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Rapid kinetic rate assay of the serum α-L-fucosidase in patients with hepatocellular carcinoma by using a novel substrate

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Abstract

Background: Developing kinetic rate assay kit for α-L-fucosidase (AFU) by using a novel substrate 2-chloro-4-nitrophenyl-α-L-fucopyranoside (CNPF) and clinical implication in the diagnosis of hepatocellular carcinoma (HCC). *Methods*: We have evaluated the new kinetic rate assay kit using a novel substrate for serum AFU employing Hitachi 7170 automated analyzer. The reaction was carried out at 37 °C, monitoring the wavelength at 405 nm. Serum samples from 884 Chinese subjects including 518 healthy adults and 366 patients were determined using this kit. *Results*: The linearity was observed up to 300 U/L. The intra-assay precision ($n = 10$) was S.D. 0.47; CV 2.6 (AFU activity, 17.8 U/L), S.D. 0.31; CV 0.79% (AFU activity, 39.71 U/L) and S.D. 0.79; CV 0.77% (AFU activity, 102 U/L). The day-to-day assay precision ($n = 10$) was 3.96% (AFU activity: 41.1 U/L). There was no significant interference of various substances such as ascorbic acid (6 g/L), hemoglobin (220 mg/L) and bilirubin (200 mg/L). Serum AFU value for healthy adults was estimated at 22.8 ± 7.1 U/L. No significant difference was found between male and female groups ($p > 0.05$). The mean value of serum AFU activity in patients with HCC was significantly higher than in patients with cirrhosis, chronic hepatitis, other malignant neoplasm, other diseases and control subjects. No significant differences were found between controls and patients with cirrhosis, chronic hepatitis, patients, other malignant neoplasm and other diseases. The sensitivity and specificity was 81.5% and 85.4%, respectively. *Conclusions*: Serum α-L-fucosidase is a useful maker in the diagnosis of HCC. The assay had high sensitivity and specificity. The procedure determined is simple, rapid, convenient, and can be adapted to automated clinical analyzers for use in large-scale screening for early diagnosis of HCC.

Keywords: α-L-fucosidase; Kinetic rate assay; Hepatocellular carcinoma; 2-chloro-4-nitrophenyl-α-L-fucopyranoside; Chronic hepatitis; Cirrhosis

1. Introduction

Hepatocellular carcinoma (HCC) is one of the commonly encountered malignant neoplasm. The early detection is important in the management of this cancer. α-L-fucosidase (AFU, EC 3.2.1.51) is found widely present in a variety of tissues, cells and fluids [1,2]. Many experimental and clinical studies have demonstrated that serum AFU activity is a valuable test for diagnosis of HCC [3–5].

In most cases, AFU activity is determined by using *p*-nitrophenyl-α-L-fucoside (PNPF) as colorimetric

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substrate [6,7], which requires a long incubation time and sample and reagent blanks. The AFU activity is also determined with fluorescent 4-methyl-umbelliferyl-α-L-fucosidase (4-MUF) substrate [8,9], which requires a spectrofluorimeter.

In 1992, Kasai et al. [10] first reported the synthesis of 2-chloro-4-nitrophenyl-α-L-fucopyranoside (CNPF). Comparing PNPF, CNPF has a more rapid hydrolytic rate than 4-NPF in the presence of AFU; we attempted to use CNPF as a novel substrate for the rate assay of serum AFU activity, to overcome difficulties caused by previous methods. Therefore, we synthesized CNPF by a modifying procedure [10]. Using our previous study on the physicochemical properties of the novel substrate, we found the optimum condition of the AFU hydrolytic reaction. The hydrolytic reaction was monitored at 405 nm in the medium of 0.1 mol/l citrate-disodium phosphate buffer, optimum pH 4.8. The hydrolytic product is 2-chloro-4-nitrophenol, its change rate in absorbency at 405 nm depends on AFU activity. In this work, we describe a kinetic rate assay method for the determination of serum AFU activity, and design a new reagent kit for clinical application.

2. Materials and methods

2.1. Chemicals

A novel substrate 2-chloro-4-nitrophenyl-α-L-fucopyranoside (CNPF) was synthesized by improving the procedure [10]. Briefly, the preparation of *O*-1,2,3,4-tetra~acetyl-a-L-fucose was from L-fucose. Secondly, the condensation of 2-chioro-4-nitrophenol was with l,2,3,4-tetra-*O*-acetyl-a-L-fucose by the catalysis of $ZnCl_2$ to produce 2-chloro-4-nitrophenyl-2,3,4-tri-*O*-acetyl-a-L-fucoside. Finally, the deacetylation of 2-chloro-4-nitrophenyl-*O*-2,3,4-tri-acetyl-α-L-fucoside was to 2-chloro-4-nitrophenyl-a-L-fucoside (CNPF). 4-CNPF is the colorless needle crystallization, $C_{12}H_{14}ClO_7N$, MW: 319.5, MP: 71–73 °C.

The AFU reagent was prepared by our laboratory, consisting of 0.1 mol/l of citrate-phosphate buffer (pH 4.8) containing 30 mmol/L of NaCl, 8 mmol/L of $MgCl_2$, 5 mmol/L of 2-chloro-4-nitrophenyl-α-L-fucopyranoside, 1 mmol/L of dithiothreitol (DTT), 0.02% of NaN_3; dithiothreitol (DTT) was from Promega (Madison, WI). Ascorbic acid, bilirubin, hemoglobin, NaN_3, α-L-fucosidase from human placenta, 4-nitrophenyl-α-L-fucopyranoside (PNPF), and other all chemicals were from Sigma (St. Louis, MO).

2.2. Serum sample collection

Serum samples ($n = 518$) from healthy adults were obtained from different hospitals, including 267 males (51.5%) and 251 females (48.5%), ages 19–90 years with a mean age of 45.1 years during 2001. The healthy adult had normal liver function test results, including values for aspartate aminotransferase <40 U/L (normal 8–40 U/L), alanine aminotransferase <40 U/L (normal 5–40 U/L), alkaline phosphatase <150 U/L (normal 40–150 U/L), γ-glutamytransferase: men <50 U/L (normal 11–50 U/L), women <32 U/L (normal 7–32 U/L), bilirubin <20.5 μmol/l (normal 3.4–20.5 umol/l) and albumin <55 g/L (normal 35–55 g/L). We used only noninfectious (negative for hepatitis B, hepatitis C, and HIV virus) samples and measured them on Hitachi 7170 analyzer.

Serum samples of patient blood donors ($n = 366$) were obtained from different hospitals. Six groups of subjects were studied. The first group consisted of 148 patients with HCC. All the HCC samples are from HCC patients without any pretreatment of outpatient or inpatients. The diagnosis of HCC was based on ultrasonographic examination, percutaneous fine-needle aspiration biopsy and characteristic echographic appearances. All data were obtained from hospital. Some of them had a high α-fetoprotein (AFP) level (>400 ng/ml). The second included 46 inpatients with cirrhosis. The diagnosis of cirrhosis was established by ultrasonographic examination, histologic findings and biochemical liver test results. Documents from the hospitals indicated in all cirrhotic patients showed that there was no clinical or echographic evidence of HCC. The third included 70 patients with chronic hepatitis. Documents from hospital showed their aspartate aminotransferase was >40 U/L, alanine aminotransferase >40 U/L and HbeAg(+), HbsAb(+), HbcAb(+). The fourth consisted of 73 patients with a variety of other malignant neoplasm, including 27 patients with gastric carcinoma, 30 patients with lung cancer, 1 patient with gallbladder cancer, 2 patients with colon cancer, 2 patients with cystitis cancer, 1 patient with pancreato-

duo cancer, 8 patients with esophageal cancer and 2 patients with bile duct. The fifth included 29 patients with other diseases, including 10 patients with abdominal pain, 8 patients with cholecystitis, 2 patients with diarrhea, 4 patients with alimentary bleed, and 5 patients with running fever. All patients also had ultrasonographic examination. Five hundred eighteen healthy adult serum samples, as controls constituted the sixth group. Table 1 summarizes the main characteristics of the subjects in the six groups.

Serum from all fasting subjects were stored at − 20 °C and assayed within 30 days after collection because AFU activity levels are known to decrease after this period.

2.3. Assay of serum AFU activity

Serum AFU was determined by the developing rate assay kit. The reaction was as follows:

$$\text{CNPF (colorless)} \xrightarrow{\text{AFU}} \text{CNP(yellow)} + L - \text{fucoside}$$

The assay was carried out on the Hitachi 7170 automated biochemical analyzer. Twenty-five microliters of serum sample was added to 250 μl of reagent solution, and then the absorbency change rate at 405 nm at 37 °C was recorded. Assay point: rate A/3-10 point. One unit will hydrolyze 1.0 μmol of CNPF to 2-chloro-nitrophenol (CNP) and L-fucoside at pH 4.8 and 37 °C. At present, there is no control serum for AFU activity determination. In our work, human placenta α-L-fucosidase (Sigma) was used as calibrator at about 500 U/L. It was diluted prior to use.

$$\text{AFU(U/L)} = \frac{\Delta A/\text{min}\,\text{Vt}}{\varepsilon \text{Vs} d} \times 1000$$

Table 1
Characteristics of subjects

Subjects	Case	Male/female	Age range	Avery (mean ± S.D.)
HCC	148	104/44	24–85	50.1 ± 14.0
Cirrhosis	46	21/10	37–74	56.5 ± 12.1
Chronic hepatitis	70	40/30	28–56	42.2 ± 9.8
Other malignant neoplasm	73	41/32	49–78	66.6 ± 8.8
Other sickness	29	16/13	18–45	33.5 ± 12
Healthy adult	518	267/251	19–90	45.1 ± 16.3

HCC: hepatocellular carcinoma; S.D.: standard deviation.

where, Vt (ml), total volume; Vs (ml), sample volume; $\varepsilon = 4.930 \times 10^3$ l/mol cm; d: light path = 1 cm.

Serum AFU activity was determined by the endpoint method. Serum AFU activity (normal values < 400 nmol/ml/h) was assay by modification of the method of Troost et al. [7], 0.25 ml of serum was added to 0.75 ml of 0.2 mol/l HAC buffer (pH 5.2) containing 4 mmol/L *p*-nitrophenyl-α-L-fucoside (PNPF). The mixture was incubated at 37 °C for 60 min and the reaction was stopped by the addition of 2 ml of 0.2 mol/l glycine buffer. Blanks were prepared in the same way. The absorbency of *p*-nitrophenol (PNP) was read at 405 nm.

AFP was measured by ELISA assay method using AFP regents kits (Kanghua Biotech, Led, China). The cutoff values was 400 ng/ml.

2.4. Statistical analysis

All data were expressed as mean value ± standard deviation. To delineate the distinction between normal subjects and patients with HCC, AFU cutoff value was defined as the mean value of controls + 2S.D. The Student's *t*-test was used to compare data. A $P < 0.05$ was considered significant.

3. Results

3.1. The kinetic rate assay method with CNPF as a substrate

For the determination of linearity, high AFU activity serum samples were obtained from patients with HCC. Fig. 1 shows that after serial dilution with 0.9% of NaCl the linearity was observed up to 300 U/L.

For the determination of precision, serum samples were obtained from samples with low, medium, and high AFU activity. Aliquots were stored at − 20 °C. Results showed the intra-assay precision ($n = 10$) was 2.6% (AFU activity: 17.8 U/L); CV 0.79% (AFU activity: 39.71 U/L) and 0.77% (AFU activity: 102 U/L) as determined by measuring the absorbance of the solution. The day-to-day assay precision ($n = 10$) was 3.96% (AFU activity: 41.1 U/L).

Serum AFU activities in 40 samples from different healthy individuals and patients were determined by the developing rate assay and endpoint method. As

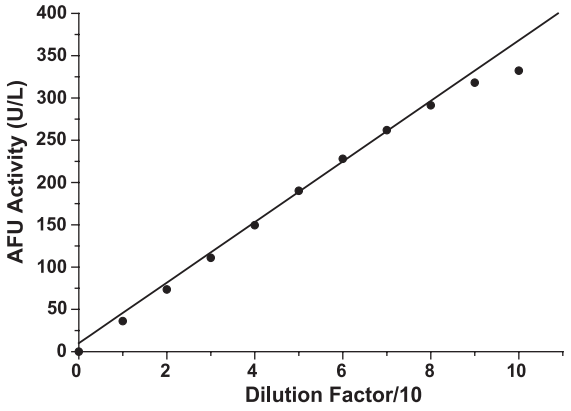

Fig. 1. Linearity of serum alpha-L-fucosidase using rate assay method.

shown in Fig. 2, we found correlation between this method and endpoint method, γ: 0.9603, $Y = 3.8325X - 3.8924$, $N = 40$, Y was the AFU activity determined by the rate assay with substance CNPF method on Hitachi 7170, X was the AFU activity determined by endpoint method with substrate PNPF on Hitachi U3000. As shown in Fig. 2, AFU value obtained with the CNPF substrate (rate assay) was higher than those obtained with the PNPF substrate (endpoint method). Depending on above analysis, we

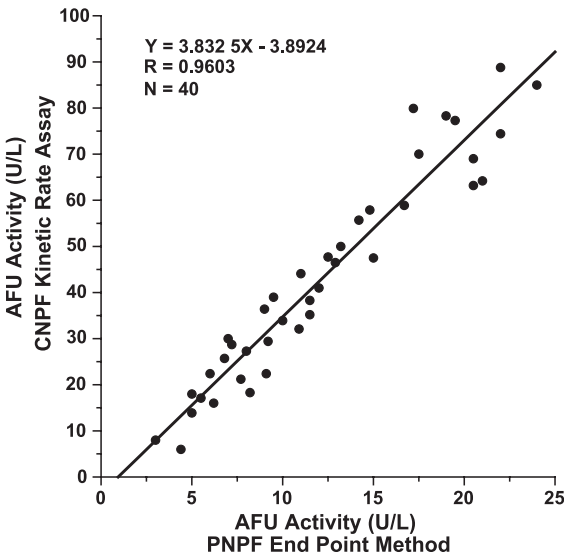

Fig. 2. Comparison of the rate assay method and the endpoint method. CNPF, 2-chloro-4-nitrophenyl-alpha-L-fucopyranoside; PNPF, *p*-nitrophenyl-alpha-L-fucopyranoside.

Table 2
Serum AFU activity in different sex

Sex	Case	AFU activity (U/L)
Male	267	23.1 ± 7.2
Female	251	22.5 ± 6.9
Sum	518	22.8 ± 7.1

AFU: alpha-L-fucosidase. Values are expressed as mean ± standard deviation. *P* value not significant versus male and female groups.

also determined 10 other patients with HCC, 10 patients with liver neoplasm and 6 patients with transfer liver carcinoma. Their positive rate is 70%, 60% and 0%, respectively for the rate assay (cutoff 37 U/L), but 50%, 50% and 0%, respectively for endpoint method (cutoff 10.5 U/L), indicating that the positive rate by kinetic assay method was higher than that by endpoint assay method.

In order to further study the accuracy of the developed rate assay method for AFU activity, we determined the recovery rate. Dilutions (0–10 times) of human placental AFU were added to serum samples to determine AFU activity. The recovery rate of AFU activity in 10 samples ranged from 97.2 % to 105.8% (mean 101%). We found no interference with bilirubin up to 200 mg/L ascorbic acid (6 g/L), or with hemoglobin (220 mg/L).

3.2. The clinical assay of serum AFU activity for healthy adults

Employing the developed rate assay method with new subtract, we evaluated the performance of 518 healthy adults. No significant difference was found between male and female groups and age (p>0.05;

Table 3
Serum AFU activity in different subject patients

Subjects	No.	Enzyme activity		positive rate	
		Range	Mean ± S.D.	>37 U/L*	%
HCC	148	10–197	59.7 ± 39	125	81.8
Cirrhosis	46	19–114	25.4 ± 8.9	12	26.1
Chronic hepatitis	70	6–45	26.2 ± 9.8	5	7.1
Other Neoplasm	73	4–52	27.6 ± 9.5	5	6.8
Other sickness	29	5–23	16.5 ± 5.8	0	0
Healthy adult	518	3–35	22.8 ± 7.1	0	0

AFU: alpha-L-fucosidase. The cutoff values for AFU is 37 U/L (Beijing area). HCC: hepatocellular carcinoma; S.D.: standard deviation. In detail, see Materials and methods.

Table 2). The serum AFU value for healthy adults was estimated at 22.8 ± 7.1 U/L (Table 3).

3.3. The clinical assay of serum AFU activity for patients

We evaluated the performance of 366 patients' serum samples. The distribution of results in HCC patients and other patients is showed in Fig. 3. The mean value of serum AFU activity in patients with HCC was significantly higher (59.7 ± 39 U/L) than patients with cirrhosis (25.4 ± 8.9 U/L, $P < 0.01$), chronic hepatitis (26.2 ± 9.8 U/L, $p < 0.01$), other malignant neoplasm (27.6 ± 9.5 U/L, $p < 0.01$), other diseases (16.5 ± 5.8 U/L, $P < 0.01$) and controls (22.8 ± 7.1 U/L, $P < 0.0$l). No significant differences were found between controls and patients with cirrhosis, chronic hepatitis, other malignant neoplasm, or other diseases (Table 3). From Table 3, the sensitivity and specificity for developed rate assay are 81.8% and 85.4%, respectively.

Table 4 shows the values of serum AFP (α-fetoprotein) and AFU in patients with HCC. The AFP cutoff values of 400 ng/ml are considered the most effective in Chinese population. Of the 58 patients with HCC, 39 had levels >400 ng/ml. The AFU cutoff values are considered as 37 U/L in the population of Beijing area. Of the 58 patients with HCC, 48 had levels greater than 37 U/L. The comparison between AFU and AFP in patients with HCC showed positive results for both tests in 31 of 58 patients and negative results for 2 of 58 patients of the remaining patients with HCC, 17 had positive

Table 4
Comparison of AFU and AFP in patients with HCC

	AFU (U/L)	AFP (ng/ml)		Total sensitivity	Specificity
	<400	>400	Case	(%)	(%)
AFU	<37	2	8	10	
	>37	17	31	48	
Total case		19	39	58	
AFU>37			48	82.5	88.2
AFP>400			39	67.2	75.0
AFU + AFP			56	96.5	

HCC: hepatocellular carcinoma; AFU: alpha-L-fucosidase; AFP: alpha-fetoprotein. The cutoff values for AFU is 37 U/L (Beijing area) and 400 ng/ml for AFP. AFU + AFP represent the positive results that both AFU and AFP activity were examined simultaneously.

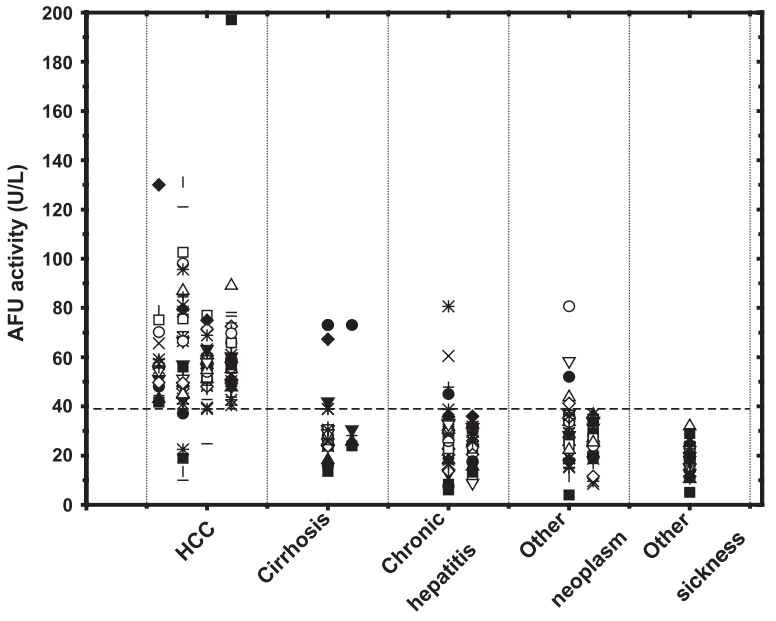

Fig. 3. Serum alpha-L-fucosidase activity in patients with hepatocellular carcinoma (HCC), cirrhosis, chronic hepatitis, other malignant neoplasm and other diseases. The horizontal line represents the cutoff value.

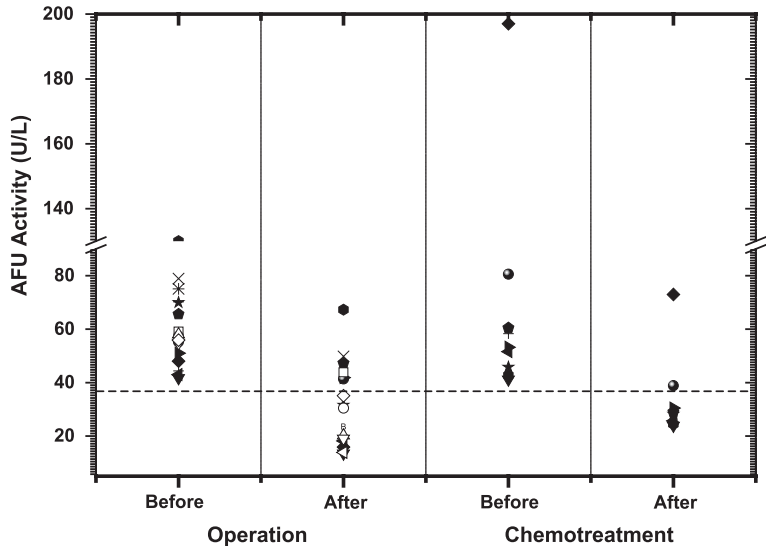

Fig. 4. Serum alpha-L-fucosidase activity in patients with hepatocellular carcinoma (HCC) before and after treatment. Serum alpha-L-fucosidase activity was determined after 2 weeks following the treatment. The horizontal line represents the cutoff value.

results for AFU and negative results for AFP, and 8 had negative results for AFU and positive results for AFP. From Table 4, in the negative case for AFP, sensitivity and specificity of AFU was calculated as sensitivity of 82.5% and specificity of 88.2%; but AFP had a sensitivity of 67.3% and specificity of 75.0%. The sensitivity and specificity of AFU in the patients with HCC was higher than that of AFP (Table 4). If both AFU and AFP activity were examined simultaneously, the diagnostic positive rate of the patients with HCC rises to 96.5%.

Fig. 4 shows the values of serum AFU in patients with HCC after 2 weeks following different treatments. Compared with before treatment, the AFU activity in all patients had a decrease after chemotreatment or operation. In about 1 week to 1 month, the AFU activity decreased to normal level. Hence, positive case and positive rate also decreased. It suggests that AFU is a useful marker of HCC for diagnosis and post-treatment monitoring.

4. Discussion

In this work, we developed a kinetic rate assay kit of the serum AFU activity by a novel substrate CNPF and applied this kit for marker of the diagnosis of HCC. Using this reagent kit, we evaluated the performance in Chinese healthy adults and inpatients. The results were comparable to those reported by other authors [9,14]. The positivity rate by our kinetic assay was higher than that by endpoint assay. The kinetic assay method did not require any blank determination and was adapted to a variety of automated chemical analyzers. This method, therefore, can be used in large-scale screening for AFU activity.

In this work, six groups of subjects were studied. In the patients with HCC, serum AFU activity levels were significantly higher than in the other five subjects tested (patients with cirrhosis, chronic hepatitis, malignant neoplasm other than HCC, other diseases and healthy subjects). Our results confirm previous reports of an increased serum activity of AFU in patients with HCC; AFU is a useful marker of HCC for diagnosis [9,11–14]. It was observed that the AFU activity in HCC patients had a significant decrease after chemo-treatment or operation, in about a week to 1 month; the AFU activity decreased close to the normal level. Fig. 4 shows that serum AFU activity appeared to be correlated with curative effect, suggesting that this might has an important value in post-treatment monitoring of HCC patients.

All the HCC samples are from HCC patients, outpatient or inpatient. They received any pretreatment, and most were in late stages. We noted that some of the patients with especially high AFU activity were in late stages, and the tumor size also was bigger. However, the high AFU activity seems to be not correlated with stage or tumor size and were unable to test more patients. Some reports demonstrated that the high AFU activity was not correlated with stage or tumor size [15].

In Table 4, we compared the values of serum AFP and AFU in 58 patients with HCC. These samples were from 148 HCC patients abovementioned. AFP only was performed on some of the subjects in Table 3. The comparison between AFU and AFP in 58 patients with HCC showed AFU sensitivity and specificity for the patients with HCC which is significantly higher than that of AFP. If both AFU and AFP were examined simultaneously, the diagnostic positive rate of the patients with HCC will rise to 96.5%. Until now, AFP has been considered the best marker in detecting early HCC. However, not all HCC secrete AFP, and AFP levels may be normal in as many as 40% of patients with early HCC [16,17]. Our results indicate that serum AFU activity is a useful marker for early detection of HCC patients, particularly in those with AFP-low or negative HCC patients.

In summary, the assay of serum AFU activity is a useful marker for the early detection of HCC patients, especially in those with early HCC AFP-negative patients. The new kinetic rate assay method by a novel substrate CNPF overcomes the defects of previous methods for determining AFU activity resulting in improved AFU sensitivity and specificity. This method is simple, rapid, convenient, and can be adapted to a variety of automated chemistry analyzers, and can be used for large-scale screening of populations at risk for HCC.

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