Validation Study of a Domestic Abnormal Prothrombin Diagnostic Reagent: A Multicenter Prospective Clinical Trial

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Abstract

Objective To verify the testing consistency between the domestic abnormal prothrombin diagnostic reagent and the original product. Methods A prospective clinical trial was conducted at three hospitals. From November 15, 2021, to April 24, 2024, a total of 1,329 blood samples were collected from the laboratories of three hospitals. Based on inclusion and exclusion criteria, 1,278 samples met the trial protocol requirements, with 385, 334, and 559 samples included from the three laboratories, respectively. After assigning serial numbers, samples were tested using both the domestic abnormal prothrombin diagnostic reagent (test reagent) and the original abnormal prothrombin diagnostic reagent (control reagent). In accordance with the 'Technical Guidance Principles for Clinical Trials of In Vitro Diagnostic Reagents,' the regression coefficient, intercept, and correlation coefficient were used as clinical evaluation indicators. The Wilcoxon test, linear regression, Passing-Bablok regression, and Bland-Altman analysis were employed to determine testing consistency between the two products. Spearman's method was used for correlation analysis. Univariate and multivariate analyses were applied to identify factors influencing the differences.

Full Text

Validation Study of Domestic Protein Induced by Vitamin K Antagonist-II Diagnostic Reagents: A Multicenter Prospective Clinical Trial

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Abstract

Objective: To verify the detection consistency between domestically produced protein induced by vitamin K antagonist-II (PIVKA-II) diagnostic reagents and the original reference product.

Methods: A prospective clinical trial was conducted across three hospitals. From November 15, 2021, to April 24, 2024, a total of 1,329 blood samples were collected from the laboratories of these three institutions. According to inclusion and exclusion criteria, 1,278 samples met the trial protocol requirements, with 385, 334, and 559 samples included from the three laboratories, respectively. After assigning serial numbers, samples were tested using both the domestic PIVKA-II diagnostic reagent (test reagent) and the original PIVKA-II diagnostic reagent (control reagent). Following the *Guideline of Clinical Trial Techniques for In Vitro Diagnostic Reagents*, regression coefficient, intercept, and correlation coefficient were used as clinical evaluation indices. Wilcoxon test, linear regression, Passing-Bablok regression, and Bland-Altman analysis were employed to determine test consistency between the two products. Spearman correlation analysis was used to assess the correlation between PIVKA-II concentrations detected by the two reagents. Univariate and multivariate analyses were conducted to identify factors influencing differences between the two diagnostic reagents.

Results: All 1,278 protocol-compliant samples were included in statistical analysis, including 483 liver tumor samples. The Wilcoxon test revealed no statistically significant differences between test and control reagents in either the entire population or the liver tumor population (entire population: $Z = -1.414$, $P = 0.157$; liver tumor: $Z = -1.238$, $P = 0.216$). The correlation coefficient between test and control reagents in the entire population was 0.9985. Detection values showed statistically significant differences across gender, laboratory, and disease diagnosis (all $P < 0.05$). Bland-Altman analysis demonstrated good symmetry in both absolute and relative bias plots, with no more than 5% of samples exceeding the 95% consistency limits. Linear regression and Passing-Bablok regression indicated good correlation between the two reagents. Using control reagent results as the independent variable and test reagent results as the dependent variable, the linear regression fitted equation was $y = 1.0007x +$

0.215 ($R^2 = 0.9999$, $P < 0.05$), while the Passing-Bablok regression fitted equation was $y = 1.000x + 0.040$ ($R^2 = 0.9999$, $P < 0.05$). The detection differences between the two reagents exhibited a peak-height distribution. After logarithmic transformation, univariate analysis showed that gender, disease diagnosis, and laboratory factors influenced the differences. Logarithmic transformation and generalized linear regression analysis revealed that disease diagnosis and detection values had statistically significant effects on differences, while interfering samples had no statistically significant impact.

Conclusions: The consistency between the two diagnostic reagents is good, and PIVKA-II detection values and differences are mainly influenced by the patient's disease status.

Key words: Liver neoplasms; Reagent kits, diagnostic; Protein induced by vitamin K antagonist-II; In vitro diagnostic reagent; Clinical trial

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Serum abnormal prothrombin, also known as protein induced by vitamin K absence or antagonist-II (PIVKA-II), is an important indicator for liver tumor diagnosis that complements alpha-fetoprotein. PIVKA-II primarily exists in the serum of liver cancer patients and represents incomplete carboxylation of hepatic prothrombin precursors. When carboxylase inhibitors appear in hepatocytes, serum PIVKA-II levels generally increase, indicating hepatocellular carcinogenesis. PIVKA-II detection can effectively predict liver tumor risk, and the *Guidelines for Diagnosis and Treatment of Primary Liver Cancer (2024 Edition)* recommends PIVKA-II as an early diagnostic marker for liver cancer, particularly in populations with negative serum alpha-fetoprotein. Studies have also shown that PIVKA-II has value in evaluating short-term efficacy of transcatheter arterial chemoembolization (TACE) in liver tumor patients. The diagnostic kit for this indicator was developed and marketed by Abbott Germany GmbH. Due to its high price and special instrument requirements, its 普及性 remains insufficient, making the development of domestic equivalent products aligned with the R&D concept of "unmet clinical needs." The original product's instructions recommend establishing normal value ranges for each laboratory based on sex, age, and ethnicity. However, literature remains divided on the extent to which biological characteristics affect this indicator's levels. Therefore, we conducted a consistency comparison between the original product (control reagent) and the domestic reagent (test reagent) and analyzed factors influencing detection results to provide evidence for rational application in clinical testing.

Methods

Study Design Study Protocol. This diagnostic clinical trial evaluated the consistency between test reagent detection results and control diagnostic reagent results using samples collected at a single time point. The study collected residual blood samples after routine clinical testing. While test reagent detection was performed, subjects also underwent routine clinical diagnosis and laboratory testing. Test reagent results were not used for patient management and did not affect clinical decision-making. The trial was conducted at three hospitals in China—two general hospitals and one specialized cancer hospital. The medical laboratory specialties and principal investigators at all three hospitals completed registration in the National Medical Products Administration's Medical Device Clinical Trial Institution Filing Management Information System before the trial began. The clinical trial protocol was written according to the *Guideline of Clinical Trial Techniques for In Vitro Diagnostic Reagents* and submitted to local drug administration departments for registration before trial initiation, with no updates or revisions during the trial (filing number: 20210030).

Ethics Review. In accordance with the *Regulations on the Management of Human Genetic Resources of the People's Republic of China*, the trial project received approval from the China Human Genetic Resources Management Office before initiation (approval number: Guoke Yiban Shenzi [2021] CJ2266). This study was approved by the Medical Ethics Committee of the Second Hospital of Tianjin Medical University (approval number: 2021K138) and the ethics committees of participating institutions: Shanxi Cancer Hospital Drug and Medical Device Clinical Trial Ethics Committee (approval number: QX2021005) and Inner Mongolia Medical University Affiliated Hospital Ethics Committee (approval number: SY2021042). The study followed the 2013 Declaration of Helsinki. Initiated in 2021, according to Article 39 of the *Measures for Ethical Review of Biomedical Research Involving Humans (2016 Edition)*—"using identifiable human materials or data for research when the subject can no longer be found, and the research project does not involve personal privacy or commercial interests"—this project was approved for waiver of informed consent by the ethics committees.

Quality Control. The National Health Commission's Clinical Testing Center has not yet organized inter-laboratory quality assessment for serum PIVKA-II. However, all three laboratories participate in and pass inter-laboratory quality assessments for other tumor marker projects organized by the National Health Commission's Clinical Testing Center. Both test and control reagents used quality control materials provided with their respective kits for daily quality control and passed. All three laboratories followed a unified trial protocol, using identical reagents and instruments. Participating researchers were authorized by their respective principal investigators and received training on the protocol and sample detection standard operating procedures before trial initiation. Blood samples were tested regularly according to collection and storage periods, with each batch completing calibration, quality control, and testing processes.

Test Materials Sample Size Calculation. With an expected positive rate (π) of 30% and estimated error (E) generally not exceeding 10% (set at 5% for this trial), the minimum sample size per center (n) was calculated using formula (1) at 95% confidence interval: n = 1.962 × 0.3(1-0.3)/0.052 = 322.69. Considering a 5% sample rejection rate, the minimum sample size per clinical trial center was set at 340 cases, with a total minimum sample size of 1,020 across three centers.

Sample Collection. From November 15, 2021, to April 24, 2024, residual serum samples from routine clinical testing or frozen samples for clinical testing were collected from the Second Hospital of Tianjin Medical University, Affiliated Hospital of Inner Mongolia Medical University, and Shanxi Cancer Hospital. Samples were screened based on clinical diagnosis information and potential departments, with eligible samples identified through data entry into case report forms according to inclusion and exclusion criteria.

Diagnostic Information and Sample Requirements: (1) $ $300 samples with positive PIVKA-II detection results (outside normal reference range); (2) $ $540 samples from patients with liver-related diseases (e.g., hepatitis B, cirrhosis) and other diseases including renal insufficiency, hyperlipidemia, osteoporosis, rheumatoid arthritis, respiratory failure, anemia, arrhythmia, pneumonia, intestinal obstruction, cytopenia, lupus erythematosus, cerebral infarction, etc.; (3) $ $40 samples with potential specific interference (hemolysis, lipemia, jaundice, rheumatoid factor, or other types); (4) $ $60 samples positive for potentially interfering tumor markers (including alpha-fetoprotein, CA125, CA153, CA19-9, or other types) from gastric, endometrial, rectal, esophageal, lung, ovarian, breast, nasopharyngeal, bladder, cervical, prostate, and other tumors.

Inclusion Criteria: (1) Complete sample information including sample number, sex, age, and clinical diagnosis; (2) Each serum sample volume $ $0.5 mL; (3) Samples from hospitalized patients, outpatients, and physical examination personnel covering the test reagent's detection range, including high, medium, and low value samples (no proportional requirements, determined based on diagnostic information).

Exclusion Criteria: (1) Contaminated samples; (2) Samples collected at different times from the same patient (retaining the first collected sample, excluding duplicate samples).

Rejection Criteria: (1) Samples with insufficient volume due to operational errors; (2) Samples unable to complete the entire testing process due to other factors; (3) Samples missing any required information from original clinical research records before statistical analysis; (4) Samples deemed necessary to exclude by researchers (e.g., exceeding detection upper limit).

Sample Storage and Coding. Collected residual samples were serum type. Samples were stable at 10-30°C for 24 hours, 2-8°C for 5 days, and below -20°C for 6 months, with no more than 2 freeze-thaw cycles and thorough mixing after thawing. Collected samples were coded by researchers not involved in testing.

Each sample was assigned a unique serial number for testing with both test and control reagents. Laboratory personnel performing tests were blinded to sample diagnostic information.

Test Equipment Diagnostic Reagents. The test reagent was the PIVKA-II diagnostic kit (magnetic particle chemiluminescence method) produced by Tianjin Huaketai Biotechnology Co., Ltd., with batch number 20220415 and linear range 20.00-20,000.00 mAU/mL. Matching calibrator (batch number 20220411) and quality control material (batch number 20220411) were produced by the same company. The control reagent was the PIVKA-II assay kit (chemiluminescent microparticle immunoassay) produced by Abbott Germany GmbH, with batch number 37941LP36 and linear range 0.00-30,000.00 mAU/mL, covering the test reagent's linear range. Matching calibrator (batch number 28945LP23) and quality control material (batch number 28947LP23) were produced by the same company. The control reagent demonstrated good sensitivity and specificity, with linear detection range and sensitivity similar to the test product. The normal reference value for both reagents was <40 mAU/mL.

Detection Instruments. The test reagent was matched with the Shine i2100 automatic chemiluminescence immunoanalyzer produced by Shenzhen Yingkai Biotechnology Co., Ltd. The control reagent was matched with the Alinity i analyzer produced by Abbott Germany GmbH.

Statistical Methods Data were analyzed using R 4.4.2 statistical software. Normally distributed quantitative data were expressed as mean ± standard deviation (s x ±), with independent samples t-test or ANOVA for inter-group comparisons. Non-normally distributed quantitative data were expressed as median (P25, P75), with Mann-Whitney U test or Kruskal-Wallis H test for inter-group comparisons. Categorical data were expressed as frequency and percentage. Comparison of detection values between test and control reagents used paired t-test or Wilcoxon test based on normal distribution. According to the *Guideline of Clinical Trial Techniques for In Vitro Diagnostic Reagents*, correlation analysis of quantitative detection results used Pearson or Spearman correlation based on data distribution normality. Bland-Altman method was used to calculate consistency limits and evaluate consistency between the two detection results. Linear regression and Passing-Bablok regression were used to evaluate consistency between the two detection methods, with control reagent results as the independent variable and test reagent results as the dependent variable. Outliers were assessed by calculating the average of absolute differences between test and control reagent results: $= \Sigma|d\_i|/n$, where d_i is the absolute difference between the two reagents for sample i, with outlier detection limit $= 4$. Similarly, the average of relative differences was calculated: $= \Sigma|r\_i|/n$, where r_i is the relative difference for sample i, with relative outlier detection limit $= 4$. Any sample exceeding both absolute and relative difference detection limits was determined as an outlier. Univariate and multivariate analyses were used

to identify factors influencing detection differences. Two-sided tests were used, with $P < 0.05$ considered statistically significant.

Results

Sample Characteristics A total of 1,329 samples were obtained during the study period. Among them, 48 exceeded detection range, 3 lacked clinical diagnosis, leaving 1,278 samples meeting inclusion criteria. Sample and corresponding patient characteristics are shown in Table 1 . Significant differences in patient sex and diagnosis existed among the three trial centers (all $P < 0.001$). PIVKA-II detection concentrations did not follow normal distribution (Kolmogorov-Smirnov test: $D = 0.406$, $P < 0.001$; Shapiro-Wilk test: $W = 0.368$, $P < 0.001$).

Comparison of Two Reagents Since PIVKA-II detection results were not normally distributed, Spearman correlation analysis was applied. The overall correlation coefficient between test and control reagent detection results was 0.9985. Correlations within each laboratory were also good. Paired Wilcoxon test showed no statistically significant differences between test and control reagents, with no significant differences within each laboratory either (Table 2).

Outlier Analysis Results The average sum of absolute differences between the two reagents was 9.749, with outlier detection limit = 38.994, and 96 samples exceeded the absolute difference detection limit. The average sum of relative differences was 0.018, with relative outlier detection limit = 0.071, and 21 samples exceeded the relative difference detection limit. However, no sample simultaneously exceeded both absolute and relative difference detection limits.

Bland-Altman Analysis Results Both absolute and relative bias plots showed good symmetry (Figure 1 [Figure 1: see original paper]). The absolute difference between the two diagnostic reagents was 0.0400 (-0.5100, 0.5800) mAU/mL, with 95% consistency limits of (-61.212, 63.018) mAU/mL. 4.38% of samples fell outside the 95% consistency limits. The relative difference between the two diagnostic reagents was 0.0013 (-0.0121, 0.0138), with 95% consistency limits of (-0.056, 0.060). 1.88% of samples fell outside the 95% consistency limits.

Regression Analysis Results Scatter plots of test and control reagent detection results are shown in Figure 2 [Figure 2: see original paper]. Using control reagent results as the independent variable and test reagent results as the dependent variable, linear regression yielded the fitted equation: $y = 1.0007x + 0.215$ ($R^2 = 0.9999$, $P < 0.05$). Passing-Bablok regression yielded the fitted equation: $y = 1.000x + 0.040$ ($R^2 = 0.9999$, $P < 0.05$), indicating positive correlation

between the two reagents' test results with good overall and intra-laboratory correlations.

Normality of Detection Differences Absolute detection differences did not follow normal distribution (Kolmogorov-Smirnov test: $D = 0.366$, $P < 0.001$; Shapiro-Wilk test: $W = 0.437$, $P < 0.001$). The absolute difference histogram showed a peak-height distribution, with Q-Q plot showing an S-shape (Figure 3 [Figure 3: see original paper]). Relative differences also did not follow normal distribution (Kolmogorov-Smirnov test: $D = 0.106$, $P < 0.001$; Shapiro-Wilk test: $W = 0.699$, $P < 0.001$). After logarithmic transformation, normality of absolute differences improved in both histogram and Q-Q plot. Absolute differences showed logarithmic correlation with test results: $y = 0.3965\ln(x) - 1.7991$ ($R^2 = 0.7288$).

Factors Associated with Detection Differences Univariate analysis showed that sex, laboratory, and disease diagnosis were not associated with absolute and relative differences between the two reagents (all $P > 0.05$) (Table 3 , Figure 4 [Figure 4: see original paper]). However, after logarithmic transformation of absolute differences, associations with sex, laboratory, and disease diagnosis became significant (all $P < 0.05$). Interfering factors were not associated with detection results (all $P > 0.05$) (Table 4). The selected samples covered all age groups (Table 5). High-density scatter plots of age versus absolute differences are shown in Figure 5 [Figure 5: see original paper]. Spearman correlation analysis showed correlation coefficients of $R = 0.001$ ($P = 0.967$) between age and test reagent, $R = -0.001$ ($P = 0.995$) between age and control reagent, and $R = -0.0241$ ($P = 0.389$) between age and absolute differences between the two reagents.

To reduce multiplicity and collinearity, multivariate analysis examined only absolute differences. Using both logarithmic transformation and Gamma distribution generalized linear regression, and considering univariate analysis results and other study findings, sex, age, disease diagnosis, PIVKA-II detection value, and interference sample variables were included in multivariate analysis. Inter-laboratory differences, being within the scope of inter-laboratory quality assessment, were not included as multivariate analysis variables. Separate multivariate analysis for each laboratory showed that PIVKA-II detection value and disease diagnosis had statistically significant effects on differences (all $P < 0.05$) (Table 6).

Discussion

As a liver tumor marker, PIVKA-II diagnostic reagents must have good sensitivity and specificity, with corresponding clinical trials required before market approval. This study's consistency comparison between domestic and original reagents followed the *Guideline of Clinical Trial Techniques for In Vitro Diagnostic Reagents*, conducting correlation analysis, paired tests, outlier assessment,

Bland-Altman analysis, and regression analysis. Since the difference distribution between the two diagnostic reagents showed a peak-height distribution and did not meet normal distribution assumptions, not fully satisfying Bland-Altman analysis conditions, we nevertheless employed the recommended Bland-Altman analysis given good distribution symmetry, supplemented with segmented statistics. As PIVKA-II detection values showed skewed distribution, both linear regression and Passing-Bablok regression were performed. Compared with linear regression, Passing-Bablok regression does not require normal distribution assumptions and is more suitable for comparing consistency between two reagents. This study was simultaneously conducted in three different types of medical institution laboratories, including both general and specialized cancer hospitals, with collected samples demonstrating considerable diversity. Therefore, consistency analysis examined not only overall consistency between the two reagents but also intra-laboratory consistency, with correlation and regression analyses both indicating good consistency between the diagnostic reagents.

This study also examined whether differences between the two diagnostic reagents were related to age, sex, and laboratory factors. Consistent with reported results in apparently healthy populations, PIVKA-II detection results differed by sex and disease status, with sex distribution varying across diseases. No significant correlation existed between age and detection results. Univariate nonparametric analysis showed these factors were not associated with differences. However, considering that detection difference data did not follow normal distribution, making nonparametric methods less sensitive, logarithmic transformation was applied, after which sex and disease status were not associated with detection differences. Multivariate analysis results showed that after adjusting for detection value effects, sex was not associated with detection differences between the two reagents. Different laboratories in this study enrolled patients with varying disease distributions, which may explain laboratory differences observed in univariate analysis.

From an economic perspective, the control reagent's matching instrument has a winning bid price of approximately 600,000 RMB, while the test reagent's matching instrument, though approved for registration, has no public quotation, with similar products priced around 200,000 RMB. Studies have shown that domestic reagents are generally priced lower than imported reagents. Although the test reagent used in this study has not been priced, the overall trend suggests price advantages for domestic reagents and instruments.

Due to existing literature, this study did not analyze differences in laboratory normal value ranges for apparently healthy populations, focusing instead on target indications, related diseases, and interference populations. Only Chinese population blood samples were included, making it impossible to evaluate the relationship between ethnicity and detection differences. This study's protocol was designed according to guidance principles, examining consistency between domestic and original reagents, with selected samples covering relevant disease subgroups. However, age and sex subgroups were not prespecified, with ex-

ploratory multivariate analysis used during data analysis to evaluate factors associated with differences. All three participating centers followed identical protocols, but tested blood samples only from their own centers without cross-testing, requiring inter-laboratory quality assessment to verify whether laboratory factors affect detection differences.

Conflict of Interest: All authors do not hold stocks in Tianjin Huaketai Biotechnology Co., Ltd., nor do they serve as consultants or provide advisory training to the company. Tianjin Huaketai Biotechnology Co., Ltd. provided reagents, instruments, and trial funding during the trial period without affecting trial implementation or data analysis.

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Author Contributions: Li Xiaohua: study design, statistical analysis, manuscript writing; Men Kun: trial implementation, data collection; Tian Xuezhi, Zhang Baoping: trial implementation, data collection; Cao Yang: study design, protocol review, manuscript revision.

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Note: Figure translations are in progress. See original paper for figures.