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Original article

Combined effects of neoadjuvant letrozole and zoledronic acid on γδT cells in postmenopausal women with early-stage breast cancer

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ABSTRACT

Introduction: Adjuvant bisphosphonates lead to better prognosis in postmenopausal breast cancer. However, the association between clinical outcomes and immune modulation by them is still unclear.
Methods: In this prospective, open-label phase II study, postmenopausal women with estrogen receptor-positive and human epidermal growth factor receptor 2-negative early-stage breast cancer received neoadjuvant letrozole (LET) for one month, followed by treatment with a single dose of zoledronic acid. The patients underwent an additional 5 months of treatment with LET prior to surgery. The primary endpoint was the tumor objective response rate (ORR) determined by diameter via MRI. The association between the ORR and γδT cell frequencies was assessed as a secondary endpoint.
Results: Out of sixty patients, 55 patients were evaluable for response by MRI. The ORR for LET with zoledronic acid was 38.2% (21/55), which was comparable to that of historical controls (45%). A decrease in the frequency of the Vδ2 T cell subset was observed throughout treatment, and Vδ2 T cells were activated for 6 months. In planned subgroup analyses, patients with low frequencies of Vδ2 T cells prior to zoledronic acid infusion experienced a favorable tumor response compared to those with high frequencies (59.3% [16/27] vs 17.9% [5/28], p = .002). There were no serious adverse events with this treatment regimen.
Conclusion: These results showed that neoadjuvant LET with zoledronic acid could not achieve overall effect for local tumor response. However, patients with a low frequency of γδ T cells would benefit from the treatment including zoledronic acid. (UMIN 000008701).

1. Introductions

Breast cancer is a common disease worldwide, and estrogen receptor (ER)-positive breast cancer accounts for 70% of the total cases. Adjuvant hormone therapy is currently the standard of care for ER-positive breast cancer, and aromatase inhibitors have greater benefits than tamoxifen in the treatment of postmenopausal women with early-stage breast cancer [1]. However, it is still necessary to maximize the efficacy of adjuvant endocrine therapy to decrease the mortality rate in ER-positive breast cancer. Neoadjuvant endocrine therapy is accepted as a feasible option for postmenopausal patients with highly endocrine-responsive disease

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[2]. Moreover, this treatment modality can provide an opportunity to better understand the molecular determinants of neoadjuvant endocrine therapy and identify subgroups that may benefit from this treatment [3].

Accumulating evidence from previous studies has demonstrated that adjuvant bisphosphonates can improve the prognosis of breast cancer [4–6]. The Early Breast Cancer Trialists' Collaborative Group (EBCTCG) meta-analysis shows that adjuvant bisphosphonates improve the survival rate in postmenopausal women [7]. The addition of zoledronic acid to neoadjuvant chemotherapy [8–11] and endocrine therapy [12] achieved better and modest clinical outcomes, respectively. The systemic anti-tumor effect of zoledronic acid was only observed in postmenopausal women, but the precise mechanism remains unclear.

Among immune cells, human γδ T cells account for 1–5% of the total peripheral T cells and play an important role in anti-infectious and anti-tumor immunity [13]. The subset of Vδ2-bearing Vγ9Vδ2 (also known as Vγ2Vδ2) T cells accounts for 50–95% of γδ T cells in healthy adults. Activated Vδ2 T cells exhibit cytotoxic activity against tumors, and the infiltration of Vδ2 T cells into tumor sites has been observed in various tumors, including lung [14], renal [15] and breast cancer [16]. Modest therapeutic effects were reported in breast cancer for both adoptive and active immunotherapies targeting Vγ9Vδ2 T cells [17].

Nitrogen-containing bisphosphonates including zoledronic acid are taken up not only by myeloid lineage cells but also by tumor cells through fluid phase endocytosis. Zoledronic acid inhibits farnesyl diphosphate synthetase (FDPS) in the mevalonate pathway, thereby inducing cell death by blocking prenylation of small GTPases [18]. Inhibition of FDPS also results in the accumulation of its upstream metabolite, isopentenyl pyrophosphate, which stimulates and activates Vγ9Vδ2 T cells in a butyrophilin 3A1-dependent manner [19].

Taken together, we hypothesize that the clinical efficacy of adjuvant bisphosphonates may be related to the immune modulatory effects on γδ T cells. In the present study, we performed a single-arm prospective trial to investigate the association between the clinical efficacy and immune modulation of γδ T cells for neoadjuvant letrozole (LET) combined with zoledronic acid in postmenopausal women with early-stage breast cancer.

2. Methods

2.1. Study design, patients and intervention

The study design and patient population were previously described [20]. Briefly, the patients enrolled in the present study were postmenopausal women with ER-positive, human epidermal growth factor receptor 2 (HER2)-negative, and clinical T1 or T2 N0M0 breast cancer. After registration, patients were started on 2.5 mg/day of LET orally and received a single dose of zoledronic acid at one month. The dose of zoledronic acid was reduced based on renal function. The patients continued to take LET every day for the following 5 months until undergoing breast surgery within two weeks.

2.2. Measurements

The patients were clinically evaluated according to the trial schedule summarized in supplementary Table 1. The primary endpoint is the objective response rate (ORR) based on the MRI diameter. The secondary endpoints are the changes in tumor volume via MRI, and the ORR evaluated using a caliper and ultrasonography (US). The frequencies of immune cells, including γδ T cells, were measured throughout the treatment. MRI diameter and volume were assessed at baseline and at 3 and 6 months by an independent central committee. Investigators evaluated the ORR using a caliper and US. Serum interferon (IFN)-γ was subject to enzyme-linked immunosorbent assays (ELISA) within hours after the administration of zoledronic acid. The frequencies of immune cells were analyzed by two-color flow cytometry prior to the administration of zoledronic acid and at 2 and 6 months. The following monoclonal antibodies (mAbs) were used: anti-CD3, anti-Vδ1, anti-Vδ2, anti-CD4, anti-CD8, anti-CD25, and anti-NKG2D mAb as previously described [20].

2.3. Statistical analysis

The present study was a single-arm phase II study. The ORR of neoadjuvant LET was reported as 45%, whereas the ORR of zoledronic acid-combined neoadjuvant LET was expected to be 60%. A sample size of 69 was chosen using the Bayesian predictive probability criterion based on a previous design [21], with an expected ORR of 60%, a null hypothesis of 45% [22], and non-informative analysis prior and pre-specified probability thresholds of 95% (akin to a significance level of 5%) and 80% (akin to power). Therefore, a sample size of 75 was determined, considering ineligible patients.

The primary endpoint was analyzed using the Bayesian method as previously described. As a reference, Clopper-Pearson's exact confidence intervals (CIs) were provided for the ORR. For the pre-planned subgroup analyses, the γδ T cell frequency and immune-related properties were assessed in context with the ORR using Fisher's exact test. Associations between the γδ T cell frequency and clinical pathological and immune-related factors were planned as exploratory analyses. A two-sided significance level of 5% was used to calculate CIs throughout the exploratory analyses. No adjustment was made for multiple comparisons for any of the endpoints or subgroups analyzed. All analyses were performed using SAS statistical software, version 9.4 (SAS Institute, Cary, NC).

3. Results

3.1. Patient characteristics

The first patient was enrolled in August 2013, and data collection ended in October 2015. The trial was planned to recruite 75 patients, of which 61 postmenopausal women with ER-positive early-stage breast cancer recruited from 6 centers in Japan were enrolled to receive neoadjuvant LET. Patient disposition is summarized in supplementary Table 2. One patient who received an overdose of zoledronic acid was excluded from the full analysis set due to a major protocol violation. Five of the 60 patients did not receive either zoledronic acid or surgery. Fifty-one patients received surgery and were provided the surgical specimen. The safety analysis set comprised 56 patients. Patient demographics and baseline tumor characteristics are summarized in Table 1.

3.2. Objective response rate

Out of 60 patients, 55 individuals underwent MRI assessment of tumor response. The calculated ORR was 38.2% (21/55; 95% CI, 25.4 to 52.3), and the Bayesian posterior probability of exceeding the threshold of 45% was 16%; this value did not meet the pre-specified probability threshold of 95%. The ORR at the end of treatment, measured by caliper (n = 56) and by ultrasound (n = 58) was 50.0% (28/56; 95% CI, 36.3 to 63.7) and 51.7% (30/58; 95% CI, 38.2 to 65.0), respectively (Table 2). The median volume reduction on MRI from the baseline value was 51.0% (95% CI, 34.7 to 59.3) at 6 months. A

Table 1
Baseline characteristics of the patients

Characteristics (n = 60)	
Age , year (range)	65 (54–74)
Body mass index, kg/m^2 (range)	23.6 (18.6–35.5)
Tumor stage, n (%)	
T1	32 (53.3)
T2	28 (46.7)
Quantity of estrogen receptor, % (range)	95 (5–100)
<50%	2 (3.3)
≧50%	58 (96.7)
Progesterone receptor status, n (%)	
Positive	58 (96.7)
Negative	2 (3.3)
Ki 67 labeling index, % (range)	12.0 (1.2–70)
<14%	31 (52.5)
≧14%	28 (47.5)

Table 2
Summary of best overall tumor response

Modalities	n (%)	95% CI
MRI diameter (n = 55)		
CR	2 (3.6)	
PR	19 (34.5)	
CR+PR	21 (38.2)	25.4 to 52.3
Caliper (n = 56)		
CR	7 (12.5)	
PR	21 (37.5)	
CR+PR	28 (50.0)	36.3 to 63.7
Ultrasound (n = 58)		
CR	0 (0)	
PR	30 (51.7)	
CR+PR	30 (51.7)	38.2 to 65.0
MRI volume (n = 52)		
Median volume reduction , %	51.0	34.7 to 59.3
≧ 50% reduction	29 (55.8)	

greater than 50% reduction in tumor volume was observed for 55.8% of the patients (29/52).

3.3. Changes in clinico-pathological factors during treatment and outcomes of surgery

The core biopsies, obtained prior to starting therapy and at 3 months after the start of therapy (mid-course), and surgical specimens were subjected to analyses of biomarkers. Whereas the expression of ER did not change during treatment, the positive progesterone receptor rate and Ki67 proliferation index were decreased at mid-course (at 3 months) compared to those prior to treatment (96.7% vs 60.0% and 16.7% vs 6.8%, respectively; supplementary Table 3). Serum IFN-γ was detected in 7 (12.7%) out of 55 patients (mean 321 pg/ml; ranging from 0 to 8963) within 6 h after zoledronic acid treatment, and 17 patients (30.9%) experienced fever after zoledronic acid treatment.

3.4. The regular T cell properties during the treatment

The absolute number of T cells was assessed based on peripheral blood mononuclear cell counts and the proportion of CD3-expressing cells measured through flow cytometry. The frequency of the different subsets of T cells, including CD4 and CD8 T cells, was calculated as the proportion of cells expressing specific markers among CD3 T cells. The neoadjuvant treatment of LET combined with zoledronic acid did not exert any changes in either the absolute number of T cells or the frequency of CD4 and CD8 T cells at any time point (Table 3).

3.5. In vivo effect of zoledronic acid on γδ T cell properties

Next, we analyzed the proportion of γδ T cell subsets during treatment. The frequency of Vδ1 T cells among CD3 T cells did not change during treatment, while a decrease in Vδ2 T cell frequencies (3.7% at two months and 3.5% at six months) was observed when compared with the baseline value of 4.8% (Table 3). These decreases in frequencies were −1.1% (95% CI, −1.9 to −0.4; p = .004) and −1.5% (95% CI, −2.4 to −0.6; p = .002) at 2 and 6 months, respectively. The Vδ2 T cell attrition was independent of Ki-67, IFN-γ release and fever. A decrease in Vδ2 T cells was observed in the subgroup of overweight patients (body mass index > 25 kg/m^2). Natural killer receptors of NKG2D and the interleukin-2 (IL-2) receptor of CD25 are expressed by a subset of activated γδ T cells. To evaluate the active state of γδT cells, the frequencies of these activation markers were analyzed among a subset of Vδ2 T cells. There was a trend towards an increased frequency of NKG2D-positive Vδ2 T cells at two and six months. In contrast, CD25-positive Vδ2 T cells also increased during treatment, and an increase in CD25-positive cells was observed at six months (4.0%; 95% CI, 1.0 to 7.0, p = .010).

3.6. Association between ORR and γδ T cells and immune-related properties

To examine the correlation between the treatment and immune-modulatory properties, we evaluated the association between ORR and the frequency of Vδ2 T cells, serum IFN-γ, and a history of fever. The median frequency of circulating Vδ2 T cells was

Table 3
Frequency of immune cells and activation markers

	1 month	2 months	6 months
No. of patients	55	55	52
CD3, cells x10^5/ml	5.1	5.4	5.0
Regular T cells, % (95% CI)			
CD4/CD3	62.2 (59.8 to 68.6)	66.2 (61.8 to 70.6)	66.4 (61.9 to 70.9)
CD8/CD3	31.7 (28.8 to 34.6)	30.4 (27.6 to 33.2)	30.5 (27.4 to 33.7)
γδT cells, % (95% CI)			
Vδ1/CD3	2.5 (2.1 to 2.9)	2.6 (2.2 to 3.0)	2.7 (2.2 to 3.2)
Change from baseline at 1 month		0.1 (−0.2 to 0.4), p = 0.499	0.1 (−0.3 to 0.5), p = 0.769
Vδ2/CD3	4.8 (3.3 to 6.3)	3.7 (2.4 to 4.9)	3.5 (2.1 to 4.9)
Change from baseline at 1 month		−1.1 (−1.9 to −0.4), p = 0.004	−1.5 (−2.4 to −0.6), p = 0.002
CD25/Vδ2	11.5 (8.8 to 14.2)	13.4 (10.5 to 16.3)	15.0 (11.5 to 18.5)
Change from baseline at 1 month		1.9 (−1.0 to 4.8), p = 0.189	4.0 (1.0 to 7.0), p = 0.010
NKG2D/Vδ2	39.0 (33.4 to 44.6)	41.3 (36.2 to 46.3)	41.6 (36.4 to 46.8)
Change from baseline at 1 month		2.2 (−1.9 to 6.3), p = 0.277	1.3 (−4.6 to 7.2), p = 0.661

P value using paired t-test compared with the baseline at 1 month.

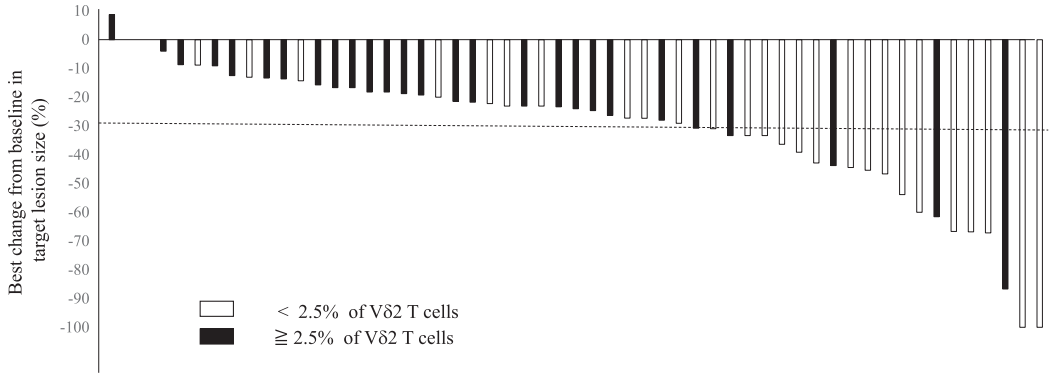

Fig. 1. Best percentage change from baseline in target lesion by MRI diameter. The line at 30% represents boundary for determination of partial response.

Table 4
Tumor response in the subgroups according to Vδ2 T cell frequency

	n (%)	MRI[a]	Caliper[b]	US[c]
Vδ2/CD3 (%)				
< 2.5	27	16 (59.3)	16 (59.3)	20 (74.1)
≧2.5	28	5 (17.9)	12 (44.4)*	10 (35.7)

*Twenty-seven patients had available data for caliper; estimated differences between groups, [a]41.4%, p = 0.002; [b]14.9%, p = 0.414; [c]38.4%, p = 0.007; p values were calculated by chi-square test.

2.5% at baseline, prior to zoledronic acid treatment. Patients with lower levels (<2.5%) of Vδ2 T cells experienced higher ORR compared to those with higher levels of Vδ2 T cells for MRI diameter (59.3% [16/27] vs 17.9% [5/28], p = .002). The change in tumor size in each patient is summarized in Fig. 1. A favorable tumor response was also observed on US (74.1% [20/27] vs 35.7% [10/28], p = .007) but not on caliper (59.3% [16/27] vs 44.4% [12/27], p = .414; Table 4). However, Vδ2 T cell frequencies at two and six months were not associated with clinical outcomes. There was no association between ORR and other immune-related properties. These results show that a low frequency of Vδ2 T cells prior to the administration of zoledronic acid is a predictive factor for the use of neoadjuvant LET therapy combined with zoledronic acid.

3.7. Safety

A total of 30 patients (53.6%) experienced adverse events (AEs), of which most AEs were grade 1 or 2, consistent with the reported profile. Only one grade 3 AE (1.8%), a sudden hearing loss, was observed. The most common AEs attributed to zoledronic acid were fever (25%) and myalgia (10.7%). Overall, no serious AEs were observed. Neither clinically significant laboratory values nor surgical complications were recorded.

Serum bone-specific alkaline phosphatase (BAP), a bone turnover marker, did not change until month 3, at which time the marker gradually declined (14.8 μg/L at baseline to 9.3 μg/L at 6 months), whereas the serum N-telopeptide of type I collagen (NTx) decrease began 2 months after zoledronic acid treatment (16.7 nmol BCE/L at baseline and was 11.7 nmol BEC/L at 2 months). The results demonstrated that a single dose of zoledronic acid maintains bone health for 6 months.

4. Discussion

The present study is the first prospective study to explore the relationship between the clinical outcomes and immune-modulatory effects on γδ T cells by neoadjuvant endocrine therapy combined with zoledronic acid in postmenopausal women with breast cancer. The results were regarded as exploratory for the primary endpoint, and no improvement in ORR was noted overall compared to the historical control. However, a favorable tumor response was observed in women with low frequencies of γδ T cells prior to the administration of zoledronic acid compared to those with high γδ T cell frequencies.

The use of an aromatase inhibitor for neoadjuvant setting was shown to induce clinical responses in 37–70% of patients and down-staging to breast conserving surgery in 43–86% [22–25]. This wide range of clinical outcomes was due to patient characteristics and imaging modalities used to assess tumor response. For the evaluation of residual disease in women receiving neoadjuvant chemotherapy, the MRI imaging technique was superior in accuracy to conventional clinical assessment [26,27]. The treatment in the present study failed to achieve a significant clinical response on the basis of MRI diameter, caliper and US measurements. While the tumor volume from baseline was decreased on MRI, the volume reduction was modest. A favorable tumor response in the subgroup with low frequencies of γδ T cells was observed for 59.3% on MRI and for 74.1% on US compared with the subgroup with high γδ T cell frequencies, but was not observed on caliper. Although these response rates were comparable to those reported in previous studies, MRI and US might be better imaging modalities to evaluate the responders among the patients categorized based on the frequency of γδ T cells.

The inhibitory dose of zoledronic acid varies among tumor cells [28], and the synergism with anti-cancer agents depends on the drug sequence. Neville-Webbe et al. [29] reported that synergistic anti-tumor activity of LET and zoledronic acid was observed in the sequence of LET followed by zoledronic acid, which was confirmed by the pharmaco-dynamics in the mevalonate pathway. In the present study, the intervention was designed with the first month of LET treatment, followed by a single dose of zoledronic acid throughout 6 months neoadjuvant LET. The previous administration of LET prior to zoledronic acid was aimed for the better drug sequence and to ensure the low level of serum estrogen in postmenopausal women. A single dose of zoledronic acid was expected to be effective because frequent zoledronic acid infusion caused exhaustion of γδ T cells leading to a decrease in the frequency of peripheral Vδ2 T cells [30]. The modest activation of NKG2D receptor was observed, and the CD25 expression was significantly enhanced at six months. These results demonstrated that the treatment induces the activation of circulating Vδ2 T cells, and its immuno-modulatory effect is long-lasting. Nevertheless, the treatment could not achieve the improvement of local response, and the optimal treatment dose and sequence of zoledronic acid is still unclear.

In the present study, we hypothesized that the frequency of γδ T cells could be a relevant biomarker to predict clinical outcomes in breast cancer patients receiving neoadjuvant therapy with LET and zoledronic acid. However, a local response was unexpectedly observed in patients with lower frequencies of γδ T cells. The most likely explanation for this paradox is that the responsiveness to the treatment varies among the patients with high frequencies of γδ T cells. Patients with high frequencies of Vδ2 T cells are a heterogeneous population, and patients may or may not respond to the treatment. This idea is supported by our personal observation that Vδ2 T cells fully expanded with zoledronic acid in vitro fail to vigorously respond to target cells. The other explanation is that activated Vδ2 T cells could infiltrate from the periphery to the tumor site, which might lead to a decrease in Vδ2 frequency in the periphery. Santini et al. reported that treatment with a single dose of zoledronic acid in vivo caused a significant decrease in naïve and central memory γδ T cells [31]. Recently, Spitzer et al. [32] elucidated that peripheral effector cells were activated but decreased in the mice that respond to immunotherapy. These results indicate that the frequency of circulating effector cells does not always reflect the active state of immune cells but the results of the redistribution of effector cells from the periphery to the tumor site.

There are limitations in the present study. As the present study was designed as a single arm study in the absence of a reference with LET alone, it was difficult to evaluate the additive effects of zoledronic acid on neoadjuvant LET. Moreover, the frequency of γδ T cells was not measured at baseline, which should be meaningful for comparison between LET alone and LET plus zoledronic acid for immune modulation. However, the frequency of Vδ2 T cells progressively decreased, and bone turnover markers were improved, whereas significant changes in the number of Vδ1 T and αβ T cells were not observed. Although the immunological effects of LET alone on γδ T cells could not be excluded, these results indicated that the combined treatment might have some effects on part of the immune response.

In conclusion, the present study did not demonstrate a significant clinical efficacy of neoadjuvant LET combined with zoledronic acid for local tumor response. The present results, however, suggest that patients with a low frequency of Vδ2 T cells would benefit from the treatment. As adjuvant bisphosphonates can prevent metastases outside bone and breast cancer-related death, more precise studies focusing on immuno-oncology are warranted to explore the anti-tumor effect of adjuvant bisphosphonates.

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Conflict of interest statement

Tomoharu Sugie received a research grant from Novartis and honoraria from Novartis, AstraZeneca and Chugai. Norikazu Masuda received honoraria from AstraZeneca and Chugai. Masakazu Toi received research grants and honoraria from Novartis and Daiichi-Sankyo. The other authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.breast.2017.12.017.

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