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CD8+ lymphocyte infiltration is an independent favorable prognostic indicator in basal-like breast cancer

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Abstract

Introduction

Tumor infiltrating lymphocytes may indicate an immune response to cancer development, but their significance remains controversial in breast cancer. We conducted this study to assess CD8+ (cytotoxic T) lymphocyte infiltration in a large cohort of invasive early stage breast cancers, and to evaluate its prognostic effect in different breast cancer intrinsic subtypes.

Methods

Immunohistochemistry for CD8 staining was performed on tissue microarrays from 3992 breast cancer patients. CD8+ tumor infiltrating lymphocytes were counted as intratumoral when in direct contact with tumor cells, and as stromal in adjacent locations. Kaplan-Meier functions and Cox proportional hazards regression models were applied to examine the associations between tumor infiltrating lymphocytes and breast cancer specific survival.

Results

Among 3403 cases for which immunohistochemical results were obtained, CD8+ tumor infiltrating lymphocytes were identified in an intratumoral pattern in 32% and stromal pattern in 61% of the cases. In the whole cohort, the presence of intratumoral tumor infiltrating lymphocytes was significantly correlated with young age, high grade, estrogen receptor negativity, human epidermal growth factor receptor-2 positivity and core basal intrinsic subtype, and was associated with superior breast cancer specific survival. Multivariate analysis indicated that the favorable prognostic effect of CD8+ tumor infiltrating lymphocytes was significant only in the core basal intrinsic subgroup

(Hazard ratio, HR = 0.35, 95% CI = 0.23-0.54). No association with improved survival was present in those triple negative breast cancers that lack expression of basal markers (HR=0.99, 95% CI = 0.48-2.04) nor in the other intrinsic subtypes.

Conclusions

CD8+ tumor infiltrating lymphocytes are an independent prognostic factor associated with better patient survival in basal-like breast cancer, but not in non-basal triple negative breast cancers nor in other intrinsic molecular subtypes.

Introduction

Immune response may play an important role in cancer progression. Tumor infiltrating lymphocytes (TILs) reflect a local immune response, and could be a key mechanism in controlling tumor progression [1, 2]. A number of studies demonstrate that TILs are associated with clinical outcome in carcinoma and melanoma patients [3-8]. Tumor infiltrating lymphocytes have been found to be mainly T-lymphocytes, with the majority expressing a cytotoxic effector phenotype (CD8+) [9-11]. CD8+ T cell-mediated type 1 immune responses can enhance accumulation of distinct endogenous CD8+ and CD4+ T cells, and facilitate their antitumor function within the tumor micro-environment [12, 13]. Studies in ovarian carcinomas and colon cancer show that high levels of CD8+ lymphocyte infiltration are associated with better prognosis in these diseases [3, 14]. In breast cancer, some studies have reported that inflammation and cytotoxic lymphocyte infiltration are associated with better survival [15-17]. In contrast, other groups have reported that high numbers of tumor infiltrating lymphocytes are related to worse overall

survival [18, 19], whereas still other studies did not find any significant association of TILs with patient outcome [20, 21]. A recent publication reported that a high ratio of CD8+ TILs to FOXP3+ regulatory T cells had a significant relation to improved patient survival in breast cancer [22]. Two other studies have tested larger series: one using a retrospective cohort of 1,334 primary breast cancer patients diagnosed from 1987 to1998 in the United Kingdom showed that total CD8+ TILs were independently associated with better survival in breast cancer [23], while another study with 1,953 breast cancer cases treated in the University Hospital Basel in Switzerland between 1985 and 1996 demonstrated that the independent favorable prognostic effect of total CD8+ TILs was observed only in those with estrogen receptor negative (ER-) tumors (whereas in univariate analyses CD8+ TILs had an unfavourable effect on outcome in ER positive (ER+) breast cancers) [24]. Thus it has remained controversial the extent to which TILs contribute to tumor progression and clinical outcome in breast cancer, possibly because the effect is limited to certain subgroups of patients.

Breast cancer is a heterogeneous disease composed of different intrinsic subtypes, each with distinctive biological and prognostic behavior and response to therapy. Although the introduction of adjuvant systemic therapy has led to a significant reduction in breast cancer mortality, many patients do not benefit. Gene expression studies suggest that predictive indicators should be developed for different breast cancer subtypes [25, 26]. The interaction between immune response, intrinsic subtype and treatment strategy all likely contribute to the outcome of the disease. The development of molecular diagnostic techniques has facilitated better understanding of the heterogeneity of breast cancer and opened the possibility for more personalized

therapy [27, 28]. Hormone receptor and human epidermal growth factor receptor-2 (HER2) molecular status are currently used to guide adjuvant systemic therapy strategies for the luminal and HER2+ intrinsic subgroups, but no targeted therapy for the basal-like subgroup is currently available. Basal-like breast cancer comprises about 15% of all invasive breast cancers and is likely to be high grade, occur in young women and have an aggressive clinical course [29]. Although a majority of basal-like tumors carry a clinical triple negative phenotype (TNP: ER-, PR-, HER2-), they are not synonymous [30], and triple negative breast cancers include many cases which lack expression of basal markers – the so-called “five-marker negative phenotype” (5NP: ER-, PR-, HER2-, EGFR-, CK5/6-) – which have been shown to have significantly better outcomes than core basal cases [31, 32]. Gene expression profiling data suggests that medullary breast tumors (a rare histologic subtype with prominent lymphocytic reaction and good prognosis) are a specific subgroup within the basal-like class, indicating that the overall poor survival of basal-like breast cancer might be mitigated in cases where there is a strong immune response [33-35]. On the other hand, a separate body of research has highlighted that recruitment of chronic inflammatory cells including macrophages can actually promote cancer progression [36]. Different types of immune response in different subtypes of breast cancer might explain apparently contradictory results. However, to date no large immunohistochemistry study has explored the prognostic effect of an immune response in breast cancer stratified by breast cancer intrinsic subtype.

Therefore, there is a clear need for studies with sufficient power for subgroup analysis, employing validated measurements of immune response, to evaluate the

significance of tumor infiltrating lymphocytes in breast tumors. The aim of this study was to examine the prognostic significance of CD8+ TILs in different breast cancer intrinsic subtypes, in a large population-based cohort with long term follow-up. Our hypothesis was that CD8+ lymphocyte infiltration has distinct prognostic effects in different intrinsic molecular subtypes of breast cancer.

Materials and Methods

Study population

The study population includes 3,992 female patients diagnosed with invasive breast cancer between 1986 and 1992 in the province of British Columbia. This cohort was collected from the Breast Cancer Outcomes Unit database maintained by the British Columbia Cancer Agency (BCCA). During the study period, 75% of breast cancer patients in the province were referred to the BCCA; non-referred patients were generally elderly or those without indications for adjuvant therapy [37, 38]. Of the patients referred to the BCCA, approximately 25% had available FFPE blocks with sufficient tumor tissues for tissue microarray (TMA) construction. Thus, the study cohort represents about 20% of all the diagnosed breast cancer patients in the province during the study era. Mean age of the cohort at diagnosis was 58.9 years (23-95 years), with a median follow-up of 12.6 years. Baseline clinical information of the study population includes age at diagnosis; histology; grade; tumor size; number of involved axillary nodes; lymphovascular invasion (LVI); and dates of diagnosis, recurrence, death and cause of death (breast cancer vs. other). As shown in table 1, among the study cases, approximately half (51.1%, 2040/3992) were poorly differentiated tumors (grade 3),

47.3% (1888/3992) had breast tumors over 2 cm, 43.1% (1719/3992) were node positive, and 42.8% (1710/3992) had lymphovascular invasion (LVI). Histological categorization on these cases, including assignment to the medullary subtype, was determined by central review of full sections performed at the time of referral to the BCCA. During the time period of this study cohort, most breast cancer patients were treated according to the provincial guidelines developed by the British Columbia Cancer Agency based on patient age, tumor size, nodal status and LVI. Patients were defined as high risk if their lymph nodes were positive, if there was presence of LVI or if the tumor was both > 2 cm and ER negative at the time of diagnosis. High risk patients were treated with adjuvant systemic therapy (AST) according to their age and menopausal status. Low risk patients were not given any AST. This study and the use of de-identified data were approved by the Clinical Research Ethics Board of the BCCA and University of British Columbia. We were permitted access to the de-identified patient outcome information from the Breast Cancer Outcomes Unit database, maintained by the BCCA. In compliance with the Canadian Tri-Council Policy Statement for ethical research involving human subjects, the requirement for informed consent was waived as this study was limited to anonymous archival specimens.

Tissue microarray and immunohistochemistry

The Vancouver General Hospital's centralized provincial laboratory retained single archival archival blocks for each of the 3992 patients. Using one 0.6 mm core per patient, seventeen tissue microarrays (TMAs) representing these samples were constructed, and immunohistochemistry and scoring for ER, progesterone receptor

(PR), HER2, the Ki67 proliferation marker, epidermal growth factor receptor (EGFR) and cytokeratin 5/6 (CK5/6) was performed as previously described [31, 37, 39-42]. Immunohistochemistry for CD8+ TILs was performed using the antibody against human CD8 (clone C8/144B, Dako Cytomation, dilution 1:100), according to the manufacturer's protocol. Intrinsic breast cancer subtypes were determined by the immunohistochemical expression of ER, PR, HER2, Ki67, EGFR and CK5/6. Luminal A was defined as ER+ or PR+, HER2-, and low Ki67 (<14%); luminal B as ER+ or PR+, and HER2- with high Ki67 (≥14%); luminal/HER2 subgroup as ER+ or PR+, and HER2+; HER2+/ER- as HER2+ with ER- and PR- [42]; and triple negative subgroup (TNP) as ER-, PR-, and HER2-. The core basal subgroup was defined as triple negative with either EGFR+ or CK5/6+, and the five negative phenotype (5NP) as triple negative as well as EGFR- and CK5/6- [31]. The 3992 breast cancer patients were thereby categorized as follows: 38.0% (1518/3992) luminal A, 20.8% (829/3992) luminal B, 5.6% (223/3992) luminal/HER2, 6.3% (250/3992) HER2+/ER-, and 15.8% (630/3992) triple negative, of which 8.3% (330/3992) could be categorized as core basal, 4.1% (162/3992) as 5NP; the remainder had partial or unassignable subtype due to missing or ambiguous biomarker data (Table 1).

CD8+ tumor infiltrating lymphocytes: scoring and quantification

Stained tissue microarray slides were digitally scanned and CD8+ TILs were visually scored by a pathologist who was blinded to the clinical characteristics and outcomes of the patients. Scoring and quantification of CD8+ TILs was carried out as described in a recent study [24]. In brief, intratumoral CD8+ tumor infiltrating

lymphocytes (iTIL) were defined as CD8+ lymphocytes located within tumor cell nests or in direct contact with the breast carcinoma malignant epithelial cells, whereas stromal CD8+ tumor infiltrating lymphocytes (sTIL) were defined as CD8+ lymphocyte in the adjacent peritumoral stroma without direct contact with the carcinoma cells. Total CD8+ tumor infiltrating lymphocytes (tTIL) were measured by combining the counts of iTIL and sTIL for each tissue core. To assess the reproducibility and reliability of the scoring, 490 cases were repeatedly scored by the same pathologist after a period of time (4 weeks), and 200 cases were randomly selected from the whole cohort and iTIL was re-scored by a second pathologist. Pearson correlation analysis was used to check the reliability of the repeated scoring by the same scorer, and intra-class correlation coefficient (ICC) was used to assess the reliability of re-scoring by the two scorers. High correlation coefficients were obtained (Pearson $r \geq 0.94$; ICC = 0.74).

Statistical analysis

The outcome variable in this study was breast cancer specific survival (BCSS). Optimal cutoff points for TIL counts against BCSS were chosen based on recently published findings from an independent series [24], and checked by receiver operating characteristic (ROC) curve analysis using 10-year BCSS as the endpoints, as described in the supplemental method section (Additional file 1, Supplemental method). The optimal cutoff points for iTIL, sTIL and tTIL used in this study was 1, 3 and 2, respectively. To specify, CD8+ iTIL expression was categorized as low when iTIL = 0 (no CD8+ iTIL counted), and high when iTIL ≥ 1 (1 or more CD8+ iTIL in the assessed

tissue core); sTIL low means less than 3 CD8+ sTIL per core, and tTIL low when less than 2 CD8+ tTIL were identified in a core.

Analysis of the association between TILs and clinicopathologic variables was performed using SPSS version 19.0 and R 2.11.1. Because the distributions of the outcome variable (BCSS) were not normal in the study cohort, nonparametric Wilcoxon testing was used to check the bivariate relationship between BCSS and TILs, and other potential confounding variables including age at diagnosis, grade, tumor size, involvement of lymph nodes, lymphovascular invasion, and intrinsic subtypes. Chi-square testing was used to check the relationship between TILs and those potential confounding variables. For survival analysis, the event under the study was death from breast cancer. Breast cancer specific survival time was defined as the number of years between the date of diagnosis of breast cancer and the date of death attributable to breast cancer. Survival time was censored at the time a patient died from another cause, or the follow-up period ended. For univariate survival analyses, the Kaplan-Meier function analysis was performed to estimate probabilities of breast cancer specific survival. Log-rank testing was used to assess differences in BCSS among different subgroups. For multivariate survival analyses, Cox proportional hazards regression models were built to estimate the hazard ratio (HR) of TILs adjusted by the potential confounding variables, based on the partial maximum likelihood estimation. Smoothed, rescaled Schoenfeld residuals plots were performed to test proportional hazards assumptions. Only cases with sufficient information for all covariates were included in the multivariate analysis. Wald statistics were used to test the significance of individual coefficients. Interactions between TILs and some co-variables were checked by building

Cox regression models for different levels of those variables, and comparing hazard ratios of TILs. All the tests were two-sided at a significance level of 0.05.

Supplementary analyses were also performed using relapse-free survival (RFS) as an outcome variable, defined as the number of years between the date of diagnosis of breast cancer and the date of any type of relapse including local, regional and distant relapse of the disease.

Results

CD8+ tumor infiltrating lymphocyte counts and distributions in breast cancer

Among the 3992 breast tumor cases, intact cores bearing infiltrating breast carcinoma sufficient for interpretation of immunohistochemical data for CD8 staining were available from 3403 (85.2%) tumors. Median counts of CD8+ TILs per 0.6 mm tissue microarray core were 0 for iTIL [Interquartile Range (IQR), 0 – 1], 2 for sTIL (IQR, 0 – 10), and 3 for tTIL (IQR, 0 – 12). Of the 3403 interpretable cases, 32.4% had tumor infiltrated with at least one CD8+ iTIL, and 60.6% by at least one CD8+ sTIL (Additional file 2, Figure S1). The distributions of CD8+ iTIL and sTIL were both significantly and positively skewed (Additional file 3, Figure S2). Because analytical results from all types of TIL interpretation were broadly similar, results presented in this paper are primarily based on iTIL analysis, which is the fastest and simplest to perform. As shown in table 1, the presence of iTIL is significantly associated with young age, high grade, medullary histology, ER negativity, HER2 positivity and with the core basal intrinsic subgroup, the category which has the highest prevalence of cases displaying intratumoral lymphocytes.

Prognosis of CD8+ iTIL in breast cancer patients (whole cohort)

To examine the prognosis of CD8+ TILs in the study population, we first applied univariate Kaplan-Meier function survival analysis in the whole cohort. The results did not show a significant difference in breast cancer disease-specific survival between breast cancer patients with iTIL ≥ 1 and iTIL = 0 ($p = 0.761$). Since the distribution of iTIL was associated with patient age at diagnosis, tumor grade, ER and HER2 status, we next assessed the survival functions of iTIL associated with BCSS in groups with different age, tumor grade, ER and HER2 status. Figure 1 showed that, in younger patients (< 50 years) and in those with ER- tumors, cases with iTIL had significantly better BCSS than those without. Reversed associations were observed in patients with age ≥ 50 years or ER+. No significant associations were detected in cases stratified by grade (grade1+ 2 vs. grade 3), or HER2 status (HER2+ vs. HER2-). These results indicated that age and ER status could have an interaction with the association between iTIL and patient survival in breast cancer.

We built Cox proportional hazards regression models to estimate the hazard ratio for iTIL. Smoothed, rescaled Schoenfeld residuals plots showed that iTIL and most other covariables satisfied the proportional hazards assumptions well during the period of follow-up. Only iTIL in the luminal A subgroup varied slightly during longer follow-up.

Results from the univariate Cox regression model analysis showed that iTIL was not a significant prognostic factor associated with BCSS in the cohort as a whole (HR=1.02, 95% CI = 0.89, 1.17). To take into consideration potential confounders, a multivariate Cox regression model was built to assess the association between iTIL and BCSS, including the covariates of age at diagnosis, tumor grade and size, lymph node

status, lymphovascular invasion, and intrinsic subtype. Table 2 showed that the adjusted HR of iTIL was 0.79 (95% CI = 0.68, 0.91) meaning that, in the whole cohort, the probability of BCSS among iTIL≥ 1 patients was 21% (1-0.79) higher than among those with iTIL= 0, adjusted for age, grade, tumor size, lymph node status, lymphovascular invasion, and intrinsic subtypes. Besides iTIL, tumor grade and size, nodal status, lymphovascular invasion, and intrinsic subtype each had significant effects on BCSS. To examine the effect of interaction between age, ER status, and iTIL, we built multivariate Cox regression models for iTIL at different levels of age and ER status. These analyses showed that the adjusted HRs for iTIL were 0.65 (95% CI = 0.51, 0.84) for those age < 50 and 0.89 (95% CI = 0.74, 1.06) for those ≥ 50 years; for ER- tumors the adjusted HRs was 0.61 (95% CI = 0.47, 0.77), whereas it was 0.91 (95% CI = 0.77, 1.11) for those with ER+ tumors. Therefore, interactions between iTIL and age and ER status might modify the effect size for iTIL in the unstratified whole cohort of breast cancer patients.

Association of CD8+ iTIL with breast cancer specific survival in different breast cancer intrinsic subgroups

We further assessed the association of CD8+ TILs with patient survival in different breast cancer intrinsic subtypes, first using univariate Kaplan-Meier function survival analysis. No difference in BCSS was detected between those with iTIL ≥ 1 and iTIL = 0 within the luminal A and luminal B subgroups (Figure 2A and 2B). Although we observed an apparent difference between the two groups among HER2+/ER- cases, this was not statistically significant (p = 0.064, Figure 2C). However, as shown in Figure 2D, a large and significant difference in BCSS was found between iTIL ≥ 1 and iTIL = 0

cases among triple negative breast cancers. By stratifying triple negatives into core basal and 5NP subgroups, we observed a much larger difference in BCSS between iTIL ≥ 1 and iTIL = 0 cases in the core basal intrinsic subgroup. Patients with iTIL ≥ 1 basal-like tumors had significantly better survival than those with iTIL = 0 (mean survival time, 14.5 years vs. 11.0 years, $p < 0.001$, Figure 2E). No such association was found among triple negative, non-basal (5NP) cases (Figure 2F). We also performed survival analysis in all ER- patients excluding the core basal cases, and found no significant difference in BCSS between iTIL ≥ 1 and iTIL = 0 cases ($p = 0.434$).

To confirm the association between iTIL and BCSS and to assess the independent prognostic effect size in different breast cancer intrinsic subgroups, multivariate Cox proportional hazards regression models were built to estimate the hazard ratios of iTIL, adjusted by the potential confounders. Results in table 3 showed that the hazard ratios of iTIL were not significant in the luminal A, luminal B, and HER2+/ER- intrinsic subgroups. However, iTIL was demonstrated to be a significantly independent favorable factor for BCSS in triple negative cases, because of a strong effect in the core basal subgroup (Table 4). Among core basal cases, the presence of any intratumoral CD8+ lymphocytes (iTIL≥ 1) was associated with a 65% higher probability of BCSS than among those tumors lacking intratumoral CD8+ lymphocytes (iTIL= 0), statistically significant even after adjusting for age at diagnosis, grade, tumor size, lymph node status, and lymphovascular invasion. Considering that medullary breast carcinoma, a histologically-evident subtype known to carry a good prognosis, usually has a core basal immunophenotype and could be responsible for some of the observed effect, we repeated the multivariate Cox regression analysis for core basal

cases by excluding those with medullary carcinoma (27 cases). The results still showed a similar and significant hazard ratio (HR = 0.38, 95% CI = 0.24, 0.59), which therefore could not be attributed to medullary histology. In contrast, the multivariate analysis did not show any association between iTIL and BCSS in 5NP subgroup (i.e. triple negative breast cancers which do not express basal markers). These results demonstrated that the prognostic effect of iTIL was significantly different in these two subgroups of triple negative cases, indicating that the association of iTIL with breast cancer specific survival primarily exists only in the core basal intrinsic subgroup.

Association of CD8+ sTIL and tTIL with clinical outcome

To confirm the prognostic value of CD8+ TILs in breast cancer, we also evaluated the distributions of stromal tumor infiltrating lymphocytes (sTIL) and total tumor infiltrating lymphocytes (tTIL) in relation to patient and tumor characteristics, and the associations of sTIL and tTIL with survival. Similar results were obtained as those from the analysis with iTIL. In brief, high expressions of sTIL and tTIL were significantly correlated with young age, high grade, larger tumor size, medullary histology, ER negativity, HER2 positivity and with the core basal phenotype (Additional file 4, Table S1), and again were significantly associated with better breast cancer specific survival only in the core basal intrinsic subgroup (Additional file 5, Figure S3; Additional file 4, Table S2-S3).

Discussion

The prognostic significance of tumor infiltrating lymphocytes in breast cancer has been debated, but no consistent conclusion has yet been drawn. We implemented this study, using a particularly large, well-annotated cohort comprising nearly four thousand patients, in an attempt to definitively assess the clinical implication of TILs in breast cancer. In addition to addressing the question of whether immune response (as measured by CD8+ tumor infiltrating lymphocytes) has a prognostic role in breast cancer in general, we also examined the effect of TILs in the major breast cancer intrinsic biological subtypes. To our knowledge, this is the first study sufficiently powered for multivariate analysis to investigate the association of CD8+ TILs with patient survival within the breast cancer intrinsic subtypes. Our results demonstrate that the presence of iTILs is independently associated with a significantly superior outcome in women diagnosed with core basal tumors. Although the presence of CD8+ iTILs is also an independent prognostic indicator for improved patient survival in triple-negative breast cancers, this favorable prognostic effect cannot be detected among those lacking expression of basal biomarkers (5NP). In the core basal subgroup, patients having tumors with CD8+ iTIL survived, on average, 3.5 years longer than did patients with basal tumors lacking evidence of a CD8+ iTIL immune response.

Breast cancer is both clinically and molecularly heterogeneous, in practice stratified by hormonal receptors (ER and PR), by HER2 status, and increasingly also by expression of other biomarkers such as Ki67 or by gene expression profiling methodologies. Dissecting the heterogeneity of breast cancer is critically important for understanding the underlying mechanisms of the disease, and for identifying subpopulations which are most likely to respond to particular therapies [43]. In general,

ER- breast cancers have worse prognosis than those that are ER+, but not all ER- breast cancer patients have poor survival. Teschendorf et al applied an integrative analysis of three gene expression datasets to assess the prognostic value of molecular signatures, and found that most prognostic markers of better prognosis in ER- breast cancer are associated with activation of immune response pathways [44]. Furthermore, a seven-gene immune response classifier was constructed and showed significant good prognostic value in ER- patients [45]. Meta-analytic studies of clinical and gene expression data have demonstrated that Immune response is significantly associated with prognosis in breast cancer [46], primarily in rapidly-proliferating [47] and ER- subgroups [48, 49]. Results from some studies indicate that TILs could be a protective factor reducing the likelihood of distant metastasis in patients with triple-negative breast tumors [50] and among those with medullary carcinoma [17]. Moreover, two recently published gene expression profiling studies demonstrated that effective immune (particularly cytotoxic T-cell) response plays favorable prognostic role in basal breast cancer subgroups [51, 52]. In our study, the multivariate analysis clearly demonstrates that the presence of CD8+ iTIL has different prognostic value in breast cancer with different intrinsic biological subtypes. Even among the triple negative cases, immune response has different meanings in core basal versus “five negative” phenotypes. Evidence from previous studies has shown that core basal-like tumors are associated with a poorer prognosis and appear biologically different from 5NP tumors [31, 32]. Our results suggest that local immune response characterized by CD8+ lymphocytes infiltration might be considered an important factor differentiating the core basal from 5NP breast tumors within the class of triple negative breast cancers.

Tumor infiltrating lymphocytes and macrophages are thought to be molecular determinants of clinical outcome in breast cancer. Although cytotoxic T lymphocytes and natural killer cells have been found to have antitumor activity, some lymphocytes such as B cells exhibit bipolar roles in breast cancer development. Distinct cell-mediated immune responses also play antagonistic roles in disease prognosis. T-helper cell-1 (Th1) mediated immune response pathways are considered to have inhibitory effect; whereas T-helper cell-2 (Th2) immune response pathways may promote development and metastasis of breast cancer. It has been found that CD4+ T lymphocytes can promote metastasis by activating EGFR signaling pathway in a Th2-type tumor microenvironment [53]. Identification of interactions between immune response and other molecular pathways may define novel prognostic subtypes. In ER-beast cancer, those characterized with high expression of EGFR and low expression of Th1 mediated pathway related markers such as IL12 and IFNG were found to have poor prognosis [54]. TILs in the tumor microenvironment are predominantly CD8+ T cells [55, 56], which are considered to be the effector cells in Th1 anti-tumor immune responses. CD8+ T cells produce interferon gamma through interaction with tumor related antigens, potential leading to tumoricidal activity by induction of apoptosis and/or macrophage tumor killing activity [57]. Studies indicate that tumor-specific or even non-cancer-specific antigens such as p53 and β-actin are common targets of cytotoxic T-lymphocytes and can induce immunological and clinical effect in breast cancer patients [58-60]. Findings from our study suggest that core basal-like breast cancer is more immunogenic than other intrinsic subgroups, as measured by CD8+ T cell infiltration. Tumors of this subtype have high expression of basal markers, some of which (such as

EGFR) may interact with T-cell medicated immune response to affect clinical outcome in breast cancer. We would suggest a hypothesis that certain “basal proteins”, expressed on the cell surface can be recognized as tumor antigens, and the consequent induction of adaptive basal marker specific immunity can enhance the local Th1 mediated anti-tumor immune response in these breast cancers. The absence of these surface markers in 5NP breast cancers could underpin the observed difference in prognostic significance of TILs in core basal compared with 5NP breast cancers.

Recent studies have suggested that a pre-existing immune response can strengthen the effect of conventional chemotherapy [61, 62], enhancing destruction of tumor cells [63], and this favorable effect could become stronger in patients with highly immunogenic tumors, perhaps including the core basal group. Basal-like breast cancers have distinctive survival patterns, with many relapses and deaths during the first 5 years after diagnosis, but fewer events after this period [32], indicating that basal-like breast cancers encompass both poor and good prognostic subgroups responding variably to conventional therapies. In our cohort, systemic treatment decisions were not randomized, making outcomes stratified by treatment difficult to interpret; nevertheless, an exploratory analysis suggests that pre-treatment CD8+ lymphocyte infiltration is an independent favorable predictive indicator of good outcomes in basal-like cases treated with chemotherapy (HR = 0.29, 95% CI = 0.16-0.55, $p < 0.001$, n = 107; Additional file 4, Table S4). Our results indicate that efforts toward developing immuno-stimulative therapies might be best directed to the core basal group. The recognition of tumor-associated antigens by CD8+ cells is a significant contributor to the detection and ultimate destruction of tumor cells [64]. Basal-like breast cancer could be particularly

suitable for targeted immunotherapy. The lack of success of prior attempts at immunotherapy for breast cancer may be attributable in part to the lack of focus on appropriate breast cancer subtypes. A better understanding of the interaction between immune response, intrinsic subtype, adjuvant systemic therapy and patient outcome is critical to more effective and targeted clinical management for breast cancer patients, especially those with basal-like breast tumors.

Studies on TILs in breast cancer have come to inconsistent conclusions. We believe that one of the underlying reasons could be inconsistency in defining and measuring tumor infiltrating lymphocytes. Some research considered only the presence of peritumoral stromal lymphocytes [65, 66], and many considered all T lymphocytes (which might include larger numbers of regulatory T-cells that could in some cases reflect immune suppression instead of activation). In our study, specific immunohistochemistry was used with a mouse monoclonal anti-human CD8 antibody to detect cytotoxic effector CD8+ TILs in intratumoral and stromal locations for each tumor tissue core. We evaluated the reliability of repeated scoring by the same scorer and between different scorers, and it was demonstrated that our visual CD8+ TIL scoring was highly reliable (Additional file 6, Figure S4). Analyses with intratumoral, stromal and total CD8+ TIL were conducted and consistent results obtained. We also did analyses using relapse-free survival as an outcome and obtained similar results with that using breast cancer specific survival as the outcome (Additional file 7 and 8, Figure S5-S6; Additional file 4, Table S5-S7). We are thus confident that the identification and quantification of TILs, and the assessment of the association of TILs with clinical outcome in breast cancer are reliable and valid in this study. One potential limitation of

our methods is that TMAs may not adequately represent breast tumor heterogeneity. Several studies nevertheless have shown that findings from TMAs are consistent with those from full-face tissue sections [67, 68]. Although we observed a trend to a favorable prognostic effect of CD8 TILs in the HER2+/ER- subgroup, which is consistent with a gene expression study [69], the effect was not statistically significant in our univariate or multivariate analyses. Research with more power particularly for this subgroup needs to be done to draw more definitive conclusions among HER2 positive cases. We were not able to measure changes in immune response induced by chemotherapy, as all the tissue samples were collected before patients received systemic therapy. Further studies would need to be conducted to assess the interaction of TILs with chemotherapy, ideally in randomized trials.

Conclusions

This study provides strong evidence that CD8+ lymphocyte infiltration is an independent factor associated with improved survival in breast cancer patients. The favorable prognostic effects of TILs mostly occur in the basal-like intrinsic subgroup.

Abbreviations

5NP: five negative phenotype; AJCC: American Joint Committee on Cancer; AST: adjuvant systemic therapy; BCSS: breast cancer specific survival; CBP: core basal phenotype; CK: cytokeratin; EGFR: epidermal growth factor receptor; ER: estrogen receptor; HER2: human epidermal growth factor receptor-2; HR: hazard ratio; iTIL: intratumoral tumor infiltrating lymphocytes; PR: progesterone receptor; RFS: relapse-

free survival; sTIL: stromal tumor infiltrating lymphocyte; TILs: tumor infiltrating lymphocytes; TNP: triple negative phenotype.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SLi coordinated the study, analyzed data and drafted the manuscript. JL advised on scoring and edited the paper. SLe assisted with statistical analyses. DG generated primary data. WDF provided the idea for the study, helped with data analysis and edited the paper. TON organized the study, directed data generation and analysis, and edited the paper. All authors read and approved the final manuscript.

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Figure Legends

Figure 1. Breast cancer specific survival (BCSS) by iTIL among groups with different age and ER status. (A) age < 50 year, (B) age ≥ 50 year; (C) ER-, and (D) ER+.

Figure 2. Breast cancer specific survival (BCSS) by iTIL in different breast cancer intrinsic subgroups. (A) luminal A, (B) luminal B, (C) HER2+/ER-, (D) triple negative (TNP), (E) core basal (CBP), and (F) five negative (5NP) subgroups.

Tables

Table 1. Clinico-pathologic characteristics and distribution of CD8+ intratumoral lymphocytes (iTIL) in the study population

Characteristics	No. patients (%)	iTILs (≥1)	
		Prevalence %	p-value
Age at diagnosis (year)			**<0.001**
< 40	294 (7.4)	38.9 (98/252)	
40-49	844 (21.1)	37.5 (273/728)	
50-65	1,425 (35.7)	31.3 (377/1203)	
> 65	1,429 (35.8)	29.2 (356/1220)	
Grade			**<0.001**
1 (well differentiated)	209 (5.2)	24.4 (40/164)	
2 (moderately well or partially differentiated)	1,563 (39.2)	26.9 (361/1342)	
3 (poorly differentiated)	2,040 (51.1)	37.2 (652/1754)	
Unknown	180 (4.5)		
Tumor size (cm)			**0.076**
≤ 2	2,078 (52.1)	30.5 (540/1768)	
> 2-5	1,667 (41.8)	34.1 (494/1449)	
> 5	221 (5.5)	34.9 (59/169)	
Unknown	26 (0.6)		
Nodal status			**0.051**
Negative	2,265 (56.7)	31.0 (593/1911)	
Positive	1,719 (43.1)	34.3 (509/1484)	
Unknown	8 (0.2)		
Lymphovascular invasion (LVI)			**0.638**
Negative	2,106 (52.8)	32.5 (576/1770)	
Positive	1,710 (42.8)	31.8 (474/1492)	
Unknown	176 (4.4)		
Histology			**<0.001**
Medullary	66 (1.7)	78.4 (40/51)	
Not Medullary	3926 (98.3)	31.7 (1064/3352)	
AJCC stage			**0.004**
I	1,393 (34.9)	28.8 (337/1172)	
II	2,255 (56.5)	34.6 (677/1959)	
III	317 (7.9)	32.9 (83/252)	
Unknown/missing	27 (0.7)		
Adjuvant systemic therapy (AST)			**0.012**
No AST	1,676 (42.0)	21.2 (302/1427)	
Tamoxifen only	1,276 (32.0)	18.6 (206/1105)	
Chemotherapy only	727 (18.2)	27.2 (169/622)	
Tamoxifen + chemotherapy	297 (7.4)	29.4 (73/148)	
Other	16 (0.4)	21.4 (3/14)	
ER			**<0.001**
Negative	1,200 (30.1)	39.9 (370/927)	
Positive (≥1% nuclei stained)	2,761 (69.1)	29.6 (728/2456)	
Uninterpretable/missing	31 (0.8)		
HER2			**<0.001**
Negative	3,316 (83.1)	31.3 (907/2902)	
Positive	498 (12.5)	39.6 (176/444)	
Uninterpretable/missing	178 (4.4)		
Subtype			**<0.001**
Luminal A	1,518 (38.0)	25.4 (353/1392)	
Luminal B	829 (20.8)	36.9 (285/773)	
Luminal/HER2	224 (5.6)	39.8 (82/206)	

Luminal not further assigned	244 (6.1)	21.5 (37/172)
HER2+/ER-	250 (6.3)	39.6 (90/227)
TNP	630 (15.8)	42.2 (226/535)
Core basal	330 (8.3)	49.2 (151/307)
5NP	162 (4.1)	35.2 (50/142)
TNP not assignable	138 (3.4)	29.1 (25/86)
Unassignable	297 (7.4)	31.6 (31/98)
Total	**3,992 (100)**	**32.4 (1104/3403)**

Table 2. Hazards for breast cancer specific survival in the whole cohort with univariate and multivariate analysis

Variable	Univariate analysis		Multivariate analysis n=3144	
	HR (95% CI)	**p**	**HR (95% CI)**	**p**
Age				
≥ 50 vs. < 50	0.85 (0.75 - 0.96)	0.011	1.01 (0.88 - 1.16)	0.884
Grade				
3 vs. (1 and 2)	2.12 (1.87 - 2.41)	< 0.001	1.57 (1.35 - 1.82)	< 0.001
Tumor size (cm)				
> 2 vs. ≤ 2 cm	2.17 (1.92 - 2.45)	< 0.001	1.59 (1.36 - 1.83)	< 0.001
Nodal Status				
Positive vs. negative	2.79 (2.48 - 3.15)	< 0.001	2.05 (1.76 - 2.39)	< 0.001
LVI				
Positive vs. negative	2.25 (1.99 - 2.54)	< 0.001	1.29 (1.10 - 1.51)	0.001
Subtype				
Luminal B vs. luminal A	2.08 (1.78 - 2.45)	< 0.001	1.75 (1.46 - 2.09)	< 0.001
HER2+/ER- vs. luminal A	2.98 (2.40 - 3.70)	< 0.001	2.51 (2.99 - 3.19)	< 0.001
Core basal vs. luminal A	2.30 (1.87 - 2.84)	< 0.001	2.02 (1.58 - 2.58)	< 0.001
5NP vs. luminal A	1.65 (1.30 - 2.10)	0.002	1.49 (1.12 - 1.97)	0.011
iTIL				
≥ 1 vs. 0	1.02 (0.89 - 1.17)	0.761	0.79 (0.68 - 0.91)	< 0.001

Table 3. Hazards for breast cancer specific survival with multivariate analysis in the luminal A, luminal B, and HER2+/ER- intrinsic subgroup

Variable	LumA (n = 1276)		LumB (n = 709)		HER2+/ER- (n = 216)	
	HR	**p**	**HR**	**p**	**HR**	**p**
Age	1.38		1.04		1.13	
≥ 50 vs. < 50	(1.02 - 1.86)	0.037	(0.81 - 1.35)	0.750	(0.75-1.70)	0.564
Grade	1.75		1.28		2.13	
3 vs. (1 and 2)	(1.36 - 2.25)	<0.001	(0.99 - 1.67)	0.062	(1.21 - 3.76)	0.009
Tumor size	1.64		1.49		1.73	
> 2 cm vs. ≤ 2 cm	(1.28 - 2.11)	<0.001	(1.14 - 1.95)	0.004	(1.11 - 2.68)	0.015
Nodal Status	2.20		1.75		1.75	
Positive vs. negative	(1.65 - 2.95)	<0.001	(1.31 - 2.32)	<0.001	(1.07 - 2.83)	0.025
LVI	1.12		1.33		1.36	
Positive vs. negative	(0.84 - 1.49)	0.444	(0.99 - 1.77)	0.056	(0.84 - 2.18)	0.211
iTIL	1.14		0.85		0.76	
≥ 1 vs. 0	(0.86 - 1.50)	0.357	(0.66 - 1.11)	0.235	(0.50 - 1.15)	0.194

Table 4. Hazards for breast cancer specific survival with multivariate analysis in TNP, core basal and 5NP groups

Variable	TNP (n = 496)		core basal (n = 287)		5NP (n = 130)	
	HR	p	HR	p	HR	p
Age	0.90		0.91		1.07	
≥ 50 vs. < 50	(0.66 - 1.22)	0.488	(0.62 - 1.35)	0.648	(0.54 - 2.14)	0.830
Grade	1.74		1.54		1.81	
3 vs. (1 and 2)	(1.11 - 2.70)	0.015	(0.80 - 2.97)	0.201	(0.74 - 4.41)	0.191
Tumor size	1.66		1.85		1.49	
> 2 cm vs. ≤ 2 cm	(1.19 - 2.30)	0.003	(1.23 - 2.79)	0.003	(0.71 - 3.12)	0.293
Nodal Status	2.00		2.16		1.58	
Positive vs. negative	(1.42 - 2.83)	<0.001	(1.39 - 3.35)	0.001	(0.73 - 3.42)	0.244
LVI	1.55		1.52		3.13	
Positive vs. negative	(1.08 - 2.21)	0.017	(0.97 - 2.36)	0.065	(1.27 - 7.77)	0.013
iTIL	0.48		0.35		0.99	
≥ 1 vs. 0	(0.34 - 0.67)	<0.001	(0.23 - 0.54)	<0.001	(0.48 - 2.04)	0.986

Additional files

Additional file 1: Supplemental method.pdf

Validation of the cutoff points of TILs

The Supplemental method section explained how the receiver operating characteristic (ROC) analysis was used to validate the optimal cutoffs of TILs chosen from an independent study. To take into consideration that outcome variable, breast cancer specific survival, is a time to event endpoint, X-tile software was also used to validate the optimal cut-offs, and the same cutoff points of iTIL and sTIL were obtained as those from the ROC method.

Additional file 2: Figure S1_image of CD8.pdf

CD8+ TILs in breast cancer

This image showed some examples of CD8+ iTIL and sTIL in a breast tumor sample (scale bar: 50 μm). Information with respect to availability of all of our CD8 staining images were provided in the figure legend.

Additional file 3: Figure S2_distribution of TILs.pdf

Distributions of CD8+ iTIL and sTIL in the whole cohort

Histograms were used to show the distributions of CD8+ iTIL and sTIL in the whole study population. Values on the X-axis represent absolute counts of CD8+ iTIL (A) or sTIL (B) per tissue microarry core.

Additional file 4: Supplemental tables.pdf

Supplemental tables

Table S1 showed the distributions of CD8+ sTIL and tTIL in relation to patient and tumor characteristics. Table S2 showed the hazard ratios (HRs) of sTIL and tTIL in the whole cohort with multivariate Cox regression analysis, adjusted by age at diagnosis, tumor grade and size, lymph node status, lymphovascular invasion, and intrinsic subtype. Table S3 showed the HRs of sTIL and tTIL in triple negative (TNP), core basal (CBP), and five negative (5NP) breast cancer intrinsic subgroups in multivariate analysis. Table S4 showed the HRs of iTIL, sTIL and tTIL in patients without adjuvant systemic therapy (AST) and with chemotherapy in multivariate analysis. Table S5 showed HRs of iTIL in the whole cohort with univariate and mulvariate analysis, using relapse-free survival (RFS) as the outcome variable. Tables S6 and S7 showed the HRs of iTIL in different intrinsic subgroups with multivariate Cox regression analysis using RFS as the outcome variable.

Additional file 5: Figure S3_sTIL and tTIL.pdf

Breast cancer specific survival (BCSS) by sTIL and tTIL in different breast cancer intrinsic subgroups

Kaplan-Meier function survival analysis of association of TILs with BCSS: (A) sTIL in triple negative (TNP), (B) tTIL in TNP, (C) sTIL in core basal (CBP), (D) tTIL in CBP, (E) sTIL in five negative (5NP), and (F) tTIL in 5NP.

Additional file 6: Figure S4_scatter plot of rescoring.pdf

Correlation of re-scoring of CD8+ TILs by the same and different pathologists

The scatter plots demonstrated correlations of repeated scoring for 490 cases by the same pathologist for CD8+ iTIL (A) and sTIL (B), and re-scoring of CD8+ iTIL for 200 cases by two pathologists (C).

Additional file 7: Figure S5_RFS_age and ER.pdf

Relapse-free survival (RFS) by iTIL among groups with different age and ER status

Kaplan-Meier function survival analysis of association between iTIL and RFS in: (A) age < 50 year, (B) age ≥ 50 year; (C) ER-, and (D) ER+.

Additional file 8: Figure S6_RFS_subtype.pdf

Relapse-free survival (RFS) by iTIL in different breast cancer intrinsic subgroups

Kaplan-Meier function survival analysis of association between iTIL and RFS in: (A) luminal A, (B) luminal B, (C) HER2+/ER-, (D) Triple negative, (E) core basal, and (F) five negative subgroups.

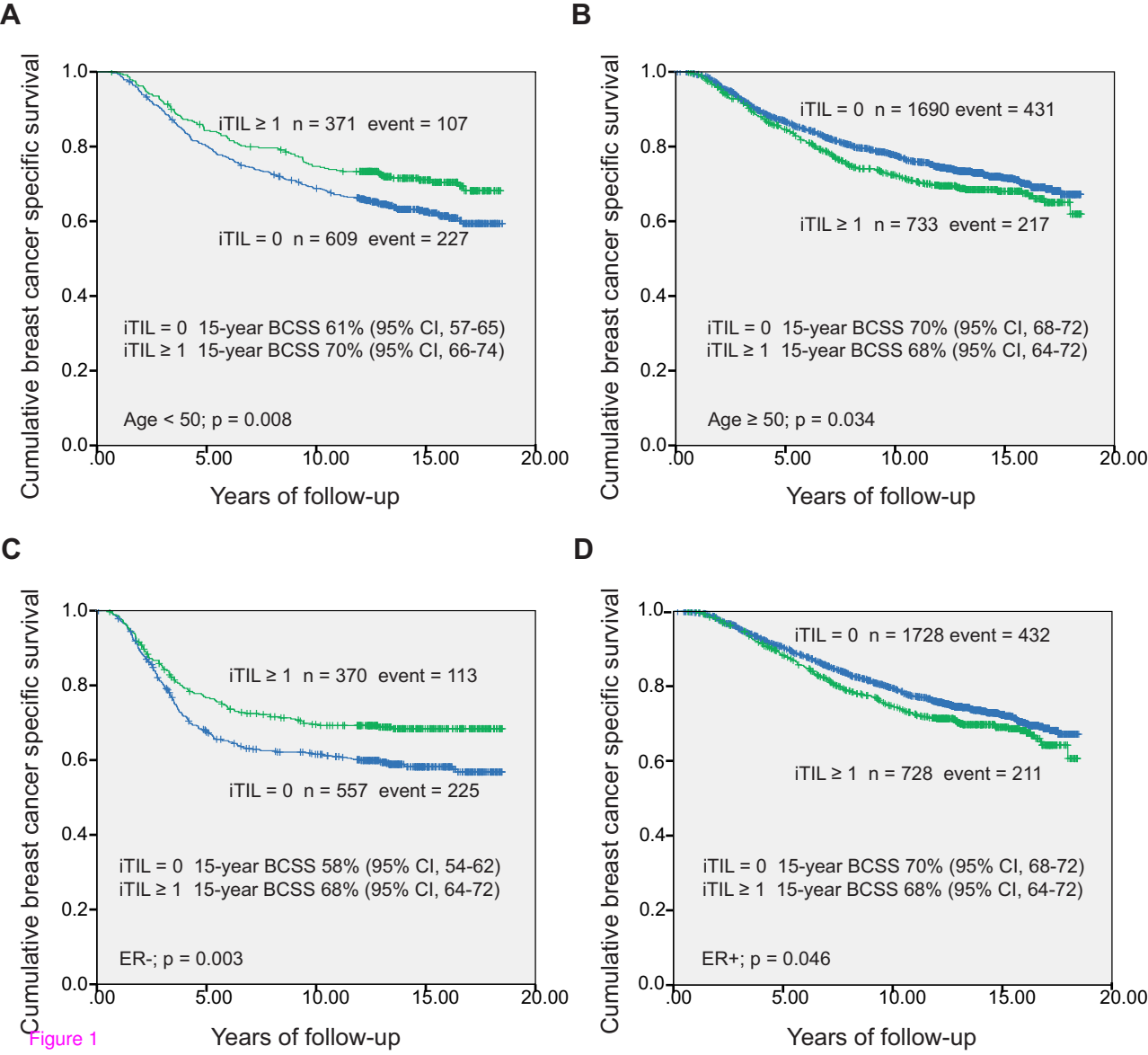
A
Cumulative breast cancer specific survival
1.0
0.8
0.6
0.4
0.2
0.0
iTIL ≥ 1 n = 371 event = 107
iTIL = 0 n = 609 event = 227
iTIL = 0 15-year BCSS 61% (95% CI, 57-65)
iTIL ≥ 1 15-year BCSS 70% (95% CI, 66-74)
Age < 50; p = 0.008
.00
5.00
10.00
15.00
20.00
Years of follow-up
B
Cumulative breast cancer specific survival
iTIL = 0 n = 1690 event = 431
iTIL ≥ 1 n = 733 event = 217
iTIL = 0 15-year BCSS 70% (95% CI, 68-72)
iTIL ≥ 1 15-year BCSS 68% (95% CI, 64-72)
Age ≥ 50; p = 0.034
Years of follow-up
C
Cumulative breast cancer specific survival
iTIL ≥ 1 n = 370 event = 113
iTIL = 0 n = 557 event = 225
iTIL = 0 15-year BCSS 58% (95% CI, 54-62)
iTIL ≥ 1 15-year BCSS 68% (95% CI, 64-72)
ER-; p = 0.003
Years of follow-up
D
Cumulative breast cancer specific survival
iTIL = 0 n = 1728 event = 432
iTIL ≥ 1 n = 728 event = 211
iTIL = 0 15-year BCSS 70% (95% CI, 68-72)
iTIL ≥ 1 15-year BCSS 68% (95% CI, 64-72)
ER+; p = 0.046
Years of follow-up

Figure 1

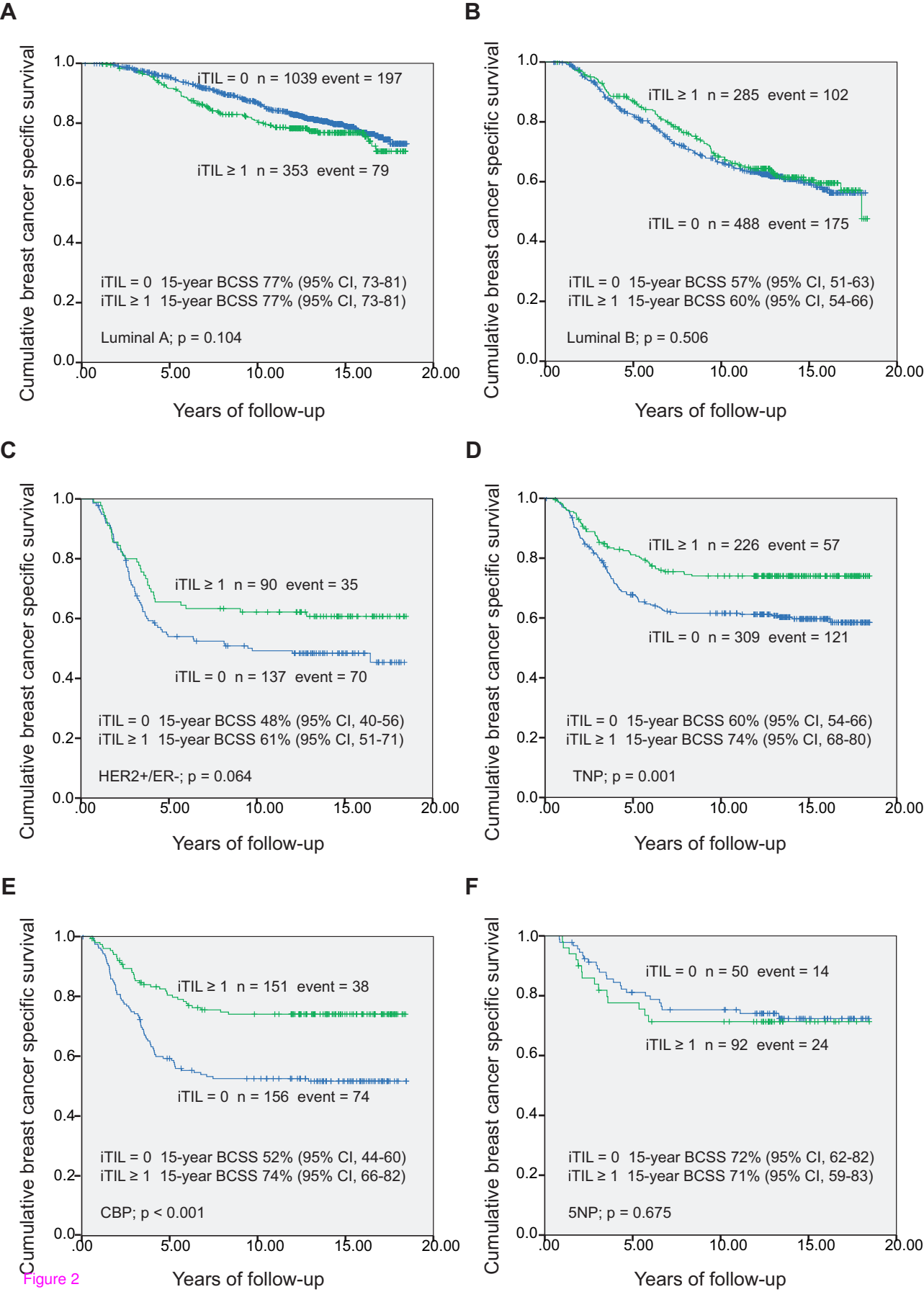
A
Cumulative breast cancer specific survival
1.0
0.8
0.6
0.4
0.2
0.0
iTIL = 0 n = 1039 event = 197
iTIL ≥ 1 n = 353 event = 79
iTIL = 0 15-year BCSS 77% (95% CI, 73-81)
iTIL ≥ 1 15-year BCSS 77% (95% CI, 73-81)
Luminal A; p = 0.104
.00
5.00
10.00
15.00
20.00
Years of follow-up
B
Cumulative breast cancer specific survival
iTIL ≥ 1 n = 285 event = 102
iTIL = 0 n = 488 event = 175
iTIL = 0 15-year BCSS 57% (95% CI, 51-63)
iTIL ≥ 1 15-year BCSS 60% (95% CI, 54-66)
Luminal B; p = 0.506
Years of follow-up
C
Cumulative breast cancer specific survival
iTIL ≥ 1 n = 90 event = 35
iTIL = 0 n = 137 event = 70
iTIL = 0 15-year BCSS 48% (95% CI, 40-56)
iTIL ≥ 1 15-year BCSS 61% (95% CI, 51-71)
HER2+/ER-; p = 0.064
Years of follow-up
D
Cumulative breast cancer specific survival
iTIL ≥ 1 n = 226 event = 57
iTIL = 0 n = 309 event = 121
iTIL = 0 15-year BCSS 60% (95% CI, 54-66)
iTIL ≥ 1 15-year BCSS 74% (95% CI, 68-80)
TNP; p = 0.001
Years of follow-up
E
Cumulative breast cancer specific survival
iTIL ≥ 1 n = 151 event = 38
iTIL = 0 n = 156 event = 74
iTIL = 0 15-year BCSS 52% (95% CI, 44-60)
iTIL ≥ 1 15-year BCSS 74% (95% CI, 66-82)
CBP; p < 0.001
Years of follow-up
F
Cumulative breast cancer specific survival
iTIL = 0 n = 50 event = 14
iTIL ≥ 1 n = 92 event = 24
iTIL = 0 15-year BCSS 72% (95% CI, 62-82)
iTIL ≥ 1 15-year BCSS 71% (95% CI, 59-83)
5NP; p = 0.675
Years of follow-up

Figure 2

Additional files provided with this submission:

Additional file 1: Supplemental method.pdf, 52K
http://breast-cancer-research.com/imedia/2110819639695544/supp1.pdf
Additional file 2: Figure S1_image of CD8.pdf, 3772K
http://breast-cancer-research.com/imedia/8414407869554487/supp2.pdf
Additional file 3: Figure S2_distribution of TILs.pdf, 192K
http://breast-cancer-research.com/imedia/6124460676955457/supp3.pdf
Additional file 4: Supplemental tables.pdf, 108K
http://breast-cancer-research.com/imedia/4612354369554589/supp4.pdf
Additional file 5: Figure S3_sTIL and tTIL.pdf, 477K
http://breast-cancer-research.com/imedia/9867592766955460/supp5.pdf
Additional file 6: Figure S4_scatter plot of rescoring.pdf, 302K
http://breast-cancer-research.com/imedia/1933825101691688/supp6.pdf
Additional file 7: Figure S5_RFS_age and ER.pdf, 927K
http://breast-cancer-research.com/imedia/1589044055691689/supp7.pdf
Additional file 8: Figure S6_RFS_subtype.pdf, 643K
http://breast-cancer-research.com/imedia/1947974913691690/supp8.pdf