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Multiplexed Quantitative Analysis of CD3, CD8, and CD20 Predicts Response to Neoadjuvant Chemotherapy in Breast Cancer

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Abstract

Purpose—Although tumor infiltrating lymphocytes have been associated with response to neoadjuvant therapy, measurement is typically subjective, semi-quantitative and does not differentiate between subpopulations. Here we describe a quantitative objective method for analyzing lymphocyte subpopulations and assess their predictive value.

Methods—We develop a quantitative immunofluorescence (QIF) assay to measure stromal expression of CD3, CD8, and CD20 on one slide. We validate this assay by comparison to flow cytometry on tonsil and assess predictive value in breast cancer on a neoadjuvant cohort (n = 95). Then each marker is tested for prediction of pathologic complete response (pCR) compared to pathologist estimation of percentage lymphocyte infiltrate.

Results—Lymphocyte percentage and CD3, CD8, and CD20 proportions were similar between flow cytometry and QIF on tonsil. Pathologist TIL count predicted pCR (p = 0.043, OR: 4.77[1.05–21.6]) despite fair interobserver reproducibility (κ = 0.393). Stromal AQUA scores for CD3 (p = 0.023, OR: 2.51[1.13–5.57]), CD8 (p = 0.029, OR: 2.00[1.08–3.72]), and CD20 (p = 0.005, OR: 1.80[1.19–2.72]) predicted pCR in univariate analysis. CD20 AQUA score predicted pCR (p = 0.019, OR: 5.37[1.32–21.8]) independently of age, size, nuclear grade, nodal status, ER, PR, HER2, and Ki-67, whereas CD3, CD8, and pathologist estimation did not.

Conclusions—We have developed and validated an objective, quantitative assay measuring tumor infiltrating lymphocytes in breast cancer. While this work provides analytic validity, future larger studies will be required to prove clinical utility.

Introduction

Neoadjuvant chemotherapy facilitates breast conserving surgery and enables early evaluation of response, allowing for discontinuation of ineffective treatment (1). Moreover, patients who achieve pathologic complete response (pCR), elimination of invasive tumor

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following therapy, demonstrate better disease-free and overall survival (2, 3). However, most patients do not achieve pCR when treated with standard neoadjuvant chemotherapy (4, 5).

Tumor infiltrating lymphocytes (TILs) are an important immune component of the response to cancer (6). Lymphocyte subpopulations are recognized by expression of specific cell surface biomarkers. CD3, a T-cell receptor protein, instigates a signaling cascade after antigen recognition to activate T cells (7). CD8, a co-receptor for the T-cell receptor, recognizes the major histocompatibility I complex and is expressed on cytotoxic T lymphocytes, the predominant TIL subpopulation in breast cancer (8, 9). CD20, a transmembrane protein expressed on B cells but not plasma cells, regulates B cell activity, proliferation, and differentiation (10, 11). B cells drive the humoral, antibody-driven, immune response and often co-localize with T cells in tumors (11, 12). Previously, lymphocyte infiltration has predicted improved response to chemotherapy and trastuzumab, and CD3, CD8, and CD20 have all been discussed as potential predictive biomarkers (13–18).

Assessing the predictive significance of TILs is challenging due to differing methods of measurement (19). TIL measurement has been reported as TIL count (8, 20), as density (TILs per high powered field) (13, 18), in semi-quantitative scales (21, 22), and as percentage of stromal infiltrate (14, 23). TIL location is also not assessed in a standardized manner. Locations can be defined as intratumoral or stromal, which is further subdivided into adjacent and distant stroma (13). Another concern is variable selection of and number of fields of view analyzed, especially considering TIL heterogeneity (18). Perhaps the most significant issue plaguing TIL quantification is subjectivity and non-reproducibility. Several assays attempt to objectively quantify TILs, but many consider only one biomarker, precluding relationships between TIL subpopulations (24–28). Moreover, these assays are often challenging on large cohorts, where they are unable to account for location, and measure TILs semi-quantitatively.

This study aims to objectively measure TILs on a cohort of biopsies obtained before neoadjuvant chemotherapy. We develop and validate an immunofluorescence-based quantitative approach for measuring CD3, CD8, and CD20 expression within the stroma adjacent to the tumor. We objectively analyze expression in all fields of view, determine which TIL biomarkers predict pathological complete response, and compare their predictive value.

Materials and Methods

Tissue Collection and Patient Cohort

Freshly resected tonsil tissue was collected from four patients who underwent tonsillectomies at Yale-New Haven Hospital in 2013. Upon collection, each tonsil specimen was divided into two approximately equal halves. One half was formalin fixed for 24–48 hours before paraffin embedding. The other half was placed in RPMI media on ice for single cell suspension preparation.

Paraffin embedded pre-therapeutic core biopsies were collected from 105 consecutive invasive breast cancer patients that received neoadjuvant therapy, primarily anthracycline and taxane-based, as described previously (29). The distribution of treatment regimens, nuclear grade, tumor size, hormone receptor status, and HER2 status is described in Table 1. HER2 status was determined by IHC, with 0 and 1+ classified as HER2 negative and 3+ as HER2 positive. Cases that were HER2 2+ were tested by FISH and then classified into positive or negative groups as per the 2013 ASCO/CAP guidelines. Tissue was used after written patient consent. The study was performed according to the Yale University Institutional Review Boards protocol #9505008219.

Flow Cytometry

Single cell suspensions were extracted from tonsil tissue and filtered through a 70- μ m nylon strainer (Corning). The resulting suspension was centrifuged and reconstituted to 10^7 cells/mL in PBS with 5% FBS and 0.1% sodium azide. Cells were then divided into five equal proportions of 10^6 cells and incubated for 20 min on ice with Fc Receptor Binding Inhibitor (eBioscience). One proportion was incubated with a mix of anti-CD3 FITC-conjugated (0.2 mg/mL, eBioscience), anti-CD8 PE-conjugated (0.05 mg/mL, eBioscience), and anti-CD20 APC-conjugated antibodies (0.012 mg/mL, eBioscience) for 30 minutes in the dark on ice. The other four proportions were compensation controls, one unstained and three stained in parallel with the antibody mix. After washing, flow cytometry analysis for CD3, CD8, and CD20 positive subpopulations was done on a LSRII Flow Cytometer (BD Biosciences) at the Yale Cell Sorter Core Facility, and data was collected using FACSDiva software. Flow cytometry plots were generated and analyzed with FLOWJO software (Tree Star).

Pathologic Assessment of TILs

Histopathologic analysis was performed on hematoxylin and eosin-stained sections of 103 core biopsies from the cohort. Sections when available were obtained from archives or otherwise stained prior to reading. Analysis was conducted by two pathologists (V.B. and C.N.), both blinded to clinical parameters and response. TILs were quantified as percentage estimate of the tumor stroma area that contained lymphocytic infiltrate (14). Percentages were reported in increments of 10 percent, with greater than 50 percent infiltrate denoted as lymphocyte predominant breast cancer (LPBC) (23).

Multiplexed Immunofluorescence Staining for TILs

In situ detection of CD3, CD8, and CD20 with cytokeratin and DAPI were conducted on the same slide (Supplemental Figure 1). Briefly, slides were deparaffinized and rehydrated before pH8 EDTA antigen retrieval. Endogenous peroxidase activity was blocked with dual endogenous peroxidase block (Dako) and non-specific antigens were blocked with 0.3% bovine serum albumin in Tris-buffered saline/Tween. Primary monoclonal antibodies against CD3 (1:100, Novus, Rabbit IgG Clone NB600-1441), CD8 (1:250, Dako, Mouse IgG1 Clone C8/144B), and CD20 (1:150, Dako, Mouse IgG2a Clone L26) were co-incubated for 1 hour at room temperature. Slides were incubated sequentially with three HRP-conjugated secondary antibodies for one hour at room temperature prior to tyramide-based HRP activation for 10 minutes, followed by 1mM benzoic hydrazide with 0.15%

hydrogen peroxide to quench HRP activation. The secondary antibodies were anti-rabbit Envision reagent (Dako), anti-mouse IgG1 (Abcam, 1:100), and anti-mouse IgG2a (Abcam, 1:200). HRP activators were biotinylated tyramide (PerkinElmer, 1:50), TSATMPlus Fluorescein tyramide (PerkinElmer, 1:100), and Cy-5 tyramide (PerkinElmer, 1:50), respectively. Subsequently, slides were incubated in Alexa 750-conjugated streptavidin for one hour (1:100, Invitrogen). A rabbit polyclonal anti-cytokeratin antibody (Dako, 1:100) and goat anti-rabbit secondary (1:100, Invitrogen) identified tumor epithelium. 4'6-Diamidino-2-phenylindol (DAPI) identified nuclei.

Quantitative Immunofluorescence Using AQUA

Automated quantitative analysis (AQUA[®]) objectively and accurately measures protein expression within the tumor and subcellular compartments, as described previously (30, 31). Fields of view (FOV) were selected that were cytokeratin positive or adjacent to a cytokeratin positive FOV in a previously acquired low resolution image. For each FOV, five monochromatic, high resolution images were captured at wavelengths matching DAPI, FITC, Cy-3, Cy-5, and Cy-7 fluorophores using a PM-2000 image workstation (Genoptix, Carlsbad, CA).

For analysis with AQUA software, staining artifact and FOVs without invasive breast carcinoma were manually removed. A total compartment, consisting of all nuclei, and a tumor mask were generated by dichotomizing DAPI signal and CD3 signal, respectively, so that each pixel was “on” or “off.” The stromal compartment excluded the tumor mask from the total compartment. AQUA scores were calculated by dividing the summated pixel intensity within each compartment by area. Only cases with three or more cytokeratin positive FOVs were included.

Statistical Analysis

A weighted kappa test assessed interobserver variability between pathologist estimates. Pearson's correlation coefficient (R) compared AQUA scores within different compartments. ANOVA testing was used for comparison of AQUA scores to histopathologic assessment and clinicopathologic characteristics and for analysis of response, and log-rank P values are reported. Logistic regression was used for univariate and multivariate analyses, with the CD3, CD8, and CD20 AQUA scores analyzed on a continuous scale. To generate a ratio of the likelihood of pCR for high TIL populations compared to low TIL populations, AQUA scores were split into low and high populations at a cutpoint objectively determined by Joinpoint software (NCI Surveillance Research). All statistical analysis was performed using Statview software (SAS Institute) and QuickCalcs (GraphPad Software).

Results

Histopathologic TIL Assessment

Two pathologists estimated TIL infiltration in the tumor stroma on hematoxylin and eosin stained slides (H&E TILs) for 93 cases (Figure 1A–C). TIL infiltrate was reported in increments of 10% with fair interobserver variability (weighted $\kappa = 0.393$) (Figure 1D). 8

patients (8.6%) exhibited lymphocyte predominant breast cancer (LPBC) with moderate interobserver variability of LPBC vs. non-LPBC ($\kappa = 0.501$). Of the patients with LPBC, 5 (62.5%) achieved pCR. TIL percentages between pathologists were averaged for further analysis, and H&E TILs were significantly higher in the patients who achieved pCR compared to those who did not achieve pCR ($p = 0.0075$, Figure 1E).

Validation of Quantitative Immunofluorescence of CD3, CD8, and CD20

Four tonsil specimens were analyzed for CD3, CD8, and CD20 expression by both quantitative immunofluorescence and flow cytometry. CD3 and CD8 demonstrated membranous staining and primarily extrafollicular localization, whereas CD20 expression was membranous and intrafollicular (Figure 2A). 28 to 292 fields of view (FOVs) were analyzed and percentage lymphocytic infiltrate was calculated for each FOV (Figure 2A) and averaged across the entire tonsil specimen. Flow cytometry on the same specimens demonstrated a preponderance of lymphocytes. CD3 and CD20 positive lymphocyte populations were distinct, but CD3 and CD8 positive populations overlapped (Figure 2B). Lymphocyte percentage ranged from 79.5% to 93.2% by flow cytometry and 64.8% to 82.6% by immunofluorescence (Figure 2C). Lymphocyte percentage was consistently greater by flow cytometry, although within the error margin. By both methods, most lymphocytes were CD20 positive, and CD8 positive lymphocytes were a small fraction of CD3 positive cells (Figure 2D–F).

Objective Assessment of TILs by Quantitative Immunofluorescence on the Neoadjuvant Cohort

Of 93 slides analyzed, 87 had sufficient FOVs for analysis, ranging from 4 to 118 (mean 33.5, median 27). Stromal AQUA scores were averaged across all FOVs for each case. Correlation between AQUA scores within the stromal and total compartments for CD3, CD8, and CD20 was very strong and better than between tumor mask and total compartment AQUA scores, especially for CD20 (Supplemental Figure 2). Index breast tissue microarrays stained alongside the cohort were reproducible on serial sections for all markers (Supplemental Figure 3).

Analysis of each FOV allows visualization of intratumoral heterogeneity. Some FOVs had minimal TILs (Figure 3A), whereas others had moderate TILs with low expression (Figure 3B) or numerous TILs (Figure 3C). Heat maps were constructed to visualize this intratumoral heterogeneity (Figure 3D).

Association of TILs with Clinicopathologic Characteristics

Next, TIL subpopulations were compared with standard breast cancer classifiers including ER, PR, HER2, and triple negative status. CD20 AQUA score was significantly higher ($p = 0.0051$), and CD8 expression trended higher in ER negative patients. CD3 ($p = 0.0301$), CD8 ($p = 0.0168$) and CD20 ($p = 0.0145$) expression were also significantly higher in PR negative tumors. No markers were significantly correlated with HER2, although CD3 stromal expression trended higher with HER2 positivity. Although only 24 patients (27.6%) had tumors negative for ER, PR, and HER2, CD8 ($p = 0.0052$) and CD20 ($p = 0.0058$)

stromal expression were significantly higher in triple negative tumors (Supplemental Figure 4).

Association of TILs with Response to Neoadjuvant Chemotherapy

To dichotomize TILs to assess relationship with response, cutpoints between high and low expression of all four markers were determined by generating three possible Joinpoints (32) and selecting the middle cutpoint (Supplemental Figure 5). Cutpoints for CD3, CD8, and CD20 were above the median AQUA score, and the rate of pCR above the cutpoint was 41.9% for CD3, 35.1% for CD8, and 39.4% for CD20 compared to pCR rates of 16.1%, 18.0%, and 16.7% below these cutpoints, respectively. When analyzed as a continuous variable, increased stromal expression of CD3 ($p = 0.0172$), CD8 ($p = 0.0225$), and CD20 ($p = 0.0004$) were all significantly associated with pCR, and all three markers ($p < 0.0001$) strongly correlated with pathologist assessment of LPBC (Figure 4).

High stromal CD3 ($p = 0.023$, OR: 2.51[1.13–5.57]), CD8 ($p = 0.029$, OR: 2.00[1.08–3.72]), and CD20 ($p = 0.0053$, OR: 1.80[1.19–2.72]) expression also predicted pCR in univariate analysis (Table 2). LPBC had a higher likelihood of response than non-LPBC tumors ($p = 0.043$, OR: 4.77[1.05–21.6]), although the 95% confidence interval overlapped with odds ratios for CD3, CD8, and CD20 scores (Table 2). Multivariable analysis with patient age, tumor size, nodal metastases, ER, PR, and HER2 positivity, and Ki-67 AQUA score from a previous study (29) was conducted for LPBC and CD3, CD8, and CD20 continuous AQUA scores. Only CD20 independently predicted pCR ($p = 0.0186$, OR: 5.368[1.32–21.8]), and high CD20 expressers had 5.5 times the rate of pCR (Table 2). With Ki-67 AQUA score removed, small tumor size, node negative status, HER2 positivity, and increased CD3 ($p = 0.0329$) and CD20 ($p = 0.0064$) expression all significantly predicted pCR. LPBC and high CD8 expression trended with pCR (Data Not Shown).

Receiver operating characteristic curves were generated to assess the sensitivity and specificity of CD3, CD8, and CD20 AQUA score and H&E TILs for predicting pCR. The most sensitive and specific marker was CD20 (AUC 0.685) whereas CD3 (AUC 0.626), CD8 (AUC 0.653), and H&E TILs (AUC 0.672) were somewhat less sensitive and specific (Supplemental Figure 6).

Analysis Stratified by Molecular Subtype

Association of CD3, CD8, and CD20 expression with pCR was stratified by ER, HER2, and triple negative status. High CD8 expression trended with pCR among ER negative patients, and increased CD20 AQUA score was significantly predictive ($p = 0.0015$) among ER positive patients. Higher CD8 ($p = 0.0005$) and CD20 ($p = 0.0021$) expression predicted pCR among HER2 negative cases, whereas higher CD20 expression also predicted pCR ($p = 0.0386$) among HER2 positive patients. Among triple negative patients, increased CD8 expression significantly predicted pCR ($p = 0.0386$), whereas higher CD3 and CD20 expression trended with pCR (Supplemental Figure 7).

Discussion

In an effort to objectively assess TILs we have developed an automated, reproducible method for in situ measurement of lymphocyte infiltrate, quantifying expression of up to three TIL subpopulations within the tumor and adjacent stroma. We validated this method by comparison to flow cytometry on tonsil, and demonstrated reproducibility on breast tissue. For breast tumors before neoadjuvant treatment, high stromal expression of CD3, CD8, and CD20 all predicted pCR in univariate analysis. Moreover, CD20 predicted pCR in multivariate analysis independently of age, tumor size, nuclear grade, nodal metastasis, ER, PR, and HER2 status, and Ki-67 AQUA score. Agreement between this automated objective assay and traditional semi-quantitative pathologist estimates is very good.

This is the first study to significantly correlate CD20 expression with response to neoadjuvant chemotherapy in both univariate and multivariate analysis. Previously, this association has been equivocal. Although one study demonstrated this correlation in univariate analysis (14), another study demonstrated no significant association and that genetic T cell markers but not B cell markers predicted chemotherapeutic response (17). This discrepancy could be attributed to localization of CD20 positive lymphocytes. In this study, CD20 expressing lymphocytes were often clustered in a few FOVs and therefore could be easily missed in tissue microarrays with minimal stroma. In another study, B cell deficient mice were capable of an immune response, whereas T cell deficient mice were not (33). Although that study suggests that B cells are nonessential for an immune response against cancer, B cells may have antitumor effects through several mechanisms, including generation of autoantibodies against the tumor, direct cytotoxicity by granzyme B production, pro-immunogenic cytokine secretion, and antigen presentation to T cells (11). The increased sensitivity and specificity of CD20 compared to CD3 and CD8 in our study supports an important role for B cells in response to chemotherapy. Since our study is relatively small, we look forward to future studies to validate this result.

Flow cytometry is commonly used to quantify and characterize lymphocytes and cytokines in several diseases including cancer (12, 34, 35) and can rapidly analyze up to 18 parameters. However, in solid tumors, it has not seen broad adoption in the clinical setting. One reason for this may be the importance of the architectural location of the infiltrating cells. Whether by traditional methods or our automated method, the spatial relationships are preserved, facilitating assessment of infiltrating cells in the context of the adjacent tumor. We differentiate tumor epithelium from stroma, whereas flow cytometry reveals nothing about stromal or intratumoral localization or about heterogeneity within a lymphocyte population (35). Here, we accurately localized lymphocyte subpopulations in tonsil, with CD20 positive B cells exclusively in the germinal centers and CD3 and CD8 positive T cells primarily in the interfollicular areas. Moreover, the in situ assay does not require a cell suspension, where disperse and collagenase could disrupt cell surface biomarker expression (36, 37). In our quantitative assay, lymphocyte quantification by immunofluorescence-based multiplexing and flow cytometry was concordant. While not perfect, minor differences could be attributable to tissue processing, including formalin fixation. Interestingly, this variability was minimized in samples with more FOVs.

Recently, an international group led by J. Galon proposed an “Immunoscore” that utilizes immune infiltration to develop a quantitative assay with prognostic and predictive power, feasibility, inexpensiveness, robustness, reproducibility, and standardization (19). Their proposed Immunoscore measures CD3 and CD8 infiltration at the central tumor and invasive margin using automated SpotBrowser image software to quantify immune infiltrate, ultimately producing a discrete score from I0 to I4 (24). This effort is notable but is specifically designed for colon cancer. Breast cancer is a unique disease and similar efforts are underway in breast cancer. In contrast, the method proposed here has advantages over traditional methods and the Galon “Immunoscore” in that our assay reports lymphocyte infiltration as continuous data. Moreover, our assay analyzes three TIL biomarkers and addresses the contribution of each lymphocyte subpopulation to prognostic and predictive studies. Future studies will be necessary to assess the generality of our assay including assessment in multi-institutional trials and across quantitative platforms.

This work has a number of limitations. Perhaps most significant is the relatively small size of the cohort and non-uniform patient treatment. Future larger studies are required to validate these results prior to introduction into the clinic. A second and also significant limitation is the fact that this score is dependent on a quantitative immunofluorescent platform. While many institutions have these platforms, they are almost exclusively in the research domain. However, at least two commercial CLIA labs now provide patient data to clinicians based on quantitative immunofluorescence. We are optimistic that the increased reproducibility and the increased objectivity of the data will lead to increased popularity and adoption of these approaches. Another limitation is that TIL quantification is not reported as a traditional percentage, but rather an AQUA score. This score represents intensity divided by the area, thus simulating a concentration. Since our method measures activation markers of T and B cells, concentration may be relatively more representative than percentage. However, further data is required to verify this assertion.

In summary, we have developed an objective immunofluorescence-based assay for detecting and quantifying TILs in situ. This method measures CD3, CD8, and CD20 expression on one slide and reflects the location and heterogeneity of different lymphocyte subpopulations. We successfully validated this assay by comparison to flow cytometry on tonsil tissue and compared it to pathologist TIL estimates. We demonstrated that CD3, CD8, and CD20 predict pCR following neoadjuvant chemotherapy, with CD20 as the most sensitive and specific marker. We believe this method offers potential advantages over existing lymphocyte quantification methods, including objectivity and reproducibility. However, future use at multiple institutions in a range of clinical settings will be required to determine its true value.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of Translational Relevance

Tumor infiltrating lymphocytes (TILs) have previously been identified as prognostic and predictive biomarkers in several cancers, including breast cancer. Despite these findings, assessment of TILs remains challenging, as existing methods are often subjective or semi-quantitative, poorly reproducible, and cannot distinguish TIL subpopulations. Here, we develop and validate an objective immunofluorescence-based quantitative approach for measuring up to three TIL subpopulations per tissue section. The quantitative assay demonstrates improved reproducibility and similar specificity and sensitivity compared to traditional TIL classification. We hope this initial report stimulates further validation of this objective method to determine its clinical utility in breast and other cancers.

Figure 1. Histopathologic Assessment of Tumor Infiltrating Lymphocytes

(A) An example of a case with few TILs, as determined by both pathologists. (B) An example of a case with an intermediate number of TILs (30%) as determined by both pathologists. (C) An example of a case that was determined to be lymphocyte predominant breast cancer (LPBC) by both pathologists. (D) Interobserver variability between the two pathologists was moderate. Darker squares correspond to more cases upon which there was agreement. (E) Comparison between pathologist TIL counts and pathologic complete response (pCR).

Figure 2. Validation of quantitative immunofluorescence assay for measuring lymphocyte infiltration and subpopulations by comparison to flow cytometry on tonsil

(A) In this representative field of view from quantitative immunofluorescence on tonsil tissue, CD3 is recognized in the Cy7 channel (purple, top left), CD8 in the FITC channel (green, top right), and CD20 in the Cy5 channel (red, bottom left). All three markers demonstrate the expected membranous expression. Multiplexing CD3, CD8, and CD20 (bottom right) demonstrates the differential localization between the three markers. (B) In this representative example of flow cytometry on tonsil tissue, lymphocytes, which were most of the cells analyzed (left), were gated into distinct CD3 and CD20 populations (right, top) and overlapping CD3 and CD8 populations (right, bottom). (C) Percentage of cells that were lymphocytes was similar when determined by flow cytometry and quantitative immunofluorescence. Flow cytometry consistently resulted in a greater proportion of lymphocytes, albeit within the margin of error. (D) The percentage of lymphocytes positive for CD3 was similar between the two methods. (E) In all cases, only a small proportion of tonsil lymphocytes were positive for CD8. (F) The majority of lymphocytes were B cells, and percentage of B cells was similar when determined by both methods.

Figure 3. AQUA analysis of TILs in the stroma and demonstration of heterogeneity

Selected images of CD3 staining are taken from different fields of view from the same biopsy. These include (A) a field with minimal CD3 positive TILs, (B) a field with CD3 positive TILs but low intensity staining, and (C) a field with CD3 positive TILs and high intensity staining. (D) A heat map of CD3 Stromal AQUA scores for all fields from this biopsy specimen demonstrates heterogeneity.

Figure 4. Distribution of AQUA scores for TIL biomarkers and comparison to pathologist TIL assessment

For all markers the cutpoint between low expression and high expression determined by Joinpoint software is indicated. Red columns indicate the patients who achieved pathologic complete response, whereas blue columns signify those who did not. The cutpoint for (A) CD3 AQUA score in the stromal compartment is slightly above the median, and (B) correlation with pCR and (C) with pathologist assessment of TILs on H&E slides from the same cohort is excellent. (D) CD8 AQUA score in the stromal compartment demonstrates a similar distribution and is significantly (E) correlated with pCR and (F) correlated with pathologist assessment of LPBC. (G) The distribution of stromal CD20 AQUA score is skewed more negatively than for CD3 and CD8. (H) Nonetheless, this marker is significantly correlated with pCR, and (I) correlation with pathologist TIL estimates is still excellent.

Table 1

Clinical Characteristics of Patients in the Neoadjuvant Cohort

Characteristic	N (Total: 95)	%
Age at Diagnosis		
< 50	56	58.9
50	39	41.1
Treatment		
Adriamycin – Based Therapy	72	75.8
Carboplatin + Paclitaxel + Herceptin	13	13.7
Carboplatin + Abraxane + bevacizumab	6	6.3
Cytosan + Taxotere	2	2.1
Navelbine + Herceptin	1	1.1
Carboplatin + Etoposide + Taxol	1	1.1
Tumor Size		
< 2 cm	10	10.5
2–5 cm	68	71.6
5 cm	16	16.8
Unknown	1	1.1
Nuclear Grade		
Grade 1	3	3.2
Grade 2	46	48.4
Grade 3	43	45.3
Unknown	3	3.2
Nodal Status		
Node Positive	48	50.5
Node Negative	33	34.7
Unknown	14	14.7

Table 2

Univariate and Multivariate Logistic Regression Analysis of the Prediction of Pathological Complete Response by Histopathologic Assessment and AQUA Analysis for Markers of Tumor Infiltrating Lymphocytes

Variable	Univariate		Multivariate LPBC		Multivariate CD3 AQUA Score		Multivariate CD8 AQUA Score		Multivariate CD20 AQUA Score	
	Odds Ratio (95% CI)	P-value	Odds Ratio (95% CI)	P-value	Odds Ratio (95% CI)	P-value	Odds Ratio (95% CI)	P-value	Odds Ratio (95% CI)	P-value
Age										
< 50 (n = 56)			1				1		1	
50 (n = 39)			4.92 (0.666 – 36.4)	0.118	3.340 (0.508–22.0)	0.210	3.053 (0.486–19.2)	0.234	6.322 (0.579–69.0)	0.131
Tumor Size										
< 2 cm (n = 10)			30.837 (1.28–746)	0.035	25.597 (0.805 –814)	0.066	24.527 (0.789 – 762)	0.068	237.504 (1.85 – 30463)	0.027
2–5 cm (n = 68)			1		1		1		1	
5 cm (n = 16)			8.471 (0.340– 211)	0.193	9.429 (0.364 – 245)	0.177	10.436 (0.403 – 270)	0.158	37.342 (0.465 – 2999)	0.106
Nuclear Grade										
Grade 1 – 2 (n = 49)			1		1		1		1	
Grade 3 (n = 43)			0.460 (0.057 – 3.71)	0.466	0.570 (0.068 – 4.79)	0.605	0.786 (0.113 – 5.46)	0.808	0.097 (0.005 – 1.93)	0.126
Nodal Status										
Node Negative (n = 33)			1		1		1		1	
Node Positive (n = 48)			0.244 (0.041 – 1.46)	0.123	0.180 (0.032–1.01)	0.051	0.185 (0.033– 1.04)	0.055	0.209 (0.027– 1.61)	0.133
ER Status										
ER Negative (n = 35)			1		1		1		1	
ER Positive (n = 58)			0.542 (0.034 – 8.77)	0.667	0.548 (0.032 – 9.36)	0.678	0.766 (0.050 – 11.7)	0.848	1.211 (0.061 – 24.0)	0.900
PR Status										
PR Negative (n = 43)			1		1		1		1	
PR Positive (n = 50)			0.373 (0.030 – 4.66)	0.444	0.309 (0.027 – 3.57)	0.347	0.267 (0.024 – 2.93)	0.280	0.100 (0.006 – 1.64)	0.106
HER2 Status										

Variable	Univariate		Multivariate LPBC		Multivariate CD3 AQUA Score		Multivariate CD8 AQUA Score		Multivariate CD20 AQUA Score	
	Odds Ratio (95% CI)	P-value	Odds Ratio (95% CI)	P-value	Odds Ratio (95% CI)	P-value	Odds Ratio (95% CI)	P-value	Odds Ratio (95% CI)	P-value
HER2 Negative (n = 67)			1	0.196	1	0.253	1	0.211	1	0.175
HER2 Positive (n = 27)			3.830 (0.499 – 29.4)		3.388 (0.417 – 27.5)		3.712 (0.475 – 29.0)		6.948 (0.421 – 115)	
Ki-67 AQUA										
Low Ki-67 (n = 41)			1		1		1		1	
High Ki-67 (n = 43)			5.372 (1.089 – 26.5)	0.039	4.706 (0.962 – 23.0)	0.056	4.902 (0.916 – 26.2)	0.063	9.791 (1.35 – 71.1)	0.024
LPBC										
non-LPBC (n = 85)	1	0.043	1	0.983						
LPBC (n = 8)	4.773 (1.05 – 21.6)		N/A							
CD3										
Low CD3 (n = 56)	1	0.023			1	0.401				
High CD3 (n = 31)	2.512 (1.13 – 5.57)				1.821 (0.449 – 7.38)					
CD8										
Low CD8 (n = 50)	1	0.029					1	0.592		
High CD8 (n = 37)	1.999 (1.08 – 3.72)						1.331 (0.468–3.79)			
CD20										
Low CD20 (n = 54)	1	0.005							1	0.019
High CD20 (n = 33)	1.799 (1.19 – 2.72)								5.368 (1.32–21.8)	