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ORIGINAL REPORT

Identification of a Low-Risk Luminal A Breast Cancer Cohort That May Not Benefit From Breast Radiotherapy

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ABSTRACT

Purpose
To determine the prognostic and predictive value of intrinsic subtyping by using immunohistochemical (IHC) biomarkers for ipsilateral breast relapse (IBR) in participants in an early breast cancer randomized trial of tamoxifen with or without breast radiotherapy (RT).

Patients and Methods
IHC analysis of estrogen receptor, progesterone receptor, human epidermal growth factor receptor 2 (HER2), cytokeratin 5/6, epidermal growth factor receptor, and Ki-67 was conducted on 501 of 769 available blocks. Patients were classified as luminal A (n = 265), luminal B (n = 165), or high-risk subtype (luminal HER2, n = 22; HER2 enriched, n = 13; basal like, n = 30; or triple-negative nonbasal, n = 6). Median follow-up was 10 years.

Results
Classification by subtype was prognostic for IBR (10-year estimates: luminal A, 5.2%; luminal B, 10.5%; high-risk subtypes, 21.3%; $P < .001$). Luminal subtypes seemed to derive less benefit from RT (luminal A hazard ratio [HR], 0.40; luminal B HR, 0.51) than high-risk subtypes (HR, 0.13); however, the overall subtype-treatment interaction term was not significant ($P = .26$). In an exploratory analysis of women with clinical low-risk (age older than 60 years, T1, grade 1 or 2) luminal A tumors (n = 151), 10-year IBR was 3.1% versus 11.8% for the high-risk cohort (n = 341; $P = .0063$). Clinical low-risk luminal A patients had a 10-year IBR of 1.3% with tamoxifen versus 5.0% with tamoxifen plus RT ($P = .42$). Multivariable analysis showed that RT (HR, 0.31; $P < .001$), clinical risk group (HR, 2.2; $P = .025$), and luminal A subtype (HR, 0.25; $P < .001$) were significantly associated with IBR.

Conclusion
IHC subtyping was prognostic for IBR but was not predictive of benefit from RT. Further studies may validate the exploratory finding of a low-risk luminal A group who may be spared breast RT.

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INTRODUCTION

Postoperative breast radiotherapy (RT) significantly reduces local relapse in women with breast cancer; however, the majority with early-stage disease will not relapse even without RT.[1] Clinicopathologic factors are limited in their ability to accurately predict the risk of breast cancer relapse or the benefit from adjuvant RT. Randomized studies evaluating the hypothesis that endocrine therapy alone is sufficient for local control have demonstrated an overall benefit from RT; however, older women with small, lower-grade tumors had a low absolute risk of breast cancer relapse and benefited the least.[2-6] Nonetheless, there remains disagreement regarding the characteristics of patients with the lowest risk of local recurrence who could be spared treatment,[7-9] and most women are still receiving breast RT.[10] Thus, the development of a personalized approach to define the optimal role for breast RT in early breast cancer would be extremely valuable.[11]

Gene expression and next-generation sequencing studies[12-14] have identified distinct molecular subtypes of breast cancer with different clinical behaviors and treatment responses. Molecular tools such as MammaPrint and OncotypeDX complement standard clinicopathologic features to predict distant relapse and chemotherapy response.[15,16] These molecular signatures are costly for large-scale implementation, but less expensive immunohistochemistry

(IHC) surrogates for major intrinsic biologic subtypes are prognostic for breast cancer relapse.[17-20] Previous studies did not include patients randomly assigned to breast-conserving surgery with endocrine therapy alone, limiting their predictive value for breast RT.

The Toronto–British Columbia (TBC) trial randomly assigned older patients with node-negative breast cancer to tamoxifen or tamoxifen and breast RT.[4] Tissue samples from trial participants were analyzed in that study by using a six-IHC-marker subtyping panel,[17,19] and the primary objective was to define intrinsic subtyping as a predictive biomarker of RT benefit. A secondary objective was to identify a group of women who may not require RT. An exploratory analysis of molecular subtyping in addition to clinicopathologic factors was undertaken to evaluate this objective. A goal of 10-year ipsilateral breast relapse (IBR) rate of less than 5% was defined, which was considered to be acceptable because many breast cancer relapses can be salvaged, and it is unlikely that this small risk would compromise survival.[1]

PATIENTS AND METHODS

Patients and Treatment

Details of the TBC randomized trial are summarized as follows[4]: women age 50 years or older with an invasive adenocarcinoma ≤ 5 cm (pT1/T2) with negative axillary nodes were randomly assigned to breast RT and tamoxifen or tamoxifen alone after breast-conserving surgery. RT dose was 40 Gy in 16 daily fractions to the breast over 3 to 4 weeks, followed by a boost of 12.5 Gy in five daily fractions to the primary site. Tamoxifen was administered at 20 mg per day for 5 years.

Tissue Microarrays

After obtaining the institution's Research Ethics Board approval, archival formalin-fixed paraffin-embedded blocks were obtained from 501 (65%) of the original 769 trial participants (Fig 1 and Appendix Table A1, online only). Slides stained with hematoxylin and eosin were reviewed by subspecialty breast pathologists (N.M., S.J.D., T.O.N., S.N.-M., and M.C.C.) to reassign grade and histologic subtype and to oversee construction of a tissue microarray (TMA). The TMA was constructed by using three 0.6-mm morphologically representative tumor cores and one adjacent normal mammary epithelial tissue core from each surgical sample. Each tissue core was assigned a unique TMA location number; in total, 12 tissue array blocks were constructed for all 501 specimens.

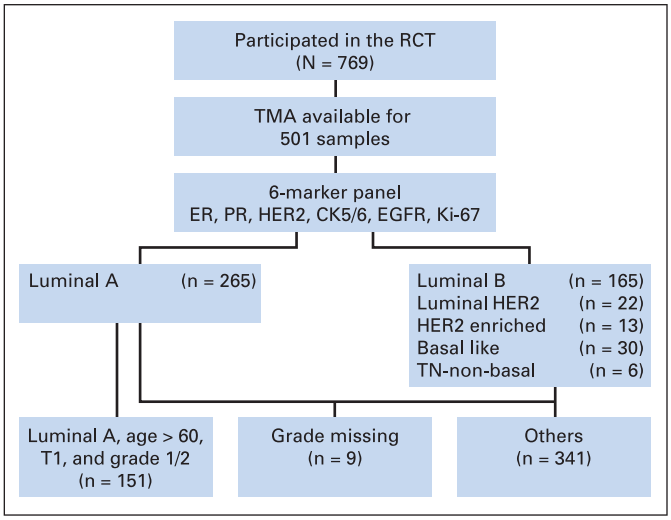

Fig 1. Participant flow schema. From the 769 participants in the original randomized clinical trial (RCT), a tissue microarray (TMA) was constructed for 501 patient samples, which was subjected to analysis for six immunohistochemical markers. The specific number of patient samples in each subtype is shown, along with the total number in the lowest risk category of luminal A, T1, age older than 60 years, and grade 1 or 2 tumors. EGFR, epidermal growth factor receptor; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor; TN, triple negative.

IHC, Human Epidermal Growth Factor Receptor 2, and Fluorescent in Situ Hybridization

IHC for estrogen receptor, progesterone receptor, human epidermal growth factor receptor 2 (HER2; SP3 and 4B5), cytokeratin 5/6 (CK 5/6), epidermal growth factor receptor, and Ki-67 was performed on TMA sections and detected by using the Ventana Autostainer (BenchMark XT; Ventana Medical Systems, Tucson, AZ) using the sources and dilutions of the primary antibodies as previously described (Appendix Methods, online only, and Appendix Table A2, online only).[17] Ten percent of samples were randomly selected for independent rescoring, demonstrating more than 90% concordance for all six immunomarkers. The 501 tumors were categorized into one of the following six molecular subtypes: luminal A, luminal B, luminal HER2, HER2 enriched, basal like, or triple-negative (TN) nonbasal (Appendix Table A1).[17]

Fluorescent in situ hybridization was performed in 53 patient samples with equivocal IHC scoring for HER2 expression (see Appendix Methods). Fluorescent in situ hybridization interpretation was performed by following the College of American Pathologists/American Society of Clinical Oncology recommendations for HER2 testing for breast cancer,[21] and the United Kingdom National External Quality Assessment Service guidelines for challenging cases.[22]

Statistical Analyses

This study was conducted according to the guidelines for a prognostic and predictive factor analysis as described by Simon et al,[23] using Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) guidelines[24] (Appendix Table A3, online only). The primary outcome was time to IBR, defined as the duration from random assignment to failure of the first ipsilateral breast treatment. Deaths without IBR were considered competing risk events, and the probability of IBR was calculated by using the cumulative incidence approach.

The primary prespecified objective was to test the predictive value of intrinsic subtyping as a biomarker of RT benefit. A secondary objective was to use intrinsic subtyping to define prognosis in addition to clinicopathologic features; these objectives were analyzed independently by the study statistician (M.P.). A Fine and Gray model with an interaction term between treatment and intrinsic subtype was used to assess the predictive significance of subtype and RT benefit.[25]

A cohort of 298 patients with tissue cores collected during the period of patient accrual was first constructed and used to develop the objectives, which were submitted for grant funding and subsequently presented at the American Society of Clinical Oncology Breast Cancer Symposium (San Francisco, CA, September 7-9, 2013). Then, 203 additional tissue samples were retrieved after Research Ethics Board approval, resulting in a total cohort of 501 patients (Table 1). High-risk luminal-HER2, HER2-enriched, basal-like, and TN-nonbasal subtypes were analyzed together because of the small numbers of patients and events in each group.

The previous analysis of the TBC trial as well as other randomized trials[2-6] indicated that T stage, grade, and age were important features associated with IBR. An exploratory analysis of the combined data sets was undertaken to assess the prognostic impact of adding molecular subtyping to these clinicopathologic factors. We hypothesized that luminal A, T1, grade 1 or 2 tumors in women older than age 60 years would have the lowest IBR and that RT in this group would have the least benefit.

The proportional hazards assumption was investigated for the prognostic analysis by inspecting the Schoenfeld residual plots and by testing the

Table 1. Distribution of Clinicopathologic Characteristics

Characteristic	Patients (n = 501)	HR	P
Age, years			
50-60	130 (26%)		
> 60	371 (74%)		
Tumor size, cm*			
< 2	346 (69%)		
2-5	153 (31%)		
Grade†			
1 to 2	365 (77%)		
3	109 (23%)		
Treatment			
Tamoxifen + RT	257 (51%)		
Tamoxifen	244 (49%)		
IBR at 10 years	9.3%		
Tamoxifen + RT	5.1%	0.32	< .001
Tamoxifen	13.7%		

Abbreviations: HR, hazard ratio; IBR, ipsilateral breast relapse; RT, radiotherapy.
*Two samples were missing information about tumor size.
†Twenty-seven samples were missing information about grade.

Table 2. Univariable Analysis of the Stratified Trial Biomarkers (T stage, age) Assessed With Histologic Grade, Treatment Arm, and Intrinsic Subtype

Variable	No.	IBR at 10 Years (%)	P
Age, years			
≤ 60	130	14.1	
> 60	371	7.4	.068
T category*			
T1	412	8.4	
T2	87	13.5	.15
Grade†			
1 to 2	365	6.8	
3	109	14.3	.015
Treatment			
Tamoxifen + RT	257	5.1	
Tamoxifen	244	13.7	< .001
Subtype			
Luminal A	265	5.2	
Luminal B	165	10.5	
Other	71	21.3	< .001

Abbreviations: IBR, ipsilateral breast relapse; RT, radiotherapy.
*Two samples were missing information about tumor size.
†Twenty-seven samples were missing information about grade.

time-dependent coefficient for each covariate. The only departure from proportionality was observed for grade, and when the time-dependent coefficient was included in the model, it did not change the significance of the other covariates. Because grade was not the covariate of interest, we chose to report the average effect for this covariate. All tests and CIs were two-sided, and a *P* value of less than .05 was considered statistically significant.

RESULTS

There were 769 eligible patients entered onto the TBC trial between December 1992 and June 2000; 386 were randomly assigned to tamoxifen plus breast RT and 383 to tamoxifen alone.[4] Median age was 68 years, and 83% had pT1 lesions (median tumor size was 1.4 cm in both groups), 94% were estrogen receptor–positive or unknown, and 83% were pathologically node negative (the remainder were clinically negative).

The median follow-up time was 10 years (range, 0.01 to 18 years); there were 130 relapses (69 were breast cancer relapses) and 137 deaths. Ten-year overall survival was 84% in both groups ($P = .83$).

Prognostic and Predictive Effects of Intrinsic Subtype

In subtyped patients (501 [65%] of 769), the 10-year IBR was similar to that for the overall group (both were 9.3%); the 10-year IBR was 13.7% for tamoxifen alone versus 5.1% for tamoxifen plus RT (hazard ratio [HR], 0.32; $P < .001$). Clinicopathologic characteristics of the subtyped patients are provided in Table 1. There was a higher proportion of T2 ($P = .044$) and grade 3 tumors ($P = .0012$) in the specimens for patients who had been subtyped compared with patients for whom blocks were no longer available (Appendix Table A4, online only).

The clinical (age, T stage) and pathologic (grade 1 or 2 *v* 3) variables were assessed with treatment and intrinsic subtype (luminal A *v* luminal B *v* high risk [luminal HER2, HER2 enriched, basal like, and TN nonbasal]) in a univariable analysis. Classification by intrinsic subtype was prognostic for IBR (10-year estimates: luminal A, 5.2%; luminal B, 10.5%; high risk, 21.3%; $P < .001$; Table 2). Comparison of luminal A patients with luminal B patients also demonstrated a significant difference in IBR on univariable analysis (HR, 0.47; $P = .038$) as did grade (1 to 2 *v* 3; $P = .015$) and treatment (tamoxifen plus RT *v* tamoxifen; $P < .001$; Table 2). On multivariable analysis, only treatment (HR, 0.32; $P < .001$) and intrinsic subtype (overall $P < .001$) were independently associated with IBR (Table 3). Multivariable analysis of the individual intrinsic subtypes demonstrated that luminal A versus high-risk subtype was significant for IBR (HR, 0.21; $P < .001$) as was luminal B versus high-risk (HR, 0.45; $P = .028$) and luminal A versus luminal B subtype (HR, 0.48; $P = .045$; Table 3).

The observed effect of subtype suggested that luminal A and luminal B subtypes derived less benefit from RT (luminal A HR, 0.40; 95% CI, 0.12 to 1.29; Fig 2A; luminal B HR, 0.51; 95% CI, 0.19 to 1.36; Fig 2B) than high-risk subtype (HR, 0.13; 95% CI, 0.03 to 0.54; Figure 2C); however, the overall subtype-treatment interaction term was not significant ($P = .26$).

Exploratory Analysis of Subtyping Combined With Clinicopathologic Features

Exploratory analysis of clinical factors was undertaken to evaluate the hypothesis that luminal A, T1, grade 1 or 2 tumors in women older than age 60 years would have the lowest IBR rate. This grouping of covariates was consistent with the previous analyses of the TBC

Table 3. Multivariable Analysis of IBR for the Stratified Trial Biomarkers, Grade, Treatment, and Intrinsic Subtype (significant variables shown)

Covariate	HR	95% CI	P
Tamoxifen + RT *v* tamoxifen	0.32	0.16 to 0.62	< .001
Subtype			
Luminal A *v* high risk	0.21	0.1 to 0.46	< .001
Luminal B *v* high risk	0.45	0.22 to 0.92	.028
Luminal A *v* luminal B	0.48	0.23 to 0.98	.045
Overall			< .001

Abbreviations: HR, hazard ratio; IBR, ipsilateral breast relapse; RT, radiotherapy.

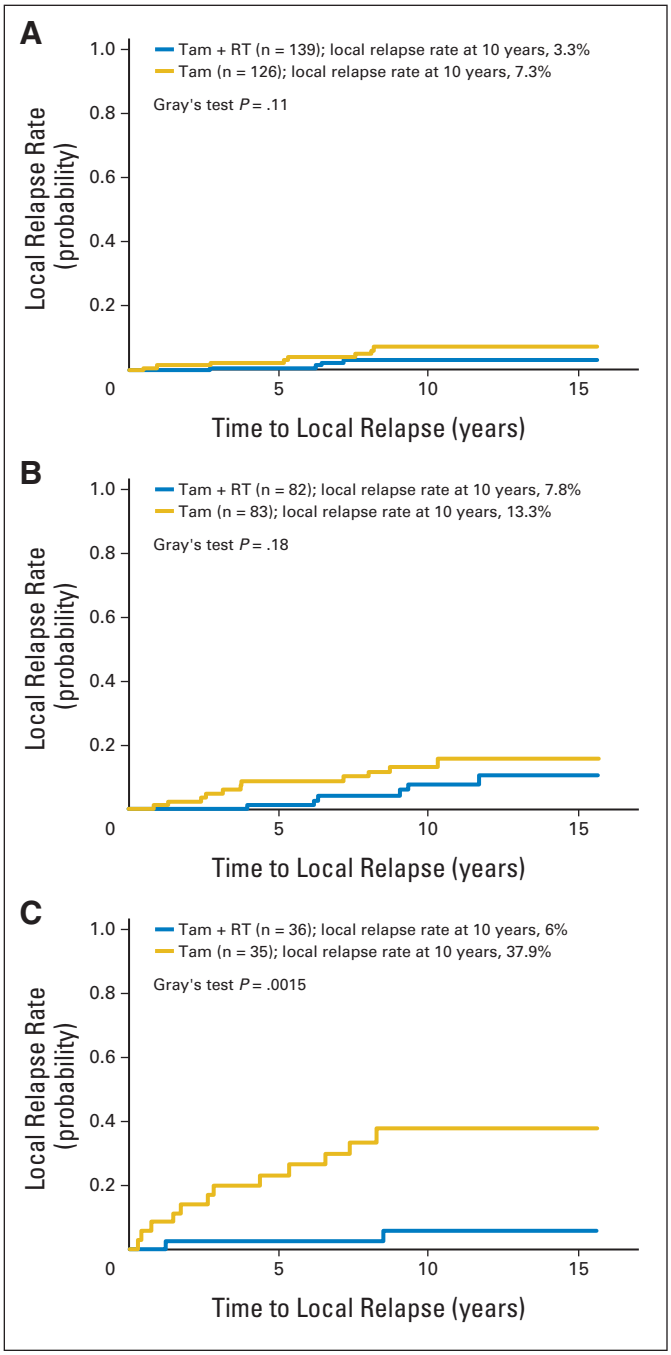

Fig 2. Cumulative incidence of ipsilateral breast relapse in the combined cohort for (A) luminal A, (B) luminal B, and (C) luminal human epidermal growth factor receptor 2 (HER2), HER2-enriched, basal-like, and triple-negative–nonbasal tumors. RT, radiotherapy; Tam, tamoxifen.

study, results from other trials,[2-6] and clinical consensus.[26] A low-risk (LR) clinical model constructed by using age older than 60 years, T1 stage, and grade 1 or 2 (n = 233; eight patients were not categorized because of missing grade) had an overall 10-year IBR of 4.6% (95% CI, 2.4% to 8.7%) compared with 13.7% (95% CI, 9.8% to 19.0%; n = 251) for the high-risk clinical group (HR, 2.75; 95% CI, 1.39 to 5.44; $P = .0036$).

Combining this LR clinical model with luminal A subtype (n = 151) resulted in a 10-year IBR of 3.1% (95% CI, 1.2% to 8.2%) versus 11.8% (95% CI, 8.6% to 16.1%) for the high-risk clinical subtype cohort (n = 341; $P = .0063$; Appendix Table A5, online only). Comparing treatment groups, LR clinical luminal A had a 10-year IBR of 1.3% (95% CI, 0.2% to 9.1%) with tamoxifen versus 5.0% (95% CI, 1.6% to 15.0%) with tamoxifen plus RT ($P = .42$). Multivariable analysis that included the clinical risk groups again demonstrated that treatment (tamoxifen plus RT *v* tamoxifen; HR, 0.31; $P <= .001$) and intrinsic subtype (overall $P = .0033$) as well as clinical risk group (HR, 2.2; $P = .025$) were independently associated with IBR (Table 4). In the patients treated with tamoxifen alone, clinical risk group (HR, 2.6; $P = .029$) and subtype (luminal A *v* high risk: HR, 0.20; $P < .001$; luminal B *v* high risk: HR, 0.35; $P = .016$; overall $P = .0022$) were significantly associated with IBR (Appendix Table A6, online only), but comparison of luminal A versus luminal B was not significant (HR, 0.56; $P = .23$).

Table 4. Multivariable Analysis of IBR Adding the Clinical Risk Groups to Treatment and Intrinsic Subtype (significant variables shown)

Covariate	HR	95% CI	*P*
Tamoxifen + RT *v* tamoxifen	0.31	0.16 to 0.62	< .001
Clinical risk groups	2.2	1.1 to 4.4	.025
Luminal A *v* high risk	0.25	0.11 to 0.56	< .001
Luminal B *v* high risk	0.51	0.24 to 1.05	.068
Luminal A *v* luminal B	0.50	0.24 to 1.05	.069
Overall			.0033

Abbreviations: HR, hazard ratio; IBR, ipsilateral breast relapse; RT, radiotherapy.

DISCUSSION

The first large-scale breast cancer gene expression profiling studies classified tumors into four major intrinsic subtypes: luminal A, luminal B, HER2 enriched, and basal like.[12,27] Luminal A was the most favorable group, with 10-year overall survival rates of approximately 95% and high local relapse-free rates of 92% after breast RT.[17] This raised the hypothesis that luminal A patients have such a favorable prognosis that breast RT might have minimal added value. In this study, a panel of six IHC biomarkers was used to define the intrinsic subtypes using the formalin-fixed paraffin-embedded blocks from participants in a randomized clinical trial comparing tamoxifen with or without breast RT for early-stage breast cancer.

The favorable biology of luminal A breast cancer has been established,[13,28] as has its relative insensitivity to chemotherapy (reviewed in Ignatiadis and Sotiriou[29]). This may be attributed to the quiet luminal A genome, in which low proliferation combined with *PIK3CA* and *MAPK* mutations could account for low rates of breast cancer recurrence.[30] In contrast, luminal B and higher-risk subtypes have more aberrant genomes, greater aneuploidy, *p53* mutations, CCND1 overexpression, DNA methylation, and other abnormalities that lead to a higher risk of IBR and greater benefit from RT.

In this study, we validated the prognostic impact of IHC subtyping on breast cancer relapse. However, we did not confirm that intrinsic subtyping was predictive for RT response, because the treatment-subtype interaction term was not significant. This suggests that the low absolute benefit from breast RT in the luminal subtypes was likely a result of the smaller number of relapses in this group.

An exploratory analysis was undertaken to define a group of women with 10-year IBR risk of less than 5% who may not require RT. Although IHC intrinsic subtyping provided valuable prognostic information, luminal A subtype alone did not meet this IBR target. However, the addition of clinicopathologic features to intrinsic subtyping resulted in an LR luminal A subgroup with a 10-year IBR of only 3.1%. Although the upper limits of the 95% CI estimated a maximum overall IBR risk of 8.2% (9.1% with tamoxifen alone), likely reflecting the small numbers of events, the benefit of RT in this selected group was also small. Thus clinical and pathologic features appear to add to intrinsic subtyping in identifying not only groups of patients with a low absolute risk of IBR but also those with minimal benefit from breast RT. Furthermore, on multivariable analysis, only luminal A subtype was significantly associated with breast cancer relapse compared with high-risk subtypes when adjusted for clinical risk group. When the analysis was limited to patients treated with tamoxifen alone, luminal A subtype remained significantly associated with IBR, as did the luminal B subtype, perhaps because of the exclusion of grade 3 tumors. However, the comparison of luminal A with luminal B subtype was not significant, although this result should be viewed with caution, given the small number of events and wide CIs for IBR.

The luminal A group represented a significant proportion of participants in this trial (53% overall [265 of 501] or 30% [151 of 501] when limited to LR clinical features (age older than 60 years, T1, grade 1 or 2 histology). Ultimately, these observations will require validation in an independent cohort of patients to determine who may be spared the inconvenience and adverse effects of breast RT. In contrast, breast RT was clearly beneficial for women with higher-risk subtypes (luminal HER2, HER2 enriched, basal like, and TN nonbasal). The luminal B subgroup had an intermediate IBR risk and impact of RT overall, which may be related to the limited sample size or differences in the Ki-67 scores in which the Ki-67 cutoff of 14% was the sole distinction between luminal A and luminal B tumors.[31] The luminal B subgroup may require additional evaluation using alternative biomarkers such as PAM50, which appears to be more robust than IHC subtyping for tamoxifen response.[32]

Although conforming to current guidelines regarding biomarker evaluation,[23] this study has limitations. Despite a relatively large population, patients with early-stage disease had low event rates in some cohorts, thus limiting the strength of our conclusions. Second, although patients in this study were treated with endocrine therapy, anti-HER2 therapy was not used, and that may have had an additional effect on IBR in the HER2 subgroup; hence the applicability to current practice in these women remains uncertain. Furthermore, it was not possible to retrieve blocks for all patients from the randomized trial.

These results suggest that when luminal A subtype is combined with clinical and pathologic factors, a subgroup of patients with a low risk of IBR may be defined for whom the benefits of RT are small. However, omitting RT and using intrinsic subtyping and clinical factors is a substantial change in care. The breast cancer community would likely require additional prospective evidence before this becomes standard of practice. To validate this observation, a prospective, single-arm clinical trial open to women age 55 years or older with pT1N0 grade 1 to 2 luminal A breast cancer has begun in Canada. Consenting patients will be treated with breast-conserving surgery and endocrine therapy without RT and will be followed with strict stopping rules if the projected risk of IBR exceeds 5%.

In conclusion, a six-IHC-biomarker panel was prognostic for IBR but not predictive of benefit from RT. Further studies may validate the exploratory finding of a low-risk group of postmenopausal women with early-stage luminal A breast cancer group who may be spared the inconvenience and adverse effects of breast RT.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Disclosures provided by the authors are available with this article at www.jco.org.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Identification of a Low-Risk Luminal A Breast Cancer Cohort That May Not Benefit From Breast Radiotherapy

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Appendix

Methods

Immunohistochemistry. Immunostained tissue microarray sections were scored by W.S. (blinded to clinical outcome), with the average score for all three cores being considered as the final result. Fifty samples were randomly selected for independent scoring and were concordant in 90% of the instances for all six immunomarkers. Staining and scoring methodology was defined a priori on the basis of previously published criteria.[19,21] Estrogen receptor or progesterone receptor positivity was defined as more than 1% of tumor nuclei staining with any intensity. Human epidermal growth factor receptor 2 (HER2) assessment was performed in accordance with College of American Pathologists/American Society of Clinical Oncology guidelines.[21] Tumor cells were considered positive (3+) for HER2 protein overexpression when more than 30% of invasive tumor cells demonstrated uniform, intense complete cytoplasmic or membrane staining. If HER2 was equivocal, it was further evaluated by HER2 fluorescent in situ hybridization (FISH). Patients were considered basal cytokeratin 5/6 (CK 5/6) positive or epidermal growth factor receptor positive if any cytoplasmic and/or membranous staining was detected in the invasive carcinoma cells on any of the cores, even if focal. Ki-67 was determined as the ratio of invasive carcinoma cells with any level of brown nuclear staining above background over the total number of invasive carcinoma nuclei present. Estrogen receptor–positive, HER2-negative patients with a Ki-67 index $\geq$ 14% were considered as luminal B.[19]

HER2 FISH

For cores with an immunohistochemical score of 2+ for HER2, a minimum of 60 morphologically clear, nonoverlapping nuclei from at least two different regions were scored by FISH using the PathVysion HER-2 DNA Probe Kit (Abbott Molecular, Abbott Park, IL) on 4-μm thick formalin-fixed paraffin embedded tissue sections, as previously described, with a slight modification to the manufacturer's instructions (Vance, et al: Arch Pathol Lab Med 133:611-612, 2009). Additional cells (30 to 60 cells) were scored for both equivocal (HER2:CEP17 ratio, 1.8:2.2; Vance, et al: Arch Pathol Lab Med 133:611-612, 2009) and challenging cases (Reinholz, et al: Lancet Oncol 10:267-277, 2009). Tumors were classified as HER2 amplified if the HER2:CEP17 ratio was more than 2.2 or the HER2 gene copy number was more than 6.0. Those with no or low HER2 gain (HER2:CEP17 ratio $<$ 1.8 or HER2 gene copy number $<$ 4.0) were designated as HER2 FISH negative. Overall HER2:CEP17 ratios of 1.8 to 2.2 were reported as HER2 FISH equivocal. Focal and/or scattered heterogeneity (5% to 50% of nuclei with HER2:CEP17 ratio $>$ 2.2; Vance, et al: Arch Pathol Lab Med 133:611-612, 2009) were noted along with patients who demonstrated an average CEP17 $\geq$ 3 or polysomy 17 (Reinholz, et al: Lancet Oncol 10:267-277, 2009).

Statistical Analyses

Training and validation sets were used to evaluate only the primary objective of intrinsic subtyping as a predictive biomarker for radiotherapy response. Because the training and validation sets were not randomly assigned, 298 patients were reselected at random to serve as a training set, and the rest served as the validation set; the treatment effect of luminal A in the two data sets was simulated 10,000 times.

Subset analyses of the prognostic effects of clinicopathologic features were evaluated in the total cohort of 501 patients. A bootstrap technique was used to investigate the robustness of the conclusions regarding the exploratory subgroup analysis; it calculated the probability of a difference in IBR at 10 years of more than 5% in 2,000 bootstrap samples (Hastie T, Tibshirani R, Friedman J: The Elements of Statistical Learning: Data Mining, Inference, and Prediction [ed 2]. New York, NY, Springer Science+Business Media, 2009). All tests and CIs were two-sided, and a *P* value of less than .05 was considered statistically significant.

The proportional hazards assumption was investigated for the prognostic analysis by inspecting the Schoenfeld residual plots and by testing the time-dependent coefficient for each covariate. The only departure from proportionality was observed for grade, and when the time-dependent coefficient was included in the model, it did not change the significance or nonsignificance of the other covariates. Because grade was not the covariate of interest, we chose to report the average effect for this covariate.

Table A1. Distribution of Clinicopathologic Characteristics and Cohorts Among the Breast Cancer Subtypes

Variable	Luminal A (n = 265)	Luminal B (n = 165)	Luminal HER2 (n = 22)	HER2 Enriched (n = 13)	Basal Like (n = 30)	TN Nonbasal (n = 6)
Age, years						
50-60	67 (25)	44 (27)	7 (32)	3 (23)	9 (30)	0 (0)
> 60	198 (75)	121 (73)	15 (68)	10 (77)	21 (70)	6 (100)
Tumor size, cm*						
< 2	205 (77)	104 (63)	13 (62)	6 (46)	16 (53)	2 (33)
2-5	60 (23)	60 (37)	8 (38)	7 (54)	14 (47)	4 (67)
Tumor grade†						
1 to 2	232 (92)	107 (69)	10 (48)	5 (38)	8 (28)	3 (60)
3	20 (8)	47 (31)	11 (52)	8 (62)	21 (72)	2 (40)
Treatment						
Tamoxifen + RT	139 (52)	82 (50)	10 (45)	6 (46)	16 (53)	4 (67)
Tamoxifen	126 (48)	83 (50)	12 (55)	7 (54)	14 (47)	2 (33)

NOTE: All data are No. (%).
Abbreviations: HER2, human epidermal growth factor receptor 2; RT, radiotherapy; TN, triple negative.
*Two samples were missing information about tumor size (one for luminal B and one for luminal HER2).
†Twenty-seven samples (5%) were missing information about grade (13 for luminal A, 11 for luminal B, one each for luminal HER2, basal like, and TN nonbasal).

Table A2. Source, Clone, and Dilution of Antibodies

Antibody	Source	Clone	Dilution
Estrogen receptor	Ventana Medical Systems/Roche	SP1	Prediluted
Progesterone receptor	Vector Laboratories	616	1:50
HER2/neo (SP3)	Thermo Fisher Scientific	SP3	1:100
HER2/neo (4B5)	Ventana Medical Systems/Roche	4B5	Prediluted
CK 5/6	Ventana Medical Systems	D5/16B4	Prediluted
EGFR	Zymed Laboratories/Life Technologies	31G7	1:100
Ki-67	NeoMarkers	SP6	1:500

NOTE. Following the College of American Pathologists/American Society of Clinical Oncology recommendations for human epidermal growth factor receptor 2 (HER2) testing for breast cancer, and the United Kingdom National External Quality Assessment Service guidelines for challenging cases.
Abbreviations: CK 5/6, cytokeratin 5/6; EGFR, epidermal growth factor receptor.

Appendix Table A3. REMARK Checklist

	Item to Be Reported	Page No.
Introduction		
1	State the marker examined, the study objectives, and any prespecified hypotheses.	2
Materials and methods		
Patients		
2	Describe the characteristics (eg, disease stage or comorbidities) of the study patients, including their source and inclusion and exclusion criteria.	2
3	Describe treatments received and how chosen (eg, randomized or rule based).	2
Specimen characteristics		
4	Describe type of biologic material used (including control samples) and methods of preservation and storage.	2
Assay methods		
5	Specify the assay method used and provide (or reference) a detailed protocol, including specific reagents or kits used, quality control procedures, reproducibility assessments, quantitation methods, and scoring and reporting protocols. Specify whether and how assays were performed blinded to the study end point.	2, Appendix Methods
Study design		
6	State the method of case selection, including whether prospective or retrospective and whether stratification or matching (eg, by stage of disease or age) was used. Specify the time period from which cases were taken, the end of the follow-up period, and the median follow-up time.	2
7	Precisely define all clinical end points examined.	2
8	List all candidate variables initially examined or considered for inclusion in models.	2
9	Give rationale for sample size. If the study was designed to detect a specified effect size, give the target power and effect size.	NA
Statistical analysis methods		
10	Specify all statistical methods, including details of any variable selection procedures and other model-building issues, how model assumptions were verified, and how missing data were handled.	2, Appendix Methods
11	Clarify how marker values were handled in the analyses. If relevant, describe methods used for cut point determination.	Appendix Methods
Results		
Data		
12	Describe the flow of patients through the study, including the number of patients included in each stage of the analysis (a diagram may be helpful) and reasons for dropout. Specifically, report the numbers of patients and the number of events both overall and for each subgroup extensively examined.	Fig. 1
13	Report distributions of basic demographic characteristics (at least age and sex), standard (disease specific) prognostic variables, and tumor marker, including numbers of missing values.	Table 1, Appendix Tables A1 and A4
Analysis and presentation		
14	Show the relation of the marker to standard prognostic variables.	Appendix Table A1
15	Present univariable analyses showing the relation between the marker and outcome, with the estimated effect (eg, hazard ratio and survival probability). Preferably provide similar analyses for all other variables being analyzed. A Kaplan-Meier plot is recommended for the effect of a tumor marker on a time-to-event outcome.	Table 2
16	For key multivariable analyses, report estimated effects (eg, hazard ratio) with CIs for the marker and, at least for the final model, all other variables in the model.	Table 3
17	Among reported results, provide estimated effects with CIs from an analysis in which the marker and standard prognostic variables are included, regardless of their statistical significance.	Table 2
18	Report results of further investigations, if they were performed, such as checking assumptions, sensitivity analyses, and internal validation.	Appendix Methods
Discussion		
19	Interpret the results in the context of the prespecified hypotheses and other relevant studies. Include a discussion of limitations of the study.	5
20	Discuss implications for future research and clinical value.	5

Abbreviation: REMARK, Reporting Recommendations for Tumor Marker Prognostic Studies.

Table A4. Distribution of Clinicopathologic Characteristics for All Samples

Characteristic	Total Tested (n = 501)	Not Tested (n = 268)*	*P*
Age, years			
50-60	130 (26%)	79 (29%)	
> 60	371 (74%)	189 (71%)	.34
Tumor size, cm†			
< 2	346 (69%)	205 (76%)	
2-5	153 (31%)	63 (24%)	.044
Tumor grade‡			
1 to 2	365 (77%)	199 (88%)	
3	109 (23%)	28 (12%)	.0012
Treatment			
Tamoxifen + RT	257 (51%)	129 (48%)	
Tamoxifen	244 (49%)	139 (52%)	.45
IBR at 10 years	9.3%	9.3%	.85

Abbreviations: IBR, ipsilateral breast relapse; RT, radiotherapy.
*Forty-one samples were missing information about grade.
†two samples were missing information about tumor size (two in the training cohort and correspondingly two in total tested).
‡Twenty-seven samples (5%) were missing information about grade in the two cohorts (nine in training cohort and 18 in validation cohort, and correspondingly 27 in total tested).

Table A5. Comparison of IBR Using the Clinical Models and Subtype Including Treatment Effects

Risk Group	No.	IBR at 10 Years (%)	95% CI	*P*
LR clinical luminal A	151	3.1	1.2 to 8.2	
High-risk clinical/subtype	341	11.8	8.6 to 16.1	.0063
LR clinical luminal A				
Tamoxifen + RT	77	5.0	1.6 to 15.0	
Tamoxifen	74	1.3	0.2 to 9.1	.42

Abbreviations: IBR, ipsilateral breast relapse; LR, low risk; RT, radiotherapy.

Table A6. Multivariable Analysis of IBR in the Tamoxifen-Treated Patients Only

Covariate	HR	95% CI	*P*
Clinical risk groups	2.6	1.1 to 6.3	.029
Luminal A *v* high risk	0.20	0.08 to 0.51	< .001
Luminal B *v* high risk	0.35	0.15 to 0.82	.016
Luminal A *v* luminal B	0.56	0.21 to 1.45	.23
Overall			.0022

Abbreviations: HR, hazard ratio; IBR, ipsilateral breast relapse.