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ORIGINAL REPORT

Loss of Nuclear Localized and Tyrosine Phosphorylated Stat5 in Breast Cancer Predicts Poor Clinical Outcome and Increased Risk of Antiestrogen Therapy Failure

Amy R. Peck, Agnieszka K. Witkiewicz, Chengbao Liu, Ginger A. Stringer, Alexander C. Klimowicz, Edward Pequignot, Boris Freydin, Thai H. Tran, Ning Yang, Anne L. Rosenberg, Jeffrey A. Hooke, Albert J. Kovatich, Marja T. Nevalainen, Craig D. Shriver, Terry Hyslop, Guido Sauter, David L. Rimm, Anthony M. Magliocco, and Hallgeir Rui

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From the Kimmel Cancer Center, Thomas Jefferson University, Philadelphia; MDR Global Systems, Windber, PA; Tom Baker Cancer Centre, Alberta Cancer Board, Alberta, Canada; Walter Reed Army Medical Center, Washington, DC; University Medical Center Hamburg-Eppendorf, Hamburg, Germany; and the Yale University School of Medicine, New Haven, CT.

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Corresponding author: Hallgeir Rui, MD, PhD, Thomas Jefferson University, 233 S 10th St, Philadelphia, PA 19107; e-mail: hallgeir.rui@jefferson.edu.

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ABSTRACT

Purpose
To investigate nuclear localized and tyrosine phosphorylated Stat5 (Nuc-pYStat5) as a marker of prognosis in node-negative breast cancer and as a predictor of response to antiestrogen therapy.

Patients and Methods
Levels of Nuc-pYStat5 were analyzed in five archival cohorts of breast cancer by traditional diaminobenzidine-chromogen immunostaining and pathologist scoring of whole tissue sections or by immunofluorescence and automated quantitative analysis (AQUA) of tissue microarrays.

Results
Nuc-pYStat5 was an independent prognostic marker as measured by cancer-specific survival (CSS) in patients with node-negative breast cancer who did not receive systemic adjuvant therapy, when adjusted for common pathology parameters in multivariate analyses both by standard chromogen detection with pathologist scoring of whole tissue sections (cohort I; n = 233) and quantitative immunofluorescence of a tissue microarray (cohort II; n = 291). Two distinct monoclonal antibodies gave concordant results. A progression array (cohort III; n = 180) revealed frequent loss of Nuc-pYStat5 in invasive carcinoma compared to normal breast epithelia or ductal carcinoma in situ, and general loss of Nuc-pYStat5 in lymph node metastases. In cohort IV (n = 221), loss of Nuc-pYStat5 was associated with increased risk of antiestrogen therapy failure as measured by univariate CSS and time to recurrence (TTR). More sensitive AQUA quantification of Nuc-pYStat5 in antiestrogen-treated patients (cohort V; n = 97) identified by multivariate analysis patients with low Nuc-pYStat5 at elevated risk for therapy failure (CSS hazard ratio [HR], 21.55; 95% CI, 5.61 to 82.77; $P < .001$; TTR HR, 7.30; 95% CI, 2.34 to 22.78; $P = .001$).

Conclusion
Nuc-pYStat5 is an independent prognostic marker in node-negative breast cancer. If confirmed in prospective studies, Nuc-pYStat5 may become a useful predictive marker of response to adjuvant hormone therapy.

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INTRODUCTION

Signal transducer and activator of transcription (Stat5) is a latent cytoplasmic transcription factor and a primary mediator of prolactin signaling in breast epithelia.[1,2] After prolactin-induced phosphorylation of Stat5 on a conserved tyrosine residue by Jak2, Stat5 dimers translocate to the cell nucleus and bind to DNA of target genes,[1] promoting growth and differentiation of mammary epithelia.[2-5] Stat5 is highly activated in terminally differentiated breast epithelial cells during lactation[2-5] and is phosphorylated at a basal level in nonpregnant mouse and human epithelia.[6] Stat5 has been implicated as a mammary tumor promoter in mice, supported by observations that tumor development was delayed in Stat5-deficient mice and was induced in mice expressing a hyper-active Stat5 transgene.[7-9] However, in vitro laboratory studies have indicated that phosphorylated Stat5 promotes cellular differentiation and inhibits invasive characteristics of human breast cancer cell lines.[10-12] Consistent with the notion of a prodifferentiation effect of Stat5 in established human breast cancer, several

immunohistochemical studies have reported that reduced levels of Stat5 protein or tyrosine phosphorylated and nuclear localized Stat5 (Nuc-pYStat5) were associated with poorly differentiated morphology, higher histologic grade, and more advanced breast cancer.[13-16] Importantly, initial tissue microarray analysis suggested that loss of Nuc-pYStat5 was a marker of poor prognosis in human breast cancer, particularly in node-negative breast cancer,[13] however, this study did not evaluate a purely prognostic cohort as at least 40% of patients received potentially confounding systemic adjuvant therapy.[13] Here, we report the novel prognostic and hormone response–predictive value of Nuc-pYStat5 based on five distinct archival cohorts of breast cancer using both traditional diaminobenzidine (DAB) chromogen immunohistochemistry (IHC) with pathologist scoring and immunofluorescence-based quantification on the Automated Quantitative Analysis (AQUA) platform.[17,18]

PATIENTS AND METHODS

Breast Tumor Specimens

Archival and deidentified formalin-fixed, paraffin-embedded breast cancer specimens representing five independent clinical cohorts were analyzed, including whole tissue sections and tissue microarrays. The use of tissues was approved by the ethics committee of the respective institutions. Demographic and clinical characteristics of patients in cohorts I, II, IV, and V (not available for progression cohort III) are presented in Table 1.

Cohort I was obtained from the National Cancer Institute's Cooperative Breast Cancer Tissue Resource as whole tissue sections from patients with node-negative invasive ductal carcinomas (IDC) who did not receive adjuvant systemic therapy (n = 233). Clinical outcome end points included breast cancer–specific survival (CSS) and time to recurrence (TTR) of either local or distant disease. Nuc-pYStat5 DAB scores were obtained for 223 tumor specimens. Cohort II was a breast cancer tissue microarray (0.6 mm cores) from Yale University pathology archives, representing 291 node-negative patients with clinical and CSS data who did not receive adjuvant systemic therapy.[19-21] Nuc-pYStat5 AQUA scores were obtained from 198 patients. A significant number of cases were not interpretable by AQUA due to loss of histospots or insufficient staining quality or tumor sampling (< 5% tumor area) required for automated analysis.[22] Cohort III was a breast cancer progression array constructed using cutting edge matrix assembly[23] representing 180 unmatched patient specimens, including 40 normal breast tissues, 20 ductal carcinoma in situ (DCIS), 100 IDC, and 20 lymph node breast cancer metastases from Thomas Jefferson University Hospital archives. Hormone receptor status of the IDCs was determined by standard IHC and identified as 20% HER2 positive, 59% estrogen receptor (ER) positive, and 42% progesterone receptor (PR) positive. Nuc-pYStat5 AQUA scores were obtained for 130 cases. Cohort IV from the Institute of Pathology at Kantonsspital Basel (Basel, Switzerland)[13] comprised 221 patients with CSS and TTR data who received antiestrogen therapy (approximately 20% of cases also received adjuvant chemotherapy). Interpretable Nuc-pYStat5 DAB staining was obtained for 166 patients. Cohort V represented a random series of patients with breast cancer identified through the Alberta Cancer Registry (Calgary, Alberta, Canada) who received adjuvant hormone monotherapy for up to 60 months (average 30 months). The tissue microarray ("Tamoxifen 50/50 array") was constructed with triplicate 0.6 mm tumor tissue cores from 50 patients who died of breast cancer, classified as resistant to antiestrogen therapy, and 50 patients with longer than 5-years follow-up without breast cancer recurrence, classified as good responders. AQUA analysis yielded informative data on Nuc-pYStat5 for 65 cases.

Detection of Nuc-pYStat5 by DAB Chromogen IHC

The specificity of mouse monoclonal antiphosphoStat5 antibody AX1 (Advantex BioReagents, El Paso, TX) recognizing the conserved phosphotyrosyl residue Y694/Y699 of Stat5a/b, has been extensively validated.[6,13] Detection of Nuc-pYStat5 with AX1 was performed as previously described[13] except AX1 incubation time was shortened to 45 minutes at a final concentration of 1.2 μg/mL to accommodate the Dako Autostainer (Dako, Carpinteria, CA). Antigen-antibody complexes were detected using biotinylated goat antimouse secondary antibody (Biogenex, San Ramon, CA) followed by streptavidin-horseradish-peroxidase complex, using 3,3-(prime) DAB as chromogen and Mayer hematoxylin as counterstain. Individual breast tumor samples were scored by a pathologist, blinded to clinical outcome, for transcriptionally active Stat5 as defined by nuclear localization and tyrosine phosphorylation of Stat5 by estimating the percent malignant cells with detectable staining and overall staining intensity on a scale ranging from 0 to 3. Positive Nuc-pYStat5 status was defined as staining intensity greater than 0 in 10% or more of the carcinoma cells, corresponding to the original report.[13]

Quantification of Nuc-pYStat5 by AQUA

Rabbit monoclonal antiphosphoStat5 antibody, E208 (Epitomics, Burlingame, CA), was used for AQUA. E208 recognizes the same phospho-antigen (Y694/Y699 Stat5a/b) as the AX1 antibody and showed similar specificity and dynamic range in side-by-side DAB chromogen IHC and AQUA testing (Figs 1C to 1E). Antigen retrieval was performed using the DAKO PT-module with citric acid buffer (pH 6.0). Immunofluorescent staining was performed on a Dako Autostainer. E208 was diluted 1:1,600 from the supplier-provided stock and coincubated for 30 minutes with mouse monoclonal antipancytokeratin (clone AE1/AE3, DAKO, 1:100 dilution) to define the tumor mask.[17] Horseradish peroxidase–conjugated antirabbit immunoglobulin G and Alexa-488-conjugated antimouse immunoglobulin G secondary antibodies were added for 30 minutes, followed by 10 minutes incubation with Cy5-tyramide (Perkin Elmer, Waltham, MA). Slides were coverslipped using 4′,6-diamidino-2-phenylindole (DAPI) -containing mounting media. The AQUA/PM2000 platform (HistoRx, New Haven, CT)[17] was used to quantify fluorescence-based immunostaining for Nuc-pYStat5. Tissue array slides were automatically scanned and fluorescent images were captured in three channels, fluorescein isothiocyanate/Alexa-488 (cytokeratin), Cy5 (Nuc-pYStat5), or DAPI (nuclei). AQUA scores, blinded to clinical data, were objectively derived by calculating the mean signal intensities within the cell nuclei of the epithelial compartment, defined by cytokeratin-positive and DAPI-positive mapping.

X-Tile Cut Point Analysis and Statistical Methods

Optimal cut points for AQUA-quantified Nuc-pYStat5 as a function of survival in prognostic cohort II and predictive cohort V were derived using X-tile software, which employs crossvalidation to produce corrected *P* values for multiple cut points.[24] End points for survival analysis were TTR (cohorts I, IV and V) and breast CSS (cohorts I, II, IV, and V) according to consensus definitions.[25] Survival analyses were performed by constructing Kaplan-Meier curves and using the log-rank test and adjusted Cox or Weibull regression models (SAS version 9.2, SAS Institute, Cary, NC). Cox regression was used when proportional hazard assumption passed (completed globally using a Wald χ^2 test for each cohort and outcome multivariate model), otherwise Weibull regression was applied (assessment made graphically). When available, variables included in the adjusted models were tumor grade, tumor size, and status of nodal involvement, ER, PR, HER2, and Nuc-pYStat5. One-way analysis of variation with Dunnett's T3 pairwise posthoc test assuming unequal variances (SPSS version 15.0; SPSS Inc, Chicago, IL) was used to test for differences in Nuc-pYStat5 levels between breast histology groups in progression cohort III.

RESULTS

Nuc-pYStat5 Is an Independent Marker of Prognosis in Node-Negative Breast Cancer

To determine whether levels of Nuc-pYStat5 would predict outcome in patients with lymph node-negative breast cancer who did not receive adjuvant therapy, we first examined the clinically relevant setting of whole tumor tissue sections from cohort I using DAB chromogen IHC and the same mouse monoclonal antipYStat5 antibody

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Table 1. Characteristics of Cohort I, II, IV, and V

Variable	Cohort I (n = 233)		Cohort II (n = 291)		Cohort IV (n = 221)		Cohort V (n = 97)	
	No.	%	No.	%	No.	%	No.	%
Discrete								
Center								
Fox Chase Cancer Center	64	27	—	—	—	—	—	—
Kaiser Permanente	79	34	—	—	—	—	—	—
University of Miami	30	13	—	—	—	—	—	—
Washington University	60	26	—	—	—	—	—	—
Race								
Asian	1	0.4	—	—	—	—	—	—
Black	16	7	3	1	—	—	—	—
White	216	93	287	99	—	—	—	—
Other	—	—	1	0.3	—	—	—	—
Unknown	—	—	—	—	221	100	97	100
Age, years								
< 50	42	18	86	30	27	12	6	6
≥ 50	191	82	205	70	194	88	91	94
Tumor size, cm								
< 2	117	50	114	39	53	24	39	40
≥ 2-< 5	107	46	133	46	142	64	43	44
≥ 5	9	4	33	11	23	10	11	11
Missing	—	—	11	4	3	1	4	4
Grade								
1	61	26	67	23	56	25	14	14
2	104	45	137	47	99	45	47	48
3	68	29	48	16	66	30	32	33
Missing	—	—	39	13	—	—	4	4
ER status								
Negative	44	19	97	33	44	20	11	11
Positive	186	80	163	56	130	59	80	82
Missing	3	1	31	11	47	21	6	6
PR status								
Negative	65	28	103	35	87	39	25	26
Positive	122	52	151	52	134	61	67	69
Missing	46	20	37	13	—	—	5	5
HER2 status								
Normal	—	—	217	75	173	78	77	79
Overexpressed	—	—	33	11	29	13	13	13
Missing	—	—	41	14	19	9	7	7
Node status								
Negative	233	100	291	100	70	32	42	43
Positive	0	0	0	0	138	62	42	43
Missing	—	—	—	—	13	6	13	13
Chemotherapy								
Untreated	233	100	—	—	171	77	95	98
Treated	0	0	—	—	45	20	1*	1
Missing	—	—	—	—	5	2	1	1
Hormone therapy								
Untreated	233	100	—	—	0	0	5	5
Treated	0	0	—	—	221	100	92	95
Radiation therapy								
Untreated	187	80	—	—	221	100	36	37
Treated	46	20	—	—	0	0	59	61
Missing	—	—	—	—	—	—	2	2
CSS events	52	22	107	37	65	29	56	58

(continued on following page)

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Table 1. Characteristics of Cohort I, II, IV, and V (continued)

Variable	Cohort I (n = 233)		Cohort II (n = 291)		Cohort IV (n = 221)		Cohort V (n = 97)	
	No.	%	No.	%	No.	%	No.	%
Nuc-pYStat5 status								
Low	129	55	84	29	93	42	10	10
High	94	40	114	39	73	33	55	57
Missing	10	4	93	32	55	25	32	33

	Mean	Median	Range	SD
Continuous				
Cohort I				
Age at diagnosis	62.3	62	31-88	12.9
Tumor size, cm	2.08	1.8	0.6-7.5	1.07
Follow-up, months	129	126	3-326	71
Nuc-pYStat5 score	5.0	0	0-40	8.6
Year of diagnosis	—	—	1974-1990	—
Cohort II				
Age at diagnosis	57.3	57	24-86	12.3
Tumor size, cm	2.53	2.0	0.4-11	1.67
Follow-up, months	165	160	1-425	106
Nuc-pYStat5 score	852	771	265-2,236	416
Year of diagnosis	—	—	1953-1980	—
Cohort IV				
Age at diagnosis	63.3	63	35-97	12.1
Tumor size, cm	3.0	2.5	0.5-13.0	1.8
Follow-up, months	59.0	60	2-137	28.0
Nuc-pYStat5 score	1	0	0-4	1.3
Positive nodes	3.8	1	0-38	6.2
Year of diagnosis	—	—	1985-1996	—
Cohort V				
Age at diagnosis	69.8	72	38-89	11.0
Tumor size, cm	2.7	2.1	0.4-11	1.7
Follow-up, months	57.5	40.7	3.6-143	40.9
Nuc-pYStat5 score	1,262	1,027	533-4,243	762
Positive nodes	2.3	0	0-22	3.7
Year of diagnosis	—	—	1990-2000	—

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; CSS, cancer-specific survival; SD, standard deviation; Nuc-pYStat5, nuclear localized and tyrosine phosphorylated Stat5.
*Patient received chemotherapy after disease relapse.

(AX1) used in our previous tissue microarray study.[13] Evaluation of TTR revealed that low levels of Nuc-pYStat5 were associated with increased risk of breast cancer recurrence (TTR; log-rank $P = .023$, n = 223, Fig 1A; univariate Cox regression hazard ratio [HR], 2.35; 95% CI, 1.18 to 4.67; $P = .015$, n = 183, Appendix Table A1, online only). Importantly, low expression of Nuc-pYStat5 also was associated with poor breast CSS (log-rank $P = .046$, n = 223, Fig 1B; univariate Weibull regression HR, 2.32; 95% CI, 1.10 to 4.89; $P = .027$, n = 183, Table 2).

Detection of Nuc-pYStat5 by AX1 gives best results after overnight incubation at 4°C and optimal sensitivity is not achieved with the short incubation times (ie, 20 to 45 minutes) required for autostainers. Therefore, we extended the analyses to include a rabbit monoclonal antipYStat5 (E208), which could be optimized for autostainer protocols. The optimized overnight AX1 and autostainer E208 protocols resulted in comparable sensitivity in detecting prolactin-induced Stat5 phosphorylation and nuclear translocation by DAB chromogen IHC (Fig 1C). Levels of Nuc-pYStat5 were also highly correlated and reflected similar dynamic ranges when quantified by AQUA using the AX1 or E208 protocols in two serial sections of an array of assorted normal and malignant breast tissues (Pearson $r = 0.774$, $P < .001$, n = 67, Figs 1D to 1E). The reproducibility of the antibodies to detect the same epitope on near-identical serial tissue sections despite differences in antigen retrieval and incubation times was further evidenced by low coefficients of variation (median, 4.7%; range, 0.1% to 9.6%). Therefore, analysis of Nuc-pYStat5 using E208 for automated, objective, and quantitative immunofluorescence was completed in an independent cohort (cohort II), a tissue microarray also limited to primary breast carcinomas from patients who were lymph node negative and did not receive systemic adjuvant therapy. Nuc-pYStat5 levels, quantified using E208 and the AQUA platform, predicted breast cancer survival also in cohort II (CSS; log-rank $P = .010$, n = 198, Fig 1F; univariate Weibull regression HR, 2.10; 95% CI, 1.26 to 3.51; $P = .004$, n = 158, Table 2). TTR data was not available for cohort II.

In multivariate analyses, Nuc-pYStat5 remained an independent marker of disease prognosis in both cohorts I and II, when adjusting for standard clinical and pathologic markers. In cohort I, patients with low Nuc-pYStat5 expression had an adjusted 2.5-fold increased risk of

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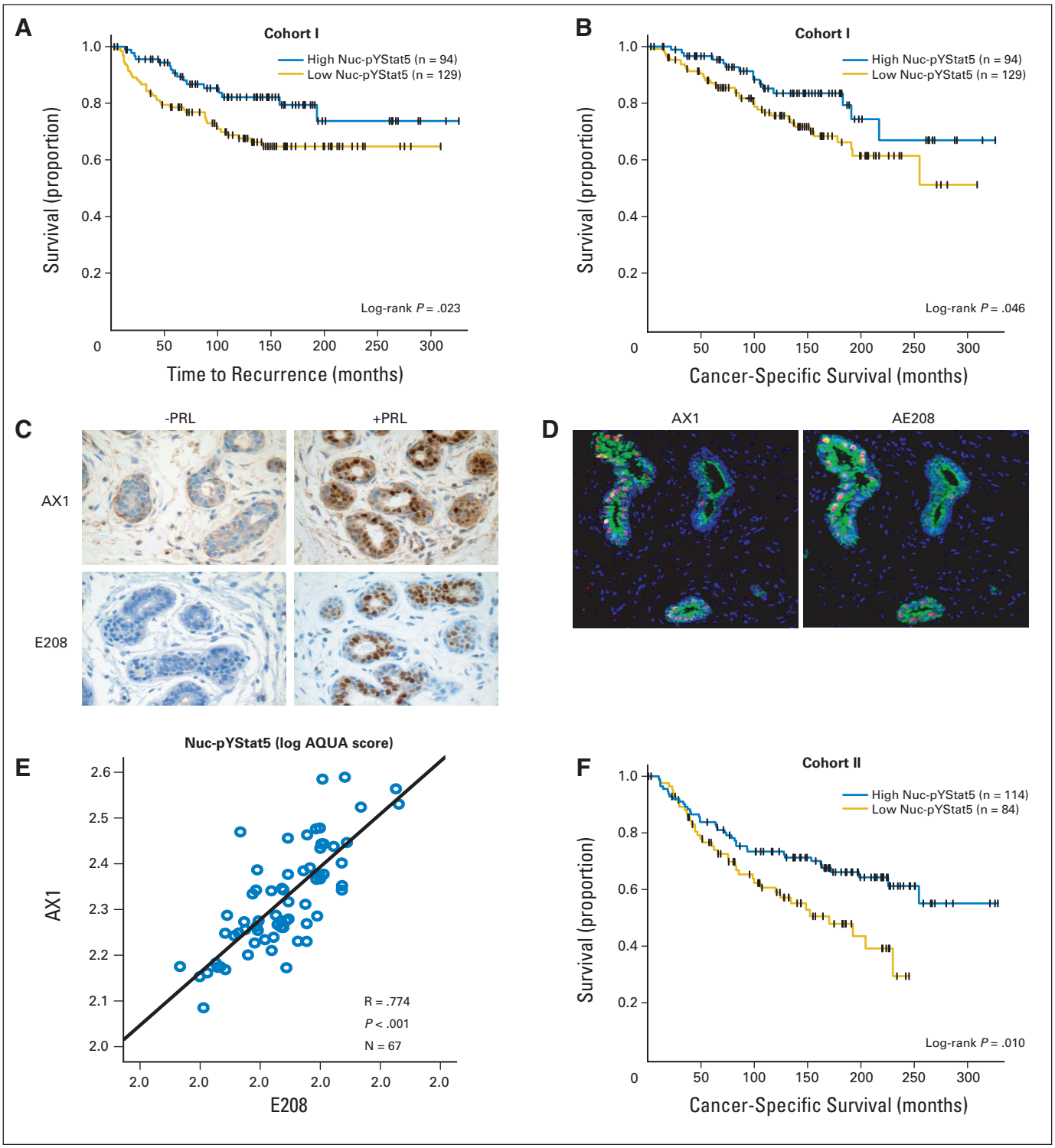

Fig 1. Low levels of nuclear localized and tyrosine phosphorylated Stat5 (Nuc-pYStat5) predict unfavorable breast cancer prognosis. (A-B) Kaplan-Meier analysis of Nuc-pYStat5, detected by mouse monoclonal antipYStat5 AX1 antibody and standard diaminobenzidine (DAB) immunohistochemistry (IHC) with pathologist review of whole tissue sections, in cohort I revealed that low expression of Nuc-pYStat5 was prognostic of (A) reduced time to recurrence of breast cancer and (B) poor breast cancer-specific survival (CSS). (C-E) Comparative validation of mouse monoclonal AX1 with rabbit monoclonal antipYStat5 antibody E208. (C) Detection of nuclear localized and tyrosine phosphorylated Stat5 by DAB chromogen IHC in healthy human breast tissue surgical explants incubated ex vivo with (positive control) or without (negative control) human prolactin (100 nmol/L; 60 minutes) using antipYStat5 antibodies AX1 (16 hours' incubation with manual staining protocol; upper panels) or E208 (20 minutes' incubation with autostainer protocol; lower panels). (D) Representative immunofluorescent images from two serial sections of a breast tissue stained with AX1 or E208 antibodies indicating comparable detection of Nuc-pYStat5. (E) Correlation of Nuc-pYStat5 expression detected by AX1 or E208 and quantified by automated quantitative analysis (AQUA) in serial sections of an assorted breast tissue microarray. (F) Kaplan-Meier analysis of breast CSS in cohort II indicated that risk of death from breast cancer was significantly elevated in patients whose tumors expressed low Nuc-pYStat5 as quantified by immunofluorescence and AQUA analysis. Censored cases (+) and number of patients per group are indicated.

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Table 2. Univariate and Multivariate Survival Analyses of Breast CSS in Cohorts I and II

Variable	No.	Multivariate Adjusted (Weibull) HR	95% CI	*P*	Unadjusted (Weibull) HR	95% CI	*P*
Cohort I (CSS)*							
Tumor grade							
1	47	1		—	1		—
2	76	1.53	0.57 to 4.10	.399	1.87	0.72 to 4.87	.198
3	60	1.61	0.56 to 4.66	.381	2.74	1.05 to 7.11	.039
Tumor size, cm							
< 2	88	1		—	1		—
≥ 2-< 5	87	2.30	1.06 to 4.99	.036	2.47	1.20 to 5.08	.014
≥ 5	8	2.50	0.62 to 10.04	.197	2.97	0.80 to 10.95	.103
ER status							
Negative	32	1		—	1		—
Positive	151	1.82	0.70 to 4.71	.216	0.72	0.34 to 1.51	.380
PR status							
Negative	64	1		—	1		—
Positive	119	0.55	0.25 to 1.20	.133	0.55	0.29 to 1.04	.065
Nuc-pYStat5							
Low (0)	105	2.38	1.13 to 5.04	.023	2.32	1.10 to 4.89	.027
High (> 0)	78	1		—	1		—
Cohort II (CSS)†							
Tumor grade							
1	34	1		—	1		—
2	90	1.25	0.66 to 2.37	.500	1.16	0.62 to 2.19	.640
3	34	0.87	0.35 to 2.19	.772	0.81	0.36 to 1.82	.616
Tumor size, cm							
< 2	60	1		—	1		—
≥ 2-< 5	80	2.49	1.33 to 4.67	.004	2.11	1.15 to 3.88	.016
≥ 5	18	3.89	1.63 to 9.26	.002	2.68	1.19 to 6.05	.018
ER status							
Negative	50	1		—	1		—
Positive	108	0.88	0.45 to 1.70	.698	1.01	0.59 to 1.73	.958
PR status							
Negative	57	1		—	1		—
Positive	101	1.26	0.69 to 2.29	.459	0.92	0.55 to 1.55	.757
HER2 status							
Normal	138	1		—	1		—
Overexpressed	20	1.37	0.61 to 3.07	.440	1.04	0.49 to 2.19	.918
Nuc-pYStat5							
Low (< 684)	67	2.39	1.37 to 4.17	.002	2.10	1.26 to 3.51	.004
High (≥ 684)	91	1		—	1		—

NOTE. Weibull regression survival analysis was used to evaluate prognostic factors. Global test for Cox regression proportional hazards assumption failed, necessitating Weibull regression. Cohort I: n = 233; 183 (79%) evaluable; 41 (22%) of 183 events. Cohort II: n = 291; 158 (54%) evaluable; 61 (39%) of 158 events.
Abbreviations: CSS, cancer specific survival; HR, hazard ratio; ER, estrogen receptor; PR, progesterone receptor; Nuc-pYStat5, nuclear localized and tyrosine phosphorylated Stat5.
*Global test: $\chi^2(5) = 12.93$, $P = .024$.
†Global test: $\chi^2(6) = 13.22$, $P = .040$.

disease recurrence (TTR; multivariate Cox regression HR, 2.49; 95% CI, 1.23 to 5.05; $P = .012$; n = 183, Appendix Table A1) and a 2.4-fold greater risk of dying from breast cancer (CSS; multivariate Weibull regression HR, 2.38; 95% CI, 1.13 to 5.04; $P = .023$, n = 183, Table 2). Likewise, when quantified by AQUA in cohort II, Nuc-pYStat5 remained an independent marker of breast CSS as reflected in a similar 2.4-fold increased risk of death (CSS; multivariate Weibull regression HR, 2.39; 95% CI, 1.37 to 4.17; $P = .002$, N = 158, Table 2). Based on two independent cohorts and analytic approaches, we conclude that Nuc-pYStat5 is an independent marker of outcome in patients with lymph node-negative breast cancer.

Levels of Nuc-pYStat5 Are Diminished During Breast Cancer Progression

E208 and AQUA were then used to quantify Nuc-pYStat5 levels in a breast tissue progression array (cohort III), which included unmatched normal breast tissue, DCIS, IDC, and breast cancer lymph node metastases. Levels of Nuc-pYStat5 detected by quantitative immunofluorescence were markedly reduced during breast cancer progression (Fig 2). Specifically, while levels of Nuc-pYStat5 remained high and unchanged between normal epithelia and DCIS, Nuc-pYStat5 was significantly reduced in IDC ($P < .001$) with even greater loss in lymph node metastases ($P < .001$). These quantitative data

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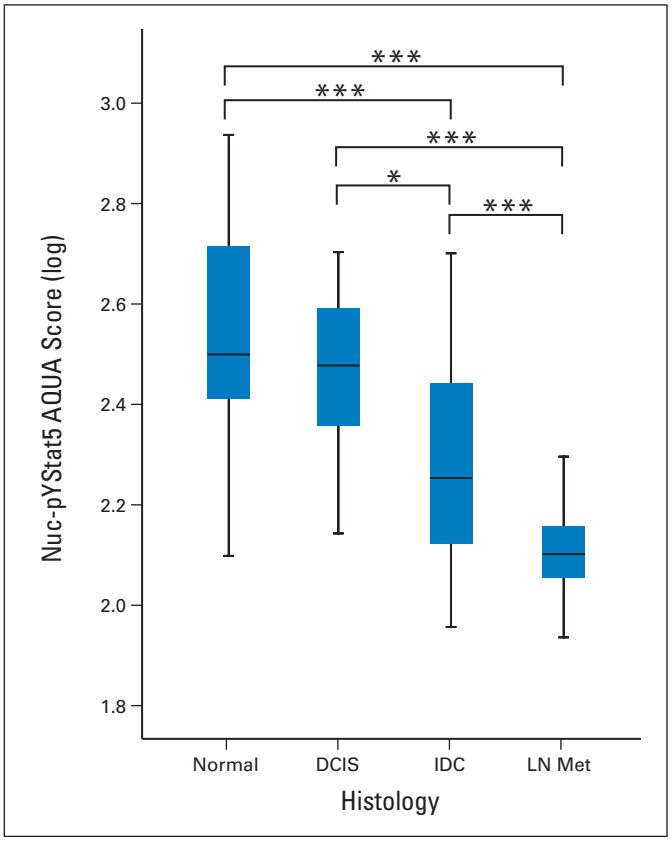

Fig 2. Loss of nuclear localized and tyrosine phosphorylated Stat5 (Nuc-pYStat5) during breast cancer progression. Levels of Nuc-pYStat5 as detected by immunofluorescence and quantified by automated quantitative analysis (AQUA) were significantly reduced in invasive ductal carcinoma (IDC; n = 72) and lymph node metastases (LN Met; n = 17) when compared with normal breast tissue (n = 27) and ductal carcinoma in situ (DCIS; n = 14) in progression array cohort III. (*) P = .012; (***) $P < .001$.

provided novel information supporting the observations of frequent loss of Nuc-pYStat5 during breast cancer progression.

Nuc-pYStat5 Predicts Responsiveness to Antiestrogen Therapy

Cohort IV comprised node-negative and node-positive breast cancer tissues from patients who received adjuvant antiestrogen therapy. Levels of Nuc-pYStat5 were assessed using AX1 and DAB chromogen IHC. The absence of detectable Nuc-pYStat5 in tumors from these patients was associated with an increased risk of breast cancer-specific death (CSS; log-rank $P = .039$, n = 166, Fig 3A; univariate Cox regression, HR, 1.83; 95% CI, 1.02 to 3.29; $P = .043$, n = 166). Breast cancer recurrence also was evaluated and revealed an increased risk of recurrence in patients with undetectable Nuc-pYStat5 (TTR; log-rank $P = .031$; n = 165; Fig 3B; HR, 1.83; 95% CI, 1.05 to 3.20; $P = .033$, n = 166). Although Nuc-pYStat5 did not remain an independent marker of response to antiestrogen therapy in multivariate analyses when detected by AX1 and DAB chromogen IHC in this limited cohort (n = 144, data not shown), univariate analyses were promising and prompted us to employ more sensitive methodology using E208 and AQUA on an independent cohort V.

Cohort V included tumors from node-negative and node-positive patients treated exclusively with antiestrogen monotherapy. The X-Tile software determined an optimal cut point that identified a subset of patients whose tumors had low levels of Nuc-pYStat5 and were at markedly increased risk of failing antiestrogen treatment and with poor breast CSS (log-rank $P < .001$; n = 65, Fig 3C). Univariate analysis indicated that approximately 15% of the patients with the lowest levels of Nuc-pYStat5 were at 7.4-fold increased risk of dying from breast cancer (CSS; univariate Cox regression HR, 7.36; 95% CI, 2.94 to 18.42; $P < .001$, n = 53, Table 3). In multivariate analysis adjusting for tumor size, tumor grade, node status, ER/PR status, and HER2 overexpression, Nuc-pYStat5 remained an independent marker of survival in cohort V (CSS; multivariate Cox regression HR, 21.55; 95% CI, 5.61 to 82.77; $P < .001$, n = 53, Table 3). Positive node status (CSS; multivariate Cox regression HR, 8.10; 95% CI, 3.03 to 21.64; $P < .001$, n = 53) and ER/PR status (CSS; multivariate Cox regression HR, 0.10; 95% CI, 0.03 to 0.39; $P = .001$, n = 53) were also independent predictors of survival (Table 3). Low levels of Nuc-pYStat5 also predicted breast cancer recurrence in these patients in both univariate (TTR; log-rank $P = .004$, n = 65, Fig 3D; Cox regression HR, 4.71; 95% CI, 1.99 to 11.16; $P < .001$, n = 53, Table 3) and multivariate analysis (TTR; Cox regression HR, 7.30; 95% CI, 2.34 to 22.78; $P = .001$, n = 53, Table 3). Collectively, these observations provide novel evidence to suggest that low levels of Nuc-pYStat5 predict failure of antiestrogen treatment and justify further analysis of Nuc-pYStat5 expression in antiestrogen-treated patients.

DISCUSSION

This study documents that low levels of Nuc-pYStat5 represent an independent marker of poor prognosis in patients with node-negative breast cancer who did not receive systemic adjuvant therapy. This was based on analyses of cancer-specific survival in two independent clinical cohorts (cohorts I and II) using two distinct antibodies and traditional pathologist scoring of whole tissue sections or quantitative AQUA analysis of a tissue microarray. These findings provide novel support for the previously proposed prognostic value of Nuc-pYStat5 in breast cancer, which was based on tissue array specimens and included patients treated with potentially confounding systemic adjuvant therapy.[13] Furthermore, loss of Nuc-pYStat5, as quantified by AQUA, was frequent across a breast cancer progression array (cohort III) with nearly undetectable levels in lymph node metastases. Importantly, analysis of two cohorts of patients with breast cancer who received antiestrogen therapy (cohorts IV and V) suggested that levels of Nuc-pYStat5 constitute a new predictive marker of response to adjuvant hormone therapy.

Antiestrogen therapy is currently guided by positive tumor expression of ERα by IHC. However, approximately 30% of patients with ER-positive breast cancer fail to respond to antiestrogen therapy due to inherent or acquired resistance.[26-29] A meta-analysis of 12 studies implicated HER2 as a modest predictor of resistance to antiestrogen therapy,[30] but American Society of Clinical Oncology guidelines do not recommend using HER2 as a predictor of response to endocrine therapy due to insufficient evidence.[31] In our multivariate analyses, HER2 status reached only borderline significance in predicting outcome in antiestrogen treated patients. In other immunohistochemical studies, markers such as epidermal growth factor receptor,[32-34] PR,[35-38] and p27[39-42] were suggested to predict failure of

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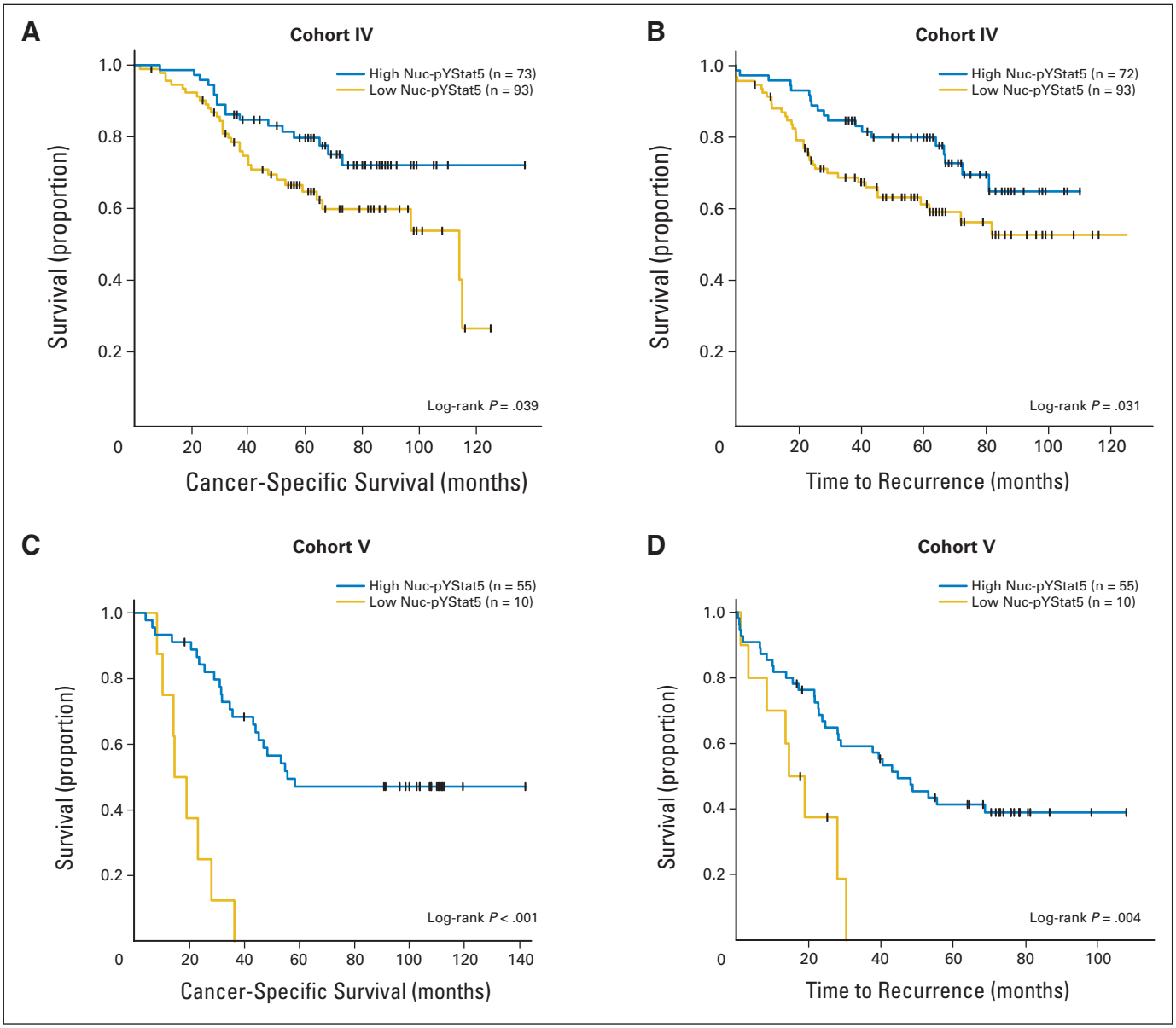

Fig 3. Low levels of nuclear localized and tyrosine phosphorylated Stat5 (Nuc-pYStat5) predict increased risk of failure of antiestrogen therapy. (A-B) Low levels of Nuc-pYStat5 detected by diaminobenzidine chromogen immunohistochemistry (IHC) and pathologist scoring in patients treated with antiestrogen therapy (cohort IV) predicted (A) poor breast cancer-specific survival (CSS) and (B) reduced time to recurrence (TTR) of breast cancer. (C-D) Immunofluorescence and quantitative automated quantitative analysis of Nuc-pYStat5 expression in breast cancer patients treated with antiestrogen monotherapy (cohort V) revealed that low expression of Nuc-pYStat5 was predictive of (C) poor CSS and (D) reduced TTR of breast cancer. Kaplan-Meier plots with censored cases (+) and number of patients per group indicated.

antiestrogen therapy and it will be of interest to expand future analyses of Nuc-pYStat5 to include epidermal growth factor receptor and p27.

Ongoing studies are exploring the individual prognostic and predictive values of the highly homologous Stat5a and Stat5b proteins, which share greater than 90% amino acid identity.[43] AntipYStat5 antibodies, including AX1 and E208 used here, do not distinguish between phosphorylated Stat5a and Stat5b due to their identical phosphotyrosyl-motifs. Increased risk of antiestrogen therapy failure was reportedly associated with loss of Stat5b protein expression in patients with breast cancer relapse.[15] However, response to classical adjuvant antiestrogen therapy was not addressed, and nuclear Stat5b was not distinguished from cytoplasmic Stat5b.[15] Therefore, it is unclear if the detected protein was transcriptionally active.

In this study, the quantitative analyses of cohort V revealed a strong predictive value of Nuc-pYStat5 for response to antiestrogen therapy. In cohort IV, the predictive value of Nuc-pYStat5 as measured by DAB chromogen IHC and pathologist scoring was statistically significant both for CSS and TTR in univariate but not in multivariate analyses. The greater predictive value of Nuc-pYStat5 in cohort V is likely attributable, at least in part, to the improved dynamic range of quantitative immunofluorescence detection over that provided by DAB chromogen and pathologist scoring[17,18,44,45] in cohort

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Table 3. Univariate and Multivariate Breast CSS and TTR Analysis of Nuc-pYStat5 in Patients With Breast Cancer Treated With Antiestrogen Monotherapy in Cohort V

		Multivariate Adjusted (Cox)			Unadjusted (Cox)		
Variable	No.	HR	95% CI	*P*	HR	95% CI	*P*
Cohort V (CSS)*							
Tumor size, cm							
< 2	25	1		—	1		—
2-< 5	23	1.24	0.42 to 3.66	.700	2.92	1.33 to 6.41	.008
≥ 5	5	0.97	0.21 to 4.41	.971	4.83	1.50 to 15.57	.008
Tumor grade							
1	8	1		—	1		—
2	27	0.60	0.19 to 1.88	.376	0.80	0.29 to 2.26	.677
3	18	0.73	0.21 to 2.55	.621	1.64	0.58 to 4.61	.347
ER/PR status							
Negative	8	1		—	1		—
Positive	45	0.10	0.03 to 0.39	.001	0.37	0.16 to 0.88	.024
HER2 status							
Normal	45	1		—	1		—
Overexpressed	8	1.99	0.71 to 5.64	.193	2.53	1.08 to 5.93	.032
Lymph node status							
Negative	28	1		—	1		—
Positive	25	8.10	3.03 to 21.64	< .001	4.72	2.19 to 10.20	< .001
Nuc-pYStat5							
Low (< 724)	8	21.55	5.61 to 82.77	< .001	7.36	2.94 to 18.42	< .001
High (≥ 724)	45	1		—	1		—
Cohort V (TTR)†							
Tumor size, cm							
< 2	25	1		—	1		—
2-< 5	23	1.56	0.56 to 4.35	.393	2.68	1.27 to 5.68	.010
≥ 5	5	1.81	0.46 to 7.21	.397	5.28	1.82 to 15.33	.002
Tumor grade							
1	8	1		—	1		—
2	27	0.46	0.16 to 1.35	.156	0.67	0.26 to 1.75	.417
3	18	0.70	0.22 to 2.26	.553	1.53	0.59 to 4.00	.384
ER/PR status							
Negative	8	1		—	1		—
Positive	45	0.16	0.05 to 0.49	.001	0.31	0.14 to 0.71	.005
HER2 status							
Normal	45	1		—	1		—
Overexpressed	8	2.02	0.74 to 5.50	.171	2.33	1.01 to 5.38	.048
Lymph node status							
Negative	28	1		—	1		—
Positive	25	5.28	2.28 to 12.18	< .001	3.65	1.80 to 7.42	< .001
Nuc-pYStat5							
Low (< 724)	8	7.30	2.34 to 22.78	.001	4.71	1.99 to 11.16	< .001
High (≥ 724)	45	1		—	1		—

NOTE. Cox regression survival analysis was used to evaluate prognostic factors. Global test for Cox regression proportional hazards passed. Cohort V CSS: n = 97; 53 (55%) evaluable; 31 (58%) of 53 events; Cohort V TTR: n = 97; 53 (55%) evaluable; 34 (64%) of 53 events.

Abbreviations: CSS, cancer specific survival; TTR, time to recurrence; Nuc-pYStat5, nuclear localized and tyrosine phosphorylated Stat5; HR, hazard ratio; ER, estrogen receptor; PR, progesterone receptor.

*Global test: $\chi^2(6) = 6.38$, $P = .38$.

†Global test: $\chi^2(6) = 5.45$, $P = .49$.

IV. In fact, as many as 56% of cases in cohort IV were scored negative for Nuc-pYStat5, while X-Tile applied to the continuous fluorescence-based AQUA data determined a cut point in cohort V that identified 15% of patients whose tumors displayed the lowest levels of Nuc-pYStat5 with distinctly elevated risk of failing adjuvant hormone therapy. Furthermore, a limitation of cohort IV is that approximately 20% of the patients received adjuvant chemotherapy in addition to hormone therapy, whereas all patients in cohort V exclusively received adjuvant hormone therapy. Additional limitations of this study include the limited cohort sizes, loss of evaluable tumor tissue that is characteristic of tissue microarrays, and missing analytic or clinical data. Close examination suggested that some data were not missing at random in the various cohorts although consistent patterns did not emerge across the various cohorts (data not shown). Furthermore, the cohorts differed with regard to period of diagnosis and this may contribute to variability. In general, predictions based on retrospective populations may not adequately reflect current therapeutic strategies.

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Collectively, this work supports the notion that Stat5 signaling is frequently lost during breast cancer progression and loss of Nuc-pYStat5 is associated with poor prognosis in node-negative breast cancer. Furthermore, novel evidence is provided suggesting that loss of Nuc-pYStat5 is associated with elevated risk of failure of antiestrogen therapy. However, conclusive validation of the response-predictive value of Nuc-pYStat5 will require quantitative analyses in tumors from patients randomized for antiestrogen therapy, and prospective analyses in a Clinical Laboratory Improvement Amendments–certified laboratory to overcome additional limitations of retrospective studies.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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AUTHOR CONTRIBUTIONS

Conception and design: Amy R. Peck, Agnieszka K. Witkiewicz, Marja T. Nevalainen, Terry Hyslop, Guido Sauter, Anthony M. Magliocco, Hallgeir Rui

Financial support: Craig D. Shriver, Terry Hyslop, Guido Sauter, David L. Rimm, Anthony M. Magliocco, Hallgeir Rui

Administrative support: Anne L. Rosenberg, Craig D. Shriver, Terry Hyslop, Guido Sauter, Anthony M. Magliocco, Hallgeir Rui

Provision of study materials or patients: Anne L. Rosenberg, Guido Sauter, David L. Rimm, Anthony M. Magliocco, Hallgeir Rui

Collection and assembly of data: Amy R. Peck, Agnieszka K. Witkiewicz, Chengbao Liu, Ginger A. Stringer, Alexander C. Klimowicz, Edward Pequignot, Boris Freydin, Thai H. Tran, Ning Yang, Anne L. Rosenberg, Jeffrey A. Hooke, Albert J. Kovatich, Marja T. Nevalainen, Craig D. Shriver, Terry Hyslop, Guido Sauter, David L. Rimm, Anthony M. Magliocco, Hallgeir Rui

Data analysis and interpretation: Amy R. Peck, Agnieszka K. Witkiewicz, Chengbao Liu, Ginger A. Stringer, Alexander C. Klimowicz, Edward Pequignot, Boris Freydin, Thai H. Tran, Ning Yang, Jeffrey A. Hooke, Albert J. Kovatich, Marja T. Nevalainen, Craig D. Shriver, Terry Hyslop, Guido Sauter, David L. Rimm, Anthony M. Magliocco, Hallgeir Rui

Manuscript writing: Amy R. Peck, Agnieszka K. Witkiewicz, Chengbao Liu, Ginger A. Stringer, Alexander C. Klimowicz, Edward Pequignot, Boris Freydin, Thai H. Tran, Ning Yang, Anne L. Rosenberg, Jeffrey A. Hooke, Albert J. Kovatich, Marja T. Nevalainen, Craig D. Shriver, Terry Hyslop, Guido Sauter, David L. Rimm, Anthony M. Magliocco, Hallgeir Rui

Final approval of manuscript: Amy R. Peck, Agnieszka K. Witkiewicz, Chengbao Liu, Ginger A. Stringer, Alexander C. Klimowicz, Edward Pequignot, Boris Freydin, Thai H. Tran, Ning Yang, Anne L. Rosenberg, Jeffrey A. Hooke, Albert J. Kovatich, Marja T. Nevalainen, Craig D. Shriver, Terry Hyslop, Guido Sauter, David L. Rimm, Anthony M. Magliocco, Hallgeir Rui

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