Validation of the Optimum Timing of Assessment of Tumor Infiltrating Lymphocytes During Preoperative Chemotherapy for Breast Cancer

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Abstract. Background/Aim: Tumor microenvironment (TME) assessment is considered to play an important role in the prediction of prognosis and therapeutic response following breast cancer treatment. No consensus has been reached regarding evaluation methods despite reports on the utilization of tumor-infiltrating lymphocytes (TILs) for immune TME (iTME) monitoring. Optimum timing of iTME assessment has not yet been established. Patients and Methods: Two hundred thirty-nine patients were treated with neoadjuvant chemotherapy (NAC). During the period from diagnostic needle biopsy to NAC initiation for breast cancer, the optimal evaluation timing was examined using a receiver operating characteristic (ROC) curve analysis. Results: A significant correlation between TILs and pathological complete response (pCR) was only observed in the short-term group (≤35 days) (p=0.033). Prognostic analysis revealed that in the short-term group, patients with high TIL levels had a significantly better survival prognosis relative to those with low TIL levels (>35 days) [disease-free survival (DFS): p=0.001, overall survival (OS): p=0.021]. TILs were identified as an independent factor affecting DFS in a multivariate analysis (p=0.008, hazard ratio=0.130). Conclusion: TIL assessment during NAC for breast cancer is a prognostic predictor only when performed at ≤35 days before NAC initiation.

Cancers not only directly affect the tumor cells, but also influence the local microenvironment (e.g., surrounding stromal cells and blood vessels) to enhance tumor cell survival. Accordingly, tumor tissues include not only cancer cells, but also inflammatory cells, immune cells, component cells of blood vessels and lymphatic vessels, fibroblasts, and fibrous tissues, which create the characteristic tumor microenvironment (TME) (1). Regarding cancer treatment, evaluation of the host TME has been considered to play an important role in the prediction of prognosis and therapeutic effects (2). Furthermore, the immune system within the host TME has been noted as immune TME (iTME). Furthermore, the strategy of treatment related to iTME may be a key factor in the future development of cancer therapies (3-5). Previous reports have described the use of tumor-infiltrating lymphocytes (TILs) in various carcinomas, including breast cancer, for monitoring the iTME (4, 6-11). However, a consensus regarding the iTME assessment method has not been reached (12). Identification of the optimal region and timing for TIL assessment will facilitate the design of optimal treatment options based on accurate iTME monitoring.

TIL is a generic designation for the lymphocytes that accumulate within tumors, and the in situ expression status of these cells is thought to play an important role in tumor-associated immune mechanisms. In breast cancer, the in situ expression status of TILs was found to be useful for predicting prognosis and chemotherapeutic effects (6, 13-15).
Therefore, an assessment of the TIL expression is important and must be done simply and reproducibly. Regarding the optimal region of TIL evaluation, the International Working Group currently recommends the tumor stroma (12). However, the iTME exhibits dynamic changes, and the timing of the assessment has not yet been optimized. Therefore, in this study, we evaluated and clinically verified the timing of assessment for predicting therapeutic effects to neoadjuvant chemotherapy (NAC), using TILs as an indicator.

Patients and Methods

**Patient background.** This is a retrospective analysis based on archival tissue samples. A total of 239 patients with resectable early-stage breast cancer received NAC between 2007 and 2015 at Osaka City University Hospital, Osaka, Japan. Patients were diagnosed with stage IIA (T1, N1, M0 or T2, N0, M0), IIB (T2, N1, M0 or T3, N0, M0), or IIIA disease (T1-2, N2, M0 or T3, N1-2, M0). Qualitative breast cancer diagnoses were confirmed histologically by core needle biopsy (CNB) or vacuum-assisted biopsy (VAB). Breast cancer stage stratification was based on the TNM Classification of Malignant tumors, Union for International Cancer Control (UICC), Seventh Edition (16). Breast cancer subtypes were classified through an immunohistochemical evaluation of estrogen receptor (ER), progesterone receptor (PgR), human epidermal growth factor receptor (HER) 2, and Ki67 expression, as follows: luminal A, ER+ and/or PgR+, HER2-, Ki67-low; luminal B, ER+ and/or PgR+, HER2+ or ER+ and/or PgR+, HER2-, Ki67-high; HER2-enriched (HER2BC), ER-, PgR-, and HER2+; and triple-negative breast cancer (TNBC), negative for ER, PgR and HER2 (17). A Ki67-labeling index was considered positive with ≥14% of tumor cells with nuclear staining (17).

All patients received NAC per a standardized protocol. The following regimen was administered: four courses of FEC100 (500 mg/m² fluorouracil, 100 mg/m² epirubicin, and 500 mg/m² cyclophosphamide) every 3 weeks, followed by 12 courses of 80 mg/m² paclitaxel administered weekly (18-20). Patients with HER2-positive breast cancer additionally received weekly (2 mg/kg) or tri-weekly (6 mg/kg) trastuzumab therapy (21).
Patients underwent mastectomy or breast-conserving surgery following NAC (22). The anti-tumor effects of NAC were assessed in accordance with the Response Evaluation Criteria in Solid tumors (RECIST) (23). A pathological complete response (pCR) was defined as an absence of residual invasive carcinoma in both the breast and lymph nodes of surgically resected specimens, regardless of residual intraductal carcinoma (24). Patients who underwent breast-conserving mastectomy were administered adjuvant radiotherapy to the residual breast. Standard postoperative adjuvant therapy was administered according to the breast cancer subtype. Disease-free survival (DFS) was defined as the period from the initiation of NAC to the occurrence of any local, locoregional, and/or distant recurrence or death from any cause. Overall survival (OS) was defined as the period from the initiation of NAC to the time of death from any cause. The median follow-up periods were 3.4 years (range=0.1-6.0 years) for DFS and 3.7 years (range=0.1-6.0 years) for OS.

Immunohistochemistry. Immunohistochemical staining for ER, PgR, HER2, and Ki-67 was performed on formalin-fixed, paraffin-embedded tumor tissues obtained before and after chemotherapy. Primary monoclonal antibodies directed against ER (clone 1D5, dilution 1:80; Dako, Cambridge, UK), PgR (clone PgR636, dilution 1:100; Dako), HER2 (HercepTest™; Dako), and Ki67 (clone MIB1, dilution 1:100; Dako) were used. The cut-off values for ER and PgR positivity were set at 1%. HER2 status was evaluated according to the ASCO/CAP HER2 Test Guideline Recommendations (25). Briefly, breast cancers were defined as HER2 positive with an immunohistochemical score of 3+, or 2+ with HER2 gene amplification indicated by fluorescence in situ hybridization.

Region of histopathological TIL evaluation. A histopathological assessment of predictive factors was performed using biopsy specimens collected at the time of breast cancer diagnosis. The optimal region for TIL assessment was selected according to the recommendations of the International Working Group (12). Specifically, TILs were measured by examining the occupation ratio of immune cells present in the tumor stroma of hematoxylin and eosin (HE) stained specimens at 400× magnification (9, 26). Necrotic tissue and surrounding normal tissues were not included in the evaluation region. Proportional scores of 3, 2, 1, and 0 were given if the area of stroma containing lymphoplasmacytic infiltration around invasive tumor cell nests comprised >50%, >10-50%, ≤10%, and 0%, respectively (Figure 1). A score of ≥2 was considered positive for TILs, whereas scores of 1 and 0 were considered negative. Two pathologists evaluated the TILs independently and were blinded to the patient information. If the evaluations were discordant, the slides were reviewed, and a final score was reached by consensus.

Timing of histopathological evaluation of TILs. The time of biopsy for the diagnosis of breast cancer and the time of NAC initiation were set as the start and end points, respectively. The interval between the start and end points was calculated, and a receiver operating characteristic (ROC) curve for DFS was used to determine a cut-off value of 35 days as the optimum timing of assessment. This cut-off value was used to assign subjects to either a short-term (≤35 days) or long-term group (>35 days). We compared prognosis between the groups and conducted clinicopathological background and prognostic analyses based on the TIL expression status.

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Statistical analysis. We used the SPSS version 19.0 statistical software package (IBM, Armonk, NY, USA) for all statistical analyses. Associations between the TILs and clinicopathological variables were examined using the chi square or Fisher’s exact test. The Cox proportional hazards model was used to compute univariate and multivariate hazard ratios (HR) with 95% confidence intervals (CIs) for the study parameters, and a backward stepwise method was used for variable selection in the multivariate analyses. DFS and OS were estimated using the Kaplan–Meier method and compared using the log-rank test. Differences were considered statistically significant at p-values of <0.05.

Results

Prognostic analysis of the long-term and short-term patient groups. Regarding TIL evaluation, the mean interval from the time of biopsy for breast cancer diagnosis to the start of NAC was 39 days (median: 35 days, range=13-98 days). As noted above, the ROC curve analysis of DFS yielded a cut-off value of 35 days (sensitivity: 57.4%, specificity: 38.5%, area under the curve: 0.592, p=0.050, 95% CI=0.500-0.685), and used to stratify patients into short-term (≤35 days) and long-term groups (>35 days) (Figure 2). Of the 239 NAC patients, 118 (49.4%) and 121 (50.6%) were classified into the short-term and long-term groups, respectively. DFS was significantly longer in the short-term group than the long-term group (p=0.020, log-rank) (Figure 3). OS was significantly longer in the short-term group than the long-term group (p=0.010, log-rank).
Prognostic analysis of the short-term and long-term groups based on TIL evaluation. We further confirmed the correlation between clinicopathological background factors and TILs. In the short-term group, 63 (53.4%) and 55 patients (46.6%) had high and low TIL expression statuses, respectively, whereas in the long-term group, 66 (54.5%) and 55 patients (45.5%) had high and low TIL expression statuses, respectively. In the short-term group, the pCR rate was higher among patients with high TIL expression ($p=0.042$), whereas no correlations with other factors were noted (Table I). By contrast, in the long-term group, no factors were found to correlate with the TIL expression status.

A prognostic analysis revealed that in the short-term group, those with high TIL levels had a significantly prolonged survival prognosis, compared to those with low TIL levels ($p=0.001$ and $p=0.021$, respectively; log-rank). In addition, no effect of TIL expression on the prognosis was observed in the long-term group (DFS: $p=0.529$, OS: $p=0.457$; log-rank) (Figure 4). TILs were also found to be a significant factor affecting DFS in the univariate analysis ($p=0.004$, HR=0.115), as well as an independent factor affecting DFS in a multivariate analysis ($p=0.008$, HR=0.130) (Table II) (Figure 5).

Discussion

Cancer cells were thought to proliferate autonomously and survive as a consequence of various genetic abnormalities; however, the surrounding environment (i.e., TME) has been found to greatly affect cancer cells and the formation of cancer-specific characteristics (27). Furthermore, the importance of iTME control has recently been recognized, as this factor affects not only the efficacy of immunotherapy, but also the efficacies and prognosis related to other treatments, such as anti-cancer chemotherapy (3, 28). Various types of immune cell infiltration are observed in tumor tissues; however, the infiltration of immune suppressor cells [regulatory T cells (Tregs), myeloid derived suppressor cells (MDSCs), etc.] and production of immunosuppressive substances establishes immunosuppression and facilitates tumor immune escape (27, 29).

Cancer immunoediting comprises three phases: the elimination phase (that is, cancer immune surveillance), equilibrium phase, and escape phase (29-33). Of these, the escape phase results from the ability of cancer cells to escape recognition and elimination by immune surveillance mechanisms, and thus represents the clinical manifestation of cancer (27). These escape mechanisms are thought to be effectively controlled by immune checkpoint inhibitors, and accordingly the utility of anti-PD-1 and anti-CTLA-4 antibodies, among others, have been demonstrated by large-scale clinical trials (33-35). Even in clinical practice, OS is extended in melanoma and lung cancer, and its effect is expected also in breast cancer. Clinical trials of anti-PD-1 and anti-PD-L1 antibodies are ongoing in breast cancer. In brief, the cancer immune response changes dynamically, and an understanding of the iTME, and particularly its strong immunosuppressive network, may be the key to future anticancer therapies.
predictive marker of the therapeutic effects of NAC. The iTME has been noted to facilitate monitoring of the iTME. Therefore, if the interval from breast cancer diagnosis by biopsy to NAC initiation was short, it may be considered of optimum timing when utilizing TILs as a predictive biomarker of the therapeutic effects of NAC.

When using TILs to evaluate the dynamically changing iTME, a standard optimum region and timing must be set. The iTME fluctuates dynamically along with cancer progression, leading to the concept of a cancer immunoarray, which chronologically depicts the cancer-related immune response (37). First, as tumor antigens are released from tumor cells, antigen-presenting cells (APCs) such as dendritic cells take up and present these antigens on surface MHC molecules and migrate to the lymph nodes. Once in the lymph node, the APCs present the tumor antigens to T cells, leading to the activation of antigen-specific T cells. Subsequently, these activated T cells migrate to and invade tumor tissues. This latter step is observed during the assessment of TILs in situ. tumor cells that have been attacked and killed by T cells can also release new tumor antigens. The evaluation of TILs in situ may allow the observation of some steps in this cancer immunity cycle and facilitates monitoring of the iTME. Therefore, if the interval from biopsy to treatment initiation is short, it may be suitable as an optimal evaluation region (36).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Short-term group (n=118)</th>
<th>p-Value</th>
<th>Long-term group (n=121)</th>
<th>p-Value</th>
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<tr>
<td></td>
<td>High (n=63)</td>
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<tr>
<td>Age at chemotherapy ≤55</td>
<td>30 (47.6%)</td>
<td>0.970</td>
<td>36 (54.5%)</td>
<td>0.841</td>
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<tr>
<td>Age at chemotherapy &gt;55</td>
<td>33 (52.4%)</td>
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<td>30 (44.5%)</td>
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<td>Menopause</td>
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<tr>
<td>Negative</td>
<td>25 (39.7%)</td>
<td>0.868</td>
<td>28 (42.4%)</td>
<td>0.893</td>
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<tr>
<td>Positive</td>
<td>38 (60.3%)</td>
<td></td>
<td>38 (57.6%)</td>
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<tr>
<td>Tumor size ≤2 cm</td>
<td>11 (17.5%)</td>
<td>0.874</td>
<td>12 (18.2%)</td>
<td>0.263</td>
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<tr>
<td>Tumor size &gt;2 cm</td>
<td>52 (82.5%)</td>
<td></td>
<td>54 (81.8%)</td>
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<td>Lymph node status Negative</td>
<td>21 (33.3%)</td>
<td>0.104</td>
<td>18 (27.3%)</td>
<td>0.825</td>
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<td>Positive</td>
<td>42 (66.7%)</td>
<td></td>
<td>48 (72.7%)</td>
<td></td>
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<tr>
<td>Nuclear grade 1, 2</td>
<td>49 (77.8%)</td>
<td>0.525</td>
<td>54 (81.8%)</td>
<td>0.793</td>
</tr>
<tr>
<td>Nuclear grade 3</td>
<td>14 (22.2%)</td>
<td></td>
<td>12 (18.2%)</td>
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<tr>
<td>Ki67 ≤14%</td>
<td>24 (38.1%)</td>
<td>0.074</td>
<td>27 (40.9%)</td>
<td>0.615</td>
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<tr>
<td>Ki67 &gt;14%</td>
<td>39 (61.9%)</td>
<td></td>
<td>39 (59.1%)</td>
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<tr>
<td>Intrinsic subtype TNBC</td>
<td>21 (33.3%)</td>
<td>0.779</td>
<td>21 (31.8%)</td>
<td>0.180</td>
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<tr>
<td>Non-TNBC</td>
<td>42 (66.7%)</td>
<td></td>
<td>45 (68.2%)</td>
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<tr>
<td>Intrinsic subtype HER2BC</td>
<td>11 (17.5%)</td>
<td>0.724</td>
<td>14 (21.2%)</td>
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<td>Non-HER2BC</td>
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<td>52 (78.8%)</td>
<td></td>
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<tr>
<td>Intrinsic subtype HRBC</td>
<td>31 (49.2%)</td>
<td>0.990</td>
<td>31 (47.0%)</td>
<td>0.331</td>
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<tr>
<td>Non-HRBC</td>
<td>32 (50.8%)</td>
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<td>35 (53.0%)</td>
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<tr>
<td>Pathological response pCR</td>
<td>26 (41.3%)</td>
<td>0.042</td>
<td>26 (39.4%)</td>
<td>0.105</td>
</tr>
<tr>
<td>Non-pCR</td>
<td>37 (58.7%)</td>
<td></td>
<td>40 (60.6%)</td>
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</tbody>
</table>

TILs: Tumor-infiltrating lymphocytes; 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.
possible to determine the optimal timing for an accurate evaluation of TILs.

In the future, iTME evaluations are expected to play a key role in the development of individualized cancer treatments; however, whether an appropriate immune response can be evaluated using TILs remains an issue for future studies. Despite individual differences in the cancer immunologic conditions in human, the implementation of a clinically appropriate iTME monitoring using TILs would be expected to contribute to improvements in therapeutic effects and prognosis. However, the present study is limited by its retrospective design and the small number of subjects. A prospective review of therapeutic effects and prognosis in the context of an investigation of the optimal region and timing for TILs evaluation should be conducted in the future.

Conclusion

TIL assessment during NAC for breast cancer may be a useful prognostic factor only if the interval from biopsy to treatment initiation is short (≤35 days).

Conflicts of Interest

All of the Authors have no conflicts of interest to disclose regarding this study.

Authors’ Contributions

All Authors were involved in the preparation of this manuscript. SK collected the data and wrote the manuscript. SK, YA, KT, WG, RK, AY, YT, TM and KO performed the operation and designed the
study. SK, MS, and HT summarized the data and revised the manuscript. 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**

The Authors thank Tomomi Okawa (Department of Breast and Endocrine Surgery, Osaka City University Graduate School of
Medicine, Osaka, Japan) for the helpful advice regarding data management. This study was funded by grants from the Japan Society for the Promotion of Science (Tokyo, Japan) (KAKENHI, Nos. 20K08938, 26461957, and 17K10559) to Shinichiro Kashiwagi.

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Received March 14, 2022
Revised May 15, 2022
Accepted May 16, 2022

451

Kashiwagi et al: Optimum Timing of TILs During NAC in Breast Cancer