Serum DKK1 as a protein biomarker for the diagnosis of hepatocellular carcinoma: a large-scale, multicentre study

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Summary

Background Hepatocellular carcinoma (HCC) is prevalent worldwide and improvements in timely and effective diagnosis are needed. We assessed whether measurement of Dickkopf-1 (DKK1) in serum could improve diagnostic accuracy for HCC.

Methods We analysed data for patients with HCC, chronic hepatitis B virus (HBV) infection, liver cirrhosis, and healthy controls, recruited from two Chinese centres between December, 2008, and July, 2009. A validation cohort matched for age and sex was recruited from another centre in China between July, 2010, and June, 2011. DKK1 was measured in serum by ELISA by independent researchers who had no access to patients’ clinical information. We used receiver operating characteristics (ROC) to calculate diagnostic accuracy.

Findings We assessed serum DKK1 in 831 participants: 424 with HCC, 98 with chronic HBV infection, 96 with cirrhosis, and 213 healthy controls. The validation cohort comprised 453 participants: 209 with HCC, 73 with chronic HBV infection, 72 with cirrhosis, and 99 healthy controls. Levels of DKK1 in serum were significantly higher in patients with HCC than in all controls. ROC curves showed the optimum diagnostic cutoff was 2·153 ng/mL (area under curve [AUC] 0·848 [95% CI 0·820–0·875], sensitivity 69·1%, and specificity 90·6% in the test cohort; 0·862 [0·825–0·899], 71·3%, and 87·2% in the validation cohort). Similar results were noted for early-stage HCC (0·865 [0·835–0·895], 70·9%, and 90·5% in the test cohort; 0·896 [0·846–0·947], 73·8%, and 87·2% in the validation cohort). Furthermore, DKK1 maintained diagnostic accuracy for HCC who were α-fetoprotein (AFP) negative (0·841 [0·801–0·882], 70·4%, and 90·0% in the test cohort; 0·869 [0·815–0·923], 66·7%, and 87·2% in the validation cohort), including for patients with early-stage HCC (0·870 [0·829–0·911], 73·1%, and 90·0% in the test cohort; 0·893 [0·804–0·983], 72·2%, and 87·2% in the validation cohort). Compared with all controls, raised concentrations of DKK1 in serum could differentiate HCC from chronic HBV infection and cirrhosis (0·834 [0·798–0·871], 69·1%, and 84·7% in the test cohort; 0·873 [0·832–0·913], 71·3%, and 90·6% in the validation cohort). Moreover, measurement of DKK1 and AFP together improved diagnostic accuracy for HCC versus all controls compared with either test alone (0·889 [0·866–0·913], 73·3%, and 93·4% in the test cohort; 0·888 [0·856–0·920], 78·5%, and 87·2% in the validation cohort).

Interpretation DKK1 could complement measurement of AFP in the diagnosis of HCC and improve identification of patients with AFP-negative HCC and distinguish HCC from non-malignant chronic liver diseases.

Funding National Key Basic Research Programme of China, National Key Sci-Tech Special Projects of Infectious Diseases, National Natural Science Foundation of China, Research Fund for the Doctoral Programme of Higher Education of China.

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignant disease and the third leading cause of cancer-related death worldwide.1 In 2008, half of all new cases of liver cancer and related deaths worldwide were estimated to occur in China.2 HCC accounts for 70–80% of all liver cancers. Across all countries, 5-year overall survival is only 3–5%.3 This dismal outcome is due partly to the lack of an effective method for timely diagnosis, which leads to only 30–40% of patients with HCC being suitable for potentially curative treatments at the time of diagnosis.4,5 α-Fetoprotein (AFP) is the best serum biomarker for diagnosis of HCC, but sensitivity is low (25–65%) at the commonly used cutoff of 20 ng/mL, particularly in detection of early-stage HCC.6,7 In addition, many patients with non-malignant chronic liver disease have raised AFP concentrations in serum, including 15–58% of patients with chronic hepatitis and 11–47% with liver cirrhosis.8,9 Therefore, novel and reliable diagnostic biomarkers to complement AFP are urgently needed to improve clinical outcomes.

Dickkopf-1 (DKK1), a secretory antagonist of the canonical Wnt signalling pathway,10–14 was identified in 1998.15 It is hardly expressed in normal human adult tissues, except in placental and embryonic tissues.16,17 We have shown that DKK1 is overexpressed in HCC tissue but is not detectable in corresponding non-cancerous liver tissue.18,19 We have also shown that high levels of secreted DKK1 could be specifically measured in culture media derived from multiple human-cancer cell lines,
such as HCC, lung cancer, breast cancer, glioma, cervical
cancer, and in the sera of patients with HCC. Therefore, we and other researchers proposed that DKK1 had potential as a diagnostic and prognostic biomarker for multiple human cancers, including HCC. Yu and colleagues showed upregulated DKK1 in HCC tissues on microarray analysis and suggested it had prognostic value for HCC, especially in patients with early-stage disease or AFP-negative status. Sato and colleagues noted that concentrations of DKK1 in the sera of patients with liver cancer were higher than those in healthy volunteers. Tung and co-workers calculated sensitivity of 34% and specificity of 100% for DKK1 in the diagnosis of HCC. However, all these reports had limitations, such as assessments being done not in serum but only at tissue level, small study size, single-centre study design, absence of healthy controls or controls with non-malignant chronic liver disease, and no independent validation. Therefore, we designed a large-scale, multicentre validation study to assess the diagnostic accuracy of DKK1 as a serum protein marker for HCC, as part of the National Cancer Institute’s Early Detection Research Network (EDRN)-defined phase 2 biomarker study.

Methods

Study population

We recruited consecutive patients with HCC to a test cohort, from the Liver Cancer Institute, Zhongshan Hospital, Fudan University, Shanghai, China, from December, 2008, to June, 2009. We also recruited consecutive patients with chronic hepatitis B virus (HBV) or liver cirrhosis and healthy controls from the Department of Infectious Disease, First Affiliated Hospital of Soochow University, Suzhou, China, from April to July, 2009. A validation cohort comprising patients with HCC, chronic HBV infection, and cirrhosis and healthy controls was recruited from Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai, China, from July, 2010, to June, 2011.

HCC was defined on the basis of ultrasound, CT, or MRI characteristics and biochemistry (AFP serology and liver function enzymes), and was confirmed by histopathology, according to the American Association for the Study of Liver Diseases guidelines. Tumour stage was defined according to the Barcelona Clinic Liver Cancer (BCLC) staging system: for the purpose of this study, we classified tumours with BCLC stage 0+A as early-stage HCC. Diagnosis of chronic HBV infection included the presence of HBsAg for the previous 6 months, HBV DNA concentrations higher than 10³ copies per mL, and raised concentrations of alanine aminotransferase in serum, according to the guidelines of prevention and treatment of chronic HBV infection. The diagnosis of cirrhosis was based on histopathology of liver biopsy samples, and on clinical, laboratory, and imaging evidence where possible, including nodular liver contour, presence of ascites, portal hypertension, varices, enlargement of the caudate lobe, splenomegaly, and collateral portal-venous anastomoses. Patients with cirrhosis who had raised AFP concentrations were required to have undergone imaging by multiple methods (ultrasoundography, CT, or MRI) and to have had no evidence of a hepatic mass for at least 3 months before enrolment. The healthy controls were eligible blood donors with normal liver biochemistry, no history of liver disease, no viral hepatitis, and no malignant disease. Patients who had a history of other solid tumours were excluded from the study.

We matched the groups in the two cohorts for age and sex as far as possible. Data collection and analyses were undertaken by three independent researchers (JF, YT, and WZ).

Approval for the study was obtained from the institutional ethics review committee at each study centre. Informed consent was obtained from participants, according to the committees’ regulations.

Testing of blood and liver-tissue samples

Peripheral blood samples collected into anticoagulant-free tubes at the time of diagnosis were centrifuged and stored at –70°C until testing. Assays for serum DKK1 were done by two researchers (QS and WC) at the Shanghai Cancer Institute, Shanghai, China, and had no access to patients’ clinical information. A commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA) was used, according to the manufacturer’s recommendations. Briefly, 96-well Nunc-Immuno microtitre plates with MaxiSorp surface (Nalge Nunc, Penfield, NY,
USA) were coated with 100 μL monoclonal antibody to DKK1 supplied with the ELISA kit (4 μg/mL) and incubated at 4°C overnight. The reaction was blocked with 1% bovine serum albumin. Sera diluted with 10% neonatal calf serum were incubated for 2 h at 37°C. The detection antibody, biotinylated goat anti-human DKK1 (50 ng/mL), was incubated for 2 h at 37°C, followed by the addition of 100 μL 1:200 dilution of streptavidin-horseradish peroxidase for 20 min. Colour development was achieved with 100 μL per well 3,3,5,5-tetramethylbenzidine and hydrogen peroxide as a substrate, and sulphuric acid (1 mol/L) was added to stop the reaction. The optical density was measured at 450 nm and referenced to 570 nm on a Synergy 2 multimode plate reader (Biotek, Winooski, VT, USA). The concentrations of DKK1 were obtained with a four-parameter logistic curve, fitted for the standard value and multiplied by the dilution factor. When the concentration of DKK1 was less than 0·03 ng/mL (the lowest limit of the standard curve), the value was set as equal to zero. All measurements were done in duplicate.

AFP concentrations were measured with commercially available ELISA (R&D Systems), according to the manufacturer’s recommendations. When the concentration of AFP was less than 0·31 ng/mL (the lowest limit of the standard curve), the value was set as equal to 0. All measurements were done in duplicate.

To compare expression status of DKK1 and AFP, parallel expression profiles of these proteins were analysed at messenger RNA (mRNA), protein, and secreted protein levels in HCC-tissue and non-cancerous liver-tissue samples and in corresponding serum samples. We took samples from 16 patients with HCC (eight with AFP concentrations of 20 ng/mL or less and eight with concentrations higher than 20 ng/mL) and four healthy controls recruited at Zhongshan Hospital, and eight patients with cirrhosis recruited at the First Affiliated Hospital of Soochow University. The tissue

**Figure 2:** DKK1 and AFP concentrations in serum in the test and validation cohorts
(A) DKK1 for test cohort. (B) DKK1 for validation cohort. (C) AFP for test cohort. (D) AFP for validation cohort. Black horizontal lines are means, and error bars are SEs. 1210 ng/mL was used as the upper limit for AFP for any concentrations higher than this value. DKK1=Dickkopf-1. HC=healthy control. CHB=chronic hepatitis B virus infection. LC=liver cirrhosis. HCC=hepatocellular carcinoma. AFP=α-fetoprotein.
positive

Proportion (%)

Sensitivity

1-Specificity

ROC curve for DKK1, AFP, or both, in all patients with HCC, and for DKK1 by AFP status, in the validation cohort. (E) ROC curve for DKK1, AFP, or both, for all patients with HCC versus controls at risk of HCC in the test cohort. (F) ROC curve of DKK1, AFP, or both, for patients with chronic HBV infection or cirrhosis, and for DKK1 by AFP-positive status, in the validation cohort. ROC-receiver operating characteristics. HCC=hepatocellular carcinoma. HC=healthy control.

Figure 3: Diagnostic outcomes for serum DKK1 in the diagnosis of HCC
(A) ROC curve for DKK1, AFP, or both for all patients with HCC versus all controls in the test cohort. (B) ROC curve for DKK1, AFP, or both for all patients with HCC versus all controls in the validation cohort. (C) The rate of positive results for AFP, DKK1, or both, in all patients with HCC, and for DKK1 by AFP status, in the test cohort. (D) The rate of positive results for AFP, DKK1, or both, in all patients with HCC, and for DKK1 by AFP status in the validation cohort. (E) ROC curve of DKK1, AFP, or both, for all patients with chronic HBV infection or cirrhosis, and for DKK1 by AFP-positive status, in the test cohort. (F) ROC curve of DKK1, AFP, or both, for all patients with HCC versus controls at risk of HCC in the validation cohort. (G) The rate of positive results for AFP and DKK1 for patients with chronic HBV infection or cirrhosis, and for DKK1 by AFP-positive status, in the validation cohort. ROC-receiver operating characteristics. HCC=hepatocellular carcinoma. HC=healthy control. HBV=hepatitis B virus. CHB=chronic hepatitis B virus infection. LC=liver cirrhosis.

The following primers were used in the semi-quantitative reverse-transcription PCR and quantitative real-time PCR analyses: DKK1 (GenBank NM_001101.3) forward primer: 5′-GACCCAGGTGGCAAAATGTGAC-3′; reverse primer: 5′-CCTGAGCAATCCAGCACAT-3′; β-actin (ACTB, GenBank NM_001134.1) forward primer: 5′-TTGTTACAGGAAGTTCCCTTGCC-3′; reverse primer: 5′-ATGCTATCAGCTCCCCTGTTG-3′. All the experiments were done in triplicate.

To assess whether levels of DKK1 in serum change after surgical resection of HCC, we collected serum samples were obtained immediately after surgical resection and were snap-frozen in liquid nitrogen and stored at -70°C. The serum samples were also stored at -70°C until assay.

Total RNA was extracted with TRIzol reagent (Invitrogen, CA, USA). Reverse transcribed complementary DNA was synthesised by a PrimerScript RT reagent kit (Takara Bio, Shiga, Japan). Semi-quantitative reverse-transcription PCR experiments were undertaken with a HotStarTaq DNA polymerase (Qiagen, Shanghai, China). We used β-actin as an internal control. The cycling conditions were 5 min at 94°C, 28 (β-actin) or 32 (DKK1 or AFP) cycles of 30 s at 94°C, 30 s at 57°C, 1 min at 72°C, with a final extension of 10 min at 72°C, in a 96-well Veriti Thermal Cycler system (Applied Biosystem, Carlsbad, CA, USA). We analysed amplified products by 2% agarose gel electrophoresis. Quantitative real-time PCR was done with SYBR Premix Ex Taq (Takara Bio) in an ABI-Prism 7500 sequence detection system (Applied Biosystems). Relative concentrations of mRNA were calculated on the basis of threshold cycle (Ct) values, corrected by β-actin expression, with the following equation for DKK1

\[ 2^{-\Delta Ct} = \frac{Ct(DKK1) - Ct(\beta-actin)}{Ct(DKK1)} \]

or with the following equation for AFP

\[ 2^{-\Delta Ct} = \frac{Ct(AFP) - Ct(\beta-actin)}{Ct(AFP)} \]

The following primers were used in the semi-quantitative reverse-transcription PCR and quantitative real-time PCR analyses: DKK1 (GenBank NM_001101.3) forward primer: 5′-GACCCAGGCTGGCAAAATGTGAC-3′; reverse primer: 5′-CCTGAGCAATCCAGCACAT-3′; β-actin (ACTB, GenBank NM_001134.1) forward primer: 5′-TTGTTACAGGAAGTTCCCTTGCC-3′; reverse primer: 5′-ATGCTATCAGCTCCCCTGTTG-3′. All the experiments were done in triplicate.

To assess whether levels of DKK1 in serum change after surgical resection of HCC, we collected serum
samples from HCC patients in the test cohort before and after surgery.

Statistical analysis

Statistical analyses were done with SPSS for Windows (version 16.0) and MedCalc (version 10.4.7). Differences between two independent groups were tested with the Mann-Whitney U test (continuous variables and non-parametric analyses). Receiver operating characteristics (ROC) curves were constructed to assess sensitivity, specificity, and respective areas under the curves (AUCs) with 95% CI. We investigated the optimum cutoff value for diagnosis by maximising the sum of sensitivity and specificity and minimising the overall error (square root of the sum of squared errors).

### Table 1: Results for measurement of serum DKK1, AFP, or both,* in the diagnosis of HCC

<table>
<thead>
<tr>
<th>Test</th>
<th>Validation</th>
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<tbody>
<tr>
<td>AUC (95% CI)</td>
<td>Sensitivity (%)</td>
</tr>
<tr>
<td>HCC vs CHB, LC, and HC</td>
<td>0.848 (0.820–0.875)</td>
</tr>
<tr>
<td>DKK1</td>
<td>0.834 (0.798–0.871)</td>
</tr>
<tr>
<td>AFP</td>
<td>0.767 (0.698–0.832)</td>
</tr>
<tr>
<td>DKK1+AFP</td>
<td>0.860 (0.827–0.892)</td>
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</tbody>
</table>

DKK1=Dickkopf-1. AFP=alpha-fetoprotein. AUC=area under curve. PPV=positive predictive value. NPV=negative predictive value. LR=likelihood ratio. HCC=hepatocellular carcinoma. CHB=chronic hepatitis B virus infection. LC=liver cirrhosis. HC=healthy controls. *The diagnostic cutoff values of serum DKK1 and AFP were 2.153 ng/mL and 20 ng/mL, respectively.

### Table 2: Results for measurement of DKK1 in serum in the diagnosis of HCC

<table>
<thead>
<tr>
<th>Test</th>
<th>Validation</th>
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<tbody>
<tr>
<td>Test</td>
<td>Validation</td>
</tr>
<tr>
<td>AUC (95% CI)</td>
<td>Sensitivity (%)</td>
</tr>
<tr>
<td>HCC vs CHB, LC, and HC</td>
<td>0.841 (0.801–0.882)</td>
</tr>
<tr>
<td>HCC vs CHB and LC</td>
<td>0.830 (0.785–0.875)</td>
</tr>
<tr>
<td>Early-stage HCC vs CHB, LC, and HC</td>
<td>0.830 (0.785–0.875)</td>
</tr>
<tr>
<td>Early-stage HCC vs CHB and LC</td>
<td>0.857 (0.812–0.903)</td>
</tr>
</tbody>
</table>

DKK1=Dickkopf-1. AFP=alpha-fetoprotein. AUC=area under curve. PPV=positive predictive value. NPV=negative predictive value. LR=likelihood ratio. HCC=hepatocellular carcinoma. CHB=chronic hepatitis B virus infection. LC=liver cirrhosis. HC=healthy controls. *The diagnostic cutoff value was 2.153 ng/mL.
of the sum $[1-\text{sensitivity}]^2 + [1-\text{specificity}]^2$, and by minimising the distance of the cutoff value to the top-left corner of the ROC curve. To test the diagnostic accuracy when both DKK1 and AFP were measured, we estimated the regression equations for all comparisons are provided in the appendix.

### Role of the funding source

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

### Results

We recruited 1284 participants overall, 831 in the test cohort and 453 in the validation cohort (figure 1). Clinicopathological characteristics of HCC, chronic HBV infection, and cirrhosis in patients in the test and validation cohorts are summarised in the appendix. The cohorts were well matched for age and sex overall, except that fewer women with HCC were enrolled into the test cohort than into the validation cohort.

DKK1 concentrations on ELISA were significantly higher in patients with HCC in the test cohort than in all controls (median 3·08 ng/mL, IQR 1·75–4·57; mean 3·48, SD 2·33 ng/mL; p<0·0001; figure 2, appendix); values did not differ significantly between the three control groups (figure 2). Although the median concentration of AFP in serum was increased for patients in the HCC group compared with that in healthy controls, as expected (p<0·0001), significant increases were also seen in patients with chronic HBV infection and cirrhosis (p<0·0001, figure 2, appendix). Expression of DKK1 was frequently higher than that of AFP in liver-tissue samples and corresponding serum samples from patients with HCC, compared with patients with chronic HBV infection and cirrhosis.

ROC curves showed the optimum diagnostic cutoff for DKK1 was 2·153 ng/mL (AUC 0·848, 95% CI 0·820–0·875, sensitivity 69·1%, specificity 90·6%; figure 3, table 1). The optimum cutoff value for AFP was 15·35 ng/mL (AUC 0·830, 0·802–0·858, sensitivity 59·4%, specificity of 87·4%). As the sensitivity and specificity of the sum [1−sensitivity]$^2 + [1−specificity]^2$, and by minimising the distance of the cutoff value to the top-left corner of the ROC curve. To test the diagnostic accuracy when both DKK1 and AFP were measured, we estimated functions of the combined marker by binary logistic regression, and the values of these functions were used as one marker and subjected to ROC analysis, as previously described. The correlation between DKK1 concentrations in serum and clinicopathological characteristics was analysed with Pearson’s χ² test or Fisher’s exact test. We compared DKK1 levels in serum before and after surgical resection in patients with HCC with the independent samples t test and the paired t test.

To assess whether the combined use of DKK1 and AFP measurement was better than either of these two biomarkers alone, a new variable predicted probability (p) for HCC was created on the basis of an equation obtained by binary logistic regression (all HCC versus all control groups in the test cohort):

$$\log \left( \frac{p}{1-p} \right) = -6·152 + 5·517 \times \text{DKK1} + 6·867 \times \text{AFP}$$

The regression equations for all comparisons are provided in the appendix.

We took p values lower than 0·05 (two sided) to be significant.
specificity were similar to those for the recommended clinical cutoff of 20 ng/mL (57·8% and 88·0%, respectively; p=0·104), we chose 20 ng/mL as the cutoff value for AFP in this study. Predictive values and likelihood ratios for DKK1 and AFP in the diagnosis of HCC are shown in table 1.

A greater proportion of patients with HCC in the test cohort were positive for DKK1 than for AFP (293 [69·1%] vs 245 [57·8%] of 424 patients; figure 3). Furthermore, 126 (70·4%) of 179 AFP-negative patients with HCC had positive DKK1 results (figure 3). The rate was similar (167 [68·2%] of 245) in AFP-positive patients (figure 3). The ROC curves for DKK1 indicated a diagnosis of HCC irrespective of AFP status (table 2, appendix). DKK1 concentrations in serum correlated with tumour size (p=0·001, appendix).

In the assessment of differential diagnostic accuracy, serum DKK1 had greater AUC, sensitivity, and specificity values than did AFP in patients with HCC compared with chronic HBV infection and cirrhosis controls (figure 3, table 1). The proportions of patients with chronic HBV infection or cirrhosis who had positive DKK1 results (figure 3). The ROC curves for DKK1 indicated a diagnosis of HCC irrespective of AFP status (table 2, appendix).

In patients with HCC who had only one tumour of 2 cm or less, the AUC for DKK1 was 0·805 (95% CI 0·740–0·869) with sensitivity of 58·5% and specificity of 84·7%, compared with all controls (appendix). When HCC patients were compared with chronic HBV infection and cirrhosis patients, the AUC for DKK1 was larger than that for AFP (0·794, 0·727–0·861 vs 0·669, 0·589–0·748, p=0·019; appendix).

ROC analysis showed that testing of both DKK1 and AFP increased the diagnostic accuracy for HCC compared with either test alone (AUC 0·889, 95% CI 0·866–0·913, sensitivity 73·3%, and specificity 93·4%, figure 3; DKK1 plus AFP vs DKK1 alone, p<0·0001; DKK1 plus AFP vs AFP alone, p<0·0001; table 1). 371 (88%) of 424 patients with HCC had positive results when DKK1 and AFP were tested together (figure 3). Diagnostic accuracy of the combination of DKK1 and AFP remained improved when only early-stage HCC
Measurement of DKK1 and AFP together was also proven to improve diagnostic accuracy for HCC. Furthermore, the test could distinguish HCC from non-malignant chronic hepatitis B or liver cirrhosis, particularly in AFP-positive patients. Our findings indicate that DKK1 has the potential to be a serum protein biomarker for HCC and should be investigated further.

We used the following terms to search PubMed and the Chinese Biomedical Literature database, without date restrictions, for original research articles: “DKK1 OR DKK-1 AND (cancer OR tumor OR neoplasm) AND diagnosis”, and “DKK1 OR DKK-1 AND (hepatocellular carcinoma OR liver cancer)”. We found no large-scale, multicentre studies that had assessed diagnostic relevance of serum dickkopf-1 (DKK1) in hepatocellular carcinoma (HCC) or other human cancers, but five small, single-centre studies, published in Chinese or English, had explored DKK1 as a potential biomarker for HCC. However, whether measurement of DKK1 in serum had diagnostic value for patients with HCC remained to be revealed.

We did a comprehensive assessment of the diagnostic accuracy of serum DKK1 in HCC in a large-scale, multicentre investigation with validation. The sensitivity and specificity of serum DKK1 were high, and the predictive values and likelihood ratios were satisfactory for the diagnosis of HCC, including for early-stage disease and in patients with α-fetoprotein (AFP)-negative status. Measurement of DKK1 in serum could, therefore, complement that of AFP to improve diagnostic accuracy for HCC. Furthermore, the test could distinguish HCC from non-malignant chronic hepatitis B or liver cirrhosis, particularly in AFP-positive patients. Our findings indicate that DKK1 has the potential to be a serum protein biomarker for HCC and should be investigated further.

Discussion

We have shown that measurement of serum DKK1 has diagnostic value for HCC better than that of AFP, especially for patients with AFP-negative status and early-stage HCC. 30–40% of all patients with HCC are AFP negative, and diagnosis and assessment of treatment response are difficult with current methods.5,9 Thus, combined testing of DKK1 and AFP concentrations in serum could improve results.

Results were negative for DKK1 in most AFP-positive patients with chronic HBV infection or cirrhosis and, therefore, patients with these chronic non-malignant diseases could be distinguished from those with HCC. Around 2 billion people have been infected with HBV worldwide, of whom about 350 million have chronic infections.7,8 AFP concentrations are raised in 11–58% of patients with chronic hepatitis or cirrhosis in the absence of HCC.8,9 Therefore, measurement of DKK1 in serum can help to make a differential diagnosis of HCC in patients in these high-risk populations.

Identification of novel serum biomarkers is an important goal in the diagnosis of cancer, especially for detection and screening in early-stage cancer.27 In the past decade, many discoveries of non-protein serum markers have been documented—eg, mutated DNAs, methylated DNAs, RNAs,28,29 However, protein markers measurable in serum are the most applicable for clinical routine assessments and population studies30–32 because generally such tests are non-invasive, require less than 100 μL serum, have low dependence on operator expertise, are low cost, have high reproducibility, and samples need no pretreatment (eg, extraction, purification or reverse transcription). Although many serum protein markers for cancer diagnosis have been proposed, few have been introduced into the clinic over the past 15 years,27,33 mainly because they have not met the following criteria: specific overexpression in cancer cells but not in corresponding normal cells; secreted proteins that can be easily detected in serum; and rare expression in human adult normal tissues except in embryonic tissues. DKK1 meets these criteria: it is a secretory protein, is specifically overexpressed in cancer cells,25,34 and is hardly detectable in human adult normal tissues except in placenta and embryonic tissues.13,14,16,34,35 Therefore, this protein might have potential as a cancer-specific serum biomarker for various human cancers, including HCC. Thus, the integration of measurement of DKK1 in serum into the diagnostic work-up for HCC along with information on AFP and HBV or hepatitis C virus infection status, image examinations, and other clinicopathological features should be considered.

We based our study in clinical centres in China where most cases of HCC are related to cirrhosis or HBV infection. This pattern differs from that in the USA, Europe, and Japan.15 Thus, the diagnostic value of DKK1 still needs further investigation. Nonetheless, a report from Japan noted raised concentrations of DKK1 in...
serum in patients with liver cancer, which indicates that measurement might be useful in patients with non-HBV-related HCC. Of note, we found that concentrations of DKK1 in serum were higher in HCC patients without cirrhosis than in those with cirrhosis, which was unexpected and needs to be confirmed. The molecular mechanism and clinical importance of this difference also need to be further explored.

Our study is cross-sectional and retrospective in nature and, therefore, we plan to do a prospective study to assess whether use of DKK1 can be validated in patients with HBV-related and non-HBV-related HCC.

The striking decrease in DKK1 concentrations in serum after surgery suggests that this protein will be a useful surveillance biomarker to assess the therapeutic response of HCC patients. To further explore this potential role, we are undertaking long-term follow-up of the HCC patients who underwent surgery in this study.

The sample size and the proportion of patients with early-stage HCC in our validation cohort were smaller than those in the test cohort (figure 1, appendix). Therefore, the groups differed to some degree in results for diagnostic performance (tables 1, 2). For instance, the positive and negative predictive values of serum DKK1 for differential diagnosis of AFP-negative early-stage HCC from HBV infection and cirrhosis were obviously different because the validation cohort had only 18 AFP-negative patients with early-stage HCC, compared with 130 in the test cohort (table 2, appendix). The sensitivity, specificity, and positive likelihood ratio of serum AFP also differed between cohorts (table 1). Despite these differences, the diagnostic capabilities of serum DKK1 and AFP were generally similar in the test and validation cohorts. We used BCLC stage 0-A to define early-stage HCC. Most patients belonged to BCLC stage A (225 [79%] of 285 in the test cohort and 63 [97%] of 65 in the validation cohort). To stage patients in the BCLC system, multiple clinical indexes, such as Child-Pugh score, Eastern Cooperative Oncology Group performance status, Okuda stage, liver function (portal hypertension, bilirubin), and tumour status (tumour size and number, vascular invasion, lymphatic metastasis, distance metastasis, etc.), are taken into account. As a result, although serum DKK1 level correlated with tumour size, it had no correlation with BCLC stage (appendix). Consequently, the diagnostic accuracy of serum DKK1 was similar in the early-stage HCC and all HCC patients, as well as in the two cohorts, irrespective of the different proportions of patients. Another possible reason for the similarity is that DKK1 is preferentially overexpressed at the early stage of prostate cancer and, therefore, it might also be overexpressed in early-stage HCC. However, this hypothesis needs to be explored further. We believe, though, that serum DKK1 has great potential to be a diagnostic protein biomarker for HCC because its accuracy in the test cohort was proven in an independent validation cohort, even with a different constitution.

To our knowledge, this is the first large-scale, multicentre study to report the clinically diagnostic relevance of DKK1 as a serum protein marker for HCC in a test cohort and an independent validation cohort (panel). Our results indicate that serum DKK1 could potentially be used to diagnose HCC, especially early-stage disease, and will help to resolve the deficiencies of AFP in the testing of AFP-negative patients, and can be used to make differential diagnoses.

Contributors
QS designed the study, did the experiments, analysed and interpreted the data, and wrote the manuscript. JF and X-RY provided patients’ samples and clinical data, analysed and interpreted the data, and wrote the paper. YT and WZ provided, analysed, and interpreted patients’ samples and clinical data. YX, YN, YN, ZW, JZ, SJ-Q, Y-HS, NT, WC, MW, and JW provided patients’ samples, clinical data, or both. BY, ZZ, SY, JG, and HW advised on the conception and design of the study. WQ conceptualised and designed the study, supervised the project, and revised the paper. All authors vouch for the respective data and analysis, approved the final version and agreed to publish the manuscript.

Conflicts of interest
We declare that we have no conflicts of interest.

Acknowledgments
We thank Huamao Wang, Hua Jiang, and Shile Sheng for technical support. This study was funded by the National Key Basic Research Program of China (2009CB521803), National Key Sci-Tech Special Projects of Infectious Diseases (2012ZX10002011-004 and 2012ZX10002011-002), National Natural Science Foundation of China (10973492, 81030038, 81000927, 81071661, and 81172777), and Research Fund for the Doctoral Program of Higher Education of China (20100071120064).

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