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Elucidation of the Usefulness of Glypican-3, a Hepatocellular Carcinoma Biomarker, with the Fully Automated LUMIPULSE® System

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***Background*: Glypican-3 (GPC3) is expressed in most of hepatocellular carcinoma (HCC) and GPC3 immunohistochemical staining is widely used in the clinical setting, but it has not been recommended as a blood biomarker, mainly due to its heterogeneous nature and the lack of established assay system. Here, we developed and evaluated the basic performance of fully automated GPC3 immunoassay kits which detect the full-length or the N-terminal fragments. We analyzed the molecular forms of GPC3 in HCC serum and evaluated the diagnostic performance of GPC3 and other biomarkers. *Methods*: We examined the analytical performance of the GPC3 kits. Then, the automated GPC3 assays were compared with an established ELISA kit. Afterwards, we determined the clinical cutoff of GPC3 and compared its diagnostic performance to alpha-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist-II (PIVKA-II) using 180 serum samples from clinically diagnosed patients. *Results*: GPC3 assays showed good analytical performance. The level of GPC3 in HCC was higher than recorded in healthy or other liver diseases' sera. The AUC of GPC3 was 0.90, whereas the AUCs of AFP and PIVKA-II were 0.89 and 0.76, respectively. *Conclusion*: Automated GPC3 assays with stable performance against GPC3 in screening HCC have been established and the diagnostic accuracy of GPC3 was as good as AFP.**

Key words glypican-3, tumor marker, HCC, CLEIA

INTRODUCTION

Glypican-3 (GPC3) belongs to the glypican family of glycosylphosphatidylinositol (GPI)-anchored heparan sulfate proteoglycans, which plays an important role in cellular growth, cell differentiation, and cell migration.[1–3] There is growing evidence that GPC3 is highly expressed in hepatocellular carcinoma (HCC) but not in cholangiocarcinoma or normal liver tissue, suggesting that GPC3 is a new tumor marker for HCC.[2–5] Indeed, GPC3 is a well-established immunohistological marker.[4,6–8]

GPC3 is a disulfide-linked 70-kDa protein, composed of a 40-kDa N-terminal subunit and a 30-kDa C-terminal subunit.[9] GPC3 is released from the cell membrane by Notum-mediated GPI-anchor cleavage (hereafter this released form is referred to as full-length GPC3 (FL-GPC3)).[10] The N-terminal subunits also are released as soluble GPC3 (N-GPC3), although the underlying molecular mechanism remains unknown.[9,11,12]

Several studies[4,13–16] using assay formats including Enzyme-linked Immunosorbent Assay (ELISA), competitive radioimmunoassay and chemiluminescence enzyme immunoassay, showed that serum GPC3 was a promising blood biomarker for HCC. However, the diagnostic accuracy and value of serum GPC3 remains controversial because contrary results have been reported.[17] Furthermore, a meta-analysis showed that as HCC biomarker, the performance of serum GPC3 measured with ELISA kits was inferior to that of alpha-fetoprotein (AFP).[18] The difference in sensitivity between serological and histological assay platforms could be one of the reasons why the performance of serum GPC3 hasn't been as good as that of GPC3 immunohistochemical staining. Moreover, the variation of target molecules in each assay format could also have affected the performance. Therefore, we hypothesized that the heterogeneous nature of GPC3 could have caused the discrepancy between the studies reporting the clinical utility of serum GPC3.

To evaluate the performance of serum GPC3 as a HCC marker, we developed chemiluminescent enzyme immunoassay (CLEIA) kits for two molecular forms of serum GPC3, FL-GPC3 and N-GPC3, on the fully automated LUMIPULSE platform. For the FL-GPC3 assay, a combination of antibodies that recognizes the N-terminal subunit and the C-terminal subunit was used. Meanwhile, two antibodies that both reacts with the N-terminal subunit were used for the N-GPC3 assay. We compared the basic performance of these assays and analyzed GPC3 molecular forms in HCC serum. We also evaluated the clinical performance of serum GPC3 along with other HCC biomarkers, namely AFP and protein induced by vitamin K absence or antagonist-II (PIVKA-II).

MATERIALS AND METHODS

Description of the Fully Automated Immunoassay for GPC3 The GPC3 assays were developed on a fully auto-

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mated chemiluminescent enzyme immunoassay system, LUMIPULSE L2400 (FUJIREBIO INC., Tokyo, Japan). Two assays were developed; one detects the N-terminal of GPC3 (N-GPC3), and the other detects the full-length form of GPC3 (FL-GPC3). Briefly, magnetic beads were coated with a monoclonal anti-GPC3 mouse antibody which recognizes the N-terminal. The GPC3 molecules present in the specimens were captured by the magnetic beads, generating stable immune complexes. The magnetic beads were then washed to eliminate unbound material and incubated with another monoclonal anti-GPC3 mouse antibody conjugated with alkaline phosphatase (ALP). After a second wash, the 3-(2'-spiroadamantyl)-4-methoxy-4-(3"-phosphoryloxy)-phenyl-1,2-dioxetane (AMPPD) substrate was added to the reaction mixture and the resulting luminescence was measured at 477 nm. The intensity of the luminescent reaction is directly proportional to the concentration of GPC3 in the test sample. The sample volume needed for the assay is 20 µL (plus 100 µL of dead volume on the analyzer) and results are available in 25 min (Supplementary Fig. 1). Both assays used the full-length recombinant human glypican 3 protein (R&D systems, Minneapolis, MN, USA) as a reference standard. The concentration of GPC3 was calculated by a three-parameter equation with a quantification range of 0–10,000 pg/mL.

Evaluation of Analytical Performance of the GPC3 Assays

Limit of Detection and Limit of Quantitation The limit of blank (LOB), limit of detection (LOD) and limit of quantitation (LOQ) of the GPC3 assays were evaluated as follows. The LOB was calculated as the value corresponding to the sum of the mean and 1.645*standard deviation (SD) of 20 consecutive replicates of saline. The LOD was calculated as the sum of the LOB and 1.645*SD of 20 replicates of an inpatient serum pool with the lowest measurable GPC3 value. The LOQ was defined as the lowest GPC3 concentration that could be determined with a coefficient of variation (CV) ≤ 10%. This value was calculated by preparing serial dilutions in sample buffer (i.e., 1:2; 1:4; 1:8; 1:16 and 1:32) of an inpatient serum sample with a low GPC3 concentration. The CV for each dilution was calculated after measurement of 20 replicates. A model fit was then developed to extrapolate the GPC3 value with 10% imprecision.

Imprecision Studies The imprecision studies were carried out using three serum pools with low, intermediate, and high concentrations of GPC3. Each serum pool was obtained by pooling 10 anonymized serum samples. The pools were then thoroughly mixed and divided into 21 identical aliquots of 2 mL, which were stored below −70°C. The intra-assay imprecision was evaluated by performing 6 sequential measurements of one of the stored aliquots of each pool, whereas the inter-assay imprecision was assessed by measuring the remaining stored aliquots over 7 consecutive working days (i.e., one aliquot of each pool per day). The results were reported as CV.

Dilution Linearity Dilution linearity (recovery) was assessed by serial dilution of three patient samples (> 150 pg/mL). Dilutions were carried out with LUMIPULSE Specimen Diluent 1 (FUJIREBIO INC.). The results were then plotted and the expected vs. observed values were analyzed by linear regression.

Comparison of GPC3 Measurements Across Different Assay Formats

Size Exclusion Chromatography Specimens from HCC patients were mixed with PBS and then subjected to size exclusion chromatography (SEC) performed on a Superdex200 increase 10/300 column (Cytiva, Tokyo, Japan). Each sample was fractionated at a flow rate 0.5 mL/min in buffer containing 100 mM phosphate buffer, 150 mM NaCl, 5 mM EDTA, and 0.1% NaN_3 (pH 6.0). The total amount of GPC3 in each fraction was then measured with the developed GPC3 immunoassays. The molecular mass of each fraction was calibrated with Conalbumin (75 kDa) and Ovalbumin (44 kDa).

Comparison of GPC3 Assays The developed automated GPC3 assays and an ELISA immunoassay, CanAg Glypican-3 EIA (Fujirebio Diagnostics AB, Goteborg, Sweden), were simultaneously used to analyze 50 commercially available serum samples (Precision for Medicine, Maryland, MD, USA). The correlations between measurement methods were assessed with ordinary least-square regression and Pearson's correlation coefficient (r).

Comparison of GPC3 Assay with HCC Biomarkers Along with GPC3, AFP and PIVKA-II were sequentially measured in 180 commercially available serum samples including 70 HCC, 50 nonalcoholic steatohepatitis (NASH) cases and 60 normal controls (NC) (Precision for Medicine). AFP and PIVKA-II were measured with LUMIPULSE Presto AFP and LUMIPULSE Presto PIVKA-II (FUJIREBIO INC.), respectively, with cutoff points set at 10 ng/mL and 40 mAU/mL, respectively. Study procedures were compliant with the principles of the Declaration of Helsinki.

Statistics All statistical analyses were performed with R version 4.05 (R Foundation for Statistical Computing, Vienna, Austria, URL http://www.R-project.org/.). Continuous variables were summarized into median, first quartile (Q1) and third quartile (Q3). Group comparisons for continuous variables were performed using the Wilcoxon rank-sum test with Bonferroni correction. Pearson correlation coefficients (r) or Spearman correlation coefficients (ρ) were calculated. The significance level of the statistical analyses was set at $p < 0.05$. A receiver operating characteristics (ROC) curve analysis was used to assess the diagnostic performance of GPC3, AFP and PIVKA-II. Cutoffs were derived using the Youden Index. AUC differences were assessed with DeLong's test.

RESULTS

Evaluation of Analytical Performance of the GPC3 Assays We developed GPC3 assays which target FL-GPC3 and N-GPC3 and evaluated the basic performance of these assay kits. LOD of FL-GPC3 and N-GPC3 assays were 2.2 pg/mL and 1.3 pg/mL, respectively (Table 1). LOQ of FL-GPC3 and N-GPC3 assays were 2.2 pg/mL and 3.3 pg/mL, respectively (Table 1). The within-run CVs were 1.2~2.8% for the FL-GPC3 assay, and 2.0~2.3% for the N-GPC3 assay (Supplementary Table 1). The between-day CVs were 4.9~7.3% for the FL-GPC3 assay, and 4.9~8.1% for the N-GPC3 assay (Supplementary Table 2). The imprecisions of the kits were less than 10% CV, and both GPC3 assays showed favorable results. The dilution linearity was determined using serial dilutions (from 1:2 to 1:32) of 3 patient samples. The equations obtained by linear regression showed a correlation coefficient (r) of 1.000 (Supplementary Table 3).

Comparison of GPC3 Measurements Across Different Assay Formats The schema in Fig. 1A shows the structure of GPC3 protein. The SEC analysis identified two GPC3 molecular forms in the HCC patient specimens. GPC3 immunoreactivities to both immunoassays were observed in the fractions apparently corresponding to the FL-GPC3 (70 kDa) on SEC.

Table 1. Features of Each of the Measurement System

	FL-GPC3 (CLEIA)	N-GPC3 (CLEIA)
Capture mAb	N-terminal	N-terminal
Detector mAb	C-terminal	N-terminal
Measurement time	25 min	25 min
Measurement range	2.2 pg/mL–10,000.0 pg/mL	3.3 pg/mL–10,000.0 pg/mL
Sample volume	20 μL	20 μL
LoD	2.2 pg/mL	1.3 pg/mL
LoQ	2.2 pg/mL	3.3 pg/mL

However, only the N-GPC3 assay showed reactions toward the N-terminal GPC3 (40 kDa) fractions on SEC (Fig. 1B and Supplementary Fig. 2).

We then compared the results of GPC3 measurement using automated GPC3 CLEIAs with those obtained with CanAg Glypican-3 EIA. The scatterplot shows good correlations between these kits (Fig. 1C). It is noteworthy that the value at the lower limit of the CLEIA's measurement range is much smaller than that of CanAg Glypican-3 EIA. The correlation coefficient (r) between CanAg Glypican-3 EIA and FL-GPC3 results was 0.84 ($p < 0.001$), while the CanAg Glypican-3 EIA and N-GPC3 correlation value was 1.00 ($p < 0.001$) (Fig. 1C), indicating that the N-GPC3 measurements were equivalent to CanAg kit. However, the FL-GPC3 levels in the specimens were lower than that of N-GPC3. These findings indicate that the N-GPC3 assay could measure both the full-length forms and the N-terminal fragments in the serum samples, suggesting it could be more robust than the FL-GPC3 assay. Therefore, the N-GPC3 assay was used for the subsequent comparison of GPC3 assay with other HCC biomarkers.

Comparison of GPC3 Assay with HCC Biomarkers We evaluated the clinical performance of the N-GPC3 assay, AFP and PIVKA-II using commercially available samples. Age and gender distributions of the subjects from which these samples were obtained are shown in Table 2. The levels of biomarkers in the analyzed samples ranged from 1.0 to 22,347.0 ng/mL for AFP, 16 to 10,463.1 pg/mL for N-GPC3, and 11.2 to 30,809.7 mAU/mL for PIVKA-II (Fig. 2). The median [Q1, Q3] AFP values of NC, NASH, and HCC subjects were 3.0 [2.2, 4.9], 3.7 [3.0, 6.5], and 119.1 [9.9, 383.5], respectively. The N-GPC3 median [Q1, Q3] values of NC, NASH, and HCC subjects were 50.6 [33.2, 68.2], 61.4 [43.6, 83.5], and 140.5 [94.4, 327.3], respectively. Meanwhile, for PIVKA-II, the median [Q1, Q3] values of NC, NASH, and HCC subjects were 22.6 [17.5, 28.8], 44.8 [27.9, 117.9], and 56.7 [32.5, 413.0], respectively.

Compared to NC, HCC subjects showed statistically significantly higher concentrations for all biomarkers (Fig. 2). There were significant differences between NASH and NC subjects in the AFP and PIVKA-II assays, but not in N-GPC3 assay, suggesting that the N-GPC3 assay could be more suitable for HCC screening (Fig. 2). For each biomarker, the number of NC, NASH and HCC subjects showing value elevated enough to be considered as outliners was also investigated. Among the specimen from NASH patients, 20%, 6% and 0% showed outliner values for PIVKA-II, N-GPC3 and AFP, respectively (Supplementary Table 4). Moreover, the analysis of the gender- and age-based distributions of the GPC3 values of NC and NASH subjects showed no significant differences between groups (Supplementary Fig. 3).

The ROC curves for differentiating HCC from NASH and NC, the cutoff, the associated sensitivity, specificity, overall percent agreement (OPA), and area under the curve (AUC) of all biomarkers are shown in Fig. 3. The AUCs for distinguishing between HCC and non-HCC were 0.89 (95% confidence interval [CI] 0.84–0.95), 0.90 (95% CI 0.86–0.95), and 0.76 (95% CI 0.69–0.83) for AFP, N-GPC3, and PIVKA-II, respectively (Fig. 3). In this sample set, AFP and N-GPC3 showed significantly higher accuracy than PIVKA-II ($p < 0.001$), but there was no significant difference between the AUCs of AFP and N-GPC3.

The correlation plots showed weak correlations between N-GPC3 and AFP, N-GPC3 and PIVKA-II, AFP and PIVKA-II (Fig. 4A–C). Moreover, the Venn's diagrams illustrating the

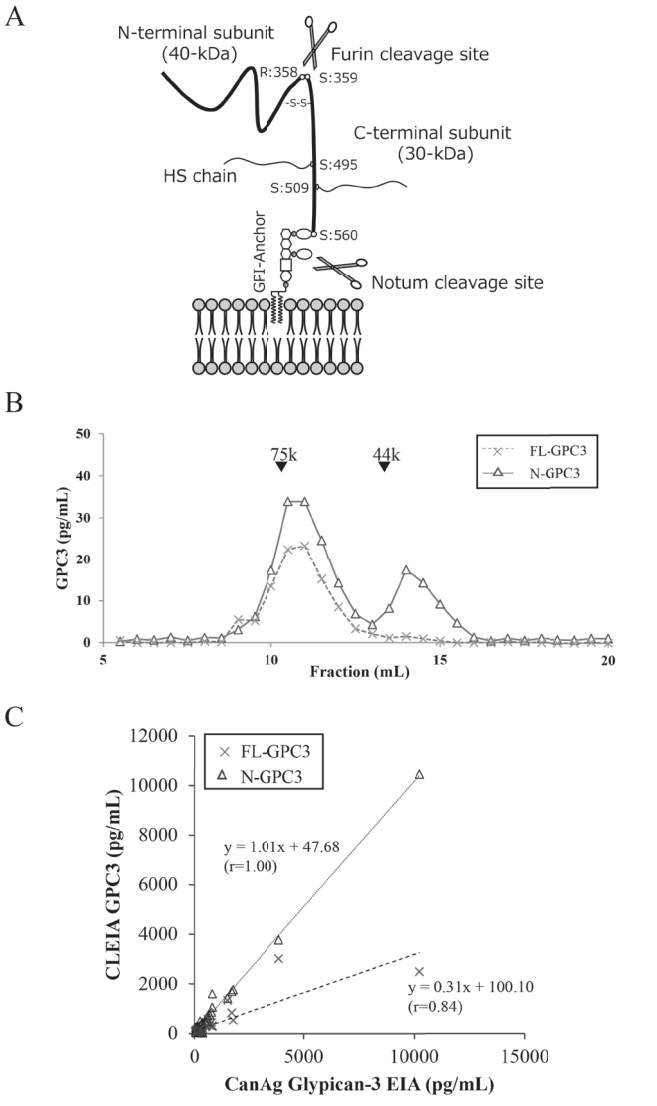


Fig. 1. Molecular Characterization of GPC3

(A) The structure of GPC3 protein. GPC3 consists of a core protein and a heparan sulfate chain. It binds to the cell membrane via a glycosylphosphatidylinositol (GPI) anchor. GPC3 has a cleavage site between Arg358 and Ser359 for Furin protease. Cleavage by Furin results in a 40-kDa N-terminal subunit and a 30-kDa C-terminal subunit. These two subunits can be linked by a disulfide bond. Two heparan sulfate (HS) side chains occur near the C-terminal of GPC3 (Ser495 and Ser509). Ser560 of GPC3 inserts into the lipid bilayer and anchors the protein to the bilayer by phosphatidylinositol. Notum, an extracellular lipase, cleaves the GPI anchor of GPC3, releasing it from the cell membrane. (B) Size exclusion chromatography analysis of GPC3 proteins with specimen from HCC patients on a Superdex2000 increase column. (C) Correlation plot of the relationship between CanAg Glypican-3 EIA and FL-GPC3 (cross), or between CanAg Glypican-3 EIA and N-GPC3 (triangle) with superimposed linear regression lines. The statistical method of Pearson's correlation coefficient (r) was used.

Table 2. Characteristics of All Specimens By Clinical Status

Characteristic		NC	NASH	HCC
N		60	50	70
Sex (%)	F	32 (53.3)	34 (68.0)	22 (31.4)
	M	28 (46.7)	16 (32.0)	48 (68.6)
Age†		40.5 [30.8, 54.3]	63.5 [56.0, 71.5]	59.0 [50.0, 67.0]

†Median [Q1, Q3].

performance of the biomarkers in non-HCC and HCC groups (Fig. 4D–E) demonstrated weak relationships between AFP, N-GPC3, and PIVKA-II positivity. Altogether, these data suggest that the expression of these tumor markers might be controlled differently.

DISCUSSION

In this study, we established GPC3 assays with the LUMIPULSE platform, and demonstrated their good analytical performances. We then analyzed GPC3 molecular forms in HCC patient specimens using SEC and showed that the N-GPC3 assay could detect both the full-length GPC3 and the N-terminal GPC3. As a result, the GPC3 concentration obtained with the N-GPC3 assay was higher than that of the FL-GPC3 assay. These findings are consistent with a previous report on GPC3 forms in the sera of HCC patients.[11] The correlation coefficient between the N-GPC3 assay and CanAg Glypican-3 EIA, which is a CE-marked product, was substantially good. These data suggest that because GPC3 can be fragmented, the N-GPC3 assay could be a more robust assay than the FL-GPC3. However, further investigations of GPC3 fragments, e.g. studies on the mechanism generating the full-length and N-terminal fragments in cells and the stability of these fragments in blood, might be required to clarify this point.

We evaluated the clinical performance of the N-GPC3 assay and compared it with other tumor markers, AFP and PIVKA-II. Although a meta-analysis report showed that serum GPC3 was inferior to AFP in the differential diagnosis between HCC and liver cirrhosis,[18] in this study, the N-GPC3 assay could separate HCC subjects from NASH and NC groups with an accuracy equivalent to AFP. One possible explanation for these contrasting results is that the datasets in the meta-analysis were generated with manually operated ELISA systems which targeted different analytes. On the other hand, the fully automated GPC3 assay is more sensitive. Therefore, although the N-GPC3 assay was already established in ELISA,[12] LUMIPULSE platform assay could allow us, in terms of improved accuracy and reproducibility, elimination of the impact of technical skill, labor and cost reductions, to precisely evaluate the clinical performance of GPC3 for HCC diagnosis.

Previous studies reported that there was no correlation between AFP, PIVKA-II, and GPC3, suggesting that these parameters are functionally independent.[13,16] Consistent with these reports, we found weak correlations between GPC3 and AFP, between GPC3 and PIVKA-II, and between AFP and PIVKA-II, suggesting that they might reflect the differ-

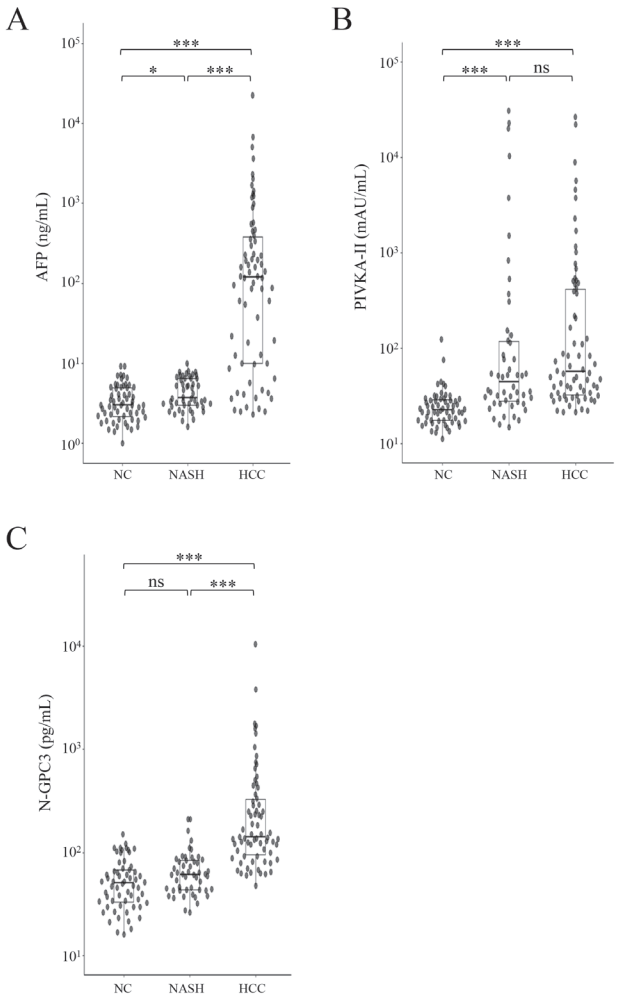
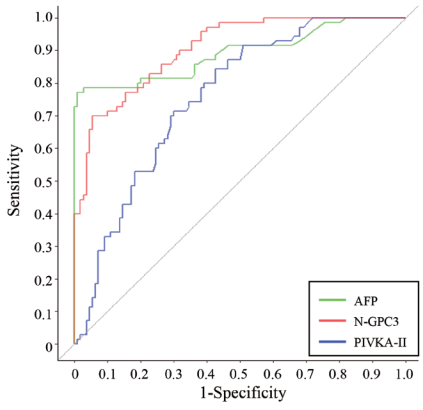


Fig. 2. Distribution of Biomarkers

Beeswarm boxplot of each biomarker, AFP (A), PIVKA-II (B), and N-GPC3 (C). The box plots display the median values with the interquartile range (lower and upper hinge) and ± 1.5-fold the interquartile range from the first and third quartile (lower and upper whiskers). Data were analyzed using Wilcoxon signed-rank tests with Bonferroni correction. ns: not significant; *p < 0.05; **p < 0.01; ***p < 0.001.



	Cut-off	Sensitivity	Specificity	OPA	AUC
AFP	10 ng/mL	72.9% (61.5-81.9)	99.1% (95.0-99.8)	88.9% (83.5-92.7)	0.89 (0.84-0.95)
N-GPC3	120 pg/mL	68.6% (57.0-78.2)	94.5% (88.6-97.5)	84.4% (78.4-89.0)	0.90 (0.86-0.95)
PIVKA-II	40 mAU/mL	64.3% (52.6-74.5)	71.8% (62.8-79.4)	68.9% (61.8-75.2)	0.76 (0.69-0.83)

Fig. 3. Receiver Operator Characteristic (ROC) Curves of Biomarkers for the Discrimination Between HCC and Non-HCC Subjects

For ROC analysis, individuals were dichotomized into HCC and non-HCC groups. For each assay, the table indicates the cutoff values and associated sensitivity, specificity, overall percent agreement (OPA) and area under the ROC curve (AUC). 95% confidence intervals are included in parentheses.

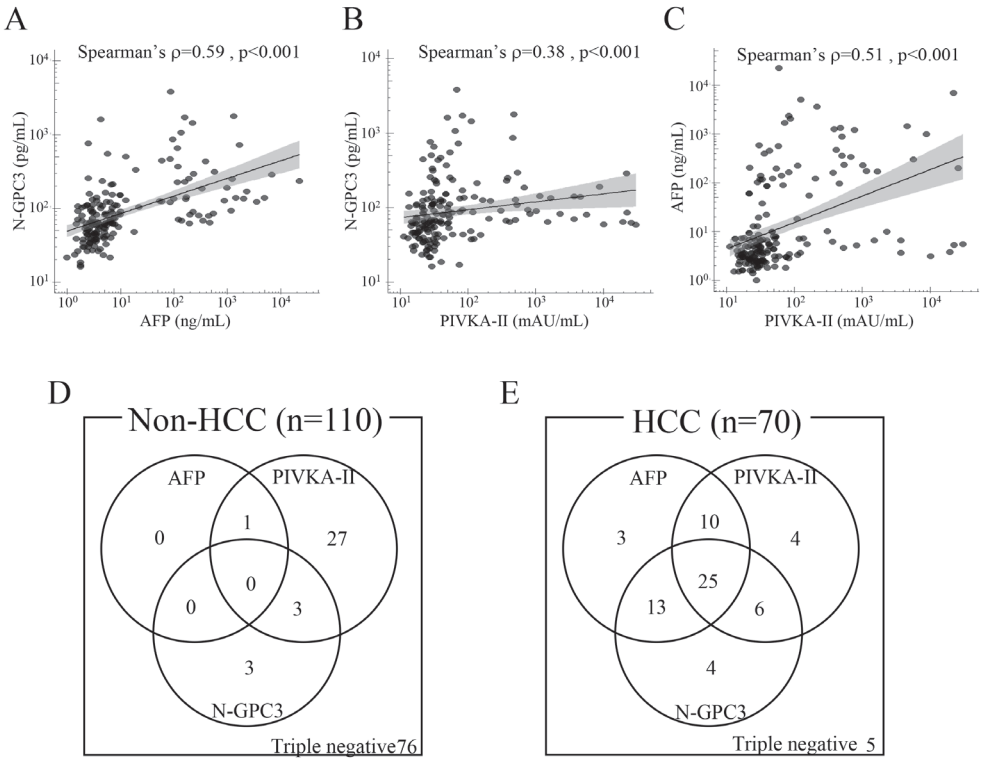


Fig. 4. The Performance of the Biomarkers

(A–C) Correlations between N-GPC3 and AFP (A), N-GPC3 and PIVKA-II (B), and AFP and PIVKA-II (C) are illustrated with superimposed linear regression lines (with 95%CI). The values for ρ and p-value (Spearman rank correlation) are included in each figure. (D, E) Venn's diagrams showing the performance of the biomarkers in non-HCC group (D) and HCC group (E). Numbers indicate the intersection of positivity of the biomarkers.

ent features that contribute to cancer development. Thus, the combination of assays for these tumor markers would provide a potentially promising tool to better differentiate HCC from benign liver disorders, and from other malignant tumors.[13,15,18,19] Indeed, it has been reported that the combination of GPC3 and AFP increased diagnostic accuracy.[15,18] Further studies are required to identify the combination which shows the best diagnostic utility with this fully automated platform.

A limitation of this study is that since the information about the stage or tumor size of the HCC patients, was not available, we could not assess whether GPC3 can detect the early stage of HCC. Also, the accuracy of AFP in this study was higher than previously reported,[16,18,20] suggesting that the sample set in this study might have some bias. Moreover, although age and gender did not have significant effects on the GPC3 values of NC and NASH subjects, the age and gender biases among the NC, NASH and HCC groups might have influenced our findings. To finish, we couldn't compare GPC3 performance with the combination of ultrasonography and AFP. Therefore, to address these points, further studies investigating these tumor markers with age-, gender-matched and well-defined sample sets, are required.

In conclusion, in this study, a clinically applicable and GPC3-specific CLEIA assay was successfully developed and validated using clinical samples. It has been reported that Wnt signaling plays a major role in HCC pathology and that GPC3 activates the Wnt canonical pathway, thereby stimulating HCC progression.[21] As a result, various inhibitors targeting GPC3 are currently under investigation. Indeed, monoclonal antibodies targeting GPC3 or GPC3-derived peptide/DNA vaccines are potentially attractive options for treating HCC.[22] Therefore, appropriate patient screening will be required for therapeutic intervention. Our GPC3 assays could be useful for such purpose.

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Conflict of interest H.N., T.N., P.F.A.A.M., and S.Y. are employees of FUJIREBIO INC.. K.A. is a board member of FUJIREBIO INC. and a president & CEO of Advanced Life Science Institute, Inc.. K.I. and S.Y. are employees of Advanced Life Science Institute, Inc.. This study was funded by FUJIREBIO INC..

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