Development of a Clinical Chemiluminescent Immunoassay for Serum GPC3 and Simultaneous Measurements Alone With AFP and CK19 in Diagnosis of Hepatocellular Carcinoma

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Background: Glypican-3 (GPC3) is an oncofetal antigen that shows great promise as a biomarker for diagnosis of hepatocellular carcinoma (HCC), but there is no reliable kit that can be used to detect it in clinics. The aim of this study is to develop a stable performance kit for GPC3 detection in clinics. **Design and methods:** The paired antibodies were identified through cycle-screening methods based on our previous research. Then, a double antibodies sandwich chemiluminescent immunoassay for detecting serum GPC3 was developed. The performance of the developed GPC3 diagnostic kit was evaluated by detecting the concentration of serum GPC3 and assessing its single or combined use with alpha fetoprotein (AFP) and cytokeratin 19 fragment (CK19) for HCC diagnosis. **Results:** The assay demonstrated a linear range of 10–800 ng/ml, the cross-reactivity rate at 0.018% (AFP), 0.020% (carcino-embryonic

antigen), and 0.021% (CK19), respectively. The minimum detectable concentration was 0.05 ng/ml; the intraassay coefficient of variation (CV) and interassay CV were both less than 10%, with good stability and reproducibility. GPC3 has a high sensitivity (54.2%) and specificity (99.4%) in diagnosing HCC. The level of GPC3 in HCC was robust higher than that in healthy or other liver diseases' sera (108.67 ± 230.04 ng/ml vs. 3.99 ± 7.68 ng/ml). The diagnostic sensitivity of GPC3 single or combined with CK19 and AFP for HCC was evaluated, and the rates were 54.2 and 90.6%, respectively. **Conclusions:** An applicable chemiluminescent immunoassay with stable performance against GPC3 in diagnosing HCC has been established and the combination of GPC3 with CK19 and AFP could improve the diagnostic sensitivity for HCC. J. Clin. Lab. Anal. 29:85–93, 2015. © 2014 Wiley Periodicals, Inc.

Key words: glypican-3; hepatocellular carcinoma; chemiluminescent immunoassay

INTRODUCTION

Hepatocellular carcinoma (HCC) remains a common malignant cancer worldwide. There are often no symp-

toms of HCC until the later stages, which last against surgery that has been a standard treatment. Much worse, HCC does not respond to most chemotherapy drugs. Alpha fetoprotein (AFP) has been used as a “gold-standard” diagnostic biomarker for HCC according to several

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clinical practice guidelines for several decades, but the sensitivity is still poor, around 30–60%. There is an urgent need to identify new biomarkers for the development of novel diagnostic approaches (1). Glypican-3 (GPC3) belongs to the glypican family of glycosylphosphatidylinositol-anchored heparan sulfate proteoglycans, which plays an important role in cellular growth, cell differentiation, and cell migration (2). There is a growing body of evidence to support that GPC3 is a new tumor marker for HCC since its first identification in 1988 by Filmus et al. GPC3 was found highly expressed in HCC but not in CCA or normal liver tissue, as well as in serum (3,4). The positive expression rates in HCC tissues can reach 70–90% and 40–60% in serum, with the specificity more than 90%. GPC3 may have a better utility value in detecting small and early HCC than AFP (5). The combined use of serum GPC3 and AFP can significantly increase the sensitivity of the diagnosis up to 80% since there may be an approximately 40% rate of GPC3-positive but AFP-negative diagnosis (6, 7). Furthermore, GPC3 is also a promising candidate for HCC therapy (8). Both novel recombinant humanized antibody and conformation-specific single-domain antibody against GPC3 and GPC3-derived peptide vaccine are found in-man phase I trial (9–11). So, GPC3 has emerged as a new HCC diagnosis biomarker both for early diagnosis and company diagnosis.

But there is still a long way to go for the transfer of a new biomarker into routine use in clinics (12, 13). Several methods such as ELISA (3), competitive radioimmunoassay (14), and chemiluminescence enzyme immunoassay (15) have been developed for serum GPC3 detection in HCC patients, based on the monoclonal antibody (mAb) or polyclonal antibody raised against human GPC3 (e.g., mAb 1G12 from Santa Cruz Biotechnology, California, US). However, the sensitivity and working curve varied a lot. Yasuda et al. evaluated a commercially available ELISA kit with GPC3 antibody 1G12 and found it was not useful in the diagnosis of HCC (16). A better diagnosis kit with greater sensitivity, low noise signal, broader linearity, less assay time, and easy-to-use is urgently needed to be developed for a new promising biomarker in translational medicine.

In the previous studies, we have developed 11 mAbs specific against GPC3 (17). The mAb 7D11 was used to establish an immunohistochemical method and was validated in HCC patients (18). Here, we reported the development of a chemiluminescence immunoassay (CLIA) kit for serum GPC3 detection based on the paired mAbs from our own laboratory, on the pilot production level according to the guidelines of IVD (in vitro diagnostic products). Sera from HCC, gastric cancer, colorectal cancer, hepatitis, esophageal cancer, cirrhosis, and normal healthy people were also collected for the valuation compared with AFP and CK 19.

MATERIALS AND METHODS

Chemicals, Immunoreagents, and Apparatus

Thirteen GPC3 mAbs and GPC3 antigen were prepared based on our previous study (17). Eu³⁺-labeled kit, acridinium ester (AE), and magnetic beads were purchased from Wallac Company (Finland). Bovine serum albumin (BSA), bovine γ -globulin, N-hydroxysulfosuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 4-morpholineethanesulfonic acid (MES; CAS: 71119-23-8) were purchased from Sigma-Aldrich (US). Dedicated fluorescence enhancement solution was purchased from the US Perkin Elmer, prestimulate solution A and stimulate solution B were purchased from Promega Corporation (Wisconsin, US). AFP (cat number: DR-CM-A001) and cytokeratin 19 fragment (CK19, cat number: DR-CM-A012) time-resolved immunofluorescence assay kits were purchased from Da An Gene Co., Ltd. (Guangdong, China). Ninety-six-well microplates were purchased from Labsystem (Finland) (size: 96 to 8 × 12). Chromatographic column-packing SephadexTM G-50 was purchased from Amersham Pharmacia (Sweden). Ultrafiltration centrifuge tube was the product of Millipore Corporation. The chemicals were of analytical reagent grade.

EM-1 protein nucleic acid detector, 1327 protein recorder, 2110 part of the collection device, and ED-1 constant speed pump were US Bio-Rad products; DR-M6601 time-resolved fluorescence immunoassay detector was purchased from Dagro Antibody Engineering Technology. Sirius L single-tube chemiluminescence analyzer was purchased from Berthold Detection Systems GmbH, Pforzheim, Germany. Magnetic separator was obtained from Tianjin Baseline Chromtech Research Center.

Solutions

Twenty millimoles per liter Tris-HCl buffer (pH 7.4) was used in this study. Blocking solution contained 50 mmol/l Tris-HCl buffer (pH 7.2) plus 1% BSA, 3% trehalose, filtered. Washing buffer was 0.1 M PBS (pH 6.5), containing 0.9% NaCl, 0.02% NaN₃, and 0.03% Tween-80. Eu³⁺-labeling buffer (pH 9.5) contained 50 mmol/l Na₂CO₃ and 0.03% NaN₃, filtered. Assay buffer (pH 7.8) had 0.05% NaN₃, 0.01% Tween-20, 50 μ M diethylene triamine pentaacetic acid (DTPA), 0.9% NaCl, 0.5% BSA, 0.02% bovine γ -globulin, fully dissolved, filtered (0.22 μ m), and stored at 4°C. Magnetic beads coated buffer (pH 5.0) was 0.1 M MES. AE-labeled buffer was 0.1 M PB (pH 6.5). TBST (pH 7.2) solution contained 0.1 M Tris-HCL, 0.15 M NaCl, 0.5% BSA, and 0.05% Tween-20. The antigen solution buffer (pH 7.2) contained 0.1 M Tris-HCl, 1.5% BSA, 0.9% NaCl, and 0.05% Tween-20.

Serum Samples

Serum samples from 518 patients were collected from the Southern Hospital (Guangzhou, China). Among the enrolled patients, 192 patients (172 males and 20 females, ages 50.48 ± 11.04 years) were classified as HCC, 54 patients (42 males and 12 females, ages 56.48 ± 12.63 years) as gastric cancer, 92 patients (72 males and 20 females, ages 55.81 ± 13.26 years) as colorectal cancer, 57 patients (46 males and 11 females, ages 38.73 ± 13.61 years) as hepatitis, 31 patients as esophageal cancer (20 males and 11 females, ages 55.71 ± 13.41 years), and 44 patients as cirrhosis (36 males and 8 females, ages 51.89 ± 13.15 years). Additionally, 48 healthy peoples' sera were also collected (39 males and 9 females, ages 50.67 ± 12.53 years). One hundred and ninety-two serum samples were collected from HCC patients before any therapeutic approach. Approval was obtained from the regional ethics committee, and all subjects gave informed consent to participate. All of the samples were diagnosed according to the pathological standard.

The GPC3-Matching Antibodies Screening

One milligram GPC3 mcAbs were processed to be concentrated through a concentration membrane (cat number: UFC5050BK) produced by Millipore Corporation. GPC3 mcAbs' concentration was adjusted to 5 mg/ml. According to the labeled kit's instructions of Wallac Company (1 mg antibody needs 0.2 mg of labeling reagent, the mass ratio is 1:5), the labeled antibody and europium-labeling reagent were mixed at 4°C for 8 hr; then, the labeled antibody was purified by the gel chromatography. The double antibody sandwich method of time-resolved fluoroimmunoassay technology was selected to cycling screen paired antibody after marking procedure completion.

Preparation of Magnetic Beads Coated With Capture Antibody

Ten milligram magnetic beads were prepared and washed six times with 600 μ l, 0.1 M MES. Then, 300 μ l, 0.1 M EDC buffer and 220 μ l, 0.1 M NHS buffer were added to activate the surface carboxyl groups on the magnetic beads for 30 min at room temperature. Then, the activation solution was removed, and the beads were washed five times with 0.1 M MES.

GPC3 mcAbs (0.2 mg) were concentrated by centrifugation at 8,000 rpm for 6 min and the concentration was adjusted to 1 mg/ml by 0.1 M MES. It was added to 10 mg activated magnetic beads and mixed well by rotary mixer at 4°C, for 8 hr. The next day, the beads were washed with TBST to remove the free antibody and the

antibody-conjugated magnetic beads were diluted to 400 μ g/ml with TBST and stored at 4°C.

Preparation of AE-Labeled Detection Antibody

GPC3 mcAbs (0.5 mg) were concentrated by centrifugation in 8,000 rpm, for 6 min, and the antibody concentration was adjusted to 1 mg/ml by 0.1 M PB; then, 5 μ l AE solution (10 mg/ml) was added to it and mixed well, the reaction does happen in the darkness for 8 hr at 4°C. The labeled antibody was purified by Sephadex TM G-50. The purified labeled-antibody was stored at 4°C.

Preparation of Different Reference Concentrations of GPC3 Antigen

The original concentration of GPC3 recombinant antigen was determined as 1.25 mg/ml and the purity as 80% by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). After that, the concentration was gradiently diluted to six series concentrations of 0, 10, 50, 100, 400, and 800 ng/ml, respectively.

Preparation of Blocked Microplate

Three hundred microliter blocking solution was added to each well on a 96-well microplate. The microplate was then covered with plastic film and incubated overnight at 4°C. The next day, the blocking solution was removed and the microplate was dried at 37°C 4 hr, then covered with plastic film and stored at 4°C.

The CLIA Operation Procedure

- (1) Reagents and the microplates were set at room temperature to balance.
- (2) Fifty microliter magnetic beads coated with capture antibody, 25 μ l GPC3 antigen, and 50 μ l assay buffer were added. Then, they were covered with plastic film and incubated at 37°C for 15 min.
- (3) After the first incubation, 200 μ l washing buffer was injected two times into each well to remove the free antigen. Then, 50 μ l, 1:100, AE-labeled detection antibody was added to each well and mixed well. It was followed by incubation at 37°C for 10 min.
- (4) After the second incubation, the beads were washed four times with 200 μ l washing buffer.
- (5) One hundred microliter prestimulate solution A was added to each well; then, the Sirius L single-tube chemiluminescence analyzer was automatically added to 100 μ l stimulate solution B and immediately to detection reagent.

Optimization of Reaction Conditions

The reaction volumes of the antibody-conjugated magnetic beads were set at four levels as 30, 50, 70, and 90 μ l in the experimental operation, that is, 10, 20, 28, and 36 μ g per reaction. AE-labeled GPC3 antibody concentrations were set at three levels: 1:50, 1:100, 1:200. GPC3 antigen volume was set at four levels: 15, 25, 35, and 45 μ l. The optimum reaction conditions were evaluated by orthogonal test.

Evaluation of the Detection Reagent

Linear range and hook effect

The standard curve for GPC3 was prepared using the antigen concentrations (nanograms per milliliter) as the abscissa (x axis) and the luminescence value as ordinate (y axis). A total of 22 points (concentration range: 0–7,000 ng/ml) was selected to evaluate the reagent's hook effect.

Analysis of the accuracy

The analytical accuracy was evaluated by added recovery and dilution recovery. The GPC3 antigen was added to normal human serum with the theoretical concentrations 2, 5, 50, 100, 200, 300, and 500 ng/mL. The dilution samples were prepared by 4,000, 2,000, and 1,000 ng/ml and were diluted twofold, fivefold, and tenfold, respectively. Then, the ratio between the measured and theoretical values (the ratio should be between 90.00 and 110.0%) was calculated.

Analysis of the minimum detectable concentration

Zero reference standard was measured 20 times, the mean ($\bar{x}$) and standard deviation (SD) were calculated and substituted with $\bar{x} + 2SD$ into the standard curve equation to obtain the minimum detectable concentration.

Specificity test

The cross-reactivity of this method with several tumor markers associated with HCC, including AFP (1,000 ng/ml, from the AFP time-resolved immunofluorescence assay kit, cat number: DR-CM-A001), CEA (carcinoembryonic antigen; 1,000 ng/ml, from Meridian Life Science, Inc., catalog number: A32321H), and CK19 (800 U/ml, from the CK19 time-resolved immunofluorescence assay kit, cat number: DR-CM-A012), added these markers to normal human serum, respectively; then the luminescence values were measured.

Interfering test

The interference immunity of the CLIA reagents developed in this experiment was added to the common serum constituents known as causing interference in immunoassays (hemolytic, high cholesterol, high bilirubin). The human hemoglobin (1,000, 500 ng/ml), triglycerides (1,000, 500 ng/ml), and bilirubin (50, 25 ng/ml) were added to the GPC3 antigen samples, respectively. The GPC3 antigen was diluted to 50, 100, and 500 ng/ml for the test. The above solutions were tested and the recovery rate was calculated.

Test of stability

GPC3 reagent was placed at 37°C incubator for 7 days or 4°C for 6 months. Then, the physical appearance was observed and the performance was detected during various stages.

Detection of clinical specimens

One hundred ninety-two patients with HCC, 57 with hepatitis, 54 with gastric cancer, 92 with colorectal cancer, 31 with esophageal cancer, 44 with liver cirrhosis, and 48 cases of healthy human serum samples were evaluated using the GPC3-CLIA reagent.

Determination of serum AFP and CK19

Expression of AFP in clinical serum samples was tested by AFP time-resolved detection kit. The CK19 was detected by CK19 time-resolved detection kit, operated according to the manufacturer's instructions.

Statistical methods

Statistical analysis of the results was performed using SPSS 13.0. The t -test was used for statistical analysis. Significant difference was defined as $P < 0.05$.

RESULTS

Results of Paired Antibody Screening

Thirteen GPC3 mAbs were cycling paired, it was found that the highest fluorescence efficiency was produced using the 8G6 mAb as the coated antibody and 7D11 mAb as the detection antibody (Fig. 1). Six series GPC3 antigen concentrations, 0, 31.25, 62.5, 125, 250, and 500 ng/mL, were labeled as standard. The antigen concentration as the abscissa (x axis) and luminescence value as vertical axis (y axis) showed the matching antibodies can obtain a good linear curve. The working curve equation was $y = 3,386.4x + 9,357.5$, $R^2 = 0.9984$.

Fig. 1. The standard curve of paired antibody screening.

Development of the GPC3 CLIA

Reaction conditions

According to the results of the orthogonal experiment (Fig. 2), the best reaction conditions were as follows: bead-coated GPC3 capture antibody, 400 $\mu\text{g}/\text{ml}$;

pipette, 50 $\mu\text{l}/\text{reaction}$, namely 20 $\mu\text{g}/\text{reaction}$; AE-labeled GPC3 detection antibody, diluted to 1:100; and pipette, 50 $\mu\text{l}/\text{reaction}$. GPC3 antigen or analytic sample pipette was 25 $\mu\text{l}/\text{reaction}$. To draw the curve of the human GPC3-CLIA, the reference standard concentrations (0, 10, 50, 100, 400, and 800 ng/mL) were set as the

Fig. 2. Optimization of the added volume of standard. Bead-coated GPC3 capture antibody, 400 $\mu\text{g}/\text{ml}$; pipette, 50 $\mu\text{l}/\text{reaction}$; AE-labeled GPC3 detection antibody, diluted to 1:100; and pipette, 50 $\mu\text{l}/\text{reaction}$; the standard pipette, 15, 25, 35, and 45 $\mu\text{l}/\text{reaction}$, respectively. The standard curve can attain the best at 25 $\mu\text{l}/\text{reaction}$ of standard.

Fig. 3. The hook effect of GPC3-CLIA.

TABLE 1. Added Recovery Rates of GPC3 Tested in Normal Human Serum

GPC3 added (ng/ml, $n = 4$)	GPC3 assayed (ng/ml)	Recovery (%)
2.000	1.890 ± 0.221	94.50
5.000	5.130 ± 0.863	102.6
50.00	53.40 ± 3.752	106.8
100.0	101.6 ± 5.926	101.6
200.0	195.0 ± 5.641	97.50
300.0	290.5 ± 9.190	96.83
500.0	523.0 ± 17.15	104.6

$n = 4$, each concentration repeated four times.

abscissa (x axis), the luminescence value as vertical axis (y axis), and the working curve equation was $y = 2,998.2x + 1,222.2$, $R^2 = 0.9999$ (Fig. 2). The curve became nonlinear at 3,000 ng/ml, and at 6,000 ng/ml there was an obvious signal decrease (Fig. 3).

Analysis of accuracy

Recovery rates were calculated as the measured concentration divided by the theoretical concentration $\times 100\%$. The added recovery rates were from 94.5 to 106.8%, the average rate was 100.63% (Table 1). The dilution recovery rates were from 93.0 to 107.5% and the average rate was 101.44% (Table 2).

The minimum detectable concentration and specificity

The mean of zero reference standard determination values plus two times SD ($\bar{x} + 2SD$) was used to calculate

TABLE 2. Dilution Recovery Rates of GPC3 Tested in Normal Human Serum With Two-, Five-, and Tenfold Dilution Factor

Theoretical concentration	Dilution factor	Tested concentration (ng/ml)	Recovery (%)
4,000	2	2,089	104.4
	5	785	98.12
	10	413	103.2
2,000	2	1,062	106.2
	5	387	96.75
	10	186	93.00
1,000	2	534	106.8
	5	215	107.5
	10	97	97.00

TABLE 3. Cross-Reactivity Rates Detected by GPC3-CLIA Kit

Antigen	Clinical cutoff value	Tested value	Cross-reactivity (%)
AFP	<20 ng/mL	1,000 ng/ml	0.015
		500 ng/ml	0.018
CEA	<15 ng/mL	1,000 ng/ml	0.020
		500 ng/ml	0.016
CK19	<37 U/mL	800 U/ml	0.019
		400 U/ml	0.021

the minimum detectable concentration. The minimum detectable concentration was 0.05 ng/mL. The results of cross-reactivity of AFP, CEA, and CK19 are shown in Table 3. The measured values for each of the added markers were far less than the sensitivity of the GPC3 reagent (0.05 ng/ml); the specificity values reached 0.018, 0.020, and 0.021%, respectively (Table 3). These results showed

TABLE 4. Interference From Addition of Hemolysate, Triglyceride, and Bilirubin to Patients' Serum Samples

Concentration of GPC3 in serum (ng/ml)	Interferent	Concentration (ng/ml)	Measured value (ng/ml)	Recovery rate (%)
50	Hemoglobin	0	49.86	99.72
		500	50.96	101.92
		1,000	51.06	102.12
	Triglycerides	0	49.83	99.66
		500	51.37	102.74
		1,000	52.64	105.28
	Bilirubin	0	50.02	100.04
		25	51.45	102.90
		50	52.27	104.54
100	Hemoglobin	0	99.59	99.59
		500	103.42	103.42
		1,000	105.25	105.25
	Triglycerides	0	100.43	100.43
		500	104.87	104.87
		1,000	106.48	106.48
	Bilirubin	0	101.13	101.13
		25	104.29	104.29
		50	105.46	105.46
500	Hemoglobin	0	501.03	100.21
		500	506.89	101.38
		1,000	513.48	102.70
	Triglycerides	0	510.45	102.09
		500	514.98	103.00
		1,000	521.36	104.27
	Bilirubin	0	509.83	101.97
		25	518.54	103.71
		50	524.62	104.92

that there was little similarity of those biomarkers with the accuracy of the self-made GPC3 reagent.

Interference experiments

The recovery rates were tested between 99.59 and 106.48% (Table 4) in this experiment. The results demonstrated that there was no interference by these substances.

Stability test

The performance was validated when placed at 37°C for 7 days or at 4°C for 6 months. Correlation coefficient (R^2) of the working curve was greater than 0.99. The recovery rates were between 102.14 and 109.51% by detecting three serological markers with high, medium, and low GPC3 concentrations. The sensitivity is lower than 0.05 ng/ml, and there was no cross-reaction with AFP, CEA, and CK19. The intraassay coefficient of variation (CV) and interassay CV were still less than 10 and 15%, respectively (Table 5). All these results met the stability requirements.

GPC3 expression in the serum of healthy people, benign liver disease, HCC, and other cancer

The results about serum GPC3 levels in different serum samples are shown in Figure 4. Serum GPC3 levels were 108.67 ± 230.04 ng/ml in HCC, significantly higher than that in the healthy samples (3.99 ± 7.68 ng/ml, $P < 0.05$, t -test), liver cirrhosis (6.87 ± 9.10 ng/ml, $P < 0.05$), colorectal cancer (5.96 ± 6.29 ng/ml, $P < 0.05$), esophageal cancer (6.27 ± 6.09 ng/ml, $P < 0.05$), gastric cancer (6.00 ± 6.72 ng/ml, $P < 0.05$), and hepatitis (7.55 ± 7.91 ng/ml, $P < 0.05$). Serum GPC3 level in HCC was about 100-fold higher than in healthy samples, and about fivefold higher than in cirrhosis. Thus, serum GPC3 level in human serum can well differentiate HCC from healthy samples and other liver diseases.

Evaluation and comparison of the diagnostic sensitivity and specificity about HCC using GPC3, AFP, and CK19

The diagnostic sensitivity of GPC3 for HCC was 54.2% (104/192) when the cutoff was set at 30 ng/ml, however, with negative expression in that of hepatitis, gastric cancer, colorectal cancer, and esophageal cancer patients. In addition, there were only two GPC3(+) cases (4.5%) among 44 liver cirrhosis patients, the sensitivity and specificity of GPC3 reached 54.2 and 99.4%. The diagnostic sensitivity of the combination with GPC3 and AFP could be significantly increased to 80.7%. GPC3 and CK9 expression levels have significant difference ($P = 0.004$), but do not have consistency ($\kappa = -0.022$, $P = 0.747$); the diagnostic sensitivity of the combination with GPC3 and CK19 was 72.4%. When all of these three markers were combined, the diagnostic sensitivity increased to 90.6%.

DISCUSSION

So far, the early diagnosis and surgical resection have made significant contribution to improve HCC patient's survival. However, the 5-year survival of HCC patients is still fairly poor for lack of effective detection methods. Although AFP is the most commonly used marker for HCC screening, the diagnosis rates could not meet the clinical needs. GPC3, a membrane heparan sulfate glycoprotein, does have another enormous potential marker for HCC detection (19, 20). It affects a variety of biological effects of molecules, and thus plays a key role in cell growth, development, and during carcinogenesis. GPC3 is just highly expressed in human fetal liver, placenta, and other tissues, with no expression in normal adult liver (19, 21, 22), while in HCC, GPC3 was highly expressed and was positively correlated with tumor size and liver cancer pathological grading, playing an important role in the severity of liver cancer. Serum GPC3 level is

TABLE 5. Stability Experiment of Detection Reagent

			37°C, 1 day	37°C, 3 days	37°C, 5 days	37°C, 7 days	4°C, 1 month	4°C, 3 months	4°C, 6 months
Appearance			Normal	Normal	Normal	Normal	Normal	Normal	Normal
Working curve	R^2		0.998	0.994	0.992	0.990	0.999	0.995	0.993
Accuracy	Recovery rate (%)	Low	105.84	106.25	108.41	109.51	102.43	104.69	107.34
		Medium	104.38	104.67	104.93	105.63	102.14	103.87	106.28
		High	103.76	104.07	104.54	105.82	101.78	102.65	104.79
Sensitivity	ng/ml		0.0450	0.037	0.048	0.05	0.048	0.045	0.041
Interference	Cross-reaction with AFP, CEA, CK19		No	No	No	No	No	No	No
Precision	Intraassay CV (%)		4	6	7	9	3	5	8
	Interassay CV (%)		9	7	11	13	7	10	9

Fig. 4. Quantification of GPC3 protein in serum from 192 HCC patients and other patients with liver diseases or other cancers by GPC3-CLIA reagent. The serum GPC3 level in HCC was significantly higher than in cirrhosis, CRC, EC, GC, and hepatitis (CRC, colorectal cancer; EC, esophageal cancer; GC, gastric cancer).

significantly elevated in patients with early and advanced HCC, but it is rarely detectable in patients with nonmalignant chronic liver disease and healthy controls (23). GPC3 is expressed in AFP-negative HCC, joint detection of AFP and GPC3 could significantly increase the detection rate and accuracy of the diagnosis for HCC (24–27). Therefore, a diagnosis kit with great sensitivity, low noise signal, broad linearity, less assay time, and easy use is urgently needed to be developed for a new promising biomarker in translational medicine.

This study provided an applicable chemiluminescent immunoassay with stable performance and specificity against GPC3 in the diagnosis of HCC. The minimum detectable concentration reached 0.05 ng/ml. The added recovery rates were from 94.5 to 106.8% (Table 1) and the dilution recovery rates were from 93.0 to 107.5%. The cross-reactivity values with AFP, CEA, and CK19 were 0.018, 0.020, and 0.021%, respectively. The interference experiment results showed that there was no interference

TABLE 6. Diagnostic Sensitivity of GPC3, AFP, and CK19 for HCC

Detection		Project	Case	Sensitivity (%)
Independent detection	AFP(+)		108	56.3
	GPC3(+)		104	54.2
	CK19(+)		74	38.5
Combined detection	GPC3(+) and/or AFP(+)		155	80.7
	GPC3(+) and/or CK19(+)		139	72.4
	AFP, GPC3, CK19(+)		174	90.6

with the hemoglobin, triglycerides, and bilirubin. We developed serum GPC3 from 192 HCC patients, which was detected by GPC3-CLIA (Fig. 4). GPC3 actually showed robust expression in the serum of HCC patients (108.67 ± 460.08 ng/ml), than in the serum of 48 healthy human (3.99 ± 7.68 ng/ml). Moreover, GPC3 expression just appeared specifically robust, compared to that in the colorectal cancer patients (5.96 ± 6.29 ng/ml), hepatitis (7.55 ± 7.91 ng/ml), gastric cancer (6.00 ± 6.72 ng/ml), esophageal cancer (6.27 ± 6.09 ng/ml), liver cirrhosis (6.87 ± 9.10 ng/ml). When 30 ng/ml as the cutoff value in 192 HCC patients, serum GPC3 sensitivity was 54.2%; 57 hepatitis, 54 with gastric cancer, 92 colorectal, 31 esophageal cancer patients, and 48 healthy human serum were no expression; in 44 cases of liver cirrhosis, there were two positive cases. These results are consistent with Zhang et al.'s and Capurro et al.'s previous reports (23, 28).

In this study, the sensitivity and specificity of homemade GPC3-CLIA is 54.2 and 99.4% (Table 6). In cases of $\text{AFP} \leq 20$ ng/ml, 47 cases of patients were with AFP(–), while GPC3(+), serum. In AFP(+) patients, 51 patients showed as GPC3(–). It suggests that the combined detection of AFP and GPC3 can improve the diagnostic specificity and sensitivity in HCC patients. When AFP and GPC3 were combined, the sensitivity of diagnosis of HCC increased from 56.3 to 80.7% compared to AFP single use (Table 6). In 74 cases of CK19(+), there were 19 cases that were negative for GPC3 and AFP. The sensitivity of HCC

diagnosis was upgraded to 90.6% when three markers were used combinedly, which is similar to other previous reports. Capurro et al. reported that in most cases elevated GPC3 values do not correlate with elevated AFP values, the simultaneous use of GPC3 and AFP significantly increases the sensitivity of the diagnosis (3). A meta-analysis by Cheng et al. showed that the sensitivity of AFP combined with GPC3 has a significant elevation compared to AFP or GPC3 alone (29). Since tumor is a complex multifactorial disease, single marker in the crowd will have flaws, and the combination detection of tumor markers will greatly enhance the detection sensitivity.

In conclusion, an applicable chemiluminescent immunoassay specific against GPC3 (GPC3-CLIA) was successfully prepared for large clinical validation. Combined application with AFP, CK19, and GPC3 really enhanced the diagnostic sensitivity for HCC. The clinical trial study for further validation of GPC3-CLIA kit will be undertaken according to the registration rules issued by the State of Food and Drug Administration (SFDA) including a follow-up study on at least 100 HCC patients for monitoring serum GPC3 levels pre and posttreatment.

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