

Immunohistochemical Detection Using the New Rabbit Monoclonal Antibody SP1 of Estrogen Receptor in Breast Cancer Is Superior to Mouse Monoclonal Antibody 1D5 in Predicting Survival

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ABSTRACT

Purpose

Estrogen receptor (ER) expression predicts improved breast cancer–specific survival and reduced risk of recurrence and is targeted in breast cancer therapy. A high-quality antibody to identify ER-positive patients plays an important role in clinical decision making for women with breast cancer. This study evaluates immunohistochemistry using two anti-ER antibodies, a new rabbit monoclonal antibody (SP1) and the mouse monoclonal antibody (1D5), in relation to biochemical ER assay results and clinical data on survival and adjuvant systemic therapy.

Patients and Methods

A population-based tissue microarray series of 4,150 invasive breast cancers was constructed. All patients had staging, pathology, treatment, and follow-up information. The median follow-up was 12.4 years and the median age at diagnosis 60 years. Survival analysis and log-rank tests were used to evaluate the prognostic value of ER status and correlations with clinical data.

Results

Among the 4,105 samples interpretable for both antibodies, SP1 detected ER positivity in 69.5% and 1D5 in 63.1% of cases. Both monoclonal antibodies are demonstrated to be good prognostic indicators for breast cancer–specific and relapse-free survival. In multivariate analysis, including age, tumor size, grade, and lymphovascular and nodal status, SP1 was a better independent prognostic factor than 1D5. Among patients with discrepant ER results, the 8% of patients who were SP1 positive/1D5 negative showed good outcomes, and the 2% SP1-negative/1D5 positive had poor outcomes. Maintaining the same 92% specificity and 98% positive predictive value, SP1 is 8% more sensitive than 1D5 using biochemical assay as gold standard.

Conclusion

SP1 represents an improved standard for ER immunohistochemistry assessment in breast cancer.

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INTRODUCTION

Estrogen receptor (ER) may be the best example of a tumor biomarker with an assay that drives therapeutic decision making.¹⁻⁴ In the 1990s, immunohistochemical (IHC) evaluation of ER largely supplanted dextran-coated charcoal (DCC) ligand-binding assay because it is more economical and yields concurrent histopathologic correlation. Several anti-ER antibodies are used clinically; two mouse monoclonals (6F11 and 1D5) have been compared in clinical studies and were shown to have similar sensitivities.^{5,6} Studies have also compared 1D5 with a new rabbit monoclonal, SP1,^{7,8} which has eight-fold higher affinity.⁸ Our preliminary studies found that SP1 is more sensitive than 1D5 for

detecting ER expression in breast cancer, in both a duplicate-redundancy 431-sample TMA, and in 121 whole sections of clinical materials from multiple institutions⁹ (Tables A1 and A2, online only). We present the first population-based series comparing the IHC detection of ER by antibodies 1D5 and SP1, and results from DCC in patients with long-term clinical follow-up, to investigate the prognostic values of these assays in breast cancer.

PATIENTS AND METHODS

Study Population

The study cohort is 4,150 female patients with newly diagnosed, invasive breast cancer in British Columbia, whose tumor specimens were tested by a central (ER)

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laboratory at Vancouver Hospital between 1986 and 1992. The DCC protocol is as published¹⁰⁻¹² and reproduced on our web site (www.gpec.ubc.ca/index.php?content=papers/ER.php). Median follow-up was 12.4 years and age at diagnosis 60 years. All patients had been referred to the British Columbia Cancer Agency and have staging, pathology, treatment, and follow-up information.^{13,14} During the study era, 75% of breast cancer patients in the province were referred; nonreferred patients were generally elderly or treated by mastectomy without indications for adjuvant therapy.¹⁵

Abstracted clinical information includes age; histology; grade; tumor size; number of involved axillary nodes; lymphatic or vascular invasion (LVI); ER status by the DCC method¹¹; type of local and initial adjuvant systemic therapy (AST); and dates of diagnosis, first local, regional, or distant recurrence, and death. A subset of these patients was included in a recent population-based study validating the prognostic model ADJUVANT!¹⁶ Table A3 (online only) summarizes cohort characteristics. The study was approved by the Clinical Research Ethics Board of the University of British Columbia and BC Cancer Agency.

Tissue Microarrays and IHC

The Vancouver Hospital ER laboratory retained single archival blocks from each patient. This material had been frozen before neutral buffered formalin fixation. Slides from these blocks stained with hematoxylin and eosin were reviewed by two pathologists to identify areas of invasive breast carcinoma. Tissue microarrays (TMAs) were constructed as described^{17,18} (details reproduced at www.gpec.ubc.ca/index.php?content=papers/ER.php). Using one core per patient, 17 TMA blocks were required. Thin sections (4 μ m) were immunostained using DakoCytomation EnVision and System-HRP (Dako Corporation, Carpinteria, CA) in a two-step technique. Slides were deparaffinized with xylene and rehydrated through three alcohol changes. Endogenous peroxidase activity was quenched by incubating 5 minutes with 0.03% hydrogen peroxide/sodium azide. Slides were then incubated with one of two primary anti-ER antibodies, 1D5 (1:100; Dako Corp, Carpinteria, CA) or SP1 (1:250; LabVision, Fremont, CA), followed by the peroxidase-labeled polymer, in a Tris-HCl buffer containing stabilizing protein and an antimicrobial agent, using sequential 30-minute incubations. Staining was completed by a 10-minute incubation with 3,3'-diaminobenzidine plus substrate-chromogen. Primary antibody was omitted in negative controls. External positive controls were slides from breast cancers with previously documented ER expression. A previous study using a duplicate-redundancy 431-case TMA demonstrated 96% agreement between duplicate cores, for both 1D5 and SP1⁹ (Tables A1 and A2).

Stained TMA slides were digitally scanned and linked to a relational database,¹⁹ and are available for review (<https://www.gpecimage.ubc.ca/tma/web/viewer.php>; username, ersp11D5; password, er4150).

ER Scoring System

TMAs were scored visually by two pathologists (D.T., A.M.G.) for percentage of tumor cell nuclear positivity, and scored as negative (< 1%); positive, 1+ (1% to 25%); positive, 2+ (25% to 75%); or positive, 3+ (> 75%). For most analyses, IHC scores are dichotomized at ≥ 1 = ER positive.²⁰ Pathologists were blinded to clinical outcomes.

Statistical Analysis

Statistical analysis was performed using SPSS software version 13.0 (SPSS Inc, Chicago, IL) and R 2.1.1 (www.r-project.org). In univariate analyses, Overall survival (OS), breast cancer-specific survival (BCSS), and relapse-free survival (RFS) were estimated using Kaplan-Meier²¹ curves, and survival differences determined by log-rank tests.^{22,23} A trend test was used when three or more ordered groups were compared. For BCSS, survival time was censored at death if the cause was not breast cancer, or if the patient was still alive at the end of the study. Six patients with unknown cause of death were excluded from BCSS analysis. For RFS, survival time was also censored at death if the cause was not breast cancer or the patient was alive without relapse at the end of the study. For OS, survival time was censored if the patient was still alive at the end of the study.

Cox proportional hazards^{22,24} models were used to calculate adjusted hazard ratios (HRs) accounting for covariates. Hypothesis testing was performed using Wald's statistic. Smoothed plots of weighted Schoenfeld residu-

als were used to test proportional hazard assumptions.²⁵ κ^{26} statistics and Kendall's τ -b²⁷ tests were used to measure agreement between the two ER immunostains and DCC assay, and correlation of ER status to pathologic variables. Differences involving pathologic factors were compared using Pearson's χ^2 and Mann-Whitney U^{29} for categorical and continuous variables, respectively. All statistical tests were two-sided and $P < .05$ was considered significant.

RESULTS

Our previous study⁹ found that to detect ER expression, SP1 is more sensitive than 1D5 on both whole sections (5.3% absolute increased sensitivity) and TMAs (6.6%), and suggested that SP1 is the better prognostic marker for breast cancer survival (Tables A1 and A2). In this study we assessed the value of SP1 on a population-based series of 4,150 invasive breast cancers; 48% of patients had tumor ≥ 2 cm, 51% had grade 3 tumor, and 44% had positive nodal status (Table A3). Among the 1,838 patients undergoing lumpectomy, 91% received radiation, whereas 29% of the 2,241 patients undergoing mastectomy received radiation. Forty-one percent of patients received no AST and 33% received tamoxifen-only AST.

Comparison of ER Expression by SP1 and 1D5 With DCC and Clinicopathologic Characteristics

Table 1 summarizes the IHC staining results for SP1 and 1D5 on TMAs. Figures 1A to 1C are from the same tumor sample that was negative for ER by 1D5 but strongly positive by SP1. This sample had an ER concentration of 48 fmol/mg by DCC. Figures 1D to 1F are from another tumor sample, which was moderately positive by 1D5 but strongly positive by SP1 (ER concentration, 174 fmol/mg).

The number of interpretable samples for the two immunostains is slightly different due to occasional core dropout from TMAs during sectioning and staining. Overall, 69.5% of samples were positive by SP1 versus 63.1% positive by 1D5. In absolute terms, the SP1 antibody had 11% more strong 3+ stains than 1D5, and 1D5 had 6.4% more negative stains than SP1. There is a strong positive correlation between the two antibodies (Kendall's τ -b, 0.790; $P < 1.0 \times 10^{-17}$).

Among clinical tumor samples, 3,884 samples were originally tested by DCC assay and categorized into four groups^{10,11}: negative (≤ 1 fmol/mg), low (2 to 9 fmol/mg), moderate (10 to 159 fmol/mg), and high (≥ 160 fmol/mg). Frequencies were 2.9% (111 of 3,884), 17.5% (678 of 3,884), 41.1% (1,596 of 3,884), and 38.6% (1,499 of 3,884), respectively. Values of DCC ≥ 10.0 fmol/mg were considered

Table 1. Frequencies of ER Antibodies SP1 and 1D5 Immunostaining Expression in 4,150 Breast Cancer Patient Samples

| Expression Level | SP1 | | 1D5 | |
|------------------------------|-----------------|------|-----------------|------|
| | No. of Patients | % | No. of Patients | % |
| Negative (< 1%) | 1,255 | 30.2 | 1,520 | 36.6 |
| 1%-25% | 358 | 8.6 | 409 | 9.9 |
| 25%-75% | 1,272 | 30.7 | 1,420 | 34.2 |
| > 75% | 1,231 | 29.7 | 775 | 18.7 |
| Uninterpretable/missing core | 34 | 0.8 | 26 | 0.6 |
| Total | 4,150 | 100 | 4,150 | 100 |

Abbreviation: ER, estrogen receptor.

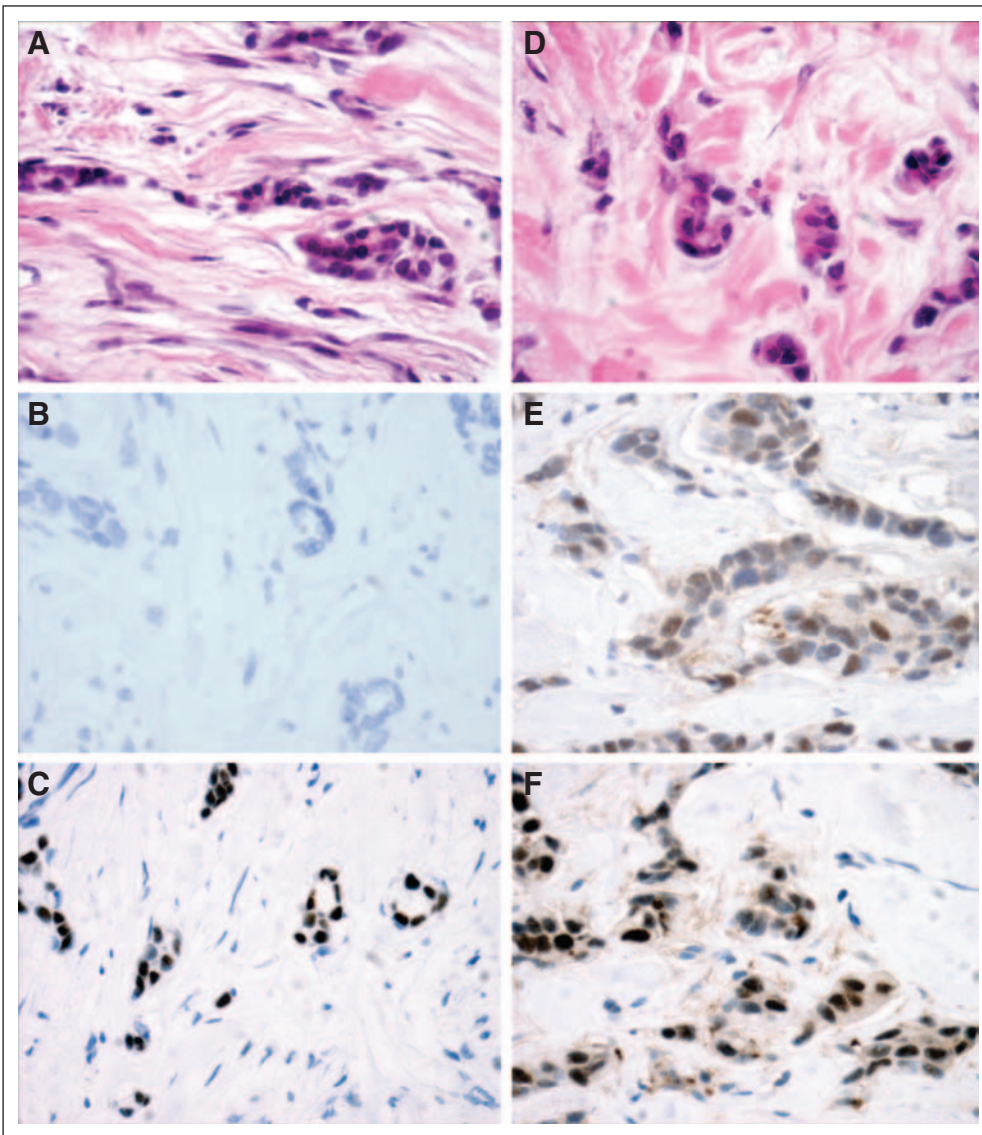


Fig 1. (A to C) Patient 953, an infiltrating ductal carcinoma: (A) hematoxylin and eosin (H and E) histology; (B) tumor cells completely negative for expression of estrogen receptor (ER) using 1D5 monoclonal antibody; (C) 3+ immunostaining for ER expression using SP1 monoclonal antibody. (D to F) Patient 963, an infiltrating ductal carcinoma: (D) H and E histology; (E) 2+ moderate immunostaining by 1D5; (F) 3+ immunostaining for ER using SP1.

positive (the clinical cut point).^{11,12} ER status by SP1 (κ , 0.654; sensitivity, 86%; specificity, 92%; $P < 1.0 \times 10^{-17}$) agreed better with DCC than did 1D5 (κ , 0.536; sensitivity, 78%; specificity, 92%; $P < 1.0 \times 10^{-17}$; Table 2).

A total of 4,105 samples were interpretable for both antibodies. SP1 stained 8% more positive samples that otherwise were negative by 1D5 (Table 3). For these 337 discrepant samples, 92% were DCC positive (median concentration, 67.0 fmol/mg). Among the 77 discrepant cases identified as negative by SP1 but positive by 1D5, 65%

Table 2. Comparison of ER Status as Detected by SP1 and 1D5 Immunohistochemistry, Using DCC as the Gold Standard

| ER Status | Result | ER Concentration Detected by DCC (fmol/mg) | | Predictive Value |
|-----------------|--------------|--|------------------|------------------|
| | | < 10 | ≥ 10 | |
| Detected by SP1 | SP1 negative | 716 | 445 | Negative, 62% |
| | SP1 positive | 64 | 2,628 | Positive, 98% |
| | | Specificity, 92% | Sensitivity, 86% | |
| Detected by 1D5 | 1D5 negative | 718 | 687 | Negative, 51% |
| | 1D5 positive | 64 | 2392 | Positive, 97% |
| | | Specificity, 92% | Sensitivity, 78% | |

Abbreviations: ER, estrogen receptor; DCC, dextran-coated charcoal.

Table 3. Frequencies of SP1 and 1D5 Discrepancies Among Patient Samples Interpretable for Both SP1 and 1D5 Antibodies

| | | SP1 | | Total |
|-----|----------|----------|----------|-------|
| | | Negative | Positive | |
| 1D5 | Negative | 1,176 | 337 | 1,513 |
| | Positive | 77 | 2,515 | 2,592 |
| | Total | 1,253 | 2,852 | 4,105 |

NOTE. SP1 identifies reclassifies 8.2% (337 of 4,105) positive of patient samples that would have been considered negative by 1D5, and 1.9% (77 of 4,105) negative of patient samples considered positive by 1D5.

were DCC positive (median concentration, 34.0 fmol/mg). The SP1-positive/1D5-negative discordant samples had significantly higher ER concentrations by DCC ($P = .021$) and significantly lower tumor grades ($P = .022$) than the SP1-negative/1D5-positive discordant samples; there was no significant difference in tumor size ($P = .110$). Among the SP1-negative/1D5-negative group, 64% were DCC negative; among the SP1-positive/1D5-positive group, 98% were DCC positive.

SP1-positive, 1D5-positive, and DCC-positive status all correlate with older age at diagnosis (Kendall's τ -b, 0.149 [$P < 1.0 \times 10^{-17}$], 0.173 [$P < 1.0 \times 10^{-17}$], and 0.165 [$P < 1.0 \times 10^{-17}$], respectively), but correlate inversely with grade (Kendall's τ -b, -0.241 [$P = 9.96 \times 10^{-60}$], -0.196 [$P = 1.88 \times 10^{-38}$], and -0.282 [$P = 3.25 \times 10^{-86}$]) and tumor size (Kendall's τ -b, -0.107 [$P = 4.56 \times 10^{-12}$], -0.083 [$P = 7.42 \times 10^{-8}$], and -0.108 [$P = 1.2 \times 10^{-11}$]).

Prognostic Values of SP1-, 1D5-, and DCC-Positive Expression

To assess the prognostic value of ER in a general population, we examined SP1, 1D5, and DCC in the entire cohort. Both immunostains and DCC identified ER-positive patients as having an improved BCSS. DCC assay demonstrates the most significant and strongest linear trend for expression: the higher expression, the better BCSS (Fig 2A [SP1], $P = 4.78 \times 10^{-13}$; Fig 2B [1D5], $P = 1.65 \times 10^{-7}$; and Fig 2C [DCC], $P = 6.08 \times 10^{-16}$). A similarly significant trend is observed for RFS (SP1, $P = 3.98 \times 10^{-8}$; 1D5, $P = 3.71 \times 10^{-6}$; and DCC, $P = 9.25 \times 10^{-14}$) but not OS (SP1, $P = .132$; 1D5, $P = .511$; and DCC, $P = .154$). The 5- and 10-year survival probabilities of binarized ER status from Kaplan and Meier analyses are listed in Table A4 (online only). The increased survival for ER-positive patients starts to attenuate after 5 years. The same phenomenon is seen with all three detection methods.

AST for this cohort was prescribed according to guidelines based on age, tumor size, LVI, nodal status, and DCC-determined ER level.¹⁶ High risk was defined as node positive, or if node negative, the presence of LVI or tumor more than 2 cm and ER negative (DCC < 10 fmol/mg). Patients deemed low risk at the time of diagnosis were not given any AST; they had a better outcome (10-year BCSS, 82%; 95% CI, 80% to 84%) than patients receiving AST such as the tamoxifen group (10-year BCSS 69%; 95% CI, 66% to 71%; $P = 8.4 \times 10^{-21}$). Therefore, survival analyses based on ER status were done separately in these two subgroups.

For the pure prognostic subset of patients receiving no AST, SP1-positive status was associated with 14% absolute increased BCSS ($P = 5.0 \times 10^{-8}$) at 10 years, 1D5-positive status was associated with 9% ($P = 2.2 \times 10^{-4}$), and DCC-positive status was associated with 19% ($P = 2.3 \times 10^{-12}$; Table A4). For RFS, SP1-positive and DCC-positive status were increased significantly by 6% ($P = .002$) and 13% ($P = 5.1 \times 10^{-7}$) at 10 years. 1D5-positive status showed 3% increased 10-year RFS ($P = .06$).

Among 1,377 patients receiving tamoxifen as their only AST, only 84 were ER negative by DCC. SP1, 1D5, and DCC all identified ER-positive patients as having better outcomes than ER-negative patients. The absolute increased 10-year BCSS for SP1-, 1D5-, and DCC-positive status are 14% ($P = 4.7 \times 10^{-6}$), 10% ($P = 1.5 \times 10^{-4}$), and 24% ($P = 3.3 \times 10^{-7}$); the increased 10-year RFS for SP1-, 1D5-, and DCC-positive status are 10% ($P = 1.7 \times 10^{-4}$), 7% ($P = .001$), and 18% ($P = 3.9 \times 10^{-5}$), respectively (Table A4).

Concordant and Discordant Cases Between SP1 and 1D5

As expected, patients with SP1/1D5 double-positive status have better BCSS (HR, 0.667; 95% CI, 0.592 to 0.753) compared with patients with SP1/1D5 double-negative status ($P = 5.00 \times 10^{-11}$; Fig 3). The SP1-positive/1D5-negative group has a HR of 0.647 (95% CI, 0.423 to 0.989; $P = .044$) compared with the SP1-negative/1D5-positive group. Importantly, there are no significant survival differences between the SP1-positive/1D5-positive and SP1-positive/1D5-negative patients ($P = .408$). The SP1-negative/1D5-positive group does not have significantly different survival from the SP1-negative/1D5-negative group (HR, 0.93; 95% CI, 0.63 to 1.36; $P = .698$) but seems inferior to the SP1-positive group (HR, 1.45; 95% CI, 0.99 to 2.11; $P = .055$). In relapse-free survival analysis, the SP1-negative/1D5-positive patients have a similar hazard compared with the SP1-negative/1D5-negative group, with a HR of 0.972 (95% CI, 0.683 to 1.38), whereas SP1-positive/1D5-negative patients have relapse-free hazard similar to that of SP1-positive/1D5-positive patients (HR, 1.07; 95% CI, 0.885 to 1.28; (Fig A1, online only). The same pattern in BCSS and RFS is seen in both the subsets of patients not treated with AST or those treated only with tamoxifen (data not shown). These results also support that SP1 antibody is a better prognostic marker than the commonly used ER antibody 1D5.

Multivariate Analysis

Smoothed, rescaled Schoenfeld residuals plots were used to test proportional hazards assumptions. All covariates followed proportional hazards, except ER status, which varied slightly during the long period of follow-up. The hazard rate of breast cancer death and relapse is different in the first 5 years, consistent with data reported by Hess et al.³⁰ Table A5 summarizes the adjusted HRs of ER-positive status detected by the two immunostains and DCC; the reported values were determined from Cox regression models including age, tumor size, grade, LVI, and nodal status as covariates for BCSS and RFS (Table A5). The results show that SP1, 1D5, and DCC all work efficiently as independent prognostic factors for BCSS and RFS in a general population after adjusting for the listed clinicopathologic prognosticators. However, SP1, 1D5, or DCC did not remain significant among the low-risk patients receiving no AST.

For patients receiving tamoxifen as their only AST, SP1, 1D5, and DCC each retained significance when added individually to a model including the same clinical parameters listed above as covariates. The BCSS HRs for SP1-positive, 1D5-positive, and DCC-positive status were 0.636 (95% CI, 0.499 to 0.811; $P = 2.55 \times 10^{-4}$), 0.697 (95% CI, 0.559 to 0.870; $P = 1.37 \times 10^{-3}$), and 0.649 (95% CI, 0.450 to 0.935; $P = 2.03 \times 10^{-2}$), respectively. The RFS HRs for SP1-positive, 1D5-positive, and DCC-positive status were 0.683 (95% CI, 0.540 to 0.864; $P = 1.45 \times 10^{-3}$), 0.744 (95% CI, 0.601 to 0.921; $P = 6.59 \times 10^{-3}$), and 0.709 (95% CI, 0.491 to 1.03; $P = .068$), respectively. To test which detection method was more significantly associated with prognosis, a Cox regression model with age, tumor size, grade, LVI, and nodal status was fitted with each of SP1, 1D5, and DCC. SP1 was the most significant prognostic factor among the three ER detection methods, both in BCSS (HR, 0.664; 95% CI, 0.517 to 0.852; $P = 1.27 \times 10^{-3}$) and RFS (HR, 0.699; 95% CI, 0.550 to 0.888; $P = 3.40 \times 10^{-3}$) in this subgroup. The HRs of the clinicopathologic covariates are listed in Tables 4 and 5.

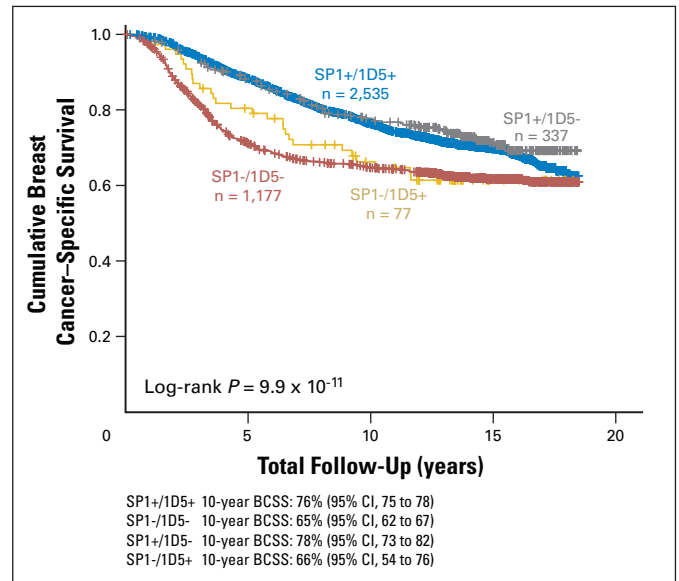
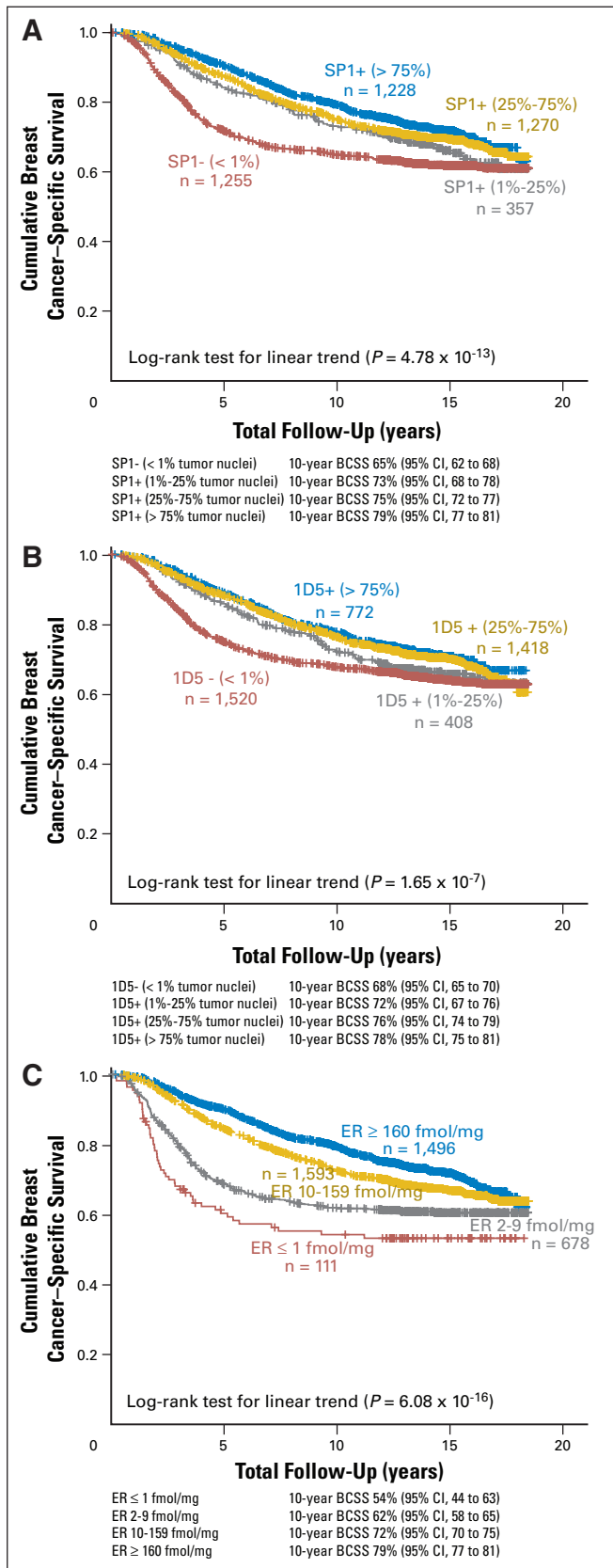


Fig 3. Breast cancer-specific survival (BCSS) of SP1 and 1D5 concordant and discordant cases (total, n = 4,128). The 10-year BCSS estimates are labeled accordingly. The SP1-negative/1D5-positive group has a worse BCSS survival compared with the expected outcome from the rest of ER-positive patients.

DISCUSSION

Using population-based TMAs of more than 4,000 patients with long-term follow-up, this study not only validates the prognostic value of ER immunostaining in breast carcinomas with and without adjuvant tamoxifen, but also demonstrates the superiority of the SP1 rabbit monoclonal antibody to the standard 1D5 mouse monoclonal, both in agreement with DCC assay and in correlation with outcome. ER is a well-established determinant of survival and correlate of other clinicopathologic variables.³¹ Accordingly, the vast majority of our analyses are confirmatory validations rather than tests of hypotheses, and a Bonferroni correction would be inappropriate.

Because of reduced cost, shorter turnaround time, morphologic correlation, and ease of specimen handling, IHC testing for ER has largely replaced DCC assays³²; so high-quality antibodies play an important role in clinical decision making. Developed against an N-terminal epitope of ER- α , the mouse monoclonal antibody 1D5 is currently in wide use.²⁰ Another mouse monoclonal, 6F11, has similar sensitivity.^{33,34} Previous studies using limited numbers of patients without outcome data suggested superior clinical sensitivity of the recently developed rabbit monoclonal antibody SP1, recognizing the ER- α C-terminal portion.⁷

We show that SP1 is superior to 1D5 in identifying ER-positive patients who have a good prognosis, in the whole cohort (representing a British Columbia population), in patients receiving no AST (a pure prognostic group), and in patients receiving only tamoxifen AST. Our data suggest that 1D5 fails to identify some ER-positive patients who would benefit from adjuvant tamoxifen, thus potentially denying a well-tolerated, efficacious treatment to approximately 6% of breast cancer patients.

SP1 more closely approximates the prognostic value of DCC assay than does 1D5. This suggests that IHC using SP1 is a better substitute for the previous gold standard in a clinical

Table 4. Cox Proportional Hazard Regression Analysis to Test the Best ER Detection Methods (SP1, 1D5, and DCC) on Patients Receiving Tamoxifen Only as Their AST: BCSS

| Characteristic | BCSS (n = 1,150) | | |
|-------------------------|------------------|----------------|-----------------------|
| | HR | 95% CI | P |
| Age at diagnosis, years | | | |
| 40-49 v < 40 | 0.067 | 0.008 to 0.536 | 1.09×10^{-2} |
| 50-65 v < 40 | 0.049 | 0.007 to 0.367 | 3.28×10^{-3} |
| > 65 v < 40 | 0.061 | 0.008 to 0.450 | 6.11×10^{-3} |
| Grade | | | |
| II v I | 1.19 | 0.599 to 2.35 | .623 |
| III v I | 2.20 | 1.12 to 4.33 | 2.23×10^{-2} |
| Tumor size, cm | | | |
| 2-5 v ≤ 2 | 1.53 | 1.23 to 1.90 | 1.54×10^{-4} |
| > 5 v ≤ 2 | 3.11 | 2.16 to 4.49 | 1.23×10^{-9} |
| LVI | | | |
| Positive v negative | 1.15 | 0.925 to 1.44 | .204 |
| Nodal status | | | |
| Positive v negative | 2.04 | 1.60 to 2.59 | 7.65×10^{-9} |
| ER status* by SP1 | | | |
| Positive v negative | 0.664 | 0.517 to 0.852 | 1.27×10^{-3} |

NOTE. Age at diagnosis, grade, tumor size, LVI, and nodal status were included as covariates. Patient samples with missing values in any of the covariates or ER status were excluded in the analysis. Hazard estimates were not computed for the insignificant test variables.

Abbreviations: ER, estrogen receptor; DCC, dextran-coated charcoal; AST, adjuvant systemic therapy; BCSS, breast cancer-specific survival; HR, adjusted hazard ratio; LVI, lymphovascular invasion.

*In a model including SP1, 1D5, and DCC, SP1 is selected as the most significant contributor to BCSS (1D5, $P = .267$; DCC, $P = .372$). In a model including 1D5 as the only ER measure, 1D5-positive HR, 0.699 (0.557 to 0.877), $P = 1.93 \times 10^{-3}$. In a model including DCC as the only ER measure, DCC-positive HR, 0.648 (0.450 to 0.934), $P = .0200$.

Table 5. Cox Proportional Hazard Regression Analysis to Test the Best ER Detection Methods (SP1, 1D5, and DCC) on Patients Receiving Tamoxifen Only As Their AST: RFS

| Characteristic | RFS (n = 1,104) | | |
|-------------------------|-----------------|----------------|-----------------------|
| | HR | 95% CI | P |
| Age at diagnosis, years | | | |
| 40-49 v < 40 | 0.151 | 0.019 to 1.17 | 7.04×10^{-2} |
| 50-65 v < 40 | 0.071 | 0.010 to 0.522 | 9.41×10^{-3} |
| > 65 v < 40 | 0.080 | 0.011 to 0.589 | 1.32×10^{-2} |
| Grade | | | |
| II v I | 1.55 | 0.786 to 3.05 | .206 |
| III v I | 2.64 | 1.35 to 5.18 | 4.75×10^{-3} |
| Tumor size, cm | | | |
| 2-5 v ≤ 2 | 1.50 | 1.22 to 1.84 | 9.92×10^{-5} |
| > 5 v ≤ 2 | 2.65 | 1.81 to 3.87 | 4.73×10^{-7} |
| LVI | | | |
| Positive v negative | 1.23 | 0.995 to 1.52 | 5.52×10^{-2} |
| Nodal status | | | |
| Positive v negative | 1.84 | 1.47 to 2.29 | 7.64×10^{-8} |
| ER status* by SP1 | | | |
| Positive v negative | 0.699 | 0.550 to 0.888 | 3.40×10^{-3} |

NOTE. Age at diagnosis, grade, tumor size, LVI, and nodal status were included as covariates. Patient samples with missing values in any of the covariates or ER status were excluded in the analysis. Hazard estimates were not computed for the insignificant test variables.

Abbreviations: ER, estrogen receptor; DCC, dextran-coated charcoal; AST, adjuvant systemic therapy; RFS, relapse-free survival; HR, adjusted hazard ratio; LVI, lymphovascular invasion; BCSS, breast cancer-specific survival.

*In a model including SP1, 1D5, and DCC, SP1 is selected as the most significant contributor to BCSS (1D5, $P = .555$; DCC, $P = .577$). In a model including 1D5 as the only ER measure, 1D5-positive HR, 0.754 (0.606-0.939), $P = .0116$. In a model including DCC as the only ER measure, DCC-positive HR, 0.709 (0.490-1.03), $P = .0675$.

diagnostic setting. Although IHC assays in general can suffer from interlaboratory variability, ER IHC has been shown to be robust and reproducible.³⁵

In this study, ER expression correlates with substantially greater improvements in disease-specific and relapse-free survival among tamoxifen-treated patients than among patients receiving no AST. In the latter group, although ER-positive patients (detected by any method) had better survival probabilities, their survival advantage slowly decreased and eventually crossed at 18 years (Table A4 and Fig A2, online only). This suggests some ER-positive patients have slow growing but high-risk tumors not related to tamoxifen resistance.

Quantitative DCC results showed the best positive linear trend for better survival, and DCC is better than semiquantitative visual IHC to identify the dose effect of ER on outcome. Quantitative image analysis may be necessary for IHC techniques to match DCC assay in this regard.^{32,35,36}

Because of the exceptionally large study size, TMAs were constructed of single cores. Previous data from our laboratory found 96% agreement between duplicate cores for SP1 and for 1D5 immunostains⁹ (Table A1). The almost five-fold difference in the numbers of SP1-positive/1D5-negative versus SP1-negative/1D5-positive discordant results in the current larger study argues strongly that the SP1-positive/1D5-negative patients (illustrated in Fig 1) do not merely represent false-negative 1D5 results. Because the applicability of these

results to whole tissue sections might be questioned, we performed studies comparing SP1 and 1D5 in an unselected multi-institutional series of 121 whole-section⁹ breast cancer specimens, and the absolute ER positivity rate was still 5.3% higher for SP1 compared with 1D5.

This study also tested the two antibodies on a single-institution, 431-patient, formalin-fixed breast cancer TMA independent of the one presented here; as might be expected, the absolute rates of ER positivity were found to be higher in whole sections than in tissue microarrays (SP1, 83.5% [whole section] v 79.3% [TMA]; 1D5, 78.2% [whole section] v 72.7% [TMA]; Table A1), but importantly, the relative increase in sensitivity of SP1 versus 1D5 was maintained in both whole sections (5.3%) and the TMA format (6.6%), and agrees with the results presented here on 4,150 patients (6.4%). Although it is difficult to compare absolute rates because the contributing patient populations were different, in this 4,150-patient series, both antibodies did show lower absolute rates of positivity, which might reflect the fact that the source tissues (as per DCC protocol) were frozen before fixation. The fact that DCC assay was positive in 36% of the tumors, which were negative by both 1D5 and SP1 IHC, supports loss of ER immunoreactivity in tissues frozen before fixation.

We provide evidence that the new rabbit monoclonal antibody SP1 represents an improved standard for IHC ER assessment in breast cancer. Recent studies have made accurate assessment of ER status even more critical, given its implications for predicting response to systemic therapies.³⁷

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Appendix

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

Authors' Disclosures of Potential Conflicts of Interest

Although all authors completed the disclosure declaration, the following author or immediate family members indicated a financial interest. No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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