Ki67 Index, HER2 Status, and Prognosis of Patients With Luminal B Breast Cancer

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Background Gene expression profiling of breast cancer has identified two biologically distinct estrogen receptor (ER)-positive subtypes of breast cancer: luminal A and luminal B. Luminal B tumors have higher proliferation and poorer prognosis than luminal A tumors. In this study, we developed a clinically practical immunohistochemistry assay to distinguish luminal B from luminal A tumors and investigated its ability to separate tumors according to breast cancer recurrence-free and disease-specific survival.

Methods Tumors from a cohort of 357 patients with invasive breast carcinomas were subtyped by gene expression profile. Hormone receptor status, HER2 status, and the Ki67 index (percentage of Ki67-positive cancer nuclei) were determined immunohistochemically. Receiver operating characteristic curves were used to determine the Ki67 cut point to distinguish luminal B from luminal A tumors. The prognostic value of the immunohistochemical assignment for breast cancer recurrence-free and disease-specific survival was investigated with an independent tissue microarray series of 4046 breast cancers by use of Kaplan–Meier curves and multivariable Cox regression.

Results Gene expression profiling classified 101 (28%) of the 357 tumors as luminal A and 69 (19%) as luminal B. The best Ki67 index cut point to distinguish luminal B from luminal A tumors was 13.25%. In an independent cohort of 4046 patients with breast cancer, 2847 had hormone receptor–positive tumors. When HER2 immunohistochemistry and the Ki67 index were used to subtype these 2847 tumors, we classified 1530 (59%, 95% confidence interval [CI] = 57% to 61%) as luminal A, 846 (33%, 95% CI = 31% to 34%) as luminal B, and 222 (9%, 95% CI = 7% to 10%) as luminal–HER2 positive. Luminal B and luminal–HER2-positive breast cancers were statistically significantly associated with poor breast cancer recurrence-free and disease-specific survival in all adjuvant systemic treatment categories. Of particular relevance are women who received tamoxifen as their sole adjuvant systemic therapy, among whom the 10-year breast cancer–specific survival was 79% (95% CI = 76% to 83%) for luminal A, 64% (95% CI = 59% to 70%) for luminal B, and 57% (95% CI = 47% to 69%) for luminal–HER2 subtypes.

Conclusion Expression of ER, progesterone receptor, and HER2 proteins and the Ki67 index appear to distinguish luminal A from luminal B breast cancer subtypes.

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Breast cancer is a molecularly heterogeneous disease that appears to include at least four major tumor subtypes (1–3). Currently, the choice of adjuvant systemic therapy is based on patient's age, tumor size, histological grade, lymph node involvement, hormone receptor status, and HER2 status. The only predictive markers with an associated targeted therapy are the estrogen receptor (ER) and HER2. The approximately 15% of patients with breast cancer who have HER2 overexpressing and/or amplified tumors are treated with a combination of trastuzumab, a monoclonal antibody targeting HER2, and adjuvant chemotherapy (4). For the two-thirds of breast cancers that are positive for ER and/or progesterone receptor (PR),

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endocrine therapy with tamoxifen (5) or aromatase inhibitors (6,7) is generally indicated. The application of adjuvant systemic therapy has contributed to a recent decrease in breast cancer mortality (8). When patients with hormone receptor–positive tumors were treated with adjuvant tamoxifen, their risk for the composite outcome of recurrence or death was reduced by more than 30% (8). However, many patients with lymph node–positive, ER-positive breast tumors gain minimal benefit from adjuvant chemotherapy (9).

Although adjuvant systemic therapy fails to prevent recurrence in some patients with high-risk hormone receptor–positive breast cancer, many lower risk patients may have been subjected to side effects without benefit from such interventions. Thus, there is a need to distinguish patients with hormone receptor–positive tumors at high risk for recurrence despite current treatment protocols from patients at low risk for whom adjuvant hormonal therapy alone may be sufficient.

Breast cancers expressing high levels of Ki67, a nuclear marker of cell proliferation, are associated with worse outcomes (10–12). Ki67 is not included in routine clinical decision-making because of a lack of clarity regarding how Ki67 measurements should influence clinical decisions. Recent studies (13,14) indicate that changes in Ki67 expression after neoadjuvant endocrine treatment may predict long-term outcome.

Gene expression studies have identified five molecularly distinct subtypes of breast cancer that have prognostic value across multiple treatment settings (3,15–17). These subtypes are termed ER-positive–luminal A (luminal A), ER-positive–luminal B (luminal B), HER2-enriched (ie, tumors that overexpress ERBB2-associated genes but do not express genes that define the luminal subtype), basal-like, and normal breast-like. HER2-enriched and basal-like subtypes are hormone receptor negative and have poor prognosis (1–3). In contrast, the expression of ER-associated genes characterizes the luminal breast cancers, with luminal B tumors having poorer outcomes than luminal A tumors. Although some luminal B tumors can be identified by their expression of HER2, the major biological distinction between luminal A and B is the proliferation signature, including genes such as *CCNB1*, *MKI67*, and *MYBL2*, which have higher expression in luminal B tumors than in luminal A tumors (15,18). The recurrence score (19), which is derived from quantitative reverse transcription–polymerase chain reaction (qRT-PCR) assays of the expression of 16 discriminator genes, divides ER-positive, lymph node–negative tumors into prognostic subgroups. The expression of genes that are involved in proliferation, including *MKI67* (encoding Ki67), is the most heavily weighted component in calculating the recurrence score derived from that assay. Thus, a distinction between luminal A and B tumors that is based on proliferation status among ER-positive luminal patients may be important to breast cancer biology and prognosis.

The high cost of gene expression profiling has limited its incorporation into most randomized clinical trials, and therefore, DNA microarray–defined proliferation status is not used to provide prognostic information in general practice. To date, there is no available immunohistochemistry-based surrogate assay that can distinguish between luminal A and luminal B tumors. However, our previously defined immunohistochemistry surrogate for identifying the basal-like subtype (20,21), which includes ER, PR, and HER2 status, does identify hormone receptor–positive, HER2-positive patients as having a worse outcome. To further facilitate proliferation measures by immunohistochemistry, technically excellent rabbit monoclonal antibodies for Ki67 are now available (22), and thus, the Ki67 labeling index may serve as a clinically valuable biomarker for the luminal B subtype.

Consequently, we sought to determine 1) the optimal cut point for the percentage of Ki67-positive invasive breast cancer cells that would distinguish between luminal A and luminal B tumors, by comparing gene expression profile assignments and immunohistochemistry data from formalin-fixed paraffin-embedded tissues; 2) the prognostic value of the Ki67 index (percentage of invasive cancer cell nuclei that are positive for Ki67 immunostaining over total invasive cancer cell nuclei present in a histological sample) among hormone receptor–positive tumors from a large independent series of patients who had not received adjuvant systemic therapy; and 3) the association between the Ki67 index and outcome among patients with hormone receptor–positive tumors who were treated with tamoxifen as their sole adjuvant systemic therapy. We tested the hypothesis that the immunohistochemical determination of ER, PR, and HER2 status and the Ki67 index is able to distinguish the poor prognosis luminal B subgroup of breast cancers from the good prognosis luminal A subgroup.

CONTEXT AND CAVEATS

Prior knowledge

Two biologically distinct estrogen receptor–positive subtypes of breast cancer have been identified by gene expression profiling of breast cancers: luminal A and luminal B, with luminal B tumors having a higher rate of tumor cell proliferation and poorer prognosis than luminal A tumors.

Study design

A group of tumors from patients with invasive cancer was subjected to gene expression profiling to determine the breast cancer subtype. Another group of hormone receptor–positive breast cancers was assessed by immunohistochemistry for the expression of a panel of four biomarkers for breast cancer (ie, estrogen receptor, progesterone receptor, HER2, and Ki67); patients in this group were separated by subtype determined by immunohistochemistry, and their survival was analyzed.

Contribution

Luminal A and B breast cancers appear to be distinguished by the expression of estrogen receptor, progesterone receptor, HER2, and Ki67 proteins.

Implications

An immunohistochemistry test of four biomarkers appears to be able to separate breast cancer patients by subtype. Additional research is warranted to determine whether this assay could have clinical utility.

Limitations

This study has limitations typical of immunohistochemical approaches, including limited technical reproducibility, subjective interpretation, and qualitative readouts. The false-positive and false-negative rates of the assay were approximately 25%.

From the Editors

Patients, Materials, and Methods

Cohorts of Patients

The training set contained a total of 357 formalin-fixed paraffin-embedded tissues from invasive breast carcinomas from the

University of British Columbia and Washington University at St Louis (hereafter referred to as the UBC-WashU series). This series was chosen to include high- and low-risk patient groups, so that each of the major intrinsic breast cancer subtypes was represented by adequate numbers of specimens. In the combined cohort of 357 tumors, 137 (38%) were from patients with lymph node–positive disease, 200 (56%) were larger than 2 cm in diameter, and 133 (37%) were grade 3 by the Bloom and Richardson method (23) (*see* Table 1).

The validation set contained a total of 4046 formalin-fixed paraffin-embedded tissues. All patients had been referred to the British Columbia Cancer Agency from January 1, 1986, through September 30, 1992, and had staging, pathology, treatment, and follow-up information available (*see* Table 1); this cohort is hereafter referred to as the BCCA series. The median follow-up time was 12.5 years. In British Columbia, most patients were treated with adjuvant systemic therapy according to provincial cancer management guidelines set by the British Columbia Cancer Agency (24). The guidelines provided criteria for defining high-risk patients who could benefit from adjuvant systemic therapy. A high-risk patient was defined as being lymph node positive, having lymphovascular invasion, or having a tumor larger than 2 cm in diameter if it was also ER negative. Patients who were considered to be at clinically low risk at the time of diagnosis during the study era, which included approximately 40% of the study cohort, were not recommended to receive any adjuvant systemic therapy. Patients who were considered to be at high risk were recommended to receive tamoxifen if their tumor was ER positive and if they were older than 65 years, to receive chemotherapy if their tumor was ER negative or if they were younger than 50 years, and to receive both if their tumor was ER positive and if they were between ages 50 and 65 years.

Biomarker studies on the anonymized archival specimens and clinical data were approved by the Clinical Research Ethics Board of the British Columbia Cancer Agency and the Human Research Protection Office of Washington University. Tissues were collected according to institutional review board–approved protocols of the Alvin J. Siteman Cancer Center Tissue Procurement Core and at the British Columbia Cancer Agency.

In the BCCA series of 4046 breast tumors, a total of 2847 tumors were hormone receptor positive, of which 2598 had complete immunohistochemistry data for ER, PR, HER2, and Ki67 (*see* Table 1). We found no statistically significant differences in age, tumor size, lymph node status, or adjuvant systemic therapy between patients with complete immunohistochemistry data and patients with missing data; however, the missing data status was associated with less lymphovascular invasion ($P = .010$) and with a marginal tendency for lower grade (grade 1 or 2 vs grade 3) ($P = .048$) compared with the complete data available status.

Gene Expression Profiling by qRT-PCR to Define Breast Cancer Subtypes

The 357 tumor samples in the UBC-WashU series were subjected to qRT-PCR. The strategy used to develop and implement a breast cancer specimen classifier is detailed in Figure 1. The 50-gene PAM50 subtype predictor, as described by Parker et al. (25), was used to assign intrinsic subtypes to each tumor specimen in the training set from gene expression profiles that had been generated by qRT-PCR. Total RNA was extracted from pathologist-guided core samples of the paraffin-embedded tissue blocks (two core punches, each 1.5 mm in diameter, taken from areas adjacent to the cores that were extracted for tissue microarrays) by use of a High Pure RNA Paraffin kit (Roche Applied Science, Indianapolis IN). After treatment with Turbo DNase (Ambion, Austin, TX), total RNA yield was assessed with the ND-1000 Spectrophotometer (NanoDrop Technologies, Rockland, DE). Gene expression by qRT-PCR was determined as described previously (25,26). In brief, first-strand cDNA was synthesized from 1.2 μg of total RNA with Superscript III reverse transcriptase (First Strand Kit; Invitrogen, Carlsbad, CA) and a mixture of random hexamers and gene-specific primers. PCR amplification was carried out on 1.25 ng of the resulting cDNA in the presence of SYBRGreen I Master Mix (Roche Applied Science). Double-stranded DNA, which incorporated the SYBRGreen 530-nm fluorophore, was detected by use of the Roche LightCycler 480 (Roche Applied Science). Melting curve analysis was used to verify the specificity of the amplified products for each gene. The relative gene copy number was determined by following the manufacturer's directions. PCR primers optimized for use on formalin-fixed paraffin-embedded materials were thereby used to make quantitative measurements for a panel of 50 discriminatory genes selected for the capacity to discriminate luminal A, luminal B, HER2-enriched, basal-like, and normal breast-like expression profiles [the PAM50 bioclassifier panel, as described in detail in Parker et al. (25)].

Tissue Microarrays

Tissue microarrays were constructed, as described previously (20,27,28), by use of the 4046 formalin-fixed paraffin-embedded archival blocks from the validation dataset. Slides from these blocks that were stained with hematoxylin–eosin were reviewed by two pathologists to identify areas of invasive breast cancer. Single 0.6-mm cores were extracted from each tumor block to construct tissue microarrays. A total of 17 tissue array blocks were built to assemble the complete 4046 tumor series.

Immunohistochemistry and Fluorescent In Situ Hybridization

The 4046 tumors assembled into tissue microarrays, in BCCA series, were examined with immunohistochemistry and fluorescent in situ hybridization. Immunohistochemistry for ER, PR, HER2, and Ki67 was performed concurrently on serial sections with the standard streptavidin–biotin complex method with 3,3′-diaminobenzidine as the chromogen. Staining for ER, PR, and HER2 interpretation was as described previously (20). Briefly, the Ki67 antibody (clone SP6; ThermoScientific, Fremont, CA) was applied at a 1:200 dilution for 32 minutes, by following the Ventana Benchmark automated immunostainer (Ventana, Tucson AZ) standard Cell Conditioner 1 (CC1, a proprietary buffer) protocol at 98°C for 30 minutes. ER antibody (clone SP1; ThermoFisher Scientific, Fremont CA) was used at 1:250 dilution with 10-minute incubation, after an 8-minute microwave antigen retrieval in 10 mM sodium citrate (pH 6.0). Ready-to-use PR antibody (clone 1E2; Ventana) was used by following the CC1 protocol as above. HER2 staining was done with the SP3 antibody

(ThermoFisher Scientific) at a 1:100 dilution after antigen retrieval in 0.05 M Tris buffer (pH 10.0) with heating to 95°C in a steamer for 30 minutes. For HER2 fluorescent in situ hybridization assay, slides were hybridized with probes to LSI (locus-specific identifier) HER2/neu and to centromere 17 by use of the PathVysion HER-2 DNA Probe kit (Abbott Molecular, Abbott Park, IL) according to manufacturer's instructions, with modifications to pretreatment and hybridization as previously described (29). Slides were counterstained with 4′,6-diamidino-2-phenylindole, stained material was visualized on a Zeiss Axioplan epifluorescent microscope, and signals were analyzed with a Metafer image acquisition system (Metasystems, Altlussheim, Germany). Biomarker expression from immunohistochemistry assays was scored by two surgical pathologists (T. O. Nielsen and D. Gao), who were blinded to the clinicopathological characteristics and outcome and who used previously established and published criteria for biomarker expression levels that had been developed on other breast cancer cohorts (12,30). Tumors were considered positive for ER (27) or PR (31) if immunostaining was observed in more than 1% of tumor nuclei, as described previously. Tumors were considered positive for HER2 if immunostaining was scored as 3+ according to HercepTest criteria, with an amplification ratio for fluorescent in situ hybridization of 2.0 or more being the cut point that was used to segregate immunohistochemistry equivocal tumors (scored as 2+) (32). Ki67 was visually scored for percentage of tumor cell nuclei with positive immunostaining above the background level by two pathologists (T. O. Nielsen and D. Gao). Tissue microarray core samples with fewer than 50 tumor cells were considered uninterpretable (27,28). All the stained tissue microarrays were digitally scanned, and primary image data are available for public access (http://www.gpecimage.ubc.ca; username, luminalB; password, luminalb).

Statistical Analysis

All statistical analyses were carried out in SPSS version 16.0 (SPSS, Inc., Chicago, IL) and R version 2.6.0 (www.r-project.org). For breast cancer subtype prediction with qRT-PCR data, the 50-gene PAM50 classifier, as described in detail by Parker et al. (25), was used to assign breast cancer subtypes to the 357 training samples. The algorithm maps the gene expression in each specimen to centroids (a multidimensional average expression of the 50 discriminatory genes) that were previously constructed from prototypical examples of the five breast cancer subtypes (luminal A, luminal B, HER2-enriched, basal-like, and normal breast-like) (25). We assigned a subtype to each tumor specimen tested by calculating the distances to each of the subtype centroids with the Spearman rank correlation test. Tumors for which the difference between Spearman rank correlation coefficients for the luminal A and B centroids was less than 0.1 were considered borderline.

Expression of ER and ER-associated genes is a characteristic of luminal breast cancers as defined by microarray expression profiling (1–3). Approximately 30% of tumors in the luminal B cluster expressed HER2 and associated genes, and in this study, we defined tumors that expressed hormone receptor proteins (ER or PR) and were positive for HER2 as being of the luminal–HER2-positive subtype. However, the remaining 70% of luminal B tumors primarily differed from better prognosis luminal A tumors by virtue of higher expression of proliferation genes (15,18). We investigated whether addition of the proliferation marker Ki67 to the immunopanel of ER, PR, and HER2 could distinguish luminal B tumors (ie, hormone receptor–positive, HER2-negative, and Ki67-high tumors) from luminal A tumors (ie, hormone receptor–positive, HER2-negative, and Ki67-low tumors). In the UBC-WashU series, we used the 50-gene PAM50 classifier to identify tumors as being either luminal A or luminal B, to determine the optimal cut-point value for the Ki67 index. We then compared quantitative data from visual assessment of Ki67 immunohistochemical labeling against these gene expression profile–based assignments for hormone receptor–positive, HER2-negative tumors. The optimal cutoff value for Ki67 was selected by use of the receiver operating characteristic (ROC) method, by minimizing the sum of the observed false-positive and false-negative errors with bootstrapping methodology (33). In this fashion, the cutoff value was selected against the gold standard of gene expression profiling, as opposed to assigning a cut point against clinical outcome (which can be difficult to extrapolate to other patient populations with differences in treatment and risk).

The immunopanel thereby defined (ie, ER, PR, HER2, and Ki67) was used to assign tumors of the BCCA validation series to breast cancer subtypes and to assess clinicopathological characteristics and the relation to patient outcome. We estimated 95% confidence intervals (CIs) with bootstrapping methodology (32) for the reported percentages of luminal subtypes as defined by the immunopanel. Differences in clinicopathological characteristics, including age, tumor grade, tumor size, and lymph node status, among breast cancer subtypes were examined by use of χ^2 tests. For univariate survival analysis, relapse-free survival and breast cancer–specific survival were estimated by use of Kaplan–Meier curves (34), and the statistical significance of survival differences was assessed with a log-rank test (35). For relapse-free survival, survival time was censored at death if the cause was not breast cancer or if the patient was alive without relapse on June 30, 2004. For breast cancer–specific survival, survival time was censored at death if the cause was not breast cancer or if the patient was still alive on June 30, 2004 (the date for outcome data collection). Patients with unknown cause of death were excluded from breast cancer–specific survival analysis. For multivariable survival analyses, Cox regression models (36) were used to estimate the association between the Ki67 index and breast cancer subtypes, with adjustment for with standard clinicopathological variables, including age at diagnosis (as a continuous variable), histological grade (grade 3 vs grade 2 or 1), tumor size (>2 vs ≥2 cm), lymphovascular invasion (positive vs negative), and number of positive axillary lymph nodes as a percentage of the total numbers examined (coded in three categories, in which 0%–25% was compared with 0%, and >25% was compared with 0%). We classified patients with breast cancer in the British Columbia population by using the percentage of positive lymph nodes as a continuous variable in the Cox model because this variable was shown to be more prognostic than a categorical variable of one to three positive lymph nodes vs four or more than positive lymph nodes (37). Only patients with information for all the covariates were included in the Cox regression analyses. Smoothed plots of weighted Schoenfeld residuals were used to test proportional hazard assumptions (38), and no evidence that these assumptions were

invalid was observed. All statistical tests were two-sided, and *P* values of less than .05 were considered statistically significant. The data were assembled to provide more than 80% power for testing hypotheses regarding the biomarkers in all patients combined and for patient subgroups that were defined by the adjuvant therapies received.

Results

Determination of Ki67 Index to Identify Luminal B Breast Cancers

Among the 357 breast tumors with qRT-PCR gene expression profiles (Figure 1) and tumor subtypes as assigned by the 50-gene PAM50 predictor (25), 101 (28%) were classified as luminal A, 69 (19%) luminal B, 62 (17%) HER2-enriched, 98 (28%) basal-like, and 27 (8%) normal breast-like. From the gene expression profile data (1–3), we expected that tumors classified as ER positive and HER2 negative by immunohistochemistry would include a mixture of luminal A and luminal B subtypes that might be distinguished by Ki67 index. By linking the available immunohistochemical data with the expression profile assignments, we identified 84 hormone receptor–positive and HER2-negative tumors as luminal A and 60 tumors as luminal B.

Ki67 index is a continuous variable, but a biologically and clinically relevant cut point can be determined by the ROC method and a gene expression profile–defined gold standard. Among the 144 luminal A or B tumors in the training set, the best cutoff value for the immunohistochemically determined Ki67 index to distinguish luminal B was 13.25% (Figure 2, A). The sensitivity of the Ki67 index with this cutoff value was 72% (95% CI = 59% to 82%), and the specificity was 77% (95% CI = 67% to 85%). Among these 144 tumors, 17 were considered borderline by virtue of having a less than 0.100 difference between Spearman rank correlation coefficients for the luminal A and B centroids (meaning that the 50-gene profile correlated with luminal A and B types almost equally well). When we excluded these borderline tumors and restricted the ROC analysis to the 74 unambiguously luminal A and 53 unambiguously luminal B tumors, we still found that the best cutoff value for Ki67 immunohistochemistry data was 13.25%, with a sensitivity of 77% (95% CI = 64% to 87%) and a specificity of 78% (95% CI = 68% to 87%) (Figure 2, B).

Defining a visually assessable cut point allows assignment of standard pathology breast cancer specimens into luminal A and B subtypes by use of immunohistochemical approaches. We selected a Ki67 index of 14% or more Ki67-positive tumor nuclei as the best cut point for human visual assessment. That is, we defined the luminal A subtype as being ER and/or PR positive, HER2 negative, and Ki67 low (ie, a Ki67 index of <14%) and the luminal B subtype as being ER and/or PR positive, HER2 negative, and Ki67 high (ie, a Ki67 index of ≥14%). Although expression profiling also classifies ER-positive, HER2-positive tumors as luminal B, patients with HER2-positive breast cancer currently receive a different therapy regimen (that incorporates targeted anti-HER2 therapy) than patients with other luminal breast cancer subtypes. We therefore separated luminal tumors into three groups—luminal A, luminal B, and luminal–HER2 positive—in subsequent survival analyses.

Predicting Survival Among Hormone Receptor–Positive Breast Cancers With the Surrogate Immunopanel of ER, PR, HER2, and Ki67

When we used HER2 and Ki67 immunohistochemistry to subtype the 2598 hormone receptor–positive tumors in the BCCA series, we classified 1530 (59%, 95% CI = 57% to 61%) as luminal A, 846 (33%, 95% CI = 31% to 34%) as luminal B, and 222 (9%, 95% CI = 7% to 10%) as luminal–HER2-positive tumors. In comparison with luminal A, both luminal B and luminal–HER2-positive tumors (Table 1) were statistically significantly associated with younger age at diagnosis, higher grade, larger tumor size, positive lymph node involvement, and lymphovascular invasion (all $P < .001$).

Among the tumors from the 943 clinically low-risk patients in the BCCA series who were lymph node negative at diagnosis and did not receive adjuvant systemic therapy, the surrogate immunohistochemical panel classified 625 as luminal A, 263 as luminal B, and 55 as luminal–HER2-positive tumors. The 10-year relapse-free survival was 78% (95% CI = 75% to 82%) for patients with luminal A tumors, 67% (95% CI = 61% to 73%) for those with luminal B tumors, and 64% (95% CI = 52% to 78%) for those with luminal–HER2-positive tumors (Figure 3, A) (for luminal B vs luminal A survival, log-rank $P < .001$; and for luminal–HER2-positive tumors vs luminal A survival, log-rank $P = .02$). The 10-year breast cancer–specific survival was 92% (95% CI = 90% to 94%) for those with luminal A tumors, 79% (95% CI = 74% to 85%) for those with luminal B tumors, and 78% (95% CI = 67% to 90%) for those with luminal–HER2-positive tumors (Figure 3, B) (for luminal B vs luminal A survival, log-rank $P < .001$; and for luminal–HER2-positive tumors vs luminal A survival, log-rank $P < .001$). In a multivariable Cox regression comparison (Table 2), the luminal B subtype was associated with statistically significantly poorer relapse-free survival (hazard ratio [HR] = 1.4, 95% CI = 1.1 to 1.9) and poorer breast cancer–specific survival (HR = 1.8, 95% CI = 1.3 to 2.6) than the luminal A subtype. In addition, the luminal–HER2-positive subtype was associated with statistically significantly poorer relapse-free survival (HR = 1.6, 95% CI = 1.0 to 2.5) and breast cancer survival (HR = 2.1, 95% CI = 1.2 to 3.8) than the luminal A subtype (Table 2). Ki67 and HER2 thus appear to provide additional prognostic information beyond clinical parameters currently in use as prognostic factors for the clinically low-risk patients with lymph node–negative and hormone receptor–positive breast cancer.

Among the 976 tumors from patients in the BCCA cohort treated with tamoxifen as their only adjuvant systemic therapy, we identified 584 as luminal A, 303 as luminal B, and 89 as luminal–HER2 positive by use of the surrogate immunopanel. The 10-year relapse-free survival was 70% (95% CI = 66% to 74%) for patients with luminal A tumors, 53% (95% CI = 47% to 59%) for patients with luminal B tumors, and 51% (95% CI = 41% to 63%) for patients with luminal–HER2-positive tumors (Figure 4, A) (for luminal B vs luminal A and luminal–HER2-positive tumors vs luminal A, all log-rank $P < .001$). The 10-year breast cancer–specific survival was 79% (95% CI = 76% to 83%) for patients with luminal A tumors, 64% (95% CI = 59% to 70%) for patients with luminal B tumors, and 57% (95% CI = 47% to 69%) for patients with luminal–HER2-positive tumors

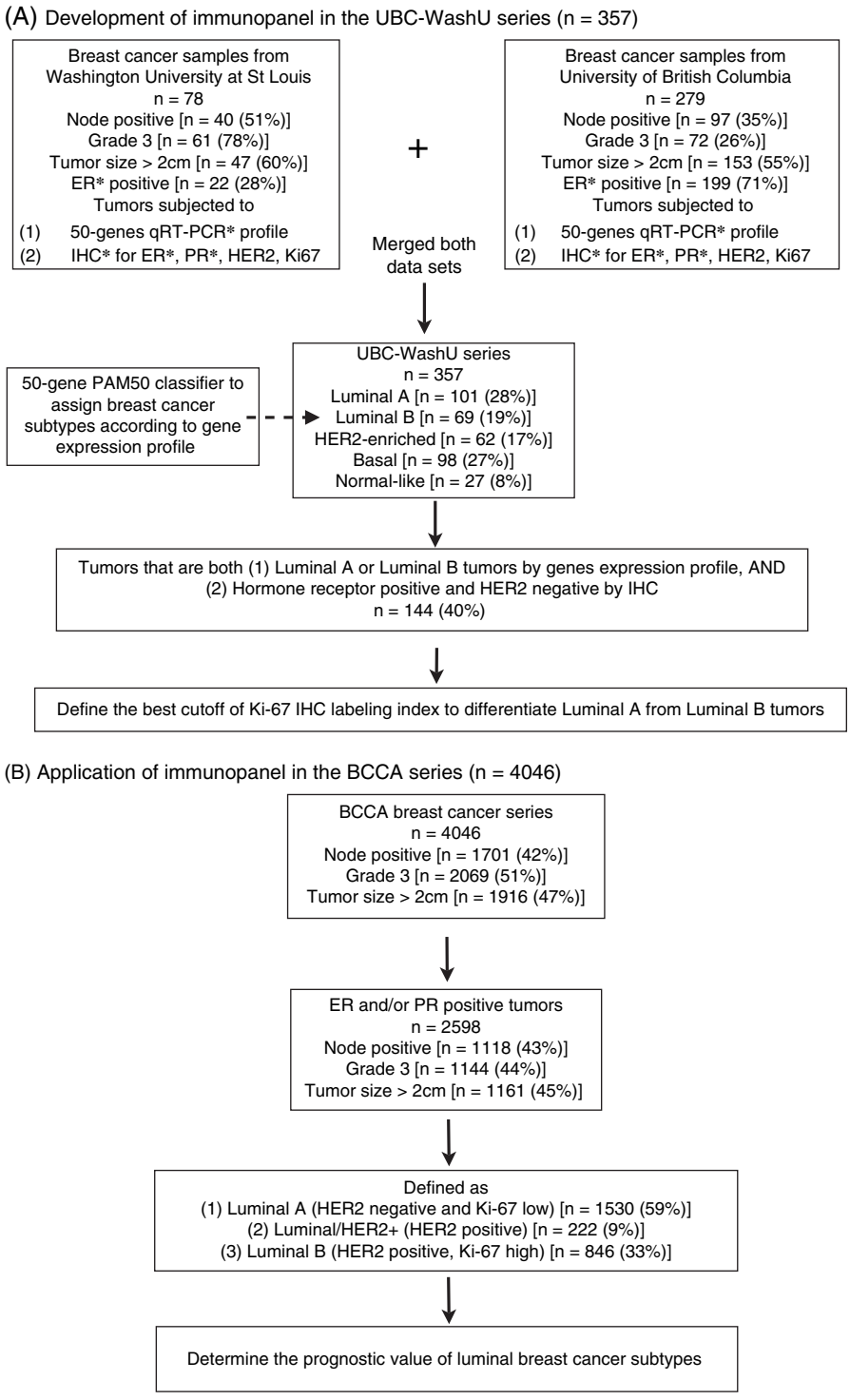

Figure 1. Flow diagram of the strategy for development and implementation of a breast cancer specimen classifier. **A**) Development with breast cancer specimens from the University of British Columbia and Washington University at St Louis (ie, the UBC-WashU series). **B**) Implementation or application to breast cancer specimens from the British Columbia Cancer Agency (ie, the BCCA series). ER = estrogen receptor; qRT-PCR = quantitative reverse transcription–polymerase chain reaction; IHC = immunohistochemistry; PR = progesterone receptor.

(Figure 4, B) (for luminal B vs luminal A and luminal–HER2-positive tumors vs luminal A, all log-rank $P < .001$). In the multivariable Cox regression analyses, the luminal B (for relapse-free survival, HR of recurrence = 1.5, 95% CI = 1.3 to 2.0, $P < .001$) and luminal–HER2-positive (for relapse-free survival, HR of recurrence = 1.6, 95% CI = 1.1 to 2.3, $P = .02$) subtypes were associated with more than 1.5 times increased risk for relapse and/or death from breast cancer compared with the luminal A subtype (Table 3). These findings remained statistically significant among patients with lymph node–negative or lymph node–positive breast cancer (Figure 4, C–F and Table 3; the number of events among patients with luminal–HER2-positive, lymph node–negative tumors was too small for accurate interpretation of this subset).

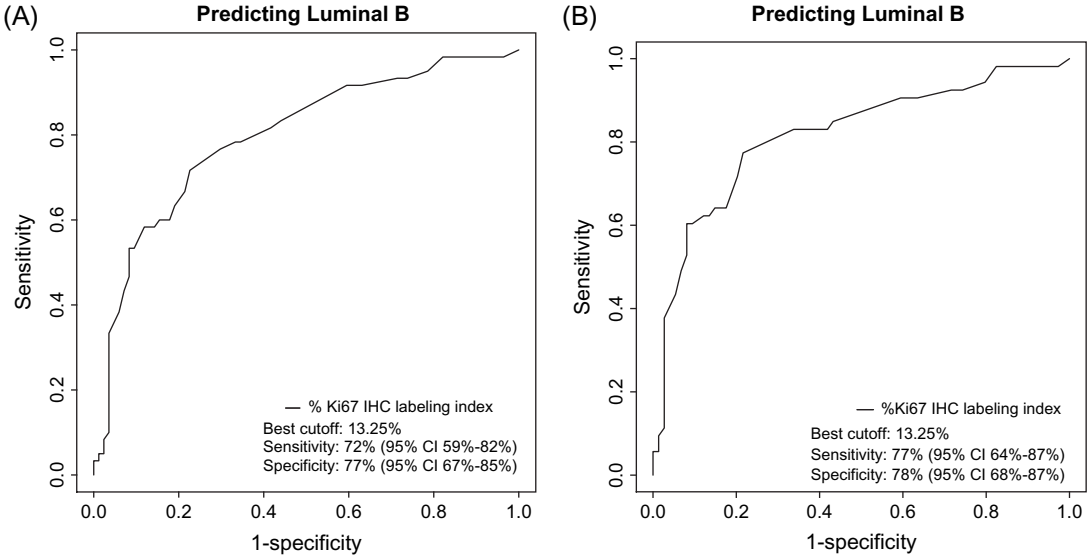

Figure 2. The x and y axes of ROC curve are true positive rate and false positive rate respectively. True positive rate equals to sensitivity and false positive rate is 1-specificity. Establishment of Ki67 cut point. True positive rate equals to sensitivity and false positive rate is 1-specificity. **A**) ROC analysis of 144 luminal A and B tumors with Ki67 IHC data to identify luminal B tumors as defined by a 50-gene classifier. Gene expression data for the classifier were obtained by quantitative reverse transcription–polymerase chain reaction. The selected best cut point for the Ki67 index was 13.25%. **B**) ROC analysis that was confined to 127 luminal A and B tumors with Spearman rank correlation coefficients of more than 0.1. CI = confidence interval; ROC = receiver operating characteristic; IHS = immunohistochemistry.

Among the 196 patients with hormone receptor–positive tumors who were treated with both adjuvant tamoxifen and chemotherapy in the BCCA cohort, 124 were treated with anthracycline-based regimens of doxorubicin and cyclophosphamide or of fluorouracil, doxorubicin, and cyclophosphamide, and 72 were treated with non-anthracycline–based regimens of cyclophosphamide, methotrexate, and fluorouracil. The majority of these tumors were high grade and larger than 2 cm in diameter, and patients had positive lymphovascular invasion and/or more than 25% positive axillary lymph nodes of the total examined at primary surgery. No statistically significant differences were found in the clinicopathological parameters or breast cancer subtype frequencies between cohorts treated with the anthracycline-based regimens of doxorubicin and cyclophosphamide or of fluorouracil, doxorubicin, and cyclophosphamide, and those treated with non-anthracycline–based regimens of cyclophosphamide, methotrexate, and fluorouracil (Table 4). When we used the immunohistochemical surrogate definition (ie, ER, PR, HER2, and Ki67) to classify these 196 hormone receptor–positive tumors, we found that 87 were luminal A, 84 were luminal B, and 25 were luminal–HER2-positive tumors from patients who received combined adjuvant treatment. The 10-year relapse-free survival was 69% (95% CI = 59% to 79%) for patients with luminal A tumors, 51% (95% CI = 42% to 63%) for those with luminal B tumors, and 42% (95% CI = 26% to 67%) for those with luminal–HER2-positive tumors (Figure 5, A) (for luminal B vs luminal A, log-rank P = .007; and for luminal–HER2-positive tumors vs luminal A, log-rank P = .006). The 10-year breast cancer–specific survival was 78% (95% CI = 69% to 87%) for those with luminal A tumors, 58% (95% CI = 48% to 70%) for those with luminal B tumors, and 44% (95% CI = 28% to 70%) for those with luminal–HER2-positive tumors (Figure 5, B) (for luminal B vs luminal A, log-rank P = .014; and for luminal–HER2-positive tumors vs luminal A, log-rank P = .001). In multivariable Cox regression analyses, breast cancer subtypes retained independent association with survival (Table 3). Both luminal B (for relapse-free survival, HR of recurrence = 2.03, 95% CI = 1.15 to 3.58, P = .015) and luminal–HER2-positive (for relapse-free survival, HR of recurrence = 2.65, 95% CI = 1.23 to 5.71, P = .013) tumors were associated with more than twofold increased risk for recurrence and death from breast cancer, compared with luminal A tumors. In this subset, breast cancer subtype was the only variable (other than >25% lymph node involvement) that was statistically significantly associated with breast cancer outcome.

Discussion

In this study, we develop an easily applied immunohistochemical surrogate for gene expression profile–defined luminal subtypes of breast cancer. We demonstrated that biological subtyping by use of this immunohistochemical surrogate panel of four biomarkers (ie, ER, PR, HER2, and Ki67) had statistically significant value that was independent of standard clinicopathological parameters (including age at diagnosis, tumor size, grade, lymphovascular invasion, and axillary lymph node status) in identifying high-risk women with hormone receptor–positive breast cancer in the settings of no adjuvant systemic therapy, adjuvant tamoxifen, and combined adjuvant tamoxifen and chemotherapy.

Gene expression profiling studies have consistently revealed biologically distinct breast cancer subtypes with different prognoses (39). Luminal B breast cancers are a clinically important subgroup associated with poor outcome in both the presence and the absence of systemic adjuvant therapy. By use of two independent cohorts of invasive breast carcinomas, our study is, to our knowledge, the first to develop a four-marker surrogate immunohistochemistry panel, including ER, PR, HER2, and Ki67, to distinguish the luminal B subtype from the luminal A subtype. We developed the immunopanel against a gold standard definition for

Table 1. Clinicopathological characteristics of breast cancers used in this study*

Characteristic	UBC-WashU series	Total BCCA series	Luminal tumors in BCCA series			
	Training set (n = 357), No. of patients (%)	Validation set (n = 4046), No. of patients (%)	No. of luminal A (%) (n = 1530)	No. of luminal B (%) (n = 846)	No. of luminal/HER2+ (%) (n = 222)	Total No. (n = 2598)
Age at diagnosis, y						
≤40	34 (10)	380 (9)	82 (5.4)	82 (9.7)	21 (9.5)	185
40–49	73 (20)	767 (19)	261 (17.1)	175 (20.7)	49 (22.1)	485
50–65	119 (33)	1435 (36)	566 (37.0)	275 (32.5)	78 (35.1)	919
>65	124 (35)	1464 (36)	621 (40.6)	314 (37.1)	74 (33.3)	1099
Unknown	7 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0
Menstrual status at referral						
Premenopausal	—	1188 (29)	363 (23.7)	281 (33.2)	66 (29.7)	710
Postmenopausal	—	2761 (68)	1139 (74.4)	548 (64.8)	145 (65.3)	1832
Pregnant	—	2 (0.1)	0 (0)	0 (0)	1 (0.5)	1
Unknown	—	95 (2)	28 (1.8)	17 (2.0)	10 (4.5)	55
Histology						
Ductal	322 (90)	3661 (91)	1390 (90.8)	781 (92.3)	216 (97.3)	2387
Lobular	27 (8)	308 (8)	133 (8.7)	59 (7.0)	5 (2.3)	197
Other	6 (2)	77 (2)	7 (0.5)	6 (0.7)	1 (0.5)	14
Unknown	2 (0.6)	0 (0)	0 (0)	0 (0)	0	0
Grade						
1	45 (13)	211 (5)	925 (60.5)†	350 (41.4)†	60 (27.0)†	1335†
2	171 (48)	1582 (39)				
3	133 (37)	2069 (51)	521 (34.1)	468 (55.3)	155 (69.8)	1144
Unknown	8 (2)	184 (5)	84 (5.5)	28 (3.3)	7 (3.2)	119
Tumor size, cm						
≤2	139 (39)	2093 (52)	943 (61.6)	384 (45.4)	90 (40.5)	1417
2–5	170 (48)	1697 (42)	580 (37.9)‡	450 (53.2)‡	131 (59.0)	1161‡
>5	30 (8)	219 (5)				
Unknown	18 (5)	37 (1)	7 (0.5)	12 (1.4)	1 (0.5)	20
Lymph node status						
Negative	183 (51)	2161 (53)	844 (55.2)	422 (49.9)	90 (40.5)	1356
Positive	137 (38)	1701 (42)				
1–3 lymph nodes			436 (28.5)	244 (28.8)	64 (28.8)	744
≥4 lymph nodes			180 (11.8)	140 (16.5)	54 (24.3)	374
Unknown	37 (10)	184 (5)	70 (4.6)	40 (4.7)	14 (6.3)	124
Lymphovascular invasion						
Positive	—	1750 (43)	590 (38.6)	423 (50.0)	133 (59.9)	1146
Negative	—	2120 (52)	874 (57.1)	385 (45.5)	84 (37.8)	1343
Unknown	—	176 (4)	66 (4.3)	38 (4.5)	5 (2.3)	109
ER status						
Positive	221 (62)	2791 (69)	1480 (96.7)	823 (97.3)	204 (91.9)	2507
Negative	109 (31)	1224 (30)	47 (3.1)	23 (2.7)	18 (8.1)	88
Uninterpretable	27 (8)	31 (1)	3 (.2)	0 (0)	0 (0)	3
PR status						
Positive	137 (40)	1846 (46)	1040 (68)	522 (61.7)	108 (48.6)	1670
Negative	142 (38)	1759 (43)	389 (25.4)	275 (32.5)	105 (47.3)	769
Uninterpretable	78 (22)	441 (11)	101 (6.6)	49 (5.8)	9 (4.1)	159
HER2 status						
Positive	35 (10)	507 (13)	0 (0)	0 (0)	222 (100)	222
Negative	277 (78)	3360 (83)	1530 (100)	846 (100)	0 (0)	2376
Uninterpretable	45 (13)	179 (4)	0 (0)	0 (0)	0 (0)	0
Local therapy						
No breast surgery	—	60 (2)	25 (1.6)	11 (1.3)	2 (0.9)	38
Mastectomy + RT	—	631 (16)	196 (12.8)	129 (15.2)	47 (21.2)	372
Mastectomy alone	—	1557 (39)	589 (38.5)	341 (40.3)	92 (41.4)	1022
Lumpectomy alone	—	135 (3)	54 (3.5)	31 (3.7)	4 (1.8)	89
Lumpectomy + RT	—	1663 (41)	666 (43.5)	334 (39.5)	77 (34.7)	1077

(Table continues)

Table 1 (continued).

Characteristic	UBC-WashU series	Total BCCA series	Luminal tumors in BCCA series			
	Training set (n = 357), No. of patients (%)	Validation set (n = 4046), No. of patients (%)	No. of luminal A (%) (n = 1530)	No. of luminal B (%) (n = 846)	No. of luminal/HER2+ (%) (n = 222)	Total No. (n = 2598)
Adjuvant systemic therapy						
None	128 (36)	1689 (42)	672 (43.9)	287 (33.9)	64 (28.8)	1023
HT only	82 (23)					
Tam only		1305 (32)	542 (38.2)	303 (35.8)	89 (40.1)	976
Ovarian ablation or HT other than Tam		7 (0.2)	2 (0.1)	2 (0.2)	0 (0)	4
Chemotherapy only	65 (18)					
AC		148 (4)	23 (1.5)	31 (3.7)	6 (2.7)	60
CMF		429 (11)	143 (9.3)	96 (11.3)	27 (12.2)	266
FAC		92 (2)	12 (0.8)	25 (3.0)	7 (3.2)	44
Other		70 (2)	7 (0.5)	13 (1.5)	4 (1.9)	24
Combination therapy	78 (22)					
AC + Tam		125 (3)	39 (2.5)	37 (4.4)	11 (5.0)	87
CMF + Tam		39 (1)	15 (1.0)	13 (1.5)	2 (0.9)	30
FAC + Tam		68 (2)	13 (0.8)	16 (1.9)	8 (3.6)	37
Chemotherapy (other) + Tam		69 (2)	20 (1.3)	18 (2.1)	4 (1.8)	42
Ovarian ablation or HT + chemotherapy		5 (0.1)	0 (0)	5 (0.6)	0 (0)	5
Unknown	4 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0

* UBC-WashU = University of British Columbia and Washington University at St Louis series; BCCA = British Columbia Cancer Agency series; RT = radiation therapy; ER = estrogen receptor; PR = progesterone receptor; Tam = tamoxifen; HT = hormone therapy; AC = doxorubicin and cyclophosphamide; CMF = cyclophosphamide, methotrexate, and fluorouracil; FAC = 5-fluorouracil, doxorubicin, and cyclophosphamide; — = not available.

† Grade 1 or 2 tumors combined for χ^2 test.

‡ Tumor size more than 2 cm used for χ^2 test.

tumor subtype that used results from gene expression profiling and demonstrated clinically significant associations with breast cancer relapse and survival.

The luminal B subtype is characterized by having increased expression of HER2-associated genes (ie, *ERBB2* and *GRB7*) and a cell proliferation signature that includes the expression of *MKI67*, *CCNB1*, and *MYBL2*, which have been associated with tamoxifen resistance (16,40). Efficient clinical identification of luminal B breast cancers would isolate a poor prognosis subgroup that could likely benefit from additional systemic therapy from among otherwise good prognosis, hormone receptor–positive tumors. As suggested from gene expression profiling, coexpression of HER2 and

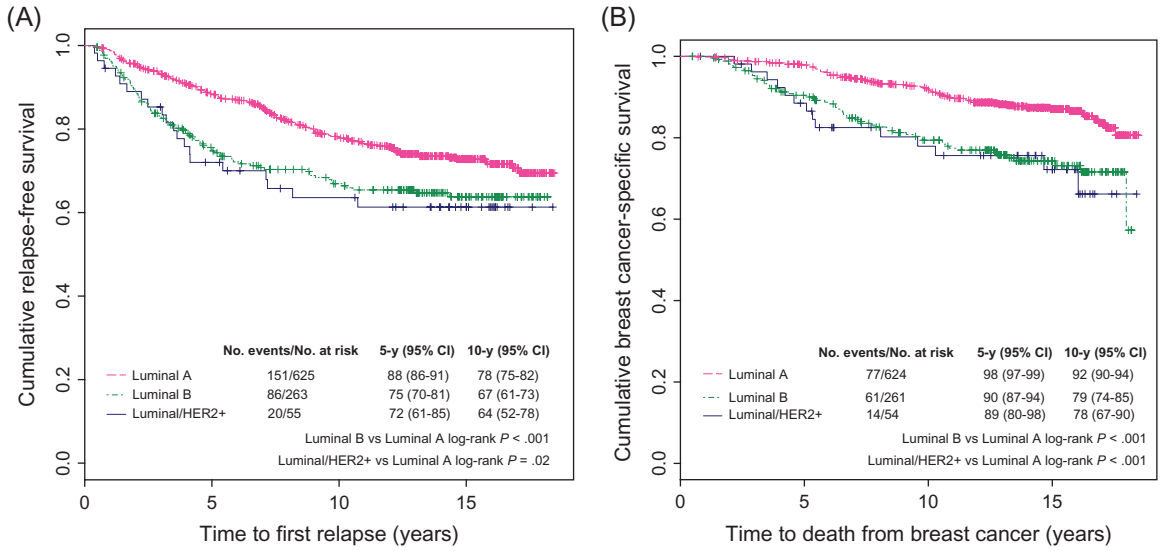

Figure 3. Univariate survival by breast cancer subtype among 943 patients with lymph node–negative, hormone receptor–positive breast cancer who received no adjuvant systemic therapy. **A**) Relapse-free survival. **B**) Breast cancer–specific survival. CI = confidence interval.

Table 2. Association of patient and tumor characteristics with relapse-free survival and breast cancer–specific survival among 883 patients with lymph node–negative, hormone receptor–positive breast cancer with complete data for covariates and who did not receive any adjuvant systemic therapy*

Characteristic and comparison	Relapse-free survival (n = 883)		Breast cancer–specific survival (n = 879)	
	HR (95% CI)	*P* value†	HR (95% CI)	*P* value†
Age at diagnosis‡	1.00 (0.99 to 1.01)	.43	1.01 (0.99 to 1.02)	.30
Grade (3 vs 2 or 1)	1.10 (0.84 to 1.44)	.50	1.24 (0.88 to 1.75)	.22
Tumor size (>2 cm vs ≤2 cm)	1.43 (1.09 to 1.86)	.010	1.59 (1.14 to 2.23)	.007
LVI (positive vs negative)	1.49 (1.04 to 2.13)	.031	1.72 (1.11 to 2.66)	.015
Breast cancer subtypes				
Luminal B vs luminal A	1.43 (1.08 to 1.90)	.013	1.84 (1.28 to 2.63)	.001
Luminal/HER2+ vs luminal A	1.57 (0.97 to 2.54)	.066	2.08 (1.15 to 3.76)	.016

* Multivariable Cox proportional hazards regression analyses were used to estimate the adjusted HRs for the breast cancer subtypes. HR = hazard ratio; CI = confidence interval; LVI = lymphovascular invasion.

† All Wald statistical tests were two-sided.

‡ Age is in years.

ER and/or PR can identify some luminal B tumors (ie, the luminal–HER2-positive group). However, only approximately 30% of luminal B tumors are HER2 positive, indicating that this clinical marker alone is not sensitive enough to identify most luminal B breast cancers. In this study, we categorized such tumors as luminal–HER2 positive because they require a distinct treatment approach involving HER2-targeted therapy (eg, trastuzumab). However, from a biological perspective (ie, in terms of their gene expression profile), these tumors belong to the luminal B subtype.

Ki67 is a well-established cell proliferation marker in cancer and an excellent candidate biomarker for luminal B tumors. Two recent meta-analyses have reported a statistically significant association between high Ki67 expression and increased risk of breast cancer relapse and death (12,41). However, assessment of Ki67 has been a matter of controversy because some studies have used 10% (42,43) or 20% (44,45) cut points, whereas others dichotomized around the mean (46) or median (30,47) value. Our study is the first to apply quantitative Ki67 visual immunohistochemistry scores to breast cancer biological subtypes that were classified by gene expression profiling. An advantage of this approach is that the optimal threshold of Ki67 immunohistochemistry (in this case 14%) was determined against an important distinction in the underlying biology of breast cancer rather than against clinical outcome or the mean or median value of the Ki67 index in the study population. By this approach, the cut point will more likely be directly applicable in other cohorts of patients with different treatment regimens and risk distributions. Although gene expression profiling remains the most sensitive method, we have demonstrated that Ki67 can be added concurrently to the standard biomarker panel of ER, PR, and HER2 to identify additional luminal B tumors that would not be identified by these three markers. The addition of epidermal growth factor receptor and cytokeratin 5/6 to this panel allows identification of the basal-like subtype of breast cancer (20,48).

We evaluated the prognostic value of our luminal B immunohistochemistry panel using an independent, regional population-based cohort of 4046 patients who were originally diagnosed with breast cancer between January 1, 1986, and September 30, 1992. These patients received adjuvant therapy according to guidelines developed and disseminated by the British Columbia Cancer Agency (24). In general, adjuvant systemic treatment was less aggressive than in contemporary practice, with consequent higher event rates. We demonstrated the prognostic value of our luminal B definition within homogeneously treated patient subsets. Among patients with hormone receptor–positive tumors who received no adjuvant systemic treatment, luminal B and luminal–HER2-positive tumors were associated with increased risk of breast cancer relapse and death. In contemporary practice, almost all patients with hormone receptor–positive breast cancer are treated with hormonal therapy (tamoxifen or aromatase inhibitors), and in this study, luminal B and luminal–HER2-positive tumors were associated with increased risk of breast cancer relapse and death in the subgroup receiving adjuvant tamoxifen, in comparison with the more common luminal A subtype.

In multivariable analysis, the luminal B and luminal–HER2-positive subtypes provided statistically significant prognostic value beyond current standard clinicopathological parameters. The Cox regression models included tumor size, age at diagnosis, grade, lymph node involvement, and lymphovascular invasion, which include the compulsory variables for calculation of the Nottingham Prognostic Index (49) and Adjuvant! Online, a computer software program that predicts breast cancer outcomes by use of SEER data and clinical trial meta-analyses to guide treatment decisions in clinical practice (50). Indeed, almost half of our patient cohort was included in an earlier study confirming that in the British Columbia population, Adjuvant! predictions are associated with observed outcomes (51), providing support that the conclusions in this study can be extended to other North American and UK populations. Luminal B status as defined by Ki67 labeling retained independent prognostic value in patients with lymph node–negative or lymph node–positive, hormone receptor–positive breast cancer who were treated with adjuvant tamoxifen. The recurrence score, a qRT-PCR–based measure of risk of breast cancer recurrence, is currently an available diagnostic test to predict distant recurrence for ER-positive breast cancers in patients with negative axillary lymph nodes who are treated with adjuvant tamoxifen (19). This score

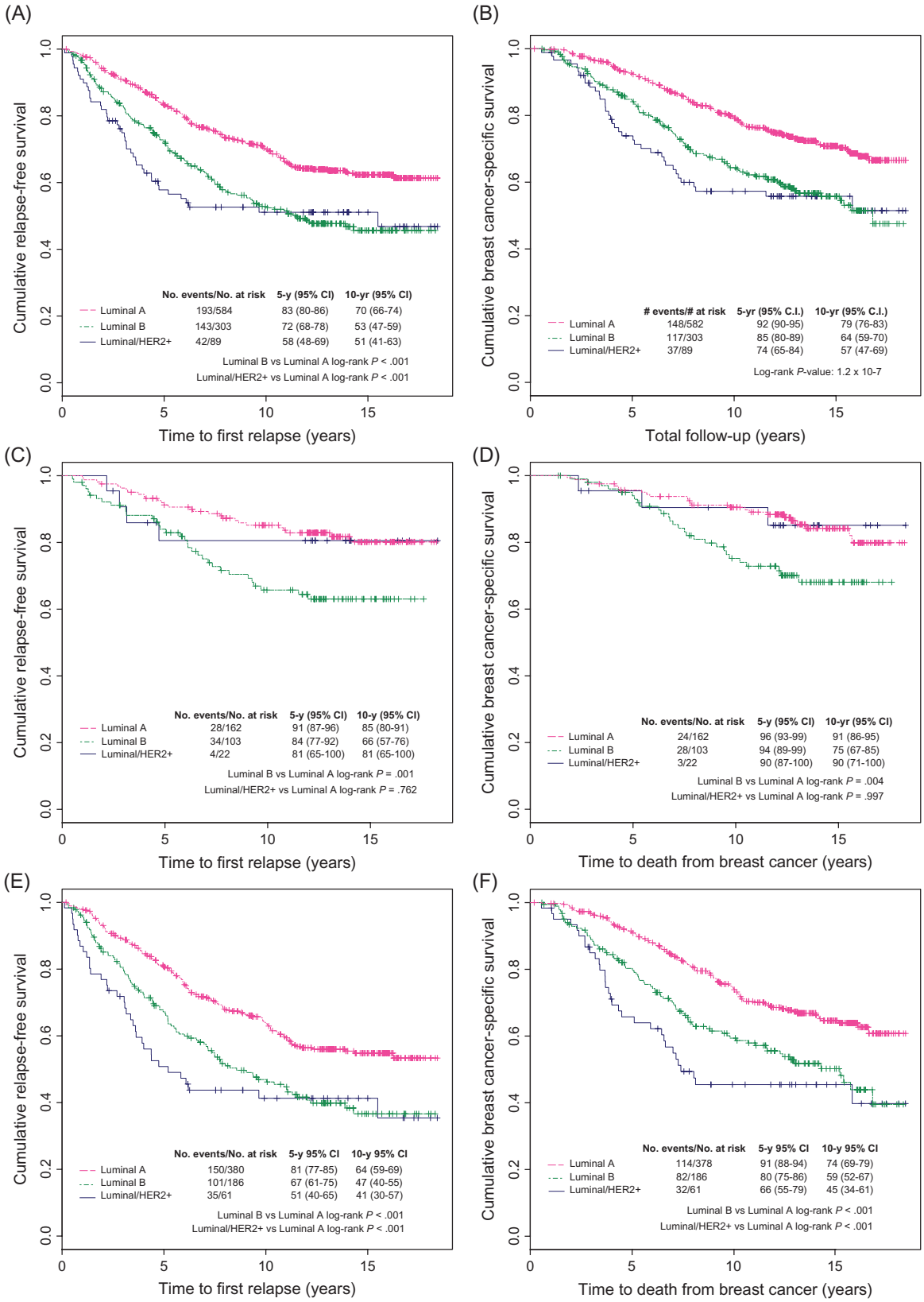

Figure 4. Univariate survival by breast cancer subtype among 976 patients with hormone receptor–positive breast cancer who received tamoxifen as their sole adjuvant systemic therapy. **A**) Relapse-free survival among all 976 patients. **B**) Breast cancer–specific survival among all 974 patients (two patients with unknown cause of death were excluded). **C)** Relapse-free survival among 287 patients with lymph node–negative disease. **D**) Breast cancer–specific survival among 287 patients with lymph node–negative disease. **E**) Relapse-free survival among 627 patients with lymph node–positive disease. **F**) Breast cancer–specific survival among 625 patients with lymph node–positive disease (two patients with unknown cause of death were excluded). CI = confidence interval.

Table 3. Association of patient and tumor characteristics with relapse-free survival and breast cancer–specific survival among 828 hormone receptor–positive patients with complete data for covariates and who received tamoxifen as their sole adjuvant systemic therapy and among 167 patients with hormone receptor–positive tumors with complete data for all the covariates and who received both tamoxifen and chemotherapy as adjuvant systemic therapies*

	Relapse-free survival		Breast cancer-specific survival	
Characteristic and comparison	**HR (95% CI)**	***P* value†**	**HR (95% CI)**	***P* value†**
Hormone receptor–positive patients who received only tamoxifen as adjuvant therapy				
Total group‡				
Age at diagnosis§	0.99 (0.98 to 1.00)	.090	1.00 (0.98 to 1.02)	.95
Grade (3 vs 2 or 1)	1.33 (1.06 to 1.68)	.016	1.35 (1.04 to 1.75)	.023
Tumor size (> 2 vs ≤ 2 cm)	1.56 (1.23 to 1.97)	<.001	1.64 (1.26 to 2.13)	<.001
LVI (positive vs negative)	1.18 (0.91 to 1.51)	.21	1.02 (0.78 to 1.34)	.88
Positive axillary lymph nodes, % of total examined				
0–25 vs 0	1.91 (1.40 to 2.61)	<.001	1.69 (1.19 to 2.40)	.004
> 25 vs 0	3.24 (2.38 to 4.42)	<.001	3.26 (2.32 to 4.57)	<.001
Breast cancer subtypes				
Luminal B vs luminal A	1.59 (1.25 to 2.03)	<.001	1.60 (1.22 to 2.10)	<.001
Luminal/HER2+ vs luminal A	1.56 (1.09 to 2.25)	.016	1.77 (1.20 to 2.62)	.004
Group with lymph node–negative disease (n = 267)				
Age at diagnosis§	1.02 (0.99 to 1.05)	.28	1.03 (1.00 to 1.07)	.06
Grade (3 vs 2 or 1)	1.63 (0.96 to 2.76)	.070	1.25 (0.70 to 2.22)	.45
Tumor size (> 2 vs ≤ 2 cm)	1.42 (0.83 to 2.41)	.20	1.54 (0.85 to 2.78)	.16
LVI (positive vs negative)	0.87 (0.51 to 1.46)	.59	0.90 (0.51 to 1.61)	.73
Breast cancer subtypes				
Luminal B vs luminal A	2.14 (1.24 to 3.67)	.006	2.22 (1.22 to 4.04)	.009
Luminal/HER2+ vs luminal A	1.07 (0.36 to 3.16)	.90	1.04 (0.30 to 3.60)	.95
Group with lymph node–positive disease ‖				
Age at diagnosis§	0.99 (0.97 to 1.00)	.062	1.00 (0.98 to 1.01)	.71
Grade (3 vs 2 or 1)	1.32 (1.02 to 1.70)	.037	1.46 (1.09 to 1.95)	.011
Tumor size (> 2 vs ≤ 2 cm)	1.67 (1.28 to 2.16)	<.001	1.75 (1.30 to 2.34)	<.001
LVI (positive vs negative)	1.33 (0.99 to 1.79)	.057	1.09 (0.80 to 1.51)	.56
Breast cancer subtypes				
Luminal B vs luminal A	1.50 (1.14 to 1.97)	.004	1.49 (1.09 to 2.03)	.013
Luminal/HER2+ vs luminal A	1.78 (1.21 to 2.62)	.004	2.03 (1.34 to 3.07)	<.001
Hormone receptor–positive patients who received both tamoxifen and chemotherapy				
Total group¶				
Age at diagnosis§	0.97 (0.94 to 1.00)	.046	0.98 (0.94 to 1.01)	.13
Grade (3 vs 2 or 1)	1.19 (0.70 to 2.03)	.52	0.94 (0.54 to 1.62)	.82
Tumor size (> 2 vs ≤ 2 cm)	1.00 (0.57 to 1.76)	1.00	1.54 (0.82 to 2.90)	.18
LVI (positive vs negative)	1.05 (0.59 to 1.87)	.86	1.00 (0.54 to 1.84)	.99
% of positive axillary lymph nodes over total examined				
0–25 vs 0	1.54 (0.70 to 3.40)	.29	2.11 (0.83 to 5.34)	.12
> 25 vs 0	2.04 (0.96 to 4.30)	.062	3.11 (1.31 to 7.39)	.010
Breast cancer subtypes				
Luminal B vs luminal A	2.03 (1.15 to 3.58)	.015	1.92 (1.05 to 3.52)	.034
Luminal–HER2+ vs luminal A	2.65 (1.23 to 5.71)	.013	3.73 (1.70 to 8.16)	.001

* Multivariable Cox proportional hazards regression analyses were used to estimate the adjusted HRs of breast cancer subtypes. HR = hazard ratio; CI = confidence interval; LVI = lymphovascular invasion.

† All Wald statistical tests were two-sided.

‡ In the total group, data from 828 patients were available for relapse-free survival and data from 826 patients were available for breast cancer–specific survival.

§ Age is in years.

‖ In this group, data from 561 patients were available for relapse-free survival and data from 559 patients were available for breast cancer–specific survival.

¶ The total group had 167 patients with complete data for all the covariates and who received both tamoxifen and chemotherapy (doxorubicin and cyclophosphamide; fluorouracil, doxorubicin, and cyclophosphamide; or cyclophosphamide, methotrexate, and fluorouracil) as adjuvant systemic therapies. Data from all 167 patients were available for both relapse-free survival and breast cancer–specific survival.

Table 4. Clinicopathological characteristics of 196 hormone receptor–positive patients who received both tamoxifen and chemotherapy as their adjuvant systemic therapy*

Characteristic	Tamoxifen + (AC or FAC) (n = 124), N (%)	Tamoxifen + CMF (n = 72), N (%)	Total (n = 196)	*P* value†
Age, y				
≤40	12 (9.7)	13 (18.1)	25	.094
40–49	50 (40.3)	18 (25.0)	68	
50–65	58 (46.8)	37 (51.4)	95	
>65	4 (3.2)	4 (5.6)	8	
Tumor size, cm				
≤2	44 (36.7)	16 (22.5)	60	.053
>2	76 (63.3)	55 (77.5)	131	
Grade				
1 or 2	60 (50.0)	35 (51.5)	95	.88
3	60 (50.0)	33 (48.5)	93	
Lymphovascular invasion				
Negative	36 (30.8)	25 (37.3)	61	.42
Positive	81 (69.2)	42 (62.7)	123	
% of positive axillary lymph nodes/total examined lymph nodes				
0	25 (20.7)	6 (9.2)	31	.067
0–25	40 (33.1)	19 (29.2)	59	
>25	56 (46.3)	40 (61.5)	96	
Breast cancer subtypes				
Luminal A	52 (41.9)	35 (48.6)	87	.332
Luminal B	53 (42.7)	31 (43.1)	84	
Luminal–HER2+	19 (15.3)	6 (8.3)	25	

* AC = doxorubicin and cyclophosphamide; FAC = fluorouracil, doxorubicin, and cyclophosphamide; CMF = cyclophosphamide, methotrexate, and fluorouracil.

† All Wald statistical tests were two-sided.

uses the expression of 16 genes, weighted heavily on *MKI67* and other proliferation-associated genes, to calculate a risk score. This recurrence score assay has not been applied to the BCCA series of tumors, which limits our capacity to do a head-to-head comparison between our immunopanel and this qRT-PCR assay (the cost of which is approximately 10-fold higher per tumor). However, Fan et al. (39) have shown that breast cancer subtype as determined by gene expression profiling and the recurrence score have statistically significant agreement in outcome predictions. This result indicates that among patients with lymph node–negative, ER-positive disease who were treated with adjuvant tamoxifen, high recurrence scores appear to track largely with luminal B cancers.

The association between the luminal B subtype and response to adjuvant systemic chemotherapy has yet to be fully elucidated. A

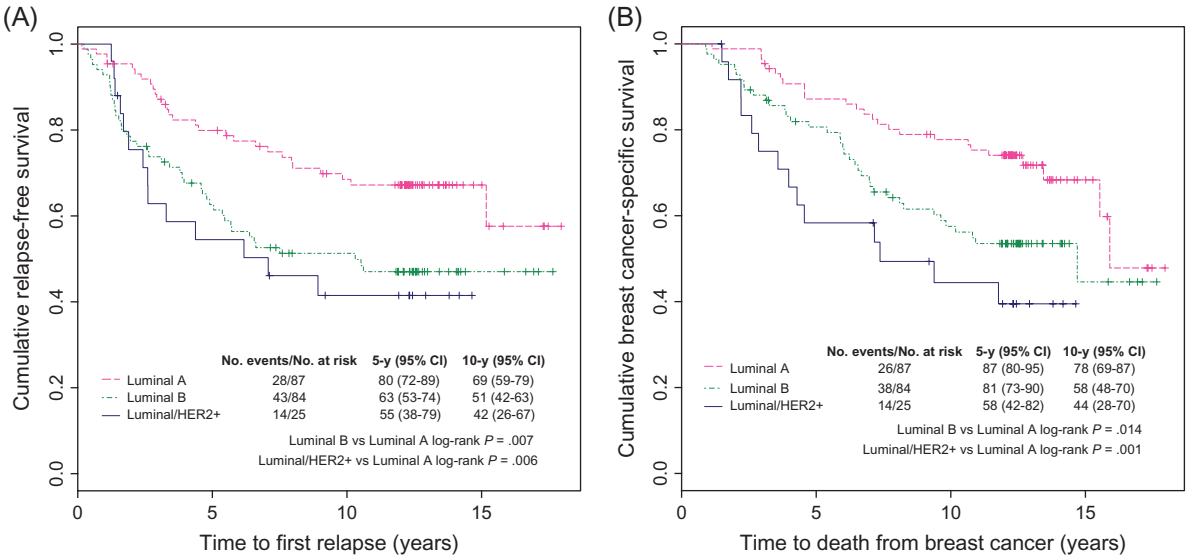

Figure 5. Univariate survival by breast cancer subtype among 196 patients with hormone receptor–positive breast cancer who were treated with both tamoxifen and chemotherapy (doxorubicin and cyclophosphamide; fluorouracil, doxorubicin, and cyclophosphamide; or cyclophosphamide, methotrexate, and fluorouracil) as adjuvant systemic treatments. **A**) Relapse-free survival. **B**) Breast cancer–specific survival. CI = confidence interval.

meta-analysis of 1521 patients with endocrine-responsive tumors enrolled in two randomized trials of adjuvant chemoendocrine therapy reported that Ki67 expression as a single marker was not associated with resistance or benefit from chemotherapy, beyond the benefit incurred with hormonal therapy alone (30). The chemotherapy regimen used in these two trials was cyclophosphamide, methotrexate, and fluorouracil, and the median value—19%—was the cut point for the Ki67 index. In contrast, we assessed the Ki67 index only in the context of hormone receptor–positive, HER2-negative tumors and used a cut point of 14%. We found that the Ki67 index and HER2 expression could be used to stratify the risk for breast cancer relapse and death among patients with hormone receptor–positive breast cancer who were treated with both tamoxifen and chemotherapy as their adjuvant systemic therapy.

This study has several limitations. The main weaknesses of immunohistochemical approaches are limited technical reproducibility, subjective interpretation, and qualitative readouts (52,53). To facilitate analysis of sufficiently large cohorts of samples, the immunostaining panel was trained and validated on tissue microarrays, whereas clinical implementation would likely occur on whole sections. It is possible that Ki67 index may demonstrate focally higher areas on whole sections that can be appreciated on tissue microarrays, although tissue microarray results have repeatedly been demonstrated to show excellent agreement with whole sections (54), including in studies of breast cancer (55) and of Ki67 (56). The distinction between luminal A and luminal B tumors is somewhat difficult to achieve, and our panel has false-positive and false-negative rates of approximately 25% (Figure 2). The need for long-term follow-up data necessitated the use of a historical cohort of old paraffin blocks, which could potentially differ from recently fixed and processed prospective specimens. Treatment recommendations at the time patients in this study were treated tended to be less aggressive than in contemporary practice, and treatment cohorts are not randomized in the population-based validation cohort.

Strengths of the study design include the use of large independent cohorts to separate development of the immunopanel (against a gold standard of gene expression profiling) from its application to a large patient series for which information on clinical outcomes was available. Our subtype definition was linked to breast cancer biology through a gene expression profiling approach in which tumor specimens were assigned to intrinsic breast cancer biological subtypes, which were previously shown to be reproducible across patient populations and gene expression platforms (25), as opposed to being linked to an expression profile classifier optimized against patient outcome (which can be problematic to extrapolate to other cohorts of patients who in general will have differences in risk profile and specific treatment). The four-biomarker immunopanel is economical, antibodies against the biomarkers are readily available, and equivalent tissue microarray approaches can be used with existing legacy specimens from clinical trials to examine predictive values. Immunohistochemistry retains the advantage of assessing protein expression in the context of tumor morphology, can be applied to tiny core needle biopsy samples in clinical and research laboratories, and has a rapid turnaround time. Although we consider breast cancer molecular subtyping by gene expression profiling to be the gold standard, we nevertheless believe that there is an immediate need for a well-defined and validated immunopanels for worldwide clinical diagnostic use.

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Notes

Dr P. S. Bernard owns founding stock in University Genomics, Inc. Dr C. M. Perou owns stock in University Genomics, Inc., which makes assays for breast cancer patient prognosis prediction. Dr M. J. Ellis is a founder of University Genomics, Inc. and owns stock in this company. Dr T. O. Nielsen is a codirector of the tissue microarray unit of the Genetic Pathology Evaluation Centre, which has an unrestricted educational grant from sanofi-aventis, Canada.

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