Fundamental Study of HISCL™ PIVKA-II Reagent for Fully Automated Immunoassay Analyzer HISCL™

Yuji HORIUCHI [1*], Mayumi IKEDA [1*], Shinji OIKAWA [1*], Kenji ABE [2*] and Akira HISHINUMA [1,3*]

[1*] *Clinical Laboratory Center, Dokkyo Medical University Hospital*

[2*] *Clinical Research Department, EDIA Co., Ltd.*

[3*] *Department of Infection Control and Clinical Laboratory Medicine, Dokkyo Medical University*

Key Words PIVKA-II, CLEIA Method, Hepatocellular Carcinoma

INTRODUCTION

Proteins induced by vitamin K absence or antagonists (PIVKAs) are a result of the abnormal synthesis of γ-carboxyglutamic acid (Gla) residues near the N-terminus of a vitamin K-dependent blood coagulation protein, where all or part of the Gla residues appear in the blood as glutamic acid residues. PIVKA-II is the PIVKA form of blood coagulation factor II (prothrombin), which is found in high concentration in the blood of patients with hepatocellular carcinoma. [1)]

PIVKA-II is measured using the MU-3 antibody, a monoclonal antibody that recognizes PIVKA-II. [2)] The MU-3 antibody has strong reactivity to PIVKA-II with a small number of Gla residues. A large amount of accumulated clinical data shows that PIVKA-II specifically increases in patients with hepatocellular carcinoma and is thus useful for assessing response to treatment or assisting in diagnosing the recurrence of the disease. [3)]

HISCL™ PIVKA-II Reagent was developed for the fully automated immunoassay analyzer HISCL™ (Sysmex Corporation, Kobe, Japan), which employs chemiluminescent enzyme immunoassay. In this paper, we report the results of a fundamental study of HISCL and its correlation with other assays using human serum samples.

I. REAGENTS AND METHODS

1. Materials

Human serum samples (purchased from a biotechnology-based company in the United States) were used for a fundamental study of the precision, dilution linearity, and the prozone phenomenon. For the evaluation of the effect of potentially interfering substances, control serum samples with two different concentrations were used. The correlation was examined using 107 human serum samples obtained from Dokkyo Medical University Hospital. We used the residual serum samples that had been anonymized in an unlinkable fashion, in accordance with the Japanese Society of Laboratory Medicine's "policy on the use of leftover laboratory test samples for other tests, education, and research." [4)] The study was approved by the hospital's ethical committee.

2. Devices and Reagents

The device studied was the fully automated immunoassay analyzer HISCL-5000 (Sysmex) with its HISCL PIVKA-II Reagent (HISCL-PIVKA II for CLEIA [EDIA]). Two control assays were used: the electrochemiluminescence immunoassay device PicoLumi with its reagent PicoLumi PIVKA-II MONO (PicoLumi-PIVKA II for ECLIA [EDIA]) and the chemiluminescent enzyme

This article is translated and republished from Japanese Journal of Medicine and Pharmaceutical Science Vol.72 No.9, 2015. (issued in Japan) (Acceptable secondary publication).

immunoassay device LumiPulse® Forte with its reagent "LumiPulse PIVKA-II EISAI" (LumiPulse-PIVKA II for CLEIA [Fujirebio]).

3. Measurement Principle

The HISCL PIVKA-II is a two-step sandwich assay using a PIVKA-II monoclonal antibody (MU-3 antibody) and a prothrombin monoclonal antibody. The measurement principle is based on a CLEIA detection method. The measurement procedures are shown in ***Fig. 1***.

1) Biotin-binding anti-PIVKA-II monoclonal antibodies (MU-3 antibodies) (mouse) in Reagent 1 (R1) specifically react with PIVKA-II in samples. They then bind to streptavidin-binding magnetic particles in Reagent 2 (R2).
2) After removing unreacted liquid, add Reagent 3 (R3). ALP-labeled anti-prothrombin monoclonal antibodies (mouse) specifically react with PIVKA-II on the magnetic particles.
3) After removing unreacted liquid, add Reagents 4 and 5 (R4 and R5). The luminescent substrates CDP-Star™ undergo enzymatic degradation via the ALP on the magnetic particles. Measure the resulting luminescence intensity.
4) The luminescence intensity increases with the concentration of PIVKA-II in the samples. By measuring the PIVKA-II level in samples containing PIVKA-II at known concentrations (HISCL PIVKA-II Calibrators C0 to C5) and preparing calibration curves, the PIVKA-II concentrations in the samples can be measured.

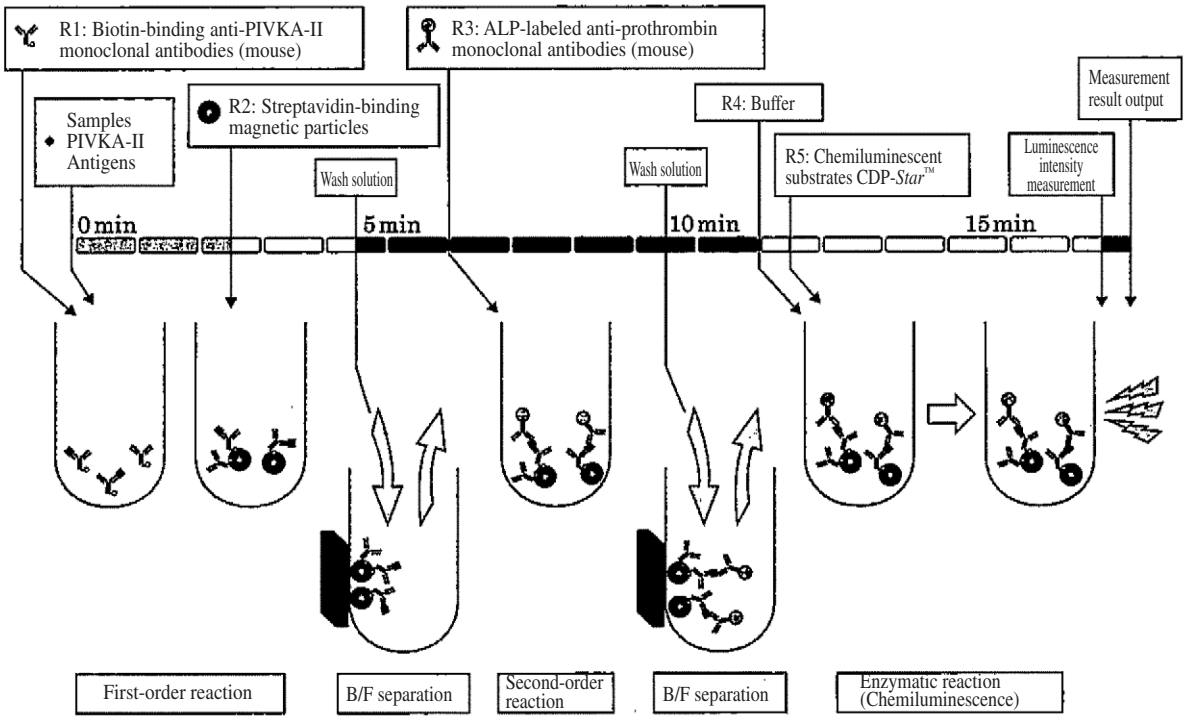

Fig. 1 *Measurement Principle of HISCL PIVKA-II Reagent*

4. Methods

To determine precision, human serum samples with three different concentrations of PIVKA-II were used to assess the within-run reproducibility in 10 repeated measurements.
The between-run reproducibility was also examined by measuring the PIVKA-II concentrations in these samples for 20 days.
For the evaluation of the minimum detection sensitivity, a reference standard with the lowest concentration of PIVKA-II (44 mAU/mL) was diluted using a reference standard with zero concentration of PIVKA-II for calibration curve preparation to obtain a dilution series of four different concentrations, including those of less than 5 mAU/mL.
Using these samples and the reference standard with zero concentration, a total of 10 repeated measurements were made to determine the mean ± 2 Standard deviation (SD) of the luminescence intensity (counts). The minimum detection sensitivity was defined as the concentration at which the mean + 2SD of the luminescence intensity for the reference standard and the mean - 2SD of that for each sample in the dilution series do not overlap.
For a dilution test, three samples with high concentrations of PIVKA-II and a HISCL sample diluent were used to prepare a dilution series of five different concentrations. The PIVKA-II concentration in each of these samples was repeatedly measured three times. Based on the measurements obtained, the dilution linearity was examined.
To assess the presence or absence of the prozone phenomenon, a dilution series was prepared using serum samples showing abnormally high levels of PIVKA-II with a calculated value of approximately 2,000,000 mAU/mL of PIVKA-II (following dilution) and a reference standard with zero concentration for calibration curve preparation. Based on measurements obtained, the effect of the prozone phenomenon was examined.
The effect of interfering substances was examined by adding Interference Check A Plus and RF Plus (Sysmex) to dedicated control sera with two different concentrations (84 and 4,349 mAU/mL) and assessing the variation in measurements.
For the evaluation of the correlation between assays, measurements from 107 human serum samples were compared between PicoLumi PIVKA-II and LumiPulse PIVKA-II.

II. RESULTS

Table 1 shows results on the within-run reproducibility. The mean and coefficient of variation (CV) of the within-run reproducibility were 91.8 mAU/mL and 2.5% for Level 1, 4,877 mAU/mL and 1.4% for Level 2, and 56,957 mAU/mL and 3.0% for Level 3, respectively. ***Table 2*** shows the between-run reproducibility determined using the same samples. The mean and CV were 93.0 mAU/mL and 4.0% for Level 1, 4,812 mAU/mL and 4.1% for Level 2, and 54,009 mAU/mL and 3.4% for Level 3, respectively.

Table 1 *Within-Run Reproducibility*

(mAU/mL)

	Level 1	Level 2	Level 3
1	92	4,829	59,236
2	94	4,899	55,992
3	95	4,856	58,519
4	89	4,969	56,182
5	88	4,915	55,582
6	92	4,761	58,188
7	94	4,986	59,321
8	90	4,834	54,261
9	93	4,859	55,711
10	91	4,864	56,573
Mean	91.8	4,877	56,957
S.D.	2.3	67.3	1,736.2
C.V. (%)	2.5	1.4	3.0

For the determination of the minimum detection sensitivity, 10 repeated measurements were performed on each sample in the dilution series prepared, and the mean and standard deviation of the luminescence intensity were obtained. At a concentration of 2.2 mAU/mL the sample had a measured mean - 2SD of 4,277 counts, a value that did not overlap the mean + 2SD of the luminescence intensity (counts) for the sample with zero concentration (3,157 counts) (***Table 3***).

When the dilution linearity was examined using three samples with a PIVKA-II concentration of 7,000 to 60,000 mAU/mL (Samples A, B, and C), all of these samples showed good linearity converging on the origin up to 60,036 mAU/mL (***Fig. 2***).

When the effect of the prozone phenomenon was examined using samples with abnormally high concentrations of PIVKA-II, no prozone phenomenon was observed up to the upper limit of measurement (75,000 mAU/mL) (***Fig. 3***).

When examining the effect of potentially interfering substances, no change in measurements was shown for bilirubin F up to 19 mg/dL, for bilirubin C up to 21 mg/dL, for hemolyzed hemoglobin up to 490 mg/dL, for chyle up to 1,450 FTU (formazinturbidity unit), and for rheumatoid factor (RF) up to 500 IU/mL, as compared with PIVKA-II-free samples (***Fig. 4***).

Table 2 *Between-Run Reproducibility*

(mAU/mL)

	Level 1	Level 2	Level 3
1	101	4,948	54,365
2	92	4,936	56,097
3	95	5,012	55,667
4	98	5,052	55,948
5	90	5,116	54,547
6	97	5,203	56,136
7	96	4,887	55,602
8	96	4,900	56,094
9	95	4,730	55,551
10	92	4,885	53,401
11	94	4,912	54,799
12	90	4,740	53,122
13	93	4,667	55,356
14	93	4,567	53,505
15	94	4,631	50,481
16	88	4,628	52,195
17	89	4,612	51,807
18	88	4,635	51,333
19	86	4,608	51,131
20	92	4,572	53,041
Mean	93.0	4,812	54,009
S.D.	3.8	196.8	1,862.2
C.V. (%)	4.0	4.1	3.4

Table 3 *Minimum Detection Sensitivity*

	HISCL PIVKA-II (mAU/mL)				
	0	2.2	4.4	8.8	44.0
1	2,937	4,516	6,359	9,764	34,926
2	2,991	4,711	6,195	9,918	34,265
3	3,091	4,573	6,288	9,679	35,090
4	2,926	4,810	6,278	9,289	34,558
5	3,011	4,664	6,221	9,543	33,884
6	3,030	4,637	6,143	9,456	34,373
7	2,926	4,659	6,036	9,582	34,185
8	2,928	4,263	5,971	9,841	33,938
9	3,164	4,454	6,345	9,228	34,625
10	2,956	4,533	6,123	9,645	35,216
Mean (counts)	2,996	4,582	6,196	9,595	34,506
S.D.	80.4	152.5	128.6	224.5	462.7
Mean −2SD		4,277	5,939	9,146	33,581
Mean +2SD	3,157				

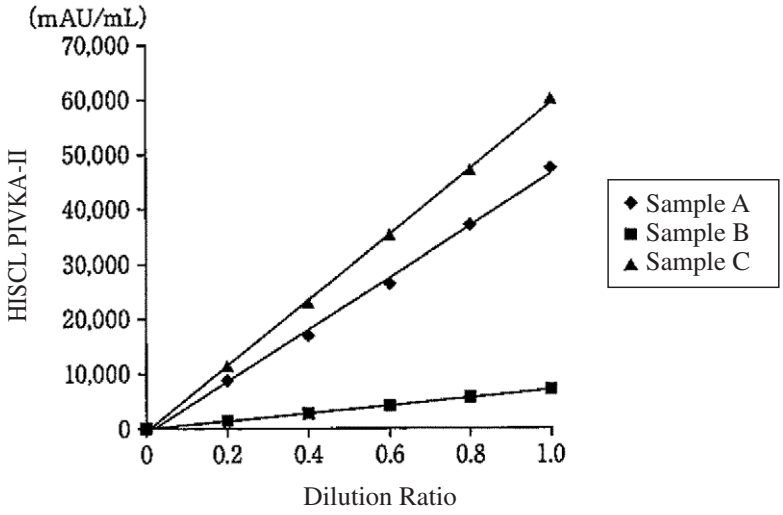

Fig. 2 *Dilution Linearity*

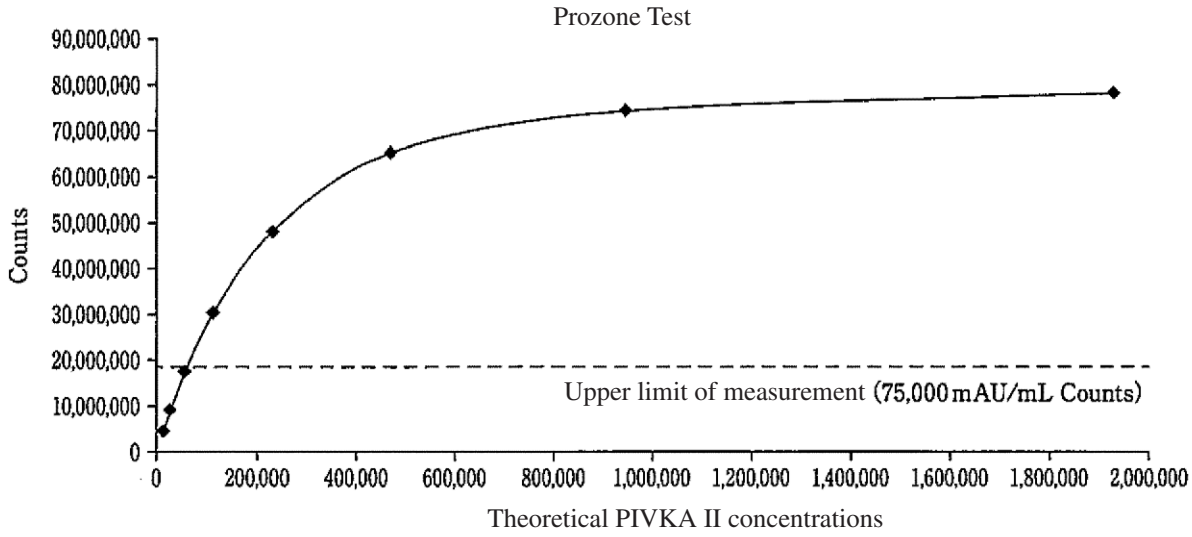

Fig. 3 *Examination of Prozone Phenomenon*

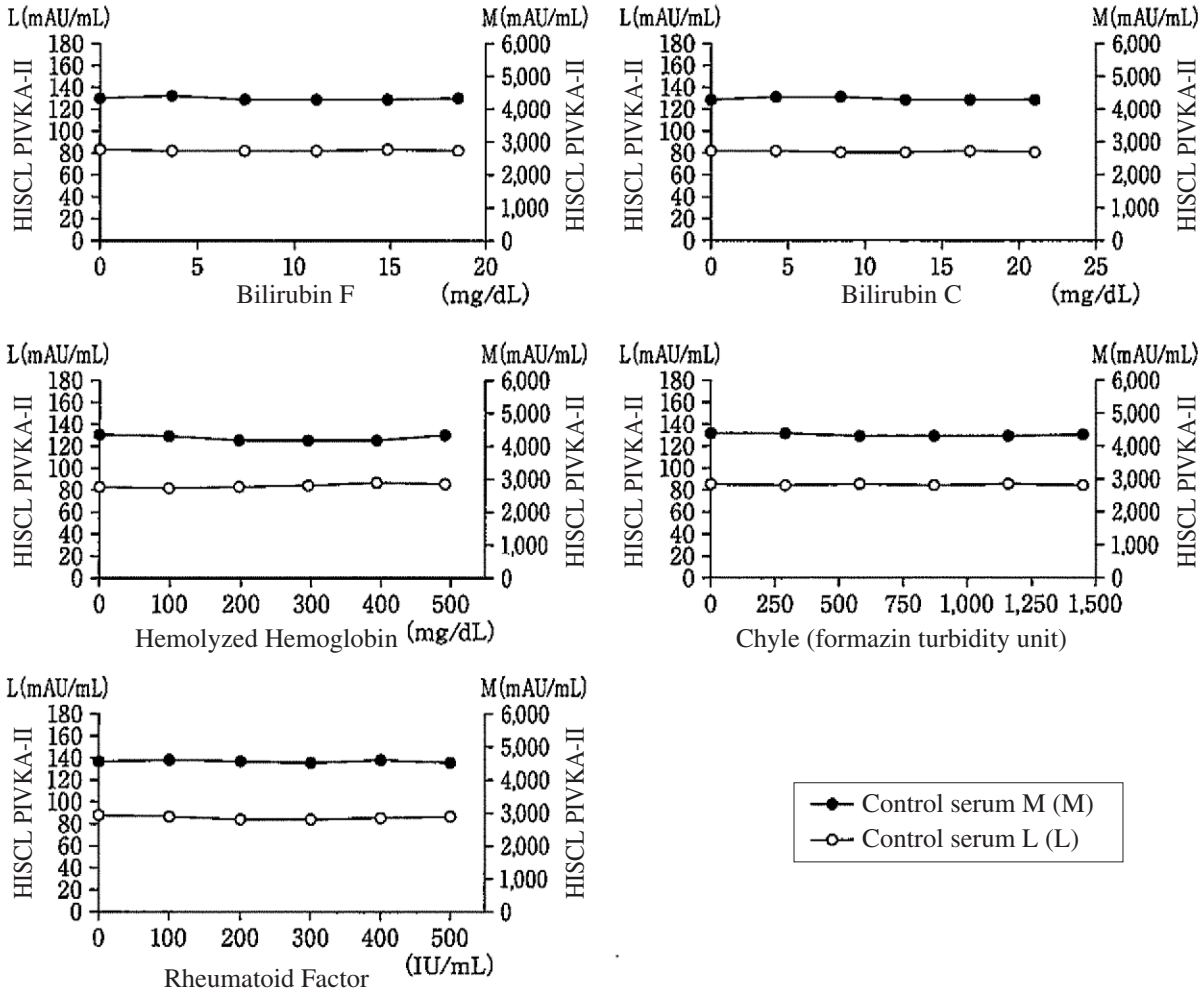

Fig. 4 *Effect of Potentially Interfering Substances*

For the correlation between PicoLumi-PIVKA II (x) and HISCL-PIVKA II (y) in 107 samples with concentrations of PicoLumi-PIVKA II ranging from 10 to 58,177 mAU/mL, the regression equation was y = 0.945x + 21.3 and the correlation coefficient was r = 0.999 (***Fig. 5a***). From these, 57 human serum samples with concentrations of 100 mAU/mL or lower were selected for comparison of measurements near the cutoff value (40 mAU/mL). [5] Results showed that the regression equation was y = 0.913x + 3.1 and the correlation coefficient was r = 0.993 (***Fig. 5b***).

When similar human serum samples were used for the evaluation of the correlation between LumiPulse PIVKA-II (x) and HISCL-PIVKA II (y), the regression equation was y = 1.043x - 11.5 and the correlation coefficient was r = 0.983 (***Fig. 6a***). A comparison of measurements using samples with concentrations of 100 mAU/mL or lower showed that the regression equation was y = 1.048x + 1.1 and the correlation coefficient was r = 0.931 (***Fig. 6b***).

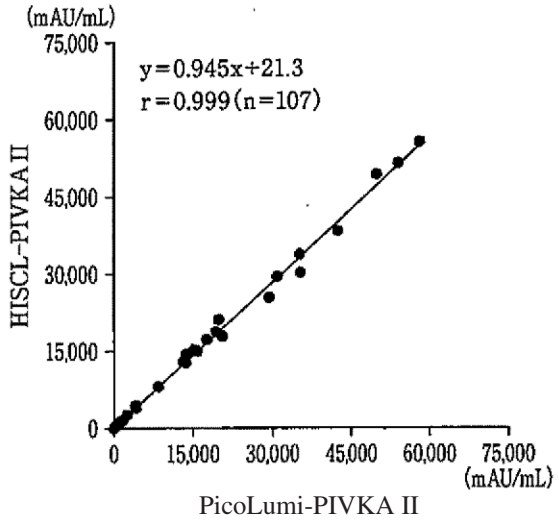

Fig. 5a *Correlation with PicoLumi-PIVKA II*

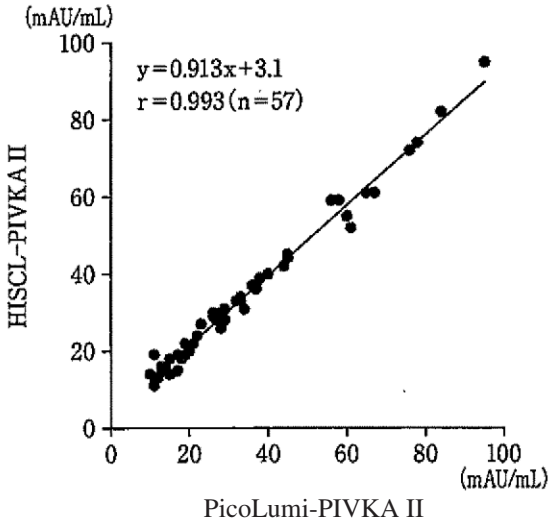

Fig. 5b *Correlation with PicoLumi-PIVKA II (100 mAU/mL or lower)*

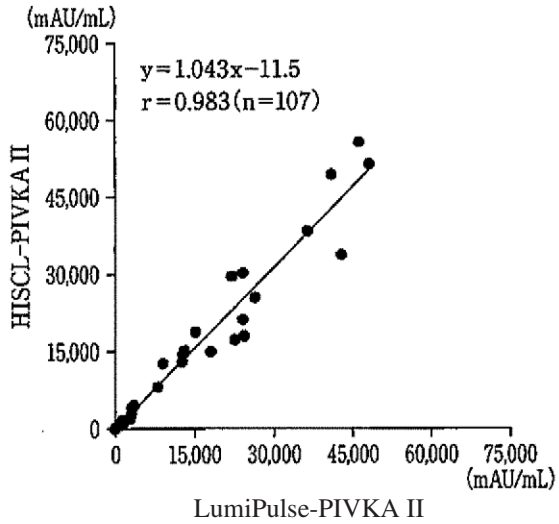

Fig. 6a *Correlation with LumiPulse PIVKA-II*

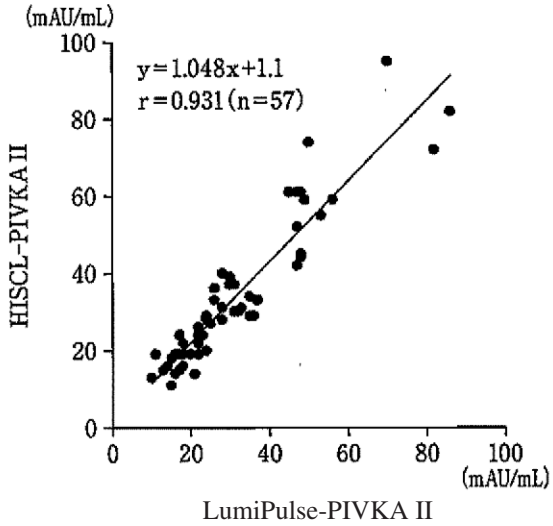

Fig. 6b *Correlation with LumiPulse PIVKA-II (100 mAU/mL or lower)*

III. DISCUSSION

Tumor markers for hepatocellular carcinoma include AFP, the AFPL3 fraction, and the PIVKA-II measurement. Of these, AFP has been most commonly used as a test subject to additional medical fee reimbursement for rapid outpatient laboratory tests. However, AFP alone has a positive predictive rate of 60% [5] in the diagnosis of hepatocellular carcinoma with the use of a cutoff value. Therefore, there has been a need for the concurrent use of AFP and PIVKA-II (positive predictive rate of 84%). [6] The Evidence-based Clinical Practice Guidelines for Hepatocellular Carcinoma 2007 recommends the measurement of at least two tumor markers for the diagnosis of small hepatocellular carcinoma. [7] The concurrent measurement of two test items has been available since the medical service fee revision in 2008. Since then, the number of requests to conduct the PIVKA-II test received by our laboratory has been increasing, with increased demand from clinicians for rapid test result reporting similar to that for AFP.

The fully automated immunoassay analyzer the HISCL-5000 facilitates rapid measurement, with a reaction time of 17 minutes. We conducted a fundamental study of the HISCL PIVKA-II Reagent for the device, and evaluated its usefulness for routine laboratory tests.

In terms of precision, the study showed favorable within- and between-run reproducibility with a CV (%) of 4.1% or lower.

The minimum detection sensitivity was shown to be 2.2 mAU/mL, which enables the measurement of samples with PIVKA-II concentrations lower than the cutoff value of 40 mAU/mL. The minimum reported value was 5 mAU/mL, which was very close to that for other assays. [5]

The dilution linearity was shown to be favorable, converging on the origin up to 60,036 mAU/mL.

The assessment of the prozone phenomenon using samples with abnormally high concentrations of PIVKA-II revealed that the luminescence intensity did not decrease up to the upper limit of measurement (75,000 mAU/mL), indicating the absence of the prozone phenomenon.

None of the following coexisting substances had any effect on the results: bilirubin F, bilirubin C, hemolyzed hemoglobin, chyle, rheumatoid factor.

HISCL-PIVKA II was shown to generally have a good correlation with each of the approved reagents for the control assays, the PicoLumi-PIVKA II (ECLIA) and the LumiPulse PIVKA-II (CLEIA).

CONCLUSION

The HISCL PIVKA-II Reagent for the fully automated immunoassay analyzer HISCL was shown to be useful in routine testing. Due to its characteristics as a rapid measurement method for PIVKA-II, the reagent is useful in the diagnosis and follow-up of hepatocellular carcinoma.

References

1) *Liebman HA, Furie BC, Tong MJ et al: Des-γ-carboxy (abnormal) prothrombin as a serum marker of primary hepatocellular carcinoma. N Engl J Med 310 (22): 1427-1431, 1984.*
2) *Naraki T, Kohno N, Saito H et al: γ-Carboxyglutamic acid content of hepatocellular Carcinoma-associated des-γ-carboxy prothrombin. Biochimica et Biophysica Acta 1586: 287-298, 2002.*
3) *Fujiyama S, Morishita T, Hashiguchi O et al: Plasma abnormal prothrombin (des-γ-carboxy Prothrombin) as a marker of hepatocellular carcinoma. Cancer 61: 1621-1628, 1988.*
4) *Hayashi S, Suehisa E, Asari S, et al.: Informed Consent for the Use of Residual Laboratory Samples for Education/ Research and Precision Control (in Japanese). The Official Journal of Japanese Society of Laboratory Medicine, 49:273-277, 2001.*
5) *Liver Cancer Follow-up Committee, Liver Cancer Study Group of Japan: 19th National Primary Liver Cancer Follow-up Report, Media Planning, Kyoto, 2014.*
6) *Mita Y Aoyagi Y, Yanagi M et al: The usefulness of determining des-γ-carboxy prothrombin by sensitive enzyme immunoassay in the early diagnosis of patients with hepatocellular carcinoma. Cancer 82: 1643-1648, 1998.*
7) *Japan Society of Hepatology: Evidence-based Clinical Practice Guidelines for Hepatocellular Carcinoma 2013 (in Japanese), 41-45, Kanehara & Co., LTD., Tokyo, 2013*