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ORIGINAL ARTICLE

ITIH4: Effective Serum Marker, Early Warning and Diagnosis, Hepatocellular Carcinoma

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Abstract Hepatocellular carcinoma (HCC) is a highly lethal malignant tumor evolved from cirrhosis. It is quite significant to seek accurate, easy markers for early warning and diagnosis of HCC. Through prospective cohort follow-up study and mass spectrometry, we discovered and verified a serum marker valuable for early warning and diagnosis. Follow-up observation was performed on cirrhosis patients. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was adopted to detect the serums of patients, and the serum polypeptides with a potential value in early HCC warning and diagnosis were screened. Electrospray ionization quadrupole time-of-flight tandem mass spectrometry (ESI-Q-TOF-MS/MS) was exploited to identify these screened polypeptides. Moreover, the serum marker concentration was determined by ELISA to validate the clinical value of the serum marker. Among 109 cirrhosis patients followed up for two years, 29 patients (26.6%) finally progressed into HCC. MALDI-TOF MS shows that the concentration of a 3155.66Da polypeptide was significantly different between the patients that progressed into HCC and those not. Through MS/MS identification, it is confirmed that the polypeptide is inter-alpha-trypsin inhibitor heavy chain 4 (ITIH4). The serum ITIH4 concentrations in two groups were measured with ELISA and compared with Alpha-fetoprotein (AFP). Results show that serum ITIH4 and AFP concentrations were negatively correlated (r=−0.263, p=0.0006), and the ITIH4 concentration had a significant intergroup difference (p=0.000). Receiver operating characteristic (ROC) curve indicates that its predictive value (area under the curve, AUC) is 0.667, superior to AFP. For the patients progressing into HCC, serum samples were separately collected when they were recruited and diagnosed as cirrhosis. Measurement on these samples reveals that ITIH4 was declining during the progression of HCC (p=0.006). By virtue of mass spectrometry, we discovered and identified a biomarker valuable for early HCC warning and diagnosis. This marker overperforms the commonly used AFP, demonstrating a bright prospect.

Keywords Mass spectrum · Liver diseases · Liver cancer · Cirrhosis patients · Biomarker · AFP

Introduction

Hepatic carcinoma is one of the major malignant tumors endangering human health. According to statistics of the WHO, some 782,000 cases of hepatic carcinoma were added globally in 2012, with 83% in developing countries and over 50% in China. Hepatic carcinoma is also the malignant tumor with the second highest mortality in the world. In 2012, nearly 746,000 persons died of this disease, with the ratio of mortality to morbidity being 0.95 [1]. As the most common primary hepatic carcinoma (accounting for 70% ~ 90%), hepatocellular carcinoma (HCC) has the third highest mortality among malignant tumors, next to gastric and esophageal carcinoma [2]. It has been reported that more than 90% of HCC cases developed from chronic liver diseases, especially the cirrhosis,

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which accounted for over 80% [3]. The American Association for the Study of Liver Diseases (AASLD) makes a strong case to screen cirrhosis patients with serum marker alpha-fetoprotein (AFP) and ultrasound. But AFP only enjoys a sensitivity of 39%–65% in HCC diagnosis [4]. The screening based on AFP alone is very likely to cause missed diagnosis. In addition, the serum AFP concentration may also increase in many patients with chronic benign tumors, so the HCC screening based on AFP probably results in a false-positive rate of 5% [5]. Due to the unsatisfactory approach of early screening, over 60% HCC patients were not diagnosed until the late stage, missing the optimal treatment period. The 5-year survival rate was less than 16% for such patients [6], but exceeded 75% for HCC patients treated earlier [7]. If timely warning and interference could be given to cirrhosis patients at the early stage of or before the occurrence of HCC, its morbidity and mortality would decline sharply. Currently, whether AFP and other serum markers related to hepatic carcinoma are valuable for early warning remains unsure. Hence, we need to exploit new technologies to seek other serum markers that can help diagnose HCC at the early stage [8–10]. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a newfashioned soft ionization mass spectrometry. Featured by high sensitivity, throughput and speed, it has been extensively applied to microbial identification and tumor marker screening [11–15]. MALDI-TOF MS demonstrates a bright prospect in discovery of novel serum markers for HCC.

Inter-alpha-trypsin inhibitor heavy chain 4 (ITIH4), a 120 kDa plasma glycoprotein, is secreted by the liver to the blood circulatory system. It belongs to acute phase proteins. At present, ITIH4 is considered to be closely related to genesis, development, invasion and metastasis of many solid tumors. Its main function is to secrete and regulate extracellular matrix (ECM) [16, 17]. In this work, we confirmed the effectiveness of ITIH4 in early warning for HCC and as a diagnostic marker, as well as its value in early screening and warning for hepatic carcinoma, through the follow-up on cirrhosis patients and MALDI-TOF MS.

Materials and Methods

Subjects

All subjects were recruited from outpatients and inpatients of the 302 Military Hospital of China and followed up for two years from January 2013 to December 2015. Inclusion criteria: (1) infected with hepatitis B virus (HBV) or hepatitis C virus (HCV) for over 5 years; (2) above 35 years old; (3) patients with clinically confirmed cirrhosis, but without malignant tumors. Exclusion criteria: (1) severe complications and other systemic diseases; (2) history of liver disease-related invasive treatment; (3) loss to follow-up or data incompleteness. The condition of patients was finally confirmed through pathological or MRI examination. According to Guidelines for the Diagnosis of Primary Hepatic Carcinoma (2015) and (Tentative) Guidelines for the diagnosis and treatment of hepatic carcinoma [18, 19], all patients have signed the informed consent document, and this study was approved by the Ethics Committee of the Hospital. Whether patients progressed into HCC was monitored during the 24-month follow-up, with the MRI result as a diagnostic reference. Patients progressing into HCC were classified as the HCC group, while the rest constituted the non-HCC group.

Sample Preparation

1. Reagents: trifluoroacetic acid (TFA) from TEDIA (U.S.A); α-Cyano-4-hydroxycinnamic acid (HCCA) and acetonitrile from SIGMA (U.S.A); Magnetic beads-based weak cation exchange (MB-WCX) kit and peptide calibration standard from Bruker Daltonies (Germany); leucine enkephalin from SIGMA (U.S.A); sodium formate (NaFA) from Beijing Chemical Reagent company; ZipTipC18 microcolumns from Millipore (U.S.A); ITIH4 ELISA kit from Wuhan EIAab Science Co. Ltd.
2. Instruments and consumables: MALDI-TOF Mass Spectrometer Autoflex from Bruker Daltonies (Germany); SYNAPT G2-S from Waters (U.S.A); several types of pipettes from GILSON (France); serum separating tubes from BD (U.S.A); centrifuge tubes and tips made in China.
3. Sample collection: Serum was sampled when patients were recruited in the study. Then MRI examination was performed semiannually. If a patient progressed into HCC, he or she would be no longer followed up and receive serum sampling again. For those who did not progress into HCC, the follow-up was terminated at the end of the second year. Sampling method is introduced below. Using a disposable blood sampler, 3 ~ 5 ml of venous blood was collected from the patient fasted from liquids and solids in the morning and injected into a separation gel vacuum tubes. After 30 min of standing at room temperature (25 °C), the blood sample was centrifuged at 3000 r/min for 15 to separate serum. The serum was added in two 1.5 ml centrifuge tubes, with 1 ml each tube, and then stored at −80 °C. Before testing, the centrifuge tubes would be taken out in advance to avoid repeated freezing and thawing

Table 1 The characteristics of subjects

Variables	HCC group (n = 29)	Non-HCC group (n = 80)
Age	57.3 ± 10.3	52.6 ± 9.3
Gender(M/F)	21/8	45/35
ALT(U/L)	52(20–253)	35.5(9–416)
AFP(ng/mL)	15.23(1.41–937.1)	6.25(0.9–1432)
Albumin(g/L)	31.7 ± 6.5	30.9 ± 6.4

4. MALDI-TOF MS: Serum sample was enriched with MB-WCX, according to product specifications. Subsequently, MALDI-TOF MS was performed under the following parameter setting: positive ion linear mode; mass range, 1000 Da - 20,000 Da; laser intensity, 60%; 500 × 6 shots were acquired at each spot and accumulated. Differential polypeptide peaks of two groups were analyzed with ClinPro Tools. The ratio of peak intensity to peak area was used as a statistic. Data that conform to a normal distribution were subjected to Student's t-test, while those not normally distributed received the Wilcoxon rank-sum test. $P < 0.05$ was considered statistically significant.
5. Differential serum peptide profiling using MS/MS: Serum sample processed by magnetic beads was loaded into a C18 column for separation and desalination, followed by HPLC in combination with electrospray ionization quadrupole time-of-flight tandem mass spectrometry (ESI-Q-TOF-MS/MS). Later, data were imported to and searched in the Mascot database (http: //www.matrixscience.com). Synapt G2-S parameters were set as follows: positive ion mode, ion source temperature of 100 °C, flow velocity of 200 nL/min; capillary voltage of 3000 V, cone voltage of 40 V; mass range of 100 Da - 2000 Da in MS, with the scanning time of 0.5 s; mass range of 50 Da - 1600 Da in MS/MS, with the scanning time of 0.2 s.
6. ITIH4 ELISA: According to specifications, reagents were balanced to room temperature. Each well was added with 100 ul of sample, and standard and blank wells were arranged. After mixing, incubation was performed at 37 °C for 120 min, with the liquid discarded. Then 100 ul of solution was added into each well, followed by 60 min of incubation at 37 °C. The liquid was discarded again, with the plate washed three times. Into each well, 100 ul of solution B was added, followed by another 60 min of incubation at 37 °C. After the plate was washed five times, 90 ul of substrate solution was added. Following 15 min of color development at 37 °C in a dark condition, 50 ul of stop solution was added. Finally, the optical density (OD) of each well was measured at 450 nm using an ELISA analyzer, and sample concentration was calculated based on the standard curve.

Statistical Analysis

ClinPro Tools software was used to analyze differential polypeptide peaks in the serum fingerprint obtained from MALDI-TOF MS. Other statistical analyses were performed in SPSS 17.0. The averages of testing data of markers from the firstly sampled serum were compared between HCC and non-HCC groups, and paired data were subjected to a paired-samples t test. Normally distributed data were subjected to Student's t-test, while the data that did not conform to a normal distribution received the Wilcoxon rank-sum test. $P < 0.05$ was considered statistically significant.

Table 2 ClinProTools peak statistics for the differential peaks between positive and negative groups

Index	Mass	DAve	PTTA	PWKW	PAD	Ave(positive)	Ave(negative)	SD(positive)	SD(negative)	CV(positive)	CV(negative)
Down-regulated peptides in positive group											
41	3155.66	9.31	0.00021	0.000033	< 0.000001	3.57	14.21	1.24	8.66	32.1	44.7
11	1281.81	8.66	0.0035	0.00022	< 0.000001	6.31	11.72	1.88	6.47	22.32	28.74
23	2660.13	6.64	0.0217	0.0117	0.0000514	5.55	13.47	1.69	5.17	41.7	66.2
Up-regulated peptides in positive group											
58	4068.99	15.74	0.0047	0.0028	< 0.000001	22.69	10.47	5.87	2.66	40.7	45.12
56	4050.90	16.55	0.024	0.0036	0.288	21.88	10.57	6.66	6.17	50.44	36.47
59	4086.99	12.03	0.042	0.007	0.0451	16.52	9.23	4.22	4.10	47.11	30.64
69	4295.6	9.34	0.667	0.0342	< 0.000001	10.49	6.33	4.87	2.66	80.63	72.11

The peaks are sorted according to the *P*-value in descending order

PTTA *p*-value of t-test or ANOVA test, *PWKW* *p*-value of Wilcoxon test or Kruskal-Wallis test, *PAD* *p*-value of Anderson-Darling test

* The identified peptide peaks with Mascot score lower than threshold value

** The identified peptide peaks with Mascot score higher than threshold value

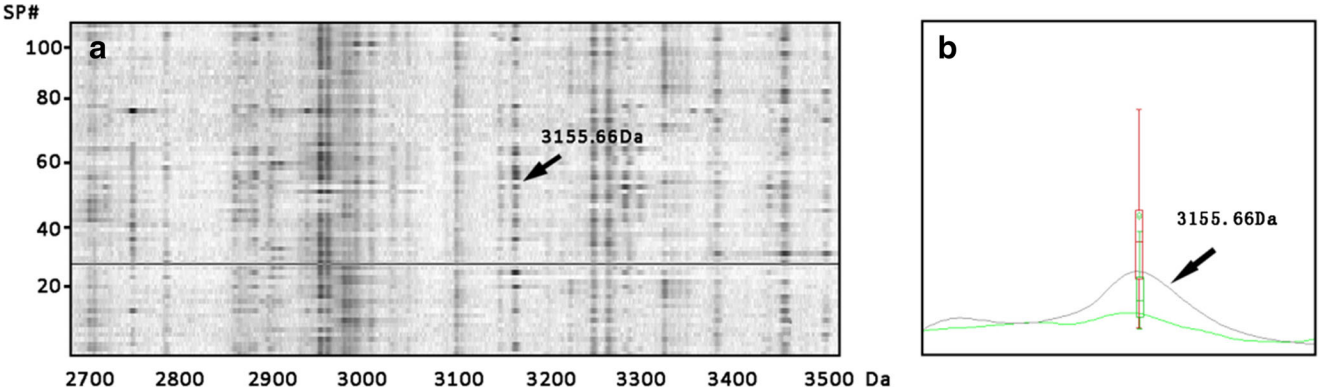

Fig. 1 **a** simulated gel electrophoretogram (top: non-HCC group; bottom: HCC group); **b** MS spectrum of the 3155.66 Da differential peak (red: non-HCC group; green: HCC group)

Results

1. Subjects

   A total of 161 cirrhosis patients were recruited, we only enrolled the outpatient and inpatient subjects, infected more than 5 years, with 52 cases eliminated by exclusion criteria (35 cases accompanied with other systemic diseases, 14 cases with severe complications, and 3 cases lost to follow-up). The remaining 109 cases were followed up for 24 months. Therein, 29 cases (26.6%) progressed into HCC, as confirmed by MRI examination. These 109 patients comprised 66 males (60.6%) and 43 females (39.4%), with an average age of 53.9 years (SD = 9.7). Among them, the cirrhosis of 103 patients (94.5%) was caused by HBV. See Table 1 for detailed patient information.

2. Screening of serum polypeptide markers for HCC warning

   All samples were classified into HCC and non-HCC groups according to follow-up results. Using MALDI-TOF MS, marker concentrations in the firstly sampled serum were measured and differential serum peptides between the two groups were analyzed. Before measurement, peptide calibration standard was used for calibration, along with repetitive tests on reagents and instruments. The intra-assay coefficient of variation (CV) was 14.97% (4.03–55.4%), and the inter-assay CV was 18.02% (4.46–32.16%). After the measurement with MALDI-TOF MS, ClinPro Tools software was used to screen all polypeptide peaks with the signal-to-noise (S/N) ratio larger than 5. As seen from Table 2, seven polypeptides were significantly differentially expressed between HCC and non-HCC groups. Therein, the difference in 3155.66 Da serum polypeptide peak was most significant (p = 0.00021), with a dramatically lower expression abundance in the HCC group (Fig. 1). It suggests that this serum protein might have a potential of becoming a marker for HCC warning.

3. Identification of differential polypeptide peaks by MS/MS

   ESI-Q-TOF-MS/MS and Mascot identification show that the 3155.67 Da peptide stems from ITIH4, with an Mscot score of 72. Its sequence is R.NVHSGSTFFKYYLQGAKIPKPEASFSPR.R. Figure 2 is the MS/MS spectrum of the peptide.

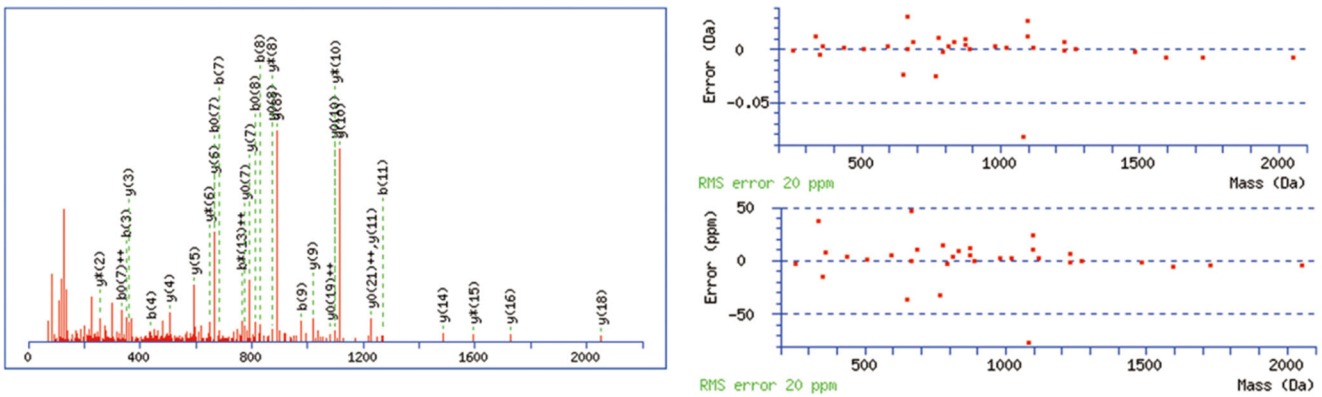

Fig. 2 MS/MS spectrum of the 3155.66 Da differential peak

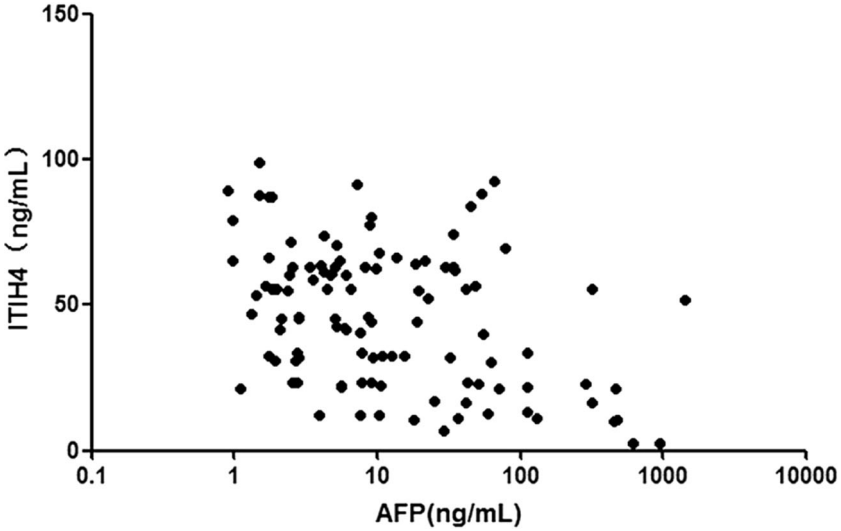

Fig. 3 Scatter plot concerning the correlation between ITIH4 and AFP concentrations

4. Correlation between ITIH4 and AFP

   Both AFP and ITIH4 in the serum samples of 109 patients were measured to determine the value of ITIH4 in early HCC warning and diagnosis. Meanwhile, the correlation between ITIH4 and AFP concentrations was analyzed. As seen in Fig. 3, the two were negatively correlated ($r = -0.263$, $p = 0.0006$).

5. Comparative study of diagnostic value of ITIH4 and AFP

   ITIH4 has a much lower concentration in the HCC group than in the non-HCC group ($p = 0.000$). Receiver operating characteristic (ROC) curve indicates that its predictive value (area under the curve, AUC) is 0.667. The optimal cut-off values which were determined with the maximum sum of sensitivity and specificity, the value of CUT-OFF is 61.35 ng/mL, the sensitivity and specificity of it are 40% and 93.1% respectively. AFP also shows a significant intergroup difference ($p = 0.021$). The predictive value of AFP is 0.646, lower than that of ITIH4 (Figs. 4 and 5; Table 3). For 17 patients progressing into HCC, serum samples were separately collected when they were recruited and diagnosed as cirrhosis. ITIH4 and AFP concentrations in the serum samples of different periods were compared. It is found that ITIH4 was declining during the progression of HCC ($p = 0.006$), but AFP had no evident variation ($p = 0.879$) (Fig. 6; Table 4). It suggests that cirrhosis patients may have a higher possibility of suffering from HCC when their ITIH4 concentration declines. ITIH4 enjoys a higher diagnostic value than AFP.

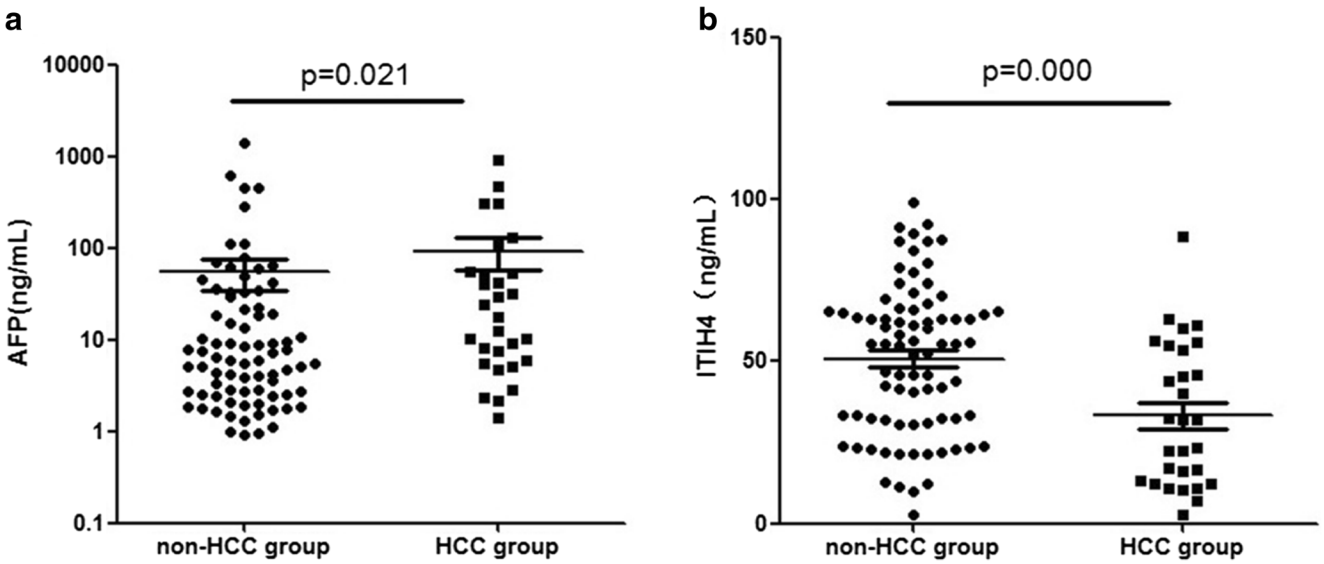

Fig. 4 Scatter plot concerning the difference between ITIH4 and AFP concentrations

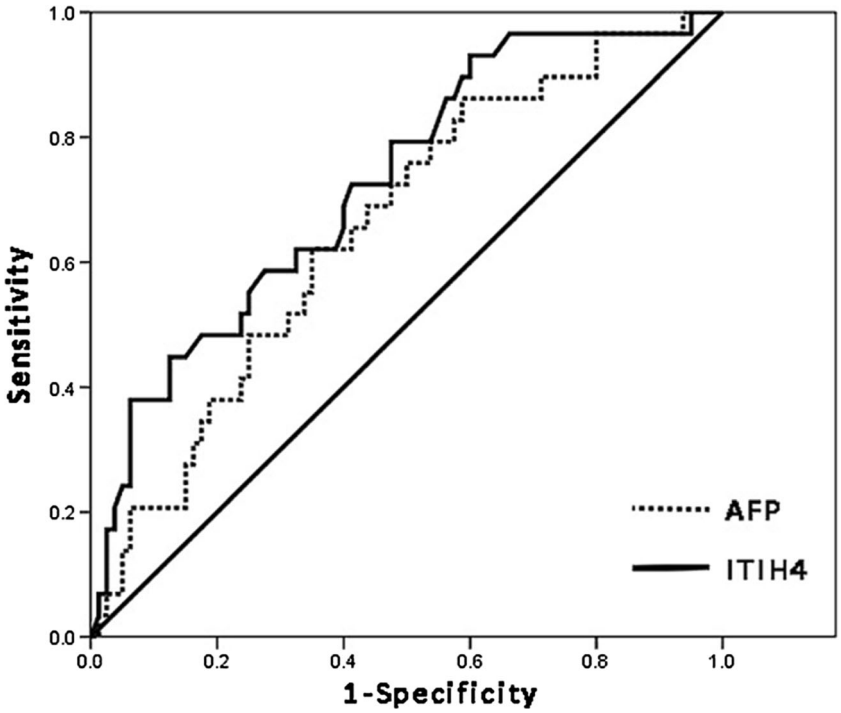

Fig. 5 Diagnostic values and ROC curves of ITIH4 and AFP

Discussion

The exploration of new diagnostic markers for HCC has been a hotspot in the tumor diagnosis field, as AFP sensitivity (39%–65%) fails to satisfy the clinical demand and costly imageological and pathological examination lower the compliance of patients [20] As a well-developed clinical diagnostic and research technique, MALDI-TOF MS has found wide applications in identification of proteins, polypeptides, polysaccharide, nucleic acids, and other biomacromolecules. Especially in bacterial identification, MALDI-TOF MS is gradually superseding conventional biochemical identification approaches, entering numerous clinical microbial laboratories in China. Moreover, the quantification of middle and low molecular weight proteins in the serum of patients by MALDI-TOF MS has also been increasingly mature. MB-WCX technology is mainly used to enrich and purify low molecular weight (1–20 kDa) polypeptides and proteins in various biological samples. After the processing by MB-WCX, the interference of high-abundance protein in MALDI-TOF MS can be eliminated. A number of studies have adopted this scheme to conduct mass spectrometric detection, and obtained satisfactory sensitivity and reproducibility [21–23]. Moreover, there are many studies on hepatic diseases based on mass spectrometry, including the diagnosis on malignant hepatic tumors using proteomic strategies [24–27].

In this work, we accumulated complete sequence samples of HCC patients through two years of follow-up and sample collection and detection. Using MALDI-TOF MS, mass spectrometry fingerprinting was performed on the serums of those followed-up patients. The 3155.66 Da polypeptide shows a significant difference between the serums of the cirrhosis patients that progressed into HCC and those not. MS/MS identification indicates that this polypeptide is ITIH4. The difference implies the potential of ITIH4 in warning and early diagnosis for HCC. ITIH4 is a 120 kDa plasma glycoprotein. It is secreted by the liver to the blood circulatory system,

Table 3 The statistical analysis of the levels of serum markers between HCC group and non-HCC group

Parameters	HCC group		Non-HCC group		T/Z values	*p* values
	N	Statistical description	N	Statistical description		
AFP(ng/mL)	29	15.23(1.41–937.1)	80	6.25(0.9–1432)	−2.299	0.021
ITIH4(ng/mL)	29	31.8 (2.7–88.5)	80	55.4(2.7–99)	−3.597	0.000

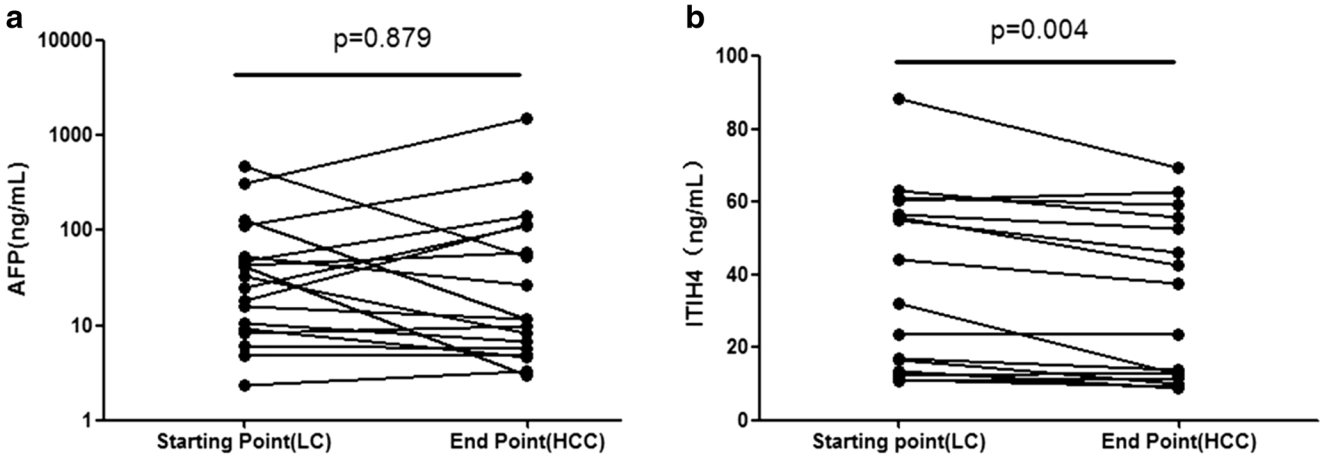

Fig. 6 Dynamics of ITIH4 and AFP concentrations during the progression of HCC

belonging to acute phase proteins. ITIH4 is one of five heavy chains (ITIH1- ITIH5) of the inter-alpha-trypsin inhibitor (ITI), with its gene at the short arm of Chromosome 3. ITIH4 has been found to be closely related to genesis, development, invasion and metastasis of many solid tumors. And its main function is to secrete and regulate extracellular matrix. In our study, ITIH4 was highly expressed in the serum of cirrhosis patients, which may be related to the involvement of this protein in extracellular matrix generation. ITIH4 participates in the genesis and development of HCC by downregulating interleukin-6 (IL-6), belonging to the serpin family. By binding to hyaluronic acid, ITIH4 also participates in the formation of extracellular matrix. As the protein is secreted by the liver to the blood circulatory system, its concentration in serum can be determined by immunological assays. Many studies have confirmed the differential expression of ITIH4 in various solid tumors. For example, Melanie et al. observed the differential proteins in murine models of colorectal adenocarcinoma using the proteomic quantification method [28]. They found that the serum ITIH4 concentration significantly changed in the progress of tumorigenesis, verifying the early diagnostic value of the protein. Based on the isobaric tags for relative and absolute quantitation (iTRAQ), Yashwanth et al. found dramatically rising serum ITIH4 in patients with gastric carcinoma [29]. Protein mass spectrometry helped Yang et al. confirm ITIH4 as a potential diagnostic marker for breast cancer [30]. Using the two-dimensional electrophoresis, Gangadharan et al., found that the serum ITIH4 concentration was much higher in patients with hepatic fibrosis compared with normal controls [31]. Noh et al. indicate that the ITIH4 level was significantly correlated with the prognosis of HCC patients [32]. Meanwhile, their data show that the serum ITIH4 level was significantly different between normal controls, cirrhosis patients, and HCC patients, with normal controls much lower than the latter two groups. But it was also lower in HCC patients than in cirrhosis patients. It means that ITIH4 may be downregulated after the genesis of HCC. That said, this report is a cross-section research, making its results less convincing.

Through a prospective follow-up study, we observed the variation of serum ITIH4 concentration in the dynamic development progress. It is confirmed that the concentration will significantly decline after the genesis of HCC. At the same time, detection on the samples of varying outcomes indicates that patients with a high HCC risk may also have low ITIH4 expression. Hence, ITIH4 can serve as a warning marker. Regular ITIH4 monitoring can achieve earlier detection of HCC and guide the prognosis of cirrhosis patients. However, the limited sample size of our research may result in a

Table 4 The dynamic change of AFP and ITIH4 concentrations before and after HCC occured

Markers	Starting point (LC)		End point (HCC)		T/Z values	*p* values
	N	Statistical description	N	Statistical description		
AFP(ng/mL)	17	74.6(2.34–469.9)	17	136.2(2.96–1501)	−0.152	0.879
ITIH4(ng/mL)	17	33.75(10.6–88.5)	17	30.55(8.71–69.3)	−2.726	0.006

Starting point: start time of this study; End Point: the time the patients were diagnosed HCC

bias in conclusion. In the future, we need to conduct long-term, multicenter follow-up study with a larger sample size to further verify the value of ITIH4 in early diagnosis and warning for HCC.

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