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Original Paper

Preoperative Alfa-Fetoprotein and Fibrinogen Predict Hepatocellular Carcinoma Recurrence After Liver Transplantation Regardless of the Milan Criteria: Model Development with External Validation

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Key Words
Hepatocellular carcinoma • Liver transplantation • Recurrence

Abstract
Background/Aims: Patient selection is critically important in improving the outcomes of liver transplantation for hepatocellular carcinoma. The aim of the current study was to identify biochemical measures that could affect patient prognosis after liver transplantation. ***Methods:*** A total of 119 patients receiving liver transplantation for hepatocellular carcinoma were used to construct a model for predicting recurrence. The results were validated using an independent sample of 109 patients from independent hospitals. All subjects in both cohorts met the Hangzhou criteria. ***Results:*** Analysis of the discovery cohort revealed an association of recurrence with preoperative fibrinogen and AFP levels. A mathematical model was developed for predicting probability of recurrence within 5 years: Y = logit(P) = -4.595 + 0.824 ×fibrinogen concentration (g/L) + 0.641 × AFP score (1 for AFP<=20ng/ml, 2 for 20<AFP<=100ng/ml, 3 for 100<AFP<=200ng/ml, 4 for 200<AFP<=400ng/ml, 5 for AFP>400ng/ml). At a cutoff score of -0.85, the area under the curve (AUC) was 0.819 in predicting recurrence (vs. 0.655 when using the Milan criteria). In the validation cohort, this model had reasonable performance in predicting 5-year overall survival (68.8% vs. 28.1% in using the -0.85 cutoff, p<0.001) and

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disease-free survival (65.7% vs. 25.9%, p<0.001). The sensitivity and specificity were 77.0% and 62.5%, respectively. The AUC of this newly developed model was similar to that with the Milan criteria (0.698 vs. 0.678). Surprisingly, the DFS in patients with score <= -0.85 under this model but not meeting the Milan criteria was similar to that in patients meeting the Milan criteria (53.8% vs. 60.0%, p=0.380). ***Conclusions:*** Preoperative AFP and fibrinogen are useful in predicting recurrence of hepatocellular carcinoma after liver transplantation.

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths worldwide, with majority of the cases in China [1]. Liver transplantation (LT) is a treatment option that offers a chance of cure and long-term survival. A major issue with LT is organ shortage. As such, optimal allocation of organs is a major challenge. The Milan criteria are the most widely used set of criteria to assess the suitability of a certain patient to LT. The criteria consists: 1) a single tumor with diameter at <=5 cm or up to 3 tumors each with diameter at <=3 cm; 2) no extra-hepatic involvement; 3) no major vessel involvement [2]. Subsequent to the Milan criteria, several more liberal selection criteria have been proposed and increasingly used in practice [3, 4]. The use of these modified criteria has generally achieved survival comparable to the Milan criteria.

Significant room and needs exist for further refinement of the existing selection criteria since tumor recurrence remains a main cause of graft loss and patient mortality [4-6]. The Milan criteria and most other expanded criteria consider only tumor burden, distant metastasis and major vessel involvement. Previous studies have demonstrated that patient prognosis after LT are also affected by other clinical factors such as AFP and fibrinogen [7]. The Hangzhou criteria consider pathological differentiation [3], and thus represent a major step towards a more biologically based set of criteria. At a technical level, however, the Hangzhou criteria are not suitable for preoperative assessment [8].

In the current study, we examined potential impact of a wide variety of routine pre-operative variables on recurrence. The first step of model development considered 12 specific items within 4 domains (demographics, tumor size, liver function, and lab results reflecting tumor biological behavior). A multivariate COX regression revealed 2 independent risk factors of recurrence: AFP and fibrinogen. In the subgroup of patients not meeting the Milan criteria, the 5-year disease free survival (DFS) in patients with a score <= -0.85 under this model was comparable to that in patients meeting the Milan criteria. Validation using an independent cohort resulted in promising results.

Materials and Methods

Patients

The discovery cohort included 119 HCC patients receiving LT at the Third Affiliated Hospital of Sun Yat-sen University during a period from 2003 to 2009. The diagnosis of HCC was confirmed by pathological examination of the explanted liver in all cases. Subjects in this cohort included 62 patients meeting the Milan criteria and 57 patients meeting the Hangzhou criteria but not the Milan criteria. Only subjects with reliable follow-up information with regards to recurrence and death were included in data analysis. Subjects who died within perioperative period due to complications to surgery were not included.

Candidate variables for model development included demographics (age, sex), pre-operative lab results (total bilirubin, albumin, prothrombin time, PT-INR, fibrinogen concentration, neutrophil and lymphocyte count and AFP) and radiographic data (number and volume of tumor nodules, status of vascular invasion). The volume of each nodule was calculated by the equation: tumor volume = $4/3\times\pi\times r^3$ (r is the maximum radius of the tumor nodule). The total tumor volume (TTV) refers to the sum of each tumor nodule. We also calculated Child-Turcotte-Pugh grade and neutrophil-to-lymphocyte ratio in each patient.

Validation was carried out in an independent sample of 109 patients: 14 from the same medical center and the remaining 95 from 3 independent centers (35 from Xijing Hospital in Xi'an; 40 from the First Affiliated Hospital of Xi'an Jiaotong University, and 20 patients from Southern Hospital in Guangzhou). Subjects in this cohort included 51 patients meeting the Milan criteria and 58 patients meeting the Hangzhou criteria but not the Milan criteria.

Surgery and postoperative management

All subjects received piggyback LT. Postoperative immunosuppression regimen included tacrolimus and/or rapamycin, glucocorticoids (discontinued within 3 months), and basiliximab (on the day of operation and 4th day afterwards).

Follow-up

The follow-up was conducted once every month in outpatient clinics, and included liver function and AFP level. Abdominal and chest CT or MRI were conducted every 3 months. Recurrence was defined as detection of tumor recurrence inside the liver and/or metastasis.

Statistical analysis

Potential association of likely factors with 5-year DFS was examined by univariate analysis. Factors with a P value of <0.10 were entered into a multivariate COX regression analysis. Risk factors identified using this approach were used to construct a mathematical model using logistic regression, with recurrence within 5 years after LT (yes or no) as the outcome measure. Overall survival (OS) and DFS were compared using the Kaplan-Meier method. A receiver operating characteristic (ROC) curve analysis was performed and the AUC, sensitivity and specificity were calculated to compare the predictive value of the proposed model. All statistical analyses were performed using the SPSS 19.0 for Windows (SPSS Inc., Chicago, IL). Statistical significance was set at P<0.05. The cut-off value was selected using the receiver operating characteristic (ROC) curve analysis. Independent sample Student's t-test was used to compare clinicopathologic features between patients in different groups.

Results

Patient demographics and outcomes

The discovery cohort included a total of 119 subjects (111 men and 8 women; mean age: 50 years). All subjects met the Hangzhou criteria; 62 also met the Milan criteria. The median follow-up was 48.0 months (range: 3-60 months). 37 subjects died during follow-up; 38 developed tumor recurrence. The 5-year OS rate was 66.0% in the overall cohort, and 76.2% in the subset meeting the Milan criteria. The 5-year DFS rate was 65.3% in the overall cohort, and 75.1% in the subset meeting the Milan criteria.

Table 1. Preoperative factors affecting DFS. HR, hazard ratio; CI, confidence interval

Category		Univariate analysis	Multivariate analysis	HR	95% CI
Gender					
Male	111	0.689	-	-	-
Female	8				
Age (years)	50.4±1.0	0.011*	0.077	-	-
Prothrombin time (s)	16.4±0.4	0.179	-	-	-
Fibrinogen concentration (g/L)	2.90±0.13	0.006*	0.010	1.282	1.061-1.548
PT-INR	1.35±0.05	0.293	-	-	-
CTP grade					
A	70				
B	34	0.188	-	-	-
C	15				
HBV-DNA					
Positive	72	0.380	-	-	-
Negative	47				
TTV (cm^3)	258.5±53.8	0.006*	0.237	-	-
Number of tumor nodules					
>3	13	0.269	-	-	-
<=3	106				
AFP (ng/ml)					
<=20	37				
20-100	22				
100-200	11	0.001*	0.002	1.391	1.127-1.715
200-400	13				
>400	36				
HBV surface antigen					
Positive	109	0.437	-	-	-
Negative	10				
NLR	3.57±0.35	0.804	-	-	-
Pre-LT treatment					
Yes	37				
TACE	13				
Liver resection	9	0.534	-	-	-
Ablation	5				
Combined TACE+resection/ablation	10				
No	82				

The validation cohort consisted 109 subjects (99 men and 10 women; mean age: 47 years). All subjects met the Hangzhou criteria; 51 patients also met the Milan criteria. The median follow-up was 30.0 months (range: 5-60 months). 57 patients died during follow-up; 61 developed recurrence. The 5-year OS arte was 44.4% in the overall cohort, and 65.5% in the subset meeting the Milan criteria, and the 5-year DFS rate was 42.2% in the overall cohort, and 60.0% in the subset meeting the Milan criteria.

Discovery cohort - identification of risk factors

Univariate analysis revealed an association of poor DFS with: older age, higher plasma fibrinogen concentration, higher AFP and larger TTV (Table 1). Multivariate COX regression analysis identified the following independent risk factors: preoperative plasma fibrinogen concentration and AFP level.

Further analysis showed that the fibrinogen concentration was strongly correlated with TTV (p<0.001), and patients who developed recurrence had significantly higher fibrinogen concentration than patients who did not develop recurrence (3.39 vs. 2.47g/L, p=0.001). Also, the fibrinogen concentration was correlated with the Child-Tercotte-Pugh grade (CTP grade) of the patients (p<0.001). In the patients with AFP <= 20ng/ml, subjects with lower fibrinogen concentration had a significantly better outcome than subjects with higher fibrinogen concentration (OS 95.7% vs. 54.7%, p=0.004, DFS 95.5% vs. 49.2%, p=0.004, Fig. 1 A and B).

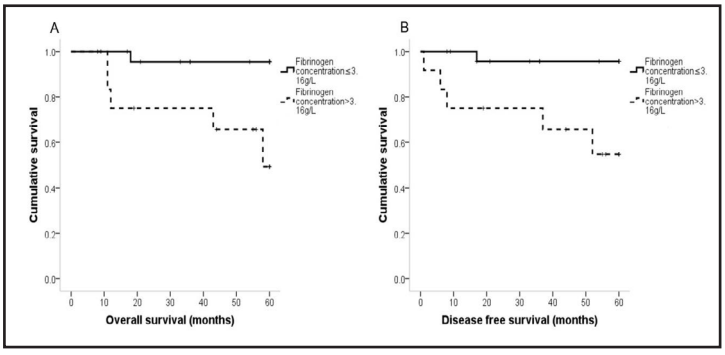

Fig. 1. In patients with AFP <= 20ng/ml, subjects with lower fibrinogen concentration had a significantly higher OS and DFS than subjects with higher fibrinogen concentration. A: The OS was significantly higher in patients with lower fibrinogen concentration than patients with higher fibrinogen concentration (95.7% vs. 54.7%, p=0.004). B: The DFS was significantly higher in patients with lower fibrinogen concentration than patients with higher fibrinogen concentration (95.5% vs. 49.2%, p=0.004).

Discovery cohort - model construction

The following 2 factors were entered into a mathematical model to predict recurrence risk: preoperative plasma fibrinogen concentration and AFP level using logistic regression. The model could be mathematically presented as: Y = logit(P) = -4.595 + 0.824 ×fibrinogen concentration (g/L) + 0.641 × AFP (score of 1 for AFP<=20ng/ml, 2 for 20<AFP<=100ng/ml, 3 for 100<AFP<=200ng/ml, 4 for 200<AFP<=400ng/ml, 5 for AFP>400ng/ml).

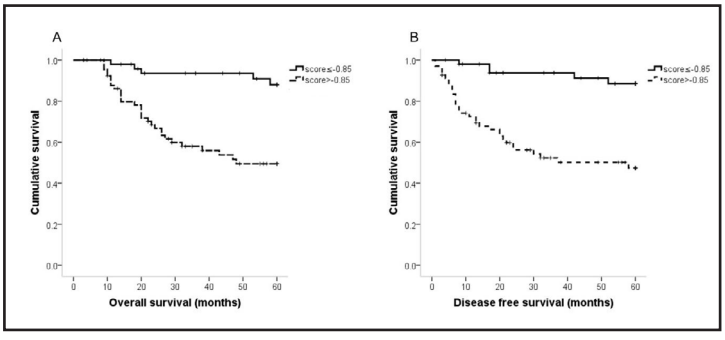

Fig. 2. At the -0.85 cutoff, the model discriminate the patients in the discovery cohort into two categories with different OS and DFS. A: Subjects with score<= -0.85in the discovery cohort had a significantly higher 5-year OS rate (88.5% vs. 49.5%, p<0.001) than in subjects with score >-0.85. B: Subjects with score<= -0.85in the discovery cohort had a significantly higher 5-year DFSrate (88.0% vs. 47.4%, p<0.001) than in subjects with score >-0.85.

Under the ROC analysis, the AUC of this model for predicting recurrence was 0.819 (95%CI 0.730-0.909, *P*<0.001). At a cut-off value at -0.85, the model produced a sensitivity of 86.5% and a specificity of 64.6%. Subjects with score <= -0.85 had a significantly higher 5-year OS rate (88.5% vs. 49.5% in subjects with score >-0.85, p<0.001, Fig. 2A) and 5-year DFS rate (88.0% vs. 47.4% in subjects with score >-0.85, p<0.001, Fig. 2B). The AUC for predicting recurrence using the Milan criteria was 0.655. We also built a normogram for predicting the probability of recurrence within 3 and 5 years with a C-index of 0.711 (Fig. 3).

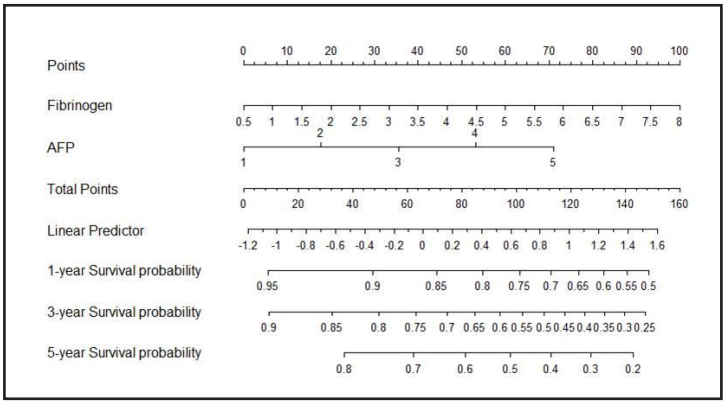

Fig. 3. Nomogram for predicting the probability of recurrence.

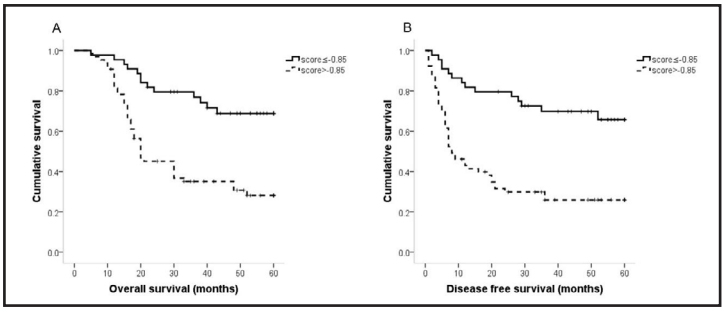

Fig. 4. At the -0.85 cutoff, the model discriminate the patients in the validation cohort into two categories with different OS and DFS. A: Subjects with score<= -0.85in the validation cohort had a significantly higher 5-year OS rate (68.8% vs. 28.1%, p<0.001) than in subjects with score >-0.85. B: Subjects with score<= -0.85in the validation cohort had a significantly higher 5-year DFS rate (65.7% vs. 25.9%, p<0.001) than in subjects with score >-0.85.

Validation cohort

An independent cohort (n=109) was used to validate the model. The 5-year OS rate was 68.8% in subjects with score <=-0.85 vs. 28.1% in patients with score > -0.85 (p<0.001, Fig. 4A). The 5-year DFS was 65.7% in subjects with score<= -0.85 vs. 25.9% in patients with score > -0.85 (p<0.001, Fig. 4B). Under the ROC curve analysis, the AUC was 0.698 (95% CI 0.596-0.799, *P*<0.001) with a sensitivity of 77.0% and a specificity of 62.5%. The AUC of the model in predicting recurrence in patients meeting the Milan criteria was 0.678.

Comparison of the current model with the Milan criteria

In the discovery cohort, 51 patients with score <= -0.85 under the proposed model had similar 5-year OS rate compared with 62 subjects who met the Milan criteria (88.5% vs. 76.2%, p=0.070) as well as 5-year DFS rate (88.0% vs. 75.1%, p=0.086) (Fig. 5A and B). In a subgroup analysis that included only patients meeting the Milan criteria in the discovery cohort, 39 patients with score <= -0.85 under the proposed model had significantly higher 5-year OS rate (88.8% vs. 54.8% in patients with score >-0.85, p=0.001, Fig. 6A) and 5-year DFS rate (88.3% vs. 54.5%, p=0.003, Fig. 6B). The proposed model also performed well in a subgroup analysis that included 57 subjects not meeting the Milan criteria in the discovery cohort: 12 patients with score <= -0.85 under the proposed model had significantly higher 5-year OS rate (87.5% vs. 46.5% in patients with score >-0.85, p=0.028, Fig. 6C) and 5-year DFS rate (88.9% vs. 41.6%, p=0.012, Fig. 6D). Notably, in patients beyond the Milan criteria in the discovery cohort, the subjects with score <= -0.85 also had similar OS (88.9% vs. 76.2%, p=0.324, Fig. 6E) and DFS (87.5% vs. 75.1%, p=0.358, Fig. 6F) with patients meeting the Milan criteria.

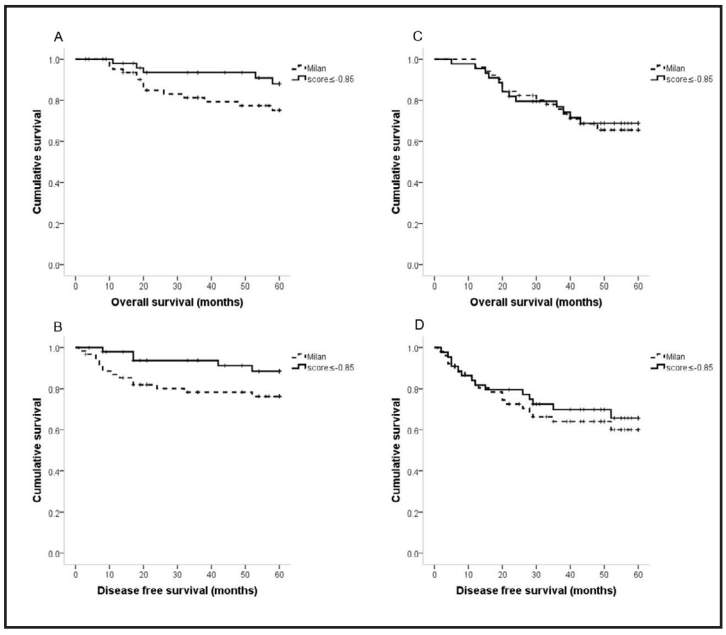

Fig. 5. Patients with score <=-0.85 had similar outcome with patients fulfilling the Milan criteria both in the discovery and validation cohort. A: Subjects with score <= -0.85in the discovery cohort had similar 5-year OS rate compared with subjects who met the Milan criteria (88.5% vs.76.2%, p=0.070). B: Subjects with score <= -0.85in the discovery cohort had similar 5-year DFS rate compared with subjects who met the Milan criteria (88.0%vs 75.1%, p=0.086). C: Subjects with score <= -0.85in the validation cohort had similar 5-year OS rate compared with subjects who met the Milan criteria (68.8% vs. 65.5%, p=0.873). D: Subjects with score <= -0.85in the discovery cohort had similar 5-year DFS rate compared with subjects who met the Milan criteria (65.7% vs. 60.0%, p=0.561).

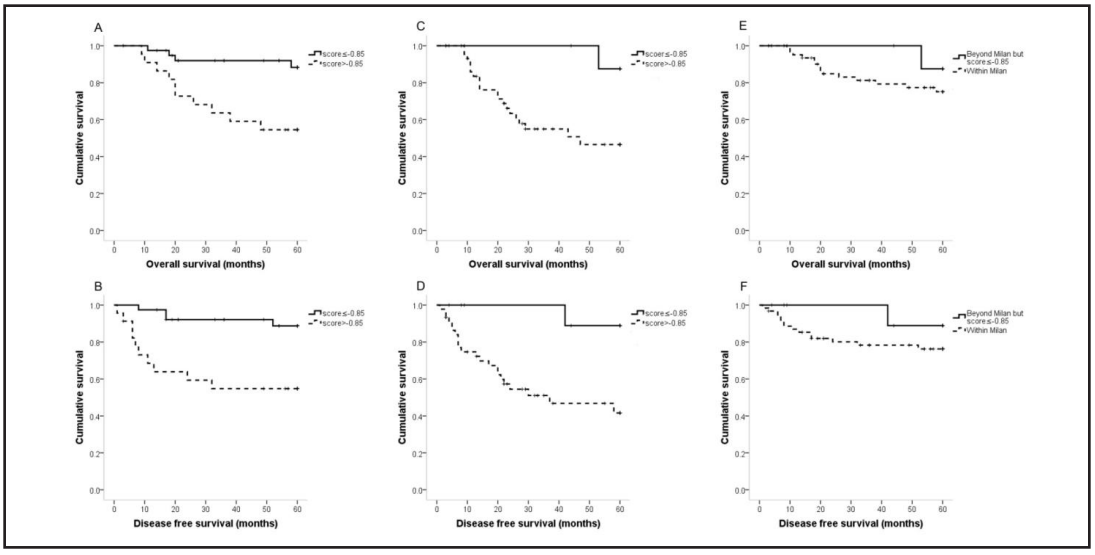

Fig. 6. In the discovery cohort, in the subgroups of patients fulfilling and exceeding Milan criteria, the model could discriminate patients into two categories with different OS and DFS, while the patients who exceeded Milan criteria but had score <=-0.85 still had similar outcome with patients fulfilling the Milan criteria. A: In patients meeting the Milan criteria in the discovery cohort, patients with score <= -0.85 had significantly higher 5-year OS rate (88.8% vs. 54.8%, p=0.001). B: In patients meeting the Milan criteria in the discovery cohort, patients with score <= -0.85 had significantly higher 5-year DFS rate (88.3% vs. 54.5%, p=0.003). C: In patients beyond the Milan criteria in the discovery cohort, patients with score <= -0.85 had significantly higher 5-year OS rate (87.5% vs. 46.5%, p=0.028). D: In patients beyond the Milan criteria in the discovery cohort, patients with score <= -0.85 had significantly higher 5-year DFS rate (88.9% vs. 41.6%, p=0.012). E: In patients beyond the Milan criteria in the discovery cohort, the subjects with score <= -0.85 had similar OS with patients meeting the Milan criteria (88.9% vs. 76.2%, p=0.324). F: In patients beyond the Milan criteria in the discovery cohort, the subjects with score <= -0.85 had similar DFS with patients meeting the Milan criteria (87.5% vs. 75.1%, p=0.358).

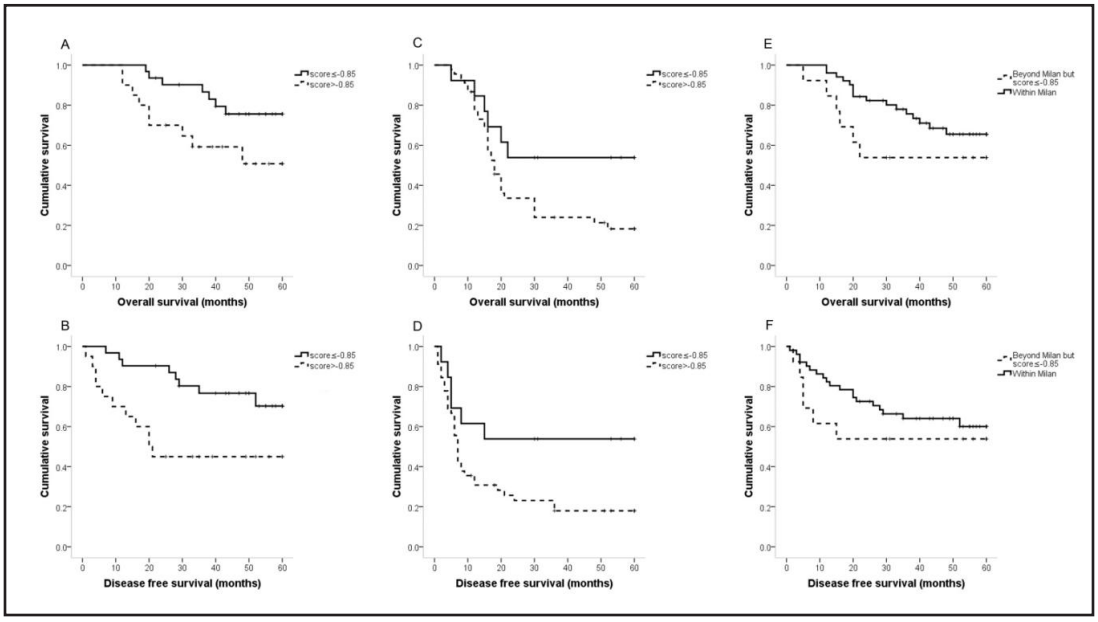

Fig. 7. In the validation cohort, in the subgroups of patients fulfilling and exceeding Milan criteria, the model could discriminate patients into two categories with different OS and DFS, while the patients who exceeded Milan criteria but had score <=-0.85 still had similar outcome with patients fulfilling the Milan criteria. A: In patients meeting the Milan criteria in the validation cohort, patients with score <= -0.85 had significantly higher 5-year OS rate (75.6% vs. 50.8%, p=0.043). B: In patients meeting the Milan criteria in the validation cohort, patients with score <= -0.85 had significantly higher 5-year DFS rate (70.3% vs. 45.0%, p=0.010). C: In patientsbeyond the Milan criteria in the validation cohort, patients with score <= -0.85 had significantly higher 5-year OS rate (53.8% vs. 18.3%, p=0.052). D: In patientsbeyond the Milan criteria in the validation cohort, patients with score <= -0.85 had significantly higher 5-year DFS rate (53.8% vs. 18.0%, p=0.043). E: In patients beyond the Milan criteria in the validation cohort, the subjects with score <= -0.85 had similar OS with patients meeting the Milan criteria (53.8% vs. 65.5%, p=0.160). F: In patients beyond the Milan criteria in the validation cohort, the subjects with score <= -0.85 had similar DFS with patients meeting the Milan criteria (53.8% vs. 60.0%, p=0.380).

In the validation cohort, 44 patients with score <= -0.85 under the proposed model had 5-year OS rate and 5-year DFS similar to that in 51 subjects who met the Milan criteria (OS: 68.8% vs. 65.5%, p=0.873; DFS: 65.7% vs. 60.0%, p=0.561) (Fig. 5C and 5D). A subgroup analysis that included only patients meeting the Milan criteria, 31 patients with score <= -0.85 also had significantly higher 5-year OS rate (75.6% vs. 50.8% in patients with score >-0.85, p=0.043, Fig. 7A) and 5-year DFS rate (70.3% vs. 45.0%, p=0.010, Fig. 7B). In 58 subjects not meeting the Milan criteria, 13 patients with model score <= -0.85 also had higher 5-year OS rate (53.8% vs. 18.3% in patients with score >-0.90, p=0.052, Fig. 7C) and 5-year DFS rate (53.8% vs. 18.0%, p=0.043, Fig. 7D). In patients beyond the Milan criteria in the validation cohort, the subjects with score <= -0.85 also had similar OS (53.8% vs. 65.5%, p=0.160, Fig. 7E) and DFS (53.8% vs. 60.0%, p=0.380, Fig. 7F) with patients meeting the Milan criteria.

Discussion

The results from the current study indicated that preoperative plasma fibrinogen concentration and AFP level are independent factors of recurrence after LT for HCC while most of the previously developed criteria for LT did not include factors that predict tumor biological behavior other than size and number of tumor [9, 10]. The scoring model based

on the 2 factors could be a useful tool to evaluate the risk of HCC recurrence in individual patients, particularly in patients not meeting the Milan criteria. With further development, the current model might also be useful in guiding post-transplantation surveillance and treatment. Compared with the Milan criteria, the model we built contained clinical variables incorporating tumor biological behavior and could predict recurrence of HCC after LT individually. The risk of recurrence was calculated for each patient, and patients with higher possibility for long-term survival could be selected before operation. An interesting finding in the current study is the promising performance of this model in patients meeting the Milan criteria: patients with model score <= -0.85 has significantly better outcome than patients with score > -0.85. Considering higher recurrence rate in patients not meeting the Milan criteria and higher recurrence rate, this model could be more useful in screening patients not meeting the Milan criteria: in patients not meeting Milan criteria, patients with model score <= -0.85 had similar outcome as patients meeting the Milan criteria. These findings suggest that the criteria for LT in HCC patients could be expanded if biologically based variables are considered.

Fibrinogen is a critical component of the hemostatic system: when the hemostatic system is activated, fibrinogen is cleaved to form fibrin by thrombin [11]. Fibrinogen plays important roles in many physiological and pathological processes, including adhesion, transendothelial cell migration and vascular formation [12]. A state of chronic inflammatory reaction and hypercoagulability in patients with malignant tumor increases plasma fibrinogen concentration [13]. Elevated plasma fibrinogen concentration has been associated with progression of many malignant tumors, including endometrial cancer, ovarian cancer, cervical cancer, gastric cancer, pancreatic cancer, colorectal cancer, lung cancer, esophagus cancer and HCC [14-24]. In comparison to normal tissues, cancer tissues of HCC patients express higher mRNA encoding γ chain of fibrinogen, and elevated plasma fibrinogen concentration has been correlated with tumor thrombosis [25]. The relationship between tumor metastasis and hemostatic system had been widely researched [20, 26]. A previous study suggests that soluble fibrin, the activated form of fibrinogen, could increase platelet adherence to tumor cells and facilitate metastatic spread [27]. There is also evidence indicating that vascular endothelial growth factor (VEGF) could bind to fibrinogen and fibrin with high affinity, which in turn could affect the localization and activity of VEGF, and stimulate endothelial cell proliferation [28]. In our study, we found that elevated preoperative plasma fibrinogen concentration is an independent risk factor of recurrence and metastasis after LT, and patients with lower fibrinogen concentration have a relatively lower risk of recurrence. Further analysis indicated that fibrinogen concentration was correlated with the CTP grade of the patients, while CTP grade was not a significant risk factor of post-operative recurrence of HCC in univariate nor multivariate analysis. The results suggest that fibrinogen concentration might be another effective indicator of not only tumor recurrence but also liver functional reserve.

AFP had been used for diagnosis and surveillance of recurrence of HCC for decades, but it had been reported that about 14% to 32% of HCC patients could present with a normal AFP of less than 20 ng/ml [29-31]. The cutoff value of AFP level for predicting recurrence of HCC varies in different studies. In the current study, we tested cutoff values (20, 100, 200 and 400 ng/ml) reported by previous studies [3, 29, 32-35]. 31.1% (37/119) and 25.7% (28/109) of the subjects presented with an AFP level of less than 20 ng/ml in the discovery and validation cohort, indicating that AFP alone was not accurate enough to predict the existence of HCC. However, when combined with preoperative fibrinogen concentration as described in our study, the accuracy in predicting recurrence of HCC increased dramatically than using AFP alone. Furthermore, we found that in patients with AFP <= 20ng/ml, patients with lower fibrinogen concentration still had better outcome. These results indicate that fibrinogen might contribute to the invasion and metastasis of HCC, and in AFP-negative HCC patients, fibrinogen concentration might be a sensitive indicator for the prediction of recurrence.

In conclusion, our study identified 2 independent risk factors of recurrence after LT in HCC patients. A logistic regression model based on these preoperative factors could predict recurrence with promising sensitivity and specificity. All the variables in this model are readily available in preoperative examinations. Validation of the model using external samples showed promising performance: the model had an AUC higher than the Milan criteria. Our study is a retrospective, small sample size study, if further validated and refined with a larger sample of more defined subjects, this model could be used in conjunction with the Milan and Hangzhou criteria for selecting liver recipients.

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Disclosure Statement

The funding sources had no involvement in study design; the collection, analysis and interpretation of data; the writing of the report; and the decision to submit the article for publication. The authors declare to have no conflict of interests.

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