Prediction of Distant Metastatic Recurrence by Tumor-Infiltrating Lymphocytes in Hormone Receptor-Positive Breast Cancer

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Abstract

Background: Breast cancer subtypes are known to have different sites of metastatic recurrence. Distant metastases are often seen during the post-operative course in patients with human epidermal growth factor receptor 2 (HER2)-enriched breast cancer (HER2BC) and triple-negative breast cancer (TNBC) while being relatively rare in those with hormone receptor-positive and HER2-negative breast cancer (HR+HER2−BC). Tumor-infiltrating lymphocytes (TILs) can serve as an index to monitor tumor immune microenvironment and possibly predict the prognosis and therapeutic effect in breast cancer. This study aimed to investigate the correlation between TIL density and recurrence site in HR+HER2−BC.

Methods: Four-hundred and seventy-one patients with HR+HER2−BC underwent surgery as the first treatment and received adjuvant endocrine therapy except adjuvant chemotherapy at the Osaka City University Hospital from April 2007 to October 2015. To evaluate tumor morphology and examine TILs, needle biopsy specimens were used. Morphological assessment was conducted using conventional hematoxylin and eosin staining.

Results: Forty-two patients had a recurrence of breast cancer. In patients with no TIL density, local recurrence was significantly less (p = 0.022), while distant metastases were significantly more (p = 0.015) compared to those in patients with TIL density. Therefore, for the prediction of distant metastases in HR+HER2−BC without chemotherapy, TILs could be used as predictors in univariate analysis (p = 0.015, odds ratio [OR] = 0.127), although not as independent factors (p = 0.285, OR = 0.144).

Conclusions: We concluded that TILs may be able to predict distant metastatic recurrence in stages I−II of HR+HER2−BC.

Background

Cancer, even if detected early and surgery performed successfully, still has the risk of recurrence. Breast cancer often has local recurrence and axillary lymph node metastasis, although distant metastases, such as to bone, lung, and liver, may occur first. Imaging procedures, such as computed tomography (CT), ultrasonography (US), and bone scintigraphy, are necessary to detect distant metastases sub-clinically. However, some prospective studies have shown early detection of distant metastases to not affect prognosis, and routine examination is not recommended in such cases [1–3]. If distant metastasis can be detected before the onset of symptoms, both therapeutic effect and quality of life (QOL) of the patients can be improved. Therefore, clinicopathological factors prone to distant metastases have been studied quite extensively till date [4, 5]; different breast cancer intrinsic subtypes have different sites where metastatic recurrence might occur [4, 6]. Distant metastases are frequently found during the post-operative course in patients with human epidermal growth factor receptor 2 (HER2)-enriched breast cancer (HER2BC) and triple-negative breast cancer (TNBC), whereas they are relatively rare in patients with hormone receptor-positive and HER2-negative breast cancer (HR+HER2−BC). Therefore, local
assessment of HR⁺HER²⁻ BC has been suggested to be vital [7]. However, HR⁺HER²⁻ BC can also be distant from the first site of recurrence.

Invasion and metastasis of cancer involve not only the cancer, but also the surrounding interstitial region, the so-called tumor microenvironment (TME) [8, 9]. TME consists of cancer-related fibroblasts, neovascular cells, and tumor-infiltrating lymphocytes (TILs). Recently, the correlation between TILs and lymph node metastasis was reported in gastric cancer and melanoma [10, 11], and that between TILs and axillary lymph node metastasis was reported in TNBC [12, 13]. In all these reports, lower TIL density was shown to more likely cause lymph node metastasis.

We hypothesized that the site of the first recurrence may vary based on the difference in TIL density for HR⁺HER²⁻ BC. Therefore, this study aimed to investigate the correlation between TIL density and recurrence site in HR⁺HER²⁻ BC for patients who did not receive chemotherapy either before or after surgery.

**Methods**

**Patient background**

A total of 709 patients with primary resectable breast cancer underwent surgery between 2007 and 2015 at the Osaka City University Hospital (Fig. 1). Four-hundred and seventy-one patients with HR⁺HER²⁻ BC underwent surgery as the first treatment and received adjuvant endocrine therapy except for adjuvant chemotherapy. Breast cancers and their subtypes were diagnosed by core needle biopsy (CNB) or vacuum-assisted biopsy (VAB). HR⁺HER²⁻ BC was defined as estrogen receptor (ER) and/or progesterone receptor (PgR)-positive and HER2-negative by immunohistological staining of the tissue. Expression of Ki67 was also examined using immunohistological staining and the cutoff value for Ki67 was 14% according to previous reports [14]. CT, US, and bone scintigraphy were used for evaluation of the stage. All patients underwent either mastectomy or breast-conserving surgery, and the latter received radiation therapy in the remaining mammary gland after surgery. For patients diagnosed with axillary lymph node metastasis by preoperative imaging, axillary lymph node dissection was also performed. In the case of breast cancer diagnosed without axillary lymph nodes metastasis, sentinel lymph node biopsy was performed using the combination of radioisotope and dye methods, as reported previously [15, 16]. Metastasis was examined pathologically by slicing sentinel lymph node at 2-mm thickness [17, 18]. Axillary lymph node dissection was performed in cases where metastasis in the sentinel lymph node was greater than 2 mm, also called macro-metastasis. Regarding survival outcomes in this study, disease-free survival (DFS) was defined as the time from surgery to recurrence or death. Progression-free survival (PFS) was defined as the time from recurrence to relapse or death due to breast cancer after the next treatment. Post-recurrence survival (PRS) was defined as the time from recurrence to death due to breast cancer. The 471 patients were followed-up for a median of 2364 days (range, 66–4446 days) post-operatively.
Histopathological Evaluation Of TIL Density

TIL density was evaluated pathologically using biopsy specimens. The definition and evaluation method of TILs followed the International TILs Working Group 2014 [19]. Specifically, the density of infiltrating lymphocytes was averaged over five random fields of the tumor stroma. The results were divided into 4 groups according to the previous report (score 3: > 50%, score 2: > 10–50%, score 1: ≤ 10%, and score 0: absent) (Fig. 2) [20–23].

Statistical analysis

All statistical analyses were performed using the JMP software package (SAS, Tokyo, Japan). For evaluating the correlation between two groups for each clinicopathological feature, Pearson's chi-square test was used. The odds ratio (OR) and 95% confidence interval (CI) were calculated using logistic analysis. Multivariable analysis was performed with the multivariable logistic regression model. Regarding the hazard ratio (HR) and 95% CI related to survival outcomes, such as PFS or PRS, Cox proportional hazards models were used for univariate analysis and Cox regression models were used for multivariate analysis. Significance was defined by a p-value < 0.05.

Results

Clinicopathological features

Four-hundred and seventy-one patients, diagnosed with HR+HER2−BC, were not treated with adjuvant chemotherapy after surgery. Forty-two of them had a breast cancer recurrence. Table 1 shows the clinicopathological features. The median age at the time of operation was 59 years (range, 37–79 years). The median tumor size was 20.5 mm (9.5–49.3 mm), and no evidence of axillary lymph node metastasis was found by pre-operative imaging, in all cases. Therefore, sentinel lymph node biopsy was performed for all. Micro-metastasis was identified in 3 cases (7.1%) and macro-metastasis was found in 7 cases (16.7%). The latter underwent axillary lymph node dissection, and pathological examination revealed less than 3 axillary lymph nodes with metastases. The expression of ER in one patient (2.4%) was negative, whereas that of PgR in the patient was positive. Three patients (7.1%) were negative for PgR; 7 (16.7%) had high Ki67. Eighteen cases (42.9%) underwent breast-conserving treatment and received post-operative radiation therapy for the residual mammary gland. None of the 42 patients received post-operative radiation therapy in the axilla or supraclavicular fossa. Post-operative pathological findings showed lymphatic invasion in 21 patients (50.0%), and venous invasion in 3 patients (7.1%). Regarding nuclear grade, 6 patients (14.3%) were diagnosed with grade 3. All patients received adjuvant endocrine therapy; tamoxifen (TAM) was selected for 10 patients (23.8%), and TAM combined with luteinizing hormone-releasing hormone (LH-RH) agonist was selected for 7 patients (16.7%). The remaining 25 patients (59.5%) received anastrozole (ANA). The median DFS time was 1462 days (range, 132–3300 days). Most of the first recurrence sites had locoregional recurrence (local recurrence: 18 patients (42.9%), regional lymph node: 13 patients (30.9%)). Eleven patients (26.2%) had distant metastases at
the first recurrence site, 7 (16.6%) of which were lung metastases. Six patients (14.3%) had TIL density higher than 10% and in the other 6 patients (14.3%), TIL density was absent.

**Correlations Between Clinicopathological Features And Recurrence Sites**

The correlations between clinicopathological features and recurrence sites are listed in Table 2. Of the seven cases with metastasis, as per sentinel lymph node biopsy, two patients had regional lymph node recurrence and five had distant metastatic recurrence. As a result, in patients with axillary lymph node metastases, local recurrence was significantly less (p = 0.012), and distant metastasis was significantly more (p = 0.003) than in patients without axillary lymph node metastases. In addition, in patients with long DFS, distant metastatic recurrence was significantly higher (p = 0.014). Further, patients who underwent mastectomy had significantly more locoregional recurrence (p = 0.020). Examining the correlation between operative procedures and clinicopathological features, the following significant differences were found in patients who underwent mastectomy; older age (p = 0.001), higher frequency of tumor with diameter more than 30 mm (p = 0.006), and lower Ki67 (p = 0.012) (Additional file 1; Table S1).

Focusing on TIL density, no significant difference was observed when the cutoff value of TIL density was 10%. However, in the 6 patients with no TIL density, 2 had regional lymph node recurrence and 4 had distant metastatic recurrence. Therefore, in patients with no TIL density, local recurrence was significantly less (p = 0.022) and distant metastasis was significantly more (p = 0.015) than in those with TIL density.

**Correlations Between Clinicopathological Features And Tils**

We examined the correlation between clinicopathological features and TILs (Table 3); TIL density tended to be absent in patients with tumor size > 20 mm (p = 0.078), and 10% or more in patients with tumor size < 20 mm (p = 0.078). Macro-metastasis was found by sentinel lymph node biopsy in 4 of the 6 patients with no TIL density. Significantly more lymph node metastasis was found in patients with no TIL than in those with TIL (p < 0.001). Significantly higher frequency of venous invasion was found in patients with TIL ≥ 10% than in those with TIL < 10% (p = 0.007).
Table 3
Correlation between TILs and clinicopathological features in HR\(^+\)HER2\(^-\) BC not received chemotherapy

| Parameters                                      | Tumor-infiltrating lymphocytes (\(n = 42\)) | \(\text{Absent} (n = 6)\) | \(\text{Not absent} (n = 36)\) | \(\text{\(p\) value}\) | \(\leq 10 (n = 36)\) | \(> 10 (n = 6)\) | \(\text{\(p\) value}\) |
|------------------------------------------------|---------------------------------------------|-----------------------------|-------------------------------|--------------------------|-----------------------|-------------------|--------------------------|
| Age at operation (years old)                   |                                             | 2 (33.3%)                   | 23 (63.9%)                    | 0.158                    | 22 (61.1%)           | 3 (50.0%)         | 0.608                    |
| \(\leq 60\)                                   |                                             | 4 (66.7%)                   | 13 (36.1%)                    |                          | 14 (38.9%)           | 3 (50.0%)         |                          |
| > 60                                           |                                             | 20 (55.6%)                  | 16 (44.4%)                    | 0.078                    | 16 (44.4%)           | 5 (83.3%)         | 0.078                    |
| Tumor size (mm) \(\leq 20.0\)                 |                                             | 1 (16.7%)                   | 20 (55.6%)                    | 0.336                    | 28 (77.8%)           | 6 (100.0%)        | 0.199                    |
| > 20.0                                         |                                             | 5 (83.3%)                   | 16 (44.4%)                    |                          | 20 (55.6%)           | 1 (16.7%)         |                          |
| Tumor size (mm) \(\leq 30.0\)                 |                                             | 4 (66.7%)                   | 30 (83.3%)                    | 0.336                    | 28 (77.8%)           | 6 (100.0%)        | 0.199                    |
| > 30.0                                         |                                             | 2 (33.3%)                   | 6 (16.7%)                     |                          | 8 (22.2%)            | 0 (0.0%)          |                          |
| Pathological lymph node metastasis             |                                             | 2 (33.3%)                   | 33 (91.7%)                    | \(< 0.001\)              | 29 (80.6%)           | 6 (100.0%)        | 0.237                    |
| pN0, pN1mic                                    |                                             | 4 (66.7%)                   | 3 (8.3%)                      |                          | 7 (19.4%)            | 0 (0.0%)          |                          |
| pN1a                                           |                                             | 2 (33.3%)                   | 34 (94.4%)                    | 0.328                    | 34 (94.4%)           | 5 (83.3%)         | 0.328                    |
| Progesterone receptor                          |                                             | 1 (16.7%)                   | 2 (5.6%)                      | 0.328                    | 2 (5.6%)             | 1 (16.7%)         | 0.328                    |
| Negative                                       |                                             | 5 (83.3%)                   | 34 (94.4%)                    |                          | 34 (94.4%)           | 5 (83.3%)         |                          |
| Positive                                       |                                             | 1 (16.7%)                   | 2 (5.6%)                      | 0.328                    | 2 (5.6%)             | 1 (16.7%)         | 0.328                    |
| Ki67 \(\leq 14\%\)                            |                                             | 6 (100.0%)                  | 29 (80.6%)                    | 0.237                    | 29 (80.6%)           | 6 (100.0%)        | 0.237                    |
| >14\%                                          |                                             | 0 (0.0%)                    | 7 (19.4%)                     |                          | 7 (19.4%)            | 0 (0.0%)          |                          |
| Surgical treatment                             |                                             | 2 (33.3%)                   | 16 (44.4%)                    | 0.611                    | 15 (41.7%)           | 3 (50.0%)         | 0.703                    |
| BCT and radiation therapy                      |                                             | 4 (66.7%)                   | 20 (55.6%)                    |                          | 21 (58.3%)           | 3 (50.0%)         |                          |

TILs: tumor-infiltrating lymphocytes. HR\(^+\)HER2\(^-\) BC: hormone receptor-positive and human epidermal growth factor receptor 2 negative breast cancer. BCT: breast conserving treatment. TAM: tamoxifen. LH-RH: luteinizing hormone-releasing hormone. ANA: anastrozole. TILs: tumor-infiltrating lymphocytes.
| Parameters                        | Tumor-infiltrating lymphocytes (n = 42) |  |  |  |  |  |
|----------------------------------|----------------------------------------|--|--|--|--|--|
|                                  | Absent (n = 6)                          | Not absent (n = 36) | p value | ≤ 10 (n = 36) | > 10 (n = 6) | p value |
| Lymphatic invasion               | 3 (50.0%)                              | 18 (50.0%)          | 1.000   | 18 (50.0%)    | 3 (50.0%)    | 1.000   |
| ly0                              | 3 (50.0%)                              | 18 (50.0%)          | 1.000   | 18 (50.0%)    | 3 (50.0%)    | 1.000   |
| ly1                              | 3 (50.0%)                              | 18 (50.0%)          | 1.000   | 18 (50.0%)    | 3 (50.0%)    | 1.000   |
| Venous invasion                  | 6 (100.0%)                             | 33 (91.7%)          | 0.463   | 35 (97.2%)    | 4 (66.7%)    | 0.007   |
| v0                               | 0 (0.0%)                               | 3 (8.3%)            | 1 (2.8%) | 2 (33.3%)     |             |         |
| v1                               | 0 (0.0%)                               | 3 (8.3%)            | 1 (2.8%) | 2 (33.3%)     |             |         |
| Nuclear grade                    | 5 (83.3%)                              | 31 (86.1%)          | 0.857   | 31 (86.1%)    | 5 (83.3%)    | 0.857   |
| 1, 2                             | 1 (16.7%)                              | 5 (13.9%)           | 1 (16.7%)          |             |           |         |
| 3                                | 1 (16.7%)                              | 5 (13.9%)           | 1 (16.7%)          |             |           |         |
| Adjuvant endocrine therapy       | 1 (16.7%)                              | 16 (44.4%)          | 0.199   | 15 (41.7%)    | 2 (33.3%)    | 0.700   |
| TAM (+ LH-RH agonist)            | 5 (83.3%)                              | 20 (55.6%)          | 0.199   | 15 (41.7%)    | 2 (33.3%)    | 0.700   |
| ANA                              | 5 (83.3%)                              | 20 (55.6%)          | 0.199   | 15 (41.7%)    | 2 (33.3%)    | 0.700   |
| Disease free survival (days)     | 1 (16.7%)                              | 20 (55.6%)          | 0.078   | 18 (50.0%)    | 3 (50.0%)    | 1.000   |
| ≤ 1462                           | 5 (83.3%)                              | 16 (44.4%)          | 0.078   | 18 (50.0%)    | 3 (50.0%)    | 1.000   |
| >1462                            | 5 (83.3%)                              | 16 (44.4%)          | 0.078   | 18 (50.0%)    | 3 (50.0%)    | 1.000   |
| Primary recurrence site          | 0 (0.0%)                               | 18 (50.0%)          | 0.022   | 15 (41.7%)    | 3 (50.0%)    | 0.703   |
| Local recurrence                 | 6 (100.0%)                             | 18 (50.0%)          | 0.022   | 15 (41.7%)    | 3 (50.0%)    | 0.703   |
| Not local recurrence             | 6 (100.0%)                             | 18 (50.0%)          | 0.022   | 15 (41.7%)    | 3 (50.0%)    | 0.703   |
| Primary recurrence site          | 2 (33.3%)                              | 29 (80.6%)          | 0.015   | 27 (75.0%)    | 4 (66.7%)    | 0.667   |
| Locoregional recurrence          | 2 (33.3%)                              | 30 (83.3%)          | 0.015   | 27 (75.0%)    | 4 (66.7%)    | 0.667   |
| Distant metastasis               | 4 (66.7%)                              | 7 (19.4%)           | 0.015   | 9 (25.0%)     | 2 (33.3%)    | 0.667   |

TILs: tumor-infiltrating lymphocytes. HR⁺HER2⁻BC: hormone receptor-positive and human epidermal growth factor receptor 2 negative breast cancer. BCT: breast conserving treatment. TAM: tamoxifen. LH-RH: luteinizing hormone-releasing hormone. ANA: anastrozole. TILs: tumor-infiltrating lymphocytes.

Prediction of distant metastases in patients with HR⁺HER2⁻BC without chemotherapy
Examining distant metastasis predictors based on the results, axillary lymph node metastasis ($p = 0.003$, OR = 16.723) and operative method ($p = 0.026$, OR = 0.044) were found to be independent factors (Table 4). TILs (absent vs. present) were predictors of distant metastases in univariate analysis ($p = 0.015$, OR = 0.127), though not independent factors ($p = 0.285$, OR = 0.144).
Table 4
Univariate and multivariate analysis with distant metastasis for HR⁺HER2⁻ BC not received chemotherapy

| Parameters                                      | Univariate analysis | Multivariate analysis |
|------------------------------------------------|---------------------|-----------------------|
|                                                 | Odd ratio           | 95% CI                | p value  | Odd ratio           | 95% CI                | p value  |
| Age at operation (years old) ≤ 60 vs > 60       | 0.455               | 0.101–2.048           | 0.299    |                    |                      |          |
| Tumor size (mm) ≤ 20.0 vs > 20.0                | 0.781               | 0.197–3.106           | 0.726    |                    |                      |          |
| Tumor size (mm) ≤ 30.0 vs > 30.0                | 0.343               | 0.037–3.161           | 0.328    |                    |                      |          |
| Pathological lymph node metastasis pN0, pN1mic vs pN1a | 12.083              | 1.880–77.665          | 0.003    | 16.723              | 1.197–520.348         | 0.050    |
| Progesterone receptor Negative vs Positive      | -                   | -                     | 0.284    |                    |                      |          |
| Ki67 ≤14% vs >14%                               | 1.156               | 0.190–7.037           | 0.875    |                    |                      |          |
| Surgical treatment BCT and radiation therapy vs Mastectomy | 0.179               | 0.039–0.821           | 0.020    | 0.044              | 0.001–0.420          | 0.026    |
| Lymphatic invasion ly0 vs ly1                   | 1.280               | 0.322–5.088           | 0.726    |                    |                      |          |
| Venous invasion v0 vs v1                        | 1.450               | 0.118–17.766          | 0.770    |                    |                      |          |
| Nuclear grade 1, 2 vs 3                         | 0.520               | 0.054–5.021           | 0.567    |                    |                      |          |
| Adjuvant endocrine therapy TAM (+ LH-RH agonist) vs ANA | 0.867               | 0.217–3.461           | 0.839    |                    |                      |          |

HR⁺HER2⁻ BC: hormone receptor-positive and human epidermal growth factor receptor 2 negative breast cancer. CI, confidence intervals. BCT: breast conserving treatment. TAM: tamoxifen. LH-RH: luteinizing hormone-releasing hormone. ANA: anastrozole. TILs: tumor-infiltrating lymphocytes.
### Univariate Analysis

|                                | Univariate analysis | Multivariate analysis |
|--------------------------------|---------------------|-----------------------|
| Disease free survival (days)   | 7.125               | 6.693                 |
| ≤1462 vs > 1462                | 1.309–38.771        | 0.954–81.406          |
| TILs density                   | 1.500               | 0.127                 |
| ≤10 vs > 10                    | 0.234–9.611         | 0.015                 |
| TILs                           | 0.127               | 0.015                 |
| Absent vs Not absent           | 0.018–0.797         | 0.003–5.060           |

HR⁺HER²⁻ BC: hormone receptor-positive and human epidermal growth factor receptor 2 negative breast cancer. CI, confidence intervals. BCT: breast conserving treatment. TAM: tamoxifen. LH-RH: luteinizing hormone-releasing hormone. ANA: anastrozole. TILs: tumor-infiltrating lymphocytes.

### Prognosis After Recurrence

Using univariate analysis with progression-free survival after recurrence, no clear predictive factor could be identified, since treatment after recurrence was variable (Additional file 2; Table S2). On the other hand, in PRS, lymph node metastasis during surgery was a poor prognostic factor as per multivariate analysis (p = 0.042, HR = 17.339) (Table 5).
Table 5
Univariate and multivariate analysis with post-recurrence survival after recurrence

| Parameters                                      | Univariate analysis |          |          | Multivariate analysis |          |          |          |
|------------------------------------------------|---------------------|----------|----------|-----------------------|----------|----------|----------|
|                                                 | Hazard ratio        | 95% CI   | p value  | Hazard ratio          | 95% CI   | p value  |
| Age at operation (years old)                    |                     |          |          |                       |          |
| ≤ 60 vs >60                                     | 0.871               | 0.040–9.101 | 0.910    | 0.871                 | 0.040–9.101 | 0.910    |
| Tumor size (mm)                                 |                     |          |          |                       |          |
| ≤ 20.0 vs >20.0                                 | 1.798               | 0.172–38.728 | 0.623    | 1.798                 | 0.172–38.728 | 0.623    |
| Tumor size (mm)                                 | -                   | -        | 0.265    | 1.000                 | 1.000    | 1.000    |
| ≤ 30.0 vs >30.0                                 |                     |          |          |                       |          |
| Pathological lymph node metastasis              | 21.520              | 1.878–488.577 | 0.016    | 17.339               | 1.112–530.855 | 0.042    |
| pN0, pN1mic vs pN1a                             |                     |          |          |                       |          |
| Progesterone receptor                           | -                   | -        | 0.545    |                       |          |
| Negative vs Positive                            |                     |          |          |                       |          |
| Ki67                                            | 1.806               | 0.084–18.864 | 0.642    |                       |          |
| ≤14% vs >14%                                    |                     |          |          |                       |          |
| Surgical treatment                              | 0.422               | 0.020–4.410 | 0.467    |                       |          |
| BCT and radiation therapy vs Mastectomy         |                     |          |          |                       |          |
| Lymphatic invasion                              | 1.906               | 0.182–41.094 | 0.589    |                       |          |
| ly0 vs ly1                                      |                     |          |          |                       |          |
| Venous invasion                                 | 3.760               | 0.174–39.725 | 0.327    |                       |          |
| v0 vs v1                                        |                     |          |          |                       |          |
| Nuclear grade                                   | -                   | -        | 0.294    |                       |          |
| 1, 2 vs 3                                       |                     |          |          |                       |          |
| Adjuvant endocrine therapy                      | 0.422               | 0.020–4.410 | 0.467    |                       |          |
| TAM (+ LH-RH agonist) vs ANA                    |                     |          |          |                       |          |

HR\textsuperscript{+}HER2\textsuperscript{-}BC: hormone receptor-positive and human epidermal growth factor receptor 2 negative breast cancer. CI, confidence intervals. BCT: breast conserving treatment. TAM: tamoxifen. LH-RH: luteinizing hormone-releasing hormone. ANA: anastrozole. TILs: tumor-infiltrating lymphocytes.
|                                | Univariate analysis | Probability | Multivariate analysis | Probability |
|--------------------------------|---------------------|-------------|-----------------------|-------------|
| Disease free survival (days)   |                     |             |                       |             |
| ≤1462 vs >1462                 | 1.229               | 0.056–13.152| 0.870                 |             |
| Primary recurrence site        |                     |             |                       |             |
| Locoregional recurrence vs     | 9.331               | 0.877–202.892| 0.063                 | 6.057       |
| Distant metastasis             |                     |             | 0.414–178.741         | 0.187       |
| TILs density                   |                     |             |                       |             |
| ≤10 vs >10                     | 3.405               | 0.158–35.582| 0.360                 |             |
| TILs                           |                     |             |                       |             |
| Absent vs Not absent           | 0.211               | 0.018–4.800 | 0.269                 |             |

HR⁺HER²⁻BC: hormone receptor-positive and human epidermal growth factor receptor 2 negative breast cancer. CI, confidence intervals. BCT: breast conserving treatment. TAM: tamoxifen. LH-RH: luteinizing hormone-releasing hormone. ANA: anastrozole. TILs: tumor-infiltrating lymphocytes.

**Discussion**

There are various reports on the prediction of distant metastasis. However, the recurrence site differs depending on the subtype, and tumor size and lymph node metastasis are common risk factors [5, 24–26]. In addition, young age [5], histopathological grade, and lymphovascular invasion have also been reported as risk factors [5, 25], although there are reports suggesting otherwise, as well. In this study, operative procedure was shown to be a predictive factor for distant metastasis, and the cause was found to be correlated with the operative procedure as well as some clinicopathological features, such as age, tumor diameter, and Ki67, also referred to as risk factors.

For the prediction of distant metastasis, some research groups have examined the molecular pathological features of cancer cells; for example, co-expression of α6β4 integrin and neuroepithelioma transforming gene 1 (Net1) [27], expression of YKL-39 (a kind of chitinase-like protein) [28], and overexpression of phosphorylated PLC-gamma-1 (one of the phosphoinositides) were reported in this regard [29]. These expression levels have been shown to promote invasion and metastasis of cancer cells both in vivo and in vitro; hence, these studies retrospectively examined the use of clinical samples as a predictor of distant metastases. There have also been reports that examined the molecular characteristics of cancer cells considering the circulating tumor cells (CTCs) that are deeply involved in distant metastasis [30, 31]. One such report suggested CTCs to possibly be an indicator of the therapeutic effect of chemotherapy or endocrine therapy [32], whereas some other studies reported them as a predictor of distant metastatic recurrence of breast cancer based on clinical data [33, 34]. Some publications have even reported the prediction of distant metastasis using genetic assays [35].
Mutations in p53, a gene involved in deoxyribonucleic acid (DNA) repair, are involved in recurrence; Narod has reported p53 accumulation to be a strong predictor of recurrence [36]. Filipits et al. predicted distant metastasis using RNA-based multigene score [37]. A genomic evaluation study shows a higher mutation burden to accompany recurrence than primary tumor [38]. These results together suggest gene mutations to potentially cause local or host immune tolerance at the distant metastatic site [4].

Formation of distant metastases in cancer generally follows the concept of “seed and soil.” While we have discussed the “seed” until now [39], TME corresponds to the “soil” as per this concept [8, 9]. TILs are also included in the cells that constitute the TME. In breast cancer, TILs have been reported to vary by subtype. In particular, HER2BC and TNBC have been reported to show significantly higher TIL density than HR⁺HER2⁻BC, and TILs have been proven to predict the therapeutic effect of chemotherapy [13, 40–42]. Conversely, there are very few reports examining the correlation between TILs and clinicopathological factors or therapeutic effects in HR⁺HER2⁻BC.

In this study, we excluded patients who had undergone neoadjuvant/adjuvant chemotherapy based on three reasons. First, chemotherapy affects the immune microenvironment, including TILs, in preoperative chemotherapy study, and related changes may affect prognosis [43]. TILs are also predictors of the therapeutic effect of adjuvant chemotherapy; therefore, adjuvant chemotherapy may also affect the tumor immune microenvironment. Secondly, axillary lymph node metastasis before adjuvant chemotherapy was diagnosed based on image only, due to which the diagnosis was not accurate. Lastly, different chemotherapy regimens are known to have different effects and neoadjuvant chemotherapy causes more local recurrence than adjuvant therapy [44].

There are some reports on the relationship between recurrence sites and TILs. Park et al. reported patients with TILs higher than 10% in early-stage TNBC to show significantly more locoregional recurrence than those with lower TILs [45]. In cervical squamous cell carcinoma, low TILs are also likely to cause distant metastasis [46]. Moreover, distant metastasis is reported to be predicted from pathological features, including lymphocytes of lymph nodes in breast cancer with lymph node metastasis, although not from the density of lymphocytes around the cancer [47]. Bidwell et al. have shown innate immune escape to promote bone metastasis, based on clinical data and experiments in mice [48]. Some studies using breast cancer cell lines in vivo had also report immunosuppression in the tumor immune environment to increase the risk of lung metastasis [49, 50]. In this study, TILs were suggested to possibly be a distant metastatic predictor, although not an independent factor. We have reported that TILs may also be involved in lymph node metastasis in HR⁺HER2⁻BC [51]. In this study, TILs were considered to be strongly correlated with lymph node metastasis, being involved in distant metastasis prediction as well as prognosis after recurrence.

The greatest limitation of this study was that very few cases of recurrence were examined. Another limitation was that the types of adjuvant endocrine therapy were different. However, TILs can be evaluated in needle biopsy specimens for the diagnosis of breast cancer and are highly useful. Filipits et al. predicted distant metastases using the above-mentioned predictors in combination [37] and TILs may
be considered to be an additional condition. When distant metastasis generates symptoms, the patient’s QOL is impaired.

**Conclusions**

This study suggested TILs to possibly be predictors of distant metastatic recurrence in stages I–II of HR⁺HER₂⁻ BC.

**Abbreviations**

BC  
breast cancer, CI: confidence interval, CNB: core needle biopsy, CT: computed tomography, CTCs: circulating tumor cells, DFS: disease free survival, DNA: deoxyribonucleic acid, ER: estrogen receptor, HER2: human epidermal growth factor receptor 2, HER2BC: human epidermal growth factor receptor 2-enriched breast cancer, HR⁺HER₂⁻ BC: hormone receptor-positive and HER2-negative breast cancer, HRs: hazard ratios, Net1: neuroepithelioma transforming gene 1, OR: odd ratio, QOL: quality of life, PFS: progression-free survival, PgR: progesterone receptor, PRS: post-recurrence survival, TNBC: triple-negative breast cancer, TILs: tumor infiltrating lymphocytes, TME: tumor microenvironment, US: ultrasonography, VAB: vacuum-assisted biopsy.

**Declarations**

**Ethics approval and consent to participate**

A written informed consent to participate in the study was obtained from each subject in accordance with the declaration of Helsinki principles. Each patient or the patient’s family was fully informed of the investigational nature of this study and provided their written, informed consent. The study protocol was approved by the Ethics Committee of Osaka City University (approve number #926).

**Consent for publication**

Not applicable.

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

**Funding**
This study was supported in part by Grants-in Aid for Scientific Research (KAKENHI, Nos. 19K18067, 17K10559, and 20K08938) from the Ministry of Education, Science, Sports, Culture and Technology of Japan.

Authors’ contributions

All authors were involved in the preparation of this manuscript. KT collected the data and wrote the manuscript. SK, YA, WG, TM, MS, and TT performed the operation and designed the study. KT and SK summarized the data and revised the manuscript. HF, KH, and MO provided a substantial contribution to the study design, performed the operation, and revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank Yayoi Matsukiyo and Tomomi Okawa (Department of Breast and Endocrine Surgery, Osaka City University Graduate School of Medicine) for helpful advice regarding data management.

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Figures
Figure 1

Consort diagram. A total of 709 patients with primary resectable breast cancer underwent surgery between 2007 and 2015 at the Osaka City University Hospital. Four-hundred and seventy-one patients with HR+HER2-BC underwent surgery as the first treatment and received adjuvant endocrine therapy except for adjuvant chemotherapy.
Figure 2

Histopathologic evaluation of the tumor-infiltrating lymphocytes (TILs) density was performed on hematoxylin and eosin-stained tumor section. TILs density was divided into four groups (>50% (A); >10–50% (B); ≤10% (C); and absent (D), respectively).

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