Clinical Chemistry 64:8
000–000 (2018)

Proteomics and Protein Markers

Clinical Assay for AFP-L3 by Using Multiple Reaction Monitoring–Mass Spectrometry for Diagnosing Hepatocellular Carcinoma

Hyunsoo Kim,[1,2,3†] Areum Sohn,[3†] Injoon Yeo,[1] Su Jong Yu,[4] Jung-Hwan Yoon,[4] and Youngsoo Kim[1,2,3*]

BACKGROUND: *Lens culinaris* agglutinin-reactive fraction of α-fetoprotein (AFP-L3) is a serum biomarker for hepatocellular carcinoma (HCC). AFP-L3 is typically measured by liquid-phase binding assay (LiBA). However, LiBA does not always reflect AFP-L3 concentrations because of its low analytical sensitivity. Thus, we aimed to develop an analytically sensitive multiple reaction monitoring–mass spectrometry (MRM-MS) assay to quantify AFP-L3 in serum.

METHODS: The assay entailed the addition of a stable isotope-labeled internal standard protein analog, the enrichment of AFP using a monoclonal antibody, the fractionation of AFP-L3 using *L. culinaris* agglutinin lectin, deglycosylation, trypsin digestion, online desalting, and MRM-MS analysis. The performance of the MRM-MS assay was compared with that of LiBA in 400 human serum samples (100 chronic hepatitis, 100 liver cirrhosis, and 200 HCC). Integrated multinational guidelines were followed to validate the assay for clinical implementation.

RESULTS: The lower limit of quantification of the MRM-MS assay (0.051 ng/mL) for AFP-L3 was less than that of LiBA (0.300 ng/mL). Thus, AFP-L3, which was not observed by LiBA in HCC samples (n = 39), was detected by the MRM-MS assay, improving the clinical value of AFP-L3 as a biomarker by switching to a more analytical sensitive platform. The method was validated, meeting all the criteria in integrated multinational guidelines.

CONCLUSIONS: Because of the lower incidence of false-negative findings, the MRM-MS assay is more suitable than LiBA for early detection of HCC.

α-Fetoprotein (AFP)[5] is a glycoprotein with a complex, single asparagine-linked sugar structure that has been widely used as a blood biomarker for hepatocellular carcinoma (HCC) *(1)*. However, the insufficient diagnostic sensitivity of AFP (33%–65%) *(1, 2)* provides little clinical value. To address this issue, several studies have been attempted to improve the diagnostic sensitivity of AFP by measuring HCC-specific heterogeneity in sugar structures. Approximately 25 sugar structures have been identified at a single site in AFP, primarily by mass spectrometry *(3)*. Among them, the *Lens culinaris* agglutinin (LCA)-reactive fraction of α-fetoprotein (AFP-L3), a glycoform with core fucosylation, has greater diagnostic sensitivity in the early diagnosis of HCC than total AFP. In clinical practice, AFP-L3% is defined as the ratio of the AFP-L3 fraction to the total AFP concentration *(4)*. AFP-L3% has superior diagnostic sensitivity (75%–97%) compared with total AFP, and increases in AFP-L3% are independent of increases in total AFP in HCC *(5, 6)*.

Liquid-phase binding assay (LiBA) is the clinical standard for measuring AFP-L3%. In particular, the micro-total assay system (μTAS; Wako™ i-30 autoanalyzer), which obtained clearance from the US Food and Drug Administration (FDA) for in vitro diagnostic use in February 2011 *(7, 8)*, is the preferred system of most major reference laboratories in the US. This assay is also available for clinical use in South Korea, Japan, and most European countries *(9)*. However, if AFP concentrations fall below a certain concentration (<0.3 ng/mL), the AFP-L3 concentration is not measured, and patients with HCC are diagnosed as normal even when AFP-L3 concentrations are high *(10, 11)*. Consequently, existing methods for quantifying AFP-L3 in serum lack the analytical sensitivity to make accurate diagnoses.

[1] Department of Biomedical Engineering; [2] Institute of Medical and Biological Engineering, Medical Research Center; [3] Department of Biomedical Sciences; [4] Department of Internal Medicine and Liver Research Institute, Seoul National University College of Medicine, Seoul, Republic of Korea.
* Address correspondence to this author at: Department of Biomedical Engineering, Seoul National University College of Medicine, 28 Yongon-Dong, Jongno-gu, Seoul 110-799, Republic of Korea. Fax +82-2-741-0253; e-mail biolab@snu.ac.kr.
† H. Kim and A. Sohn contributed equally to this work.
Received March 12, 2018; accepted May 4, 2018.
Previously published online at DOI: 10.1373/clinchem.2018.289702

[5] Nonstandard abbreviations: AFP, α-fetoprotein; HCC, hepatocellular carcinoma; LCA, *Lens culinaris* agglutinin; AFP-L3, *Lens culinaris* agglutinin-reactive fraction of α-fetoprotein; LiBA, liquid-phase binding assay; μTAS, micro-total assay system; FDA, Food and Drug Administration; MRM-MS, multiple reaction monitoring–mass spectrometry; QC, quality control; LLOQ, lower limit of quantification; ULOQ, upper limit of quantification; AUROC, area under the receiver operating characteristic.

Quantitative multiple reaction monitoring–mass spectrometry (MRM-MS) assays have been used primarily to measure abundant protein biomarkers *(12)*. However, with the advent of immunoaffinity techniques and more analytically sensitive instruments, the quantification of low-concentration proteins is becoming more common *(13, 14)*, as evidenced by the increasing use of MRM-MS-based assays for clinical applications *(15)*.

Here, we developed and validated a method for quantifying AFP-L3 in serum by capillary-flow liquid chromatography, interfaced with an MRM-MS-based assay that can overcome the low analytical sensitivity of LiBA. To improve the analytical sensitivity of AFP-L3, we added enrichment and fractionation steps using monoclonal anti-AFP antibody and LCA lectin, which has high affinity for AFP-L3, before deglycosylation and digestion. The MRM-MS assay was validated by the US FDA, European Medicines Agency, Korea FDA, and Clinical and Laboratory Standards Institute.

Materials and Methods

All solvents and reagents had the highest available purity. All materials, sample preparation step, and instrument conditions are described in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol64/issue8.

CLINICAL SAMPLE COLLECTION

All blood samples were incubated in BD Vacutainer® blood collection tubes for 30 min (clotting time, at room temperature) and centrifuged at 1200*g* for 20 min at room temperature. Supernatant aliquots (300 μL) were stored in plain tubes at −80 °C until analysis. All serum samples were collected at a single institution between 2008 and 2014 per standard operating procedures *(16)*. The use of human serum samples was approved by the Institutional Review Board of Seoul National Hospital (IRB No. 0506–150–005). One hundred samples from patients with chronic hepatitis (71 men, 29 women; median age, 56 years; range, 29–69 years), 100 samples from patients with liver cirrhosis (38 men, 62 women; median age, 58 years; range, 34–78 years), and 200 samples from patients with HCC (166 men, 34 women; median age, 59 years; range, 38–86 years) were obtained.

SERUM SAMPLE PREPARATION

All serum samples were pipetted manually, based on the volumetric method ("addition only"). The sample preparation comprised the addition of a stable isotope-labeled internal standard protein analog to the serum, AFP enrichment with monoclonal antibody, AFP-L3 fractionation with LCA lectin, deglycosylation, trypsin digestion, and online desalting, followed by MRM-MS analysis (see Fig. 1 in the online Data Supplement).

MRM-MS ASSAY

All MRM-MS analyses were performed on an Agilent 6490 triple quadrupole mass spectrometer with a Jetstream electrospray source, coupled to a 1260 Capillary LC system (Agilent Technologies). MRM-MS data were processed in Skyline (MacCoss Lab). Additional data analyses were performed using Excel (Microsoft).

LIBA

The μTAS autoanalyzer (Wako Pure Chemical Industries) is an FDA clearance device for diagnosing HCC by measuring AFP and AFP-L3 concentrations. A sample load of 100 μL was analyzed for 9 min with a 2-min interval between each sample. The AFP-L3 concentration was calculated automatically as a percentage of total AFP and printed out. The quantifiable ranges of AFP and AFP-L3 were 0.3 to 4000.0 ng/mL and 0.5% to 99.5%, respectively, using a 2-point calibrator. All serum samples were measured following the manufacturer's instructions.

Results

MRM-MS ASSAY DEVELOPMENT

The MRM-MS transitions that were used for the development of the method and the quantification are summarized in Table 1 of the online Data Supplement. Details on the development of the method are described in the online Supplemental Data file. A nonglycopeptide (GYQELLEK) and deglycopeptide (V*D*FTEIQK) were chosen as surrogate peptides to quantify the total concentration of AFP and the AFP-L3 fraction, respectively, based on our previous studies *(17)* and preliminary experiments (see Fig. 2 in the online Data Supplement).

ANALYTICAL METHOD VALIDATION

For validation of the analytical method, the following criteria were evaluated: calibration curve, analytical specificity (selectivity or interference), analytical sensitivity, carryover, precision, recovery of assay, matrix effect, recovery of immunoprecipitation, dilution integrity, stability, reproducibility, and quality control (QC) of samples and frequency. All validation procedures are detailed in the online Supplemental Data file.

A schematic diagram of the validation of the analytical method and the optimized analytical sequence and schedule are provided in Fig. 7 and Table 2, respectively, in the online Data Supplement. The analytical measurement range, encompassing the lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) of all 6 matrices, was 0.051 to 4000 ng/mL and linear ($R^2 > 0.99$) for both peptides (see Fig. 8 and Tables 3 and 4 in the online Data Supplement). The LLOQ of AFP-L3% was confirmed to be 0.132% by mixing 2 HCC samples at varying proportions (Table 1). The precision and recovery of the MRM-MS assay for the 4 QC sam-

Table 1. Lower limit of quantification of AFP-L3% in clinical samples.

Mixing ratio							
Sample A	100.0	0.0	1.0	2.0	3.0	4.0	5.0
Sample B	0.0	100.0	99.0	98.0	97.0	96.0	95.0
AFP, ng/mL							
Expected concentration, ng/mL[a]	452.816	772.943	769.742	766.540	763.339	760.138	756.937
Measured concentration, ng/mL[b]	452.816	772.943	835.358	716.627	736.629	780.700	847.419
SD	15.023	24.320	78.158	23.135	44.633	64.457	34.473
CV, %	3.318	3.146	9.356	3.228	6.059	8.256	4.068
Bias, %[c]	0.000	0.000	8.524	−6.512	−3.499	2.705	11.954
AFP-L3, ng/mL							
Expected concentration, ng/mL[a]	33.617	0.000	0.336	0.673	1.009	1.345	1.681
Measured concentration, ng/mL[b]	33.617	0.000	0.032	0.393	0.951	1.413	1.864
SD	1.887	NA[d]	0.019	0.068	0.070	0.097	0.072
CV, %	5.612	NA	59.477	17.399	7.342	6.888	3.859
Bias, %[c]	0.000	0.000	−90.616	−41.522	−5.714	5.094	10.861
AFP-L3, %							
Expected concentration, %[a]	7.422	0.000	0.044	0.088	0.132	0.177	0.222
Measured concentration, %[b]	7.422	0.000	0.004	0.055	0.129	0.181	0.220
SD	0.260	NA	0.002	0.009	0.010	0.006	0.018
CV, %	3.508	NA	56.949	16.534	7.599	3.118	8.066
Bias, %[c]	0.000	0.000	−91.569	−37.482	−2.152	2.424	−0.759

[a] Calculated by measuring the concentration of 2 unmixed samples and then applying the mixed ratio of the 2 samples.
[b] Calculated based on mean value of 3 replicates.
[c] $\text{Bias (\%)} = \frac{(\text{measured concentration} - \text{expected concentration})}{\text{expected concentration}} \times 100.$
[d] NA, not applicable.

ples, analyzed over 6 days, were <12.3% and less than ±16.0%, respectively (Table 2). The concentration of these samples was below or above the range of cutoff values for diagnosis, based on LiBA measurements *(18)*. The analytical specificity (see Fig. 9 and Table 5 in the online Data Supplement) and analytical sensitivity (see Table 6 in the online Data Supplement) satisfied all criteria when calculated with respect to the LLOQ sample. The absence of carryover was confirmed by analyzing a ULOQ sample, followed by a blank sample (see Table 7 in the online Data Supplement).

The matrix effect for the 8 calibrators and the 4 QC samples of 6 matrices was <13.2% for both peptides (except for calibrator 1, 25.1%; see Table 8 in the online Data Supplement). The recovery of spiking for the GYQELLEK and V*D*FTEIQK peptides in the 4 QC samples was 87.3% to 113.7% and 92.2% to 105.6%, respectively (see Table 9 in the online Data Supplement). The linearity ($R^2 > 0.99$) of the patient samples was confirmed by mixing 2 HCC samples at varying proportions (see Fig. 10 and Table 10 of the online Data Supplement). Icterus had less of an impact on the performance of the MRM-MS assay than lipemia and hemolysis (see Table 11 of the online Data Supplement). There was no difference in the concentrations of AFP and AFP-L3 between the 2 types of blood (serum and plasma) collection tubes (see Fig. 11 of the online Data Supplement).

The recovery of immunoprecipitation for the GYQELLEK and V*D*FTEIQK peptides in the 4 QC samples was 99.3% to 102.8% and 95.9% to 103.9%, respectively (see Table 12 of the online Data Supplement). When the sample was diluted by 2500-fold, the quantification value of the assay was acceptable (see Table 13 of the online Data Supplement). Long-term storage (up to 28 days) of the samples at −20 °C and −70 °C and freeze–thaw cycles (up to 7 cycles) did not affect the concentrations of GYQELLEK and V*D*FTEIQK peptides. However, storage of the samples for >5 days at room temperature or 4 °C resulted in unacceptable measurements (see Fig. 12 of the online Data Supplement).

The entire procedure for the MRM-MS assay was confirmed to be reproducible, based on the results of the 4 HCC samples for 6 days (see Fig. 13 and Table 14 of the online Data Supplement). The robustness of the

Table 2. Precision and recovery of assay measured in 4 QC samples (LLOQ, low, medium, and high) over 6 days.

Peptide	Measurements	QC1	QC2	QC3	QC4
	Expected concentration, ng/mL	0.051	0.154	2000.256	3600.000
GYQELLEK					
Intraassay[a]	Concentration, ng/mL	0.059	0.169	1937.411	3249.274
	SD	0.003	0.012	136.230	109.457
	CV_{intra}, %	5.149	6.893	7.032	3.369
	Recovery of assay, %[c]	115.979	109.899	96.869	90.258
Interassay[b]	Concentration, ng/mL	0.055	0.159	2015.015	3184.032
	SD	0.006	0.009	187.764	94.294
	CV_{inter}, %	11.218	5.865	9.318	2.961
	Recovery of assay, %[c]	107.210	103.507	100.749	88.445
	CV_{total}, %[d]	12.343	9.051	11.674	4.485
VDFTEIQK					
Intraassay[a]	Concentration, ng/mL	0.046	0.141	1955.724	3668.717
	SD	0.005	0.006	71.414	101.406
	CV_{intra}, %	10.280	4.078	3.652	2.764
	Recovery of assay, %[c]	90.337	91.801	97.785	101.909
Interassay[b]	Concentration, ng/mL	0.048	0.134	1912.751	3649.914
	SD	0.002	0.007	76.163	166.858
	CV_{inter}, %	4.353	5.048	3.982	4.572
	Recovery of assay, %[c]	94.317	86.935	95.636	101.387
	CV_{total}, %[d]	11.163	6.489	5.403	5.342

[a] Calculated by averaging the mean values of the 6 replicates on each day over 6 days.
[b] Calculated by averaging the first replicate of each day over 6 days.
[c] Recovery of assay (%) = measured concentration/expected concentration × 100.
[d] Total CV = $\sqrt{CV_{intra}^2 + CV_{inter}^2}$.

MRM-MS assay was verified in 4 QC samples that were analyzed at regular intervals in individual sample analysis (see Table 15 in the online Data Supplement). All results for the validation of the analytical method are summarized in Table 16 of the online Data Supplement.

METHOD COMPARISON OF THE TWO ASSAYS

The performance of the MRM-MS assay was compared with that of the μTAS autoanalyzer, the current FDA-cleared, standard LiBA system. The sample population, comprising samples from patients with chronic hepatitis, liver cirrhosis, and HCC, was analyzed by MRM-MS assay and LiBA (see Fig. 14 and Tables 17 and 18 of the online Data Supplement). The scatterplots and Deming regression equation for the comparison of AFP and AFP-L3 were LiBA = 0.799 (95% CI, 0.759–0.838) × MRM-MS + 0.286 for AFP when using 396 samples (Pearson correlation coefficient, R = 0.895; 95% CI, 0.873–0.913; $P < 0.0001$) and LiBA = 0.968 (95% CI, 0.851–1.084) × MRM-MS + 0.333 for AFP-L3 when using 141 samples (R = 0.813; 95% CI, 0.748–0.862; $P < 0.0001$), respectively (Figs. 1, A–D and 2, A–C here and see Table 19 of the online Data Supplement). In comparing the 2 assays using Bland–Altman plots, the MRM-MS assay had a mean positive bias of 0.340 (log_2-scale in ng/mL; 95% CI, −2.569 to 3.249) for AFP and a mean negative bias of 0.231 (log_2-scale in %; 95% CI, −2.200 to 1.738) for AFP-L3% (Figs. 1, E–H, and 2, D–F, here and Table 19 of the online Data Supplement). This result demonstrates that the LiBA underestimated the concentrations of AFP and AFP-L3 vs the MRM-MS assay.

DIAGNOSTIC PERFORMANCE OF THE TWO ASSAYS

As an HCC screening assay, the MRM-MS assay quantified the AFP concentration in all samples. However, LiBA failed to do so in 4 samples because the AFP concentrations were below the LLOQ (400 cases vs 396 cases). The optimal cutoff value (the maximum sum of the diagnostic sensitivity and specificity) was calculated to best distinguish between the HCC and high-risk HCC group (chronic hepatitis plus liver cirrhosis). The optimal

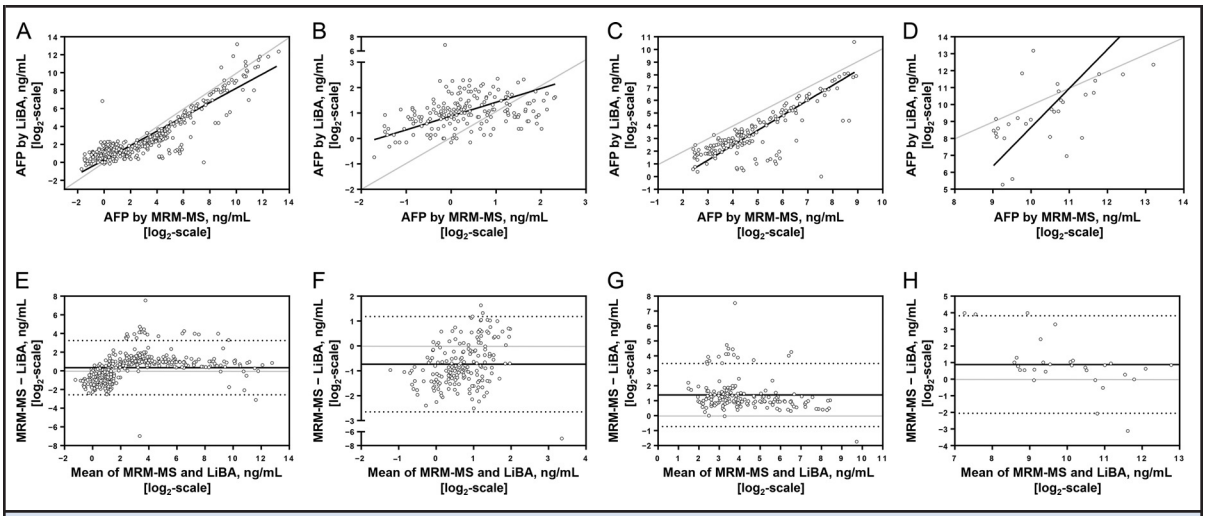

Fig. 1. Correlation between AFP values as measured by MRM-MS assay and LiBA.

MRM-MS assay was compared with LiBA regarding the concentration of AFP in 396 patients who were detectable by both assays. Deming regression (A–D) and Bland–Altman plots (E–H) were analyzed according to the concentration intervals of AFP, as measured by MRM-MS assay [entire concentration range (A, E); ≤5 ng/mL (B, F); 5–500 ng/mL (C, G); and >500 ng/mL (D, H)]. The black line represents the mean difference between the 2 assays. The gray line represents unity (y = x), and the dotted line (dashed line) indicates the 95% CI of the mean difference.

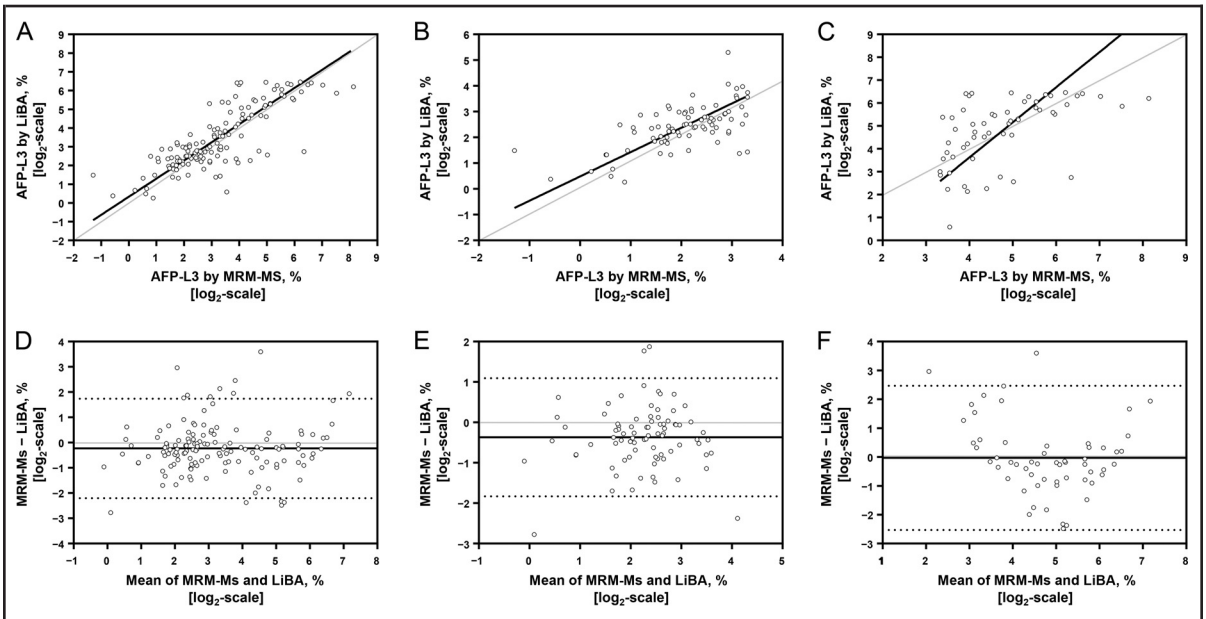

Fig. 2. Correlation between AFP-L3 values as measured by MRM-MS assay and LiBA.

MRM-MS assay was compared with LiBA regarding the concentration of AFP-L3 in 141 patients with HCC who were detectable by both assays. Deming regression (A–C) and Bland–Altman plots (D–F) were analyzed according to the concentration intervals of AFP-L3, as measured by MRM-MS assay [entire concentration range (A, D); ≤10% (B, E); and >10% (C, F)]. The black line represents the mean difference between the 2 assays. The gray line represents unity (y = x), and the dotted line (dashed line) indicates the 95% CI of the mean difference.

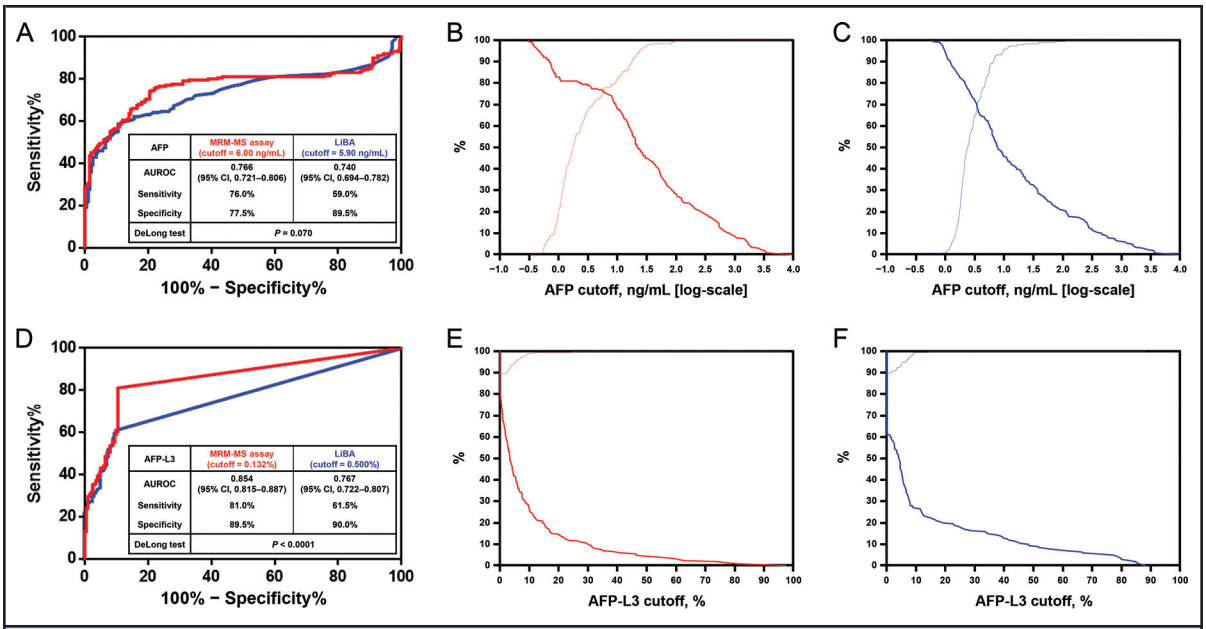

Fig. 3. Comparison of AFP and AFP-L3 measurements between MRM-MS assay and LiBA.

For AFP (A–C) and AFP-L3 (D–F), ROC curves (A and D) and plot-vs-criterion values (B, C, E, and F) were plotted for HCC vs the high-risk HCC group (chronic hepatitis plus liver cirrhosis) by MRM-MS assay (red line) and LiBA (blue line), respectively. Delong test was used to determine whether the difference in AUROC values was significant. For the plot-vs-criterion values, the solid and dashed lines indicate the diagnostic sensitivity and specificity, respectively. The ROC curves for AFP-L3% formed a straight line because of the small number of detectable samples in the high-risk HCC group.

cutoff values for the AFP measurement were 6.00 ng/mL for the MRM-MS assay and 5.90 ng/mL for LiBA. The area under the receiver operating characteristic (AUROC) value, diagnostic sensitivity, and specificity were 0.766 (95% CI, 0.721–0.806), 76.0%, and 77.5% for the MRM-MS assay and 0.740 (95% CI, 0.694–0.782), 59.0%, and 89.5% for LiBA, respectively (Fig. 3, A–C here and see Table 20 of the online Data Supplement). Regarding AFP concentrations, the DeLong test failed to conclude that the MRM-MS assay differed significantly from LiBA ($P = 0.070$) in the HCC vs high-risk HCC group.

Of those patients with chronic hepatitis, AFP-L3 was detected by MRM-MS assay and LiBA in 3 and 1 patients, respectively, vs 18 and 19 patients with liver cirrhosis. In the HCC group, AFP-L3 was measured in 162 (81.0%) and 123 (61.5%) patients by MRM-MS assay and LiBA, respectively. For AFP-L3%, the optimal cutoff values of the MRM-MS assay and LiBA were 0.132% and 0.500% (each LLOQ concentrations), respectively. The AUROC, diagnostic sensitivity, and specificity were 0.854 (95% CI, 0.815–0.887), 81.0%, and 89.5% for the MRM-MS assay and 0.767 (95% CI, 0.722–0.807), 61.5%, and 90.0% for LiBA, respectively (Fig. 3, D–F here and see Table 20 of the online Data Supplement). The MRM-MS assay outperformed LiBA in quantifying AFP-L3%, based on its significantly higher AUROC values in diagnosing HCC patients (DeLong test, $P < 0.0001$).

For AFP-L3%, 39 HCC samples were measured exclusively above the cutoff value (>0.132%) by only MRM-MS assay, not by LiBA. Of the 39 HCC samples, 24 were below the cutoff value (≤5.90 ng/mL) when AFP was measured by LiBA, constituting false-negative cases by LiBA (Fig. 4). Among 24 HCC samples, except in 1 patient, all AFP-L3% values in the 23 samples were ≤5.95% (range, 0.41%–5.95%; 22.1% for 1 patient), demonstrating that the MRM-MS assay readily identified small changes in AFP-L3 at low AFP concentrations because of the lower background noise and higher dynamic range in measuring AFP-L3 responses than with LiBA. The MRM-MS assay could determine a low cutoff value by quantifying low values of AFP-L3, resulting in a reduced false-negative rate, allowing effective HCC screening.

Discussion

The FDA has cleared novel and specific HCC serum biomarkers, such as AFP-L3, for assessing the risk of HCC *(19)*. Using a lectin affinity electrophoresis

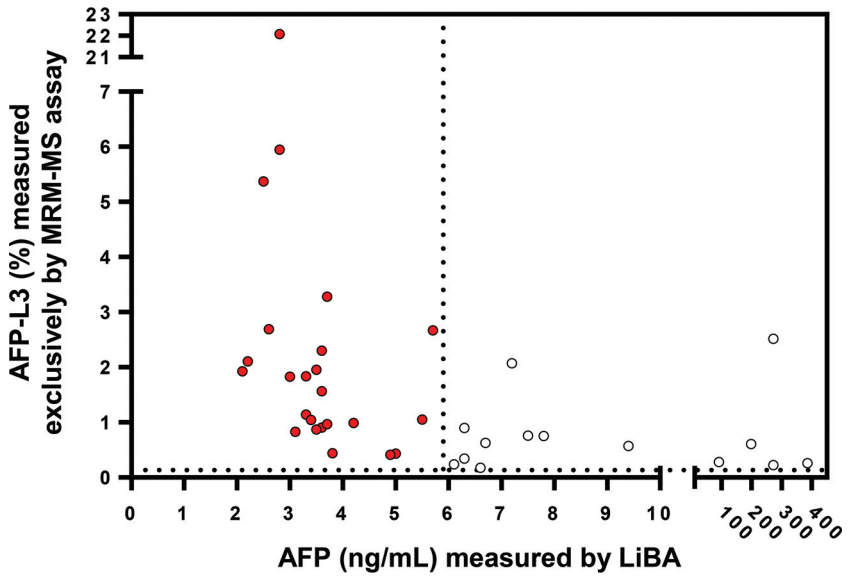

Fig. 4. AFP-L3 measurements by MRM-MS assay compared with LiBA.

AFP-L3 values (*y* axis) of the 39 HCC samples in which AFP-L3 was detected exclusively by MRM-MS assay but not LiBA, and AFP values (*x* axis) as measured by LiBA for 39 HCC samples. The range of AFP-L3% was 0.18% to 22.1%, and the dotted line indicates the optimal cutoff values, which were 5.90 ng/mL and 0.132% for AFP by LiBA and AFP-L3 by MRM-MS assay, respectively. Further, 24 HCC samples (red dotted) were estimated to be below the cutoff of AFP by LiBA but were quantified to be above the cutoff of AFP-L3% by MRM-MS assay.

method, Japan developed the first automated clinical laboratory assay on a LiBA. AFP-L3% values could be generated when AFP was >10 ng/mL, with a minimal detectable limit of AFP of 0.8 ng/mL *(20, 21)*. Since then, assay technologies have continued to evolve. Since 2009, such assays have been incorporated into microchip capillary electrophoresis and LiBA on a μTAS autoanalyzer. With the deployment of second-generation assays, the analytical sensitivity has improved and can detect AFP concentrations as low as 0.3 ng/mL. Despite this improvement, measuring AFP-L3% requires a minimum AFP concentration of 0.3 ng/mL *(22)*.

Although the analytical sensitivity of the assay has increased as its detection limit has improved, the consensus of practicing clinicians is that the high incidence of false-negative findings renders the test results unreliable *(23)*. Accurate measurements of AFP-L3% have been limited to patients with HCC who had AFP concentrations >0.3 ng/mL because of the insufficient analytical sensitivity of the instrument. On the μTAS autoanalyzer, AFP-L3% cannot be reported if the total AFP concentration is <0.3 ng/mL, even in cases of high AFP-L3 concentrations. The low analytical sensitivity of AFP-L3 has impeded its potential as an HCC-specific biomarker.

Two studies have attempted to measure AFP-L3 by mass spectrometry after fractionating AFP with lectin *(24, 25)*. A more recent study quantified low-abundance AFP-L3 using a nanoprobe approach, coupled with a mass spectrometer to improve analytical sensitivity *(26)*. Both the MRM-MS assay for the nonglycopeptide and the MS/MS assay for the glycopeptide were performed in a single tube to quantify AFP and profile the glycoforms of AFP-L3. In this work, no effort was made to simultaneously quantify the amount of nonglycopeptide and glycopeptide. When comparing the dynamic range of AFP nonglycopeptide between that study *(26)* and our report, we found that the range was lower by 1 and 2 orders of magnitude than LiBA and our MRM-MS assay (see Fig. 8 in the online Data Supplement), respectively. We were unable to compare the dynamic range of the glycopeptide because it was not measured in the other study.

In contrast to these approaches, we have developed and validated an MRM-MS assay that measures AFP-L3 in human serum for clinical applications. The improved analytical sensitivity of our MRM-MS assay allows us to detect AFP-L3 concentrations that are not observable by LiBA. This study improved the low analytical sensitivity and low reproducibility of a previous study *(17)* by immunoprecipitation using a monoclonal antibody to AFP and separately measuring AFP and AFP-L3 after fractionation with LCA lectin. This process resulted in a wider quantification range than all other AFP (0.051–4000 ng/mL) and AFP-L3 (0.132%–100%) measurement methods.

Consequently, the AFP-L3-positive rates in HCC samples by MRM-MS assay were higher compared with LiBA (see Table 18 of the online Data Supplement). Further, the MRM-MS assay identified more patients with HCC with normal AFP concentrations than LiBA (Fig. 4). Despite the inclusion of low-concentration samples with high analytical variability (MRM-MS, Table 2), the AUROC value of the MRM-MS assay was higher than that of LiBA (Fig. 3), which indicates superior clinical utility with respect to making a diagnosis. Thus, in addition to improving the analytical sensitivity of the MRM-MS assay, the use of many calibrators (8 vs 2 points) and internal standards might have improved its recovery of assay (see Table 21 in the online Data Supplement), increasing the clinical value of AFP-L3 as a biomarker in ways not possible with LiBA. Although the MRM-MS assay is not considered in diagnostic decision-making processes, if implemented, it would have the potential to benefit patients with HCC who could be misdiagnosed by LiBA.

There are 3 notable aspects of our study that distinguish it from earlier efforts to quantify (glyco)protein biomarkers by mass spectrometry. Our assay uses a monoclonal antibody to selectively enrich AFP from serum, improving the analytical sensitivity by removing most of the matrix proteins that cause interference. In general, commercially available protein antibodies are cheaper than peptide antibodies (e.g., stable isotope standards and capture by antipeptide antibodies) *(27)*, rendering them more cost-effective in quantifying low-concentration (glyco)proteins in blood.

One of the challenges of bottom-up proteomics is that quantifying endogenous proteins in human serum depends highly on the sample preparation, which is subject to extreme variability *(28, 29)*. To minimize this variability, we used a stable isotope-labeled internal standard protein analog. The advantage of using such compounds instead of peptide analogs is that they can be added at the beginning of the sample preparation, mitigating variations in enrichment, fractionation, deglycosylation, and digestion *(30)*. In addition, all sample preparation steps were performed on a volumetric basis ("addition only") to enhance their compatibility with liquid handling systems.

We validated this analytical method in accordance with integrated multinational guidelines. Table 16 in the online Data Supplement details the criteria that dictated the experimental design with respect to the validation of the analytical method. In addition, the schedule (see Table 2 in the online Data Supplement) constitutes an efficient program for conducting MRM-MS analyses, minimizing the number of injections into the equipment. We hope that this proof-of-principle application of the suggested guidelines will be helpful for validating other biomarkers as they progress through the clinical application pipeline.

In summary, our method quantifies AFP-L3, a biomarker of HCC, with greater analytical sensitivity than LiBA. We recommend implementing this MRM-MS assay, which is superior to LiBA in distinguishing HCC from non-HCC, despite it being a fundamentally disparate method from the conventional AFP-L3 assay.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.*

H. Kim, data collection and statistical analysis; A. Sohn, experiments; I. Yeo, technical support; S.J. Yu, provision of study material or patients; J.-H. Yoon, provision of study material or patients; Y. Kim, financial support, administrative support, provision of study material or patients.

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: Y. Kim, the Collaborative Genome Program for Fostering New Post-Genome Industry (NRF-2017M3C9A5031597), a National Research Foundation grant (No. 2011-0030740), the Industrial Strategic Technology Development Program (#10079271), the Korea Health Technology R&D Project (No. HI14C2640).
Expert Testimony: None declared.
Patents: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or final approval of manuscript.

Acknowledgments: The authors thank all the patients, whose blood donations made this study possible.

References

1. Taketa K, Sekiya C, Namiki M, Akamatsu K, Ohta Y, Endo Y, Kosaka K. Lectin-reactive profiles of alpha-fetoprotein characterizing hepatocellular carcinoma and related conditions. Gastroenterology 1990;99:508–18.
2. Zhu K, Dai Z, Zhou J. Biomarkers for hepatocellular carcinoma: progression in early diagnosis, prognosis, and personalized therapy. Biomark Res 2013;1:10.
3. Campbell MP, Peterson R, Mariethoz J, Gasteiger E, Akune Y, Aoki-Kinoshita KF, et al. UniCarbKB: building a knowledge platform for glycoproteomics. Nucleic Acids Res 2014;42:D215–21.
4. Leerapun A, Suravarapu SV, Bida JP, Clark RJ, Sanders EL, Mettler TA, et al. The utility of Lens culinaris agglutinin-reactive alpha-fetoprotein in the diagnosis of hepatocellular carcinoma: evaluation in a United States referral population. Clin Gastroenterol Hepatol 2007;5:394–402; quiz 267.
5. Wang SS, Lu RH, Lee FY, Chao Y, Huang YS, Chen CC, Lee SD. Utility of lentil lectin affinity of alpha-fetoprotein in the diagnosis of hepatocellular carcinoma. J Hepatol 1996;25:166–71.

6. Kumada T, Nakano S, Takeda I, Kiriyama S, Sone Y, Hayashi K, et al. Clinical utility of Lens culinaris agglutinin-reactive alpha-fetoprotein in small hepatocellular carcinoma: special reference to imaging diagnosis. J Hepatol 1999;30:125–30.
7. Yamagata Y, Shimizu K, Nakamura K, Henmi F, Satomur S, Matsuura S, Tanaka M. Simultaneous determination of percentage of *Lens culinaris* agglutinin-reactive α-fetoprotein and α-fetoprotein concentration using the LiBASys clinical auto-analyzer. Clin Chim Acta 2003; 327:59–67.
8. Fuzery AK, Levin J, Chan MM, Chan DW. Translation of proteomic biomarkers into FDA approved cancer diagnostics: issues and challenges. Clin Proteomics 2013;10:13.
9. Kurosawa T, Watanabe M. Development of on-chip fully automated immunoassay system "Mutaswako i30" to measure the changes in glycosylation profiles of alpha-fetoprotein in patients with hepatocellular carcinoma. Proteomics 2016;16:3056–61.
10. Tamura Y, Igarashi M, Kawai H, Suda T, Satomura S, Aoyagi Y. Clinical advantage of highly sensitive on-chip immunoassay for fucosylated fraction of alpha-fetoprotein in patients with hepatocellular carcinoma. Dig Dis Sci 2010;55:3576–83.
11. Toyoda H, Kumada T, Tada T, Kaneoka Y, Maeda A, Kanke F, Satomura S. Clinical utility of highly sensitive lens culinaris agglutinin-reactive alpha-fetoprotein in hepatocellular carcinoma patients with alpha-fetoprotein <20 ng/ml. Cancer Sci 2011;102:1025–31.
12. Hoofnagle AN, Becker JO, Oda MN, Cavigiolio G, Mayer P, Vaisar T. Multiple-reaction monitoring-mass spectrometric assays can accurately measure the relative protein abundance in complex mixtures. Clin Chem 2012; 58:777–81.
13. Ackermann BL, Berna MJ. Coupling immunoaffinity techniques with ms for quantitative analysis of low-abundance protein biomarkers. Expert Rev Proteomics 2007;4:175–86.
14. Dufield DR, Radabaugh MR. Online immunoaffinity LC/MS/MS. A general method to increase sensitivity and specificity: how do you do it and what do you need? Methods 2012;56:236–45.
15. Kushnir MM, Rockwood AL, Roberts WL, Abraham D, Hoofnagle AN, Meikle AW. Measurement of thyroglobulin by liquid chromatography-tandem mass spectrometry in serum and plasma in the presence of antithyroglobulin autoantibodies. Clin Chem 2013;59:982–90.
16. Tuck MK, Chan DW, Chia D, Godwin AK, Grizzle WE, Krueger KE, et al. Standard operating procedures for serum and plasma collection: early detection research network consensus statement standard operating procedure integration working group. J Proteome Res 2009;8:113–7.
17. Kim H, Kim K, Jin J, Park J, Yu SJ, Yoon JH, Kim Y. Measurement of glycosylated alpha-fetoprotein improves diagnostic power over the native form in hepatocellular carcinoma. PLoS One 2014;9:e110366.
18. Bellet DH, Wands JR, Isselbacher KJ, Bohuon C. Serum alpha-fetoprotein levels in human disease: perspective from a highly specific monoclonal radioimmunoassay. Proc Natl Acad Sci U S A 1984;81:3869–73.
19. Li D, Mallory T, Satomura S. Afp-l3: a new generation of tumor marker for hepatocellular carcinoma. Clin Chim Acta 2001;313:15–9.
20. Katoh H, Nakamura K, Tanaka T, Satomura S, Matsuura S. Automatic and simultaneous analysis of Lens culinaris agglutinin-reactive alpha-fetoprotein ratio and total alpha-fetoprotein concentration. Anal Chem 1998; 70:2110–4.
21. Yamagata Y, Katoh H, Nakamura K, Tanaka T, Satomura S, Matsuura S. Determination of alpha-fetoprotein concentration based on liquid-phase binding assay using anion exchange chromatography and sulfated peptide introduced antibody. J Immunol Methods 1998;212:161–8.
22. Kagebayashi C, Yamaguchi I, Akinaga A, Kitano H, Yokoyama K, Satomura M, et al. Automated immunoassay system for AFP-l3% using on-chip electrokinetic reaction and separation by affinity electrophoresis. Anal Biochem 2009;388:306–11.
23. Tsuchiya N, Sawada Y, Endo I, Saito K, Uemura Y, Nakatsura T. Biomarkers for the early diagnosis of hepatocellular carcinoma. World J Gastroenterol 2015;21: 10573–83.
24. Drake RR, Schwegler EE, Malik G, Diaz J, Block T, Mehta A, Semmes OJ. Lectin capture strategies combined with mass spectrometry for the discovery of serum glycoprotein biomarkers. Mol Cell Proteomics 2006;5:1957–67.
25. Nakagawa T, Miyoshi E, Yakushijin T, Hiramatsu N, Igura T, Hayashi N, et al. Glycomic analysis of alpha-fetoprotein l3 in hepatoma cell lines and hepatocellular carcinoma patients. J Proteome Res 2008;7:2222–33.
26. Dela Rosa MA, Chen WC, Chen YJ, Obena RP, Chang CH, Capangpangan RY, et al. One-pot two-nanoprobe assay uncovers targeted glycoprotein biosignature. Anal Chem 2017;89:3973–80.
27. Anderson NL, Anderson NG, Haines LR, Hardie DB, Olafson RW, Pearson TW. Mass spectrometric quantitation of peptides and proteins using stable isotope standards and capture by anti-peptide antibodies (SISCAPA). J Proteome Res 2004;3:235–44.
28. Addona TA, Abbatiello SE, Schilling B, Skates SJ, Mani DR, Bunk DM, et al. Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma. Nat Biotechnol 2009;27:633–41.
29. Hoofnagle AN. Quantitative clinical proteomics by liquid chromatography-tandem mass spectrometry: assessing the platform. Clin Chem 2010;56:161–4.
30. McAvoy T, Lassman ME, Spellman DS, Ke Z, Howell BJ, Wong O, et al. Quantification of tau in cerebrospinal fluid by immunoaffinity enrichment and tandem mass spectrometry. Clin Chem 2014;60:683–9.