RESEARCH ARTICLE

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High level of serum protein DKK1 predicts poor prognosis for patients with hepatocellular carcinoma after hepatectomy

Hepatic Oncology

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Practice points

- Surgery including tumor resection, liver transplantation and local ablation is the primary therapeutic strategy for hepatocellular carcinoma (HCC); however, the rate of recurrence is still very high.
- Current prognosis prediction is mainly based on morphological and histopathological features.
- Blood biomarkers are quite suitable for periodic surveillance for HCC patients after surgery due to its advantage of noninvasive sample acquirement.
- AFP is the only commonly used serological biomarker for HCC recurrence after treatment, while it is negative in 40–60% patients.
- Serum DKK1 showed association with overall survival and recurrence of HCC after hepatectomy and was an independent prognostic predictor.
- Serum DKK1 maintained recurrence prediction in HCC patients with relatively low recurrence risk.
- Measurement of preoperative serum DKK1 and AFP simultaneously could improve prognostic capability.
- HCC patients who harbored positive DKK1 values both before and after surgery showed a propensity of increased recurrence.

Aim: To evaluate prognostic significance of DKK1 for hepatocellular carcinoma. **Materials & methods:** We enrolled a test cohort consisting of 266 hepatitis virus B-related hepatocellular carcinoma patients who had undergone hepatectomy and a validation cohort of 95. Associations of DKK1 with overall survival and time to recurrence were determined by Cox proportional hazards regression model. **Results:** High levels of preoperative serum DKK1 were associated with poor overall survival and higher recurrence rate and DKK1 was an

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independent prognostic predictor. Moreover, DKK1 maintained ability to predict recurrence for patients with low recurrence risk. Double positives of DKK1 and AFP indicated the worst overall survival and the highest recurrence rate compared with either used alone. Patients with preoperatively and 1-day postoperatively positive DKK1 had higher recurrence rates than those whose values were both negative. Similar results were found in the validation cohort. **Conclusion:** Serum DKK1 could predict prognosis of hepatocellular carcinoma after hepatectomy.

KEYWORDS

• AFP • DKK1 • HCC
• prognosis • serum
biomarker

Hepatocellular carcinoma (HCC) represents the sixth most prevalent malignant neoplasm and the third leading cause of cancer-related death worldwide [1]. Over 50% of new cases and deaths occurred in China [1]. Advances in technical treatments have improved HCC survival to some degree, but the prognosis remains dismal [2,3]. Surgery including tumor resection, liver transplantation and local ablation is currently the primary therapeutic strategy for HCC, however, only 30–40% of patients are eligible for these options in clinics. Even patients receiving such curative strategies are not guaranteed full recovery; up to 80% among them experience recurrence or distant metastasis within 5 years, which is much higher than that of other gastrointestinal carcinomas [4,5]. Cancer classification and outcome prediction can provide important information for HCC management, no matter at the time of treatment selection or after therapy application [6]. Therefore, it is crucial to identify reliable predictors to discriminate patients with poor outcome.

Current prognostic prediction is mainly based on conventionally morphological and histopathological features such as tumor size, tumor number, vascular invasion, histological differentiation and tumor staging system. However, these markers provide limited information for predicting postoperative recurrence and survival, especially for cases diagnosed at earlier stages [7]. Biomarkers, which display ability to predict outcome and response to therapy, may be able to advance personalized medicine in cancer treatment [8,9]. Most predictive biomarkers were identified in tumor tissue rather than in body fluid. Blood biomarkers, due to the advantages of noninvasive sample acquirement and highly reproducible measurement, are extremely suitable for periodic surveillance after treatment. Up to now, AFP is the only commonly used serological protein biomarker for HCC surveillance. However, only 40–60% of patients harbor positive AFP and it is difficult to monitor tumor recurrence in patients with

negative levels of AFP. Moreover, association of AFP with HCC outcome has not yet been confirmed [10–13]. Therefore, it is imperative to develop novel and reliable serological biomarkers to identify HCC patients with high-risk of recurrence or short survival time, especially for AFP-negative patients and for HCC subgroups with low recurrence risk.

During the last decade, we have shown that DKK1, a secretory antagonist of canonical Wnt signal pathway, was overexpressed in HCC tissue by cDNA microarray and tissue microarray, and secreted DKK1 could be specifically detected in culture media from multiple cancer cell lines, including HCC [14,15]. Considering DKK1 is hardly expressed in human normal adult tissues but ordinarily is found only in placental and embryonic tissues [16,17], it might be a novel cancer-specific biomarker. Therefore, we further demonstrated that serum DKK1 was a novel serological protein marker for HCC diagnosis by a large-scale multicenter study [18]. With follow-up of the HCC patients enrolled in the above diagnostic study, here, we evaluated the capability of serum DKK1 as a prognostic predictor for HCC patients after hepatectomy. This study stringently followed the criteria of reporting recommendations for tumor marker prognostic studies (REMARK) [19].

Materials & methods

• Patients

361 patients with hepatitis virus B (HBV)-related HCC underwent local tumor resection were enrolled for prognostic analysis (Figure 1). 266 patients in a test cohort were from Zhongshan Hospital, Fudan University, and 95 in a validation cohort were from Eastern Hepatobiliary Surgery Hospital, Second Military Medical University. These patients were sorted out from the patients recruited for our previously diagnostic study [18]. Entrance criteria for prognostic analysis were: no prior anticancer treatment; curative resection; availability of complete clinical and follow-up data. Curative resection was defined as complete

Figure 1. Study design.

HCC: Hepatocellular carcinoma; OS: Overall survival; TTR: Time to recurrence.

resection of all tumor nodules with tumor margin being free of cancer by microscopic examination [20–22].

Liver function was assessed by Child–Pugh scoring system. Tumor stage was defined according to Barcelona Clinic Liver Cancer (BCLC) staging system [23]. Histological grade of tumor differentiation was determined according to Edmondson and Steiner grading system. Detailed clinicopathological characteristics are summarized in **Supplementary Table 1** (please see online at www.futuremedicine.com/doi/full/10.2217/hep.15.12).

• ELISA for DKK1

Concentrations of serum DKK1 were detected by a commercially available ELISA kit (R&D Systems, MN, USA) according to the manufacturer's instructions. Briefly, 96 well microtiter plates (Nalge Nunc, NY, USA) were coated with monoclonal anti-DKK1 antibody (4 µg/ml) and incubated at 4°C overnight. Incubation was blocked with 1% bovine serum albumin. Diluted sera were added and incubated at 37°C for 2 h, followed by the addition of biotinylated goat antihuman DKK1 (50 ng/ml) antibody for 2 h at 37°C. Then streptavidin-horseradish peroxidase was added. Color development was achieved with substrate of hydrogen peroxide and 3,3',5,5'-tetramethyl benzidine. Reaction was stopped by sulphuric acid (1M). Optical density was measured at 450 nm and referenced

to 570 nm on a plate reader (Synergy 2, Biotek, VT, USA). Concentrations of DKK1 were calculated with a four parameter logistic curve which fitted for the standard value and multiplied by the dilution factor. Measurements were done in duplicate.

• Follow-up

Surveillance for postoperative patients was performed as previously described [22,24]. Patients were monitored every 2 months with liver ultrasonography and liver function tests and AFP levels during the first year, and then every 3–4 months. After surgery, a CT scan of abdomen was also performed every 6 months. Bone metastasis was diagnosed by bone scan and MRI. Most common causes of death are recurrence, metastasis and complicated liver cirrhosis. Recurrence was confirmed by CT or MRI. When HCC recurrence was confirmed, further treatments such as second resection, radiofrequency ablation, percutaneous ethanol injection or transcatheter arterial chemoembolization were performed, based on the size, site, number of tumor nodules and liver function. If lymph node or bone metastasis was found, external radiotherapy was given. Overall survival (OS) was defined as the interval between surgery and death or the last observation point. Time to recurrence (TTR) was defined from the date of resection to the date of detection of any type of relapse (intrahepatic or extrahepatic recurrence)

or the last follow-up assessment [14,24]. Ethical approvals for this study were obtained from the institutional ethics review committee of each study center. Informed consent was obtained from each patient.

• Statistical analysis

Statistical analyses were performed with SPSS for Windows (version 16.0, SPSS Inc., USA) and GraphPad Prism software (version 5, GraphPad Software Inc., USA). Optimum cutoff value of DKK1 for prognosis was estimated by X-tile software (version 3.6.1, Yale University, CT, USA) [15]. This software provides an assessment of every possible cutoff point of dividing a population into subsets based on a biomarker expression, and assesses biological relationships between the biomarker and patients' outcome which estimated with Kaplan–Meier curve, and also produces corrected p-values to estimate statistical significance of data assessed by multiple cutoff-points. The optimum cutoff was defined as the one of the most significance. Correlation between binary DKK1 concentration and clinical variable was analyzed with Pearson's χ^2 test or Fisher's exact test. Cumulative survival time was calculated by Kaplan–Meier method and differences were assessed with log-rank test. Univariate and multivariate analyses were based on Cox proportional hazards regression model. Receiver operating characteristics curve was utilized to assess the prognostic predictive value, and differences between the areas under the curves (AUC) were compared using Medcalc software (version 10.4.7.0). p-values smaller than 0.05 (two-sided) were deemed statistically significant.

Results

• Patients population

The study design was detailed in **Figure 1**. Follow-up for 266 HCC patients in the test cohort was completed on June 2013 with a median time of 39 months (range from 1 to 52 months). Up to the last follow-up, 114 of 266 (42.9%) patients relapsed and 97 (36.5%) died, including 19 died with other diseases such as liver failure without record of recurrence. The 4-year survival rate and recurrence rate were 64.3 and 43.6%, respectively.

• Prognostic value of serum DKK1 for HCC patients after hepatectomy

In the test cohort, an optimum cutoff value of serum DKK1 for prognosis was determined as

3.28 ng/ml by X-tile software [15] (see Materials and methods, **Supplementary Figure 1**), with a positive rate of 50.8% (135/266). Based on this cutoff value, the 1-, 2-, and 4-year OS rates in DKK1-positive group were lower than those in DKK1-negative group (85.9 vs 95.4%, 74.1 vs 87.0% and 58.2 vs 70.5%, respectively; **Figure 2A**). Meanwhile, the 1-, 2-, and 4-year cumulative recurrence rates in DKK1-positive group were higher than those in DKK1-negative group (37.8 vs 15.4%, 48.4 vs 24.1% and 57.4 vs 31.4%, respectively; **Figure 2B**). The mean level of serum DKK1 was significantly higher in recurrent patients than that in nonrecurrent ones (mean [SE]: 4.29 [0.23] vs 3.35 [0.17]; $p < 0.001$) (**Figure 2C**). The sensitivity and specificity of DKK1 at 3.28 ng/ml for discriminating recurrence from nonrecurrence were 65.5 and 60.0%, respectively (**Figure 2C**). By univariate analysis, serum DKK1 level was significantly associated with OS (hazard ratio [HR]: 1.69; 95% CI: 1.12–2.54; $p = 0.01$) and TTR (2.36, [1.61–3.46]; $p < 0.001$) (**Table 1**). In addition to DKK1, the clinical variables including AFP, hepatitis B virus e-antigen, gammaglutamyl transferase, tumor encapsulation, size, number, vascular invasion, satellite lesion and Barcelona Clinic Liver Cancer (BCLC) stage were also correlated with OS and/or TTR (**Table 1**). Factors showed significance in univariate analysis were then subjected to multivariate Cox proportional hazards analysis. To avoid potential bias, BCLC stage was not included in multivariate analysis because its criterion is associated with clinical features of tumor size, nodule number, vascular invasion and liver function and performance status. Results showed in **Table 2** clearly indicated that DKK1 was an independent prognosticator for both OS (1.69 [1.10–2.60]; $p = 0.02$) and TTR (2.12 [1.43–3.15]; $p < 0.001$), along with tumor size (OS: 1.77 [1.11–2.80]; $p = 0.02$; TTR: 1.61 [1.07–2.43]; $p = 0.02$) and tumor satellite (OS: 2.37 [1.45–3.87]; $p = 0.001$; TTR 1.77 [1.15–2.72]; $p = 0.01$). The AUC of serum DKK1 for TTR was 0.63 (95% CI: 0.56–0.70), which was the largest compared with other clinical indices (**Figure 2D**). In addition, DKK1 was found to be only correlated with tumor size ($p < 0.001$) (**Supplementary Table 2**).

• Prognostic performance of preoperative serum DKK1 in HCC subgroups

We further investigated the predictive value of serum DKK1 in clinical subgroups of HCC.

Figure 2. Associations of preoperative serum DKK1 with prognosis of hepatocellular carcinoma were assessed in the test cohort. HCC patients with positive DKK1 had poor OS (A) shorter TTR (B) by Kaplan–Meier analysis. (C) Distributions of DKK1 in nonrecurrent and recurrent HCC patients and ROC curve for DKK1 in discriminating recurrence from nonrecurrence. (D) Predictive ability of serum DKK1 for TTR of HCC was compared with other clinical features by ROC curves. The AUCs with 95% CI are also shown. AUC: Area under the curve; BCLC: Barcelona Clinic Liver Cancer; HCC: Hepatocellular carcinoma; OS: Overall survival; TTR: Time to recurrence; ROC: Receiver operating characteristics.

Currently, it remains difficult to predict prognosis for HCC patients with low AFP levels [10]. In HCC patients with negative AFP, the 4-year recurrence rate in DKK1-positive group was much higher than that in DKK1-negative group (49.7 vs 28.4%; $p = 0.01$) (≤ 20 ng/ml) (Figure 3A). In addition, patients with positive serum DKK1 displayed higher risk of recurrence than those with negative DKK1 in other subgroups with relatively low recurrence risk, including patients at early stage (BCLC 0+A, 51.1% vs 28.6%; $p < 0.001$), with well-differentiated tumor (Edmondson I-II, 53.4 vs 29.7%; $p < 0.001$), without vascular invasion (51.1 vs 28.7%; $p = 0.004$), tumor size smaller than 5 cm (45.1 vs 30.7%; $p = 0.04$), single tumor (52.8 vs 27.9%; $p < 0.001$), absence of satellite lesions (53.2 vs 27.4%; $p < 0.001$) or with encapsulation (56.3 vs 31.1%; $p < 0.001$) (Figure 3B–I).

• Combination of preoperative serum DKK1 & AFP in prognosis of postoperative HCC patients

In the test cohort, the proportion of AFP-negative (≤ 20 ng/ml) was 53.8% (143 of 266). Log-rank test showed that AFP was correlated with OS ($p = 0.008$), but not with TTR ($p = 0.16$) (Supplementary Figure 2A & B). To evaluate the combined value of DKK1 and AFP for HCC prognosis, we divided patients into four groups: group I, DKK1⁻/AFP⁻ ($n = 53$); group II, DKK1⁻/AFP⁺ ($n = 78$); group III, DKK1⁺/AFP⁻ ($n = 70$) and group IV, DKK1⁺/AFP⁺ ($n = 65$). Significant differences were found in OS and TTR among these groups (Figure 4A & B). The 4-year OS rates and recurrence rates were 79.2 and 28.4% in group I, 64.6 and 33.4% in group II, 67.1 and 49.6% in group III, and 48.7 and 65.3% in group IV, respectively. Group IV showed the lowest

Table 1. Univariate analyses of factors associated with recurrence and survival in hepatocellular carcinoma patients from test and validation cohorts.

Variables	Test				Validation			
	OS		TTR		OS		TTR	
	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value
Gender (male vs female)	1.11 (0.51–2.39)	0.79	0.87 (0.45–1.66)	0.67	0.92 (0.34–2.46)	0.86	1.41 (0.59–3.36)	0.44
Age (y) (>52 vs ≤52)	0.84 (0.57–1.26)	0.41	0.73 (0.50–1.05)	0.09	0.61 (0.27–1.38)	0.23	0.91 (0.49–1.71)	0.78
HBsAg (positive vs negative)	1.34 (0.67–2.66)	0.41	0.92 (0.54–1.59)	0.78	0.63 (0.19–2.11)	0.45	1.28 (0.39–4.16)	0.68
HBeAg (positive vs negative)	1.65 (1.07–2.54)	0.02*	1.45 (0.97–2.17)	0.07	0.90 (0.36–2.26)	0.82	0.45 (0.19–1.06)	0.07
Liver cirrhosis (yes vs no)	0.97 (0.54–1.73)	0.90	0.80 (0.48–1.31)	0.37	0.44 (0.20–1.00)	0.05	0.66 (0.34–1.28)	0.22
GGT (U/l) (>54 vs ≤54)	2.04 (1.31–3.20)	0.002*	1.65 (1.12–2.42)	0.01*	0.95 (0.42–2.12)	0.89	1.32 (0.69–2.52)	0.40
ALT (U/l) (>51 vs ≤51)	0.77 (0.41–1.45)	0.42	0.86 (0.49–1.50)	0.60	0.57 (0.21–1.52)	0.26	0.61 (0.29–1.30)	0.20
AFP ng/ml (>20 vs ≤20)	1.75 (1.15–2.66)	0.009*	1.31 (0.90–1.89)	0.16	2.10 (0.78–5.64)	0.14	0.81 (0.42–1.57)	0.54
Tumor differentiation (III–IV vs I–II)	1.09 (0.70–1.70)	0.72	1.41 (0.96–2.08)	0.08	2.46 (0.84–7.22)	0.10	2.29 (1.01–5.20)	0.04*
Tumor encapsulation (none vs yes)	0.49 (0.28–0.86)	0.01*	0.68 (0.38–1.21)	0.19	2.78 (1.20–6.46)	0.02*	2.47 (1.29–4.74)	0.006*
Tumor size (>5 vs ≤5 cm)	2.45 (1.63–3.65)	<0.001*	2.19 (1.52–3.16)	<0.001*	2.61 (1.07–6.36)	0.03*	1.47 (0.77–2.82)	0.24
Tumor number (multiple vs single)	1.39 (0.83–2.32)	0.21	1.95 (1.26–3.02)	0.003*	2.88 (1.13–7.31)	0.03*	1.83 (0.80–4.16)	0.15
Vascular invasion (yes vs no)	2.20 (1.47–3.29)	<0.001*	1.62 (1.13–2.34)	0.009*	1.93 (0.79–4.73)	0.152	1.52 (0.74–3.14)	0.26
Tumor satellite (yes vs no)	3.03 (1.95–4.72)	<0.001*	2.11 (1.38–3.23)	0.001*	1.70 (0.72–4.02)	0.22	1.18 (0.59–2.38)	0.64
BCLC stage (B+C+D vs 0+A)	2.98 (1.97–4.50)	<0.001*	2.21 (1.49–3.28)	<0.001*	2.08 (0.61–7.05)	0.24	1.01 (0.49–2.08)	0.98
DKK1 (positive vs negative) [†]	1.69 (1.12–2.54)	0.01*	2.36 (1.61–3.46)	<0.001*	3.88 (1.53–9.83)	0.004*	2.91 (1.47–5.75)	0.002*
DKK1 and AFP [†] GI vs GII vs GIII vs GIV	1.43 (1.18–1.75)	0.001	1.56 (1.31–1.87)	<0.001	1.98 (1.30–3.00)	0.001	1.45 (1.08–1.93)	0.013

Univariate analysis: Cox proportional hazards regression model.

[†]Cutoff value of serum DKK1 for HCC prognosis was 3.28 ng/ml.

*Test cohort: GI, DKK1⁻/AFP⁻ ($n = 53$); GII, DKK1⁻/AFP⁺ ($n = 78$); GIII, DKK1⁺/AFP⁻ ($n = 70$); GIV, DKK1⁺/AFP⁺ ($n = 65$); Validation cohort: GI, DKK1⁻/AFP⁻ ($n = 17$); GII, DKK1⁻/AFP⁺ ($n = 33$); GIII, DKK1⁺/AFP⁻ ($n = 15$); GIV, DKK1⁺/AFP⁺ ($n = 30$).

*p-value smaller than 0.05.

BCLC: Barcelona Clinic Liver Cancer; HBeAg: Hepatitis B e-antigen; HBsAg: Hepatitis B surface antigen; HR: Hazard ratio; OS: Overall survival; TTR: Time to recurrence.

Table 2. Multivariate analyses of factors associated with survival and recurrence in hepatocellular carcinoma patients from test and validation cohorts.

Variables	OS		TTR	
	HR (95% CI)	p-value	HR (95% CI)	p-value
Test cohort				
HBeAg (positive vs negative)	1.43 (0.90–2.28)	0.13	NA	NA
GGT (>54 U/l vs ≤54 U/l)	1.40 (0.86–2.29)	0.18	1.22 (0.80–1.85)	0.36
AFP (>20 ng/ml vs ≤20 ng/ml)	1.53 (0.98–2.39)	0.06	NA	NA
Tumor encapsulation (none vs yes)	1.13 (0.59–2.19)	0.71	NA	NA
Tumor size (>5 vs ≤5 cm)	1.77 (1.11–2.80)	0.02	1.61 (1.07–2.43)	0.02
Vascular invasion (yes vs no)	1.57 (1.01–2.44)	0.04	1.33 (0.91–1.95)	0.14
Tumor satellite (yes vs no)	2.37 (1.45–3.87)	0.001	1.77 (1.15–2.72)	0.01
DKK1(positive vs negative)	1.69 (1.10–2.60)	0.02	2.12 (1.43–3.15)	<0.001
Validation cohort				
Tumor differentiation (III–IV vs I–II)	NA	NA	1.62 (0.68–3.85)	0.27
Tumor encapsulation (none vs yes)	2.17 (0.92–5.15)	0.08	1.82 (0.91–3.6)	0.09
Tumor size (>5 vs ≤5 cm)	1.86 (0.69–5.03)	0.22	NA	NA
Tumor number (multiple vs single)	3.76 (1.39–10.22)	0.009	NA	NA
DKK1 (positive vs negative)	2.79 (1.03–7.61)	0.04	2.33 (1.15–4.71)	0.02

Multivariate analysis: Cox proportional hazards regression model. Variables were adopted for their prognostic significance by univariate analysis.
HbeAg: Hepatitis B e-antigen; HR: Hazard ratio; NA: Not applicable; OS: Overall survival; TTR: Time to recurrence.

survival rate and the highest recurrence rate. Combination of DKK1 and AFP also has the largest AUCs for OS (0.614) and TTR (0.656), followed by DKK1 alone (0.567 for OS, 0.631 for TTR) and AFP alone (0.585 for OS, 0.535 for TTR) (Figure 4C & D).

• Prognostic significance of changes in serum DKK1 before & after HCC surgery

Previously, we already demonstrated that serum DKK1 levels dropped significantly after HCC surgical resection [18]. Here, in the test cohort, 93 patients among them with 1-day postoperative DKK1 value and complete follow-up information were employed to investigate associations between changes of DKK1 level and prognosis. Based on the changes of DKK1 before and after surgery, these patients were divided into four groups: (a) pre- and postoperatively negative (DKK1⁻ to DKK1⁻; n = 41); (b) preoperatively positive to postoperatively negative (DKK1⁺ to DKK1⁻; n = 23); (c) pre- and postoperatively positive (DKK1⁺ to DKK1⁺; n = 25); (d) preoperatively negative to postoperatively positive (DKK1⁻ to DKK1⁺; n = 4). The group (d) is excluded because the number is too small for analysis. Significant differences were found among the other three groups (p = 0.01, group (a) vs group (b); p = 0.08; group (a) vs group (c); p = 0.003; group

(b) vs group (c); p = 0.49, Figure 5A). The 4-year recurrence rates in groups (a), (b) and (c) were 30.0, 60.1 and 76.4%, respectively. HCC patients with pre- and postoperatively positive DKK1 showed the highest recurrence rate. The AUC of change in DKK1 is 0.644 (95% CI: 0.528–0.761) (Figure 5B).

• Confirmation of prognostic ability in an independent validation cohort

Using the 3.28 ng/ml threshold for DKK1, we observed similar prognostic results of pre-operative serum DKK1 in another independent cohort consisting of 95 HCC patients after surgery. Follow-up of the validation cohort was terminated on August 2013 with a median time of 43.9 months (range from 2.8 to 52.6 months). Up to the last follow-up point, 39 of 95 (41.1%) patients had relapsed and 24 (25.3%) had died. The positive proportion of DKK1 was 47.4% (45 of 95). Cumulative OS rates in DKK1-positive group were also lower than that in DKK1-negative group (1-, 2- and 4-year OS rates were 97.8 vs 100%, 93.3 vs 94.0% and 48.2 vs 88.0%, respectively; Figure 6A). Meanwhile, DKK1-positive group also had higher recurrence rates as compared with DKK1-negative group (1-, 2- and 4-year recurrence rates were 22.2 vs 12.0%, 26.7 vs 18.1% and 62.7 vs 24.7%, respectively;

Figure 6B). Serum DKK1 was associated with OS ($p = 0.004$) and TTR ($p = 0.002$) by univariate analysis (Table 1) and it was an independent predictor for both OS ($p = 0.04$) and TTR ($p = 0.02$) by multivariate analysis (Table 2). The mean level of preoperative serum DKK1 in recurrent patients was much higher than that in nonrecurrent cases ($p < 0.0001$). The

Figure 3. Prognostic impacts of preoperative serum DKK1 for time to recurrence in hepatocellular carcinoma subgroups with relatively low risk of recurrence were assessed in the test cohort. Prognostic outcomes of DKK1 were significant in HCC patients with AFP ≤ 20 ng/ml (A), at BCLC 0+A stage (B), with Edmondson I–II tumor differentiation (C), without vascular invasion (D), with tumor size smaller than 5 cm (E), with solitary tumor (F), absent of satellite lesion (G) or with encapsulation (H). (I) Recurrence rates of patients with positive DKK1 versus negative DKK1 in respective subgroups. BCLC: Barcelona Clinic Liver Cancer; HCC: Hepatocellular carcinoma; TTR: Time to recurrence.

Figure 4. Prognostic value of the combination of serum DKK1 and AFP was evaluated in the test cohort. HCC patients were divided into four subgroups: group I, DKK1⁻/AFP⁻ (n = 53); group II, DKK1⁻/AFP⁺ (n = 78); group III, DKK1⁺/AFP⁻ (n = 70); group IV, DKK1⁺/AFP⁺ (n = 65). (A & B) HCC patients with positive DKK1 and positive AFP had the worst OS and the shortest TTR. The data under the K-M curves were the numbers of patients of death or recurrence at corresponding months after resection. (C & D) Predictive abilities of DKK1, AFP, and both for OS and TTR were analyzed by ROC curve.

AUC: Area under the curve; HCC: Hepatocellular carcinoma; OS: Overall survival; ROC: Receiver operating characteristics; TTR: Time to recurrence.

sensitivity and specificity of DKK1 in discriminating recurrence and nonrecurrence were 69.2 and 69.6%, respectively (Figure 6C). The AUC of DKK1 for TTR was also the largest compared with other clinical features (Figure 6D). Moreover, serum DKK1 also showed prognostic capacities for HCC subgroups in the validation cohort (Supplementary Figure 3), and DKK1-positive indicated higher potential of recurrence. Similar results were found for the combination of DKK1 and AFP (Supplementary Figure 4).

Discussion

HCC is a heterogeneous disease, even patients with the same clinical diagnosis and are subjected

to the same treatment strategy may have different clinical outcomes. Therefore, it is important to identify credible biomarkers to predict which individuals will have tumor relapse after surgical treatment. After we found serum DKK1 was a promising diagnostic biomarker for HCC [18], we further assessed whether it possesses prognostic abilities for HCC patients who received liver resection. With follow-up near 5 years and survival analyses, we found that high concentrations of preoperative serum DKK1 were associated with worse survival and higher recurrence rate, and DKK1 was an independent prognostic predictor for HCC.

Methods of current prognostic system are mostly applied after biopsy or surgery. Due

to the heterogeneity of tumors, immunohistochemistry markers may be missed by detecting them only from a thin piece of tissue section. Blood tumor markers can provide information from a whole neoplasm and have many other advantages such as noninvasiveness for sample acquirement, high reproducibility for measurement and availability for periodic surveillance; therefore, they are able to compensate certain limitations of immunohistochemistry predictors. To date, AFP is still the only commonly used serum protein marker for monitoring recurrence of HCC in patients after surgical treatment. However, results of studies on prognostic significance of AFP were not consistent [10–13]. In the present study, AFP was associated with OS but not TTR in the test cohort and associated with neither in the validation cohort (Table 1 & Supplementary Figure 2). When we evaluated the prognostic performance of serum DKK1 in AFP-negative HCC, we found these patients with positive DKK1 indicated early recurrence (Figure 3A & Supplementary Figure 3A). We further evaluated the performance of DKK1 together with AFP for HCC, and found the combination was associated with both OS and TTR, and patients with double positives of the two markers had the shortest survival time and the highest recurrence rate (Figure 4 & Table 1).

These results indicated that the simultaneous measurement of DKK1 and AFP could increase the predictability of prognosis. In addition, serum DKK1 maintained ability to predict recurrence in HCC patients at early stage, or with well-differentiated tumors, or without vascular invasion, or other subgroups with relatively low recurrence risk. As we know, monitoring changes of blood biomarkers after treatment is an important approach for early recurrence detection. In the present study, patients with postoperatively positive DKK1 showed a tendency of early recurrence. Therefore, we can foresee that HCC patients with high levels of preoperative serum DKK1 or both DKK1 and AFP may have poor prognosis after tumor resection, and then an adjuvant effective treatment should be considered.

Previously, we demonstrated by tissue microarray that DKK1 could predict prognosis for HCC patients after tumor resection or orthotopic liver transplantation [25,26]. A recent meta-analysis concluded that DKK1 might be a novel common prognostic marker for human tumors, though most of these studies involved for analysis were based on immunohistochemistry results [27]. Nowadays, interest in blood DKK1 as a potential prognostic biomarker is increasing for several types of human cancers,

Figure 5. Prognostic outcome of changes in serum DKK1 before and after operation in the test cohort. (A) Prognostic significance of DKK1 for TTR in patients with pre- and post-operatively negative (DKK1⁻ to DKK1⁻) (n = 41), conversion from preoperatively positive to postoperatively negative (DKK1⁺ to DKK1⁻) (n = 23), or pre- and post-operatively positive (DKK1⁺ to DKK1⁺) (n = 25). (B) ROC curve for change of DKK1 before and after operation.

AUC: Area under the curve; ROC: Receiver operating characteristics; TTR: Time to recurrence.

Figure 6. Associations of preoperative serum DKK1 with prognosis of hepatocellular carcinoma were assessed in the validation cohort. HCC patients with positive DKK1 had poor OS (A) shorter TTR (B) by Kaplan–Meier analysis. (C) Distributions of DKK1 in nonrecurrent and recurrent HCC patients and ROC curve for DKK1 in discriminating recurrence from nonrecurrence. (D) Predictive ability of serum DKK1 for TTR of HCC was compared with other clinical features by ROC curves. The AUCs with 95% CI are also shown. AUC: Area under the curve; BCLC: Barcelona Clinic Liver Cancer; OS: Overall survival; ROC: Receiver operating characteristics; TTR: Time to recurrence.

such as lung cancer, breast cancer, gastric cancer, cervical cancer, as well as HCC [28–34]. These results indicated that DKK1 might be a cancer-specific biomarker. In this study, with a test cohort and a validation cohort, we first clarified comprehensively the prognostic values of preoperative serum DKK1 for HCC and HCC subgroups.

DKK1 could be a promising prognostic predictor of recurrence and survival for HCC patients after hepatectomy, and this indicates certain biological mechanisms exist between elevated DKK1 and tumor metastasis and invasion. DKK1 is an antagonist but also a target gene of Wnt signaling pathway [35], therefore, the overactivated Wnt signaling in HCC [36,37] can increase the expression of DKK1. However, the overexpressed DKK1 fails to inhibit abnormal Wnt signaling. This may be because of the genetic alterations of β -catenin which keeps the Wnt signaling continuously activated [25]. Of interest, a recent study reported that ectopic expression of DKK1 could stimulate β -catenin expression [38]. DKK1 was also found to be preferentially expressed in metastatic HCC cell lines and HCC patients with recurrence, and could promote migration and invasion *in vivo* and *in vitro* through RhoA and JNK or MMP7 [25–26,38–39]. Nevertheless, more mechanisms of DKK1 in HCC still need to be further investigated.

Our study also leaves some limitations. First, the sample size of the validation cohort is not sufficiently large, and that may influence the prognostic results more or less, such as when HCC patients were stratified into subgroups, the sample number of each subgroup is under 20 (Supplementary Figure 3). In addition, it seems there is no prognostic difference for OS between DKK1-positive and -negative at the first 2 years after patients received surgery (Figure 6A); this may be caused by deleting the patients we failed to contact with, which may have resulted in some bias in the analysis. Second, the follow-up time could be longer. A third limitation is that we only have concentrations of serum DKK1 before and 1-day after surgery, but no regular measurements until recurrence or death. A future study with serial measurement of serum DKK1 is needed to detect whether it increases before recurrence. Fourth, our study was performed only on HBV-related HCC, which is a dominant etiology of Asian HCC patients. Further studies are still necessary to focus on

HCC patients with other etiologies and from other areas globally.

Conclusion & future perspective

To our knowledge, this is the first comprehensive study of serum DKK1 for the prognosis of postoperative HBV-related HCC. An elevated level of serum DKK1 was associated with a poor prognosis of HCC. The combination of DKK1 and AFP could improve the prognostic ability and might be used as a combined factor for prognosis prediction. We believe the test of serum DKK1 will benefit the management for HCC patients, especially for HCC subgroups who are difficult to predict in current clinics.

Financial & competing interests disclosure

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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