predictive performance.

Results: The key prognostic features were confirmed to be Bilirubin and Albumin together with the serological cancer biomarkers, AFP-L3, AFP, and DCP. With appropriate recalibration, the model offered clinically relevant discrimination of prognosis in both the Japanese and UK data sets and accurately predicted patient-level survival.

Conclusions: The original BALAD model has been validated in an international setting. The refined BALAD-2 model permits estimation of patient-level survival in UK and Japanese cohorts.

The key features that influence prognosis in hepatocellular carcinoma (HCC) are now well recognised and can be broadly classified under the headings of tumour-related factors (such as tumour size or multiplicity), those that assess the severity of underlying liver dysfunction (such as conventional liver function tests or the Child–Pugh (C-P) classification (Child and Turcotte, 1964; Pugh *et al*, 1973)) and patient-related factors (such as symptoms or performance status). Several staging systems/prognostic scores that combine a number of these factors have been developed (Okuda *et al*, 1985; Group, 1998; Chevret *et al*,

1999; Leung *et al*, 2002; Kudo *et al*, 2003; Llovet *et al*, 2008) and, to varying degrees, validated and compared (Kudo *et al*, 2004; Marrero *et al*, 2005; Cho *et al*, 2008; Collette *et al*, 2008; Chen *et al*, 2009; Huitzil-Melendez *et al*, 2010; Chan *et al*, 2011). Some simply offer an estimate of prognosis, whereas others aim to indicate the appropriate therapy for specific disease stages (Llovet *et al*, 2008).

In an attempt to develop a more objective staging system, Toyoda *et al* (2006) have described the BALAD model that relies on two liver function tests (Bilirubin and Albumin) and three serological cancer biomarkers (AFP-L3, AFP, and DCP). They have

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shown that it is possible to achieve an excellent degree of discrimination between the proposed risk groups using such objective variables. However, the data analysis approach in the BALAD model utilised dichotomisation of the continuous variables, which raises a number of statistical issues.

In the present study, we aimed to validate the original BALAD model (built on a Japanese Cohort) in a geographically and aetiologically distinct HCC patient data set from the UK. We first confirmed that the variables in the BALAD model were identical to those independently identified in a UK data set, and assessed the discrimination achieved within the proposed prognostic groups. We then, in a collaborative Japan/UK study, took the raw data on which BALAD model was initially derived and applied a more sophisticated statistical method that treats the variables in a continuous manner and does not assume a linear relationship between predictors and outcome. The model developed here not only allows classification of patient risk, as with the original BALAD model, but also provides detailed estimation of patient-level survival in the Japanese cohort, and, with calibration, in UK patients.

A major challenge in applying the BALAD model to the UK population is the great difference in survival compared with the Japanese cohort. This problem is due to the difference in the underlying survivor function that describes hazard in relation to time; hazard could be greatest at diagnosis and then decrease over time or, conversely, the hazard at diagnosis may be low and then increase as time accumulates. Indeed, the hazard may be described by a more complicated, non-linear, and not necessarily monotonic function. To account for such differences, the methods applied in this analysis allowed interrogation of the scale and shape of the baseline hazard function.

The derived model is assessed in terms of discrimination and calibration. To assess discrimination, Harrell's *C*-statistic was measured, as described by Taktak *et al* (2007). This measures the proportion of patient pairs for which the model correctly assigns lower risk to the patient who truly survives longest (i.e. is at least risk). A model with good discriminative performance should have a high *C*-statistic. To assess calibration, graphical methods were used. These assessments compare patient level survival with the predicted values.

MATERIALS AND METHODS

The study comprised two cohorts of patients. The first included 2599 Japanese patients previously reported by Toyoda *et al* (2006) and 319 UK patients, all with HCC (Table 1). The Japanese patients were recruited from five institutions in which a total of 3725 patients were initially diagnosed as having HCC between July 1994 and December 2004, and the UK patients from among 724 patients referred to the Queen Elizabeth, Birmingham, UK, between June 2007 and January 2012. The various aetiologies were classified as hepatitis B virus-related, hepatitis C virus-related, alcoholic-related, and 'other'. The 'other' group comprised patients with hemochromatosis, primary biliary cirrhosis, non-alcoholic steatohepatitis, or cryptogenic cirrhosis. The diagnosis of chronic liver disease was made on the basis of liver biopsy and/or typical clinical and imaging features. The study protocol was approved by the institutional ethics review board at each of the institutions.

Age and gender distributions were similar in the two populations, as was the distribution of liver dysfunction as assessed by the C-P classification (Table 1). However, there were striking differences in aetiological attribution, the Japanese patients having predominantly HCV-related HCC and the UK patients having multiple aetiologies. There were also major differences in disease

stage (Table 1) and overall survival between the two cohorts. The median survival for those treated with palliative and curative therapy was 22.6 and 60.7 months for Japanese patients, respectively, with analogous figures for the UK of 13.9 and 27.5 months.

In all patients, the three serological cancer biomarkers of HCC (AFP, AFP-L3, and DCP) were measured at the time of diagnosis, and drugs that would influence the serum DCP levels, such as warfarin and vitamin K, were not taken. A standard operating procedure was applied to all blood collection. Samples were collected in the fasting state, before any treatment. Blood was allowed to clot at room temperature for 1–2 h, centrifuged at 3000 g for 20 min and the serum collected and stored at -80°C until processing. Routine liver and renal function was measured by commercially available methods. Albumin was measured by the bromocresol green method in both UK and Japan. The severity of the liver disease was defined according to C-P classification.

Patients were staged by five systems: TNM 5, TNM 6 (Sobin and Fleming, 1997; Greene *et al*, 2002; UICC, 2002; Sobin *et al*, 2011), CLIP (Group, 1998), JIS or BCLC (Llovet *et al*, 2008), or by Milan criteria (Mazzaferro *et al*, 1996). However, for this analysis that focused on prognosis, we also grouped patients on the basis of whether or not the treatment received was curative or palliative. Curative treatments included transplantation, resection, radio-frequency ablation, and percutaneous ethanol injection. Palliative treatments included transarterial chemoembolisation, any form of chemotherapy, and supportive care. Where patients were listed for transplantation but had transarterial chemoembolisation as initial treatment as a 'bridge' to transplantation, they were classified as having potentially curative therapy. For the purpose of this analysis, UK patients who underwent liver transplantation were excluded, as the survival of this group would not be expected to be influenced by the baseline features included in the model (such as bilirubin and albumin).

Assays of AFP, AFP-L3%, and DCP. AFP, AFP-L3%, and DCP were all measured in the same serum sample. The measurements of hs-AFP-L3% and DCP were achieved by using a microchip capillary electrophoresis and liquid-phase binding assay on a $\mu\text{TASWako i30}$ auto analyzer (Wako Pure Chemical Industries, Ltd, Osaka, Japan) (Kagebayashi *et al*, 2009). Analytical sensitivity of $\mu\text{TASWako i30}$ is 0.3 ng ml^{-1} AFP, and the percentage of AFP-L3 can be measured when AFP-L3 is over 0.3 ng ml^{-1} (Kagebayashi *et al*, 2009).

Statistical methods

Discrimination. We assessed discriminatory performance using Harrell's *C*-statistic, as described by Taktak *et al* (2007). In brief, this measure reports the number of comparable pairs that are correctly ordered under the risk score. That is, for a pair of comparable patients P_A and P_B , if patient P_A is known to have survived beyond P_B 's time of event (death here), then P_A should be subject to a lesser risk than P_B , that is, should be assigned a lower-risk group. This method counts all the correctly ordered pairs from those that are comparable.

Flexible parametric models. Regression analyses utilised flexible parametric models (Royston and Lambert, 2011) that enable fitting of more appropriate baseline hazard functions. The baseline hazard describes risk over time when all covariates take the value zero (rather than the hazard at time zero as sometimes stated), and is described by a restricted cubic spline function (Royston and Lambert, 2011). Here all continuous covariates are centred about their mean, and so the interpretation of the function is the hazard at the mean of all covariates. Traditionally, the baseline hazard is assumed to have a simple constant or monotonic form, as in

Table 1. Demographics and clinical data for the two cohorts

	UK (n = 319)	Japan (n = 2599)
Demographics		
Median age (IQR*)	66.4 (59.3–72.9)	67 (61.0–72.0)
Mean age (\pm s.d.)	65.4 (\pm 9.7)	66.4 (\pm 8.9)
Gender (M:F), %	82.4:17.6	71.7:28.3
Ethnicity		
Caucasian	266 (83.4%)	N/A
Other	53 (16.6%)	2599 (100%)
Aetiology		
Alcohol, %	25.1	N/A
HCV, %	12.9	74.3
HBV, %	9.1	12.4
HCV + HBV, %	0.6	1.7
Other (including those with multiple ^a aetiologies), %	48.3	11.2
Not known, %	4.1	0.4
HCC biomarkers		
AFP, ng ml ⁻¹	57 (8.7–1264.3*), n = 319	29.7 (9–208*), n = 2599
Log ₁₀ AFP, ng ml ⁻¹	1.76 (0.94–3.1*), n = 319	1.5 (0.95–2.3*), n = 2599
L3, %	16.6 (7–51.9*), n = 319	1.4 (0–18*), n = 2599
Log ₁₀ L3, %	1.22 (0.9–1.7*), n = 319	0.15 (0–1.3*), n = 2599
DCP, ng ml ⁻¹	20.07 (2.6–169.7*), n = 319	90 (26–797.5*), n = 2599
Log ₁₀ DCP, ng ml ⁻¹	1.37 (\pm 1.2), n = 319	1.95 (1.4–2.9*), n = 2599
Liver function tests		
Albumin, g l ⁻¹	38.4 (\pm 5.6), n = 318	35 (31–39*), n = 2599
ALP, UI ⁻¹	370.5 (259.5–558*), n = 318	N/A
INR	1.1 (1.0–1.2*), n = 313	1.1 (1.03–1.2*), n = 2431
Bilirubin, μ mol l ⁻¹	17 (11–28*), n = 318	15.4 (10.3–22.2*), n = 2599
Child Pugh Score		
A:B:C:NK, %	74.0:22.6:2.8:0.6	67.1:26.3:6.6:0
Tumour characteristics		
Solitary:multifocal:NK, %	44.5:50.8:4.7	52.0:45.4:2.5
Maximum tumour diameter		
<2 cm, %	5.6	26.4
2–5 cm, %	37.6	54.1
>5 cm, %	30.1	13.4
>10 cm, %	12.2	3.5
NK or not specified, %	14.4	2.7
Macrovascular invasion (No:Yes:NK), %	68.3:26.0:5.6	68.1:31.6:0.3
Milan criteria (No:Yes:NK), %	67.7:24.5:7.8	39.1:56.0:4.8
Treatments		
Curative (intended: actual), %	19.3:16.1	66.3 (actual)
Palliative (intended: actual), %	80.7:83.9	33.7 (actual)
Median survival, months	16	47.2
Abbreviations: AFP = alpha-fetoprotein; ALP = alkaline phosphatase; DCP = Des-gamma carboxyprothrombin; F = female; HBV = hepatitis B virus; HCC = hepatocellular carcinoma; HCV = hepatitis C virus; INR = international normalised ratio; M = male; N/A = not applicable; NK = not known; s.d. = standard deviation. For all continuous variables, values are presented either as median (interquartile range*) or mean (\pm s.d.), the latter for normal distributions where appropriate.		
^a For example, alcoholic and HCV positive.		

exponential or Weibull survival models, and Cox modelling does not directly model the baseline hazard function. The model as described here comprises two main components: the baseline hazard, which is described by a spline function consisting of a constant value and a function of log-time, and the covariate vector, which modifies risk based on the subject's covariate values. Each of

these components can be recalibrated (Van Houwelingen, 2000) should the model not perform as expected. Given our intention to apply the model in two geographically distinct cohorts, we assessed the baseline hazard function, as clinical insights led us to expect a difference.

Stata version 12 was used for all analyses.

Replication of BALAD results and model derivation. As an exploratory step, we validated the original BALAD model in both the Japanese and UK cohorts. Like Toyoda *et al* (2006), we fitted univariable Cox regression models to verify the set of prognostic parameters and confirmed that statistical significance was maintained when entered into a multivariable model. The steps taken by Toyoda to dichotomise the continuous data were not replicated. The BALAD score was calculated for each cohort and discrimination was assessed by fitting Kaplan–Meier (KM) curves and measuring Harrell’s C. A ‘training’ data set, which comprised 50% (Royston and Lambert, 2011) of the Japanese cohort, was used to derive the prognostic model, and the remainder was held back for validation. The random selection of the hold-back sample was stratified by treatment intention (potentially curative and palliative) such that each subset was equally represented in the training and validation data sets. A cohort of UK individuals was also used in the validation process.

New prognostic model. We then fitted flexible parametric survival models to the Japanese data, applying a more rigorous statistical approach in which the continuous form of the covariates was maintained and linearity of predictor–outcome relation was not assumed. Univariable and multivariable models were fitted to identify important prognostic factors with potential prognostic factors chosen from those that were not considered subjective. Martingale residuals were inspected to aid the choice of the appropriate covariate functional form and second-order fractional polynomials were explored, taking powers from the standard power set. Predictors were selected at the $P=0.05$ level in the multivariable modelling procedure that combined backward elimination with the selection of an FP function. Models were compared using Akaike information criteria (AIC); a 4-point reduction (per additional covariate) is indicative of an improved model. Having identified a preferred prognostic model, we then fitted a model keeping only the serological cancer biomarkers to see if similar performance could be obtained at less ‘cost’.

Development of scoring mechanisms. To assign risk groups ‘Cox cut-points’ were applied by splitting risk predictions, based on the relative part of the model only, in the training data at the 15th, 50th, and 85th percentiles. As a result, individuals were categorised into 1 of 4 levels of risk, ranging from low to high. We then calculated individual risk in the hold-back data and classified patients based on the cut-points established earlier. We refer to this discriminatory model as *BALAD-2d*. By incorporating risk as a function of time, that is, the baseline hazard function, we could estimate the probability of survival for each individual patient. We refer to this patient-level predictor as *BALAD-2p*.

Model validation. *BALAD-2d*: The prognostic model was validated using graphical methods (Royston and Lambert, 2011). A visual inspection of discrimination between the groups was performed and survival statistics were compared to assess the clinical relevance of the model. We assessed Harrell’s C of each model in a number of patient subgroups: stage of disease, treatment intention, tumour size, and BCLC (available only in UK patients).

BALAD-2p: Stata’s suite of flexible parametric modelling (stpm2) post-estimation tools were used to estimate population average survival for each validation cohort, thus allowing Kaplan–Meier curves depicting actual vs predicted survival to be plotted for each risk group. The similarity of the curves is indicative of the performance of the model. To determine if the model is appropriate for the estimation of patient-level survival in the UK validation set, or if recalibration was required, we assessed the similarity of the baseline hazard in each cohort by plotting

the function. We also demonstrate the use of the *BALAD-2p* by example and report the results by graphical means.

RESULTS

Replication of BALAD results. We confirmed that for both the Japanese and UK cohorts the measures of serological cancer biomarkers and bilirubin are associated with increased risk of mortality (results not shown); albumin is associated with a decreased risk. The Kaplan–Meier survival curves according to BALAD scores are shown in Figure 1A and B. For BALAD model in the Japanese and UK cohorts the respective Harrell’s C-statistics were 0.73 and 0.71, indicating similar discriminative performance. We note that for the BALAD model in the UK cohort there is overlap of the curves in the first 6 months and that there are very few patients in the highest-risk group. Table 2 reports the median survival with 95% confidence intervals; the estimates for the Japanese cohort are quite distinct; however, for the UK patients there is little difference in median survival for some of the groups, indicating that, from a clinical perspective, the BALAD model may have too many levels for use in the UK. The hazard ratio estimates for the BALAD score in the Japanese cohort ranged from 2.24 (95% CI, 1.85–2.72) in the lowest-risk group to 48.48 (95% CI, 30.52–77.02) in the highest-risk group. In the UK cohort, the corresponding figures were 1.93 (95% CI, 1.18–3.17) and 210.42

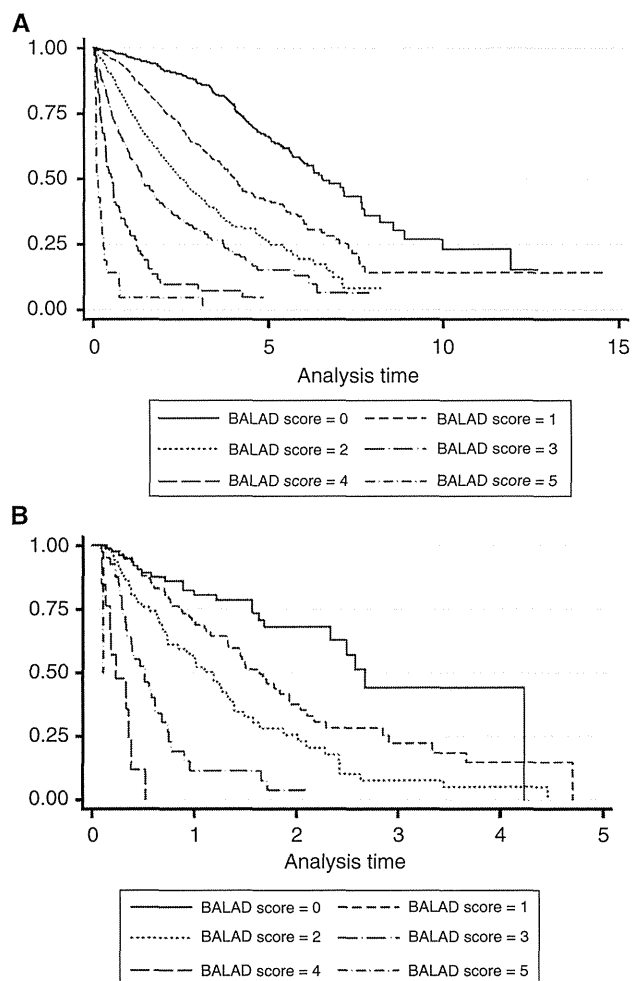


Figure 1. Survival according to the BALAD model. Kaplan–Meier curves showing survival according to the original BALAD model in (A) Japanese and (B) UK cohorts.

Table 2. Median survival times for BALAD and BALAD-2d in Japanese (validation) and UK cohorts

	Japan				UK			
	Subjects	Median (years)	95% CI		Subjects	Median (years)	95% CI	
BALAD								
0	357	6.7	5.9	8.6	79.0	2.7	2.3	
1	436	4.1	3.8	5.3	88.0	1.6	1.3	1.9
2	261	2.5	2.1	2.9	79.0	1.2	0.7	1.4
3	155	1.4	1.2	2.0	44.0	0.5	0.3	0.6
4	50	0.6	0.4	0.9	13.0	0.2	0.1	0.4
5	12	0.1	0.0	0.3	2.0	0.1	0.1	
Total	1271	3.9	3.6	4.3	305.0	1.4	1.1	1.6
BALAD-2d								
1	172	7.1	6.7		97	2.3	1.7	3.7
2	483	5.9	4.8	7.8	90	1.6	1.2	2.2
3	425	3.1	2.5	3.4	73	0.8	0.7	1.3
4	191	0.8	0.7	1	44	0.3	0.3	0.5
Total	1271	3.9	3.6	4.3	304	1.4	1.1	1.6

Abbreviation: CI = confidence interval.

Table 3. Univariable analysis in Japanese training data

Variable (x)	Transform	HR	95% CI	P-value
Gender	NA	1.177	0.974, 1.422	0.092
Major VP	NA	6.095	4.925, 7.542	<0.001
Age (years)	X	1	0.99, 1.01	0.977
INR	x^{-2}	0.254	0.171, 0.378	<0.001
AFP	$\ln(x)$	1.226	1.187, 1.267	<0.001
L3	$x^{1/2}$	1.189	1.156, 1.223	<0.001
DCP	$\ln(x)$	1.271	1.229, 1.315	<0.001
Bilirubin	$\ln(x)$	1.978	1.726, 2.267	<0.001
Albumin	X	0.903	0.889, 0.917	<0.001
Maximum tumour size (mm)	$x^{1/2}$	2.081	1.84, 2.355	<0.001

Abbreviations: AFP = alpha-fetoprotein; CI = confidence interval; DCP = Des-gamma carboxyprothrombin; HR = hazards ratio; INR = international normalised ratio.

(95% CI, 20.87–2121.74). Between these two values, each cohort indicated an increasing trend in risk.

New prognostic model. We split the 2599 Japanese patients into a 'training' set of 1327 patients and a hold-back set of 1272, and, as a result of stratification by treatment intent, each data set was approximately equal in terms of the proportion of curative (and therefore palliative) patients (33.5% training and 33.8% validation).

The univariable analysis confirmed that the variables in the original BALAD model are all highly prognostic (Table 3), and these factors maintained statistical significance in the resulting multivariable model (data not shown). The fractional polynomial transformations identified for the multivariable model were a log transform for DCP and a square-root for bilirubin. The AIC for this model was 2341. An increase in each of the markers, other than albumin, is associated with an increase in risk, and increased albumin has a beneficial effect on prognosis.

The linear predictor resulting from the multivariable analysis considering the 5-serological cancer biomarkers – bilirubin,

albumin, AFP-L3, AFP, and DCP – as potential prognostic factors is reported below. This function, the BALAD-2d score, calculates the log cumulative hazard for an individual:

Linear predictor (xb) = $0.02*(afp_c - 2.57) + 0.012*(AFP-L3 - 14.19) + 0.19*(\ln(DCP) - 1.93) + 0.17*((bili(\mu\text{mol})^{1/2}) - 4.50) - 0.09*(alb(\text{gl}) - 35.11)$

As part of the modelling procedure AFP was capped at 50 000 units. Both AFP and DCP are modelled as per 1000 units.

The multivariable model incorporating just the three serological cancer biomarkers had considerable overlap between the two lower-risk groups, indicating that the discriminative performance was considerably poorer than the 5-serological-cancer-biomarker model that included bilirubin and albumin (Harrell's C of 0.69, AIC 2536) (Figure 2A and B).

BALAD-2d validation in the Japanese cohort. Application of the Cox cut-points for the linear predictor yielded four classes (1–4) of risk. These cut-points were as follows: $xb > 0.24$ (risk 1, low), 0.24 to > -0.91 (risk 2), -0.91 to > -1.74 (risk 3) and ≤ -1.74 (risk 4, high). The KM survival curves depicting actual and predicted survival in the Japanese hold-back sample (Figure 2A) indicate that the risk groups are well discriminated (Harrell's C 0.74). The logrank test indicates a statistically significant risk difference ($P < 0.001$) and the differences in survival between the groups are clinically meaningful and distinct (Table 2). Harrell's C was approximately equal in the subgroup comparisons detailed in the foregoing. Both BALAD and BALAD-2d models perform better in patients at greater risk.

Recalibration for use in the UK cohort. Figure 3A and B describe the baseline hazard function for each of the cohorts. The baseline hazards are similar in shape but differ in height or magnitude, indicating the need for recalibration (see Supplementary Data for methodology). Adjustment to the constant term in the spline function only was deemed sufficient. Figure 4A shows that for the recalibrated model the overall predicted survival curve approximates the true survival well; here we optimised the fit between 0 and 3 years. Survival is predicted best of all in the higher-risk groups, and there is some overestimation for patients in the lowest-risk group (Figure 4B). The model has an AIC of 827 compared with 1096 prior to recalibration, an improvement of 269 points.

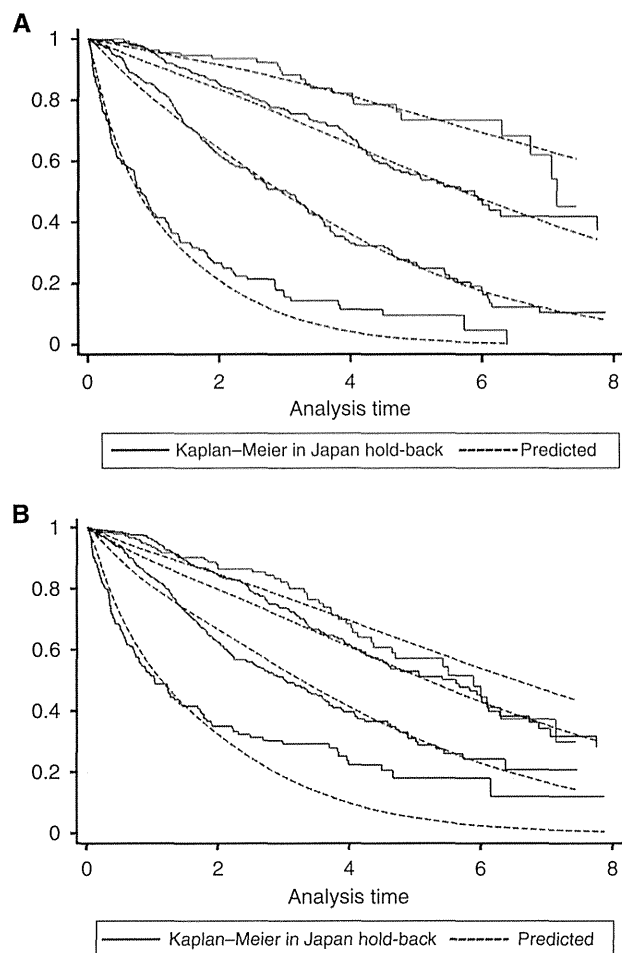


Figure 2. Comparison of five-marker and three-marker BALAD-2d model. Kaplan-Meier curves depicting actual (solid line) and predicted (dashed line) survival using a multivariable model incorporating (A) five serological cancer biomarkers and (B) three serological cancer biomarkers from the Japanese hold-back sample.

Note that the AIC in the Japanese cohort is not comparable to that in the UK.

Figure 5 demonstrates patient-level survival estimation and reports predictions of 2-year survival for increasing levels of albumin and bilirubin; all other parameters are fixed (AFP 34, AFP-L3 16.1, and DCP 1.14). Each curve describes an incrementally different level of albumin, and each point along a curve represents a change in bilirubin. As observed in the regression analysis, increased bilirubin is associated with an increase in risk and albumin is negatively correlated with risk.

DISCUSSION

We have confirmed that the biological factors identified in the original BALAD model, as described by Toyoda *et al* (2006), are highly prognostic. When applied to the UK patients, the BALAD model gave good discrimination, although performance appeared poorer among the UK cohort, particularly in groups 4 and 5, those with the worst survival (Figure 1B). Most likely the reason for the apparent limited degree of discrimination in these groups is related to the very small numbers (only 13 cases in group 4 and 20 in

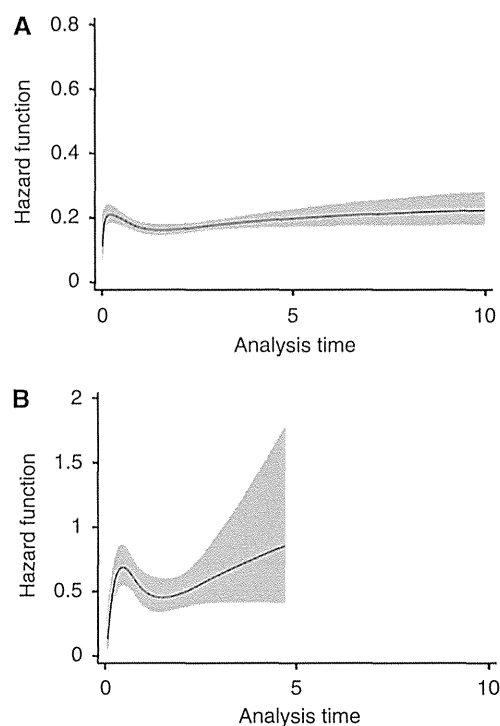


Figure 3. Baseline hazards. Plots illustrating the baseline hazard function with 95% confidence interval, CI (shaded region) for each of the (A) Japanese and (B) UK cohorts.

group 5), and the fact that survival for both these groups is only of the order of a few weeks indicates that there is little scope for clear discrimination. The overlap between risk groups is less evident for BALAD-2d, and the Harrell's C-test is, in fact, similar, indicating that there is no major difference in discriminatory performance despite the new model using just four risk groups.

One of the concerns about current staging systems is that they encompass factors that are inherently subjective, leading to a potential lack of consistency between observers. For example, one of the most widely used, the CLIP (Group, 1998), system estimates the extent of tumour as $>$ or $<$ 50% of the total liver volume, a measurement that, in clinical practice, is difficult to ascertain with any degree of certainty or consistency. Others such as CUPI (Leung *et al*, 2002) and BCLC (Llovet *et al*, 2008) demand a decision as to whether or not patients are symptomatic. Again, in practice this assessment may be highly variable between observers. Even widely used measures of liver function such as the C-P classification (Child and Turcotte, 1964; Pugh *et al*, 1973), which was developed for patients with cirrhosis rather than HCC, is remarkably subjective. Thus, presence/severity of ascites (one of the constituent variables in the C-P score) is, by some practitioners, based on whether or not subjects have ever developed ascites. Others may include ascites even when it is detectable only by radiological scanning and some may consider ascites to be 'absent' if it is controlled by diuretics. Encephalopathy may be equally difficult to grade, because of many of the early symptoms overlapping with those that may be attributable to the HCC. Such concerns have led to the development of objective measures of liver function such as the MELD score (Malinchoc *et al*, 2000; Botta *et al*, 2003), which is based solely on blood tests.

The second weakness of some of the current staging systems is that, perhaps in the pursuit of simplicity, they handle the relevant data in a categorical manner when it is, in fact, generated as a continuous variable. The loss of information consequent upon this

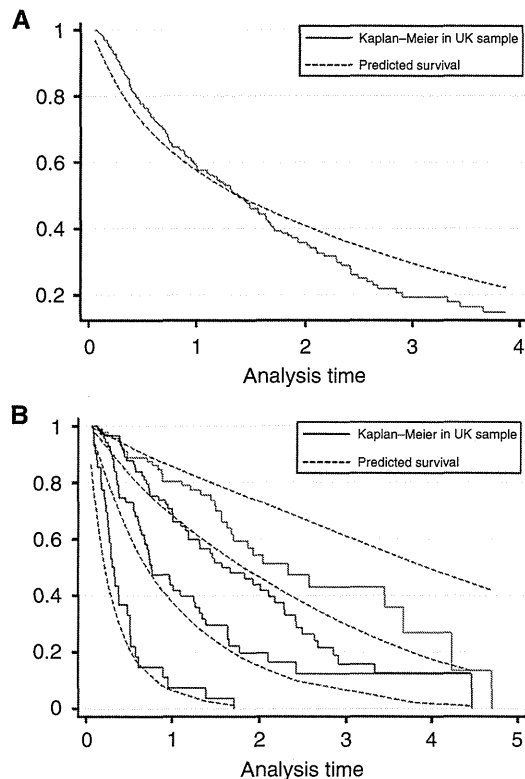


Figure 4. Kaplan-Meier curves of actual vs predicted survival in the UK cohort. Kaplan-Meier curves showing actual (solid line) vs predicted (dashed line) survival (A) overall and (B) by risk group, using the recalibrated model in the UK cohort.

approach is now increasingly well recognised, and it has been suggested that dichotomisation of continuous data in a multiple regression procedure may be associated with considerable loss of statistical power and introduction of bias (Del Priore *et al*, 1997; Royston *et al*, 2006). The most noticeable impact lies in those patients who fall around the 'cut-point', that is, just below or above the value used to define the two levels of the binary variable. They may be classified as having very different risk. Equally, in the C-P classification, a patient with a score based on a serum bilirubin level of $51 \mu\text{mol l}^{-1}$ has the same impact as one with a value of $500 \mu\text{mol l}^{-1}$ and a serum albumin of 24g l^{-1} , a similar impact as a serum albumin of 10g l^{-1} . Indeed, it has been shown that dichotomising (at the median) a normally distributed variable is equivalent to losing a third of the data. When the variable in question is exponentially distributed then such a conversion is equivalent to a loss of around half of the data (Royston *et al*, 2006). It has also been shown that in the case of logistic regression the chance of false positives is increased; as the sample size increases, so does the chance of such errors (Austin and Brunner, 2004).

Among the numerous biomarkers (Mann *et al*, 2007) that have been proposed for prognostication in HCC, the three used here have the advantage of being commercially available on a single platform and having regulatory approval in Japan, US, and Europe. All three are well documented to have prognostic significance when used individually, prognosis becoming poorer with increasing levels (Nagaoka *et al*, 2003; Toyoda *et al*, 2007; Nouse *et al*, 2011). AFP is also included in some staging systems such as CLIP (Group, 1998), where a level of $>400 \text{ng ml}^{-1}$ is an adverse feature, and in the UK guidelines for liver transplantation levels of $>10\,000$ are a contraindication to transplantation (NHSBT, 2013).

The BALAD model has an advantage over current staging systems in that it is entirely objective. The Toyoda model utilised a common method for determining the value at which continuous covariates were dichotomised, that is, multiple testing in search of the 'optimal' value. Altman *et al* (1994) appropriately refer to this approach as the 'minimum *P*-value approach', as the use of the term optimal is perhaps misleading. There are several issues with this approach. The value, if truly optimal, is likely to be such only in the derivation cohort. Furthermore, the values identified at the univariable level are not necessarily accurate in the multivariable setting. The Type I error rate is greatly increased, and as such there is a greater risk of incorrectly identifying prognostic factors. The extent of this increase is influenced by the number of values tested, and is reported to be in the region of 40% (Altman *et al*, 1994).

In this paper we have undertaken further detailed analyses of the data in its continuous form. The regression analyses were performed using flexible parametric models (Royston and Lambert, 2011), described earlier, with which we were able to examine the baseline hazard function and assess the need for model recalibration for use in cohorts outside Japan. By exploring fractional polynomials (Sauerbrei and Royston, 1999) (FPs) many of the issues associated with dichotomisation and data-driven cut-points are minimised or avoided. If appropriate, more intricate relationships between the outcome and explanatory variables can be fitted. Over-fitting of either the baseline hazard or the covariate functional form is a potential issue but is unlikely if the number of knots and the power-set for the FPs are sensibly chosen. Given the size of the Japanese cohort in particular, throughout our analyses we purposely avoided over-interpretation of *P*-values and considered more the clinical significance of the results.

At present, we cannot be certain of the reason for the markedly differing survival in the two cohorts. However, the most plausible explanation, and one supported by the data presented here, is that the Japanese patients are diagnosed at a much earlier stage, and hence are much more likely to receive potentially curative therapy. Again the most plausible explanation is that the Japanese population at risk of HCC (those with chronic liver disease) is more rigorously screened than that in the UK. Although we cannot rule out the influence of aetiology, we can be confident that ethnicity is unlikely to be responsible as survival in Japan was only 7 months in the decade 1975–85 and has risen steadily thereafter, coincidentally with the introduction of screening (Ikai *et al*, 2010).

Our analyses demonstrated that in both the Japanese and UK cohorts BALAD-2d model has a marginally better level of discrimination compared with the BALAD model despite the former having just four risk levels. Furthermore, visual inspection of the BALAD model suggested that, in the UK cohort at least, six risk levels is too many and that the BALAD-2d model is more appropriate. For risk grouping, such as BALAD or BALAD-2d, it is implicit that patients belonging to the same risk group have equal survival probability. This is of course not necessarily the case; patients at the extremes of each risk group are classified as equal but most likely have quite different chance of survival. BALAD-2p model does not suffer from this limitation.

We addressed the discrepancy in magnitude of the underlying hazard between the UK and Japanese patients through model recalibration. The relatively minor adjustments required are indicative of the transferability of the model, and we have shown that the covariate effects in the Japanese cohort are applicable in UK patients. Had recalibration beyond simple adjustment to the height of the baseline hazard been required (e.g. changes to the shape of the baseline hazard, or even the covariate vector), then the validity of BALAD-2p model would have been questionable. In this case we had no such concerns. Although the BALAD-2p model builds on the BALAD model's concept by including the ability to predict patient-level survival (Figure 5) and as such is a more powerful prognostic device, validation in other regions of the

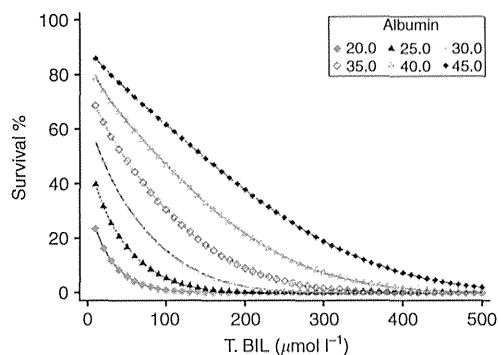


Figure 5. Example of patient-level survival estimation. Reporting predictions of 2-year survival and describing the impact of increasing albumin and bilirubin; all other parameters are fixed (AFP 34, AFP-L3 16.1, and DCP 1.14).

world, especially where the aetiology is related to Hepatitis B, is still required.

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High-sensitivity *Lens culinaris* agglutinin-reactive alpha-fetoprotein assay predicts early detection of hepatocellular carcinoma

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Abstract

Background Prognosis of patients with hepatocellular carcinoma (HCC) remains poor because HCC is frequently diagnosed late. Therefore, regular surveillance has been recommended to detect HCC at the early stage when curative treatments can be applied. HCC biomarkers, including *Lens culinaris* agglutinin-reactive fraction of alpha-fetoprotein (AFP-L3), are widely used for surveillance in Japan. A newly developed immunoassay system measures AFP-L3 % with high sensitivity. This retrospective study aimed to evaluate clinical utility of high-sensitivity AFP-L3 (hs-AFP-L3) as a predictor of early stage HCC in surveillance at a single site.

Methods Of consecutive 2830 patients in the surveillance between 2000 and 2009, 104 HCC-developed and 104 non-HCC patients were selected by eligibility criteria and propensity score matching. Samples were obtained from the HCC patients who had blood drawn annually for 3 years prior to HCC diagnosis.

Results In the present study, hs-AFP-L3 was elevated 1 year prior to diagnosis in 34.3 % of patients. The

survival rate of patients with the hs-AFP-L3 ≥ 7 % at 1 year prior to diagnosis was significantly lower than that of patients with hs-AFP-L3 < 7 %.

Conclusions Elevation of hs-AFP-L3 was early predictive of development of HCC even at low AFP levels and in absence of ultrasound findings of suspicious HCC. The hs-AFP-L3 should be added to surveillance programs with US because elevated hs-AFP-L3 may be a trigger to perform enhanced imaging modalities for confirmation of HCC.

Keywords Surveillance · A propensity score analysis · High-sensitivity AFP-L3 · DCP · HCC

Abbreviations

HCC	Hepatocellular carcinoma
AFP	Alpha-fetoprotein
AFP-L3	<i>Lens culinaris</i> agglutinin-reactive fraction of AFP
hs-AFP-L3	High-sensitivity AFP-L3
US	Ultrasound
DCP	Des-gamma-carboxy prothrombin
HBsAg	Hepatitis B surface antigen
HCV	Hepatitis C virus
ALT	Alanine aminotransferase
MRI	Magnetic resonance imaging

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Introduction

Hepatocellular carcinoma (HCC) is the third most common cause of death from cancer worldwide [1], and poor prognosis is reported because HCC is frequently diagnosed at late stages and is often untreatable. Therefore, surveillance for HCC has been advocated to detect HCC at

early stages when curative treatments can be applied [2, 3]. Global liver associations, including the American Association for the Study of Liver Disease (AASLD), the European Association for the Study of the Liver (EASL), and the Asian Pacific Association for the Study of the Liver (APASL), recommend regular surveillance on patients at high risk for HCC [4–6]. The most common tests used for surveillance are alpha-fetoprotein (AFP) tests and ultrasound (US). EASL and APASL adopt AFP and US in their guidelines, while AASLD recommends only US. Interpretation of US can be challenging when routine screening and comparison to previous imaging results are impossible or when US are performed by different institutes or instruments, whereas HCC biomarker values can be used independently with appropriate cutoff values. The Japan Society of Hepatology (JSH) has recommended not only US but also assays of three biomarkers: AFP, *Lens culinaris* agglutinin-reactive fraction of alpha-fetoprotein (AFP-L3), and des-gamma-carboxy prothrombin (DCP) [7].

However, AFP levels are often elevated even in patients with benign liver diseases. The low specificity of AFP has been a cause of concern for use as a HCC marker [8–10]. In contrast, a rate of AFP-L3 in total AFP (AFP-L3 %) has been reported to be highly specific for HCC in many studies [11–13]; however, accurate measurements of AFP-L3 % have been limited to patients having AFP >20 ng/mL by insufficient analytical sensitivity on a conventional assay system that is a liquid-phase binding assay (LiBASys) [14]. Recently, a micro-total analysis system (μ TAS) based lectin-affinity electrophoresis using microfluidics technology has enabled accurate measurements of AFP-L3 % even at low AFP [15]. The high-sensitivity AFP-L3 (hs-AFP-L3) assay has demonstrated improvement in clinical sensitivity and predicting of prognosis in HCC patients with AFP < 20 ng/mL [16–18]. The Liver Cancer Study Group of Japan has reported that 37 % of HCC patients had low AFP (<15 ng/mL) at the HCC diagnosis [19]. They also show that 34 % of patients had tumors with maximum diameter of <2 cm. Early HCC is a distinct clinical entity with a high rate of surgical cure and detection of early HCC results in long-term survival [20]. However, elevated AFP is not always observed in patients with such small tumors. Therefore, the hs-AFP-L3 assay which can measure serum levels at low AFP is expected to improve detection of HCC at the early stage. Moreover, lower cutoff values for hs-AFP-L3 has been considered to improve clinical sensitivity [16–18].

In this study, clinical utility in early prediction of development of HCC in our study cohort under surveillance using hs-AFP-L3 and analyzed retrospectively is reported.

Patients and methods

Patients

The study protocol was approved by the Institutional Ethics Committee of Ogaki Municipal Hospital in January 2009 and was in compliance with the Declaration of Helsinki. Written informed consent for use of stored serum samples for the study was obtained from the enrolled patients.

Between 2000 and 2009, a total of consecutive 2830 patients positive for hepatitis B surface antigen (HBsAg) or anti-hepatitis C virus (HCV) antibody who visited the Department of Gastroenterology and Hepatology at Ogaki Municipal Hospital were prospectively enrolled in our HCC surveillance. Of the 2830, 1214 patients met eligibility criteria: HBsAg- or HCV RNA-positive for more than 6 months, follow-up period of >3 years before HCC diagnosis, availability of sera sampled at least twice at 12-month intervals, maximal tumor diameter <3 cm and 3 nodules or less at diagnosis, and no oral intake of warfarin which is a DCP-inducing agent.

Of these 1214 patients, 114 patients had HCC and 1100 patients had no evidence of HCC during follow-up period. To reduce the confounding effects of covariates between HCC and control patients, we selected patients using propensity score matching. Six covariates including age, gender, etiology (HBV or HCV), Child-Pugh classification, platelet number, and alanine aminotransferase (ALT) except tumor markers were used. We computed the propensity score by using logistic regression with the independent variable including age (<65 years or ≤ 65 years), sex (female or male), etiology (HBV or HCV), Child-Pugh classification (A, B, or C), platelet count ($>150 \times 10^3/\text{m}^3$ or $\leq 150 \times 10^3/\text{m}^3$), and ALT activity (≤ 40 IU/mL or >40 IU/mL) as shown in previous reported cut-off values according to the previous reports [21, 22]. This model yielded a *c* statistic of 0.832 (95 % confidence interval [CI], 0.797–0.866), indicating a strong ability to differentiate between HCC and control patients. Calibration was assessed using the Hosmer–Lemeshow goodness-of-fit test [23]. The *P* value of the calculated propensity score was 0.647 based on the Hosmer–Lemeshow test and showed an absence of bias. We were able to match 104 HCC developed patients to 104 non-HCC developing patients. Table 1 shows demographics of HCC and non-HCC groups. The median of tumor size was 1.9 cm. The 69 % of HCC patients had single tumor and the 86 % of HCC patients were at TNM stage I and II.

Surveillance and diagnosis

According to Clinical Practice Guidelines for Hepatocellular Carcinoma in Japan [7], we performed US and three

Table 1 Demographics and propensity score matching

Characteristics		HCC (<i>n</i> = 104)	Non-HCC (<i>n</i> = 104)	<i>P</i> value
Age (years)	Median (range)	67 (37–81)	68 (14–84)	0.980
Gender	Male/female	58 (56 %)/46 (44 %)	58 (56 %)/46 (44 %)	0.889
Etiology	B/C/B + C	14 (13 %)/89 (86 %)/1 (1 %)	14 (13 %)/89 (86 %)/1 (1 %)	1.000
Child-Pugh classification	A/B/C	82 (79 %)/18 (17 %)/4 (4 %)	84 (81 %)/17 (16 %)/3 (3 %)	0.907
ALT (IU/L)	Median (range)	49 (7–361)	46 (12–321)	0.582
Platelet ($\times 10^4/\text{mm}^3$)	Median (range)	10.1 (3.2–34.0)	12.1 (2.1–41.4)	0.150
Tumor size (cm)	Median (25 %, 75 % quartile)	1.9 (1.5, 2.3)	NA	NA
Tumor number	Single/Multiple	72 (69 %)/32 (31 %)	NA	NA
TNM stage	I/II/III	49 (47 %)/41 (39 %)/14 (14 %)	NA	NA

biomarker studies (AFP, AFP-L3, and DCP) every 3–4 months and dynamic magnetic resonance imaging (MRI) every 12 months for cirrhosis patients under surveillance. For patients with chronic hepatitis, we performed US and three biomarker studies every 6 months. For diagnostic confirmation of HCC, patients had a dynamic MRI when US suggested progression in nodular lesion, change of echo pattern in nodules, or increased biomarkers: continuous elevation of AFP or increase to AFP 200 ng/mL or more, AFP-L3 15 % or more, or DCP 40 mAU/mL or more. The hs-AFP-L3 assay was not available for the surveillance of those days.

Forty-five patients were diagnosed as HCC histologically (surgical specimen, 39 patients; US-guided needle biopsy specimens, 6 patients). The remaining 59 patients were diagnosed as HCC as typical findings of dynamic MRI including hypervascular in the arterial phase with washout in the portal venous or delayed phase [4].

Treatments

Individual decisions for a primary treatment were generally made on the basis of the guidelines for HCC in Japan [7]. Patients were initially assessed for eligibility for resection. When patients declined or were deemed ineligible for resection, they underwent locoregional ablative therapy (LAT) as a second option or transcatheter arterial chemoembolization (TACE) as a third one. Of the enrolled 104 patients, 99 patients underwent resection (*n* = 39), LAT (*n* = 23), or TACE (*n* = 37: including patients with both LAT and TACE). Five patients did not receive any treatment for HCC. No patient underwent liver transplantation.

Imaging modalities

B-mode US was performed with an Aplio XV or XG ultrasound system (Toshiba Medical System, Tokyo, Japan) equipped with a convex probe (PUT-375BT). MR imaging was performed using a superconducting scanner

operating at 1.5 T (Signa Twin Speed; General Electric Medical Systems, Milwaukee, WI). MR images were obtained in the axial plane with a phased-array multicoil for the body. To scan whole livers, the section thickness was 8–10 mm with 2- and 3-mm intersectional gaps, depending on liver size. Breath-hold T1-weighted in-phase and out-of-phase fast spoiled gradient-recalled echo (SPGR, 200/dual echo [4.3/2.1] [TR/TE], 80° flip angle, one signal averaged) MR images were obtained with a field of view of 36–42 cm and a 256 \times 192 matrix during a 22-s acquisition time. T2-weighted fat suppression fast spin-echo (2000/85 [TR/TE], two signal averaged) MR images with respiratory synchronization were obtained with a field of view of 36–42 cm and a 352 \times 256 matrix. Breath-hold double arterial dynamic fast SPGR images (115/1.2 [TR/TE], 70° flip angle, one signal averaged) were obtained with a field of view of 36–42 cm and 512 \times 192 matrix during a 12-s acquisition time. Dynamic MR images were obtained before and after an antecubital intravenous bolus injection of 0.1 mmol/kg of gadopentetate dimeglumine (Magnevist; Bayer in Japan, Tokyo, Japan) followed by 15–20 ml of a sterile normal saline flush. The optimum timing of start of scanning was decided for each case after 1 ml test injection of gadopentetate dimeglumine. The scan times were about 25, 40, and 60 s, and 2–2.5 min after initiation of the contrast injection, representing the early hepatic artery, late hepatic artery, portal vein, and equilibrium phase, respectively. All MR images except T2-weight MR images were obtained using array spatial sensitivity encoding technique (ASSET).

Assays of hs-AFP-L3, AFP, and DCP

For this retrospective study, the measurements of hs-AFP-L3, AFP, and DCP were achieved by using a microchip capillary electrophoresis and liquid-phase binding assay on μ TASWako i30 auto analyzer (Wako Pure Chemical Industries, Ltd.) [16]. Analytical sensitivity of the μ TAS is 0.3 ng/mL AFP, and percentage of AFP-L3 can be

measured when AFP-L3 is over 0.3 ng/mL. Analytical sensitivity of LiBASys is 0.8 ng/mL AFP, but AFP-L3 % can not be calculated at AFP < 10 ng/mL.

Samples were obtained from 104 HCC patients who had blood drawn annually for 3 years prior to the HCC diagnosis and stored at -80°C until the measurements. In the HCC patients, stored serum samples at -3 years (over 30 months before, $n = 94$), -2 years (from 18 to 30 months before, $n = 97$), -1 year (from 6 to 18 months before, $n = 103$), and 0 year ($n = 104$) at the time of the HCC diagnosis were measured. In the non-HCC patients, similarly, stored serum samples at -3 years ($n = 99$), -2 years ($n = 104$), and -1 year ($n = 102$), and 0 year ($n = 104$) from the end of follow-up were measured.

Statistical analysis

To evaluate the diagnostic accuracy and predictive values of AFP, hs-AFP-L3, and DCP, sensitivity and specificity were calculated with cutoff values in the guidelines [7]. Furthermore, cutoff values of 5, 7, and 10 % for hs-AFP-L3 were used for this retrospective study according to previous reports [13, 16]. Serial changes of three biomarkers before the diagnosis of HCC were analyzed by

Wilcoxon matched pair signed rank test. For the evaluation of prognosis, the long-term survival of patients with HCC was determined by the Kaplan–Meier method and the log-rank test was used to compare the survival rates. The values were considered significant when P value was <0.05 . The analyses were performed using JMP10 statistical software (SAS Institute Japan, Japan).

The propensity score matching was performed with SPSS, version 18.0 for Windows (International Business Machines Corporation, Tokyo, Japan).

Results

Dynamic changes of biomarkers

The dynamic changes of hs-AFP-L3, AFP, and DCP in HCC patients at -3 , -2 , -1 , and 0 year before diagnosis are shown in Fig. 1a, b, and c. The levels of hs-AFP-L3 at -1 year were significantly elevated from the levels at -2 years ($P = 0.0001$). The levels of hs-AFP-L3 at -0 year were significantly elevated from the levels at -1 year ($P = 0.0003$, Table 2). AFP and DCP were significantly elevated between -1 and 0 year ($P = 0.0315$

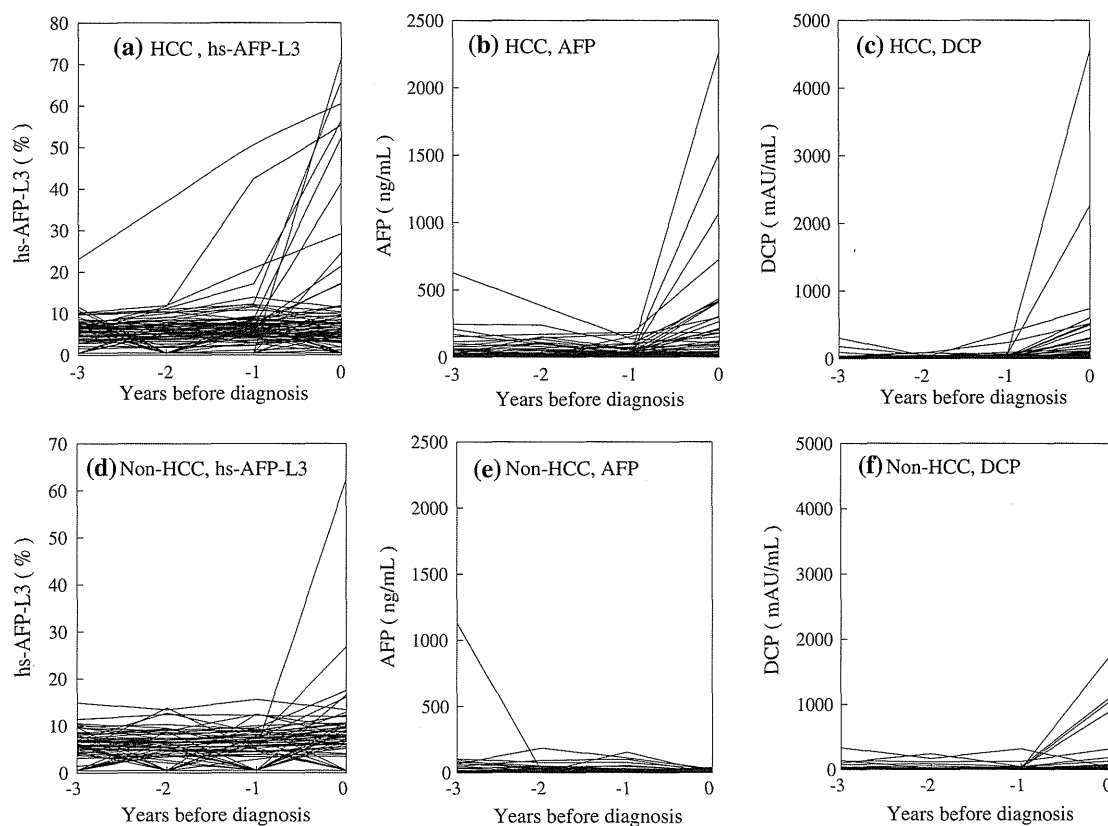


Fig. 1 Dynamic changes of biomarkers: **a** hs-AFP-L3, **b** AFP, and **c** DCP in each HCC patient ($n = 104$), and **d** hs-AFP-L3, **e** AFP, and **f** DCP in each non-HCC patient ($n = 104$)

Table 2 Serial changes of three biomarkers in HCC patients (Wilcoxon matched pair signed rank test)

Analyte	P value		
	At -3 year and -2 year	At -2 year and -1 year	At -1 year and 0 year
hs-AFP-L3	0.2935	0.0001	0.0003
AFP	0.4278	0.5359	0.0315
DCP	0.0926	0.6302	<0.0001

and $P < 0.0001$, respectively, Table 2). In non-HCC patients, no significant differences were observed for any markers (Fig. 1d–f). Only hs-AFP-L3 in HCC patients were significantly elevated 1 year prior to HCC diagnosis.

Sensitivity and specificity at diagnosis

Diagnostic sensitivity and specificity were evaluated for the hs-AFP-L3, AFP, DCP, and the combination of biomarkers (Table 3). The sensitivity was calculated by using HCC patient samples at diagnosis ($n = 104$) and the specificity was calculated by using non-HCC patient samples at -3 years ($n = 100$) to ensure that none had developed HCC for the following 3 years. Of the 104 HCC patients, 43 patients (41.3 %) had AFP < 10 ng/mL at which the conventional assay was not able to calculate AFP-L3 %. The sensitivity and specificity for hs-AFP-L3 were 11.5 and 100.0 %, respectively at a cutoff value of 15 %. A cutoff value of 7 % improved the sensitivity to 39.4 %. A combination assay with hs-AFP-L3, AFP, and DCP resulted in sensitivity of 60.6 % at diagnosis.

Sensitivity and specificity for 3 years before diagnosis

We calculated sensitivities using HCC samples at 3, 2, and 1 years prior to diagnosis. Similarly, specificities were

Table 3 Sensitivity and specificity at diagnosis

Analyte	Cutoff	Sensitivity (%)	Specificity (%)
hs-AFP-L3	5 %	50.9	51.0
	7 %	39.4	77.0
	10 %	16.3	96.0
	15 %	11.5	100.0
AFP	20 ng/mL	41.4	90.4
	200 ng/mL	12.5	99.0
DCP	40 mAU/mL	34.6	94.0
All biomarkers	7 % + 200 ng/mL	60.6	76.0
	+ 40 mAU/mL		

calculated by using non-HCC samples (Table 4). The sensitivity and specificity for hs-AFP-L3 at -1 year were 34.3 and 74.7 %, respectively. The sensitivities at -1 year for AFP and DCP were 35.0 and 12.1 %, respectively. In HCC patients, hs-AFP-L3 turned positive at 34 patients (33.3 %) and stayed in positive at 27 patients (26.2 %) for two years till the diagnosis of HCC. In contrast, hs-AFP-L3 turned positive at 25 patients (24.3 %) and stayed in positive at 22 patients (21.4 %) for 2 years till the end of follow-up in non-HCC patients.

Comparison of tumor characteristics and survival rates

Comparing tumor characteristics at detection of HCC by a level of hs-AFP-L3 at -1 year, the tumor size, the number of tumors, and TNM stage between patients with hs-AFP-L3 ≥ 7 % and < 7 % ($P = 0.064, 0.821, \text{ and } 0.504$, respectively) were not statistically significant. The number of patients receiving curative treatments such as resection and LAT was significantly higher in patients with hs-AFP-L3 < 7 % ($P = 0.020$) (data not shown).

During the follow-up period after the diagnosis that was ranged from 4 to 110 months (median of 39 months), the survival rate of patients with hs-AFP-L3 ≥ 7 % was significantly lower than that of patients with hs-AFP-L3 < 7 % by using values at -1 year ($P = 0.039$) (Fig. 2). There was no statistical significance between patients with DCP ≥ 40 mAU/mL and patients with DCP < 40 mAU/mL ($P = 0.831$). No patients had AFP > 200 ng/mL at -1 year. The survival rate of patients with hs-AFP-L3 ≥ 7 % had a lower tendency than that of patients with hs-AFP-L3 < 7 % at HCC diagnosis ($P = 0.1501$).

Triggers to perform MRI for suspicious HCC and positivity rates for hs-AFP-L3

In this study population, US was performed median of 4 times between -1 year and diagnosis day. The 104 HCC

Table 4 Sensitivity and specificity for three years before diagnosis

Analyte	Year	Sensitivity (%)	Specificity (%)
hs-AFP-L3 ≥ 7 %	-1	34.3	74.7
	-2	25.3	80.6
	-3	24.5	77.0
AFP ≥ 20 ng/mL	-1	35.0	86.4
	-2	31.0	83.0
	-3	33.0	86.0
DCP ≥ 40 mAU/mL	-1	12.1	93.9
	-2	8.4	94.9
	-3	4.3	94.0