EBioMedicine 52 (2020) 102638

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.elsevier.com/locate/ebiom

Research paper

A panel of five plasma proteins for the early diagnosis of hepatitis B virus-related hepatocellular carcinoma in individuals at risk

Kai Cheng[a,1], Jie Shi[b,1], Zixin Liu[a,1], Yin Jia[a,1], Qin Qin[a], Hui Zhang[a], Siqin Wan[b], Ziguang Niu[a], Lei Lu[a], Juxian Sun[b], Jie Xue[b], Chongde Lu[b], Xubiao Wei[b], Lei Guo[b], Fan Zhang[c], Dong Zhou[c], Yufu Tang[d], Yiren Hu[e], Yangqing Huang[f], Yang Chen[f], Wan Yee Lau[b,g], Shuqun Cheng[b,**], Shanrong Liu[a,*]

[a] Changhai Hospital, Second Military Medical University, Shanghai, China
[b] Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai, China
[c] Fujian Cancer Hospital, Fujian, China
[d] General Hospital of Northern Theater Command, Liaoning, China
[e] Wenzhou People's Hospital, Zhejiang, China
[f] Shanghai Public Health Clinical Center, Shanghai, China
[g] Faculty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong

ARTICLE INFO

Article History:
Received 28 September 2019
Revised 9 December 2019
Accepted 8 January 2020
Available online xxx

Keywords:
Peripheral blood
Multi-tumor markers
Earlier detection
Liver cancer
Multi-center study

ABSTRACT

Background: To improve the early diagnosis of hepatocellular carcinoma (HCC), more effective diagnostic biomarkers are needed. A combination of biomarkers is reported to distinguish individuals with early-stage HCC from at-risk individuals.

Methods: Participants in this study were recruited from six hospitals in China. Literature review was used to choose 19 candidate proteins, a case-control study in the discovery stage was used to identify five proteins (P5) that constituted a diagnostic model. In the training and validation stages, the effectiveness of P5 for detecting early-stage HCC was tested (cross-sectional study). Finally, a nested case-control study independent of the other stages was set up to evaluate the P5 in the preclinical diagnosis of HCC.

Findings: Between February 2013 and June 2017, a total of 1396 participants were recruited. A panel of 5 proteins (P5: OPN, GDF15, NSE, TRAP5 and OPG) showed high diagnostic accuracy when differentiating the early-stage HCC from the at-risk group, with AUCs of 0·892, 0·907 and 0·852 for the training stage, validation cohort 1 and cohort 2 data sets, respectively. In the prediction set, the sensitivity of P5 for diagnosing preclinical HCC increased with time, starting from 12 months before to the time of definitive clinical diagnosis (range, 46·15% to 86·67%).

Interpretation: The P5 panel has the potential to screen populations at high risk of developing HCC and can enable the early diagnosis of HCC.

Funding: Research supported by grants from eight funds. All sources of funding were declared at the end of the text.

1. Introduction

Hepatocellular carcinoma (HCC) is the fourth leading cause of cancer-related death worldwide [1]. More than 50% of patients with HCC are in China [2, 3]. Although surgery, liver transplantation, and local ablation are treatment options for patients with early-stage disease, the prognosis of advanced-stage HCC remains bleak [4]. Patients who are treated in the early stage of HCC have 5-year overall survival rates of up to 70%, compared to 16% in those with advanced disease [5]. Hepatitis B virus (HBV) infection and cirrhosis are the major risk factors for HCC [6, 7]. Effective and reliable biomarkers to monitor

* Corresponding author at: Changhai Hospital, Second Military Medical University, 168 Changhai Road, Shanghai 200433, China.
** Co-corresponding author at: Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, 225 Changhai Road, Shanghai 200433, China.
E-mail addresses: chengshuqun@aliyun.com (S. Cheng), liushanrong01@126.com (S. Liu).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.ebiom.2020.102638

Research in context

Evidence before this study

Early diagnosis of hepatocellular carcinoma (HCC) improves the long-term survival of patients. However, there are no good biomarkers that could be applied in the prediction and early diagnosis of HCC. To that end, we identified a panel of five proteins (P5, including OPN, GDF15, NSE, TRAP5 and OPG) to detect Hepatitis B virus-related HCC early.

Added value of this study

P5 had better diagnostic value for HCC than AFP, especially for early-stage HCC in patients with AFP-negative status. Moreover, the P5 panel was likely to predict the occurrence of HCC approximately one year ahead of time in the majority of patients.

Implications

Due to the difficulties of early diagnosis, curative treatments are not available for most HCC patients. However, it may be possible to improve the efficacy of treatment if there is a panel of biomarkers that could significantly improve the rate of early detection.

at-risk populations have been used in the early diagnosis of HCC with the aim of improving the treatment outcomes of these patients. Alpha fetoprotein (AFP) is the most widely used biomarker for the diagnosis and monitoring of HCC [8]. However, the American Association for the Study of Liver Diseases does not recommend the use of AFP alone for the early diagnosis of HCC due to its lack of sensitivity and specificity [9–11]. Better diagnostic tools are urgently needed to improve early HCC diagnosis and clinical treatment outcomes.

A single-gene biomarker cannot reflect the multistep process of cancer development, and its clinical significance is limited [12, 13]. A number of studies have demonstrated that the use of multiple biomarkers for HCC has potential merits. A measurement of seven serum miRNAs has been shown to be useful in detecting clinical and preclinical HCC in at-risk patients [14]. Three metabolic markers that were identified and validated by liquid chromatography-mass spectrometry based on large-scale metabolomics analysis techniques [15] were effective in detecting HCC among at-risk populations. However, the complicated preparation processes and informal quality control systems of these correlation technologies prevented their widespread acceptance in the clinic. The aim of this study was to identify a combination of proteins that can distinguish individuals with early-stage HCC from healthy and at-risk individuals. This combination should be more sensitive and specific than the current use of the single marker, AFP.

2. Materials and methods

2.1. Literature search

Instead of looking for new biomarkers for the early diagnosis of HCC, the aim was to find the best combination of well-established biomarkers. There are many protein chips with dozens or even hundreds of tumour markers. If all these markers are used simultaneously, the cost increases, and the specificity decreases. In this study, the Luminex® xMAP® technology based on multiple biomarkers was used to select two panels as the research target: 1, a human circulating cancer biomarker panel; and 2, a human cancer/metastasis biomarker panel. There are 34 proteins in these two panels (Supplementary Table S1).

The medical literature was searched to find the most commonly researched markers of HCC for the next experiment. PubMed was searched for articles published before 2016. The following medical subject headings terms and keywords were used: hepatocellular carcinoma, liver cancer, and liver, as well as the full names and abbreviations of the 34 proteins. Finally, 19 proteins were selected (Supplementary Table S2). These proteins were related to the different pathophysiological pathways in HCC, including cell migration and invasion, immunity, apoptosis, cell proliferation and differentiation, and metabolism (Supplementary Table S3).

2.2. Study design and subjects

Participants were recruited from six tertiary hospitals in China (Supplementary Table S4). The plasma samples in the discovery stage and training stage were collected between August 2013 and May 2016 at the Eastern Hepatobiliary Surgery Hospital and Shanghai Changhai Hospital. The validation stage consisted of two independent cohorts: cohort 1 enrolled participants at the Eastern Hepatobiliary Surgery Hospital and Shanghai Changhai Hospital between December 2014 and June 2017; and cohort 2 comprised participants recruited between January 2016 and March 2017 at four other hospitals: Fujian Cancer Hospital, General Hospital of Northern Theater Command, Wenzhou People's Hospital and Shanghai Public Health Clinical Center. The prediction set involved patients recruited at the Shanghai Changhai Hospital between February 2013 and November 2016.

Plasma samples were collected from four groups of participants: healthy volunteers (healthy controls, HC), patients with chronic hepatitis B virus infection (CHB), patients with HBV-related cirrhosis, and patients with HCC. The healthy volunteers were subjects who have underwent a physical examination at the physical examination center of Changhai Hospital. They were healthy, had no viral hepatitis, and had no history of liver or other systematic diseases. Patients with CHB were defined based on the presence of the hepatitis B surface antigen (HBsAg) (positive HBsAg >6 months), the copy number of HBV DNA (serum HBV DNA $<10^5$ copies/mL), and serum concentrations of liver function enzymes (persistent or intermittent elevation of alanine aminotransferase (ALT) levels) [16]. Abdominal ultrasound was performed in both the healthy volunteers and CHB patients to ensure that HCC was not present. Patients with liver cirrhosis had chronic hepatitis B virus infection, and cirrhosis was confirmed by two imaging modalities (abdominal ultrasound with computed tomography (CT) and/or magnetic resonance imaging (MRI)) [17]. Patients with HCC were diagnosed based on at least two imaging modalities (abdominal ultrasound with CT and/or MRI), and all HCC cases were further confirmed histopathologically according to the American Association for the Study of Liver Diseases guidelines [18]. The eligibility criteria are shown in Supplementary Table S5. The tumour stages were classified according to the Barcelona Clinic Liver Cancer (BCLC) staging system [19, 20]. Tumours with BCLC stages 0 and A were defined as early-stage HCC. HCC patients with other coexisting tumours or liver diseases were excluded from the study. The clinicopathological characteristics of all the participants in each stage were summarized in Supplementary Table S6a-e.

Approval from the Institutional Ethics Committee of each hospital and written informed consent from each participant were obtained. The data collection and analyses were undertaken by seven independent researchers (Supplementary Table S4).

2.3. Blood sample processing and analysis

Peripheral blood samples from HC, CHB and cirrhosis patients were collected between 6:00 and 8:00 AM after overnight fasting. Fasting blood samples from HCC patients were collected before surgery. Haemolytic, lipid and other unqualified blood samples were omitted from the analysis. All samples were collected into

EDTA-anticoagulant tubes and centrifuged for 10 min at 1000 × *g* within 30 min of blood collection. The separated plasma samples were then immediately stored in a −80 °C freezer and thawed on ice before analysis. Assays for the plasma proteins were performed by one researcher at the Clinical Laboratory Center, Changhai Hospital, Shanghai, China, who had no access to the patients' clinical information. When frozen samples were used, repeated (>2) freeze-thaw cycles were avoided. The samples were subjected to vortexing and centrifugation for 10 min at 1000 × *g* to remove particulates prior to use in the assay (Supplementary methods).

The concentration levels of the candidate biomarkers were assayed on a Luminex FLEXMAP 3D System using FLEXMAP 3D xPONENT software (Luminex, Austin, TX) and then analysed with Milliplex Analyst 5·1 software (EMD Millipore).

2.4. Discovery stage

The discovery stage was a case-control study, included two steps: feature selection and logistic regression. The first step was the screening of features, which was conducted according to the significance of a single feature and its area under the receiver operating characteristic curve (AUC). The differences in the levels of the candidate proteins between the groups of HCC patients and non-HCC patients (HC, patients with CHB and patients with HBV-related cirrhosis) were compared using the Wilcoxon rank sum test. These proteins progressed to the second step if the *p* values between the two groups were less than 0·05 and the AUCs were larger than 0·7.

The second step was to construct the model by logistic regression with SPSS software. The process of the logistic regression analysis was as follows. First, the goodness of fit was evaluated, and the goodness of fit test was performed. The goodness of fit was calculated using the Nagelkerke R^2 statistic. The closer the value was to 1, the better the fit of the model was. To evaluate the goodness of fit, the chi-square statistic was calculated using the Hosmer-Lemeshow method. Second, the likelihood ratio chi-square statistic was calculated to evaluate the overall significance of the regression equation. Third, the Wald test was used to determine whether a variable should be included in the model.

2.5. Training and validation stages

To test the diagnostic effectiveness of the model in early-stage HCC, we selected HCC patients and an at-risk group of patients (those with CHB and those with HBV-related cirrhosis) in the discovery group as the training group. The HCC patients were divided into early-stage HCC and advanced HCC. Cross-sectional studies were conducted in these two stages.

The two cohorts (cohorts 1 and 2) in the validation stage were independent of the training stage and of each other. They were recruited at different times from different hospitals. The effectiveness of the candidate biomarkers in diagnosing HCC was compared with that of AFP at the two commonly used cut-off levels: 20 ng/mL (AFP20) and 400 ng/mL (AFP400). The effect of the P5 test on patients who were on antiviral treatment with pegylated interferon alpha, adefovir dipivoxil, lamivudine or entecavir was also assessed.

2.6. Prediction set

Patients in the prediction set were independent of the other groups. In this nested case-control study, participants with CHB or cirrhosis underwent an ultrasound scan at the time of enrolment into the study (baseline) to exclude HCC. HCC surveillance tests were then performed once every three months and included abdominal ultrasound and serological tests for AFP, ALT, gamma-glutamyl transferase (GGT), HBsAg and HBV DNA. The tests were either biomarkers for HCC or measures of liver function. At each visit, plasma samples were collected and stored at −80 °C until analysis. Patients with new HCCs detected by ultrasound also underwent CT scans or MRI and were confirmed by histopathology. At the end of follow-up, all the newly diagnosed HCC cases were selected as the case group. The time of entry into the cohort, the time of occurrence of the disease, age, gender and other information were used as matching conditions for matching, and then the subjects (patients with CHB and those with HBV-related cirrhosis) without HCC were randomly selected from the same cohort as the control group. When the case group and the control group were identified, the retained blood samples were tested, and statistical analysis was performed according to the analysis method of the case-control study.

2.7. Statistical analysis

An a priori power calculation [21] was performed to estimate the number of HCC patients needed in the discovery stage. A total of 246 HCC participants provided 80% power at a two-tailed type 1 error rate of 0·05. All statistical analyses were performed with MedCalc software (version 15·2·2; MedCalc Software bvba) and SPSS software (version 22·0). The ROC curves were plotted, and the significance of the difference in the areas under the ROC curve (AUCs) was evaluated using SPSS version 22·0. The cut-off value was calculated using MedCalc version 15·2·2. The Chi-square test was used for analyzing the categorical variables. Continuous variables compared using the parametric test. If the data were not normally distributed, a nonparametric test was used, including the Mann-Whitney U test (two subgroups) or Kruskal-Wallis test (three or more). Pearson's χ^2 test was used to compare distributions, sensitivity, and specificity. Two-tailed p values $< 0{\cdot}05$ were considered statistically significant.

3. Results

3.1. Biomarker testing on nineteen molecules related to HCC

Up to 2016, 318 publications on HCC laboratory diagnosis using proteins were found in a medical literature search. Among 34 proteins, 19 were found to have strong correlations with important pathophysiological pathways that included cell migration and invasion [22–25], immunity [26, 27], apoptosis [28], cell proliferation and differentiation [29, 30] and metabolism [31] (Supplementary Table S2). These proteins were further used in the training set to build a predictive algorithm to detect HCC.

3.2. Number and characteristics of the recruited participants in the study

A total of 1396 participants were allocated according to the time periods in which they were managed in the study centres. 493 in the discovery stage (Participants in the training stage were derived from the discovery stage), 843 in the validation stage and 60 in the prediction set (Fig. 1). The four groups of participants (HC, CHB, cirrhosis and HCC) were well matched for age and sex. The concentrations of serum ALT and GGT did not differ significantly between the training stage and the validation stage participants.

3.3. Five proteins associated with an increased risk of HCC

We obtained 19 candidate proteins from our literature search and analysed the concentration levels of these proteins in plasma samples from participants in the discovery stage (Fig. 1). The diagnostic effectiveness of the 19 proteins was then evaluated in two steps. In the first step, the Wilcoxon rank test was used and showed that among the 19 candidate proteins, the *p* values of 10 proteins were less than 0·05 between the HCC group and non-HCC group (Supplementary Table S7a). By calculating the AUCs of these 10 proteins to compare

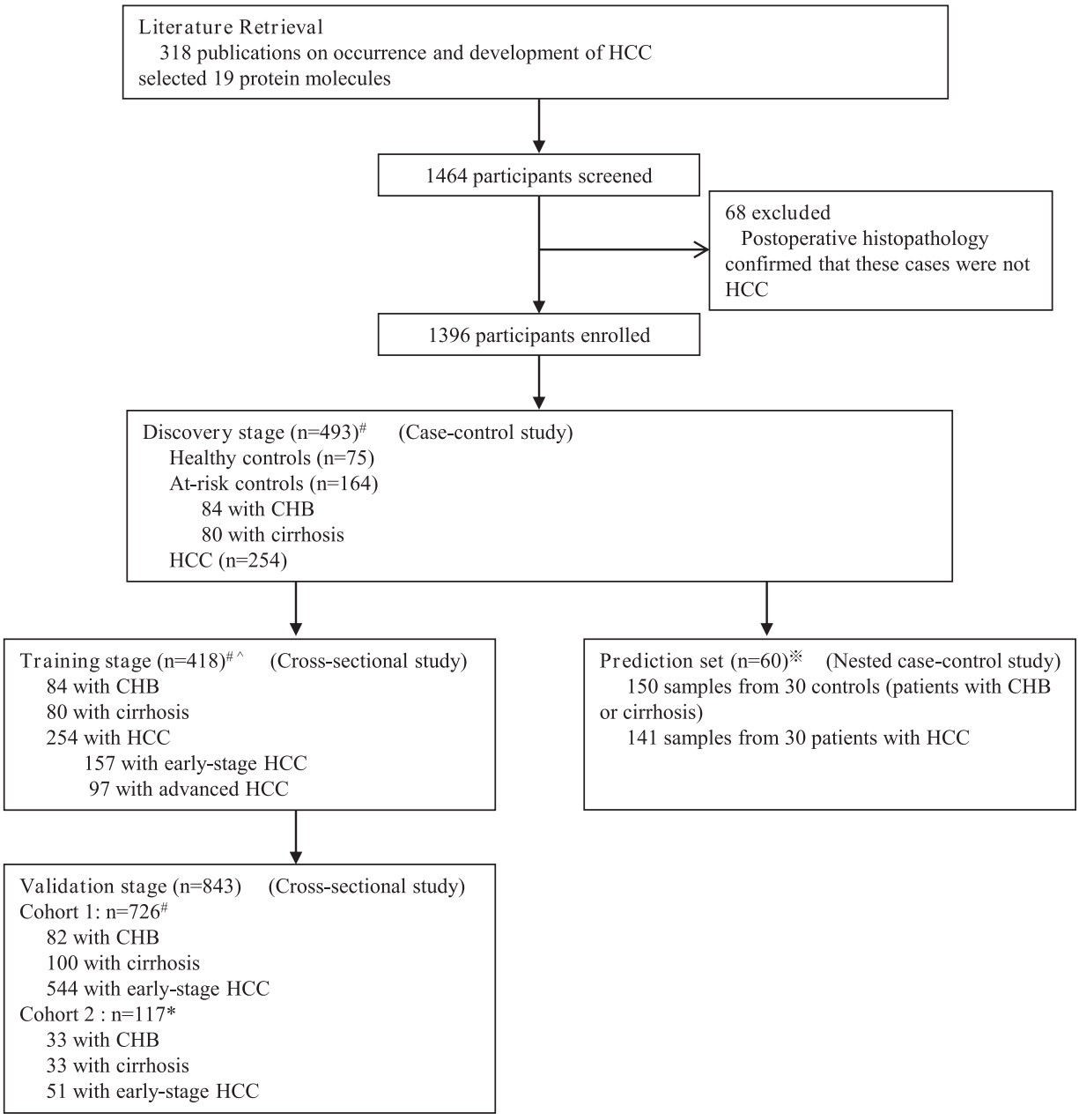

Fig. 1. Study design. #Participants at the Eastern Hepatobiliary Surgery Hospital and Shanghai Changhai Hospital. ^Participants in the training stage were derived from the discovery stage. ※ Participants at the Shanghai Changhai Hospital. *Participants at Fujian Cancer Hospital, General Hospital of Northern Theater Command, Wenzhou People's Hospital and Shanghai Public Health Clinical Center. Abbreviations: HCC, hepatocellular carcinoma; CHB, chronic hepatitis B; n, number of participants.

the two groups, it was found that the AUCs of 6 proteins were larger than 0·7, and these proteins were selected for the next step (Supplementary Table S7b).

In the second step, a logistic regression model was established, with groups (HCC group and non-HCC controls) as dependent variables and dichotomous variables as independent variables. The modelling process was as follows: as shown in Tables S7c and 7d, the Nagelkerke R^2 is 0·782, which means a better correlation of the six independent variables and the group variable, and the chi-square test of the Nagelkerke R^2 verified the fit ($p > 0·05$). Table S7e shows the significant chi-square test of the likelihood ratio ($p < 0·05$). Table S7f shows that the p value of YKL-40 is 0·791, which is nonsignificant. Therefore, a new logistic regression model was established after removing YKL-40. The remaining variables were all significant (supplementary Table S7g). Finally, the model was composed of osteopontin (OPN), growth and differentiation factor 15 (GDF15), neuron-specific enolase (NSE), thrombin receptor activator for peptide 5 (TRAP5) and osteoprotegerin (OPG), which were found to have the optimal diagnostic efficacy for HCC.

The predicted probability that HCC could be detected by the panel of five proteins was calculated as follows: logit [p = HCC] = −8·716 + 3·311 × OPN + 2·008 × GDF15 + 1·616 × NSE + 2·382 × TRAP5 + 2·940 × OPG. In this equation, the candidate protein name was substituted with the discretized value of one when the protein concentration was higher than the corresponding cut-off point (Supplementary Table S7b); otherwise, it was discretized to zero. If the result of [p = HCC] was higher than 0·5, the detected sample was predicted as HCC; otherwise, it was classified as non-HCC. In this equation, [p = HCC] was the probability of predicting

HCC by this panel; the value of the combination of five proteins (OPN, GDF15, NSE, TRAP5 and OPG), termed P5, achieved the maximum logit *p* value in the HCC group when compared with non-HCC controls. These five proteins were then selected for further verification of their HCC predictive value.

Figure S2 shows that the P5 combination displays the largest AUC value and the highest sensitivity and specificity in differentiating between the HCC group and the non-HCC group (AUC, 0·912; 95% CI, 0·883–0·941; sensitivity, 94·49%; specificity, 87·87%) (Supplementary Table S8).

3.4. Combining the five biomarkers more accurately predicted HCC

Figure S1 in the Supplementary Appendix shows that the plasma concentrations of each of the five candidate proteins were significantly different between HCC patients and at-risk controls (patients with CHB and patients with HBV-related cirrhosis), early-stage HCC and at-risk controls ($p < 0·001$ for all comparisons), supporting their potential use as biomarkers for the diagnosis of HCC. Fig. 2a shows that the AUC value of the P5 combination was 0·896 (95% CI, 0·860–0·932), with a sensitivity and specificity of 94·49% and 84·76%, respectively, when differentiating the HCC group from the at-risk group. Furthermore, the P5 panel had a large AUC (0·892, 95% CI, 0·853–0·931) and high sensitivity (93·63%) when applied to 157 subjects with early-stage HCC in the training stage to distinguish early-stage HCC patients from at-risk controls (Fig. 2b). In these two comparisons, the P5 panel was a better biomarker for distinguishing between HCC patients and at-risk controls than any one of its five constituent proteins alone.

3.5. The P5 panel distinguished early-stage HCC from at-risk controls

The diagnostic efficiency of the P5 panel for early-stage HCC was then evaluated in the validation cohort. The 843 enrolled participants included 248 at-risk controls and 595 patients with early-stage HCC. The performance of P5 for differentiating between the early-stage HCC group and the at-risk controls was evaluated (Fig. 3a and b). In cohort 1, P5 had an AUC of 0·907 (95% CI, 0·875–0·938; sensitivity, 96·14%; specificity, 85·16%) for distinguishing early-stage HCC patients from at-risk controls. In cohort 2, the AUC of P5 for distinguishing early-stage HCC from at-risk controls was 0·852 (95% CI, 0·779–0·924; sensitivity, 96·08%; specificity, 74·24%) (Table 1). In addition, in validation cohort I, 388 HCC patients were receiving antiviral treatment, and the P5 diagnostic value did not differ significantly between patients who did and did not receive antiviral therapy (chi-square test, $p = 0·105$, Supplementary Table S6a).

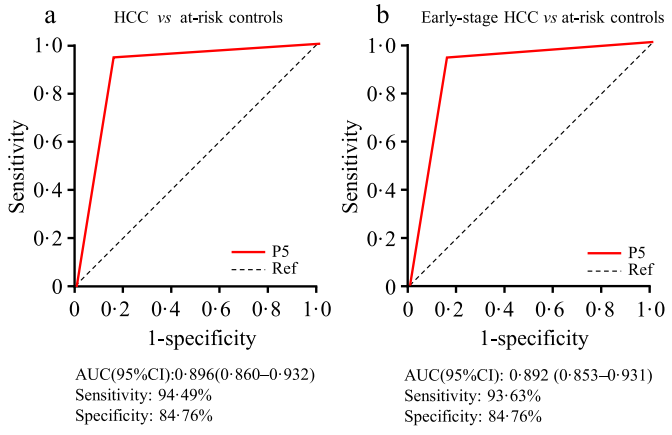

Fig. 2. Detection of HCC in the training stage. Receiver operating characteristic (ROC) curves of the training stage. (a) Performance of P5 for distinguishing individuals with HCC from at-risk controls. (b) Performance of P5 for distinguishing individuals with early-stage HCC from at-risk controls. Abbreviations: HCC, hepatocellular carcinoma; at-risk controls, patients with chronic hepatitis B and HBV-related cirrhosis; P5, the combination panel containing the five proteins, namely, OPN, GDF15, NSE, TRAP5 and OPG; Ref, reference line; AUC, area under the curve; 95% CI, 95% confidence interval.

3.6. The P5 panel outperformed AFP in diagnosing HCC

The diagnostic efficiency of P5 was compared with that of the most widely used HCC antigen marker, AFP, at the most frequently used cut-off values (AFP20 and AFP400). The performance of the P5 panel was notably better than that of AFP20 and AFP400 in distinguishing early-stage HCC patients from the at-risk group in validation cohort 1 (Fig. 3a, Table 1). The AUCs of AFP20 ($p<0·0001$) and AFP400 ($p<0·0001$) were only in the range of 0·543–0·567, which was much lower than that of P5 (0·907; 95% CI, 0·875–0·938). Furthermore, P5 had a higher sensitivity (96·14%) than AFP20 (47·43%) and AFP400 (14·71%). Similar results were observed in validation cohort 2 (Fig. 3b, Table 1). However, in these two cohorts, P5 had a lower specificity than AFP400 (85·16% vs. 93·96%; 74·24% vs. 86·36%, Table 1).

3.7. The P5 panel detected HCC independent of the AFP status

The diagnostic effectiveness of P5 was further analysed in AFP-negative HCC patients. In validation cohorts 1 and 2, AFP-negative HCC patients accounted for 85·29% (464 of 544) and 70·59% (36 of 51) of the participants, respectively. Among the patients with early-stage HCC in cohort 1, 447 of 464 AFP-negative patients (96·34%) and 76 of 80 (95·00%) AFP-positive patients had positive P5 results. In cohort 2, 34 of 36 (94·44%) AFP-negative and 15 of 15 (100%) AFP-positive patients had positive P5 results (Fig. 3c). The AUC of P5 for differentiating patients with AFP-negative HCC from at-risk controls was 0·908 (95% CI, 0·876–0·939) in validation cohort 1 and 0·843 (95% CI, 0·765–0·922) in validation cohort 2. The sensitivity was 96·34% and 94·44%, the specificity was 85·16% and 74·24%, and the false negative rate was 3·86% (21 of 544) and 3·92% (2 of 51) for P5 in validation cohorts 1 and 2, respectively (Fig. 3d and e). Collectively, these data indicated that P5 could diagnose HCC independent of the AFP status in the early stages of HCC.

3.8. The P5 panel diagnosed HCC before clinical diagnosis

To study the feasibility of the P5 panel in diagnosing HCC before clinical diagnosis, a nested case-control study was conducted with 282 at-risk patients (with CHB or cirrhosis) who were being monitored for at least one year (median 2·79 years). Thirty new cases (Supplementary Table S9) were diagnosed by imaging (3 by contrast-enhanced CT, 27 by MRI) as having developed HCC, which was later confirmed by histopathology. From the same population that was monitored, 30 age- and sex-matched at-risk individuals were enrolled to serve as controls (median follow-up 3·31 years). These patients had CHB or cirrhosis but did not progress to HCC. All subjects in this stage of the study had received antiviral treatments.

As shown in Fig. 4, the P5 panel had a larger AUC and higher sensitivity than AFP20 and AFP400. The AUC value of the P5 panel was the highest at 3 months before the clinical diagnosis of HCC, reaching 0·783 (95% CI, 0·662–0·905). At 12 months before the clinical diagnosis of HCC, the AUC was only 0·681 (95% CI, 0·536–0·825), although it was still predictive of HCC. The sensitivity of P5 for the preclinical diagnosis of HCC increased with time from 12 months before to the time of the definitive clinical diagnosis of HCC (range 46·15% to 86·67%). Conversely, AFP20 and AFP400 had significantly lower sensitivities at these time points (from 15·38% to 40·00% and from 3·85% to 16·67%, respectively, $p<0·0001$, Table 2). The false negative rate of the P5 panel was 13·33% (4 of 30), compared with 60·00% (18 of 30) for AFP20 and 83·33% (25 of 30) for AFP400. These findings suggested

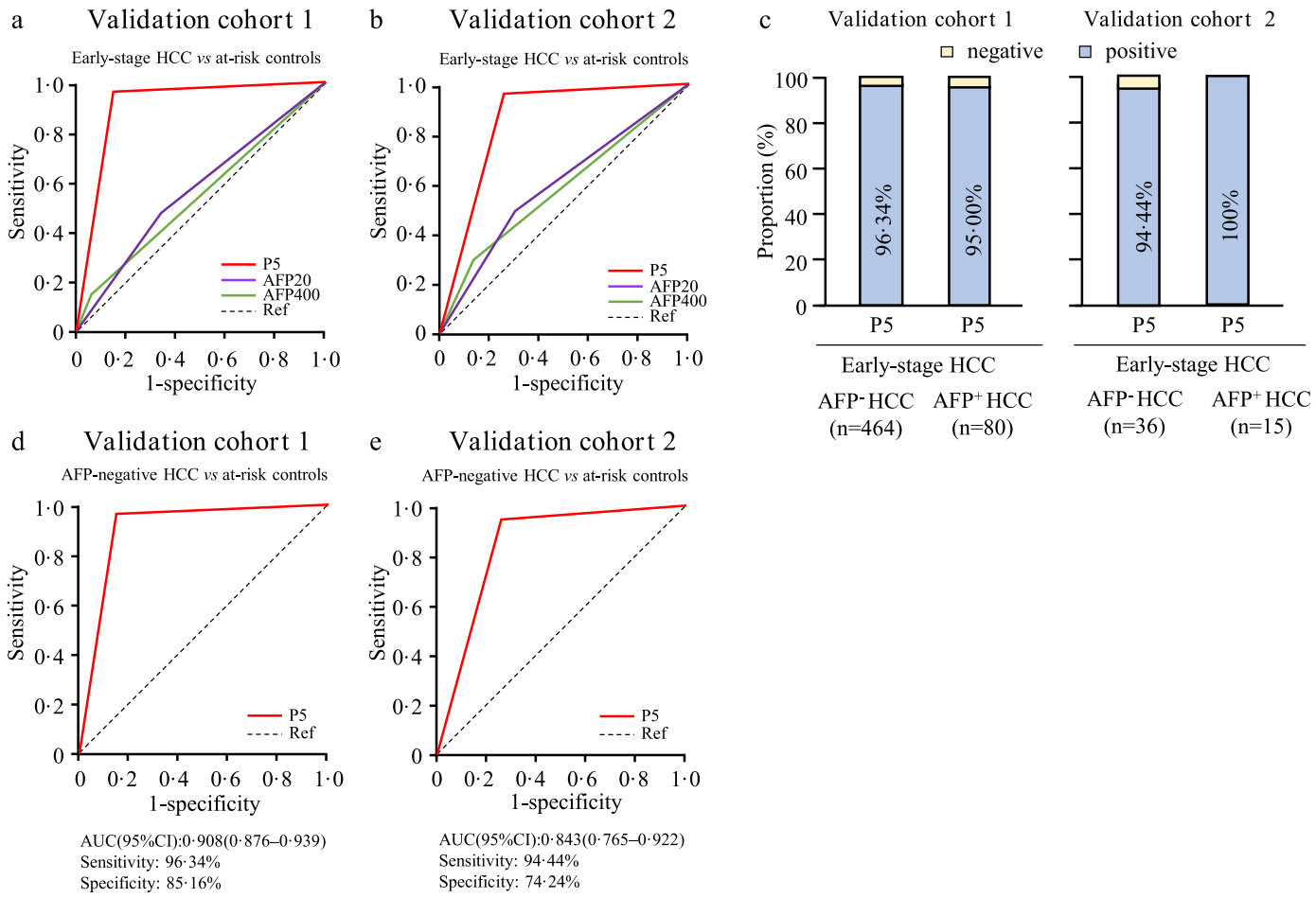

Fig. 3. Detection of early-stage HCC in the validation stage. (a) Receiver operating characteristic (ROC) curves of validation cohort 1. Performance for distinguishing individuals with early-stage HCC from at-risk controls. (b) ROC curves of validation cohort 2. Performance for distinguishing individuals with early-stage HCC from at-risk controls. (c) Rate of positive results for P5 stratified by AFP status. (d) Performance for distinguishing individuals with AFP-negative HCC (AFP ≤400 ng/mL) from at-risk controls in validation cohort 1. (e) Performance for distinguishing individuals with AFP-negative HCC (AFP ≤400 ng/mL) from and at-risk controls in validation cohort 2. Abbreviations: HCC, hepatocellular carcinoma; at-risk controls, patients with chronic hepatitis B and HBV-related cirrhosis; P5, the combination panel containing the five proteins, namely, OPN, GDF15, NSE, TRAP5 and OPG; AFP, alpha fetoprotein; AFP20, 20 ng/mL of alpha fetoprotein as a cutoff value; AFP400, 400 ng/mL of alpha fetoprotein as a cutoff value; Ref, reference line; AUC, area under curve; 95% CI, 95% confidence interval.

that the P5 panel would diagnose HCC at an earlier stage than the currently available imaging examinations.

4. Discussion

The simultaneous use of multiple biomarkers has a higher efficiency than the use of a single biomarker in the early diagnosis of cancer [32]. In our study, logistic regression was used to establish a multiple biomarker prediction model based on plasma samples to study the correlation between the five-biomarker panel and HCC. The results showed that the P5 panel had better diagnostic value for HCC than AFP, especially for early-stage HCC in patients with AFP-negative (AFP <400 ng/mL) status. In patients with a negative AFP status, AFP has no diagnostic role; therefore, the better diagnostic value of P5 is obvious. In validation cohorts 1 and 2, the P5 panel had much higher AUCs and sensitivities than AFP20 and AFP400 for the diagnosis of early-stage HCC. Moreover,

Table 1
Measurement of P5 and AFP for the diagnosis of early-stage HCC in validation cohorts 1 and 2.

	Early-stage HCC vs. at-risk controls			
	AUC (95% CI)	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	*p* value*
Validation cohort 1				
P5	0·907 (0·875–0·938)	96·14 (94·06–97·53)	85·16 (78·97–89·83)	
AFP20	0·567 (0·519–0·614)	47·43 (43·17–51·72)	65·93 (58·50–72·68)	<0·0001
AFP400	0·543 (0·497–0·590)	14·71 (11·89–18·03)	93·96 (89·17–96·79)	<0·0001
Validation cohort 2				
P5	0·852 (0·779–0·924)	96·08 (85·41–99·32)	74·24 (61·76–83·87)	
AFP20	0·594 (0·489–0·698)	49·02 (34·95–63·23)	69·70 (57·00–80·09)	<0·0001
AFP400	0·579 (0·473–0·685)	29·41 (17·91–44·02)	86·36 (75·19–93·20)	<0·0001

Abbreviations: P5, the combination panel containing five proteins, namely, OPN, GDF15, NSE, TRAP5 and OPG; AFP, alpha fetoprotein; HCC, hepatocellular carcinoma; at-risk controls, patients with chronic hepatitis B and HBV-related cirrhosis; AUC, area under the curve; 95% CI, 95% confidence interval; AFP20, 20 ng/mL of alpha fetoprotein as a cut-off value; AFP400, 400 ng/mL of alpha fetoprotein as a cut-off value.

* The *p* values indicate the statistical significance of the differences in the AUCs compared with P5.

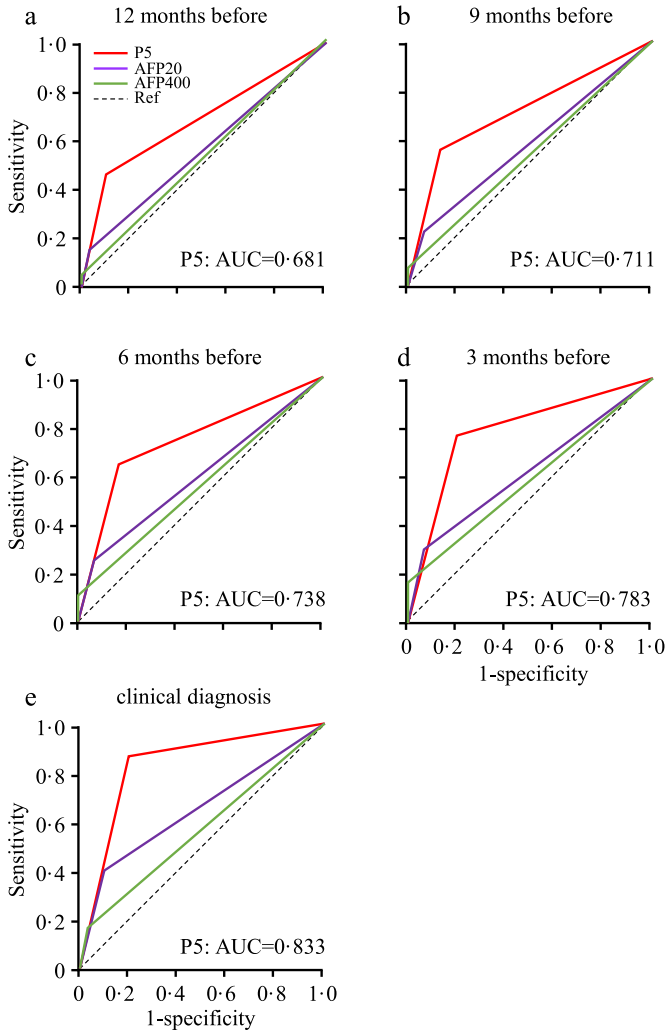

Fig. 4. Detection of preclinical HCC in the nested case-control study. (a) Receiver operating characteristic (ROC) curves for the ability to distinguish individuals with HCC from at-risk controls 12 months before clinical diagnosis. (b) ROC curves for the ability to distinguish individuals with HCC from at-risk controls 9 months before clinical diagnosis. (c) ROC curves for the ability to distinguish individuals with HCC from at-risk controls 6 months before clinical diagnosis. (d) ROC curves for the ability to distinguish individuals with HCC from at-risk controls 3 months before clinical diagnosis. (e) ROC curves for the ability to distinguish individuals with HCC from at-risk controls at the time of definitive clinical diagnosis. Abbreviations: HCC, hepatocellular carcinoma; at-risk controls, patients with chronic hepatitis B and HBV- related cirrhosis; P5, the combination panel containing the five proteins, namely, OPN, GDF15, NSE, TRAP5 and OPG; AFP20, 20 ng/mL of alpha fetoprotein as a cutoff value; AFP400, 400 ng/mL of alpha fetoprotein as the cutoff value; Ref=reference line.

in the prediction set, the P5 panel predicted the occurrence of HCC approximately one year ahead of time in the majority of patients, and the false negative rate of P5 was lower than that of AFP20 and AFP400.

The development of cancer is a complex process involving multiple factors, multiple stages and multiple gene mutations. The complexity and heterogeneity of cancers is one of the major reasons that limit the development of early diagnostic methods, especially those using only one tumour marker. As shown in Figure S1, the difference in the expression of each of the five proteins between HCC and at-risk group was statistically significant, but there seems to be some overlap. In this study, each of the five biomarkers that form the P5 panel has been shown to play important roles in promoting tumour cell proliferation and differentiation, tumour cell metabolism, actin remodelling, and tumour cell migration and invasion. This explains why P5 was more diagnostically sensitive for the early diagnosis of HCC than the conventional marker AFP.

The 30 HCC patients in the nested case-control study were first detected by ultrasound, subsequently diagnosed by imaging using contrast-enhanced CT or MRI and later confirmed by histopathology. There are two possible scenarios in the detection of HCC by P5 at a preclinical stage. First, the currently available imaging examinations were adequately sensitive, and the patients did not suffer from HCC before the clinical diagnosis. The P5 panel detected patients who were at very high risk of developing HCC. Second, the imaging methods were not sensitive enough to detect micro-carcinomas or cells that became cancerous. The P5 panel was a more sensitive method to diagnose early-stage HCC. Thus, in high-risk patients with positive P5 in the peripheral blood, the frequent use of more accurate imaging methods in diagnosing HCC, such as dynamic plus hepatobiliary phase MRI, is recommended. Hopefully, this strategy can lead to the earlier diagnosis and treatment of cancer. Due to time constraints, although 282 at-risk patients were monitored in this study, only 30 new cases of HCC were found. We believe that as the HCC surveillance programme continues, the research power will be greatly enhanced when more HCC patients are recruited into this retrospective longitudinal repository study. In addition, in order to avoid the bias caused by sample size, more control cases should be included in our follow-up studies.

Although this study recruited 1396 participants from six hospitals, more studies are needed on HCC, which is associated with various aetiologies, such as hepatitis C virus infection and alcohol-related liver disease. In validation cohort 2, P5, a new marker panel for the diagnosis of HCC, had a slightly lower specificity. We hypothesize that a larger sample size is strongly needed for verification. This approach is also useful for comparing the standard surveillance methods for other cancers by identifying panels of biomarkers that are superior to the existing single markers.

In conclusion, P5 outperformed the existing biomarker AFP for diagnosing HCC. The P5 protein markers are measurable in plasma, thus making them easily applicable for routine clinical assessments and population-wide studies [33, 34]. This test is minimally invasive and requires only a relatively small amount of plasma. Individuals who are at risk of developing HCC with a positive P5 level in their plasma should be carefully monitored with imaging studies.

Funding

This work was supported by the China National Funds for Distinguished Young Scientists (81425019), the State Key Program of National Natural Science Foundation of China (81730076), Program of Shanghai Academic Research Leader (18XD1405300), the National Natural and the Specially-Appointed Professor Fund of Shanghai (GZ2015009), the Key Project of Natural Science Foundation of China (81730097), the Chang Jiang Scholars Program (2013) of the China Ministry of Education, the National Key Basic Research Program “973 project” (2015CB554000). This work was supported in part by the Grants from the State Key Laboratory of Oncogenes and Related Genes (No.90−17−04). The funders had no role in study design, data collection, data analysis, interpretation or writing of the report.

Author contributions

Shuqun Cheng and Shanrong Liu designed the study.

Wan Yee Lau, Shuqun Cheng and Shanrong Liu supervised the study.

Kai Cheng, Jie Shi, Zixin Liu and Yin Jia analysed and interpreted the data.

Kai Cheng, Jie Shi, Zixin Liu, Yin Jia, Qin Qin, Hui Zhang, Siqin Wan, Ziguang Niu and Lei Lu collected the data.

Zixin Liu, Juxian Sun, Jie Xue, Chongde Lu, Xubiao Wei, Lei Guo, Fan Zhang, Dong Zhou, Yufu Tang, Yiren Hu, Yangqing Huang and Yang Chen collected the samples.

Kai Cheng contributed to the experimental studies.

Table 2
Measurement of the efficacy of P5 and AFP for detecting preclinical HCC stratified by the months before definitive clinical diagnosis in the nested case-control study.

	12 months before	9 months before	6 months before	3 months before	At diagnosis
P5					
AUC (95% CI)	0·681 (0·536–0·825)	0·711 (0·573–0·850)	0·738 (0·606–0·871)	0·783 (0·662–0·905)	0·833 (0·723–0·943)
Sensitivity (%) (95% CI)	46·15 (27·14–66·25)	55·56 (35·64–73·96)	64·29 (44·11–80·69)	76·67 (57·30–89·37)	86·67 (68·36–95·64)
Specificity (%) (95% CI)	90·00 (72·32–97·38)	86·67 (68·36–95·64)	83·33 (64·55–93·70)	80·00 (60·87–91·60)	80·00 (60·87–91·60)
AFP20					
AUC (95% CI)	0·560 (0·407–0·713)	0·578 (0·427–0·728)	0·592 (0·443–0·740)	0·617 (0·473–0·760)	0·650 (0·510–0·791)
Sensitivity (%) (95% CI)	15·38 (5·05–35·73)	22·22 (9·38–42·73)	25·00 (11·43–45·22)	30·00 (15·41–49·56)	40·00 (23·22–59·25)
Specificity (%) (95% CI)	96·67 (80·95–99·83)	93·33 (76·49–98·84)	93·33 (76·49–98·84)	93·33 (76·49–98·84)	90·00 (72·32–97·38)
p value[#]	<0·0001/0·280	<0·0001/0·130	0·003/0·230	0·013/0·390	0·017/0·310
AFP400					
AUC (95% CI)	0·519 (0·366–0·673)	0·537 (0·386–0·689)	0·554 (0·404–0·703)	0·583 (0·438–0·729)	0·567 (0·421–0·713)
Sensitivity (%) (95% CI)	3·85 (0·20–21·58)	7·41 (1·29–25·75)	10·71 (2·81–29·37)	16·67 (6·30–35·45)	16·67 (6·30–35·45)
Specificity (%) (95% CI)	100 (85·87–100)	100 (85·87–100)	100 (85·87–100)	100 (85·87–100)	96·67 (80·95–99·83)
p value[#]	<0·0001/0·046	<0·0001/0·010	<0·0001/0·020	<0·0001/0·040	<0·0001/0·078

Abbreviations: P5, the combination panel containing five proteins, namely, OPN, GDF15, NSE, TRAP5 and OPG; AFP, alpha fetoprotein; HCC, hepatocellular carcinoma; AUC, area under the curve; 95% CI, 95% confidence interval; AFP20, 20 ng/mL of alpha fetoprotein as a cut-off value; AFP400, 400 ng/mL of alpha fetoprotein as a cut-off value.
[#] The *p* values indicate the statistical significance of the differences in sensitivity/specificity compared with P5.

Kai Cheng, Jie Shi, Zixin Liu and Yin Jia wrote the manuscript and all authors contributed to the manuscript and approved the final manuscript.

Declaration of competing interest

The authors declare no conflicts of interest.

Acknowledgments

We thank the professors in the department of Health Statistics, Second Military Medical University for their careful support with the statistical analyses.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2020.102638.

References

[1] Global, regional, and national age-sex specific mortality for 264 causes of death, 1980-2016: a systematic analysis for the global burden of disease study 2016. Lancet 2017;390(10100):1151–210.
[2] Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, et al. Cancer statistics in China, 2015. CA Cancer J Clin 2016;66(2):115–32.
[3] Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. CA Cancer J Clin 2019;69(1):7–34.
[4] Villanueva A. Hepatocellular carcinoma. N Engl J Med 2019;380(15):1450–62.
[5] Bruix J, da Fonseca LG, Reig M. Insights into the success and failure of systemic therapy for hepatocellular carcinoma. Nat Rev Gastroenterol Hepatol 2019.
[6] Lee TY, Hsu YC, Tseng HC, Yu SH, Lin JT, Wu MS, et al. Association of daily aspirin therapy with risk of hepatocellular carcinoma in patients with chronic hepatitis B. JAMA Intern Med 2019;179(5):633–40.
[7] Seto WK, Lo YR, Pawlotsky JM, Yuen MF. Chronic hepatitis B virus infection. Lancet 2018;392(10161):2313–24.
[8] Johnson PJ. The role of serum alpha-fetoprotein estimation in the diagnosis and management of hepatocellular carcinoma. Clin Liver Dis 2001;5(1):145–59.
[9] Bruix J, Sherman M. Management of hepatocellular carcinoma: an update. Hepatology 2011;53(3):1020–2.
[10] Hesketh RL, Zhu AX, Oklu R. Radiomics and circulating tumor cells: personalized care in hepatocellular carcinoma? Diagn Interv Radiol 2015;21(1):78–84.
[11] Saffroy R, Pham P, Reffas M, Takka M, Lemoine A, Debuire B. New perspectives and strategy research biomarkers for hepatocellular carcinoma. Clin Chem Lab Med 2007;45(9):1169–79.
[12] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144(5):646–74.
[13] Hanash SM, Pitteri SJ, Faca VM. Mining the plasma proteome for cancer biomarkers. Nature 2008;452(7187):571–9.
[14] Lin XJ, Chong Y, Guo ZW, Xie C, Yang XJ, Zhang Q, et al. A serum microRNA classifier for early detection of hepatocellular carcinoma: a multicentre, retrospective, longitudinal biomarker identification study with a nested case-control study. Lancet Oncol 2015;16(7):804–15.
[15] Luo P, Yin P, Hua R, Tan Y, Li Z, Qiu G, et al. A large-scale, multicenter serum metabolite biomarker identification study for the early detection of hepatocellular carcinoma. Hepatology 2018;67(2):662–75.
[16] Terrault NA, Lok ASF, McMahon BJ, Chang KM, Hwang JP, Jonas MM, et al. Update on prevention, diagnosis, and treatment of chronic hepatitis B: AASLD 2018 hepatitis B guidance. Hepatology 2018;67(4):1560–99.
[17] Schuppan D, Afdhal NH. Liver cirrhosis. Lancet (London, England). 2008;371(9615):838–51.
[18] Marrero JA, Kulik LM, Sirlin CB, Zhu AX, Finn RS, Abecassis MM, et al. Diagnosis, staging, and management of hepatocellular carcinoma: 2018 practice guidance by the American Association for the study of liver diseases. Hepatology 2018;68(2):723–50.
[19] Llovet JM, Brú C, Bruix J. Prognosis of hepatocellular carcinoma: the BCLC staging classification. Semin Liver Dis 1999;19(03):329–38.
[20] Umberto C, Alessandro V, Francesco G, Fabio F, Alberto B, Giacomo Z, et al. Prospective validation of the Barcelona clinic liver cancer staging system. J Hepatol 2006;44(4):723–31.
[21] Obuchowski NA, Xiao-Hua Z. Prospective studies of diagnostic test accuracy when disease prevalence is low. Biostatistics 2002;3(4):477–92.
[22] Morahan BJ, Wang L, Coppel RL. No TRAP, no invasion. Trends Parasitol 2009;25(2):77–84.
[23] Xue YH, Zhang XF, Dong QZ, Sun J, Dai C, Zhou HJ, et al. Thrombin is a therapeutic target for metastatic osteopontin-positive hepatocellular carcinoma. Hepatology 2010;52(6):2012–22.
[24] Chen RX, Xia YH, Xue TC, Zhang H, Ye SL. Down-regulation of osteopontin inhibits metastasis of hepatocellular carcinoma cells via a mechanism involving MMP-2 and uPA. Oncol Rep 2011;25(3):803–8.
[25] Basten O, Bandorski D, Langner C. Hemangiosarcoma of the liver with unusual expression of neuron-specific enolase (NSE). Z Gastroenterol 2002;40(8):577–80.
[26] Zhang J, Yamada O, Matsushita Y, Chagan-Yasutan H, Hattori T. Transactivation of human osteopontin promoter by human T-cell leukemia virus type 1-encoded Tax protein. Leuk Res 2010;34(6):763–8.
[27] Moschen AR, Kaser A, Stadlmann S, Millonig G, Kaser S, Mühllechner P, et al. The RANKL/OPG system and bone mineral density in patients with chronic liver disease. J Hepatol 2005;43(6):973–83.
[28] Lu TY, Kao CF, Lin CT, Huang DY, Chiu CY, Huang YS, et al. DNA methylation and histone modification regulate silencing of OPG during tumor progression. J Cell Biochem 2009;108(1):315–25.
[29] Zimmers TA, Jin X, Gutierrez JC, Acosta C, McKillop IH, Pierce RH, et al. Effect of in vivo loss of GDF-15 on hepatocellular carcinogenesis. J Cancer Res Clin Oncol 2008;134(7):753–9.
[30] Si Y, Liu X, Cheng M, Wang M, Gong Q, Yang Y, et al. Growth differentiation factor 15 is induced by hepatitis C virus infection and regulates hepatocellular carcinoma-related genes. PLoS ONE 2011;6(5):e19967.
[31] Saito-Yabe M, Kasuya Y, Yoshigae Y, Yamamura N, Suzuki Y, Fukuda N, et al. PEGylation of osteoprotegerin/osteoclastogenesis inhibitory factor (OPG/OCIF) results in decreased uptake into rats and human liver. J Pharm Pharmacol 2010;62(8):985–94.
[32] Shariat SF, Karakiewicz PI, Ashfaq R, Lerner SP, Palapattu GS, Cote RJ, et al. Multiple biomarkers improve prediction of bladder cancer recurrence and mortality in patients undergoing cystectomy. Cancer 2010;112(2):315–25.
[33] Zhong FL, Mamai O, Sborgi L, Boussofara L, Hopkins R, Robinson K, et al. Germline NLRP1 mutations cause skin inflammatory and cancer susceptibility syndromes via inflammasome activation. Cell 2016;167(1):187–202 e17.
[34] Ruibal P, Oestereich L, Ludtke A, Becker-Ziaja B, Wozniak DM, Kerber R, et al. Unique human immune signature of Ebola virus disease in Guinea. Nature 2016;533(7601):100–4.