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Research Article

Analytical Evaluation of Latex-Enhanced Immunoturbidimetric Assay for Glypican3 and The Diagnostic Value for HCC

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Abstract

Objective: To establish a latex-enhanced immune turbidimetric assay for detecting the level of GPC3 in serum and to explore the application value of GPC3 in the clinical diagnosis of Hepatocellular Carcinoma (HCC).

Methods: The latex-enhanced immune turbidimetric assay was used on a fully automated biochemical analyzer to detect GPC3. The analytical sensitivity, repeatability, linear range, relative recovery, and interference of this method were validated. A total of 351 serum samples were collected from healthy controls, chronic hepatitis B group, untreated HCC group, and treated HCC group. The concentrations of GPC3, AFP, and PIVKA-II were measured, and statistical analysis was performed using the rank-sum test.

Results: 1). The analytical sensitivity of the latex-enhanced immune turbidimetric assay for detecting GPC3 was 29 pg/ml. The Intra-assay imprecision was <3%, and the inter-assay imprecision was <5%. The recovery rate was 99%, and there was no interference from 100 ug/ml AFP and 100 mg/ml HSA. **2)**. The levels of GPC3, AFP, and PIVKA-II in the untreated HCC group were significantly higher than those in the other groups, and the levels of these three markers were significantly decreased in the treated HCC group ($P<0.05$). **3).** The ROC curve analysis of GPC3 for the diagnosis of HCC showed an area under the curve of 0.932 (95% CI: 0.879-0.984), with a cutoff value of 81 pg/ml, and the sensitivity and specificity were 86.0% and 96.9%, respectively. **4).** In the untreated HCC group, the positive rates of GPC3, PIVKA-II, and AFP were 86.0%, 80.0%, and 76%, respectively. The positive rate of GPC3+AFP was 98%, and the positive rate of GPC3+AFP+PIVKA-II was 98%. The positive rate of GPC3+PIVKA-II was 96%. **5).** Among the 12 AFP-negative patients in the untreated HCC group, the serum GPC3 positive rate was 91.7%, and the PIVKA-II positive rate was 75%.

Conclusion: The performance characteristics of the latex-enhanced immune turbidimetric assay for detecting serum GPC3 meet the requirements of the "Guiding Principles for the Registration and Review of Analytical Performance Evaluation of Quantitative Detection In Vitro Diagnostic Reagents". Serum GPC3, PIVKA-II, and AFP detection alone or in combination have certain value in the diagnosis of liver cancer. The advantage of serum GPC3 detection is particularly evident in AFP-negative HCC, and the combined analysis of serum GPC3 and AFP significantly improves the diagnostic efficiency of HCC.

Keywords: Serum; GPC3; Latex-enhanced immunoturbidimetric assay; HCC; Early diagnosis

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Introduction

Hepatocellular Carcinoma (HCC) has become a global public health issue due to its high mortality rate [1]. In Asia, HCC secondary to hepatitis B virus is more common [2]. 10-20% of HCC patients can be treated with surgery in the early stages, and with a 5-year survival rate of 40-70%. However, most HCC cases are diagnosed at the late stage, missing the optimal treatment opportunity [3]. Therefore, early diagnosis of HCC is a breakthrough focus in HCC treatment.

The commonly used tumor marker Alpha-Fetoprotein (AFP) has limited diagnostic value for HCC patients who are AFP negative or have AFP levels less than 400 ng/ml [4]. Des-γ-Carboxyprothrombin (DCP), also known as protein induced by vitamin K absence or antagonist II (PIVKAII), can be found in the serum of patients with vitamin K deficiency or HCC [5]. However, its sensitivity for diagnosing small and early-stage HCC is still insufficient. Glypican-3 (GPC3) is a sulfated glycosaminoglycan protein on the cell membrane and has recently become a research hotspot as a tumor marker for HCC [6]. GPC3 is highly expressed in both mRNA and protein levels in HCC, while it is lowly expressed or not expressed in other tumors and benign liver diseases. It is also expressed in AFP-negative liver cancer and has high sensitivity and specificity for diagnosing HCC, as well as a certain relationship with the prognosis of HCC [7]. Currently, the commonly used method for detecting GPC3 in serum is Enzyme-Linked Immunosorbent Assay (ELISA). However, this detection method is time-consuming and involves multiple separate steps, resulting in lower accuracy and inability to achieve automatic detection, making it difficult to be widely implemented in clinical practice [8].

In this study, we developed a latex-enhanced immune turbidimetric assay suitable for fully automatic biochemical analyzers to detect serum GPC3 levels. We evaluated the analytical performance of this method according to the requirements of the "Guidelines for the Evaluation of Analytical Performance of In Vitro Diagnostic Reagents" [9] and "YY/T 1255-2015 Immune Turbidimetric Assay for Testing Reagents (Box) (Transmission Method)" [10], and explored the application value of serum GPC3 levels in the auxiliary diagnosis of HCC patients.

Materials and Methods

Patients and Plasma Samples

In this study, a total of 254 patients were selected from the outpatient and inpatient departments of the Hepatology Department of Tianjin Third Hospital from June 2022 to March 2023. Among them, there were 72 cases in the Chronic Hepatitis B (CHB) group, 50 cases in the HCC group, and 132 cases in the treated HCC group. At the same time, 97 serum samples from the health examination population at Tianjin Third Central Hospital Examination Center were collected as the Health Control (HC) group. Diagnostic and exclusion criteria: The diagnosis of HCC in this study followed the diagnostic criteria recommended by the World Health Organization; chronic hepatitis B was diagnosed according to the "Guidelines for the Prevention and Treatment of Chronic Hepatitis B (Updated in 2015)"; this study was approved by the ethics committee of Tianjin Third Central Hospital.

Reagents and Apparatus

N-Hydroxysulfosuccinimide (NHS), 4-Morpholineethanesulfonic acid (MES), and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Carboxylated polystyrene latex was purchased from JSR Corporation in Japan. Bovine Serum Albumin (BSA) was obtained from Bovogen Biologicals Pty Ltd (Victoria, Australia). Recombinant GPC3 protein and anti-GPC3 monoclonal antibodies (McAbs, 4D) were provided by Tianjin Jin Hong Biotechnology Development Co., Ltd. Alpha-fetoprotein electrochemiluminescence immunoassay kit (Roche Co., Tokyo, Japan) and PIVKA-II chemiluminescence enzyme immunoassay kit (Eisai Co., Tokyo, Japan) were used. All other chemicals and reagents were analytical grade. The Latex-Enhanced Immune Turbidimetric Assay (LEITAs) instrument used was the fully automatic Hitachi 7080 biochemical analyzer. The Hitachi CR21N high-speed centrifuge was used for latex washing and buffer exchange. The ATSE ultrasonic homogenizer (ATS Engineering Co., Suzhou, China) was used for latex dispersion and centrifugation.

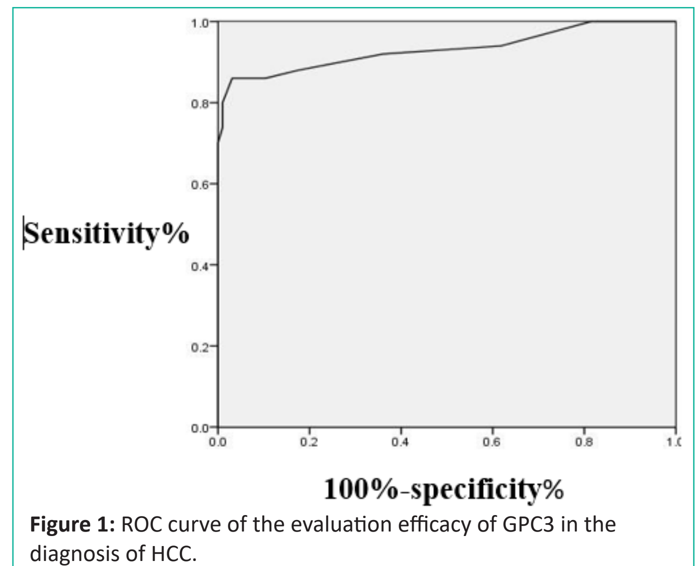

Figure 1: ROC curve of the evaluation efficacy of GPC3 in the diagnosis of HCC.

Preparation for R1 Reagent (Anti-GPC3-Latex Conjugate) and R2 Reagent

The GPC3-latex conjugate was prepared using water-soluble carbodiimide. In simple terms, 100 mg of carboxylated latex was suspended in 20 ml of MES buffer (50 mmol, containing 2% NaCl, pH 6.0), and 10 mg of EDC and 10 mg of NHS were added. The mixture was gently stirred at Room Temperature (RT) for 60 minutes. The activated latex suspension was centrifuged and the supernatant was discarded. The pellet was resuspended in 20 mL of 0.02 M PBS (phosphate-buffered saline: 0.01 μ $KH2PO4$, 0.01 μ $Na2HPO4$, pH 7.8) and incubated with 2.5 mL of GPC3 mAb (2 mg/mL) in 0.02 M PBS at RT with continuous stirring for 3 hours. The latex was then centrifuged and resuspended in 20 mL of 0.02 M PB.

Preparation of Standard Antigen

The calibration curve was calibrated using Glypican-3 antigen expressed in Escherichia coli.

Sample Collection and Preprocessing

All subjects fasted for at least 8 hours overnight. Peripheral fasting venous blood (3-5ml) was collected from the study group patients on the first day of admission. The control group subjects had fasting venous blood (3-5ml) collected during physical examination. The blood samples were centrifuged at 3000r/min (with an effective centrifugal radius of 10cm) for 15 minutes to separate the serum. The upper clear liquid was stored at -80℃for further testing.

Latex-Enhanced Turbidimetric Assay for Detecting Serum GPC3 Concentration

The GPC3 protein in the sample binds to the specific anti-GPC3 antibody (reagent R2) coated on latex particles. It triggers aggregation under the enhancement of reagent R1. The relative turbidity caused by aggregation is measured at 510nm, which is the absorbance. The absorbance value is positively correlated with the concentration of GPC3 in the sample. The experimental steps are as follows: 2 µL of serum sample is added to 150 µL of R1, mixed well, incubated at 37℃ for 5 minutes, the absorbance value (A1) is measured at 510nm. Then, 150 µL of R2 is added, incubated at 37℃ for 5 minutes, and the absorbance value (A2) is measured at 510nm. Calculate ΔA = A2-A1. ΔA is positively correlated with the concentration of GPC3 in the sample.

Methodological Validation

Limit of Detection: Use zero concentration calibration sample as the sample for detection. Repeat the measurement 20 times and calculate the mean and SD of the 20 test results. The mean

+ 2SD is the Limit of Detection.

Imprecision: Intra-assay precision: Test samples with high and low concentration levels, repeat the test 10 times, calculate the mean, standard deviation, and coefficient of variation of the measured values. Inter-assay precision: Test samples with high and low concentration levels, perform 5 tests in the morning and 5 tests in the afternoon each day, continuously test for 2 days and record the results. Calculate the mean, SD, and CV of the measured values.

Linearity: Mix high concentration samples (2930 pg/ml) close to the upper limit of the linear range and low concentration samples (0 pg/ml) close to the lower limit of the linear range to create 5 dilution concentrations. Test each dilution concentration 3 times and calculate the mean of the test results. Use the dilution concentration as the independent variable and the mean of the test results as the dependent variable to obtain the linear regression equation. Calculate the correlation coefficient (r) of the linear regression. Substitute each dilution concentration into the linear regression equation to calculate the estimated value and the percentage relative error.

Trueness: A certain volume of standard solution is added to the base sample (the volume

ratio of the standard solution to the base sample should not cause changes in the matrix; the total concentration of the sample should be within the linear range of the reagent kit after adding the standard solution). Each concentration is tested three times to calculate the mean and recovery rate. The recovery rate R = (C * (V0 + V) - C0 * V) / (V * CS) * 100%, where V represents the volume of the added standard solution, V0 represents the volume of the base sample, C represents the mean detection concentration of the base sample after adding the standard solution, C0 represents the mean detection concentration of the base sample, and CS represents the concentration of the standard solution. The preparation of recovery samples: The volume ratio of high concentration standard solution (1214pg/ml) to low concentration base sample is 1:9.

Assay Interferences: Detection of liver cancer-related serum biomarkers:

Alpha-Fetoprotein (AFP) (100ug/ml) and Human Serum Albumin (HSA) (100mg/ml). Each substance is tested three times to calculate the mean and evaluate the anti-interference ability of the detection method for AFP and HSA.

Detection of AFP in Serum

AFP is detected using electrochemiluminescence. The process is performed according to the instructions of the reagent kit. AFP > 7ng/mL is considered positive.

Detection of PIVKA-II in Serum

PIVKA-II is detected using chemiluminescent enzyme immunoassay. The process is performed according to the instructions of the reagent kit. PIVKA-II > 40 mAU/mL is considered positive.

Statistical Analysis

Data analysis is performed using SPSS Statistics 20.0. The experimental results are not normally distributed, and the data is represented as M (P25-P75). Non-parametric tests are used, with independent sample Kruskal-Wallis test for comparison between multiple groups and Steel-Dwass test for pairwise comparison. The Area Under the Curve (AUC) analysis is used to determine the diagnostic value of HCC, and sensitivity and specificity are calculated.

Results

Assay Characteristics

Limit of Blank (LoB): According to the measurement, the mean concentration of the zero-concentration calibration solution is 9pg/ml, with a standard deviation of 10pg/ml. So the LoB is 29pg/ml.

Imprecision: Intra-assay and inter-assay CVs ranged from 1% to 3% and 3 to 5%, respectively (Table 1)

Table 1: Observed intra-assay and inter-assay imprecision.

Concentration	Intra -assay			Inter-assay		
	x̄	SD	CV (%)	x̄	SD	CV (%)
Low	216	7.21	3	224	6.49	3
High	1187	12.45	1	1232	58.39	5

Table 2: Linearity validation.

Theoretical (pg/ml)	0	216	514	1214	2930
Measured (pg/ml)	6	222	526	1183	2895
Correlation coefficient	0.9999				
Slope	0.9835				
Intercept	7.7699				
Expected	8	220	513	1202	2889
Absolute/relative deviation	-1.6	2.1	3%	-2%	0%

Table 3: Recovery test results.

Measured concentration		Recovery Concentration
Concentration	236	323
(pg/ml)	222	327
	216	317
Mean	225	322
Recovery Rate	99%	

Table 4: Interference test.

Interferent	MeasuredGPC3d (pg/ml)	Interferent Concentration
Blank	9	-
AFP (100ug/mL)	7	-2
HSA (100mg/mL)	6	-3

Linearity: GPC3 shows linearity within the range of 29-2930 pg/ml, with a correlation coefficient (r) of 0.9999 (Table 2).

Trueness: Recovery test results showed a recovery of 99%, within the acceptable range of 100% ± 15% (Table 3).

Interference test: 100ug/ml AFP and100mg/ml HSA had no effect on GPC3 detection (Table 4).

Through the validation of performance indicators, this study found that the latex-enhanced immunoturbidimetric method for detecting GPC3 has an analytical sensitivity of 29pg/ml. The intra-assay and inter-assay imprecision are both <10%. The recovery rate is high and the linear range is wide, showing good linearity within the range of 29-2930 pg/ml. The concentrations of 100ug/ml AFP and 100mg/ml HSA had no effect on the results, which meets the requirements of "Guidelines for the Registration and Review of Analytical Performance Evaluation of Quantitative In Vitro Diagnostic Reagents" and "YY/T 1255-2015 Immunoassay Reagents (Kits) (Transmittance Method)". This method can meet the needs of routine clinical testing.

Evaluation of the Diagnostic Value of Serum GPC3 Concentration for HCC

Detection results of serum GPC3, AFP, and PIVKA-II in different populations: The average concentrations of serum GPC3, AFP, and PIVKA-II in each group are shown in Table 5. The concentrations of GPC3 and PIVKA-II in each group do not follow a normal distribution. According to the non-parametric rank sum test, the GPC3, AFP, and PIVKA-II levels in the HCC untreated group were significantly higher than those in the control group, chronic hepatitis group, and HCC treated group (P<0.05). In the HCC treated group, the GPC3, AFP, and PIVKA-II levels were significantly higher than those in the control group and chronic hepatitis group (P<0.05), but lower than those in the HCC untreated group (P<0.05).

Sensitivity and specificity of serum GPC3 in the diagnosis of HCC: In this study, the operating characteristic curve (ROC curve) was introduced to analyze the diagnostic value of GPC3 for HCC. According to the ROC curve results (Figure 1), the area under the curve in this study was 0.932 (95% CI: 0.879~0.984). Using a cutoff value of GPC3 concentration greater than 81pg/ml, the sensitivity and specificity of serum GPC3 in diagnosing HCC were 86.0% and 96.9%, respectively.

Table 5: Descriptive statistics for measured GPC3, AFP, PIVKA-II in patients grouped by clinical disease status [M(P25~P75)].

	N	GPC3 (pg/ml)	AFP (ng/mL)	PIVKA-II (mAU/mL)
HC	97	48(39~57)	2.30(1.74~3.18)	16.05(15.14~16.95)
CHB	72	48(39~74)	2.30(1.72~3.22)	16.59(15.42~17.76)
HCC	50	241(102~389) ab	54.01(7.31~1938.00) ab	331.50(69.75~8628.00) ab
Treated HCC	132	95(57~179) abc	30.43(4.25~243.70) abc	163.00(39.25~1037.50) abc
F value	-	15.308	0.402	3.306
P value	-	0	0.847	0.012

注：a：compared with Healthy ,P＜0.05；b：compared with HB, P＜0.05；c：compared with HCC, P＜0.05。

Table 6: Serum GPC3, AFP and PIVKA-II single or combined tests in each group [n(%)].

	N	GPC3	AFP	PIVKA-II	GPC3+PIVKA-II	GPC3+AFP	AFP+PIVKA-II	GPC3+AFP+PIVKA-II
HC	97	3(3.1)	0(0)	0(0)	3(3.1)	3(3.1)	0(0)	3(3.1)
CHB	72	12(16.7)	0(0)	0(0)	12(16.7)	12(16.7)	0(0)	12(16.7)
HCC	50	43(86.0)	38(76.0)	40(80.0)	48(96.0)	49(98.0)	47(94.0)	49(98.0)
Treated HCC	132	76(57.6)	90(68.2)	98(74.2)	118(89.4)	114(86.4)	116(87.9)	125(94.7)

Table 7: The positivity of erum GPC3 or PIVKA-II in HCC group.

		GPC3			PIVKA-II		
HCC	N	(+)	(-)	Positive %	(+)	(-)	Positive %
AFP (+)	38	32	6	84.20%	31	7	81.60%
AFP (-)	12	11	1	91.70%	9	3	75.00%
Total	50	43	7	86.00%	40	10	80.00%

Comparison of serum GPC3, PIVKA-II, and AFP positivity rates: The comparison of the positivity rates of serum GPC3, PIVKA-II, and AFP in the four groups of samples is shown in Table 6. Compared to the other groups, the HCC untreated group had the highest positivity rates for all three indicators. When comparing the three indicators, the GPC3 positivity rate was highest in the HCC untreated group (86.0%), followed by PIVKA-II (80.0%) and AFP (76%), and in the HCC treated group, it was 57.9%, lower than AFP (68.2%) and PIVKA-II (74.2%). The diagnostic value of combined detection of GPC3, AFP, and PIVKA-II for primary liver cancer is as follows: GPC3+AFP positivity rate is 98%; GPC3+AFP+PIVKA-II positivity rate is 98%; GPC3+PIVKA-II positivity rate is 96%. The positivity rates of combined detection are significantly higher than individual detection, and GPC3+AFP positivity rate is equivalent to GPC3+AFP+PIVKA-II positivity rate. In the HCC untreated group, out of 12 AFP-negative patients, the serum GPC3 positivity rate was 91.7%, higher than the PIVKA-II positivity rate (75%), as shown in Table 7.

Discussion

Currently, serum AFP is still one of the main methods for the auxiliary diagnosis of HCC. However, its sensitivity is low, and some HCC patients have low levels or negative AFP [11]. Therefore, AFP needs to be combined with other biomarkers for detection to increase sensitivity, reduce missed diagnosis rates, and improve the diagnostic performance of HCC [12]. Some recent studies have shown that Glypican-3 (GPC3) is currently recognized as an early biomarker for liver cancer diagnosis and a target for immunotherapy [7].

GPC3 is a member of the glypican family of phosphatidylinositol proteins, with a relative molecular mass of about 66 kD. Currently, the detection methods for GPC3 mainly include tissue-based GPC3 detection and blood-based GPC3 detection. Tissue-based GPC3 detection methods primarily include reverse transcription PCR [13] and immunohistochemical staining [14]. The detection methods for GPC3 in blood and the sensitivity and specificity for diagnosing liver cancer are as follows: enzyme-linked immunosorbent assay (40% and 93%) [15]; time-resolved fluorescence immunoassay (58.5% and 95.5%) [16]; reverse transcription-PCR (67.1% and 89%) [17]; flow cytometry [18]. All of the above detection methods require manual operation, which is cumbersome and time-consuming.

In order to improve the efficiency of GPC3 detection, this study established, for the first time in China, a latex-enhanced immunoturbidimetric method for detecting serum GPC3. In order to evaluate the effectiveness of this method for determining GPC3, this study analyzed the analytical sensitivity, repeatability test, recovery test, interference test, and linear range of the method. The analytical sensitivity of this method was 29 pg/ml, which can meet the clinical detection needs. The within-

batch and between-batch Coefficients of Variation (CV) were both <10%, which meet the requirements for the detection of clinical patient samples with an in vitro diagnostic reagent CV <15%. This method has strong anti-interference ability and is not affected by 100 ug/ml AFP and 100 mg/ml HSA. The linear range is 29-2930 pg/ml, within which the level of GPC3 in patients can be accurately reflected.

This study applied the GPC3 latex-enhanced immunoturbidimetric method to fully automated chemical analyzers, greatly improving the detection efficiency, reducing human errors, making the results more reliable, and also helping to promote the standardization of GPC3 detection.

To evaluate the application value of GPC3 in the clinical diagnosis of Hepatocellular Carcinoma (HCC), this study further analyzed the concentration of GPC3 in healthy control group, chronic hepatitis B group, untreated HCC group, and treated HCC group. It was found that the levels of GPC3 in HCC patients decreased by 28.1% before and after treatment ($P < 0.05$). With the progression of liver cancer, the expression level of GPC3 gradually increased, which may serve as a marker for prognosis, metastasis, and recurrence of liver cancer. Studies have found that high expression of serum GPC3 may increase the risk of in situ cancer recurrence after TACE in patients with primary liver cancer, and monitoring GPC3 levels is beneficial for clinical treatment planning and patient prognosis assessment [19]. Fu et al. also supported this view through their study. In HCC patients after surgical resection, the decrease or disappearance of GPC3 in their serum indicated a good prognosis, while high expression of GPC3 may be a potential marker for cancer recurrence or metastasis [20]. Further evaluation of the diagnostic efficacy of GPC3 in HCC through ROC curve analysis revealed a sensitivity of 86.0% and a specificity of 96.9% for serum GPC3 in diagnosing HCC. This was significantly higher than enzyme-linked immunosorbent assay, time-resolved fluorescence immunoassay, and reverse transcription-polymerase chain reaction. By analyzing the positive rates of GPC3, PIVKA-II, and AFP alone and in combination for detecting HCC, it was found that the combined detection rates were significantly higher than individual detection rates, and the positive rates were equivalent for GPC3+AFP and GPC3+AFP+PIVKA-II. This suggests that in clinical practice, only the combination of GPC3+AFP can be used to reduce the economic burden on patients to some extent. The study also found a high detection rate of GPC3 in serum AFP-negative HCC patients, especially in patients with serum AFP < 400 ng/mL, where the positive rate of GPC3 reached 48.8% [21]. Among the 12 serum AFP-negative HCC patients detected using latex-enhanced immune turbidimetry in this study, 11 had abnormally elevated serum GPC3, with a sensitivity of 91.7%, higher than the sensitivity of AFP detection alone (76%). Combined detection of serum GPC3 and AFP significantly improved the sensitivity (98%). This suggests that the combined detection of AFP and GPC3 can increase the positive detection rate of HCC in AFP-negative patients. Capurro and Filmus [22,23] compared the expression of GPC3 and AFP in the serum of patients with liver cancer and found no correlation between the two in the same patient's serum. Therefore, they believe that the combined detection of both can significantly improve the early diagnosis of liver cancer. Tangkijvanich et al. [24] found that the levels of GPC3 and AFP were not related, and they believe that serum GPC3 can be highly specific in identifying liver cancer. The combined analysis of serum GPC3 and AFP can significantly improve the differential diagnosis of hepatocellular carcinoma. In summary, the performance indicators of serum GPC3 detection using latex-enhanced immunoturbidimetry meet the requirements of the "Guiding Principles for the Evaluation and Registration Review of Analytical Performance of Quantitative Detection In Vitro Diagnostic Reagents", and its detection efficiency is improved when applied to fully automated chemical analyzers. Serum GPC3, PIVKA-II, and AFP detection alone or in combination have certain value in the diagnosis of liver cancer. The advantage of serum GPC3 detection is evident in AFP-negative HCC, and the combined analysis of serum GPC3 and AFP can significantly improve the diagnostic efficiency of HCC.

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