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RESEARCH ARTICLE

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Validation of absolutely quantitated Ki67 and cyclinD1 protein levels for prognosis of Luminal-like breast cancer patients

Guohua Yu[1] | Jiahong Lyu[2] | Yalun Li[3] | Yunyun Zhang[2] | Yan Lyu[2] | Wengfeng Zhang[2] | Jianbo Zhang[2] | Bocheng Cai[2] | Jiandi Zhang[2] | Fangrong Tang[2]

[1]Laboratory of Molecular Pathology, Department of Pathology, Affiliated Yantai Yuhuangding Hospital of Qingdao University, Yantai, China

[2]Yantai Quanticision Diagnostics, Inc., A Division of Quanticision Diagnostics, Inc of US, Yantai, China

[3]Department of Breast Surgical Oncology, Affiliated Yantai Yuhuangding Hospital of Qingdao University, Yantai, China

Correspondence
Jiandi Zhang, Yantai Quanticision Diagnostics, Inc., A Division of Quanticision Diagnostics, Inc of US., Yantai 264670, Shandong, China.
Email: jiandi.zhang@outlook.com

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Abstract

Aims: To translate a clinical research finding into daily clinical practice requires well-controlled clinical trials. We have demonstrated the usage of absolute quantitation of Ki67 and cyclinD1 protein levels to improve prognosis of Luminal-like patients based on overall survival (OS) analysis of a cohort of 155 breast cancer specimens (cohort 1). However, this finding is considered the D level of evidence (LOE) to require subsequent validation before it may be used in daily clinical practice. To set the stage for future clinical trials, our findings were validated through OS analysis of an independent cohort (cohort 2) of 173 Luminal-like patients.

Methods: Both Ki67 and cyclinD1 levels were measured absolutely and quantitatively using the Quantitative Dot Blot (QDB) method in cohort 2. The proposed cutoffs for both biomarkers from cohort 1 were re-evaluated in cohort 2 and in the merged cohort of 1 and 2, respectively, through univariate, multivariate and Kaplan–Meier survival analysis.

Results: The proposed cutoffs of 2.31 nmol/g for Ki67 and 0.44 μmol/g for cyclinD1 were validated as effective cutoffs in cohort 2 and the merged cohort through OS analysis. The combined use of both biomarkers allowed us to identify patients with both biomarker levels below the cutoffs (59.3%) with10-year survival probability (SP) of 89%, in comparison to those above the cutoffs (8.3%) with 8 year SP of 28% through OS analysis in the merged cohort.

Conclusions: This study validated our findings that absolute quantitation of Ki67 and cyclinD1 allows effective subtyping of luminal-like patients. It sets the stage for prospective or prospective-retrospective clinical studies.

KEYWORDS
absolute quantitation, breast cancer, cyclinD1, FFPE, Ki67, prognosis, QDB

Guohua Yu, Jiahong Lyu and Yalun Li contributed equally

1 | INTRODUCTION

Both cyclinD1 and Ki67 are frequently used biomarkers of tumor cell proliferation in daily clinical practice. Their expression levels are routinely examined in various cancer types, including prostate, gastric, lung, colorectal and breast cancer.[1,2] While their overexpression is in general associated with poor clinical outcome, the lack of standardization of their assessments significantly limits their usage in daily clinical practice.[3–5]

One possible culprit underlying the ambiguous roles of these biomarkers in clinical diagnosis may be the method used to assess their expression levels: immunohistochemistry (IHC). This method is known to be associated with subjectivity and inconsistency to complicate the interpretation of the prognostic roles of these protein biomarkers.[3,6,7] Yet, the IHC-based biomarker assessment remains the prevailing method in daily clinical practice.

For breast cancer patients, IHC-based surrogate assay is routinely used worldwide to guide patients for cytotoxic therapy. Patients are subtyped into Luminal-like, Her2-positive and Triple negative subtypes based on the assessment of Estrogen Receptor (ER), Progesterone Receptor (PR), Ki67, and Her2 protein levels. Luminal-like patients are further stratified into Luminal A-like ($LumA_i$) and B-like ($LumB_i$) subtypes based on IHC-based Ki67 scores.

However, seeking a practical Ki67 cutoff to stratify Luminal A-like from B-like subtypes in surrogate assay turned out to be one of the biggest challenges for clinicians in the last decade. Over the years, the proposed cutoff for IHC-based Ki67 assessment has changed from 13.25% to 14% to 20%, with 20% as the latest recommendation from St. Gallen consensus.[8–10] Some experts also recommended "5% or less, and 30% and more" recently.[3] Yet, the practicability of this recommendation remains questionable in daily clinical practice.[4,5]

Recently, Quantitative Dot Blot (QDB) method was developed to measure a broad range of protein biomarkers as absolute and continuous variables.[11–15] This method was used to measure Ki67 and cyclinD1 levels absolutely and quantitatively in a cohort of 155 Formalin Fixed Paraffin Embedded (FFPE) luminal-like breast cancer specimens (Cohort 1).[16,17] The absolutely quantitated Ki67 levels were used to replace IHC-based Ki67 scores in surrogate assay, and the patients were stratified using an outcome-based cutoff of 2.31 nmol/g into Luminal A-like ($LumA_q$) and B-like subtype ($LumB_q$). We named this modification as the adjusted surrogate assay.

For comparison, we used Ki67 score at 14% as the cutoff to stratify patients into $LumA_i$ and $LumB_i$ using a surrogate assay and compared the performance of these two methods through overall survival (OS) analysis. We showed that the 10-year survival probability (10y SP) was 91% and 63% ($p = 0.00052$) for $LumA_q$ and $LumB_q$ vs. 88% and 68% ($p = 0.031$) for $LumA_i$ and $LumB_i$ in cohort 1.[16]

Using the same cohort, we further demonstrated that cyclinD1 was an independent negative prognostic factor from Ki67 for Luminal-like breast cancer patients. An outcome-based cyclinD1 cutoff of 0.44 μmol/g was used to separate Luminal-like patients into cyclinD1 high (C_h) and cyclinD1 low subgroup (C_l). The subgroup with the expression levels of both biomarkers above the cutoffs (K_hC_h) had extremely poor prognosis with 8y SP at 26%.[17]

While these findings showed promise in daily clinical practice, they are considered level 4, or level D, for Level of Evidence (LOE) to support the clinical utility of a clinical biomarker.[18,19] These studies are considered "the results very likely to be play by chance".[18] Randomly controlled prospective (Level A) or retrospective-prospective (Level B) clinical trial is needed to validate these findings before they can be used to guide daily clinical practice. To set the stage up for future clinical trials, we needed to validate our findings, including the effectiveness of our proposed quantitative cutoffs, in an independent patient cohort. In this study, we reported our validation efforts through OS analysis of an independent cohort of 173 FFPE Luminal-like specimens from another hospital (Cohort 2).

2 | MATERIALS AND METHODS

2.1 | Human subjects and human cell lines

The inclusion criteria for this retrospective observational study was female patients diagnosed with breast cancer with FFPE specimen available at Yuhuangding Hospital from Jan 5th to Dec 30th, 2010 consecutively and nonselectively. The specimens were provided as 2×15 μm slices with minimum 50% tumor tissue based on H & E staining. A total of 246 FFPE breast cancer tissues were collected and were assigned as cohort 2. Follow-up data was available for 206 patients (83.7%) with the last follow up on Mar 31st, 2020. Cohort 1 and cell line controls are described elsewhere.[16]

All but seven patients received adjuvant therapy. The others received chemo endocrine therapy prior to mastectomy neoadjuvant therapy. Clinical information, including age, pathological lymph node status, pathological tumor size, histological grade, type of treatment [chemotherapy (Chemo), endocrine therapy (Endo), or chemo endocrine therapy (Endo & Chemo)], was collected from medical records. The end point was OS, defined as the time between breast cancer surgery and death or the last follow-up. All the missing values were treated as a new category. The cases lost to follow up were not included in the analysis. Patients still alive at the last study follow up (March 31st, 2020) were censored.

2.2 | General reagents

The general reagents for cell culture were purchased from Thermo Fisher Scientifics . The chemicals used for protein expression were purchased from Takara Inc., and the Nickel-His GraviTrap affinity column for protein purification was purchased from GE Healthcare. All the other chemicals were purchased from Sinopharm Chemicals.

Both Mouse anti-Ki67 antibody (clone MIB1) and Rabbit anti-cyclinD1 antibody (clone EP12) were purchased from ZSGB-BIO. HRP-labeled Donkey Anti-Rabbit IgG secondary antibody was purchased from Jackson Immunoresearch lab. Pierce BCA protein

quantification kit was purchased from Thermo Fisher Scientific Inc. Recombinant human Ki67 and cyclinD1 proteins were prepared in the house. QDB plate was manufactured by Quanticision Diagnostics Inc. at RTP, NC, USA.

2.3 | Preparation of FFPE and Cell Lysates

To extract total protein, two 15 μm FFPE slices were first deparaffinized and then solubilized with lysis buffer (50 mM HEPES, 137 mM NaCl, 5 mM EDTA, 1 mM $MgCl_2$, 10 mM $Na_2P_2O_7$, 1% Triton X-100, and 10% glycerol). Total protein concentration was measured using Pierce BCA protein assay kit in accordance with the manufacturer's instructions. BT474 and 293T cells were fixed in Formalin Solution for 30 mins before they were lysed in the same lysis buffer. The supernatants were collected after centrifugation and the total amount of proteins was measured using BCA protein assay kit by following the manufacturer's instructions.

2.4 | QDB analysis

The QDB process and the purification processes of recombinant Ki67 and cyclinD1 protein standards were described in detail elsewhere.[16,17] In short, the final concentration of the FFPE tissue lysates was adjusted to 0.25 μg/μl for Ki67, 0.175 μg/μl for cyclinD1 and 2 μl/unit was used for QDB analysis as well as a serially diluted recombinant protein in triplicate. The loaded QDB plate was dried for 1 h at RT and then blocked in 4% non-fat milk for an hour. Primary antibody was diluted in blocking buffer [Anti-Ki67 antibody (MIB1): 1:1000; Anti-cyclinD1 antibody (EP12): 1:500], and incubated with QDB plate at 100 μl/well overnight at 4°C. The plate was incubated next with a donkey anti-mouse secondary antibody [1:2000] for 4 h at RT. The QDB plate was inserted into a white 96-well plate prefilled with 100 μl/well ECL working solution for 3 min for quantification with Tecan Infiniti 200pro Microplate reader with the option "plate with cover".

2.5 | Statistical analysis

All the statistical analyses were performed with R version 3.6.2, using two-side statistical test. Missing values in discrete data were defined as a new category. The results were reported as mean ± standard error of the mean (SEM). p values of less than 0.05 were considered statistically significant.

The Ki67 and cyclinD1 levels measured by the QDB method were dichotomized for OS by using cutoff of 2.31 nmol/g for Ki67 and 0.44 μmol/g for cyclinD1. All the OS analyses were visualized by Kaplan–Meier method, and comparisons were performed by Log Rank test.

Univariate Cox proportional hazard models fitted for OS were employed for hazard ratio (HR) and the corresponding 95% confidence intervals (CIs) estimation. Multivariable Cox models were utilized to examine the association between subtypes and OS, adjusting for other clinical variables, such as age, pathological node status, pathological tumor size, histological grade, and type of treatment. Residuals that are analogous to the Schoenfeld residuals in Cox models were used to check the proportionality assumption.

3 | RESULTS

3.1 | Clinicopathological characteristics of the patients

The Luminal-like specimens were defined as those with ER/PR >1% based on IHC analysis. For cohort 2, among 173 Luminal-like specimens, follow-up data was available for 147 (85%) at the last follow up. The endpoint was OS of the patients, with the median time to censoring at 116 months, and the maximum at 122 months. Among 147 Luminal-like patients with follow-up data, there were 107 patients who received mastectomy including seven receiving chemo endocrine therapy prior to surgery, and 40 received breast-conserving surgery. Ninety patients were at pre-menopausal and 83 patients at postmenopausal stage. Among all patients, 26 patients were also dignosed with hypertension, four with diabetes, one with both hypertension and diabetes, four with other types of tumors, and 32 with other medical complications including appendicitis, Parkinson's disease and fibroids.

The flow chart of cohort 2 is shown in Figure 1. The clinicopathological factors of cohorts 1 and 2 and the merged cohort were listed in Table 1. As shown in Table 1, there were significant differences between these two cohorts in almost every aspect, including pathological tumor size, histological grade, and expression levels of several protein biomarkers (ER, Her2 and Ki67) assessed by IHC analysis. These drastic differences underscored the limitation of retrospective studies where specimens cannot be properly controlled.

In addition, the majority of patients in cohort 2 received endocrine (Endo) or chemo endocrine therapies (Endo & Chemo) while the majority of patients in cohort 1 received chemotherapy (Chemo). Some of the patients in cohort 2 also received aromatase inhibitors (Letrozole or Anastrozole tablet) as part of endocrine therapy. The chemotherapy regimens in cohort 1 were also different from cohort 2 as noted in Table 1.

The absolute levels of both Ki67 and cyclinD1 of each specimen in cohort 2 were measured using QDB method, and compared with those from cohort 1 (Figure 2). When analyzed using the Student's t test, the difference between Ki67 levels of cohort 1 (0 ~ 14.79 nmol/g, mean = 2.56 nmol/g) and cohort 2 (0 ~ 9.10 nmol/g, mean = 1.26 nmol/g) were statistically significant, with $p < 0.0001$ (Figure 2A). The Ki67 scores from IHC analysis in cohort 2 were ranging from 1% to 100%, with mean at 28.86%. Likewise, Ki67 scores were also different between cohorts 1 and 2 with statistical significance, with $p < 0.0001$ using the Student's t test (Figure 2B). The absolutely measured Ki67 levels from QDB method in cohort

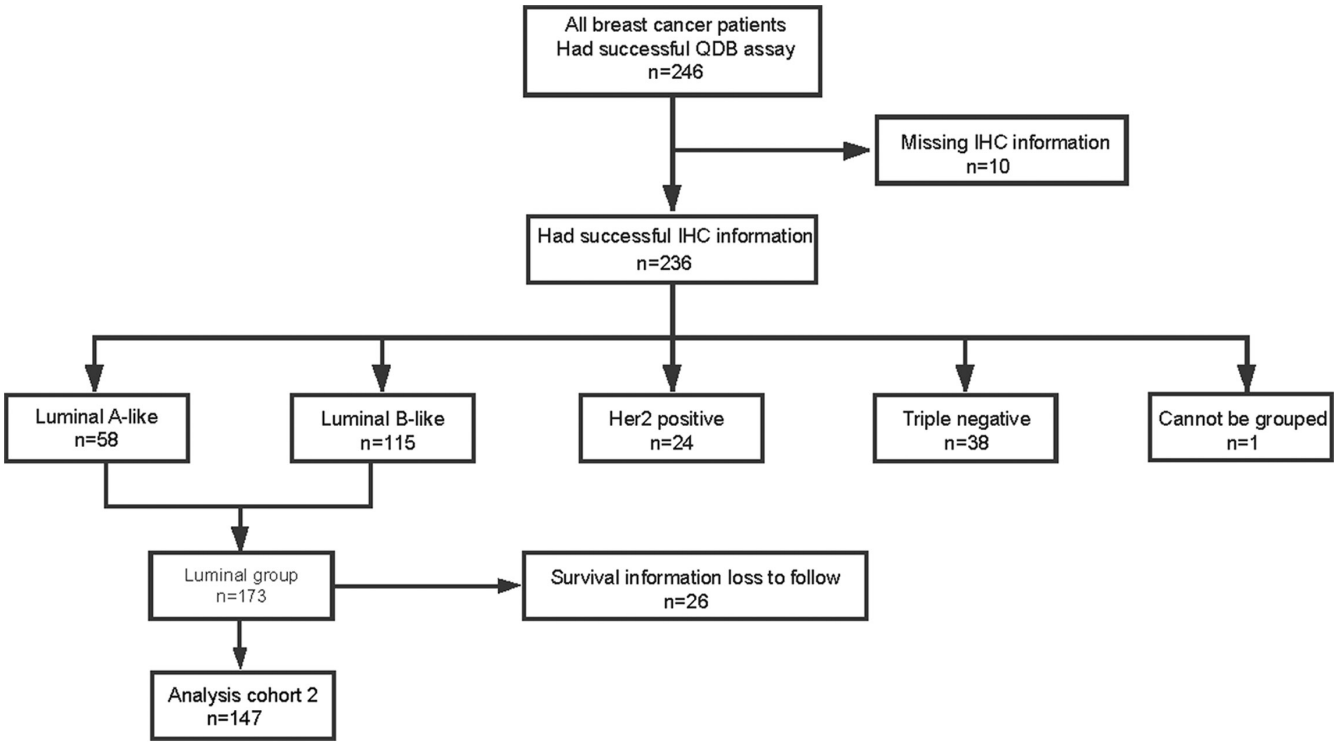

FIGURE 1 Flow chart of the cohort 2 specimens

2 were moderately correlated with Ki67 scores from IHC analysis, with $\rho = 0.53$, $p < 0.0001$ when analyzed with Spearman's correlation analysis (Figure 2C).

There was no statistically significant difference of cyclinD1 levels between cohort 1 (0.02 ~ 3.77 μmol/g, mean = 0.32 μmol/g) and cohort 2 (0.02 ~ 5.02 μmol/g, mean = 0.26 μmol/g) when analyzed with the Student's t test (Figure 2D). There was a moderate correlation between the absolute levels of cyclinD1 and Ki67 among specimens in cohort 2 when analyzed using Spearman's correlation analysis, with $\rho = 0.35$, $p < 0.0001$(Figure 2E). The moderate correlation between these two biomarkers were also observed in cohort 1, with $\rho = 0.30$, $p = 0.0003$.[17]

3.2 | Validation of 2.31 nmol/g as the optimized Ki67 cutoff for adjusted surrogate assay

The proposed 2.31 nmol/g cutoff for Ki67 defined in a previous study[16] was used to stratify specimens from cohort 2 into $LumA_q$ and $LumB_q$ in adjusted surrogate assay based on their absolutely quantitated Ki67 levels. For comparison, the same cohort was stratified based on their Ki67 scores using 14% as cutoff in surrogate assay as suggested by the latest St. Gallen consensus[10] (Figure 3A, C). The Kaplan–Meier survival analysis was used to evaluate the performance of these two assays in the prognosis of OS for Luminal-like patients. As shown in Figure 3A, B, we found that the number of $LumA_q$ specimens ($n = 107$) in adjusted surrogate assay were almost doubled over those of $LumA_i$ in surrogate assay ($n = 51$), with improved 10y SP to 88% from 84%. Consequently, the number of $LumB_q$ specimens reduced drastically to 40 from 96 for $LumB_i$, with 10y SP reduced to 74% from 84%. We calculated p value of 0.055 from adjusted surrogate assay vs. 0.96 for surrogate assay using the Log Rank test.

Both Ki67 and cyclinD1 were measured absolutely and quantitatively in cohorts 1 and 2. Thus, we combined these two cohorts into a merged cohort with 328 FFPE specimens. We again stratified the merged cohort into Luminal A-like and B-like subtypes using surrogate assay ($LumA_i$ vs. $LumB_i$) and adjusted surrogate assay ($LumA_q$ vs. $LumB_q$), respectively. OS analysis showed that surrogate assay was unable to separate $LumA_i$ from $LumB_i$ effectively ($p = 0.15$ from Log Rank test) (Figure 3C). However, adjusted surrogate assay was able to separate these two subtypes effectively with $p < 0.0001$ from Log Rank test (Figure 3D). More importantly, we observed improvements in both number (from 112 to 175) and 10y SP (85% to 88%) in $LumA_i$ vs. $LumA_q$ subtype, and reduction in number (178 to 115) and 10y SP (77% to 67%) in $LumB_i$ vs. $LumB_q$ subtype.

The prognostic effects of both surrogate assay and adjusted surrogate assay were also analyzed using both the univariate and multivariate cox regression analysis of OS of the merged cohort (Table S1 & Table S2). In both analyses, only the adjusted surrogate assay was identified as an independent prognostic factor, with HR at 3.24 (95% CI: 1.84–5.71, $p < 0.0001$) in univariate cox regression analysis, and HR at 4.52 (95% CI: 2.39–8.55), $p < 0.0001$), independent from age and pathological node status in multivariate cox regression analysis.

We also used Ki67 score of 20% as the cutoff in surrogate assay to see if this drastic difference may be attributed to wrong choice of cutoff. We observed 10y SP for $LumA_i$ at 84% ($n = 59$)

TABLE 1 Clinicopathological characteristics of cohort 1, cohort 2 and the merged cohort

Characteristics	Level	Cohort 1	Cohort 2	Merged cohort	*p*-value
N		155	173	328	
Age (median [IQR])		52.0 [45.0, 59.0]	52.0 [45.0, 58.0]	52.0 [45.0, 59.0]	0.429
Pathological Lymph Node Status, pN	pN0	77 (51.0)	104 (60.1)	181 (55.9)	0.272
	pN1	56 (37.1)	48 (27.7)	104 (32.1)	
	pN2	10 (6.6)	14 (8.1)	24 (7.4)	
	pN3	8 (5.3)	7 (4.0)	15 (4.6)	
	Unknown * Dagger	4	0	4	
Pathological Tumor Size, pT	pT1	54 (35.5)	103 (59.5)	157 (48.3)	<0.001
	pT2	91 (59.9)	69 (39.9)	160 (49.2)	
	pT3	7 (4.6)	1 (0.6)	8 (2.5)	
	Unknown * Dagger	3	0	3	
Histological Grade	G1	0 (0.0)	45 (28.5)	45 (15.3)	<0.001
	G2	84 (61.3)	98 (62.0)	182 (61.7)	
	G3	53 (38.7)	15 (9.5)	68 (23.1)	
	Unknown * Dagger	18	15	33	
Treatment Type # Dagger	Endo	2 (1.5)[1a]	41 (31.1)[1b]	43 (16.2)	
	Chemo	90 (67.7)[2a]	4 (3.0)[2b]	94 (35.5)	
	Chemo&Endo	41 (30.8)	87 (65.9)	128 (48.3)	<0.001
	Unknown * Dagger	22	41	63	
Subtype (Surrogate assay)	Luminal A	66 (42.6)	58 (33.5)	124 (37.8)	0.115
	Luminal B	89 (57.4)	115 (66.5)	204 (62.2)	
Her2 (by IHC)	0	23(14.8)	154(89.0)	177(54.0)	<0.001
	1	96(61.9)	4(2.3)	100(30.5)	
	2	21(13.5)	8(4.6)	29(8.8)	
	3	15(9.7)	7(4.0)	22(6.7)	
Ki67 (by IHC) Median [IQR]	Discrete	8.3[4.0,15.0]	20.0[10.0,50.0]	11.7[5.0,30.0]	<0.001
ER (by IHC) Median [IQR]	Discrete	80.0[70.0,90.0]	50.0[50.0,75.0]	70.0[50.0,80.0]	<0.001
PR (by IHC) Median [IQR]	Discrete	30.0[0.0,70.0]	50.0[25.0,75.0]	50.0[20.0,75.0]	0.066

Abbreviations: Chemo: chemotherapy; Chemo&Endo: chemo endocrine therapy; Endo: endocrine therapy; ER: estrogen receptor; IHC: immunohistochemistry; IQR: interquartile range; PR: progesterone receptor.

Note: # Dagger The treatment plans were developed by physicians by following the guidance issued by the Chinese Anti-Cancer Association (CACA) in 2007 at with variations at each hospital.[20] 1a: Tamoxifen or toremifene citrate tablet; 1b: Tamoxifen or Toremifene citrate tablet or aromatase inhibitor (Letrozole or Anastrozole tablet). 2a: CAF (cyclophosphamide, doxorubicin hydrochloride, and fluorouracil) or CMF (cyclophosphamide, methotrexate, and fluorouracil) or TAC (Doxorubicin Hydrochloride and cyclophosphamide with or followed by Docetaxel); 2b: TC (Taxotere and cyclophosphamide) or TE (paclitaxel/docetaxel and epirubicin) or EC (Epirubicin and cytoxan) or TEC (paclitaxel/docetaxel and epirubicin and Cytoxan) 3: one regimen from 2 followed by one regimen from 1; 4: non-standard treatments including Chinese traditional medicine or informed refusal by patients.

Note: * Dagger unknown was not treated as a category in the analysis.

vs. 84% (n = 88) for $LumB_i$, with p = 0.94 for cohort 2, and 81% (n = 165) vs. 79% (n = 125), with p = 0.86 for the merged cohort (Figure S1).

We further stratified the merged cohort based on the treatments these patients received, and evaluated the impact of the treatment on the prognosis of adjusted surrogate assay. Among patients receiving chemoendocrine therapy, the 10y SP for $LumA_q$ (n = 77) was 91% vs. 67% for $LumB_q$ (n = 40), with p = 0.0015 from Log Rank test. For patients receiving chemotherapy alone, the 10y SP for $LumA_q$ (n = 41) was 100% vs. 56% for $LumB_q$ (n = 46), with p < 0.0001 from Log Rank test. For patients receiving endocrine therapy alone, the 10y SP for $LumA_q$ (n = 25) was 87% vs. 55% for $LumB_q$ (n = 11), with p = 0.032 from Log Rank test. (Figure S2A–C).

The pathological node status also had a minimal impact on the prognosis of adjusted surrogate assay among patients in the merged cohort. Among pN_0 patients, the 10y SP was 97% for $LumA_q$ (n = 100) vs. 86% for $LumB_q$ (n = 51), with p = 0.02 from Log Rank test. For pN_1 patients, the 10y SP for $LumA_q$ (n = 52) was 87%, vs. 59% for $LumB_q$ (n = 48), with p = 0.0019 from Log Rank test (Figure S2D–E).

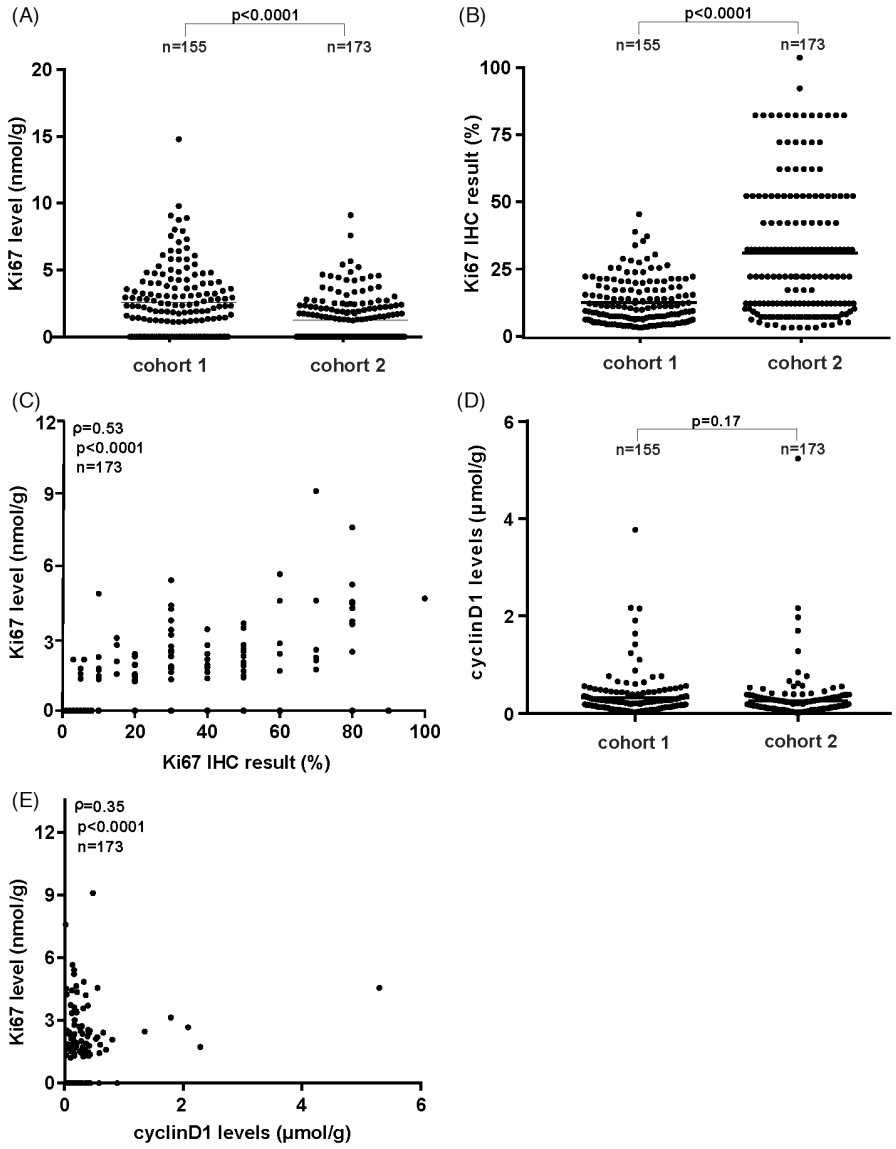

FIGURE 2 Ki67 & cyclinD1 levels in both cohorts 1 and 2. The Ki67 and cyclinD1 levels were measured using QDB method, and plotted against those from cohort 1 in the figures. (A) Distribution of absolutely quantitated Ki67 levels measured using QDB method in cohort 1 and cohort 2. There was statistical significant difference between these two cohorts, with $p < 0.0001$ from Student's t test. (B) Distribution of Ki67 scores assessed by immunohistochemistry in cohorts 1 and 2. There was a statistically significant difference between these two cohorts, with $p < 0.0001$ from Student's t test. (C) The correlation between absolutely quantitated Ki67 levels and Ki67 IHC scores analyzed using Spearman's correlation analysis, with $\rho = 0.53$, $p < 0.0001$. (D) Distribution of absolutely quantitated cyclinD1 levels in cohorts 1 and 2. (E) The correlation between absolutely quantitated Ki67 and cyclinD1 in cohort 2, assessed using Spearman's correlation analysis, with $\rho = 0.35$, $p < 0.0001$

3.3 | Evaluation of the independent prognostic role of cyclinD1 in the merged cohort

While the prognostic roles of both Ki67 and cyclinD1 were demonstrated in cohort 1 with univariate and multivariate OS analysis, we further evaluated their prognostic significance in cohort 2. However, only cyclinD1 was found to have a significant prognostic role in the univariate cox regression OS analysis. Neither of these two biomarkers were found of prognostic significance in multivariate cox regression OS analysis (Table S3).

In the merged cohort (Table 2), consistent with what we observed in cohort 1, both Ki67 and cyclinD1 were found to be negative prognostic factors, with HR at 1.21 (95% CI: 1.10–1.32, $p = 0.0001$) and 1.70 (95% CI: 1.30–2.23, $p = 0.0001$) respectively, together with age, pathological lymph node statuses and pathological tumor sizes in univariate OS analysis. Furthermore, Ki67 was found to be independent from cyclinD1 in multivariate OS analysis, with HR at 1.16 (95% CI: 1.04–1.29, $p = 0.0085$) and 1.58 (95% CI: 1.18–2.13, $p = 0.0023$), respectively. Also, both age and pathological lymph node statuses were found to be independent prognostic factors.

3.4 | Validation of 0.44 μmol/g as the optimized cyclinD1 cutoff for stratification of the patients

Having demonstrated that cyclinD1 was an independent prognostic factor from Ki67 in the merged cohort, we further evaluated the 0.44 μmol/g cutoff derived from cohort 1 as the optimized cutoff to stratify cohort 2 patients into cyclinD1 low (C_l) and cyclinD1 high (C_h) groups. The proposed cutoff was used in combination with Ki67 at 2.31 nmol/g to stratify cohort 2 specimens into four subgroups. We used C_lK_l, to indicate the subgroup with both biomarker levels below the proposed cutoffs, C_hK_h, for those with both biomarker levels above the proposed cutoffs, C_hK_l, for those with only Ki67 level below the proposed cutoff and C_lK_h, for those with only

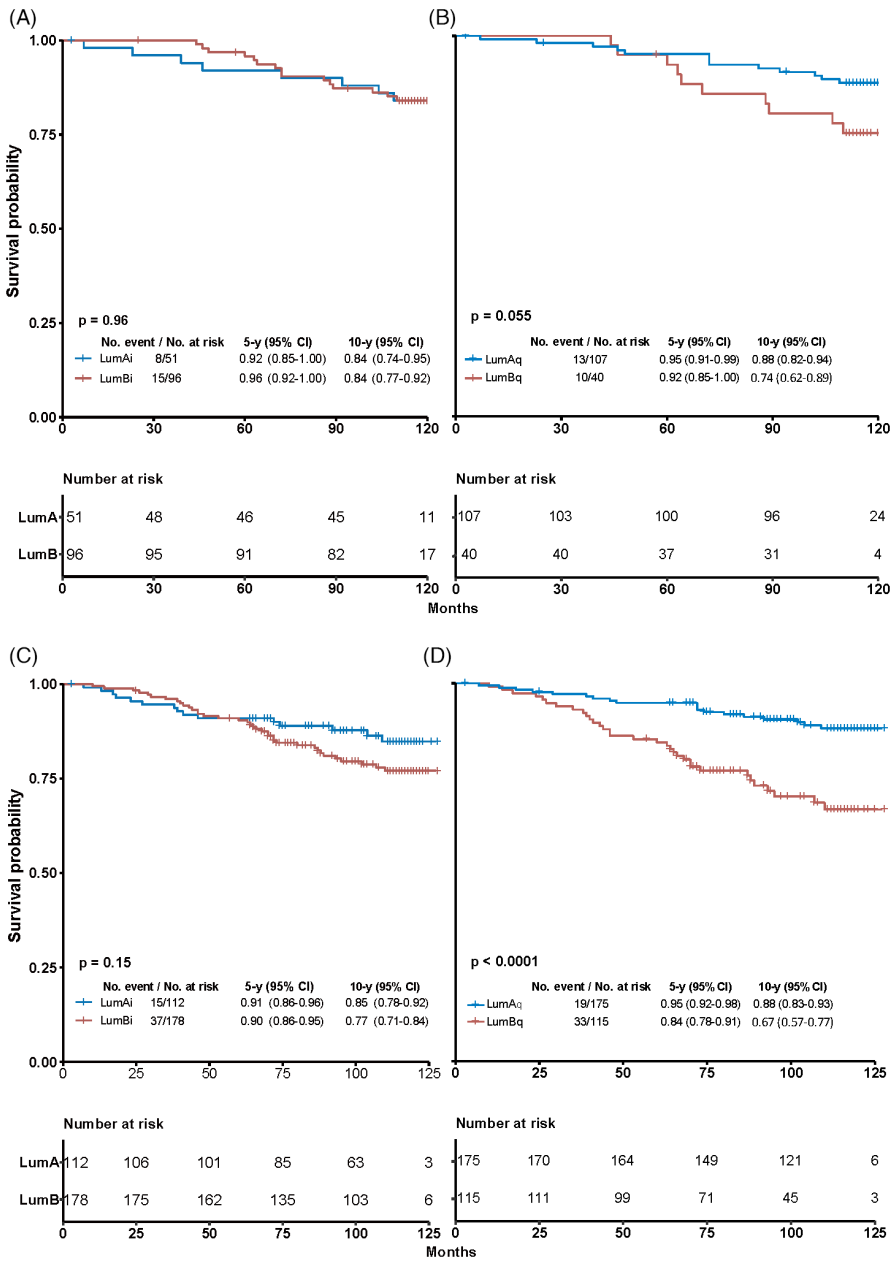

FIGURE 3 Validation of optimized cutoff of Ki67 at 2.31 nmol/g for adjusted surrogate assay. Overall survival (OS) analysis of a validate set (cohort 2) and the merged cohort (cohort 1 and cohort 2) by surrogate assay or adjusted surrogate assay. (A) and (C): The Ki67 score of 14% was used as cutoff in surrogate assay based on Recommendations from 2013 St. Gallen Consensus. (B) and (D): The Ki67 level of 2.31 nmol/g was used as cutoff in adjusted surrogate assay as defined in a previous study. The 5y and 10y SP, and the *p* values from Log Rank test were provided for both surrogate assay and adjusted surrogate assay, respectively

cyclinD1 level below the proposed cutoff. The OS analysis was performed with Kaplan–Meier survival analysis, and we were able to achieve a statistical significance with $p = 0.00034$ from Log Rank test, with 10y SP for C_lK_l ($n = 107$) at 89%, and 8y SP for C_hK_h ($n = 6$) at 33% (Figure 4A).

The same cutoffs were again used to stratify the merged cohort into four subgroups (Figure 4B). OS analysis suggested that these cutoffs were able to separate the specimens with statistical significance ($p < 0.0001$), with 10y SP at 89% ($n = 172$) for C_lK_l subgroup, and 8y SP at 28% for C_hK_h subgroup ($n = 24$).

The optimized cutoffs for Ki67 and cyclinD1 were also identified independently in the merged cohort using the same outcome-based method ("surv_cutpoint" function of the "suvminer" R package). The 2.31 nmol/g was again identified as the optimized Ki67 cutoff in the merged cohort. However, for cyclinD1, we identified 0.52 μmol/g as the optimized cutoff for the merged cohort. When this cutoff was combined with 2.31 nmol/g for Ki67 to stratify the merged cohort into C_lK_l, C_lK_h, C_hK_l and C_hK_h subgroups, we obtained the 10y SP at 89% ($n = 175$) for C_lK_l subgroup, and the 8y SP at 27% ($n = 18$) for C_hK_h subgroup, with $p < 0.0001$ from the Log Rank test. (Figure S3).

In the adjusted surrogate assay, we identified 175 patients as $LumA_q$ with 10y SP at 88% in merged cohort (Figure 3D). When we stratified the same cohort with both cyclinD1 and Ki67, we identified 172 patients of C_lK_l with 10y SP at 89% (Figure 4B). The high similarity in both number and 10y SP of these two subgroups led us to compare them individually. We found over 90% ($n = 158$) patients were included in both subgroups. Thus, the combined use of cyclinD1 and Ki67 might be viewed as further stratification of $LumB_q$ subtype to identify a high risk C_hK_h subgroup in surrogate assay.

TABLE 2 Univariate and Multivariate Cox regression of Overall Survival (OS) with both Ki67 and cyclinD1 in merged cohort

	Univariate			Multivariate		
Variable	**HR**	**95% CI**	***p* value**	**HR**	**95% CI**	***p* value**
Age	1.05	1.03–1.08	<0.0001	1.05	1.02–1.08	0.0003
Pathological Lymph Node Status, pN	2.21	1.78–2.73	<0.0001	2.00	1.58–2.54	<0.0001
Pathological Tumor size, pT	1.67	1.12–2.51	0.0129	1.50	0.88–2.55	0.1319
Histological Grade	1.26	0.92–1.72	0.1445	0.89	0.56–1.42	0.6207
Treatment Type	1.02	0.77–1.36	0.8899	0.90	0.67–1.20	0.4530
Ki67	1.21	1.10–1.32	0.0001	1.16	1.04–1.29	0.0085
cyclinD1	1.70	1.30–2.23	0.0001	1.58	1.18–2.13	0.0023

Note: Both Ki67 and cyclinD1 levels of merged cohort were measured by QDB analysis, and univariate and multivariate cox regression analyses for OS were performed for these two sets of data, respectively.

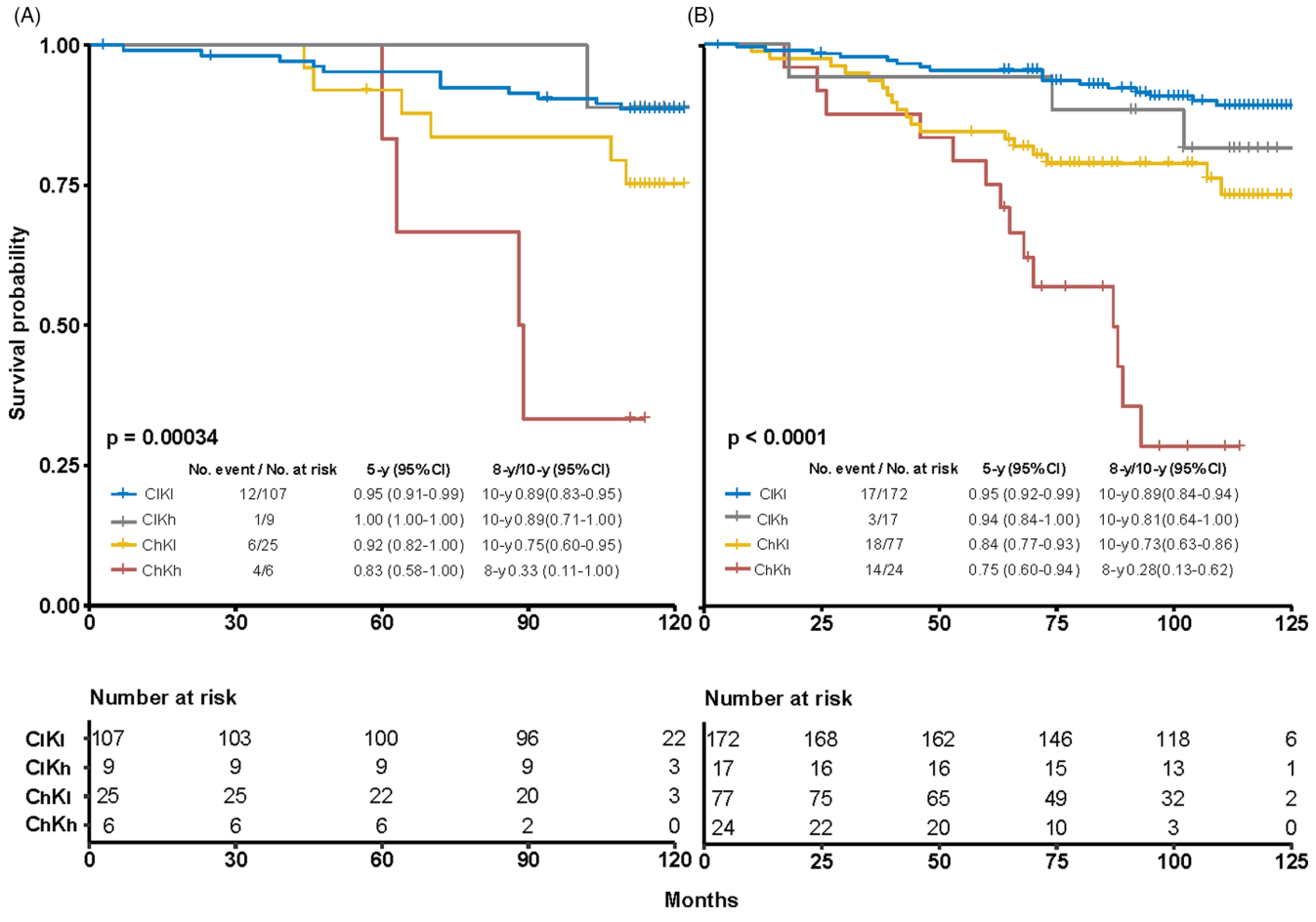

FIGURE 4 Validation of optimized cutoff of 0.44 μmol/g for cyclinD1 in cohort 2. (A) The cohort 2 was separated into four subgroups using cyclinD1 at 0.44 μmol/g and Ki67 at 2.31 nmol/g as cutoffs. C_lK_l:specimens with the protein levels of both biomarkers below the respective cutoffs; C_lK_h: Specimens with only cyclinD1 level below the recommended cutoff; C_hK_l: specimens with only Ki67 levels below the recommended cutoff; C_hK_h: specimens with both biomarkers above the respective cutoffs. (B) The merged cohort was also separated into four subgroups using cyclinD1 at 0.44 μmol/g and Ki67 at 2.31 nmol/g as cutoffs. The 5y and 10y SP, and the *p* values from Log Rank test are provided in the figure. For the high-risk subgroup of C_hK_h, we were only able to calculate 8y SP for lacking of specimens at a 10-year interval in this study

4 | DISCUSSION

IHC-based surrogate assay remains the basis of the clinical decision to utilize adjuvant cytotoxic therapy for luminal-like breast cancer patients in daily clinical practice worldwide. While this method is widely accepted as inferior to genetic assays like Oncotype Dx and PAM50,[9] any modification of this method would make profound impact on the lives of millions of breast cancer patients over the years.

Thus, caution must be taken with even the slightest change of the existing method in daily clinical practice. No matter how promising a clinical finding may look in one or more retrospective studies, a subsequent clinical trial, prospective or retrospective-prospective,[18] is needed before current guidance may be adjusted in daily clinical practice.

This is exactly the case with our efforts to modify surrogate assay using absolutely quantitated protein biomarkers. In a previous study, we showed that Ki67 protein levels as absolute and continuous variables improved the performance of the surrogate assay.[16] We also demonstrated that absolutely quantitated cyclinD1 was an independent prognostic factor from Ki67 through OS analysis, and the combined use of these two biomarkers may significantly improve the prognosis of Luminal-like patients.[17] Using two drastically different cohorts of patients, these findings, including the proposed cutoffs of Ki67 at 2.31 nmol/g and cyclinD1 at 0.44 μmol/g, were validated in the current study. We also confirmed the existence of a subgroup of patients (C_hK_h, 24/290) with 8y SP at 28%, the worst prognosis among all subgroups. To the best of our knowledge, this subgroup has never been described in the literature, warranting further attention in clinical practice in the future. It should be noted that we were only able to calculate its 8y SP due to insufficient number of specimens at a 10-year interval in the study.

However, all these clinical findings are by no means ready to be adopted in daily clinical practice. Rather, they are categorized as level 4 of LOE to require subsequent validation through prospective clinical trials. Through two previous studies and the current study, we believe we have provided sufficient support for a future prospective, or retrospective-prospective, clinical trial, with our proposed quantitative cutoffs to subtype luminal-like breast cancer patients in daily clinical practice.

The drastic differences in clinicopathological characteristics between cohorts 1 and 2 were not expected at the beginning of the study. Nonetheless, the successful validation of our findings in a drastically different cohort 2 from cohort 1 demonstrated the broad applicability of our findings among breast cancer patients. At this moment, we have no explanation why cohorts 1 and 2 showed such drastic differences in clinical characteristics, considering they were Luminal-like patients administered into two hospitals in the same city within roughly the same time period. One putative reason underlying this difference may be that the hospital accepting cohort 2 served the urban area of the city while the one accepting cohort 1 served more of the rural areas. Higher educational level and better socio-economic status associated with urban residents may translate into earlier detection of the tumor. This idea is indicated by more patients being dignosed as pT_1, pN_0, and histological grade 1 in cohort 2 than in cohort 1. Potentially for similar reasons, patients in cohort 2 may have received more up-to-date treatments than cohort 1. For example, more patients received endocrine or chemo endocrine therapies in cohort 2 than in cohort 1 (97% vs. 32.3%), and aromatase inhibitors were only used in cohort 2. In addition, there were also seven patients who received neoadjuvant therapy in cohort 2.

The observed differences may also be attributed partly to the fact that IHC results for cohort 2 were collected from medical records while those for cohort 1 were performed by three pathologists independently for the study. Thus, the consistency and reliability of the IHC results are expected to be higher in cohort 1 than in cohort 2. However, we were unable to explain the difference in HER2 expression among these cohorts, as both IHC results were confirmed as accurate based on QDB analysis [13 and unpublished data]. Clearly, these issues further underscored the necessity to launch prospective or prospective-retrospective clinical trials with tightly controlled participants.[18]

The biggest limitation of this series of studies is that they are retrospective observational studies suffering from various inherent biases. These studies lack the strict controls required for prospective or prospective-retrospective studies to offer any definite answer.[18] For example, patients from cohorts 1 and 2 received drastically different treatment regimens. A majority of patients in cohort 1 received chemotherapy while a majority of patients in cohort 2 received endocrine or chemo endocrine therapy, which is more up-to-date treatment for Luminal-like patients. This difference is expected to have a major impact on the OS of these patients. There were also seven patients in cohort 2 who received chemo endocrine therapy as neoadjuvant therapy. Its potential impact on the OS of these patients remains to be investigated.

Another limitation is that patients included in this series of studies were mainly from a mid-size city of northern China covering both rural and urban areas. It remains to be seen how representative they are for the breast cancer community worldwide. While the overall findings might be applicable, it remains unclear if the proposed cutoffs in this study need to be readjusted to suit patients in the rest of the world.

Therefore, our findings may be "play of chance".[18] No matter how many similar retrospective studies were performed, their conclusions may not be adopted in daily clinical practice without a well-controlled prospective or retrospective-prospective trial.

We also recognized that while we were able to validate 2.31 nmol/g and 0.44 μmol/g as Ki67 and cyclinD1 cutoffs in this series of studies, the readjustments of these cutoffs may be expected when more specimens are included to expand the dataset significantly in the future. In fact, we already showed in Figure S3 that a new cyclinD1 cutoff of 0.52 μmol/g was identified based on outcome analysis when the number of specimens was expanded from 155 in cohort 1 to 328 in the merged cohort of cohorts 1 and 2. However, this new cutoff offered limited advantages over the proposed 0.44 μmol/g in a previous study for identifying the high-risk subgroup of K_lC_l.

In this regard, our study demonstrated another advantage of developing biomarker cutoffs using absolutely quantitated values, as these proposed cutoffs may be constantly readjusted by merging new cohort(s) into the initial cohort. Indeed, one drawback from the current IHC-based system is that the cutoffs for protein biomarkers were derived from a limited dataset. In the case of Ki67, the 14% cutoff was identified based on a limited set of 170 luminal-like specimens.[8] It is hard to imagine that a universal applicable cutoff would

be identified from such a small dataset, considering millions of new breast cancer patients are added each year with widespread tumor heterogeneity worldwide.

5 | CONCLUSIONS

In summary, using two independent cohorts of Luminal-like specimens, we validated Ki67 cutoff of 2.31 nmol/g as an effective cutoff to significantly improve the performance of surrogate assay in daily clinical practice. We also identified, for the first time, a group of patients (Ki67 ≥ 2.31 nmol/g and cyclin D1 > 0.44 μmol/g) with worst prognosis among Luminal-like patients in the literature (8y SP at 28%). This subgroup of patients may require special attention in clinical practice in the future. Our studies set the stage for prospective or retrospective-prospective clinical trials to explore the usage of absolute quantitation of protein biomarkers in clinical diagnostics, using 2.31 nmol/g and 0.44 μmol/g as tentative cutoffs for Ki67 and cyclinD1 to subtype Luminal-like breast cancer patients.

AUTHORS CONTRIBUTIONS

GY & YLL provided clinical samples; GY performed IHC analyses and supervised all the clinical studies; YL & JBZ performed all the statistical analysis, JL, YL, YZ, WZ & FT performed all the assays and performed data analysis; BC performed data analysis, JDZ designed & supervised the overall study and drafted the manuscript; GY, JL, YL, YZ, WZ, FT & JDZ contributed to data interpretation and edited the manuscript.

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CONFLICT OF INTEREST

JL, YZ, YL, FT, WZ, BC, JBZ & JDZ are employees of Yantai Quanticision Diagnostics, Inc., a division of Quanticision Diagnostics, Inc., who own, or has filed patent applications for QDB plate, QDB method, and QDB application in clinical diagnostics. GY & YLL declared no conflict of interest.

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DATA AVAILABILITY STATEMENT

Data are available from the correspondent author upon reasonable written request.

ORCID

Jiandi Zhang https://orcid.org/0000-0002-9282-9443

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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