

Improving Prognosis of Surrogate Assay for Breast Cancer Patients by Absolute Quantitation of Ki67 Protein Levels using Quantitative Dot Blot (QDB) Method

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Abstract

Background: Immunohistochemistry (IHC) based-surrogate assay is the prevailing method in daily clinical practice to determine the necessity of cytotoxic therapy for Luminal-like breast cancer patients worldwide. It relies on Ki67 scores to separate Luminal A-like from Luminal B-like breast cancer subtypes. Yet, IHC method is plagued with subjectivity and inconsistency. We attempted to circumvent these issues by measuring Ki67 levels absolutely, quantitatively and objectively.

Methods: The Ki67 protein levels in a cohort of 253 specimens were measured with IHC and Quantitative Dot Blot (QDB) methods respectively, and used to assign these specimens into Luminal A-like and Luminal B-like subtypes accordingly. Their performances were compared with the Kaplan-Meier, univariate and multivariate survival analyses of the overall survival (OS) of Luminal-like patients.

Results: The surrogate assay based on absolutely quantitated Ki67 levels (cutoff at 2.31 nmole/g) subtyped the Luminal-like patients more effectively than those based on Ki67 scores (cutoff at 14%) (Log rank test, $p=0.00052$ vs $p=0.031$). It is also correlated better with OS in multivariate survival analysis [Hazard Ratio (HR) at 6.89 (95%CI: 2.66-17.84, $p=0.0001$) vs 2.14 (95%CI: 0.89-5.11, $p=0.087$)].

Conclusions: Our study showed the performance of surrogate assay may be improved significantly by measuring Ki67 levels absolutely, quantitatively and objectively.

Background

Microarray analysis of global gene profiling (GEP) of breast cancer tissues leads to the identification of the four intrinsic subtypes of luminal, Her2-like, basal-like and normal-like subtypes [1, 2]. This concept has been well accepted with several GEP-based genetic tests developed to determine if cytotoxic therapy is necessary for Luminal subtype patients[3].

Yet, GEP-based genetic tests remains inaccessible to a lot of patients worldwide [3]. As an alternative, surrogate assay, which is based on the protein expression levels of four biomarkers of Estrogen Receptor (ER), Progesterone Receptor (PR), Her2 and Ki67, has been used extensively all over the world. Based on 2013 St. Gallen Consensus, the patients are categorized into Luminal A-like (LumA), Luminal B-like (LumB), Her2 positive (non-luminal), and Triple negative (ductal) subtypes. LumA patients, are defined as those patients with ER+, Her2-, PR score $\geq 20\%$, and Ki67 score $< 14\%$. In some clinical practice, the cutoff of Ki67 score can also be 20%. LumA patients are expected to have the best prognosis, and in most cases, require endocrine therapy alone.

LumB patients are comprised of Her2- (LumB₁) and Her2+ (LumB₂) sub-groups. LumB₁ patients are ER+, Her2-, with Ki67 score $\geq 14\%$, or PR score $< 20\%$. LumB₂ patients are ER+ and Her2+, regardless of the Ki67 and PR statuses [3]. For these patients, cytotoxic therapy is required as part of adjuvant therapy. Clearly, the Ki67 expression levels are critical to determine if the cytotoxic therapy is necessary for Luminal-like patients[3].

In surrogate assay, all the biomarkers are assessed mainly by immunohistochemistry (IHC), a method known to be associated with subjectivity and inconsistency. Among all four biomarkers used in surrogate assay, the standardization of Ki67 may be considered most difficult. Intensive efforts have been devoted to the standardization of this biomarker, yet, there remains quite a distance away from its realization [4–8]. For example, it is still debatable if the hot spot should be included in the assessment [5, 9]. The intensity of the staining is also not considered when accessing the Ki67 levels using IHC method [6].

We hypothesized the inherent inaccuracy associated with Ki67 IHC scores may negatively affect the performance of surrogate assay for Luminal-like patients. Therefore, we attempted to measure Ki67 levels absolutely and objectively using a recently developed Quantitative Dot Blot (QDB) method in Formalin Fixed Paraffin Embedded (FFPE) specimens [10–13]. The specimens were separated into luminal A (LumA_q) and luminal B subtypes (LumB_q) based on the absolute quantitated Ki67 levels in these specimens in a retrospective study (Table 1). The prognosis of this adjusted surrogate assay was compared with that of surrogate assay to demonstrate the necessity of objective measurement of Ki67 protein levels for subtyping of Luminal-like tumors in daily clinical practice.

Table 1
Clinicopathological characteristics of 155 Luminal-like breast cancer specimens.

Characteristics	Number of Cases (%)
Age	
< 50	69(44.52)
≥ 50	86(55.48)
Treatment	
Endo	2(1.29)
Chemo	90(58.06)
Endo&Chemo	41(26.45)
Unknown	22(14.19)
Node Status	
N0	75(48.39)
N1	56(36.13)
N2	10(6.45)
N3	8(5.16)
Unknown	6(3.87)
Tumor Size	
T1	54(34.84)
T2	91(58.71)
T3	7(4.52)
Unknown	3(1.94)
Grade	
II	83(53.55)
III	52(33.55)
Unknown	20(12.9)
Surrogate Assay (14% Ki67 cutoff)	
LumA _i	66(42.58)
LumB _i	89(57.42)

Characteristics	Number of Cases (%)
LumB _{1i}	70(45.16)
LumB _{2i}	19(12.26)
Surrogate Assay (20% Ki67 cutoff)	
LumA _i	76(49.03)
LumB _i	79(50.97)
LumB _{1i}	60(38.71)
LumB _{2i}	19(12.26)

Methods

Human subjects and human cell lines

The inclusion criteria for this retrospective observational study were all patients diagnosed as breast cancer patient with FFPE tissue specimen available at Yantai Affiliated Hospital of Binzhou Medical University, Yantai, P. R. China from 2008 to 2013. The specimen must have had more than 50% tumor tissue based on H&E staining. Follow up data were available for 221 patients (87.4%) at the last follow up at April, 1st, 2019.

The clinical information including age, node status, tumor size, tumor grade, type of treatments, results of FISH analysis were all collected from medical records. The end-point was overall survival defined as the time between breast cancer surgery and death or last follow-up. All the missing values were treated as new category. The cases lost to follow-up were not included in the analysis. Patients still alive at last study follow-up (April 1st, 2019) were censored.

QDB analysis

The QDB process was described in detail elsewhere with slightly modifications[10, 13]. Additional details are provided in *Additional file 1*.

Statistical analysis

Graph Pad 7 software (La Jolla, CA, USA) was used for common data analysis, including Pearson's correlation coefficient analysis. The results were presented as Mean ± SEM. The survival analyses were done using R version 3.6.2 (<http://www.r-project.org>). The strength of the agreement among Ki67 IHC scores from three pathologists was assessed by Fleiss's Kappa analysis.

The Ki67 levels measured by QDB method or IHC method were dichotomized for OS by using optimal cutoff values determined by the “surv_cutpoint” function of the “survminer” R package respectively, with optimized cutoff at 2.31 nmol/g for QDB method and 14% for IHC method accordingly. All the OS analyses were visualized by Kaplan-Meier method, and comparisons were performed by log-rank test.

Univariate Cox proportional hazard models fitted overall survival were employed for hazard ratio (HR) and 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, node status, tumor size, tumor grade, and type of treatment. Residuals that are analogous to the Schoenfeld residuals in Cox models were used to check the proportionality assumption. P values of less than .05 were considered statistically significant.

Information about General Reagents, Purification of Ki67 recombinant fragments, QDB process, Preparation of FFPE and cellular lysates, and IHC analysis can be found in Additional file 1.

Results

Measurement of Ki67 protein levels with QDB method

A QDB-based high throughput immunoassay for absolute quantitation of Ki67 levels in FFPE specimens was developed first by defining the linear range of total tissue lysates and recombinant Ki67 protein standards using a clinically validated antibody, MIB1. The total tissue lysates from 4 FFPE specimens with Ki67 score >70% were pooled together, and diluted serially to define the linear range of the assay (Figure S2).

The Ki67 levels in all 253 FFPE specimens were measured using QDB method, and Ki67 levels were found to distribute between 0 (undetectable level) to 22.21 nmol/g, with average at 3.32 ± 0.22 nmol/g (Figure 1a). Based on a recent study [14], the Ductal Carcinoma in situ (DCIS), normal and stroma tissue were not excluded from the tissue slices, as long as more than 50% of invasive tumor was presented in the slice. In this study, the potential influence of Tumor infiltrating Lymphocytes (TIL) was also not considered.

Among 253 specimens, 244 were provide with Ki67 scores from three pathologists assessing the same set of IHC stained slides independently. Their Ki67 score averages were used throughout the study. We found the highest IHC score was at 75%, and the lowest at 1%, with average at $14.18 \pm 0.79\%$ (Figure 1b). Correlation analysis was performed using results from QDB and IHC methods with $r=0.71$, $p<0.0001$ using Pearson's correlation analysis (Figure 1c). In an attempt to reduce the potential interference from the subjectivity inherently associated with IHC analysis, we also sub-grouped these specimens by their IHC scores. As expected, the correlation between the subgroup averages of the absolute Ki67 levels from QDB method with IHC scores was increased to $r=0.93$, $p<0.0001$ using Pearson's correlation analysis (Figure 1d).

Those specimens provided with Ki67 scores were also accompanied with IHC results for ER, PR, and Her2. For specimens with Her2 score of 2+, results from FISH analysis were used to differentiate Her2+ from Her2- specimen. Based on these information, we assigned these 244 specimens into luminal-like subtype (n=155), HER2-like subtype (n=31), and Triple Negative subtype (n=53) based on 2013 St. Gallen consensus[3]. The remaining 5 specimens cannot be subtyped based on this consensus (Figure 2).

The clinicopathological parameters of the 155 luminal-like specimens were listed in table 1. For all the qualified patients, the median overall survival (OS) time to censoring was 85 months, with the maximum at 132 months. These specimens might be further divided into 66 Luminal A-like and 89 B-like subtypes using Ki67 score at 14% as cutoff, or 76 Luminal A-like and 79 Luminal B-like subtypes with Ki67 score at 20% as cutoff.

QDB-based adjusted surrogate assay vs IHC-based surrogate assay

To evaluate the influence of objectively quantitated Ki67 levels on the prognostic effect of surrogate assay, we subtyped luminal A-like from luminal B-like subtypes based on absolutely quantitated Ki67 levels, using an optimized cutoff at 2.31 nmol/g. We named this method the adjusted surrogate assay for simplicity (table 2). The 2.31 nmol/g cutoff used in adjusted surrogate assay was obtained using the “surv_cutpoint” function of the “surminer” R package in combination with the OS of these patients. This proposed cutoff was validated using an independent cohort in the accompany manuscript III [15]. In addition, we also managed to split the current cohort randomly into a training set and a validate set using RAND(“table”) function with SAS 9.4 to demonstrate its effectiveness for subtyping of Luminal-like patients (Figure S4).

Table 2

Subtyping of Luminal-like specimens.

Subtype	Surrogate Assay	Adjusted Surrogate Assay
LuminalA (LumA)	ER+, Her2-, Ki67<14% & PR≥20%	ER+, Her2-, Ki67<2.31nmol/g & PR≥20%
LuminalB (LumB)		
LuminalB ₁ (LumB ₁)	ER+, Her2-, Ki67≥14% or PR<20%	ER+, Her2-, Ki67≥2.31nmol/g or PR<20%
LuminalB ₂ (LumB ₂)	ER+, Her2+	ER+, Her2+

We also managed to obtain the optimum cutoff for Ki67 score from IHC analysis at 2.67% using the same function. However, at this value, only a small fraction of specimens were assigned to Luminal A-like subtype (n=26). Therefore, we considered 14% a more reasonable cutoff, and used this value throughout the study.

As shown in Figure 3, based on adjusted surrogate assay, the luminal A-like subtype (LumA_q) has 10 year survival probability (10y SP) at 91% vs 63% for Luminal B-like subtype (LumB_q), with p=0.00052 from Log rank test. In contrast, 10y SP for luminal A-like subtype (LumA_i) was 88% vs 68% for Luminal B-like subtype (LumB_i), with p=0.031 from surrogate assay. When Ki67 score of 20% was used as cutoff, 10y SP for LumA_i was 84% vs 70% for LumB_i, with p=0.10 (Figure S5).

The surrogate assay was compared next with adjusted surrogate assay in univariate cox regression analysis, and we found that adjusted surrogate assay provided improved prognosis for Luminal-like breast cancers with HR at 4.39 (95%CI, 1.78-10.81, p=0.0013) than that of surrogate assay with HR at 2.46 (95%CI, 1.05-5.75, p=0.0385) (Supplementary Table 1).

The prognostic values of both methods were also investigated in the multivariate cox regression analysis to include routine clinicopathological parameters including age, treatment type, node status, tumor size, tumor grade in the analysis. We found while LumB_i patients had 2.14 fold higher risk of death than LumA_i from surrogate assay (HR: 2.14, 95%CI, 0.89-5.11, p=0.0873), it is not statistically significant. On the other hand, LumB_q specimens had 6.89 fold higher risk of death than LumA_q by adjusted surrogate assay (HR: 6.89, 95%CI, 2.66-17.84, p=0.0001) (Supplementary Table 2). In addition, in both analyses, age and node status were found to be an independent prognostic factor.

Next, we tried to understand what caused this difference by comparing the luminal A-like and Luminal B-like subtypes from surrogate assay (LumA_i and LumB_i) with those from adjusted surrogate assay (LumA_q and LumB_q) in Supplementary Table 3. The specimens were named as A_iA_q or B_iB_q if they were assigned to Luminal A-like or Luminal B-like subtypes by both methods. Those assigned by surrogate assay to A-like subtype, but not by adjusted surrogate assay, was named as A_iB_q, and those assigned by adjusted surrogate assay to Luminal-A like subtype, but not by surrogate assay, were named B_iA_q. We found more specimens were assigned to Luminal A-like subtype by adjusted surrogate assay than surrogate assay (76 vs 66). The overall concordance rate between surrogate assay and adjusted surrogate assay was 75.5%.

In Figure 4, we performed the survival analyses of these four subgroups using Kaplan-Meier survival analysis. A_iA_q subgroup were found to have the best 10y SP at 91% vs B_iB_q subgroup at 59%. In addition, the 10y SP of B_iA_q, the subgroup assigned to Luminal A-like subtype only by adjusted surrogate assay, was very close to that of A_iA_q, at 90%.

Adjusted surrogate assay vs surrogate assay by various factors

We also attempted to minimize the influence of treatment on the survival probability of each subtype (Figure S6). For this purpose, only patients receiving chemotherapy were analyzed (n=85), as there were

insufficient number of specimens receiving other treatments. Consistent with the overall performance, adjusted surrogate assay presented significantly better prognosis than surrogate assay, with 10y SP at 100% for LumA_q vs 53% for LumB_q, $p < 0.0001$, in comparison to 94% for LumA_i vs 69% for LumB_i, $p = 0.037$ (Figure S6a and S6b).

The potential influence of node status was also investigated in this study by dividing patients into N0 group (no positive lymph node detected, $n = 65$) and N1 (patients with 1 to 3 positive lymph nodes, $n = 56$), and analyzed their 10y SP using Kaplan-Meier analysis. Again, adjusted surrogate assay showed better prognosis than surrogate assay in both cases. For N0 patients, 10y SP was at 97% for LumA_q vs 72% for LumB_q, $p = 0.023$, in contrast to 93% for LumA_i vs 80% for LumB_i, $p = 0.31$. Likewise, this number became 90% vs 63% for LumA_q vs LumB_q, $p = 0.026$, in contrast to 90% vs 66% for LumA_i vs LumB_i, $p = 0.10$ for N1 patients. The specimen numbers for N2 and N3 statuses were insufficient for further survival analysis (Figure S7).

Discussion

In this study, by using objectively quantitated Ki67 protein levels to replace Ki67 score in surrogate assay, we showed that inherent subjectivity and inconsistency of IHC analysis limited significantly the performance of surrogate assay for Luminal-like breast cancer patients. A revised surrogate assay is proposed to use absolutely quantitative Ki67 levels, instead of Ki67 scores from IHC analysis, for subtyping Luminal-like patients. We believe this adjustment should significantly improve the accuracy and consistency of surrogate assay in daily clinical practice worldwide.

The standardization, or lack of standardization of Ki67 in clinical practice, is a challenge facing the whole medical community. Yet, until now, no significant progress has been made so far [6, 7, 9]. The significantly improved prognosis in adjusted surrogate assay cast doubt on current standardization efforts with the IHC-based Ki67 assessment, suggesting that QDB method may be a better option for Ki67 standardization in daily clinical practice.

One culprit underlying the lack of standardization of Ki67 scores with IHC method is the widespread tumor heterogeneity. As the solution, the whole tissue is homogenized in QDB method to reduce its influence to the minimum. Although the morphological features were lost in QDB process, results from our studies suggest the overall benefits well justify this cost in daily clinical practice.

The performance of surrogate assay is also seriously affected by the subjectivity of the assay. We tried to overcome its influence by requesting three pathologists to judge the same set of IHC stained slides Ki67 independently and blindly. The IHC results from these three pathologists were analyzed with Fleiss Kappa correlation analysis with $\kappa = 0.633$. The Ki67 scores from these three pathologists were also used to assign these specimens into Luminal A-like and Luminal B-like subtypes respectively with 14% as cutoff. We obtained p values at 0.2, 0.018 and 0.1 respectively with Log rank test.

Perceivably, by including more pathologists in the analysis, the subjectivity of IHC analysis should be minimized. This assumption may find its support in Fig. 1d, where we showed significantly increased correlation between QDB and IHC when the subgroup averages of Ki67 levels from QDB method were used in our correlation analysis. Nonetheless, this requirement will inevitably place unbearable burden to the pathologists worldwide.

As the solution, the QDB-based immunoassays provided objective and quantitative measurement of Ki67 levels, safeguarded with multiple controls. The adoption of this method should translate into significance improved consistency and reliability of the results in daily clinical practice, especially in resource-limiting laboratories where IHC analysis remains a technical challenge.

Admittedly, there are several obvious limitations with this study. As a retrospective study, we were unable to evaluate the performance of surrogate assay and adjusted surrogate assay on the prognosis of the recurrence of the disease for lacking of relevant data. Our conclusions are also affected by the small sample size in this study. It remains questionable if this conclusion can be held up with more FFPE specimens in the study. Further studies are needed to validate our conclusion in the future.

We also recognized the need to validate our proposed 2.31 nmol/g cutoff in an independent cohort. The effectiveness of this proposed cutoff was validated in an independent cohort of Luminal-like breast cancer specimens from another hospital alone, and in combination with the current cohort (see accompany manuscript III[15]). We also managed to split the current cohort randomly into both trial and validation groups to demonstrate that 2.31 nmol/g was the optimized cutoff for both groups (Figure S4).

Our study also hinted that the discordance between surrogate assay and genetic assays may be smaller than we expect. The discrepancy between intrinsic subtyping and surrogate assay is clearly recognized in the field [16, 17]. That is also the driven force for the campaign of universal genetic testing for breast cancer patients. However, in this study, by merely improving the accuracy of Ki67 measurement in surrogate assay, we have significantly improved the performance of surrogate assay. Future studies are urgently needed to compare various genetic assays including PAM50 with adjusted surrogate assay.

Conclusions

In summary, the Ki67 protein levels were measured unprecedentedly in 253 FFPE specimens absolutely, quantitatively and objectively using QDB method. The measured levels were used to replace the Ki67 scores from IHC analysis in surrogate assay, using 2.31 nmol/g as cutoff, to significantly improve the prognosis for OS of Luminal-like patients. We propose QDB as a solution for standardization of Ki67 assessment in daily clinical practice to improve the prognosis of surrogate assay for breast cancer patients.

Abbreviations

QDB, Quantitative Dot Blot method; **FFPE**, Formalin Fixed Paraffin Embedded; **IVD**, In vitro Diagnostics; **ER**, Estrogen Receptor; **PR**, Progesterone Receptor; **Her2**, human epidermal growth factor receptor 2; **IHC**, immunohistochemistry; **OS**, overall survival

Declarations

Ethics approval and consent to participate

All the studies were performed in accordance with the Declaration of Helsinki, and were approved by the Medical Ethics Committee of Yantai Affiliated Hospital of Binzhou Medical University (Approval #: 20191127001), with an informed Consent Forms waiver for archived specimens.

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

YL, YZ, FT, WZ, JL, JBZ & JDZ are employees of Yantai Quantcision Diagnostics, Inc., a division of Quantcision Diagnostics, Inc., who own or has filed patent applications for QDB plate, QDB method, & QDB application in clinical diagnostics.

JH, JRZ, SX, LJ, KC & XW declared no conflict of interest.

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Authors' contributions

JH & JRZ provided clinical samples; JH, JRZ, SX & LJ performed IHC analyses; JH supervised all the clinical studies; YL & JBZ performed all the statistical analysis, YL, YZ, FT, JL & WZ performed all the assays and performed data analysis; XW & KC performed data analysis, JDZ designed & supervised the overall study and drafted the manuscript; JH, YL, YZ & JDZ contributed to data interpretation and edited the manuscript.

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Figures

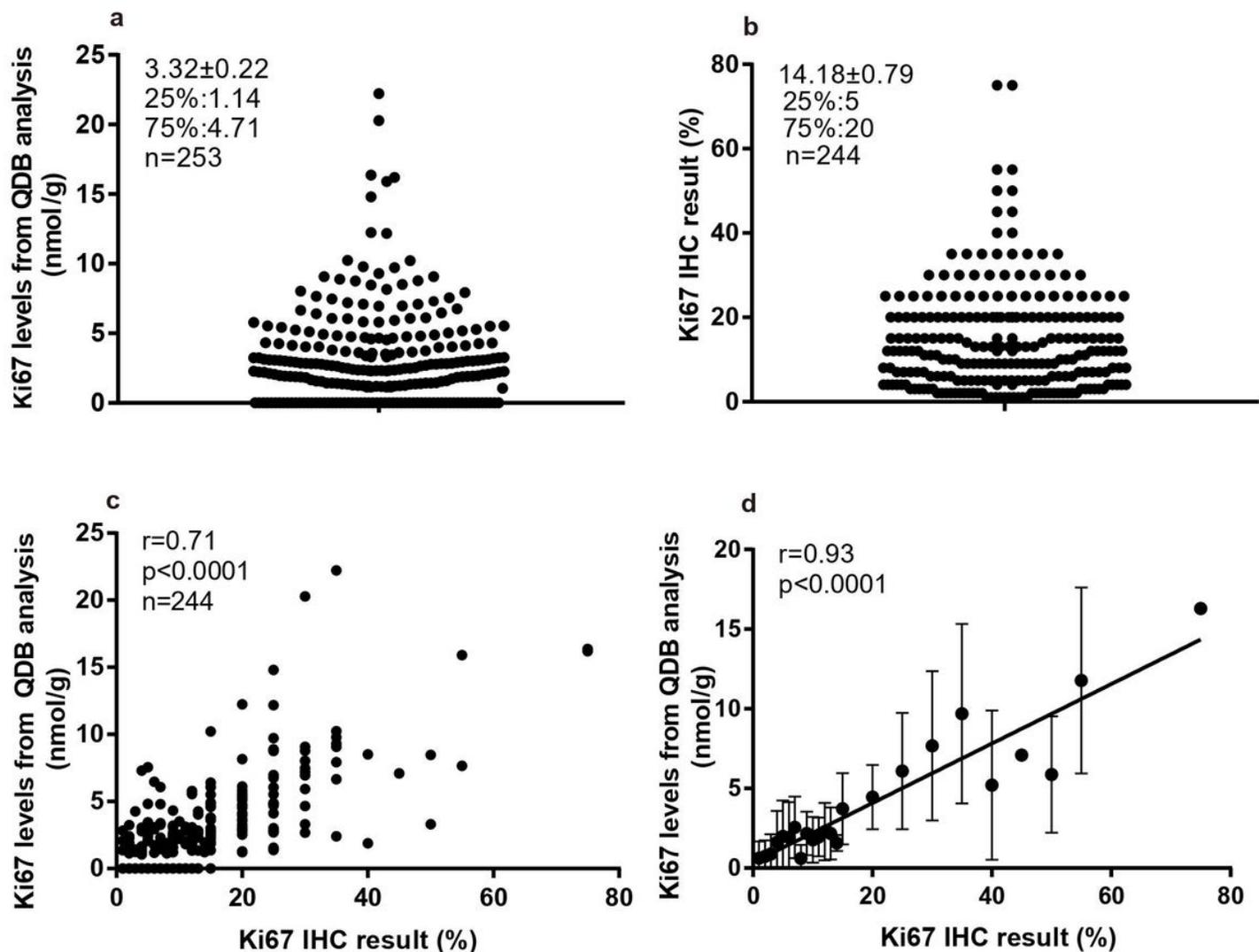


Figure 1

Ki67 levels in 253 FFPE specimens and their correlations with Ki67 scores from IHC analysis. Total lysate was extracted from 2×15 μm FFPE slices individually, and 0.5 μg/specimen was used for QDB measurement using Mouse anti-Human Ki67 monoclonal antibody (MIB1). These specimens were also assessed with IHC analysis, with each IHC stained slides assessed by three pathologists independently. The Ki67 scores used in the study were averages of three assessments. (a) Distribution of quantitatively measured Ki67 levels among these specimens. (b) Distribution of Ki67 scores from IHC analysis among 244 specimens. (c) Correlation analysis of the results from QDB and IHC analyses using Pearson's correlation analysis with $r=0.71$, $p<0.0001$; (d) these specimens were sub-grouped based on their respective Ki67 scores. The subgroup averages of the Ki67 levels from QDB measurements were used for correlation analysis with Ki67 scores from IHC analysis using Pearson correlation analysis with $r=0.93$, $p<0.0001$. The results were expressed as average±SD.

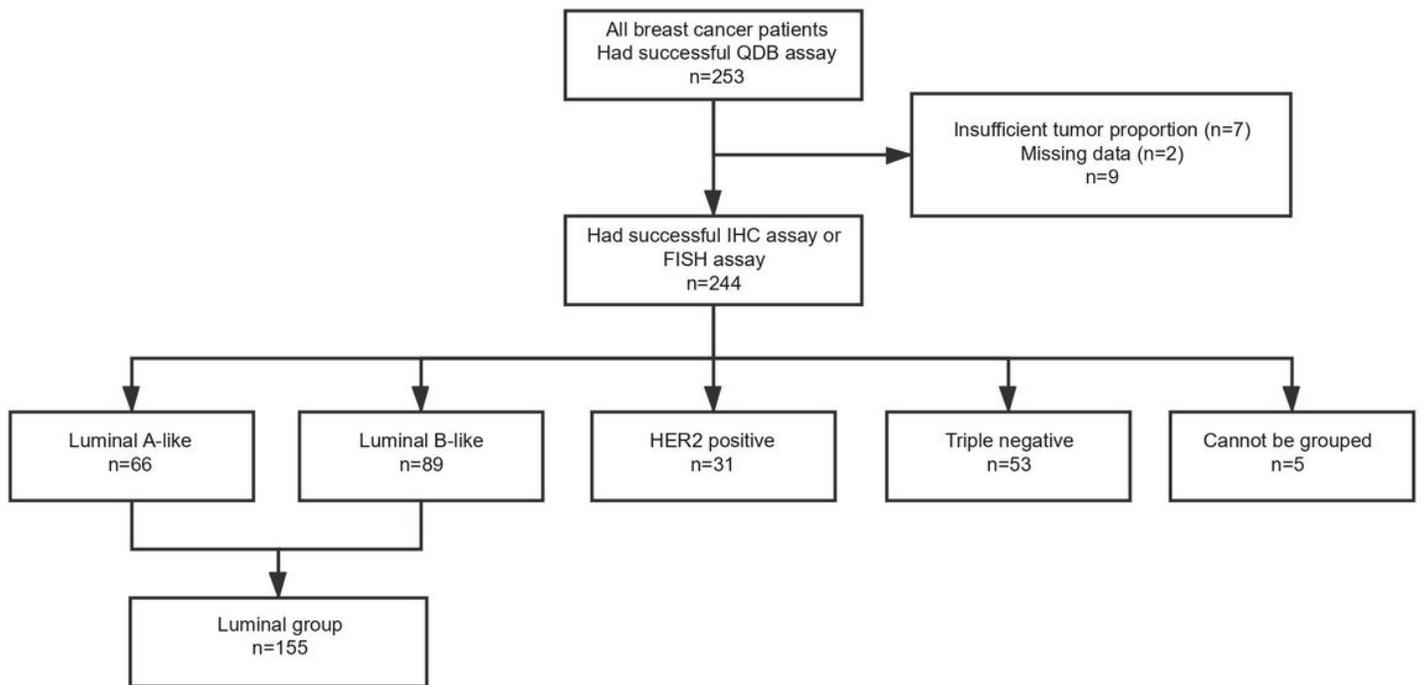


Figure 2

Flow diagram of patient selection for the study.

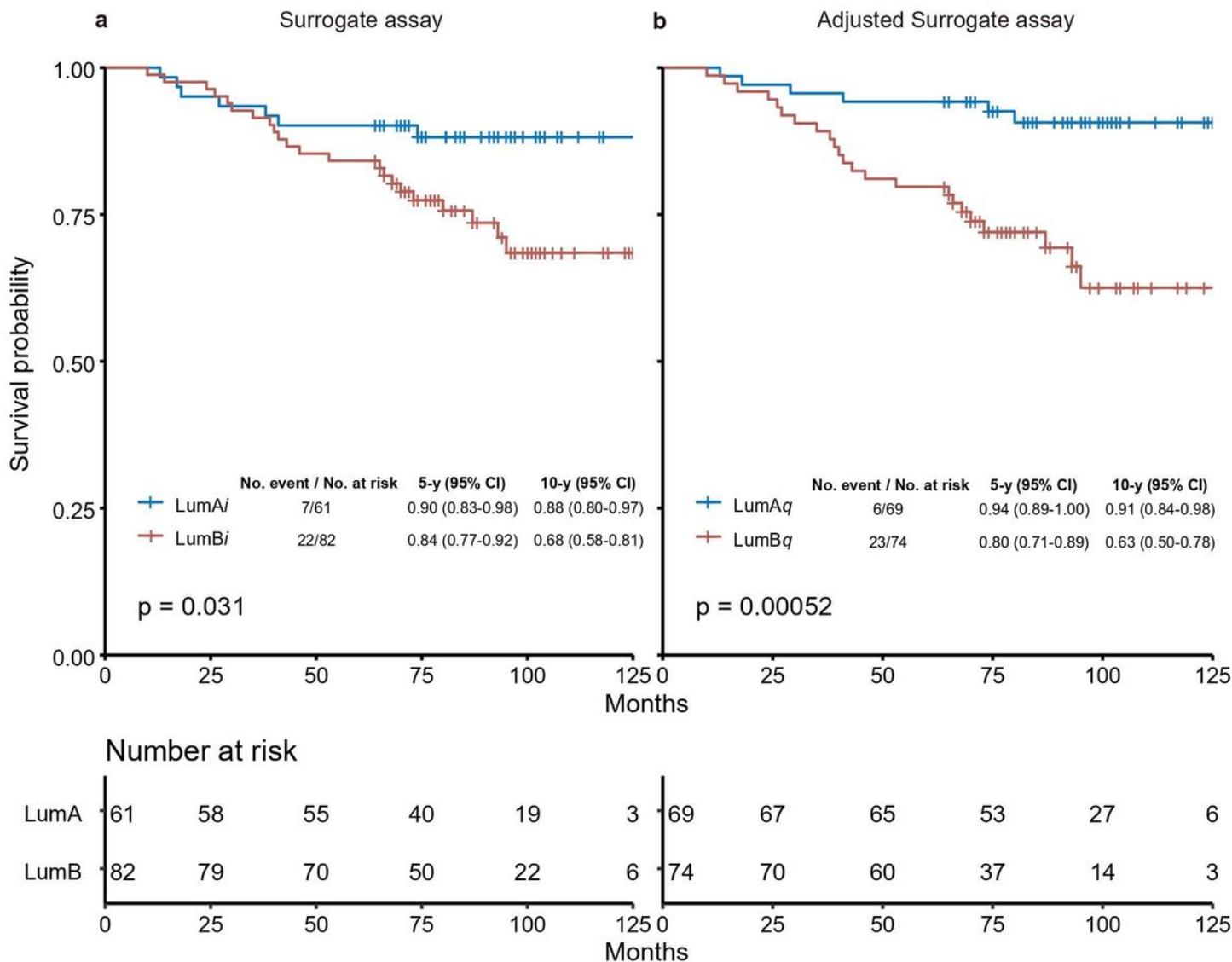


Figure 3

Overall survival analysis by surrogate assay (a) or adjusted surrogate assay (b). (a), the Ki67 score of 14% was used as cutoff in surrogate assay based on Recommendations from 2013 St. Gallen Consensus. (b), the Ki67 level of 2.31 nmol/g was used as cutoff determined by the “surv_cutpoint” function of the “survminer” R package in adjusted surrogate assay. The 5 year and 10 year survival probabilities, and the p values from Log Rank test were provided for both Surrogate assay and Adjusted Surrogate assay respectively. LumA, Luminal A-like subtype; LumB, Luminal B-like subtype; LumAi and LumBi, Luminal A-like and B-like subtypes by surrogate assay; LumAq and LumBq, Luminal A-like and B-like subtypes by adjusted surrogate assay; CI, confidence interval.

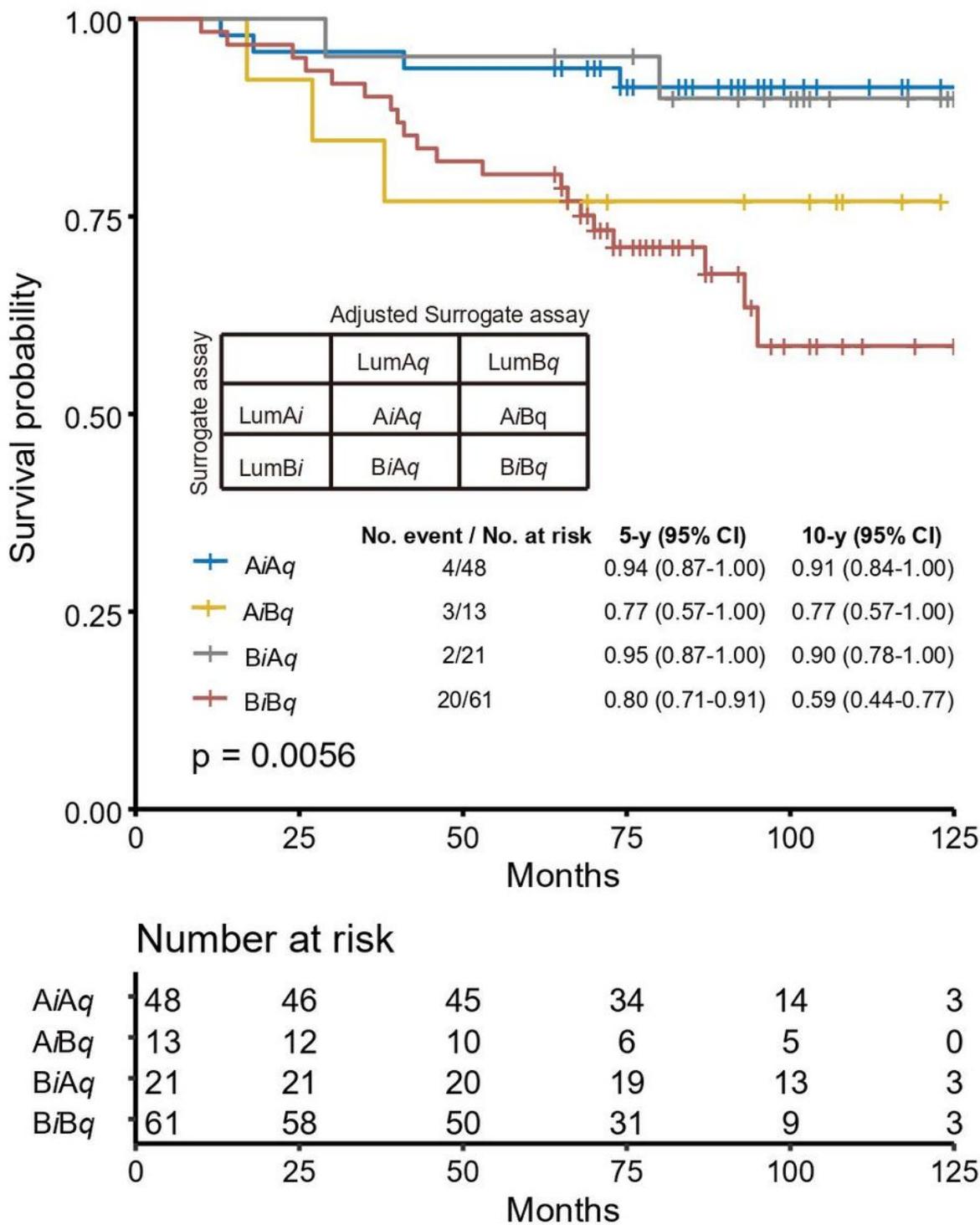


Figure 4

Comparison of the performance of surrogate assay with that of adjusted surrogate assay. The specimens were further sub-grouped into AiAq and BiBq subgroups, representing specimens assigned as Luminal A-like subtype and Luminal B-like subtype by both assays; AiBq, representing specimens assigned as Luminal A subtype by surrogate assay, but as Luminal B subtype by adjusted surrogate assay; and BiAq, representing specimens assigned as Luminal B-like subtype by surrogate assay, but as Luminal A-like

subtype by adjusted surrogate assay. The Overall survival analysis was performed with these four subgroups using Kaplan-Meier survival analysis, with survival probability for each individual subgroup provided in the figure. The p value was calculated with Log Rank test.

Supplementary Files

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- [Additionalfile4.doc](#)
- [Additionalfile5.doc](#)
- [Additionalfile6.doc](#)
- [Additionalfile7.doc](#)
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- [Additionalfile9.doc](#)
- [Additionalfile10.doc](#)