Tumour-infiltrating CD8 to FOXP3 lymphocyte ratio in predicting treatment responses to neoadjuvant chemotherapy of aggressive breast cancer

Y. Asano1, S. Kashiwagi1, W. Goto1, K. Kurata1, S. Noda1, T. Takashima1, N. Onoda1, S. Tanaka2, M. Ohsawa2 and K. Hirakawa1

Departments of 1Surgical Oncology and 2Diagnostic Pathology, Osaka City University Graduate School of Medicine, Osaka, Japan

Correspondence to: Dr S. Kashiwagi, Osaka City University Graduate School of Medicine, 1–4–3 Asahi-machi, Abeno-ku, Osaka 545–8585, Japan (e-mail: spq9ke9@view.ocn.ne.jp)

Background: Tumour-infiltrating lymphocytes (TILs) can be used to monitor the immune response, and are important in predicting treatment responses and outcomes for various types of cancer. Recently, specific TIL subsets have been reported to be clinically useful in predicting treatment responses. The CD8+/FOXP3+ TIL ratio (CFR) may be a more sensitive indicator for monitoring immune function. This study investigated the clinical significance and value of CFR as a biomarker to predict treatment responses to neoadjuvant chemotherapy for breast cancer.

Methods: Patients with resectable early-stage breast cancer treated with neoadjuvant chemotherapy at Osaka City University Hospital, Japan, between 2007 and 2013 were included. Oestrogen receptor, progesterone receptor, human epidermal growth factor receptor (HER) 2, Ki-67, CD8 and FOXP3 status were assessed by immunohistochemistry, and correlated with pathological complete response (pCR).

Results: A total of 177 patients were included, of whom 90 had a high CFR and 87 a low CFR. Triple-negative breast cancer (TNBC) was more common in the high-CFR group than in the low-CFR group (46 versus 23 per cent; P = 0.002), as was HER2-enriched breast cancer (HER2BC) (27 versus 14 per cent; P = 0.033). Among these patients, the pCR rate was significantly higher in the high-CFR group than in the low-CFR group (TNBC: P = 0.022; HER2BC: P < 0.001). In multivariable analysis high-CFR status was an independent predictor of a favourable prognosis: hazard ratio 0.24 (95 per cent c.i. 0.05 to 0.72; P = 0.015) for TNBC and 0.10 (0.010 to 0.90; P = 0.041) for HER2BC.

Conclusion: The CFR may be a useful biomarker to predict treatment response to neoadjuvant therapy in aggressive breast cancer subtypes, such as TNBC and HER2BC.

Introduction

Cancer cells have gained the capacity to proliferate autonomously and survive owing to gene mutations, but it is evident that the tumour microenvironment also greatly influences tumour progression1. The immune cells of the tumour microenvironment influence not only the response to immunotherapy, but also the response to, and outcomes after, other anticancer therapies. The importance of regulating and improving the cancer immune microenvironment can therefore play an important role in predicting treatment responses and outcomes2,3.

Tumour-infiltrating lymphocytes (TILs) can be used to monitor the immune response and are important in predicting treatment responses in many cancers4–6. TILs include various cells of the immune system. There are cells involved in eradication of cancer cells such as CD8+ T lymphocytes, natural killer cells, dendritic cells and macrophages. Other cell types, such as regulatory T (Treg) cells and myeloid-derived suppressor cells, on the other hand, promote survival and proliferation of cancer cells7. Morphological evaluation of TILs is attracting attention as they are potentially clinically useful biomarkers in breast cancer. Phase III trials including BIG 02/98, Finland Herceptin (FinHER), Eastern Cooperative Oncology Group (ECOG) 2197 and ECOG1199 have all shown the value of TILs in predicting treatment responses in patients with highly malignant breast cancer, including triple-negative…
breast cancer (TNBC) and human epidermal growth factor receptor (HER) 2-enriched breast cancer (HER2BC)8–11.

Recently, specific TIL subsets have been reported to be clinically significant and useful in predicting treatment responses. High pathological complete response (pCR) rates after neoadjuvant chemotherapy have now been reported occasionally in patients with high levels of expression of subsets of TILs such as CD3, CD4, CD8 and Forkhead box 3 (FOXP3)6,12–14. High pCR rates in TNBC have also been reported with a high CD8+/FOXP3+ TIL ratio (CFR)15,16. Cytotoxic CD8+ T lymphocytes selectively recognize and have the ability to destroy cancer cells. FOXP3, a marker of Treg cells, is a gene that regulates Treg cell generation and function. Treg cells inactivate CD8+ T lymphocytes, which subsequently reduces the function of co-stimulatory proteins on antigen-presenting cells. In other words, Treg cells suppress the induction of cytotoxic CD8+ T lymphocytes in response to cancer cells. The CFR is the ratio of CD8+ to FOXP3+ TILs. Therefore, the CFR may be a more sensitive indicator for monitoring immune function in a cancer.

The present study investigated the clinical significance and value of the CFR as a biomarker to predict treatment responses to neoadjuvant chemotherapy in breast cancer. The results were analysed with stratification for breast cancer subtypes. Changes in the CFR after breast cancer recurrence were also examined.

Methods

This study was conducted at Osaka City University Graduate School of Medicine, Osaka, Japan, according to the REporting recommendations for tumour MARKer prognostic studies (REMARK) guidelines17, and a retrospectively written research, pathological evaluation and statistical plan. Written informed consent was obtained from all patients. This research conformed to the provisions of the Declaration of Helsinki 1996. The study protocol was approved by the Ethics Committee of Osaka City University (no. 926).

Patients with resectable, early-stage, primary infiltrating ductal breast cancer diagnosed as stage IIA (T1N1M0 or T2N0M0), IIB (T2N1M0 or T3N0M0) or IIIA (T1–2 N2M0 or T3N1–2 M0), treated with neoadjuvant chemotherapy between 2007 and 2013, were included. Tumour staging was based on the International Union Against Cancer (UICC) TNM classification, seventh edition18. Breast cancer was confirmed histologically by core needle biopsy and staged by systemic imaging studies using CT, ultrasonography and bone scintigraphy. Breast cancer was classified into molecular subtypes according to the immunohistochemical expressions of oestrogen receptor (ER), progesterone receptor (PgR), HER2 and Ki-67.

Neoadjuvant therapy regimens and surgery

All patients underwent core needle biopsy before neoadjuvant chemotherapy. A standard protocol was used for neoadjuvant chemotherapy, consisting of four courses of FEC100 (500 mg/m 2 5-fluorouracil, 100 mg/m2 epirubicin and 500 mg/m2 cyclophosphamide) every 3 weeks, followed by 12 courses of 80 mg/m2 paclitaxel administered weekly19,20. Patients with HER2BC also received trastuzumab weekly (2 mg/kg) or triweekly (6 mg/kg) during paclitaxel treatment21. All patients underwent chemotherapy as outpatients. Patients underwent mastectomy or breast-conserving surgery after neoadjuvant chemotherapy. All patients who had breast-conserving surgery received postoperative radiotherapy to the remnant breast. All patients were followed up by physical examination every 3 months, ultrasonography every 6 months, and CT and bone scintigraphy annually.

Clinical endpoints

Therapeutic antitumour effects were assessed according to the Response Evaluation Criteria in Solid Tumours (RECIST)22. A pCR was defined as the complete disappearance of the invasive compartment of the lesion with or without intraductal components, including the lymph nodes. Overall survival time was the interval from the date of primary surgery to the time of death from any cause. Disease-free interval (DFS) was defined as the interval from the date of primary surgery to the first local recurrence, distant recurrence or death from any cause.

Immunohistochemistry

Immunohistochemical studies were performed as described previously on core needle biopsy specimens23. Tumour specimens were fixed in 10 per cent formaldehyde solution and embedded in paraffin, and sections 4 μm thick were mounted on glass slides. Slides were deparaffinized in xylene and heated for 20 min (105°C, 0-4 kg/m 2) in an autoclave in Target Retrieval Solution (Dako, Carpinteria, California, USA). Specimens were incubated with 3 per cent hydrogen peroxide in methanol for 15 min to block endogenous peroxidase activity, then in 10 per cent normal goat or rabbit serum to block non-specific reactions.

Primary monoclonal antibodies directed against ER (clone 1D5, dilution 1:80; Dako, Cambridge, UK),
PgR (clone PgR636, dilution 1:100; Dako), HER2 (HercepTest™; Dako), Ki-67 (clone MIB-1, dilution 1:100; Dako), CD8 (clone C8/144B, dilution 1:100; Dako) and FOXP3 (clone 236A/E7, dilution 1:100; Abcam, Cambridge, UK) were used. Tissue sections were incubated with each antibody for 70 min at room temperature or overnight at 4°C, then with horseradish peroxidase-conjugated antirabbit or antimouse Ig secondary antibodies (HISTOFINE (PO)™ kit; Nichirei, Tokyo, Japan). Slides were subsequently treated with streptavidin–peroxidase reagent and incubated in phosphate-buffered saline–diaminobenzidine and 1 per cent hydrogen peroxide (v/v), followed by counterstaining with Mayer’s haematoxylin. Positive and negative controls for each marker were used according to the supplier’s data sheet.

Immunohistochemical scoring

Immunohistochemical scoring was performed by two breast pathologists. The cut-off value for ER and PgR positivity was set at at least 1 per cent in accordance with previous studies. HER2 expression was scored according to the accepted grading system (0, no reactivity or membranous reactivity in less than 10 per cent of cells; 1+, faint/barely perceptible membranous reactivity in at least 10 per cent of cells or reactivity in only part of the cell membrane; 2+, weak to moderate complete or basolateral membranous reactivity in at least 10 per cent of tumour cells; or 3+, strong complete or basolateral membranous reactivity in at least 10 per cent of tumour cells). HER2 expression was considered positive if the immunostaining score was 3+, or where the score was 2+ and included gene amplification via fluorescence in situ hybridization (FISH). For FISH analyses, each copy of the HER2 gene and its centromere 17 (CEP17) reference were counted. The interpretation followed the criteria of the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines for HER2 immunohistochemical classification for breast cancer: positive if the HER2/CEP17 ratio was higher than 2.0. A Ki-67 labelling index with 14 per cent or more of tumour cells with nuclear staining was considered positive.

Histopathological analysis of the percentage of TILs was evaluated on a single haematoxylin and eosin-stained tumour section using criteria described by Salgado and colleagues. TILs were defined as the infiltrating lymphocytes within the tumour stroma and the tumour, and expressed in terms of the proportion of the field.

Fig. 1 CD8 and FOXP3 expression in stroma surrounding cancer cells: a–c CD8+ cells in triple-negative breast cancer (TNBC) (a), human epidermal growth factor receptor 2–enriched breast cancer (HER2BC) (b) and hormone receptor-positive breast cancer (HRBC) (c); d–f FOXP3+ cells in TNBC (d), HER2BC (e) and HRBC (f) (original magnification ×400, Mayer’s haematoxylin counterstain)
investigated. To evaluate CD8 and FOXP3 expression, five stained areas were selected, and the number of TILs in stroma surrounding the stained cancer cells was measured quantitatively in each field under 400× magnification (Fig. 1). The cut-off levels for high or low infiltration were based on the mean number of infiltrating cells per field. The CFR was defined as the number of CD8+ TILs divided by the number of FOXP3+ TILs. The cut-off value, based on previous reports, was a CFR of at least 15.

### Statistical analysis

The associations between CFR and clinicopathological variables were examined by \( \chi^2 \) test. The Kaplan–Meier method was used to estimate DFS and overall survival, and the log rank test for comparison of the results between groups. Cut-off values for different biomarkers included in this study were chosen before statistical analysis. Multivariable analysis of prognostic factors was carried out using a Cox regression model. \( P < 0.050 \) was considered significant. Statistical analysis was performed using SPSS® version 19.0 statistical software (IBM, Armonk, New York, USA).

### Results

#### Responses to neoadjuvant chemotherapy

A total of 177 patients were included in the study. The distribution of breast cancer subtypes was: TNBC in 61 patients (34.5 per cent), HER2BC in 36 (20.3 per cent), and HRBC in 50 (28.4 per cent). The distribution of clinical and pathological characteristics is shown in Table 1. Significant differences were observed in age at operation, tumour size, lymph node status, nuclear grade, and Ki-67 (%). The proportion of patients with pathological complete response (pCR) was higher in patients with high CFR than in those with low CFR (44/85 vs. 23/92, \( P < 0.05 \)).

| Clinicopathological feature | High CFR (n = 90) | Low CFR (n = 87) | \( P' \) |
|----------------------------|------------------|-----------------|--------|
| Age at operation (years)   |                  |                 |        |
| ≤ 56                       | 44 (49)          | 43 (49)         | 0.943  |
| > 56                       | 46 (51)          | 51 (59)         |        |
| In menopause               |                  |                 |        |
| No                         | 36 (40)          | 36 (41)         | 0.852  |
| Yes                        | 54 (60)          | 51 (59)         |        |
| Tumour size (cm)           |                  |                 |        |
| ≤ 2                        | 12 (13)          | 12 (14)         | 0.929  |
| > 2                        | 78 (87)          | 75 (86)         |        |
| Lymph node status          |                  |                 |        |
| Negative                   | 20 (22)          | 21 (24)         | 0.763  |
| Positive                   | 70 (78)          | 66 (76)         |        |
| Nuclear grade              |                  |                 |        |
| 1–2                        | 64 (71)          | 73 (84)         | 0.042  |
| 3                          | 26 (29)          | 14 (16)         |        |
| Ki-67 (%)                  |                  |                 |        |
| ≤ 14                       | 28 (31)          | 46 (53)         | 0.003  |
| > 14                       | 62 (69)          | 41 (47)         |        |
| Intrinsic subtype TNBC     |                  |                 |        |
| Yes                        | 41 (46)          | 20 (23)         | 0.002  |
| No                         | 49 (54)          | 67 (77)         |        |
| Intrinsic subtype HER2BC   |                  |                 |        |
| Yes                        | 24 (27)          | 12 (14)         | 0.033  |
| No                         | 66 (73)          | 75 (86)         |        |
| Intrinsic subtype HRBC     |                  |                 |        |
| Yes                        | 25 (28)          | 55 (63)         | < 0.001|
| No                         | 65 (72)          | 32 (37)         |        |
| Pathological response      |                  |                 |        |
| pCR                        | 44 (49)          | 23 (26)         | 0.002  |
| Non-pCR                    | 46 (51)          | 64 (74)         |        |

Values in parentheses are percentages. CFR, CD8+/FOXP3+ tumour-infiltrating lymphocyte ratio; TNBC, triple-negative breast cancer; HER2BC, human epidermal growth factor receptor 2–enriched breast cancer; HRBC, hormone receptor-positive breast cancer; pCR, pathological complete response. \( \chi^2 \) test.
Table 2 Univariable and multivariable Cox regression analysis with respect to disease-free survival in breast cancer subtypes

| All breast cancers (n = 177) | Univariable analysis | Multivariable analysis |
|-----------------------------|----------------------|-----------------------|
|                             | Hazard ratio         | P                     | Hazard ratio | P     |
| Tumour size (≤2 versus > 2 cm) | 1.06 (0.37, 3.05)    | 0.911                 | 0.75 (0.34, 1.66) | 0.475 |
| Lymph node status (negative versus positive) | 4.16 (0.99, 17.46)   | 0.052                 | 0.42 (0.22, 0.97) | 0.041 |
| Nuclear grade (1–2 versus 3) | 1.03 (0.44, 2.39)    | 0.954                 | 0.39 (0.19, 0.81) | 0.015 |
| Ki-67 (≤140 versus >140)     | 0.65 (0.32, 1.33)    | 0.238                 | 0.24 (0.05, 0.72) | 0.041 |
| Pathological response (pCR versus non-pCR) | 0.61 (0.28, 1.34)    | 0.217                 | 1.40 (0.15, 12.91) | 0.765 |
| CFR (high versus low)        | 0.35 (0.16, 0.76)    | 0.008                 | 0.10 (0.05, 0.90) | 0.041 |
| TNBC (n = 61)                |                      |                       |              |
| Tumour size (≤2 versus > 2 cm) | 0.55 (0.12, 2.55)    | 0.444                 |              |
| Lymph node status (negative versus positive) | 0.94 (0.20, 4.36)    | 0.939                 |              |
| Nuclear grade (1–2 versus 3) | 1.55 (0.46, 5.31)    | 0.482                 |              |
| Ki-67 (≤140 versus >140)     | 0.74 (0.22, 2.53)    | 0.630                 |              |
| Pathological response (pCR versus non-pCR) | 0.23 (0.05, 1.08)    | 0.063                 |              |
| CFR (high versus low)        | 0.14 (0.04, 0.53)    | 0.004                 |              |
| HER2BC (n = 36)              |                      |                       |              |
| Tumour size (≤2 versus > 2 cm) | 0.69 (0.08, 6.30)    | 0.744                 |              |
| Lymph node status (negative versus positive) | 3.73 (0.07, 5.05)    | 0.414                 |              |
| Nuclear grade (1–2 versus 3) | 0.04 (0.01, 5.22)    | 0.513                 |              |
| Ki-67 (≤140 versus >140)     | 0.44 (0.07, 2.62)    | 0.364                 |              |
| Pathological response (pCR versus non-pCR) | 0.48 (0.08, 2.85)    | 0.415                 |              |
| CFR (high versus low)        | 0.12 (0.18, 0.73)    | 0.021                 |              |
| HRBC (n = 80)                |                      |                       |              |
| Tumour size (≤2 versus > 2 cm) | 2.46 (0.32, 18.84)   | 0.386                 |              |
| Lymph node status (negative versus positive) | 3.68 (0.15, 10.38)   | 0.205                 |              |
| Nuclear grade (1–2 versus 3) | 1.06 (0.30, 3.81)    | 0.930                 |              |
| Ki-67 (≤140 versus >140)     | 0.60 (0.21, 1.74)    | 0.344                 |              |
| Pathological response (pCR versus non-pCR) | 1.33 (0.44, 3.97)    | 0.614                 |              |
| CFR (high versus low)        | 0.85 (0.27, 2.70)    | 0.776                 |              |

Values in parentheses are 95 per cent confidence intervals. pCR, pathological complete response; CFR, CD8+/FOXP3+ tumour-infiltrating lymphocyte ratio; TNBC, triple-negative breast cancer. HER2BC, human epidermal growth factor receptor 2–enriched breast cancer; HRBC, hormone receptor-positive breast cancer.

and hormone receptor-positive breast cancer (HRBC) in 80 (45.2 per cent). A pCR was achieved in 67 patients (37.9 per cent), a partial response in 84 (47.5 per cent) and stable disease in 19 (10.7 per cent), whereas seven patients (4.0 per cent) had progressive disease. Stratified by subtype, a pCR was achieved in 28 (46 per cent) of 61 patients with TNBC, 18 (50 per cent) of 36 with HER2BC and 21 (26 per cent) of 80 with HRBC.

Correlation between stromal and intratumoural tumour-infiltrating lymphocytes

Among all 177 patients, the stromal TILs were CD8+ in 91 (51.4 per cent) and 89 patients were FOXP3+ (50.3 per cent). The intratumoural TILs were CD8+ in 89 patients (50.3 per cent) and 80 patients were FOXP3+ (45.2 per cent). A positive correlation was observed between CD8+ and FOXP3+ lymphocyte infiltration, both in the stroma

\( r = 0.676, \ P < 0.001 \) (Fig. 2a) and in the intratumoural compartment \( r = 0.657, \ P < 0.001 \) (Fig. 2b).

CFR in stromal tumour-infiltrating lymphocytes and outcome of neoadjuvant therapy in all breast cancers

Patients who received neoadjuvant chemotherapy were divided into high- and low-CFR groups, and their clinicopathological features compared. There were 90 patients (50.8 per cent) in the high-CFR group and 87 (49.2 per cent) in the low-CFR group (Table I). TNBC \( (P = 0.002) \) and HER2BC \( (P = 0.033) \) were significantly more common, and HRBC was significantly less common \( (P < 0.001) \), in the high-CFR group than in the low-CFR group. In addition, nuclear grade \( (P = 0.042) \), Ki-67 \( (P = 0.003) \) and the pCR rate \( (P = 0.002) \) were significantly higher in the high-CFR group. No correlations between the CFR and other clinicopathological features were found.
Table 3 Correlations between tumour-infiltrating lymphocytes and clinicopathological parameters in triple-negative, human epidermal growth factor receptor 2-enriched and luminal-type breast cancers

|                     | TNBC                       | HER2BC                      | HRBC                       |
|---------------------|----------------------------|------------------------------|----------------------------|
|                     | High CFR (n=41)             | Low CFR (n=20)               | High CFR (n=24)             | Low CFR (n=12)               | P*        | High CFR (n=25)             | Low CFR (n=55)               | P*        |
| Age at operation (years) ≤ 56 | 18 (44) 10 (50)          | 0.319                        | 11 (46) 5 (42)            | 0.813                        | 0.215     | 16 (64) 27 (49)            | 0.531                        | 0.396     |
| > 56                | 23 (56) 10 (50)            | 0.310                        | 13 (54) 7 (58)            | 0.456                        | 0.531     | 9 (36) 28 (51)            | 0.547                        | 0.475     |
| In menopause        No | 13 (32) 9 (45)             | 0.584                        | 10 (42) 4 (33)            | 0.691                        | 0.544     | 13 (52) 23 (42)            | 0.547                        | 0.402     |
|                     Yes | 28 (68) 11 (55)            | 0.323                        | 14 (58) 8 (67)            | 0.222                        | 0.291     | 12 (48) 32 (58)            | 0.242                        | 0.350     |
| Tumour size (cm) ≤ 2 | 5 (12) 2 (10)              | 0.323                        | 4 (17) 2 (17)            | 0.544                        | 0.547     | 3 (12) 8 (15)             | 0.544                        | 0.475     |
| > 2                 | 36 (88) 18 (90)            | 0.323                        | 20 (83) 10 (63)           | 0.544                        | 0.547     | 22 (88) 47 (85)            | 0.544                        | 0.475     |
| Lymph node status Negative | 6 (15) 5 (25)              | 0.101                        | 7 (29) 4 (33)            | 0.162                        | 0.847     | 7 (28) 12 (22)            | 0.162                        | 0.847     |
|                     Positive | 35 (85) 15 (75)           | 0.210                        | 17 (71) 8 (67)           | 0.022                        | 0.291     | 18 (72) 43 (78)            | 0.022                        | 0.291     |
| Nuclear grade 1–2   | 27 (66) 17 (85)            | 0.355                        | 17 (71) 11 (92)           | 0.162                        | 0.847     | 20 (80) 45 (82)            | 0.162                        | 0.847     |
|                     3     | 14 (34) 3 (15)             | 0.210                        | 7 (29) 1 (8)             | 0.022                        | 0.291     | 5 (20) 10 (18)             | 0.022                        | 0.291     |
| Ki-67 (%) ≤ 14      | 10 (24) 8 (40)             | 0.210                        | 8 (33) 9 (75)            | 0.022                        | 0.291     | 10 (40) 29 (53)           | 0.022                        | 0.291     |
|                     > 14   | 31 (76) 12 (60)            | 0.210                        | 16 (67) 3 (25)           | 0.022                        | 0.291     | 15 (60) 26 (47)           | 0.022                        | 0.291     |
| Pathological response pCR | 23 (56) 5 (25)             | 0.022                        | 17 (71) 1 (8)            | < 0.001                     | 0.128     | 4 (16) 17 (31)            | 0.001                        | 0.128     |
|                     Non-pCR | 18 (44) 15 (75)           | 0.022                        | 7 (29) 11 (92)           | 0.022                        | 0.128     | 21 (84) 38 (69)            | 0.022                        | 0.128     |

Values in parentheses are percentages. TNBC, triple-negative breast cancer; HER2BC, human epidermal growth factor receptor 2-enriched breast cancer; HRBC, hormone receptor-positive breast cancer; CFR, CD8+/FOXP3+ tumour-infiltrating lymphocyte ratio; pCR, pathological complete response. χ² test.

Median follow-up for the assessment of DFS was 3.1 (range 0.1–6.0) years and that for overall survival was 3.4 (0.6–6.0) years. DFS was significantly longer in the high-CFR group than in the low-CFR group (P=0.005), but overall survival was not significantly different (P=0.057) (Fig. S1a,b, supporting information). In univariable analysis for recurrence, a high CFR was found to be a favourable prognostic factor (hazard ratio 0.35, 95 per cent c.i. 0.16 to 0.76; P=0.008) (Table 2). Multivariable analysis showed that a high CFR was an independent favourable prognostic factor (HR 0.37, 0.16 to 0.81; P=0.013)

CFR in stromal tumour-infiltrating lymphocytes and outcome in triple-negative breast cancer

Of the 61 patients with TNBC, 41 (67 per cent) were in the high-CFR group, and 20 (33 per cent) in the low-CFR group. The pCR rate was significantly higher in the high-CFR group than in the low-CFR group (P=0.022) (Table 3). Outcome analysis showed significantly longer DFS (P=0.001) and overall survival (P=0.003) in the high-CFR group (Fig. 3a,b). Patients in the high-CFR group had a significantly lower rate of recurrence in univariable (HR 0.14, 95 per cent c.i. 0.04 to 0.53; P=0.004) and multivariable (0.24, 0.05 to 0.72; P=0.015) analyses (Table 2).

CFR of stromal tumour-infiltrating lymphocytes and outcome in HER2-enriched breast cancers

Of the 36 patients with HER2BC, 24 (67 per cent) were in the high-CFR group and 12 (33 per cent) were in the low-CFR group. The Ki-67 rate (P=0.022) and pCR rate (P<0.001) were significantly higher in the high-CFR group than in the low-CFR group (Table 3). DFS was significantly longer in the high-CFR group (P=0.006), but overall survival was not (P=0.611) (Fig. 3c,d). In univariable analysis for recurrence, high-CFR status was a good prognostic factor (HR 0.12, 95 per cent c.i. 0.18 to 0.73; P=0.021). Multivariable analysis revealed high-CFR status as an independent good prognostic factor (HR 0.10, 0.10 to 0.90; P=0.041) (Table 2).

CFR in stromal tumour-infiltrating lymphocytes and outcome in hormone receptor-positive breast cancers

Of the 80 patients with HRBC, 25 (31 per cent) were in the high-CFR group and 55 (69 per cent) were in...
the low-CFR group. No correlation was found between CFR and any clinicopathological variable in these patients (Table 3). There was no correlation between CFR and outcomes (Table 2; Fig. S1c,d, supporting information).

Changes in CFR at breast cancer recurrence among patients with high CFR

Thirty patients (16.9 per cent) in this study had postoperative recurrence of breast cancer, of whom nine were in the high-CFR group and 21 in the low-CFR group. In the high-CFR group of patients with recurrence (TNBC 3, HER2BC 2, HRBC 4), three (TNBC 1, HER2BC 1, HRBC 1) had a pCR after neoadjuvant chemotherapy. Needle biopsy specimens before neoadjuvant chemotherapy, surgical specimens after neoadjuvant chemotherapy and needle biopsy specimens after recurrence (available for 7 patients) were reviewed. There were no changes in the CFR in three patients with HRBC. However, in patients with highly malignant breast cancer (TNBC 2, HER2BC 2), the CFR was decreased in needle biopsy specimens after recurrence in both intratumoural and stromal TILs.

Fig. 3 Analysis of CD8+/FOXP3+ tumour-infiltrating lymphocyte ratio (CFR) status and outcome in patients with a,b triple-negative breast cancer (TNBC) and c,d human epidermal growth factor receptor 2-enriched breast cancer (HER2BC): a,c disease-free survival and b,d overall survival. a P = 0.001, b P = 0.003, c P = 0.006, d P = 0.611 (log rank test)
Discussion

TILs serve as an indicator for regulation of the tumour immune microenvironment in the host in response to a cancer. TIL subsets include cells such as CD8+ T lymphocytes that eradicate cancer cells, and those such as Treg cells that promote survival and proliferation of cancer cells. FOXP3 expression is induced upon activation of naive CD4+ T cells. Previous studies have shown by double staining that almost all FOXP3+ cells were CD4+, and FOXP3+ cells were counted as Treg. An experimental study in mice with FOXP3-specific depletion of Treg cells showed that, by decreasing Treg cells, the antitumour immune response by CD8+ T lymphocytes was increased, and tumour proliferation suppressed. This indicates that a large number of Treg cells in the tumour microenvironment leads to an immunosuppressive effect that inhibits the function of CD8+ T lymphocytes. Therefore, to improve the tumour immune microenvironment, the number and function of Treg cells must be controlled. The CFR, as an indicator of the balance between CD8+ TILs and Treg cells in a tumour, may be useful for predicting the response to treatment with chemotherapy. Among patients with breast cancer who received neoadjuvant chemotherapy in the present study, the pCR rate was significantly higher in the high-CFR group than in the low-CFR group, and this translated into a longer DFS.

Analysis of TIL subsets in patients with breast cancer receiving neoadjuvant chemotherapy has shown higher pCR rates, with high levels of expression of CD3, CD4, CD8 and FOXP3, and higher pCR rates with a high CFR have also been reported in TNBC. However, there have been few studies with stratification for intrinsic breast cancer subtypes. In the present study, a pCR was significantly more common in patients with aggressive subtypes and a high CRF, but this was not observed for HRBC. In the patients with aggressive breast cancer subtypes, lymphocytes such as CD8+ TILs that function in tumour immunity have already infiltrated the tumour, and these are controlled by antitumour immunity through Treg cells.

Many anticancer drugs have immunosuppressive effects but, depending on their mode of administration, enhancement of the immune activity or reversal of immunosuppression is possible. Improvement in immunescape on the cancer cell side and host side is involved in the mechanism of enhancement of antitumour immunity with anticancer drugs. In the present study, a regimen of FEC followed by paclitaxel + trastuzumab, 5-fluorouracil and paclitaxel has been shown to improve immunescape by improving the decreased sensitivity of CD8+ T cells. In addition, paclitaxel also improves immunescape by inhibiting Treg cells.

The present study also examined CFR changes in needle biopsy tissue samples of lesions after a local recurrence to investigate the relationship between CFR and post-operative recurrence. The CFR decreased in recurrent TNBC and HER2BC, but remained unchanged in the HRBC subtype. This suggests that a decreased CFR may be involved in recurrences of highly malignant breast cancers, and that reduced tumour immunity in the cancer microenvironment plays a role in recurrence. As the numbers of recurrences were low in the present cohort, this finding should, however, be interpreted with caution and replicated in larger studies.

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853

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Supporting information

Additional supporting information may be found in the online version of this article:

Fig. S1 Analysis of the CD8+/FOXP3+ tumour-infiltrating lymphocyte ratio status and outcome of all patients with breast cancer and those with hormone receptor-positive breast cancer (Word document)

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