

Development and potential application of a simultaneous multiplex assay of Golgi protein 73 and alpha-fetoprotein for hepatocellular carcinoma diagnosis

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Abstract. – **OBJECTIVE:** Detecting a single serum marker, such as Golgi protein 73 (GP73) or alpha-fetoprotein (AFP), may not meet the requirements for the early diagnosis of hepatocellular carcinoma (HCC) due to low sensitivity and specificity. Therefore, this study aimed to develop a simultaneous multiplex assay of GP73 and AFP.

PATIENTS AND METHODS: Anti-human GP73- and AFP-coupled microsphere beads and biotin-labeled detectable antibodies were prepared to develop a multiplex assay of GP73 and AFP using the Luminesx xMAP technology. The assay was evaluated for cross-reactivity, standard curve, sensitivity, range of detection, and precision. Additionally, the assay was used to determine the levels of serum GP73 and AFP in healthy controls and patients with chronic hepatitis, liver cirrhosis, and HCC.

RESULTS: The multiplex assay was successfully developed to simultaneously detect GP73 and AFP without cross-reactivity. The sensitivity for GP73 detection was 0.215 ng/mL and that for AFP detection was 0.666 ng/mL. The ranges of GP73 and AFP detection were 0.98-861.08 ng/mL and 2.01-1848.73 ng/mL, respectively. The intra- and inter-assay coefficients of variation (CVs) were <10%, indicating good precision, with recovery rates of 75-125%. The levels of serum GP73 in healthy controls, chronic hepatitis patients, liver cirrhosis patients, and HCC patients were 61.64 ± 30.60 ng/mL, 208.4 ± 99.42 ng/mL, 183.7 ± 82.78 ng/mL, and 214.1 ± 160.5 ng/mL, respectively. The levels of serum AFP in healthy controls, chronic hepatitis patients, liver cirrhosis patients, and HCC patients were 24.87 ± 14.52 ng/mL, 134.4 ± 216.5 ng/mL, 66.45 ± 133.4 ng/mL, and 891.4 ± 1278 ng/mL, respectively. The receiver operating characteristic (ROC) results

showed that the area under the curves (AUC) for the combination of GP73 and AFP was 0.972, which was larger than the AUC for each marker. The sensitivity and specificity of the combined detection of GP73 and AFP for the diagnosis of HCC were 90.91% and 98.86%, respectively. The multiplex assay demonstrated a good correlation with enzyme-linked immunosorbent assay (ELISA), with correlation coefficients of 0.818 and 0.982 for GP73 ($p < 0.001$) and AFP ($p < 0.001$), respectively.

CONCLUSIONS: A multiplex assay for the simultaneous detection of GP73 and AFP with high sensitivity and accuracy was developed for the diagnosis of HCC. This assay may provide a reliable reference for the early diagnosis of HCC.

Key Words

Golgi protein 73, AFP, Hepatocellular carcinoma, Diagnosis.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide, and it is the second leading malignancy with high mortality rates in China^{1,2}. HCC is usually diagnosed at an advanced stage and often arises from a background of hepatitis and cirrhosis, resulting in poor prospects of surgical treatment^{3,4}. The five-year survival rates of HCC patients have been reported to exceed 75% with appropriate treatments^{5,6}. Therefore, early diagnosis and

timely treatment of HCC significantly impact the survival of patients.

The diagnosis of HCC is usually determined using serum biomarkers and instrumental tests, including hepatic ultrasonography, computed tomography, magnetic resonance imaging, and biopsy, which are costly. Alpha-fetoprotein (AFP) is the most widely used biomarker for the diagnosis of HCC. However, AFP does not meet the requirements for the early diagnosis of HCC due to its low sensitivity and specificity⁷⁻⁹. Golgi protein 73 (GP73), a Golgi type II transmembrane protein, has been proposed as a biomarker for the diagnosis of HCC¹⁰. Scholars^{11,12} have reported that the combined detection of serum biomarkers can improve the early diagnosis of HCC. However, clinical methods for detecting serum markers usually include enzyme-linked immunosorbent assay (ELISA), which can only detect one protein at a time and has several limitations. Thus, a multiplex immunoassay for biomarker detection has become necessary. The Luminex xMAP technology is a bead-based multiplex immunoassay system that can simultaneously detect several proteins in a single reaction. It has the advantages of reducing sample volume, labor cost, and assay time, as well as a higher sensitivity than ELISA, which make it suitable for clinical application. Several studies have demonstrated the application of the Luminex assay for disease diagnosis^{13,14} and biomarker screening^{15,16}, and several products based on the Luminex technology have been approved by the Food and Drug Administration (FDA). Thus, the use of multiplex immunoassays, such as Luminex, is expanding in both basic and applied research¹⁷.

In this work, we developed a simultaneous multiplex assay of GP73 and AFP using the Luminex xMAP technology, evaluated its performance, and estimated the effectiveness of combined GP73 and AFP detection in HCC diagnosis.

Patients and Methods

Materials and Instruments

MagPlex Microspheres, the Amine Coupling Kit, the ProteOn Amine Coupling Kit, streptavidin-R-phycoerythrin (SAPE), and sheath were purchased from Bio-Rad (Hercules, CA, USA). The 4-morpholineethanesulfonic acid hydrate (MES) and Bovine Serum Albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). AFP antigen, anti-human AFP monoclonal

antibody, horse reddish peroxidase (HRP)-conjugated anti-human AFP antibody, and biotin-conjugated anti-human AFP antibody were purchased from US Biologicala (Swampscott, MA, USA). The EZ-Link Sulfo-NHS-LC Biotinylation kit was purchased from Thermo Scientific (Waltham, MA, USA). GP73 antigen and anti-human GP73 monoclonal antibodies were prepared in our laboratory¹⁸. Microplates were purchased from Greiner (Frickenhausen, Germany).

The Luminex-200 Analyzer and Bio-Plex Pro™ wash station were from Bio-Rad (Hercules, CA, USA). The Bead Counter Auto2000 was from Nexcelom (Lawrence, MA, USA). The plate shaker was from IKA (Staufen, Germany). The microplate reader was from Bio-Tek (Winooski, VT, USA).

Patients and Samples

This study included 229 patients from Guangdong Provincial People's Hospital and 88 healthy controls from the First Affiliated Hospital of Guangzhou Medical University. The serum samples were collected from November 2015 to November 2017. A total of 317 serum samples were collected from 41 patients with chronic hepatitis (median age: 37 years old; 31 males and 10 females), 89 patients with liver cirrhosis (median age: 53 years old; 65 males and 24 females), 99 patients with HCC (median age: 52 years old; 84 males and 15 females), and 88 healthy controls (median age: 31 years old; 49 males and 39 females). The serum samples were thawed on ice and centrifuged at 12000 rpm for 20 minutes at 4°C, and then, the supernatant was pipetted into tubes, while the remaining samples were stored at -80°C. This investigation was approved by the Ethical Committee for Medical Research at Guangdong Provincial People's Hospital (01, 20160906), and the informed consent was obtained from all participants.

ELISA

Microplates were coated with 100 µL/well anti-human GP73 (final concentration of 2 µg/mL) or anti-human AFP (final concentration of 1 µg/mL) antibodies and stored at 4°C overnight. The wells were then washed twice and blocked with phosphate-buffered saline (PBS) containing 2% BSA. One hundred microliters of two-fold serial dilutions of purified GP73 and AFP, which were prepared as the standard, were added into individual wells in duplicate. Diluted serum samples (1:10) were added to the sample wells in duplicate and incubated for one hour at 37°C. The wells were washed twice, and 100 µL of HRP-con-

jugated antibodies was added to each well and incubated for 30 minutes at 37°C. The wells were washed five times, and 100 μL of tetramethylbenzidine (TMB) substrate (Thermo Fisher Scientific, Waltham, MA, USA) was added to each well and incubated for 10 minutes at 37°C. Then, 50 μL of 1 M H_2SO_4 solution was added to each well to stop the reaction. The optical density at 450 nm (OD₄₅₀) was read using a microplate reader, and the GP73 or AFP concentration in each sample was determined using calibration curves with purified GP73 and AFP, respectively.

Luminex Bead Coupling

Bead coupling was performed according to the manufacturer's instructions (Bio-Rad Amine Coupling kit, Hercules, CA, USA). First, 100 μL of resuspended beads ($1 \times 10^7/\text{mL}$) was transferred into a microcentrifuge tube. The beads were washed with 50 mM MES (pH 5.0). Then, 10 μL of 50 mg/mL Sulfo-NHS and 50 mg/mL of EDC were added into the tubes to activate the beads. The beads were resuspended and incubated for 20 minutes at room temperature on a shaker at 800 rpm. The beads were then washed twice with MES to remove excess Sulfo-NHS and EDC. Then, 4 μg of anti-human GP73 or anti-human AFP monoclonal antibodies was added to the tubes at a total volume of 500 μL with MES. The beads were resuspended and incubated for two hours at room temperature on a shaker at 800 rpm. The beads were then washed twice with MES and blocked with storage buffer for 30 minutes at room temperature on a shaker at 800 rpm. The coupled beads were then washed twice with MES and stored in the storage buffer at 2–8°C in the dark.

Biotin Labeling

Biotin labeling was performed according to the manufacturer's instructions (EZ-Link sulfo-NHS-LC Biotinylation kit). Immediately before use, 10 mM Sulfo-NHS-LC-Biotin was prepared by dissolving 2.2 mg of the reagent in 400 μL of ultrapure water. Then, 27 μL of newly prepared biotin was added to 2 mg of anti-human GP73 or anti-human AFP monoclonal antibodies. The reaction was incubated on ice for two hours, and the biotinylation reagent was removed using a desalting column. The biotin-labeled antibodies were stored with a 1:1 volume of glycerol at –20°C in the dark.

Luminex Assay

Anti-human GP73 and anti-human AFP antibodies were precoupled with magnetic beads.

The coupled beads were prepared by diluting the stock solutions to a final concentration of 3000 beads/50 μL assay buffer. The bead mixture was added to each well and washed twice. Then, purified GP73 and AFP were diluted to concentrations of 2000 ng/mL and 4000 ng/mL, respectively, and mixed at a 1:1 volume. Then, 50 μL of four-fold serial dilutions of the mixture was added to individual wells in duplicate to obtain standard curves, while diluted serum samples (1:5) were added into sample wells in duplicate, and the plate was incubated for one hour at 37°C on a plate shaker at 900 rpm. The wells were then washed twice, and 50 μL of detection antibody mixture was added to each well and incubated for one hour at 37°C on a plate shaker at 900 rpm. Subsequently, the wells were washed twice, and 50 μL of SAPE was added into each well and incubated for 15 minutes at 37°C on a plate shaker at 900 rpm. Next, the wells were washed three times, and 100 μL of assay buffer was used to resuspend the beads. The median fluorescence intensity (MFI) was read using a Luminex-200 Analyzer. The data were fitted using a weighted five-parameter logistic model using Bio-Plex Manager™ 6.1 software.

Cross-Reactivity

Cross-reactivity reflects the specificity of captured antibodies (evaluated using mixed coupled beads, individual antigens, and mixed detectable antibodies), the specificity of detectable antibodies (evaluated using mixed coupled beads, mixed antigens, and individual detectable antibodies), and the specificity of antigens (evaluated using mixed coupled beads, mixed antigens, and mixed detectable antibodies). The MFI was used to analyze the cross-reactivity.

Sensitivity, Precision, and Accuracy

Blank controls at a concentration of 0 ng/mL GP73 and AFP in 20 replicates were used to determine the sensitivity. The mean value of x and the standard deviation (SD) of s were obtained, and then the values of $x + 2s$ were used in the equations of the standard curves to obtain the sensitivity.

Three different concentrations of purified GP73 (0.98 ng/mL, 15.60 ng/mL, and 250.00 ng/mL) and AFP (1.95 ng/mL, 31.25 ng/mL, and 500.00 ng/mL) in eight replicates were used to determine the intra-assay variation. These solutions were analyzed using three independent assays, with each concentration in eight replicates. Precision was evaluated by intra/inter-assay coefficients of

variation (CVs) (%), with intra-assay CV <10% and inter-assay CV <20% being acceptable.

Accuracy was evaluated by recovery. The observed and expected concentrations of GP73 and AFP in intra- and inter-assay were used to analyze the recovery. A recovery of 75-125% was acceptable.

Evaluation of the Luminex Multiplex Assay Versus ELISA

The multiplex assay and ELISA were used to compare 40 clinical serum samples in duplicate, including samples from patients with chronic hepatitis (n = 10), cirrhosis (n = 10), and HCC (n = 10), and healthy controls (n = 10).

Statistical Analysis

Statistical analysis was performed using Graph-Pad Prism 5.0 (La Jolla, CA, USA) and Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA). The correlation assay was performed using the Pearson's correlation coefficient. The group differences in GP73 and AFP values were compared using the one-way ANOVA, followed by the Post-Hoc Test (Least Significant Difference), with *p*-values <0.05 considered to be statistically significant. Receiver operating characteristic (ROC) curves were constructed to assess the sensitivity, specificity, and respective areas under the curves (AUC) with 95% confidence intervals (CI). The optimum cut-off values of GP73 and AFP for HCC diagnosis were defined by the maximum sensitivity and specificity¹⁹. Differentiation of HCC patients from healthy controls by independent variables was assessed in univariate and multivariate analyses with binary logistic regression.

Results

Cross-Reactivity

The MFI of captured antibodies with non-specific antigens or detectable antibodies was low, while the MFI of captured antibodies with specific antigens or detectable antibodies was high. The assay results (Table I) were specific, with no cross-reactivity between the protein targets.

Standard Curves

Standard curves are critical for quantitation measurements. The standard curves for GP73 and AFP (Figure 1) were evaluated using a weighted five-parameter logistic model. The concentration of GP73 was calculated using the following formula: $FI = -37.06 + (4648.23 + 37.06) / ((1 + (\text{Concentration} / 325495)^{-1.97169})^{0.405})$.

Sensitivity, Precision, and Accuracy

The MFI of the blank controls, with 0 ng/mL GP73 and AFP in 20 replicates, was used to evaluate the sensitivity using the described formulae for GP73 and AFP. The sensitivity for the detection of GP73 was 0.215 ng/mL and that for AFP was 0.666 ng/mL.

The precision was calculated using three different concentrations of purified GP73 and AFP. The intra-assay CV and the inter-assay CV were both <10% (Table II).

We analyzed the observed and expected concentrations of GP73 and AFP in intra- and inter-assay. The recovery rates of the two proteins were between 75% and 125% (Table II).

Table I. MFI of the cross-reactivity tests.

Coupled beads	Antigens	Biotin-labeled antibodies	MFI	
			GP73	AFP
GP73+AFP	AFP	GP73+AFP	65.75	894.75
GP73+AFP	AFP	–	14.5	23
GP73+AFP	GP73	GP73+AFP	3922	40.5
GP73+AFP	GP73	–	18.5	21.75
GP73+AFP	GP73+AFP	GP73	3976	36.25
GP73+AFP	GP73+AFP	AFP	15.5	897
GP73+AFP	GP73+AFP	–	18	20
GP73+AFP	–	GP73+AFP	29.25	32.53

$FI = -162.982 + (9693.12 + 162.982) / ((1 + (\text{Concentration} / 92453.2)^{-1.274})^{0.623})$, and the concentration of AFP was calculated using the formula:

$FI = -37.06 + (4648.23 + 37.06) / ((1 + (\text{Concentration} / 325495)^{-1.97169})^{0.405})$. As shown in the standard curves, the range of GP73 detection was 0.98~861.08 ng/mL, and that of AFP was 2.01~1848.73 ng/mL.

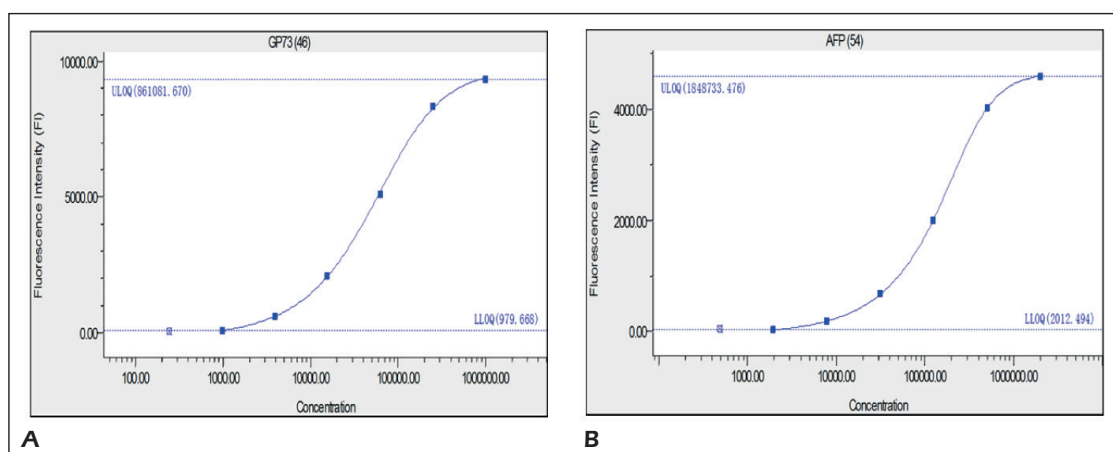


Figure 1. Standard curves for the multiplex assay. **A**, Standard curve for GP73. **B**, Standard curve for AFP.

Evaluation of the Luminex Multiplex Assay Versus ELISA

The Luminex Multiplex assay and ELISA were used to compare 40 clinical serum samples in duplicate. The correlation coefficients for GP73 and AFP were 0.818 and 0.982, respectively, $p < 0.001$ (Figure 2).

Determination of Serum GP73 and AFP Levels Using the Luminex Multiplex Assay

Serum samples were assessed for GP73 and AFP levels using the multiplex assay. Dot plots comparing the values of GP73 and AFP for a total

of 317 samples are shown in Figure 3. The mean \pm SD values of serum GP73 (sGP73) levels in healthy controls, chronic hepatitis patients, liver cirrhosis patients, and HCC patients were 61.64 ± 30.60 ng/mL, 208.4 ± 99.42 ng/mL, 183.7 ± 82.78 ng/mL, and 214.1 ± 160.5 ng/mL, respectively. The mean \pm SD values of serum AFP (sAFP) levels in healthy controls, chronic hepatitis patients, liver cirrhosis patients, and HCC patients were 24.87 ± 14.52 ng/mL, 134.4 ± 216.5 ng/mL, 66.45 ± 133.4 ng/mL, and 891.4 ± 1278 ng/mL, respectively. The distributions of sGP73 concentration in chronic hepatitis, liver cirrhosis, and HCC patient samples were significantly higher

Table II. Precision and accuracy of GP73 and AFP.

Concentration (ng/mL)	GP73					
	Intra assay			Inter assay		
	Mean (ng/mL)	CV (%)	Recovery (%)	Mean (ng/mL)	CV (%)	Recovery (%)
250.00	191.92	2.30	76.77	310.09	3.01	124.04%
15.60	14.81	3.63	94.94	17.21	6.66	110.32%
0.98	0.92	5.27	93.88	1.03	8.59	105.10%
Concentration (ng/mL)	AFP					
	Intra assay			Inter assay		
	Mean (ng/mL)	CV (%)	Recovery (%)	Mean (ng/mL)	CV (%)	Recovery (%)
500.00	427.90	3.98	85.58	514.33	4.57	102.87%
31.25	32.19	2.90	102.69	34.76	5.22	111.23%
1.95	1.73	8.36	88.72	1.90	6.95	97.44%

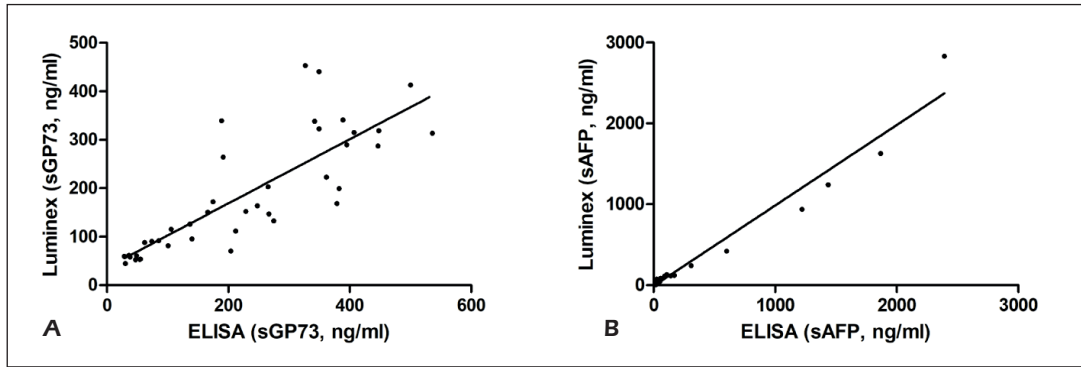


Figure 2. Correlation between the Luminex multiplex assay and ELISA. **A**, The correlation coefficient for GP73 was 0.818 ($p < 0.001$). **B**, The correlation coefficient for AFP was 0.982 ($p < 0.001$).

than the distribution of sGP73 concentration in healthy control samples. However, there was no significant difference among chronic hepatitis, liver cirrhosis, and HCC patient samples. In contrast to the level of sGP73, the level of sAFP in HCC patients was significantly higher than that in healthy controls, chronic hepatitis patients, and liver cirrhosis patients (Figure 3).

Diagnostic Value of Serum GP73 in Combination with Serum AFP for HCC

ROC curves were plotted to determine the optimal cut-off values for identifying the sensitivity and specificity of sGP73, sAFP, and combined sGP73 and sAFP for the differentiation of HCC patients from healthy controls (Figure 4). The ROC curves showed that the optimal diagnostic

cut-off value was 105.2 ng/mL for GP73 and 65.15 ng/mL for AFP. Moreover, compared with a single marker, the AUC for GP73 was 0.925, with a sensitivity of 78.79% and a specificity of 96.59%, and the AUC for AFP was 0.773, with a sensitivity of 60.61% and a specificity of 97.73%, while the combination of GP73 and AFP yielded the best AUC (0.972), with a sensitivity of 90.91% and a specificity of 98.86% (Table III).

Discussion

In the past few decades, several candidate biomarkers for HCC diagnosis have been reported^{20,21}. However, most of these biomarkers have not been employed in clinical diagnosis due to several limitations, such as low sensitivity, low specificity,

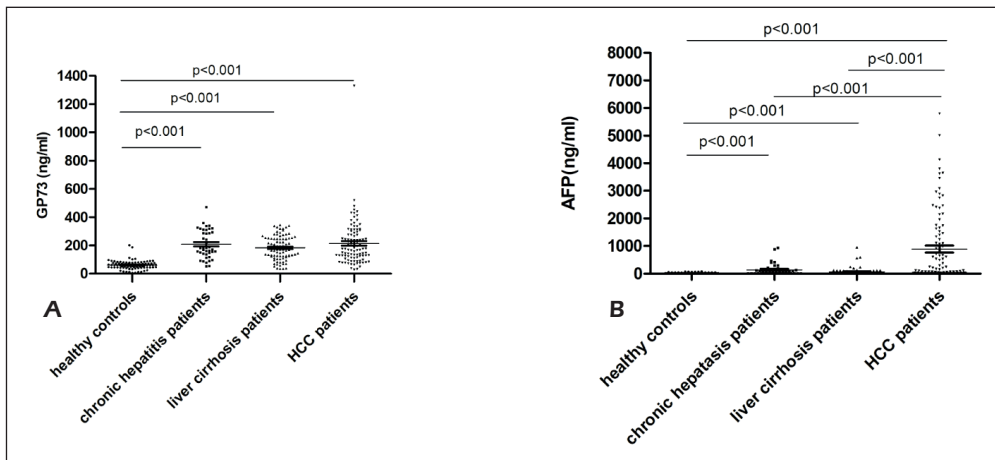


Figure 3. Dot plots comparing the levels of sGP73 and sAFP. **A**, Comparison of the levels of sGP73 in healthy controls, chronic hepatitis patients, liver cirrhosis patients, and HCC patients. **B**, Comparison of the levels of sAFP in healthy controls, chronic hepatitis patients, liver cirrhosis patients, and HCC patients.

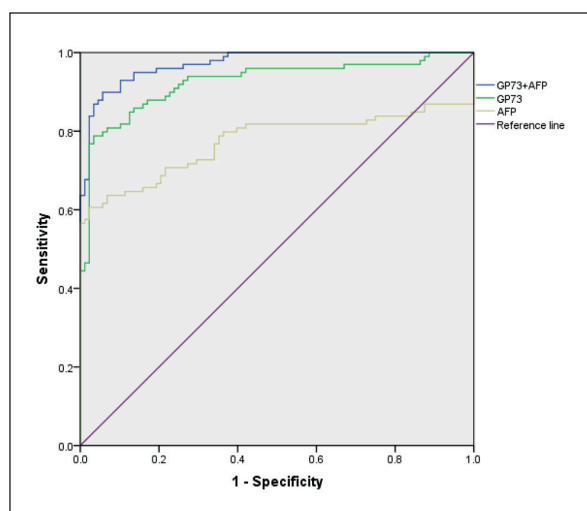


Figure 4. ROC curves for GP73, AFP, and GP73+AFP for the diagnosis of HCC.

and high cost²². To date, AFP level detection and imaging technologies are the main methods for the clinical diagnosis of HCC. Nevertheless, analyses of recent studies^{8,9} showed that the AFP test lacks adequate sensitivity and specificity for effective surveillance. GP73, a novel potential biomarker in liver disease²³⁻²⁵, showed a broad range of diagnostic accuracy values as a single marker²⁶. Several studies^{11,12} have shown that the accuracy of a combination of several biomarkers for HCC diagnosis was higher than that of a single biomarker, but these combinations are not commonly used in clinical practice due to the lack of a convenient quantitative assay. Therefore, we developed a method to simultaneously detect sGP73 and sAFP levels to improve the diagnostic value.

In our study, monoclonal antibodies were used while preparing coupled beads and detectable antibodies to avoid nonspecific interactions. The specificity of these monoclonal antibodies without cross-reactivity was verified, which was critical for developing a multiplex assay. The multiplex assay demonstrated that the sensitivity for the detection of GP73 and AFP was 0.215 ng/mL

and 0.666 ng/mL, respectively. The range of detection of GP73 and AFP was 0.98-861.08 ng/mL and 2.01-1848.73 ng/mL, respectively, making the detection of broader GP73 and AFP levels, which have large variations even at the same sample dilution, more convenient. The intra-assay CV and the inter-assay CV were both <10%, indicative of precision, and the recovery rates of GP73 and AFP in this assay were between 75% and 125%. These results indicated that the multiplex assay was accurate for detecting sGP73 and sAFP levels. Additionally, the multiplex assay required only 10 µL of serum samples to detect GP73 and AFP levels within 3-4 hours. Thus, the assay was more efficient and required a smaller volume of sample than ELISA, with good correlation.

Although Wu et al²⁷ reported the development of a multiplex assay for detecting AFP and GP73, it determined the plasma AFP and GP73 levels. In this study, we collected serum samples from healthy controls and patients with chronic hepatitis, liver cirrhosis, and HCC to determine the GP73 and AFP levels using the multiplex assay. The results showed that the levels of sGP73 in chronic hepatitis, liver cirrhosis, and HCC groups were significantly higher than those observed in the healthy control group. This finding was consistent with the findings of previous researches²⁸, indicating that GP73 is elevated not only in HCC but also in other chronic liver diseases, such as hepatitis and cirrhosis. However, no significant difference was found among chronic hepatitis, liver cirrhosis, and HCC patient groups in our work. In contrast to the level of sGP73, the level of sAFP in HCC patients was significantly higher than that in healthy controls, chronic hepatitis patients, and liver cirrhosis patients, although no significant difference was found among the healthy controls, chronic hepatitis patients, and liver cirrhosis patients. Additionally, the results of the comparison of the ROC curve for the combination of GP73 and AFP with that for each biomarker showed that the combination had the best AUC (0.972), with a sensitivity of 90.91% and a

Table III. Diagnostic value of serum GP73 in combination with AFP for HCC diagnosing.

Items	AUC (95%CI)	Sensitivity %	Specificity %	PPV %	NPV %
GP73	0.927 (0.887-0.967)	78.79%	96.59%	96.29% (78/81)	80.19% (85/106)
AFP	0.821 (0.755-0.887)	60.61%	97.73%	96.77% (60/62)	68.80% (86/125)
GP73+AFP	0.983 (0.968-0.997)	90.91%	98.86%	98.90 (90/91)	90.62% (87/96)

PPV: Positive predictive value; NPV: Negative predictive value.

specificity of 98.86%. Since most advanced HCC cases can be diagnosed with AFP, GP73 may be expected to play a supplementary role in the early diagnosis of HCC^{29,30}. However, this conclusion should be validated using a larger cohort of patients before its clinical application. Serial samples following the progression from hepatitis and cirrhosis to HCC could also be used to search for suitable biomarkers for early HCC diagnosis.

Conclusions

We used Luminex xMAP technology to develop a convenient assay to simultaneously detect GP73 and AFP levels with high sensitivity and accuracy for the diagnosis of HCC. Nevertheless, prospective validation studies to establish more efficient and cost-effective methods should be continued.

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Conflict of Interest

The authors declare that they have no competing interests.

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