Efficacy, safety, and biomarker analysis of nivolumab in combination with abemaciclib plus endocrine therapy in patients with HR-positive HER2-negative metastatic breast cancer: a phase II study (WJOG11418B NEWFLAME trial)

Jun Masuda,1,2 Hitomi Sakai,3,4 Junji Tsurutani,3 Yuko Tanabe,2 Norikazu Masuda,5,6 Tsutomu Iwasa,4 Masato Takahashi,7,8 Manabu Futamura,9 Koji Matsumoto,10 Kenjiro Aogi,11 Hiroji Iwata,12 Mari Hosonaga,1 Toru Mukohara,13 Kiyoshi Yoshimura,14 Chiyo K Imamura,3 Sakiko Miura,15 Toshiko Yamochi,15 Hitetaka Kawabata,16 Hiroyuki Yasojima,6 Nobumoto Tomioka,7 Kenichi Yoshimura,17 Toshiko Takano1,2

ABSTRACT

Background  Hormone receptor (HR)-positive breast cancer is a disease for which no immune checkpoint inhibitors have shown promise as effective therapies. Cyclin-dependent kinase 4 and 6 (CDK4/6) inhibitors synergistically increased the effectiveness of antiprogrammed cell death protein-1 (anti-PD-1)/programmed death-ligand 1 (PD-L1) antibodies in preclinical studies.

Methods  This non-randomized, multicohort, phase II study evaluated the efficacy and safety of the anti-PD-1 antibody nivolumab 240 mg administered every 2 weeks in combination with the CDK4/6 inhibitor abemaciclib 150 mg twice daily and either fulvestrant (FUL) or letrozole (LET) as a first-line or second-line treatment for HR-positive HER2-negative metastatic breast cancer. The primary end point was the objective response rate (ORR), and secondary end points were toxicity, progression-free survival, and overall survival. Blood, tissue, and fecal samples were collected at multiple points for correlative studies to evaluate immunity biomarkers.

Results  From June 2019 to early study termination due to safety concerns on July 2020, 17 patients were enrolled (FUL: n=12, LET: n=5). One patient with a prior treatment history in the FUL cohort was excluded. ORRs were 54.5% (6/11) and 40.0% (2/5) in the FUL and LET cohorts, respectively. Treatment-emergent (TE) adverse events (AEs) of grade ≥3 occurred in 11 (92%) and 5 (100%) patients in the FUL and LET cohorts, respectively. The most common grade ≥3 TEAEs were neutropenia (7 (58.3%) and 3 (60.0%) in the FUL and LET cohorts, respectively, followed by alanine aminotransferase elevation (5 (41.6%) and 4 (80.0%)). One treatment-related death from interstitial lung disease occurred in the LET cohort. Ten patients developed liver-related grade ≥3 AEs. Liver biopsy specimens from 3 patients showed hepatitis characterized by focal necrosis with predominant CD8+ lymphocyte infiltration. Marked elevation of tumor necrosis factor-related cytokines and interleukin-11, and a decrease in peripheral regulatory T cells (Tregs), were observed in patients with hepatotoxicity. These findings suggest that treatment-related toxicities were immune-related AEs likely caused by proinflammatory cytokine production and suppression of Treg proliferation due to the addition of abemaciclib to nivolumab therapy.
CONCLUSIONS
Although the combination of nivolumab and abemaciclib was active, it caused severe and prolonged immune-related AEs.

Trial registration number JapicCTI-194782, JRCT2080224706, UMIN000036970.

BACKGROUND
Hormone receptor (HR)-positive HER2-negative breast cancer accounts for approximately 70% of breast malignancies.1 The combination of endocrine therapy (ET) and cyclin-dependent kinase 4 and 6 (CDK4/6) inhibitors (abemaciclib, palbociclib, or ribociclib) is a standard first-line or second-line treatment for HR-positive HER2-negative metastatic breast cancer,2-4 and provides a clinical benefit rate of about 80% (defined as the proportion of patients with a confirmed complete or partial response or stable disease for ≥24 weeks), and >2 years of progression-free survival (PFS) when used in the first-line setting.5 However, since most patients develop endocrine resistance in the clinical course, there is an unmet need for therapies with higher response rates and extended efficacy.

Immunotherapy of breast cancer remains a significant challenge despite the great promise it holds. In HR-positive breast cancer, only a small subset of patients responds to immunotherapy. The response rate of antiprogrammed cell death protein-1 (anti-PD-1)/programmed death-ligand 1 (PD-L1) antibody monotherapy was reported to be 12.0% in a PD-L1-positive HR-positive breast cancer population6 and 2.8% in a non-selected HR-positive population.7 The immunosuppressive tumor microenvironment of HR-positive breast cancer is generally considered the potential cause. HR-positive breast cancer has less tumor-infiltrating lymphocyte (TIL) infiltration than other subtypes of breast cancer,8 with generally lower tumor mutational burden and PD-L1 expression.9 10 However, emerging evidence indicates that roughly 10% of patients with HR-positive HER2-negative breast cancer have a high TIL density of ≥60%.11

To overcome the immune microenvironment of HR-positive breast cancer, several studies have explored combination therapy of anti-PD-1/PD-L1 antibodies with other targeted therapies. Recent preclinical studies have shown that CDK4/6 inhibitors promote antitumor immunity and enhance tumor immunogenicity through enhanced tumor antigen presentation,12-14 suppressed proliferation of regulatory T (Treg) cells,14 15 enhanced effector T-cell activation,14 and induction of T-cell memory.15 16 17

In the present multicenter, multicohort, non-randomized, open-label phase II study (Nivolumab Evaluation With endocrine therapy, Fulvestrant or Letrozole, and AbeMaciclib: NEWFLAME trial), we evaluated the efficacy and safety of the combination of nivolumab and abemaciclib plus ET as a first-line or second-line treatment for patients with HR-positive HER2-negative metastatic breast cancer, in conjunction with correlative studies to investigate the mechanism underlying adverse events (AEs).

METHODS
Study design and patients
The NEWFLAME trial was a multicenter, multicohort, non-randomized, open-label phase II study to evaluate the efficacy and safety of the combination of nivolumab, abemaciclib, and ET (fulvestrant (FUL) or letrozole (LET)) in patients with HR-positive HER2-negative metastatic breast cancer (online supplemental file 2). The safety of the combination of nivolumab, abemaciclib, and ET (FUL or LET) has not been established, although the full doses of pembrolizumab and abemaciclib had been shown tolerable.14 Therefore, a phase II study including a safety cohort of first 6 subjects with each ET was planned. The study was conducted in 11 institutions affiliated with the West Japan Oncology Group (WJOG) in Japan. The first six patients in each cohort were enrolled in the safety phase of the trial and evaluated for dose-limiting toxicities (DLTs). Correlative studies were conducted to evaluate immunity biomarkers (WJOG111418BTR).

Eligible patients were those with histologically confirmed HR-positive HER2-negative metastatic breast cancer. Other criteria included age ≥20 years, Eastern Cooperative Oncology Group performance status ≤1, and adequate organ function. The LET cohort included only postmenopausal women, whereas the FUL cohort included women regardless of menopausal status.

Patients in the FUL cohort had exhibited disease progression while receiving ET or adjuvant ET, or at ≤12 months after adjuvant ET. Patients allocated to the LET cohort were not allowed to have prior systemic therapy in the metastatic setting, and only ET as an adjuvant therapy was permitted if patients had a disease-free interval of >12 months after the completion of ET; patients had to have measurable lesions based on Response Evaluation Criteria in Solid Tumors Revised guidelines V.1.1. Major exclusion criteria included prior treatment with everolimus or CDK4/6 inhibitors, the presence of visceral crisis, and evidence or history of central nervous system metastasis.

The coordinating committee oversaw the conduct of the study. An independent data and safety monitoring committee (iDSMC) reviewed the safety data quarterly.

Treatment
Patients received 240 mg nivolumab every 2 weeks, 150 mg abemaciclib twice daily, and either 500 mg FUL on days 1, 15, 29, and every 4 weeks thereafter (FUL cohort) or 2.5 mg LET once daily (LET cohort). Study treatment was continued until disease progression, unacceptable toxicity, or a decision to discontinue by either the patient or investigator. Dose reduction and discontinuation for toxicity were specified in the study protocol.

End points
In the safety cohort, the primary end point was the incidence of DLTs, and the secondary end point was the incidence of AEs. The period of assessment of DLTs was the first 4 weeks of the investigational treatments. DLT
was defined as the occurrence of any of the following criteria for toxicity, except when the causal relationship between toxicity and treatment can be ruled out: grade 3 non-hematological toxicity (excluding nausea, vomiting, diarrhea, and electrolyte abnormalities); grade 3 or higher nausea, vomiting, or diarrhea that remains uncontrollable despite appropriate supportive therapy; grade 4 hematological toxicity lasting for >5 days; grade 3 or higher febrile neutropenia; and a discontinuation rate of abemaciclib or ET exceeding 50% during the DLT evaluation period. If DLTs causally related to the treatment were observed in ≤2 patients out of the first 6 patients, the experimental treatment was deemed feasible, and proceeded to the efficacy cohort. In the efficacy cohort, the primary end point was the centrally assessed objective response rate (ORR). Objective responses were confirmed by at least two consecutive assessments performed at least 4 weeks apart. Secondary end points were toxicity, disease control rate (DCR), PFS, and overall survival (OS). DCR was determined as the proportion of patients with a best response of complete response, partial response, or stable disease. PFS was defined as the period from enrollment to the day of disease progression or death from any cause. OS was defined as the period from enrollment to the day of death from any cause. The median duration of liver-related AEs was calculated using the longest duration of liver-related AEs in each patient.

Sample collection and preparation
Blood, tissue, and fecal samples were collected for correlative studies at multiple time points. The blood collection schedule is summarized in online supplemental table 1. Pretreatment tissue samples were obtained. Fecal samples were obtained on day 1 of cycle 1 and cycle 3. Detailed sample collection and preparation methods are described in online supplemental table 1 and online supplemental file 1.

Immunohistochemistry and liver biopsy evaluation
Liver biopsy specimens were fixed in 10% buffered formalin and embedded in paraffin following standard procedures at each study site. Immunostaining and evaluation were performed by two pathologists. Histological evaluation of the liver focused on the extent and distribution of inflammation and necrosis and the type of inflammatory cells. Immunostaining with primary monoclonal antibodies against CD3, CD4, CD8, and FOXP3 was performed using an automated immunostainer (BOND-III: Leica Biosystems, Newcastle, UK) according to the manufacturer’s protocol. The antigen retrieval procedure was performed by heat-induced epitope retrieval with BOND epitope retrieval solution 1 (Leica Biosystems). Referencing a previous report, the numbers of lymphocytes positive for each marker were counted at three hot spots and calculated as the average per high power field.18 Ratios of CD20+/CD3+ and CD4+/CD8+ cells were also calculated. Scoring of staining was performed by two pathologists. The antibodies used for immunohistochemistry are summarized in online supplemental table 2.

Flow cytometric analysis
Cells were preincubated with an unlabeled anti-CD16/32 monoclonal antibody (mAb) to prevent the antibodies from nonspecifically binding to Fc receptors, and then incubated with PE-conjugated or APC-conjugated mAb. After washing twice with FACS buffer, stained cells (live-gated on the basis of forward and side scatter profiles) were analyzed on a BD LSRFortessa X-20 machine (BD Biosciences). The antibodies used for flow cytometric analysis are summarized in online supplemental table 2.

Multiplex analysis of cytokines
Serum samples obtained at baseline and post-treatment time points were evaluated. Serum cytokines were measured using the Bio-Plex Pro Human Inflammation Panel 1, 37-Plex assay (Bio-Rad Laboratories, Hercules, California, USA) and Bio-PlexTM 200 (Bio-Rad Laboratories) according to the manufacturer’s instructions.

16S metagenome sequencing
Genomic DNA was extracted using the QIAamp PowerFecal Pro DNA Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions from fecal samples, which were collected using a stool collection kit containing guanidine (TechnoSurasu Laboratory, Shizuoka, Japan) and stored at 80°C until analysis. Metagenome analysis was performed on a next-generation sequencer (MySeq: Illumina, San Diego, California, USA) to analyze the 16S V3 and V4 regions of ribosomal RNA genes. Quiime2 (https://qiime2.org/) was used to identify bacteria.

Statistical analysis
In the safety cohort, AEs and DLTs were monitored in a timely manner. After the first six patients had completed one cycle of treatment, the number of patients with DLTs was counted. These six patients were also assessed and included for all outcomes using the same schedule and criteria as subsequent patients. In the efficacy cohort, the ORR of abemaciclib plus FUL in patients with HR-positive HER2-negative metastatic breast cancer with measurable disease was previously reported to be 48.1%.19 The threshold and expected ORRs for the FUL cohort were 45% and 60%, respectively. Based on these, the number of patients needed to achieve 80% power at a one-sided significance level of 0.20 was calculated to be 32. Taking ineligible patients into account, the sample size was set at 35. The ORR of CDK4/6 inhibitor plus LET in patients with HR-positive HER2-negative metastatic breast cancer with measurable disease was previously reported to be 52.7%–59.2%.20 21 The threshold and expected ORRs for the LET cohort were 55% and 75%, respectively. Accordingly, the number of patients needed to achieve 80% power at a one-sided significance level of 0.20 was calculated to be 16. Taking ineligible patients into account, the sample size was set
at 18. We planned to analyze ORRs and DCRs with exact 95% CIs using the Clopper-Pearson method. Efficacy was assessed in the full analysis set (FAS) comprising all registered patients except those who were found to be ineligible. Safety was evaluated in the safety analysis set (SAS), which included all patients who received at least one dose of treatment.

For correlative studies, one-way analysis of variance and analysis of covariance were performed with \( p < 0.01 \) considered statistically significant. Results are presented as mean±SE. JMP software V.14.3.0 and SAS V.9.4 (both SAS Institute, Cary, North Carolina, USA) were used for safety and efficacy analyses. JMP V.16.0 (SAS Institute) was used for statistical analyses for correlative studies. \( p < 0.05 \) was considered statistically significant.

Further methods are described in online supplemental file 1.

RESULTS

Patient characteristics

From June 2019 to early study termination in December 2019, 17 patients (FUL cohort, n=12; LET cohort, n=5) from 11 institutions in Japan were enrolled in the study. One patient with a prior treatment history in the FUL cohort was excluded from the efficacy analysis. Data cut-off occurred on 31 July 2020. Baseline patient characteristics are summarized in table 1. In the FUL cohort, most patients had visceral disease, and equal proportions of premenopausal and postmenopausal patients were included. All patients were evaluated as PD-L1 negative with PD-L1 expression <1% in tumor-infiltrating immune cells.

Tolerability and AEs

In the safety cohort consisting of the first six patients, DLT was observed in the first patient (grade 3 pancreatitis) in the FUL cohort, and the experimental treatment was deemed feasible. The 10th patient experienced DLTs (grade 3 interstitial lung disease (ILD) and grade 3 dehydration) in the LET cohort. At the time of safety review in December, 6 of the first 17 patients treated with the study regimen experienced grade \( \geq 3 \) hepatotoxicity, leading to treatment discontinuation. Enrollment in each cohort was temporarily suspended based on a decision by the iDSMC in December 2019. After other clinical trials of abemaciclib in combination with ICIs reported increases in severe ILD, the iDSMC decided to discontinue the NEWFLAME trial on July 31, 2020.

An overview of safety by cohort is presented in table 2. Treatment-emergent (TE) grade \( \geq 3 \) AEs were observed in 11 (91.6%) and 5 (100%) patients in the FUL and LET cohorts, respectively. One treatment-related patient death occurred in the LET cohort (immune-related ILD). Seven (58.3%) and 3 (60.0%) patients in the FUL and LET cohorts, respectively, discontinued the treatment due to AEs (seven with liver-related toxicities, one with ILD, one with acute pancreatitis, and one with erythema multiforme).

The most frequent non-hematological grade \( \geq 3 \) TEAE was ALT elevation, which was observed in 5 (41.6%) and 2 (40.0%) patients in the FUL and LET cohorts, respectively (table 3).

Antitumor activity

The FAS comprised 16 patients (FUL cohort, n=11; LET cohort, n=5), with 1 patient with a prior treatment history in the FUL cohort being excluded as ineligible. In the FUL cohort, five patients (45.4%) and one (9.0%) patient achieved partial response and complete response, respectively, accounting for an ORR of 54.5% (95% CI 28.0 to 78.7). In the LET cohort, two (40.0%) patients achieved partial response, accounting for an ORR of 40.0% (95% CI 11.7 to 76.9). DCRs were 90.9% (10/11, 95% CI 62.2 to 98.3) and 80.0% (4/5, 95% CI 37.5 to 96.3) in the FUL and LET cohorts, respectively. Waterfall plots of investigator-assessed responses are shown in figure 1. Due to early termination, PFS and OS were not determined.

### Table 1 Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>FUL cohort (n=12)</th>
<th>LET cohort (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age, years (range)</td>
<td>52 (43–70)</td>
<td>68 (52–83)</td>
</tr>
<tr>
<td>ECOG performance status, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3 (25.0)</td>
<td>4 (80.0)</td>
</tr>
<tr>
<td>1</td>
<td>9 (75.0)</td>
<td>1 (20.0)</td>
</tr>
<tr>
<td>Disease setting, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>De novo metastatic</td>
<td>1 (8.3)</td>
<td>3 (60.0)</td>
</tr>
<tr>
<td>Metastatic recurrent</td>
<td>11 (91.6)</td>
<td>2 (40.0)</td>
</tr>
<tr>
<td>Metastatic site, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>4 (33.3)</td>
<td>1 (20.0)</td>
</tr>
<tr>
<td>Lung</td>
<td>9 (75.0)</td>
<td>2 (40.0)</td>
</tr>
<tr>
<td>Bone</td>
<td>8 (66.6)</td>
<td>3 (60.0)</td>
</tr>
<tr>
<td>Brain</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Menopausal status, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Premenopause/Perimenopause</td>
<td>6 (50.0)</td>
<td>–</td>
</tr>
<tr>
<td>Postmenopause</td>
<td>6 (50.0)</td>
<td>5 (100.0)</td>
</tr>
<tr>
<td>Prior perioperative chemotherapy, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>8 (66.6)</td>
<td>1 (20.0)</td>
</tr>
<tr>
<td>No</td>
<td>4 (33.3)</td>
<td>4 (80.0)</td>
</tr>
<tr>
<td>Prior endocrine therapy, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>–</td>
<td>3 (60.0)</td>
</tr>
<tr>
<td>Aromatase inhibitor</td>
<td>7 (58.3)</td>
<td>2 (40.0)</td>
</tr>
<tr>
<td>Other ET</td>
<td>5 (41.6)</td>
<td>0</td>
</tr>
<tr>
<td>PD-L1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>12 (100.0)</td>
<td>5 (100.0)</td>
</tr>
<tr>
<td>Positive</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ECOG, Eastern Cooperative Oncology Group; ET, endocrine therapy; FUL, fulvestrant; LET, letrozole; N, number; PD-L1, programmed death-ligand 1.</td>
<td></td>
<td></td>
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</table>
Liver-related all-causality AEs are summarized in online supplemental table 3. Liver-related grade ≥3 AEs were observed in seven (58.3%) and three (60.0%) patients in the FUL and LET cohorts, respectively. None of the patients met Hy’s law criteria (ALT 3×upper limit of normal and bilirubin>2×upper limit of normal). To examine the characteristics of patients with liver-related AEs associated with nivolumab plus abemaciclib, we summarized the clinical course of 10 patients with liver-related grade ≥3 AEs (table 4). The median duration of liver-related AEs was 215 days, which was 3 times as long as those reported previously in patients with melanoma and lung cancer. The onset of liver-related AEs was noted approximately 1–3 months after the initiation of treatment in most patients. Corticosteroids were used in most cases, and immunosuppressive agents were administered to half of the patients. Five patients were recovered/resolved and three were recovering/resolving at data cutoff, but the remaining two were not. Test results of viral hepatitis were all negative in those patients.

A liver biopsy was not mandatory but performed on three patients with severe hepatotoxicity as deemed necessary by the physician. F07 and F05 are patients in the FUL cohort, and L02 is a patient in the LET cohort. The course of treatment with the study regimen (ie, abemaciclib, nivolumab, and ET) and immunosuppressive therapy for hepatitis and changes in serum AST and ALT levels in these patients are summarized in figure 2A. In L02 and F07, AST and ALT levels decreased once following treatment discontinuation but then increased, even though the treatment was not resumed. In F05, AST and ALT levels decreased after treatment discontinuation, and the levels rapidly increased again on resumption of the treatment.

Representative microscopic images of H&E and immunohistochemical staining of liver biopsy specimens are shown in figure 2B. All three patients had lobular injury consisting of liver-related AEs.
of randomly scattered foci of focal necrosis. There were no typical findings of isolated centrilobular zonal necrosis. Infiltrating cells were predominantly lymphocytes; eosinophils, plasma cells, and neutrophils were not conspicuous in any of the cases. No epithelioid granuloma was observed. There was no evidence of bile duct injury and no findings suggestive of sclerosing cholangitis.

Immunohistochemistry results for infiltrating lymphocytes are summarized in figure 2C. In all cases, CD3+ and CD8+ lymphocytes predominated, while CD20+ or CD4+ lymphocytes were less abundant. The number of FOXP3+ lymphocytes varied. As indicated by the ratios of CD4/CD8 in the liver specimens, hepatotoxicity was likely immune-related.

Analysis of cytokines and chemokines in immune-related AEs

To further confirm that hepatotoxicity induced by the combination of nivolumab and abemaciclib is immune-related, we performed multiplex cytokine analyses of serum samples and compared data of baseline samples with data of samples closest to the onset date of the worst grade toxicity. Patients with grade ≥2 hepatotoxicity had significantly elevated sCD30/TNFRSF8, Thymic stromal lymphopoietin (TSLP), IL-11, IL-12 (p40), pentraxin-3, sTNF-R2, sTNF-R1, IL-34, and interferon-β levels and significantly reduced TWEAK/TNFSF12 levels, whereas levels of TWEAK/TNFSF12 were significantly reduced, compared with pretreatment levels (figure 3B and table 5). The numbers of cases of worst-grade hepatotoxicity, gastrointestinal toxicity, and all AEs are summarized in online supplemental table 4.

Flow cytometric analysis of immune cell subsets

Previous studies have suggested that the disruption of peripheral immune tolerance may be involved in the development of immune-related AEs (irAEs).23–24 Accordingly, we analyzed the numbers of Tregs in peripheral blood mononuclear cells (PBMCs) obtained from patients with grade ≥2 hepatotoxicity, those with grade ≥2 gastrointestinal toxicity, and all patients.

The percentage of PD-1-positive effector Tregs (Fr. II) in PBMCs obtained from patients with grade ≥2 hepatotoxicity or gastrointestinal toxicity was significantly decreased compared with pretreatment values. A decrease in the percentage of PD-1-positive effector Tregs (Fr. II) was also observed in PBMCs obtained from all patients at...
### Table 4  Summary of cases of liver-related grade ≥3 AEs

<table>
<thead>
<tr>
<th>Patient</th>
<th>Cohort</th>
<th>Type of AE</th>
<th>Grade</th>
<th>Onset (day)</th>
<th>Duration (days)</th>
<th>Peak AST/ALT (U/L)†</th>
<th>Corticosteroid</th>
<th>MMF</th>
<th>AZA</th>
<th>Causality of nivolumab</th>
<th>Causality of abemaciclib</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-01</td>
<td>FUL</td>
<td>Elevated AST</td>
<td>3</td>
<td>94</td>
<td>293</td>
<td>218/123</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>Related</td>
<td>Related</td>
<td>Recovered/Resolved</td>
</tr>
<tr>
<td>F-02</td>
<td>FUL</td>
<td>Elevated ALT</td>
<td>3</td>
<td>58</td>
<td>314</td>
<td>90/181</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>Related</td>
<td>Related</td>
<td>Recovered/Resolution</td>
</tr>
<tr>
<td>F-03</td>
<td>FUL</td>
<td>Elevated AST</td>
<td>3</td>
<td>44</td>
<td>335</td>
<td>127/484</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Related</td>
<td>Related</td>
<td>Recovered/Resolution</td>
</tr>
<tr>
<td>F-04</td>
<td>FUL</td>
<td>Elevated ALT</td>
<td>3</td>
<td>36</td>
<td>256</td>
<td>415/218</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>Related</td>
<td>Related</td>
<td>Recovered/Resolution</td>
</tr>
<tr>
<td>F-05</td>
<td>FUL</td>
<td>Elevated AST</td>
<td>3</td>
<td>85</td>
<td>121</td>
<td>333/684</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>Related</td>
<td>Related</td>
<td>Not recovered/Not resolved</td>
</tr>
<tr>
<td>F-06</td>
<td>FUL</td>
<td>Acute hepatitis</td>
<td>3</td>
<td>85</td>
<td>71</td>
<td>93/198</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>Related</td>
<td>Related</td>
<td>Recovered/Resolved</td>
</tr>
<tr>
<td>F-07</td>
<td>FUL</td>
<td>AIH</td>
<td>3</td>
<td>15</td>
<td>108</td>
<td>373/525</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>Related</td>
<td>Related</td>
<td>Recovered/Resolved</td>
</tr>
<tr>
<td>L-01</td>
<td>LET</td>
<td>Elevated AST</td>
<td>3</td>
<td>31</td>
<td>18</td>
<td>163/146</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Not related</td>
<td>Not related</td>
<td>Recovered/Resolved</td>
</tr>
<tr>
<td>L-02</td>
<td>LET</td>
<td>Elevated ALT</td>
<td>3</td>
<td>111</td>
<td>57</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Not related</td>
<td>Related</td>
<td>Recovered/Resolved</td>
</tr>
<tr>
<td>L-03</td>
<td>LET</td>
<td>Elevated AST</td>
<td>3</td>
<td>43</td>
<td>50</td>
<td>330/533</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>Related</td>
<td>Related</td>
<td>Recovered/Resolved</td>
</tr>
</tbody>
</table>

*Type of AE and its grade were documented and evaluated based on the CTCAE V.5.0 by investigators.†Peak AST/ALT were recorded based on the electronic data capture or serious AE report.
AE, adverse event; AIH, autoimmune hepatitis; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AZA, azathioprine; FUL, fulvestrant; LET, letrozole; MMF, mycophenolate mofetil; γ-GTP, gamma-glutamyl transpeptidase.
Figure 2  Hepatotoxicity in patients treated with nivolumab plus abemaciclib. (A) Clinical course of three patients who received liver biopsy. The number in the circle indicates how many times the drug has been administered. (B) Images of liver biopsy specimens. (C) Immunohistological findings. ALT, alanine aminotransferase; AST, aspartate aminotransferase; FUL, fulvestrant; LET, letrozole; MMF, mycophenolate mofetil; mPSL, methylprednisolone; PSL, prednisolone.
Figure 3  Correlative analysis of immune-related adverse events. (A) Multiplex analysis of cytokines in patients with hepatotoxicity. Comparison of pretreatment levels and levels at the onset of hepatotoxicity (n=15). (B) Multiplex analysis of cytokines in patients with grade ≥2 adverse events. Comparison of pretreatment levels and levels at onset of adverse events (n=17). (C) Flow cytometric analysis of regulatory T cells. Comparison of pretreatment levels and levels at onset of hepatotoxicity (n=15). (D) Flow cytometric analysis of regulatory T cells. Comparison of pretreatment levels and levels at onset of adverse events (n=17). In (A)-(D), P, H, and W represent measurement points. P indicates pretreatment, H indicates onset of hepatotoxicity, and W indicates the occurrence of adverse events at the worst grade. The results of one-way analysis of variance are summarized as ***p<0.001, **p<0.01, *p<0.05. (E) Differences between pretreatment and post-treatment gut microbiota compositions. Relative abundance of bacterial operational taxonomic unit (OTU) compositions at the genus level is shown. When identification at the genus level was not possible, identity at the family level is provided. Bacteria that increased in abundance after treatment compared with before are represented as ‘post’ (black); those that decreased in abundance after treatment compared with before are represented as ‘pre’ (gray). Only those with a change of ≥0.25% at the genus/family level are indicated. IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.
the onset of worst-grade AEs (figure 3C and D and online supplemental figure 3).

**Analysis of gut microbiota**
Growing evidence suggests a relationship between the gut microbiota and immunotherapy response and toxicity. Thus, we compared the gut microbiota in stool samples before and after treatment. After treatment, the most abundant bacteria were Eggerthella, Dorea, and Bifidobacterium (figure 3E). Lachnospiraceae, Enterobacteriaceae, and Ruminococcaceae species were identified at the family level, as it was difficult to identify them at the genus level.

**Genotyping of HLA and ABCG2**
Previously, specific HLA polymorphism or ABCG2 genotypes are related to the toxicity of target therapies. Abemaciclib and its active metabolite M2 are substrates for the breast cancer resistance protein transporter encoded by ABCG2, and their blood concentrations correlates the ABCG2 genotype. Therefore, we speculated the incidence and severity of the investigational treatment might be related to the ABCG2 genotype. Although statistical analysis was not performed, there were no apparent denominators of HLA or ABCG2 in patients who experienced ILD or ALT elevation (online supplemental figure 4).

**Next-generation sequencing RNA-sequencing-based assay**
To assess whether patients with HR-positive HER2-negative metastatic breast cancer have a tumor microenvironment that is potentially sensitive to immunotherapy, we performed RNA-sequencing-based tumor subtype classification and the DetermaIO 27-gene signature test. Fifteen of 17 tumor tissue samples were classified into 5 subtypes: 3 BL1, 2 BL2, 1 LAR, 6 M, and 1 MSL (2 non-qualified). Binary scores for DetermaIO were positive for three tumors, indicating an immune-active tumor microenvironment (online supplemental table 5). In patients with immuno-oncology (IO)+ disease (n=3), two had stable disease and one was not evaluable. In patients with IO− disease (n=11), one had a complete response, seven had a partial response, two had stable disease and one was not evaluable.

**DISCUSSION**
The present study evaluated the efficacy and safety of the combination of nivolumab and abemaciclib plus ET in patients with HR-positive HER2-negative metastatic breast cancer. Although the experimental therapy elicited an antitumor response, it was associated with a high incidence of immune-related hepatotoxicity, and one patient developed fatal ILD. After other clinical trials of abemaciclib in combination with ICIs reported an increase in severe ILD, the iDSMC decided to terminate the study. The present study demonstrated for the first time that a CDK4/6 inhibitor could augment ICI-mediated immune reactions in a clinical setting, as assessed by pathological evaluation of the liver, serum cytokine analysis, and flow cytometry of PBMCs. Most AEs observed during the study period were immune-related, as confirmed by the assessment of the clinical course and correlative studies.

The incidence of grade ≥3 hepatotoxicity was higher than those reported previously in studies of anti-PD-1 antibody therapy with or without chemotherapy. Hepatotoxicity was severe and long-standing and responded to corticosteroids and immunosuppressive agents. The terminal half-life (t½) of nivolumab is 12–20 days, and nivolumab binding to T lymphocytes, which could induce long-lasting irAEs, was detected at ≥20 weeks after the last infusion of nivolumab in a previous report. In the present study, two of three patients who underwent liver biopsy showed improvement in liver enzymes on interruption of nivolumab, followed by exacerbation (figure 1). Repeated exacerbations and remissions have been reported in many cases of immune-related hepatotoxicity. Previous reports have also shown that immune-related hepatotoxicity has a predominantly lymphocytic infiltrate with low CD4+/CD8+ ratios. The histopathological findings of liver biopsies were consistent with these findings. Nonetheless, the specimens did not show typical findings of centrilobular zonal necrosis, which has been reported to be another feature.

In clinical trials, immune-related hepatotoxicity could occur independently of the type of CDK4/6 inhibitor (abemaciclib or palbociclib) or ICI (anti-PD-1 or anti-PD-L1 antibody). The combination of abemaciclib and pembrolizumab, an anti-PD-1 antibody, reportedly caused severe toxicity in patients with HR-positive breast cancer and non-small cell lung cancer. The combination of pembrolizumab, pembocibl, and ET in patients with HR-positive breast cancer was well-tolerated, with only a 17% incidence of grade ≥3 hepatotoxicity. The combination of palbociclib,
avelumab, and ET in patients with HR-positive breast cancer were also well tolerated, with the following liver enzyme elevations: AST of any grade at 11.3% (grade 3/4 at 1.9%), and ALT of any grade at 9.4% (grade 3/4 at 1.9%). In contrast, neoadjuvant nivolumab combined with palbociclib and ET for primary breast cancer led to treatment discontinuation (9 of 21 patients) due to toxicity, mainly grade ≥3 hepatic TRAEs.45

Marked cytokine elevation was observed in patients with hepatotoxicity. The elevation pattern of cytokines due to hepatotoxicity suggests the involvement of TNF-related cytokines and IL-1.6 15 46 In addition, pentraxin 3, a molecule that induces inflammation via macrophages,49 may have been involved in the exacerbation of hepatitis. Proinflammatory cytokines might be related to the worsening of AEs induced by the combination of nivolumab and abemaciclib. The tumor necrosis factor α (TNF-α) blocker infliximab is not recommended because of concerns about hepatotoxicity, and mycophenolate mofetil (MMF) is generally administered for glucocorticoid-refractory immune-related hepatotoxicity.50 Although MMF does not directly inhibit TNF-α production like infliximab, it was reported to inhibit TNF-α release in a murine model.51 In the present study, only 4 of 10 patients who experienced liver-related grade ≥3 AEs received MMF treatment. Further evidence of the efficacy and safety of early MMF treatment or other immunosuppressive treatment to control cytokine release is needed.

It is noteworthy that PD-1-positive effector Tregs (Fr. II) were significantly decreased in the peripheral blood of patients with hepatotoxicity. PD-1-positive Tregs in the tumor microenvironment are related to resistance to PD-1 blockade therapy.12 Another preclinical study showed that the proliferation of Tregs was more suppressed by CDK4/6 inhibitors than other T cells, possibly due to the higher CDK6 expression of Tregs.15 Furthermore, a reduction of circulating effector Tregs (CD4+CD25+FOX-P3highCD45RA−) was observed in patients with HR-positive HER2-negative metastatic breast cancer who responded to CDK4/6 inhibitors. Based on these data, the suppression of PD-1-positive effector Tregs by abemaciclib may be the mechanism underlying the synergistic effects of nivolumab plus abemaciclib therapy, leading to the development of severe AEs.

The microbiome analysis revealed that Eggerthella, Dorea, and Bifidobacterium may play a particularly important role in the development of irAEs associated with nivolumab plus abemaciclib therapy. Eggerthella is an intestinal bacterium that has been reported to cause ulcerative colitis, liver and anal abscesses, and possible systemic bacteremia.53 54 Dorea is a Gram-positive, non-spore-forming bacterial genus belonging to the Lachnospiraceae family, which occurs in human feces55 and has some unknown impact on cancer and immunity. Bifidobacterium is a bacterial genus found in the inflamed tumor microenvironment56; it has been reported that dietary fiber intake increased the efficacy of anti-PD-1 antibody administration, while additional administration of Bifidobacterium-containing probiotics resulted in an impaired treatment response.57 Microbiome composition may have affected irAEs and efficacy in patients who received nivolumab and abemaciclib.

Results of the DetermaIO test revealed that tumor tissues from three patients were immunomodulatory-positive, implying the presence of a tumor microenvironment sensitive to immunotherapy in some HR-positive HER2-negative metastatic breast cancers. Although there is no previous report of DetermaIO tests being conducted in patients with HR-positive breast cancer, the correlation of DetermaIO score to high levels of TILs in TNBC has been reported.58 The result of DetermaIO tests in the present study are consistent with a previous study of high-TIL cases in HR-positive HER2-negative breast cancer.11 In a patient with a negative DetermaIO Binary Score, the best response to nivolumab in combination with abemaciclib showed complete response, providing hope that combination therapy with anti-PD-1/PD-L1 antibodies and other targeted therapies may overcome the immune microenvironment of HR-positive metastatic breast cancer.

This study has several limitations. First, the sample size was small due to early termination of the study, and given the non-comparative design, we could not determine to what extent the addition of abemaciclib contributed to enhancing irAEs. Second, liver biopsy revealed similar pathological patterns in three patients, but whether this is a coincidence or a real association remains unclear due to the small sample size. Third, characterizing immune activation solely based on cytokine release profiles in patients with irAEs may not represent immune activation against cancer. Future studies will be needed to address these aspects.

Despite these limitations, the prolonged clinical course, focal necrosis of the liver tissue with CD8 infiltration, marked elevation of serum cytokines, and reduced peripheral effector Tregs support the speculation that long-lasting hepatotoxicity observed during nivolumab plus abemaciclib combination therapy is due to immune-related toxicity.

CONCLUSION

The combination of nivolumab and abemaciclib triggers hyperproduction of proinflammatory cytokines and causes severe irAEs. Further development of the therapy is not warranted unless methods to control proinflammatory cytokine production and immune-related AEs are developed.

Author affiliations

1 Department of Breast Medical Oncology, The Cancer Institute Hospital of Japanese Foundation for Cancer Research, Koto-ku, Tokyo, Japan
2 Department of Medical Oncology, Toranomon Hospital, Minato-ku, Tokyo, Japan
3 Advanced Cancer Translational Research Institute, Showa University, Shinagawa-ku, Tokyo, Japan
4 Department of Medical Oncology, Kindai University Faculty of Medicine, Osaka-Sayama, Osaka, Japan
Abemaciclib induces a T cell inflamed tumor Microenvironment and collected the data. KEY and JM developed the statistical analysis plan. MF, KM, KA, HI, MH, TM, HK, HY, TT, and investigators recruited the patients and collected the data. KEY and JM developed the statistical analysis plan. KIY performed the immunological analysis and evaluated the data. SM and TY performed the pathological evaluation. CKI performed the genotyping of HLA and ABCG2. HS summarized the correlative studies. JT oversaw the conduct of the clinical trial and all safety and clinical response analyses. HS and JM wrote the draft of the manuscript. All authors interpreted the data, contributed to the writing, and provided final approval to submit the manuscript for publication. TT is the guarantor of the study.

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