*Influence of Tamoxifen on Endometrial Thickness, Bone Mineral Density, and Cholesterol Levels According to CYP2D6*10 or CYP2C19*2,*3 Genotype*

Because it is known that tamoxifen affects endometrial thickness, BMD, and serum total cholesterol, we also studied the impact of CYP2D6 and CYP2C19 genetic polymorphisms on these effects in postmenopausal patients. Changes from baseline in BMD (L2-L4), total cholesterol levels, and endometrial thickness 1 year after the start of tamoxifen are shown in Figure 3 for CYP2D6 and in Figure 4 for CYP2C19. A significant increase ($P < .05$) in BMD and a significant decrease ($P < .05$) in total cholesterol levels were observed regardless of CYP2D6 genotype after 1 year of treatment with tamoxifen; however, there was no significant difference in the extent of changes in BMD and total cholesterol levels between patients with the CYP2D6 *10/*10 genotype and those with the CYP2D6 wt/wt or wt/*10 genotype. Endometrial thickness increased significantly ($P < .05$) after a 1-year treatment with tamoxifen regardless of CYP2D6 genotype; however, there was no such significant difference in endometrial thickness between patients with the CYP2D6 *10/*10 genotype and those with the CYP2D6 wt/wt or wt/*10 genotype. Similar to the results obtained for the CYP2D6 genotype, there was no significant difference between CYP2C19 PM patients and CYP2C19EM patients in the extent of changes in BMD, total cholesterol levels, or endometrial thickness 1 year after the start of tamoxifen treatment.

Table 3. Univariate and Multivariate Analyses of the Cytochrome P450 (CYP) 2D6*10 and CYP2C19*2,*3 Polymorphisms on Recurrence-free Survival Rates

Variable	CYP2D6	CYP2C19
Univariate analysis		
HR	0.94	0.45
95% CI	0.34-2.60	0.13-1.55
P	.95	.2
Multivariate analysis†		
HR	0.6†	0.37†
95% CI	0.18-1.92	0.08-1.76
P	.39	.21

CYP2D6 indicates cytochrome P450 family 2, subfamily D, polypeptide 6; CYP2C19, cytochrome P450 family 2, subfamily C, polypeptide 19; HR, hazard ratio; CI, confidence interval.
† Adjusted for tumor size, lymph node status, histologic grade, progesterone receptor status, human epidermal growth factor receptor 2 status, and adjuvant therapy.

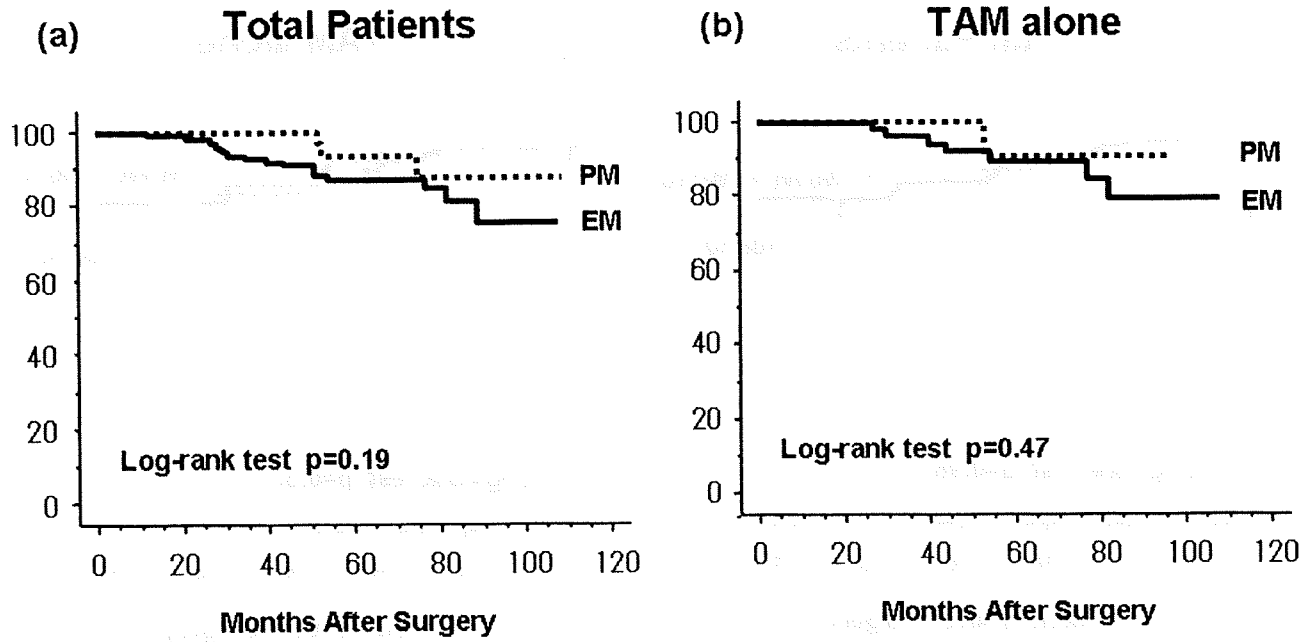

FIGURE 2. The prognosis of patients who received adjuvant tamoxifen (TAM) according to cytochrome P450 (CYP) family 2, subfamily C, polypeptide 19, allele *2,*3 (CYP2C19*2,*3) genotype. Recurrence-free survival rates were calculated with the Kaplan-Meier method according to CYP2C19 genotype, ie, poor metabolizers (PM) or extensive metabolizers (EM), for all patients who received adjuvant tamoxifen (a) and for patients who received adjuvant tamoxifen alone (b).

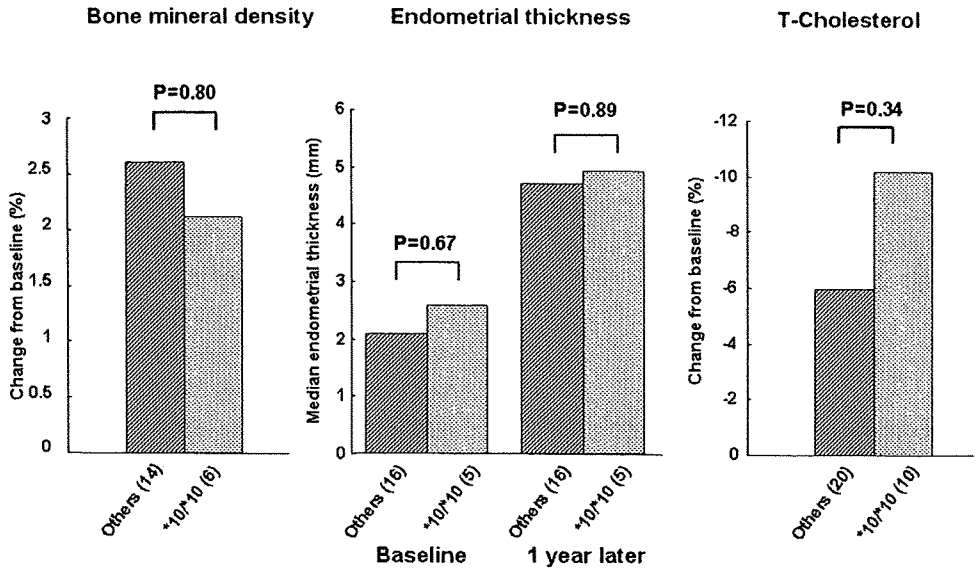

FIGURE 3. The influence of tamoxifen on bone mineral density, endometrial thickness, and total cholesterol (T-Cholesterol) levels by cytochrome P450 (CYP) family 2, subfamily D, polypeptide 6, allele *10 (CYP2D6*10) genotype in patients who received adjuvant tamoxifen. Changes in the percentages of bone mineral density, endometrial thickness, and total cholesterol levels after 1 year of tamoxifen treatment are shown according to CYP2D6 genotype, ie, the CYP2D6 *10/*10 genotype and the CYP2D6 wild-type (wt)/wt or wt/*10 genotype. Numbers in parentheses indicate the numbers of patients examined.

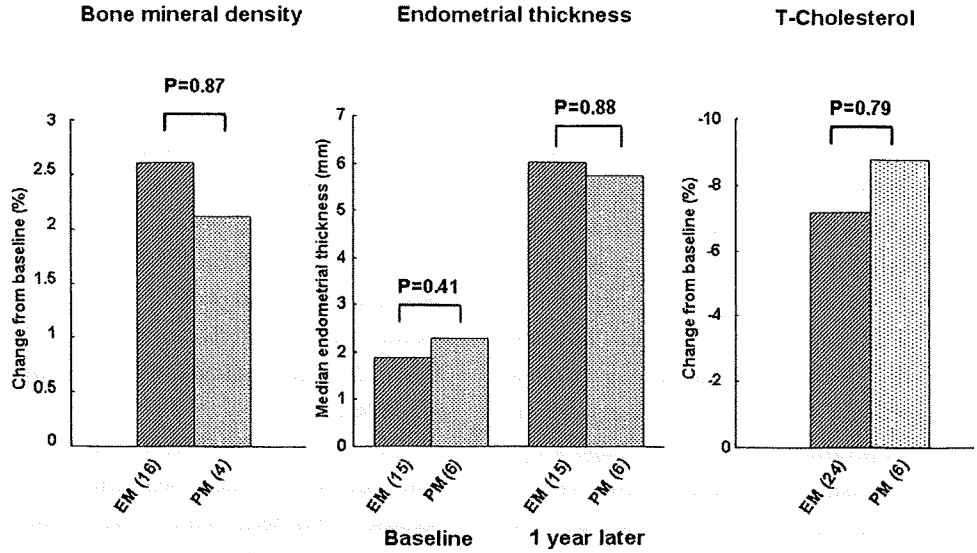

FIGURE 4. The influence of tamoxifen on bone mineral density, endometrial thickness, and total cholesterol (T-Cholesterol) levels in patients who received adjuvant tamoxifen according to cytochrome P450 (CYP) family 2, subfamily C, polypeptide 19, allele *2,*3 (CYP2C19*2,*3) genotype. Changes in the percentages of bone mineral density, endometrial thickness, and total cholesterol levels after 1 year of tamoxifen treatment are shown according to CYP2C19 genotype, ie, patients were designated as either poor metabolizers (PM) or extensive metabolizers (EM). Numbers in parentheses indicate the numbers of patients examined.

DISCUSSION

Because tamoxifen is used widely as 1 of the standard treatments in the metastatic and adjuvant settings, evidence of the suspected impact of CYP2D6*10 polymorphisms on the efficacy of tamoxifen would be of major consequence for clinical practice, especially in the case of Asian patients with breast cancer, because it is believed that these patients possess CYP2D6 *10/*10 homozygotes

Table 4. Meta-Analysis of the Prognostic Impact of Cytochrome P450 (CYP) 2D6*10/*10 Polymorphism

Study	No. of Patients	HR	95% CI
Kiyotani 2008[10]	58	10.04	1.17-86.27
Xu 2008[11]	152	4.7	1.1-20.0
Current study	173	0.6	0.18-1.92
Total	383	1.86	0.80-4.32

HR indicates hazard ratio; CI, confidence interval.

at a relatively high frequency (approximately 20%). However, unlike 2 previous reports, which claimed to demonstrate that the CYP2D6 *10/*10 genotype is associated with a poor prognosis, we did not observe any significant difference in RFS rates between the CYP2D6 *10/*10 genotype and the CYP2D6 wt/wt or wt/*10 genotype. The confidence intervals (CI) cited in those 2 previous reports revealed a wide range; ie, the hazard ratio was 10.04 (95% CI, 1.17-86.27) in the study by Kiyotani et al[10] and 4.7 (95% CI, 1.1-20.0) in the study by Xu et al.[11] Therefore, we used the method described by Parmar et al[16] to conduct an exploratory meta-analysis that included the 2 previous reports[10,11] and the current study. Our meta-analysis produced a hazard ratio of 1.86 (95%CI, 0.80-4.32) (Table 4), indicating that there was no significant association between RFS rates and CYP2D6 genotypes. In addition, it recently was reported that patients with the CYP2D6 *4/*4 genotype (null activity) are not associated not necessarily with a poor prognosis but, in fact, with a better prognosis.[17] Thus, it may be too early to conclude that the CYP2D6 *10/*10 or CYP2D6 *4/*4 genotypes have a clinically significant impact on the prognosis of patients who receive adjuvant tamoxifen. Although a significant decrease in endoxifen and 4OH-TAM levels in the blood has been reported for patients who have the CYP2D6 *10/*10 or CYP2D6 *4/*4 genotype compared with patients who have the CYP2D6 wt/wt genotype,[6,9,11] these tamoxifen metabolites still may be effective at such low levels, or the involvement of tamoxifen itself in the growth inhibition of breast tumors may be much greater than imagined.

It is known that tamoxifen has estrogenic effects on the endometrium, bone, and liver (total cholesterol levels), and these effects are thought to be mediated by its metabolites, endoxifen and 4OH-TAM. For this reason, we studied the impact of the CYP2D6 *10/*10 genotype on the effect of tamoxifen on these organs. Because normal organs are expected to respond to tamoxifen more homogeneously than tumors with their inherently heterogeneous response, the impact of the CYP2D6 *10/*10 genotype on these normal organs, if any, would be more evident than that on tumors. Consistent with the findings of previously reported studies,[18-20] tamoxifen treatment for 1 year in our study resulted in a significant increase in endometrial thickness and BMD as well as in a significant decrease in the total cholesterol levels. However, the extent of such an increase or decrease in any of these parameters was not significantly different between the CYP2D6 *10/*10 genotype and the CYP2D6 wt/wt or wt/*10 genotype. These results indicate that the CYP2D6 *10/*10 genotype has essentially no impact on the effects of tamoxifen in these target organs, which is consistent with our finding that the CYP2D6 *10/*10 genotype has no impact on the effect of tamoxifen on recurrence.

In the current study, we also investigated the association of CYP2C19 variant alleles *2 and *3 with the prognosis of patients who received adjuvant tamoxifen. Because the CYP2C19 *2 and *3 alleles possess no activity, patients with the CYP2C19 *2/*2, *3/*3, or *2/*3 genotype can be expected to have lower levels of endoxifen and 4OH-TAM, which would lead to higher recurrence rates when these patients are treated with adjuvant tamoxifen. However, such an association was not observed in our study. In addition, there was no association between these genotypes and endometrial thickness, BMD, or total cholesterol levels, indicating that the CYP2C19 *2/*2, *3/*3, or *2/*3 genotype is unlikely to influence the effect of tamoxifen.

In this study, we include patients who received adjuvant tamoxifen, and chemotherapy, and/or goserelin, because it has been demonstrated that tamoxifen significantly improves RFS rates even in the presence of these concomitant treatments[21,22] and because CYP2D6 and CYP2C19 essentially are not involved in the metabolism of epirubicin, methotrexate, or 5-FU, which were included in the chemotherapy. It is known that cyclophosphamide is activated by CYP2C19; thus, it has been speculated that cyclophosphamide, like tamoxifen, may be less effective for patients with the CYP2C19 *2/*2, *3/*3, or *2/*3 genotype. However, the prognosis for patients with any of these genetic polymorphisms was

similar to that for the patients with the CYP2C19 wt/wt, wt/*2, or wt*3 genotype, indicating that any of the CYP2C19 polymorphisms are unlikely to have an impact on the effects of either tamoxifen or cyclophosphamide.

In conclusion, in the current study, we were able to demonstrated that neither the CYP2D6 *10/*10 genotype nor the CYP2C19 *2/*2, *2/*3, or *3/*3 genotype was associated with a poor prognosis for breast cancer patients who were treated with adjuvant tamoxifen. Moreover, we demonstrated that these genetic polymorphisms are not associated with endometrial thickness, BMD, or total cholesterol levels. When taken together, these results suggest that the effects of tamoxifen are unlikely to be influenced by these genetic polymorphisms. At least, it may be too early to conclude that either the CYP2D6 *10/*10 genotype or the CYP2C19 *2/*2, *2/*3, or *3/*3 genotype has a clinically significant impact on disease recurrence in patients who receive adjuvant tamoxifen. However, our current findings need to be verified by future studies that include larger numbers of patients.

Conflict of Interest Disclosure

This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labor, and Welfare of Japan.

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ORIGINAL PAPER

Incidence of joint symptoms and bone fractures in Japanese postmenopausal breast cancer patients treated with adjuvant anastrozole

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Abstracts

Purpose Incidence of joint symptoms and bone fractures as well as changes in bone mineral density (BMD) in Japanese postmenopausal breast cancer patients treated with adjuvant anastrozole were investigated to determine whether there is an ethnic difference from Caucasian patients in the incidence of these adverse events of anastrozole.

Methods Adjuvant anastrozole was used to treat 348 postmenopausal breast cancer patients for a median period of 22 months. Adverse events of anastrozole including joint symptoms, loss of BMD, and bone fracture were investigated by means of chart review.

Results Joint symptoms developed in 96 (27.5%) patients. Age (younger than 65) and prior chemotherapy was strongly associated with an increased risk of joint symptoms. Annual fracture incidence was 0.86 and 0.85% and lumbar BMD decreased by 1.3 and 2.8% at 1 and 2 years, respectively. In comparison, the ATAC trial reported corresponding figures of 2.0 and 2.7 and of 2.2 and 4.0%.

Conclusion Incidence and risk factors of joint symptoms are similar for Japanese and Caucasian patients, but the former tend to show a smaller decrease in BMD and a lower incidence of bone fractures, probably due to ethnic difference in the hormonal milieu.

Keywords Breast cancer · Anastrozole · Bone mineral density · Fracture · Joint symptoms

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Introduction

For more than a decade, 5-year treatment with tamoxifen has been the gold standard for hormonal therapy for postmenopausal patients with hormone receptor positive breast cancer. However, the ATAC trial has clearly shown that anastrozole, a potent third-generation aromatase inhibitor, is superior to tamoxifen in terms of improved disease-free survival (Baum et al. 2002; Howell et al. 2005). As a result, anastrozole is now accepted as a standard treatment for postmenopausal patients with hormone receptor positive breast cancer.

Besides anastorozole's superior efficacy it seems to cause fewer adverse events because the incidence of tamoxifen-related serious adverse events such as endometrial cancer, thrombophlebitis, and ischemic cerebrovascular disease, etc. is significantly lower for patients receiving anastrozole rather than tamoxifen (Baum et al. 2002; Howell et al. 2005; Buzdar et al. 2006). It has been reported, however, that patients being treated with anastrozole show a higher incidence of joint symptoms (joint pain and stiffness), loss of bone mineral density (BMD) and bone fractures (Howell et al. 2005; Buzdar et al. 2006). These reports are based on clinical trials involving Caucasian breast cancer patients. Since BMD and bone fracture incidence are different for Japanese and Caucasian postmenopausal woman (Ito et al. 1997) and since hormonal milieus of these two ethnicities also differ, i.e., serum estradiol levels are about in Caucasian postmenopausal women twice as high as in their Japanese counterparts (Shimizu et al. 1990), we considered it quite possible that the adverse effect of anastrozole on bone might also be different.

We published a preliminary report on the influence of anastrozole on BMD in Japanese postmenopausal breast cancer patients, and suggested that the negative impact of

anastrozole on bone might be milder in Japanese than Caucasian women, based on the observation that BMD loss 1 year after anastrozole treatment is 1.2% for Japanese patients but reportedly 2.2% for Caucasian patients (Yoneda et al. 2006). In the study presented here, we investigated the actual incidence of joint symptoms and bone fractures for Japanese postmenopausal breast cancer patients treated with adjuvant anastrozole also studied changes in BMD after 2 years of this treatment.

Materials and methods

Patients

Between April 2002 and April 2007, 348 hormone receptor [estrogen receptor (ER) and/or progesterone receptor (PR)]-positive postmenopausal breast cancer patients were treated at our hospital with adjuvant anastrozole (1 mg/day) for a median period of 22 months ranging from 1 to 60 months. Adverse events of anastrozole on these patients, that is, joint symptoms, loss of BMD, and bone fracture, were investigated by means of chart review. Characteristics of the patients analyzed in this study are shown in Table 1. We also recorded the anastrozole-related joint symptoms (≥grade 1 according to Common Terminology Criteria for Adverse Events version 3.0) and fractures at any site.

Of the 348 patients, 122 had their lumbar spine (L2-4) BMD measured by means of dual-energy X-ray absorptiometry before the start of anastrozole treatment. Patients were categorized into three groups: normal bone mineral density [young adult mean (YAM ≥ 80%) ($n = 85$), osteopenia (80% > YAM ≥ 70%) ($n = 21$), or osteoporosis (YAM < 70%) ($n = 16$)]. Patients who were not osteopenic and those whose BMD was not measured before the start of anastrozole were not treated with preventive medication such as bisphosphonates, vitamin D, or calcium. However, patients who were osteopenic at the start of anastrozole and those who developed osteoporosis during anastrozole treatment received one or a combination of these medications.

Statistical analysis

The incidence of anastrozole-related joint symptoms was compared among various subgroups with the χ^2 test. Cumulative incidence of bone fractures was calculated with the Kaplan–Meier method. Changes from baseline in lumbar spinal BMD 1 and 2 years after the start of anastrozole treatment were assessed with the paired t test. Stat View software (Version 5.0 for Windows, SAS Institute Inc., Cary, NC, USA) was for statistical analysis. A P value less than 0.05 was considered to indicate statistical significance.

Table 1 Patient characteristics

	No. of patients
Demographics	
Age (years)	62 (8.2)[a]
Weight (kg)	56 (8.6)
Height (cm)	155 (4.7)
Body mass index (kg/m^2)	23 (3.2)
Stage	
I	140 (40.0%)
II	174 (49.7%)
III	24 (6.9%)
Hormone-receptor status	
ER positive	344 (98.2%)
PR positive	259 (74.0%)
Adjuvant chemotherapy	
None	226 (64.9%)
Epirubicin[b]	47 (13.4%)
Taxane[c]	7 (2.0%)
Epirubicin → Taxane[d]	63 (18%)
CMF	5 (1.5%)

[a] SD

[b] Epirubicin-containing regimens (epirubicin 75 mg/m^2 or 100 mg/m^2, 4–6 cycles, q3w)

[c] Taxane includes paclitaxel (80 mg/m^2, 12 cycles, q1w) or docetaxel (60 mg/m^2, 4 cycles, q3w)

[d] Epirubicin-containing regimens followed by paclitaxel or docetaxel

Results

Joint symptoms

Of the 348 patients, 96 (27.5%) developed joint symptoms (Table 2). The median time until development of joint symptoms was 3 months, ranging from 1 to 20 months. Of these 96 patients, 79 (82%) showed spontaneous resolution of joint symptoms with a median time until resolution of 3 months (range 1–18 months) even though anastrozole was continued without any medication for the symptoms. The symptoms of the remaining 17 (18%) patients deteriorated, so that anastrozole was discontinued and replaced with exemestane in, for two, tamoxifen or toremifene for 12, and no further treatment for three patients.

Risk factors for joint symptoms are displayed in Table 3. Age <65, but not BMI, was strongly associated with a higher risk of joint symptoms ($P = 0.0004$). Presence of adjuvant chemotherapy was also strongly correlated with an increased risk of joint symptoms regardless of the regimen, since the incidence of joint symptoms was 21.2% for the patients not treated with chemotherapy, 44.2%

Table 2 Number of patients (%) by type of joint symptoms

	No. of patients
Joint symptoms	
Morning stiffness	44 (45.8%)
Arthralgia	44 (45.8%)
Myagia	6 (6.2%)
Others	2 (2%)
Total	96 (100%)

Table 3 Risk factors of anastrozole-related joint symptoms

No. of patients	Total	With joint symptoms	%	*P*
Age, years				
<65	227	77	33.9	0.0004
≥65	121	19	15.7	
BMI				
<23	199	61	30.6	0.137
≥23	149	35	23.4	
Adjuvant chemotherapy				
None	226	48	21.2	
Taxane or Epirubicin → Taxane	70	31	44.2	0.0004
Epirubicin	110	47	42.7	<0.0001
CMF	5	0	0	0.166

($P = 0.0004$) for those treated with taxane or sequential epirubicin and taxane therapy, and 42.7% ($P = 0.0004$) for those treated with epirubicin.

Bone fractures

Eight fractures occurred in the 348 patients with the fracture sites as shown in Table 4. Annual fracture rates were 0.86% (3/348) and 0.85% (2/234) during the first and second year, respectively, after the start of treatment with anastrozole (Fig. 1). Since the median follow-up period was 22 months and the number of patients treated with anastrozole for more than 2 years was so small, annual fracture rates are shown only for the first two years. These rates are lower than those reported for the Caucasian breast cancer patients in the ATAC trial (2.0 and 2.7%, respectively) (Buzdar et al. 2006).

Table 4 Bone fracture sites

	No. of patients
Bone fracture sites	
Shoulder	1
Clavicle	2
Wrist	2
Exfoliation	1
Spine	1
Jaw	1
Total	8

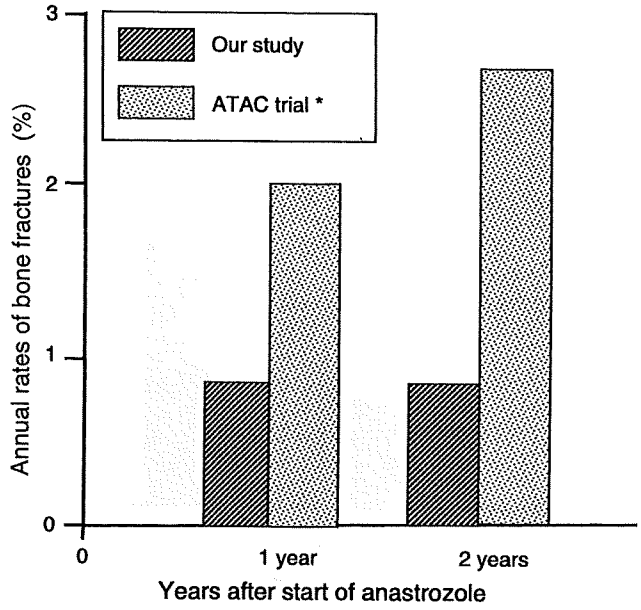

Fig. 1 Annual rates of bone fracture after start of anastrozole treatment (* Buzdar et al. 2006)

Infuluence of anastrozole on BMD

The 39 patients who showed normal BMD of the lumbar spine at baseline had their BMD measured serially 1 and 2 years after the start of anastrozole treatment (patients who were osteopenic or osteoporosis at baseline were excluded from this analysis). As shown in Fig. 2, lumbar BMD decreased by 1.3 and 2.8%, respectively. These reductions in lumbar BMD are smaller than those reported for the Caucasian breast cancer patients in the ATAC trial (2.2 and 4.0%, respectively) (Buzdar et al. 2006).

Discussion

According to the results of the ATAC trial, which compared the effects of adjuvant anastrozole and tamoxifen for Caucasian postmenopausal breast cancer patients, 35.6% of patients experienced joint symptoms, and the majority of the symptoms developed within 24 months after the start of treatment with a peak occurrence at 6 months. The median time to resolution of joint symptoms was reported as 5.5 months. Our study obtained essentially similar results for the incidence and clinical course of joint symptoms. Other studies have reported a similar incidence of

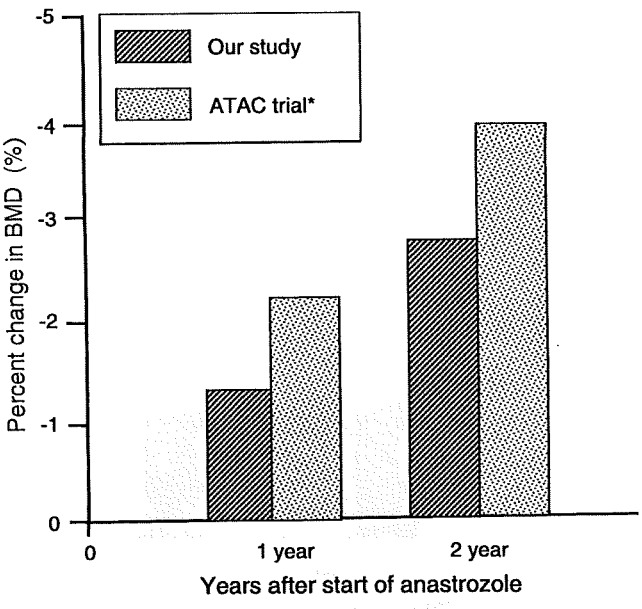

Fig. 2 Influence of anastrozole on BMD (* Buzdar et al. 2006)

anastrozole-related joint symptoms for Japanese women (Ohsako et al. 2006). It can therefore be concluded that the incidence of anastrozole-related joint symptoms and their clinical course are similar for Japanese and Caucasian postmenopausal breast cancer patients.

Although the exact mechanism of anastrozole-related joint symptoms is still unclear, Donnellan et al. suggest that a precipitous fall in estrogen levels is one cause of the observed joint symptoms (Donnellan et al. 2001). Andersen et al. also reported estrogen deficiency accelerates cartilage turnover and increases cartilage surface erosion (Andersen et al. 2004), suggesting that anastrozole-induced decrease in estrogen levels might play an important role in the pathogenesis of joint symptoms. Crew et al. reported that patients who have received prior taxane chemotherapy are more than four times more likely to develop anastrozole-related joint symptoms than those who have not (Crew et al. 2007). In the study presented here, we obtained similar results in that patients who received prior taxane or taxane-containing chemotherapy are at a higher risk for developing joint symptoms, and we also found that epirubicin-containing regimens can also be a significant risk factor for joint symptoms (Table 4).

Since BMD and bone fracture incidence are reported to be different for Japanese and Caucasian postmenopausal women in a general population (Ross et al. 1995) and since the hormonal milieu is also different, it was considered possible that the adverse effect of anastrozole on bone might also be different for these two ethnicities. The ATAC trial reports that annual rates of bone fractures are 2.0 and 2.7% for the first and second year, respectively, after the start of anastrozle treatment for Caucasian patients. In our study, however, we found that the corresponding rates for Japanese patients are 0.86 and 0.85% and thus appear to be lower than those reported for Caucasian women. Since the median follow-up period of our study is still short (22 months), the long-term adverse effect of anastrozole on bone fractures need to be investigated. Nevertheless, our results seem to indicate the possibility that there is an ethnic difference in bone fracture rates between Japanese and Caucasian patients.

We studied changes in BMD over time after the start of anastrozole treatment for patients with normal BMD and found that they lose 1.3 and 2.8% of BMD 1 and 2 years, respectively, after the start of the treatment. The subprotocol of the ATAC trial, on the other hand, shows that Caucasian women lose an average of 2.2 and 4.0% of BMD 1 and 2 years, respectively, after the start of anastrozole therapy. These results seem to suggest that Japanese women are likely to show a smaller reduction in BMD than Caucasian women do, which is compatible with our observation that Japanese patients have a lower incidence of bone fractures than Caucasian patients, although the difference in the patient population and in the method for BMD measurement used in these two studies makes a direct comparison inaccurate. It has been reported, however, that postmenopausal Caucasian women have higher serum estrogen levels and higher BMD than postmenopausal Japanese women (Shimizu et al. 1990; Ito et al. 1997). It is therefore reasonable to speculate that anastrozole treatment is likely to induce a more precipitous fall in estrogen levels in Caucasian patients than in Japanese patients, resulting in a greater reduction in BMD and a subsequent higher incidence of bone fractures in the former.

In conclusion, we have been able to show that the incidence of joint symptoms and their clinical course and risk factors is very similar for Japanese and Caucasian breast cancer patients treated with adjuvant anastrozole but that Japanese patients have a smaller reduction in BMD and a lower incidence of bone fractures than Caucasian patients after anastrozole treatment, probably due to ethnic differences in hormonal milieu. The possible ethnic difference in the adverse effect of anastrozole on bone considered in our study may be of considerable clinical importance and thus needs to be investigated in more detail and with a larger number of patients.

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Good Response to Paclitaxel Predicts High Rates of Pathologic Complete Response for Breast Cancer Patients Treated Preoperatively with Paclitaxel Followed by 5-Fluorouracil, Epirubicin and Cyclophosphamide

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Key Words
Breast cancer · Neoadjuvant chemotherapy · Predictor factor · Response

Abstract
Objective: Predictors of pathologic complete response (pCR) to neoadjuvant chemotherapy for breast cancers have been studied extensively. Here, we focused on reduction rate after paclitaxel administration for prediction of pCR to paclitaxel followed by 5-fluorouracil, epirubicin, and cyclophosphamide (FEC). ***Methods:*** This study included 115 patients with tumors ≥3.0 cm or with node-positive disease who were treated preoperatively with paclitaxel (80 mg/m^2, once a week, 12 cycles) followed by FEC (500/75/500 mg/m^2, every three weeks, 4 cycles). Reduction rate was measured with magnetic resonance imaging. ***Results:*** Tumor size (≤5.0 cm) ($p = 0.014$), estrogen receptor (ER) negativity ($p = 0.013$), and human epidermal growth factor receptor 2 positivity ($p = 0.020$), but not histologic type, histologic grade, or progesterone receptor, were significantly associated with pCR, while association of reduction rate ≥80% was highly significant ($p = 0.0003$). Multivariate analysis identified negative ER ($p = 0.022$) and reduction rate ($p = 0.003$) as independent predictors of pCR. Finally, patients with reduction rate ≥80% showed a significantly higher favorable outcome ($p = 0.014$) than others. ***Conclusions:*** Good response (reduction rate ≥80%) to paclitaxel seems to be a clinically useful predictor of pCR as well as a favorable prognosticator for patients treated preoperatively with paclitaxel followed by FEC.

Introduction

Neoadjuvant chemotherapy (NAC) has become the standard of care not only for inoperable but also for operable locally advanced breast cancer patients [1–3]. NAC offers the advantage that tumors can be downstaged, making previously inoperable tumors operable and enhancing the feasibility of breast-conserving surgery [4, 5]. In addition, several lines of evidence have demonstrated that patients who attain pathologic complete response (pCR) to NAC show a better prognosis than those without pCR, so that pCR is now considered to be the best prognosticator for patients treated with NAC [1–3, 6, 7]. Thus, many attempts have recently been made to develop clinically useful predictors of post-NAC pCR, including a variety of clinicopathologic parameters such as histo-

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logic grade, estrogen receptor (ER), progesterone receptor (PR) status, human epidermal growth factor receptor 2 (HER2) as well as gene-expression profiling [8–11]. These parameters have been evaluated for their ability to predict pCR and some associations of these parameters with pCR have been reported, but their clinical value remains unconvincing so that a more precise predictor of pCR needs to be developed.

Sequential chemotherapy consisting of paclitaxel and an anthracycline-based regimen (5-fluorouracil, epirubicin or doxorubicin, and cyclophosphamide), known as FEC or FAC, is now widely accepted as a highly efficacious NAC [2, 3, 7, 8, 12, 13]. Our study focused on response to initial paclitaxel therapy as a predictor of pCR after administration of sequential paclitaxel and FEC. Since tumors which respond well to initial chemotherapy are generally more likely to show a more favorable response to subsequent chemotherapy than initial nonresponders, we hypothesized that responsiveness to initial chemotherapy would be a good predictor of response to second-line chemotherapy.

The aim of this study was therefore to examine the association between response to initial paclitaxel therapy and pCR after completion of paclitaxel followed by FEC and to compare its value as a pCR predictor with that of other conventional predictors. Since we considered a highly accurate evaluation of the response to initial paclitaxel therapy as essential, we monitored tumor size before and after paclitaxel with magnetic resonance imaging (MRI), which is considered to be the most accurate modality for assessment of tumor size after NAC [14–16].

Patients and Methods

Patients

Primary breast cancer patients with tumors ≥3.0 cm in diameter or with cytologically confirmed lymph node involvement, but excluding those with stage IV diseases, were recruited for this study. All patients underwent a vacuum-assisted core needle biopsy to confirm the presence of invasive breast cancer before NAC. After informed consent was obtained, patients were treated with paclitaxel (80 mg/m^2, weekly for 12 cycles) followed by FEC (500/75/500 mg/m^2, every three weeks, 4 cycles) [13]. After NAC, patients were treated with breast-conserving surgery followed by radiation therapy or mastectomy (radiation therapy for the chest wall was added for more than 3 positive lymph nodes).

All patients with hormone-receptor-positive (ER and/or PR positive) tumors were given adjuvant hormone therapy (tamoxifen plus goserelin for premenopausal, and tamoxifen or anastrozole for postmenopausal patients) essentially according to the guidelines of the St. Gallen consensus meeting [17]. No patients received chemotherapy after surgery. Of the 115 patients, 110 patients completed NAC as scheduled, but 2 patients received 11 cycles of paclitaxel and 3 patients received 3 cycles of FEC. Dose was not reduced in any patients. No patient developed cardiac adverse events. Of 32 patients with HER2-positive tumors, no patients were treated with trastuzumab preoperatively, but 11 pa-

Table 1. Patient characteristics (n = 115)

Category	
Age, years	
Range	23–72
Mean	49.5
Menopausal status	
Premenopausal	58 (50%)
Postmenopausal	57 (50%)
Clinical stage	
IIA	26 (23%)
IIB	45 (39%)
IIIA	28 (24%)
IIIB	14 (12%)
IIIC	2 (2%)
Tumor size	
≤5.0 cm	78 (68%)
>5.0 cm	37 (32%)
Histology	
Invasive ductal cancer	105 (91%)
Invasive lobular cancer	8 (7%)
Others	2 (2%)
Histologic grade[1]	
I	13 (11%)
II	77 (68%)
III	24 (21%)
ER	
Positive	70 (61%)
Negative	45 (39%)
PR	
Positive	40 (35%)
Negative	75 (65%)
HER2[2]	
Negative	82 (72%)
Positive	32 (28%)
Surgery	
Mastectomy	86 (75%)
Breast-conserving surgery	29 (25%)
Adjuvant endocrine therapy	
No	37 (32%)
Tamoxifen	14 (12%)
Tamoxifen + LHRH analog	20 (17%)
Aromatase inhibitors	44 (38%)
Adjuvant trastuzumab	
Yes	11 (10%)
No	104 (90%)

[1] One sample was not available.
[2] One sample was not evaluated.

Table 2. Association between clinicopathologic parameters and pCR

Parameters	Category	pCR		p value[1]
		yes (n = 29)	no (n = 86)	
Menopausal status	pre-	11 (19%)	47 (81%)	0.119
	post-	18 (32%)	39 (68%)	
Tumor size, cm	≤5.0	25 (32%)	53 (68%)	0.014
	>5.0	4 (11%)	33 (89%)	
Histologic type	IDC	28 (27%)	77 (73%)	0.177
	ILC	0 (0%)	8 (100%)	
	mucinous	1 (50%)	1 (50%)	
Histologic grade[2]	1	4 (31%)	9 (69%)	0.847
	2	18 (23%)	59 (77%)	
	3	6 (25%)	18 (75%)	
ER	positive	12 (17%)	58 (83%)	0.013
	negative	17 (38%)	28 (62%)	
PR	positive	7 (18%)	33 (83%)	0.164
	negative	22 (29%)	53 (71%)	
HER2[2]	negative	16 (20%)	66 (80%)	0.020
	positive	13 (41%)	19 (59%)	
ER/HER2	(+)/(−)	8 (15%)	46 (85%)	0.010
	(+)/(+)	4 (25%)	12 (75%)	
	(−)/(−)	8 (29%)	20 (71%)	
	(−)/(+)	9 (56%)	7 (44%)	
WHO criteria	non-responders	2 (9%)	21 (91%)	0.041
	responders	27 (29%)	65 (71%)	
RECIST	non-responders	4 (13%)	26 (87%)	0.081
	responders	25 (29%)	60 (71%)	
Reduction rate after paclitaxel	<80%	7 (11%)	54 (89%)	0.0003
	≥80%	22 (41%)	32 (59%)	

IDC = invasive ductal carcinoma; ILC = invasive lobular carcinoma.

[1] p value was evaluated with the χ^2 test, and considered significant when $p < 0.05$.

[2] One sample was not evaluated.

tients received trastuzumab postoperatively. Median follow-up after surgery was 20 months, ranging from 6 to 53 months. Patient characteristics are summarized in table 1.

Hormone Receptor and HER2 Assay

ER and PR were determined by immunohistochemistry (IHC) with a cut-off value of 10%. HER2 expression was examined with IHC (Herceptest®; Dako Corporation, Carpenteria, Calif., USA) or fluorescence in situ hybridization (FISH) (Pathvysion®; Vysis Inc., Downers Grove, Ill., USA). Tumors with IHC (3+) or FISH (+) were considered HER2 positive.

Evaluation of Clinical and Pathologic Response to Chemotherapy

Clinical response of primary breast tumors to paclitaxel was evaluated with dynamic MRI before and after paclitaxel administration. Response to paclitaxel was evaluated with ultrasonography in 17 patients who did not undergo dynamic MRI. Reduction rate was calculated with the following formula: reduction rate (%) = (tumor area before paclitaxel − tumor area after paclitaxel)/tumor area before paclitaxel × 100. Pathologic response was evaluated in specimens obtained at surgery. pCR was defined as absence of residual invasive foci and no lymph node involvement.

Statistical Methods

Associations of clinicopathologic parameters with pCR were evaluated with the χ^2 test. Multivariate analysis of the relationship of negative ER and reduction rate (≥80%) with pCR was determined using a logistic regression method to obtain the odds ratio and 95% confidence interval. Relapse-free survival (RFS) rates were estimated with the Kaplan-Meier method, and differences were evaluated with the log-rank test. All test results with a p value of less than 0.05 were considered significant. All statistical analyses were performed with StatView™ software (SAS Institute Inc., Cary, N.C., USA).

Results

Association of Clinicopathologic Parameters with pCR

The association of clinicopathologic parameters with pCR was analyzed in 115 patients, and pCR was observed in 29 (25%) of them. Table 2 shows associations between pCR and various clinicopathologic parameters. Menopausal status, histologic type, histologic grade, and PR status were not significantly associated with pCR, although it was noteworthy that none of the 8 invasive lobular carcinomas attained pCR. Tumors ≤5.0 cm (pCR rate: 32%), ER-negative tumors (38%), and HER2-positive tumors (41%) were significantly more likely to attain pCR than tumors >5.0 cm (11%, p = 0.014), ER-positive tumors (17%, p = 0.013), and HER2-negative tumors (20%, p = 0.020). Analysis of ER and HER2 combined showed that ER-negative and HER2-positive tumors were most likely (56%), and ER-positive and HER2-negative tumors most unlikely to attain pCR (15%). ER-positive and HER2-positive tumors (25%) and ER-negative and HER2-negative tumors (29%) showed intermediate pCR rates.

Association of Reduction Rate after Paclitaxel with pCR

There was a significant association between MRI-evaluated reduction rates after paclitaxel and pCR rates (fig. 1). Tumors with a reduction rate ≥50%, equivalent to complete response and partial response by WHO criteria, were likely to attain pCR compared with those with

a reduction rate <50% (pCR rate, 29 vs. 9%, p = 0.041), and those with a reduction rate ≥80% were significantly more likely to attain pCR than those with a reduction rate <80% (pCR rate, 41 vs. 11%, p = 0.0003) (fig. 2, 3, table 2). Responders (complete response + partial response) classified by Response Evaluation Criteria In Solid Tumors (RECIST) had a tendency to attain pCR compared with nonresponders, but the difference was not statistically significant (pCR rate, 29 vs. 13%, p = 0.081) (table 2).

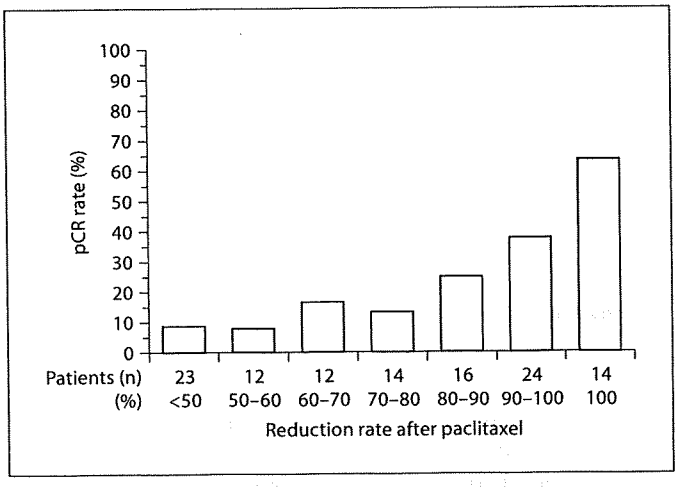

Fig. 1. pCR rates for paclitaxel followed by FEC in relation to reduction rate after paclitaxel. pCR rates were 64% for a reduction rate of 100%, 38% for a 100% > reduction rate ≥ 90%, 25% for a 90% > reduction rate ≥ 80%, 14% for an 80% > reduction rate ≥ 70%, 17% for a 70% > reduction rate ≥ 60%, 8% for a 60% > reduction rate ≥ 50%, and 9% for a 50% > reduction rate.

Univariate and Multivariate Analysis of Predictors for pCR

Univariate analysis demonstrated that small tumor size (p = 0.020), ER negativity (p = 0.015), HER2 positivity (p = 0.023), and a reduction rate ≥80% after paclitaxel (p = 0.001) are significantly associated with pCR. Multivariate analysis showed that ER (p = 0.022) and reduction rate (p = 0.003) are significant and independent predictors of pCR (table 3).

Association of pCR or Reduction Rate after Paclitaxel with Prognosis

None of the 29 patients who attained pCR developed recurrences, whereas 14 of the 86 patients who could not attain pCR did (p = 0.026). Patients with a reduction rate ≥80% after paclitaxel showed significantly better RFS rates than those with a reduction rate <80% (events, $^{2}/_{54}$ vs. $^{12}/_{61}$, p = 0.014).

Discussion

Many studies have focused on the development of predictors of pCR for NAC. Several investigators have demonstrated that ER-negative or HER2-positive tumors are

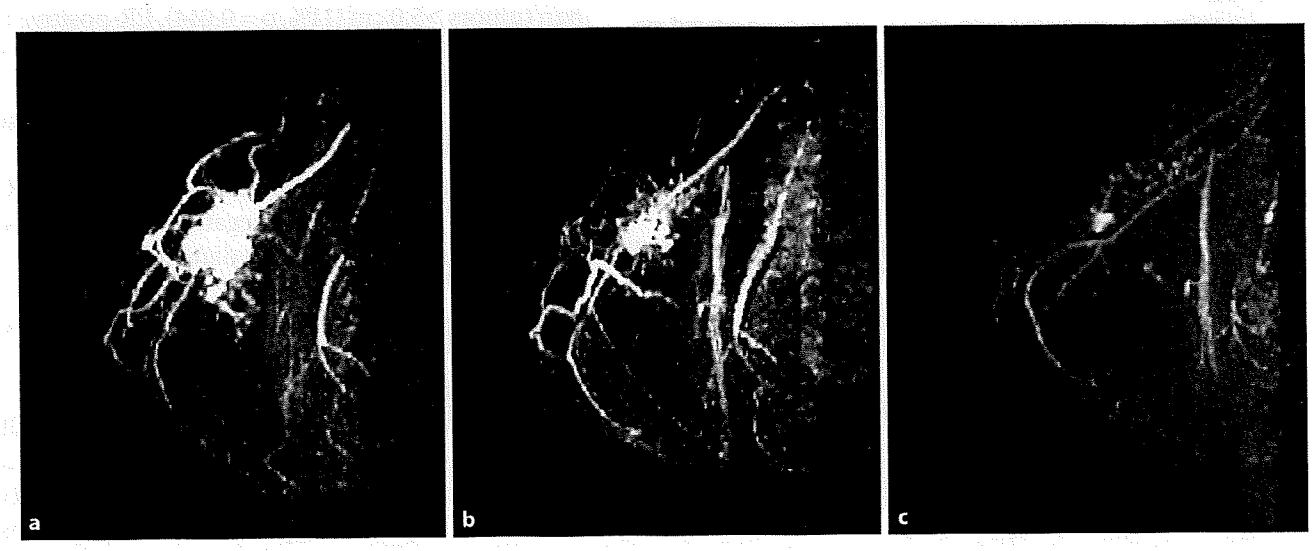

Fig. 2. Contrast-enhanced MRI images in a 72-year-old woman with <80% of tumor shrinkage after paclitaxel. **a** Before paclitaxel. **b** Reduction rate was 66% after paclitaxel. **c** The final reduction rate was 93% after the completion of paclitaxel followed by FEC. Her tumor did not attain pCR.

significantly associated with pCR [7–10]. In addition, recent review works have shown that not only anthracycline-based regimens but also taxane-containing regimens provide more benefit in patients with HER2-positive tumors than in those with HER2-negative tumors [18, 19]. Our results also showed that ER-negative and HER2-positive tumors are significantly more likely than ER-positive and HER2-negative tumors to attain pCR to paclitaxel followed by FEC (pCR rate, 56 vs. 15%), indicating that ER and HER2 can serve as significant predictors of pCR to paclitaxel followed by FEC. However, the percentage of patients with ER-negative and HER2-positive disease is small (14%) in all patients.

Besides ER and HER2, we focused on response to paclitaxel as a novel predictor of pCR to paclitaxel followed by FEC because tumors with pCR after the completion of this neoadjuvant regimen often show a good response to initial paclitaxel. Using common criteria, i.e., WHO or RECIST, as many as approximately 80% of patients were classified as responders (complete response + partial response) to paclitaxel and such clinical responses did not show a strong correlation with pCR (table 2). Therefore, we employed a stricter cut-off value (reduction rate ≥80%). pCR was observed in 22 (41%) of the 54 tumors with a reduction rate ≥80% in response to paclitaxel and in 7 (11%) of the 61 with a reduction rate <80%. These results indicate that a good response to paclitaxel can serve as a significant predictor of pCR. Multivariate analysis demonstrated that response to paclitaxel is a significant predictor of pCR, which is independent of ER and HER2. In addition, we were able to show that response to paclitaxel also serves as a prognostic indicator, i.e., patients with a good response show a significantly better prognosis than those with a poor response. However, our present data should be interpreted with great caution due to the following reasons. Firstly, 41% of patients with tumors with pCR had ER-positive disease and

Table 3. Uni- and multivariate analyses of pCR

	Univariate			Multivariate		
	OR	95% CI	p	OR	95% CI	p
Tumor size						
≤5.0 cm vs. >5.0 cm	3.9	1.2–12.2	0.020	3.0	0.9–10.2	0.076
ER						
(–) vs. (+)	2.9	1.2–7.0	0.015	3.2	1.2–8.7	0.022
HER2						
(+) vs. (–)	2.8	1.2–6.9	0.023	1.9	0.7–5.4	0.226
Reduction rate after paclitaxel						
≥80% vs. <80%	5.3	2.0–13.8	0.001	4.9	1.7–14.1	0.003

p value was considered significant when $p < 0.05$. OR = Odds ratio; CI = confidence interval.

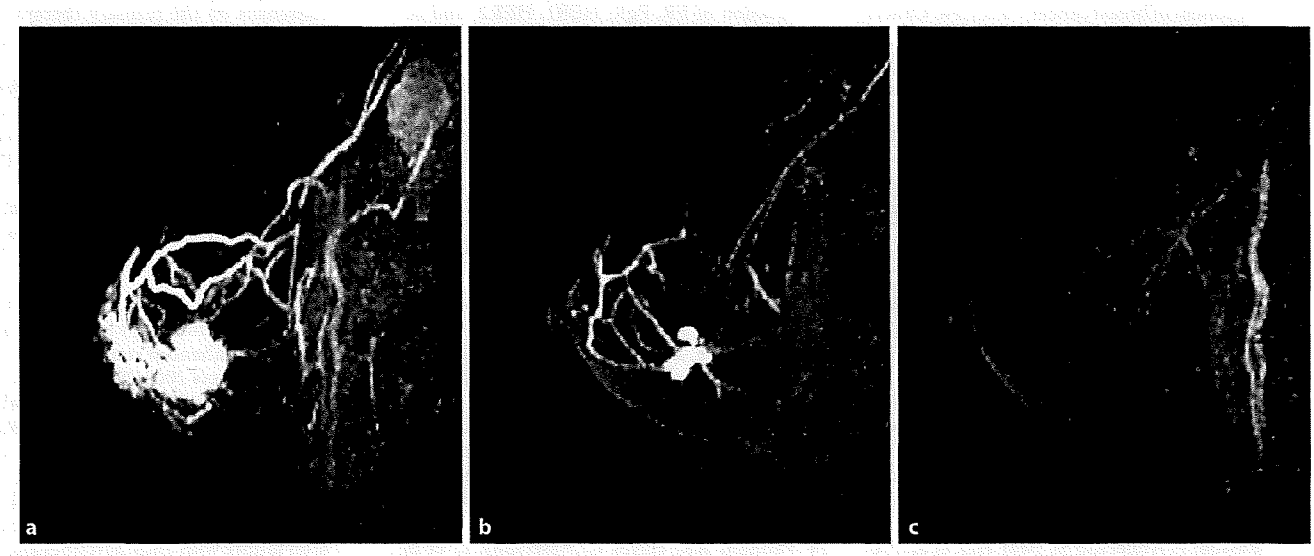

Fig. 3. Contrast-enhanced MRI images in a 60-year-old woman with ≥80% of tumor shrinkage after paclitaxel. **a** Before paclitaxel. **b** Reduction rate was 80% after paclitaxel. **c** The final reduction rate was 100% after the completion of paclitaxel followed by FEC. Her tumor attained pCR.

were treated with adjuvant endocrine therapy. Secondly, the present study is a retrospective study at a single institute, and lastly, this study has a very short follow-up period.

Our observations seem to be consistent with the results of the Aberdeen neoadjuvant trial [20], where patients who showed a good response to the initial anthracycline-based chemotherapy and switched to subsequent docetaxel attained a high pCR rate (34%) while those who showed a poor initial response and switched to docetaxel attained a low pCR rate (2%). Our results together with those of the Aberdeen neoadjuvant trial suggest that breast tumors responsive to taxane- and those responsive to anthracycline-based chemotherapy show considerable overlap, and such overlapping tumors can be expected to show a rather good response to sequential taxane- and anthracycline-based chemotherapy, with pCR as the result. Thus, patients who show a good response to paclitaxel are definite candidates for subsequent FEC since they have a very good chance to attain pCR, but those who show a poor response to paclitaxel can be expected to attain a very low pCR rate as well as a poor prognosis. For the latter group, administration of other chemotherapies proven to be effective for tumors resistant to paclitaxel- and anthracycline-based chemotherapy, i.e., capecitabine, vinorelbine, or gemicitabine, should be considered [21–23].

In conclusion, we were able to show that, in addition to ER and HER2, response to paclitaxel can serve as a significant predictor of pCR for patients preoperatively treated with paclitaxel followed by FEC. Prediction of pCR based on response to initial paclitaxel might be useful for choosing the second-line chemotherapy and might serve as a surrogate marker of prognosis, although our findings need to be validated by a future study including a larger number of patients.

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PRECLINICAL STUDY

Growth-inhibitory effect of adiponectin via adiponectin receptor 1 on human breast cancer cells through inhibition of S-phase entry without inducing apoptosis

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Abstract Adiponectin is one of the most important adipocytokines secreted from adipose tissue. In addition to its effects on glucose and fatty acid metabolism, it has been reported that adiponectin has a direct growth-inhibitory effect on breast cancer cells. However, it still remains to be established how adiponectin affects cell cycle and apoptosis and whether or not its inhibitory effect is mediated through adiponectin receptors. Here, we demonstrated that adiponectin treatment resulted in a significant dose-dependent growth inhibition of both MDA-MB-231 and T47D cells. In both cell lines, the G0/G1 population significantly increased after adiponectin treatment, but apoptosis was not induced. High expression of mRNA and protein of adiponectin receptor 1 was observed, but expression of adiponectin receptor 2 was very low in both cell lines. Treatment with small interference RNA against adiponectin receptor 1 significantly reduced the growth inhibition induced by adiponectin in both cell lines. Taken together, adiponectin decreases breast cancer cell proliferation by inhibiting the entry into S-phase without inducing apoptosis, and this inhibitory effect is mediated through adiponectin receptor 1.

Keywords Adiponectin · AdipoR1 · AdipoR2 · Breast cancer · Cell cycle

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Abbreviations

AdipoR1	Adiponectin receptor 1
AdipoR2	Adiponectin receptor 2
PBS	Phosphate-buffered saline
SDS	Sodium dodecyl sulfate
TBST	Tris-buffered saline with Tween-20
TUNEL	Terminal deoxynucleotidyl transferase biotin-dUTP Nick End Labeling
WST1	Water-soluble tetrazolium

Introduction

Adiponectin is one of the most important adipocytokines secreted from adipose tissue. In contrast to other adipocytokines such as tumor necrosis factor-α and leptin, serum adiponectin concentration correlates inversely with obesity. This peptide hormone plays a preventive role in the pathogenesis of diabetes through the modulation of glucose and fatty acid metabolism and insulin sensitivity in various stromal and epithelial cells, and in the pathogenesis of atherosclerosis through the inhibition of vascular smooth muscle and endothelial cell proliferation.

In addition, we have recently shown that low adiponectin concentration is significantly associated with an increased risk of breast cancer [1], a finding that was recently confirmed by Mantzoros et al. [2]. It has also been demonstrated that adiponectin concentration is inversely related to the risk of endometrial cancer [3], prostate cancer [4], and gastric cancer [5]. These results seem to suggest that the well-established association of obesity with a high risk for various types of cancer might be explained at least in part by the downregulation of adiponectin, and that adiponectin might have a growth-inhibitory effect on various types of cancer.

Recently, two adiponectin receptor isoforms, AdipoR1 and AdipoR2, have been cloned [6]. In mice, AdipoR1 is expressed in various organs such as skeletal muscle, lung, and spleen, and AdipoR2 is expressed predominantly in the liver [6]. In humans, AdipoR1 and AdipoR2 are expressed in the islet cells of the pancreas, macrophages, adipocytes and vascular smooth muscle [7–9]. Very recently, we have been able to show that both AdipoR1 and AdipoR2 are expressed in breast cancer cells. Although it has been reported that adiponectin inhibits the proliferation of breast cancer cells in vitro, it still remains to be established whether or not this inhibitory effect is mediated through these adiponectin receptors. It also remains to be studied how adiponectin affects cell cycle and apoptosis. In the present study, we have investigated these issues using human breast cancer cells in vitro.

Materials and methods

Cell culture and reagents

MDA-MB-231 cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12. MCF-7, T47D and HepG2 cells were maintained in RPMI 1640 medium. All media were purchased from Sigma (St. Louis, MO) and supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT) and 1% antibiotic–antimycotic solution (Gibco/Invitrogen Carlsbad, CA). One normal breast epithelial cell line (MCF-10A) was maintained in mammary epithelial growth medium (Cambrex Bio Science Walkersville Inc., Walkersville, MD) according to the manufacturer's instructions. All cell lines were maintained in a humidified incubator at 37°C in a 5% CO_2 atmosphere.

Recombinant adiponectin protein was obtained from Biovendor (Heidelberg, Germany). Antibodies against AdipoR1 and AdipoR2 were obtained from Immuno-Biological Laboratories (Gunma, Japan).

Cell viability assay

Cells were seeded at a concentration of 2×10^3 cells/well in 100 μl of culture medium into 96-well culture plates and incubated for 24 h. Cells were washed and fresh culture medium containing various concentrations of adiponectin (10 ng/ml–30 μg/ml) was added. After adiponectin treatment, 10 μl of the cell proliferation reagent WST-1 (Chemicon International, Inc., Temecula, CA) was added in 100 μl of culture medium to each well, and the plates were incubated for 1.5 h. Absorbance of the samples was measured at 440 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

Flow cytometry

Cells were exposed to 10 μg/ml adiponectin for 24, 48, or 72 h, harvested by trypsinization, and centrifuged at 200*g* for 5 min. Cells were washed with phosphate-buffered saline (PBS), fixed in 70% ethanol at −20°C for 1 h, and stained with 50 μg/ml propidium iodide in PBS-glucose containing ribonuclease A (Sigma, 2 kU/ml) for 1 h. The DNA content of the cells was measured using a FACSCalibur flow cytometer and analyzed with ModFit software (BD Biosciences, San Jose, CA).

RNA extraction and quantitative RT-PCR for adiponectin receptors

Total RNA was extracted from the cultured cell with TRIZOL reagent according to the manufacturer's protocol (Molecular Research Center, Cincinnati, OH). Three micrograms of total RNA underwent RT for single-strand cDNA using oligo(dT)15 primer and Superscript II (Life Technologies, Inc., Bethesda, MD) and was scaled up to a final volume of 50 μl. The RT reaction was performed at 42°C for 50 min, followed by heating at 70°C for 15 min.

Real-time PCR for AdipoR1 and AdipoR2 was carried out using the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, CA). The sequence of the primers and probes for AdipoR1 and AdipoR2 as well as the reaction conditions were described in our previous report [10]. β-Glucoronidase transcripts for quantitative control were used to normalize the transcript content of the sample. The primer and probe mixture for β-glucoronidase was purchased from Perkin-Elmer Applied Biosystems and used according to the manufacturer's protocol. The standard curves for AdipoR1, AdipoR2 and β-glucoronidase mRNA were generated using serially diluted solutions of each PCR product (10^{-12} μg PCR product for AdipoR1 and AdipoR2 and 10^{-8} μg PCR product for β-glucoronidase) as a template. Real-time PCR assays were conducted in duplicate for each sample, and the mean value was used for calculation of the relative expression levels. The final expression levels of AdipoR1 and AdipoR2 mRNA were expressed as ratios to those of β-glucoronidase.

Protein expression analysis of adiponectin receptors

At 60–70% confluence, cells were harvested by trypsinization and centrifuged at 200*g* for 5 min. The pellets were

lysed with lysis buffer (RIPA buffer: 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 2 mm phenylmethylsulfonyl fluoride, 1 mm sodium orthovanadate), and centrifuged at 10,000*g* for 5 min at 4°C. The resulting membrane extracts were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA) and blocked in blocking buffer for 1 h at room temperature. Membranes were incubated overnight at 4°C with rabbit polyclonal anti-AdipoR1 antibody or with rabbit polyclonal anti-AdipoR2 antibody (1:500 dilution, Immuno-Biological Laboratories) in Tris-buffered saline with Tween-20 (TBST). Membranes were then incubated with the secondary HRP-conjugated anti-rabbit IgG (1:5,000 dilution, DakoCytomation, Glostrup, Denmark) in TBST. Signal detection for each protein was performed using an ECL Western Blotting Reagents kit (GE Healthcare Bio-Science, Uppsala, Sweden).

Detection of apoptosis by Terminal deoxynucleotidyl transferase biotin-dUTP Nick End Labeling (TUNEL) assay

MDA-MB-231 and T47D cells were plated in slide flasks and grown for 24 h before adiponectin treatment. Cells were treated with or without 10 μg/ml of adiponectin for 48 h, then rinsed twice with PBS, and subjected to the TUNEL assay using the DeadEnd Colorimetric TUNEL System (Promega, Madison, WI). Paclitaxel (PTX) treatment (20 nM) was used as a positive control for the induction of apoptosis.

Small interfering RNA (siRNA) transfection

We obtained siRNA against AdipoR1 and against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from Dharmacon Research, Inc. (Lafayette, CO). Each siRNA was transfected into breast cancer cell lines with DharmaFECT transfection reagents in accordance with the manufacturer's instructions.

Results

Effect of adiponectin on cell proliferation and cell cycle progression

MDA-MB-231 and T47D cells were treated with various concentrations of adiponectin for 24, 48, or 72 h. Adiponectin treatment resulted in a significant dose-dependent growth inhibition of both MDA-MB-231 and T47D cells (Fig. 1a). DNA flow cytometry was performed on cells treated with 10 μg/ml adiponectin for 24, 48, or 72 h. In both cell lines, the G0/G1 population increased to about 80% after 72 h (Fig. 1b).

Influence of adiponectin on apoptosis

We examined whether adiponectin could induce apoptosis in breast cancer cell lines (MDA-MB-231 and T47D). Cells were treated with adiponectin or paclitaxel for 48 h and then subjected to the TUNEL assay. Apoptotic cells were frequently observed after treatment with paclitaxel in both cell lines, whereas apoptotic cells were rarely observed after treatment with adiponectin (Fig. 2).

Expression of adiponectin receptors (AdipoR1 and AdipoR2)

Expression of AdipoR1 and AdipoR2 mRNA was determined in five cell lines, i.e., three human breast cancer cell lines (MDA-MB-231, MCF-7, T47D), one normal breast epithelial cell line (MCF-10A), and one human hepatocellular carcinoma cell line (HepG2). AdipoR1 and AdipoR2 mRNA expression was detected in all cell lines (Fig. 3a). The expression level of AdipoR1 mRNA was similar for all cell lines. The expression level of AdipoR2 mRNA was much lower than that of AdipoR1 mRNA in MDA-MB-231, MCF-7, T47D, and MCF10A similar to that of AdipoR1 in HepG2.

Expression of AdipoR1 and AdipoR2 protein was determined in these cell lines by western blot analysis. Consistent with the expression levels of mRNA, AdipoR1 protein expression was observed at similar levels in all cell lines while AdipoR2 protein expression was much lower than AdipoR1 protein expression in MDA-MB-231, MCF-7, T47D, and MCF10A, but similar in HepG2 (Fig. 3b).

Effect of AdipoR1 knock-down on adiponectin-induced growth-inhibition

Since AdipoR1 was preferentially expressed in human breast cancer cell lines, we attempted to investigate the influence of AdipoR1 knock-down by siRNA on the growth-inhibitory effect of adiponectin. Treatment with siRNA against AdipoR1 mRNA resulted in a decrease in AdipoR1 protein expression after 48 h in MDA-MB-231 and T47D (Fig. 4a). Treatment with siRNA against GAPDH decreased GAPDH protein level but not AdipoR1

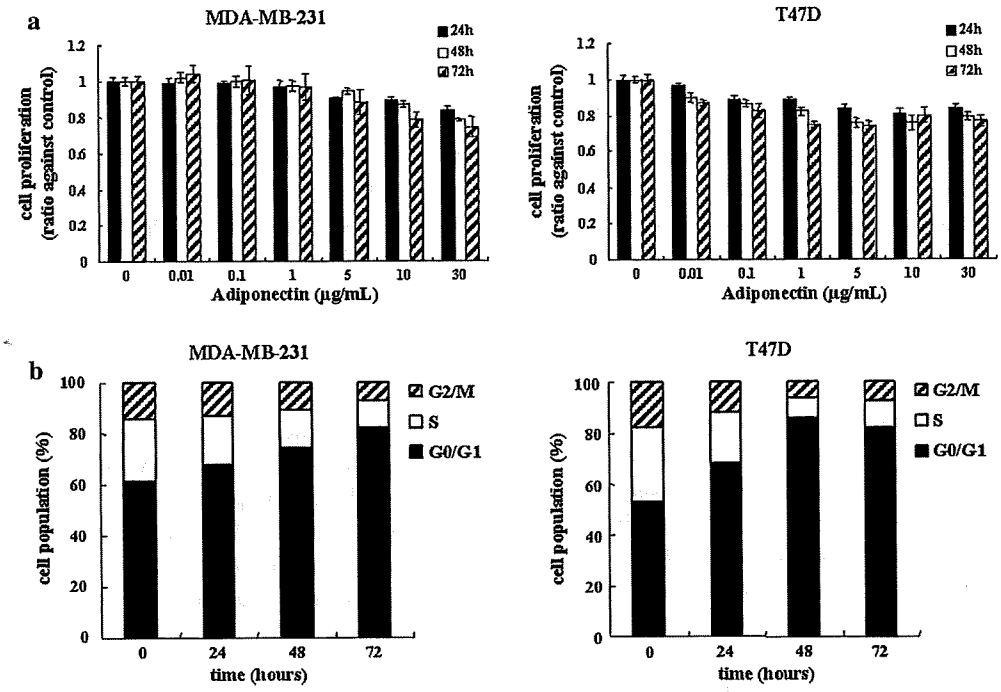

Fig. 1 Effect of adiponectin on cell proliferation and cell cycle in human breast cancer cell lines. (**a**) MDA-MB-231 and T47D cells were plated at 2×10^3 into 96-well plates and left overnight. Cells were treated with concentrations of adiponectin from 10 ng/ml to 30 μg/ml for the times indicated. The relative number of viable cells was estimated using the WST1 assay. A value of 100% was assigned to the absorbance value of each cell culture without adiponectin. Bars: mean + SD of three determinations. (**b**) Cells were treated with 10 μg/ml adiponectin and harvested at various times as indicated, fixed, and stained with propidium iodide. The cell cycle distribution of MDA-MB-231 and T47D cells was detected by DNA flow cytometry. The protocol for DNA analysis is described in Materials and Methods

protein level, and mock treatment did not affect either AdipoR1 or GAPDH protein levels.

Adiponectin treatment induced a growth inhibition in both mock-treated cell lines showing 23% reduction in MDA-MB-231 cells and 26% reduction in T47D cells. Treatment with siRNA against AdipoR1 significantly reduced the growth inhibition induced by adiponectin in both cell lines. The extent of reduction was more prominent in T47D cells than MDA-MB-231 cells (Fig. 4b).

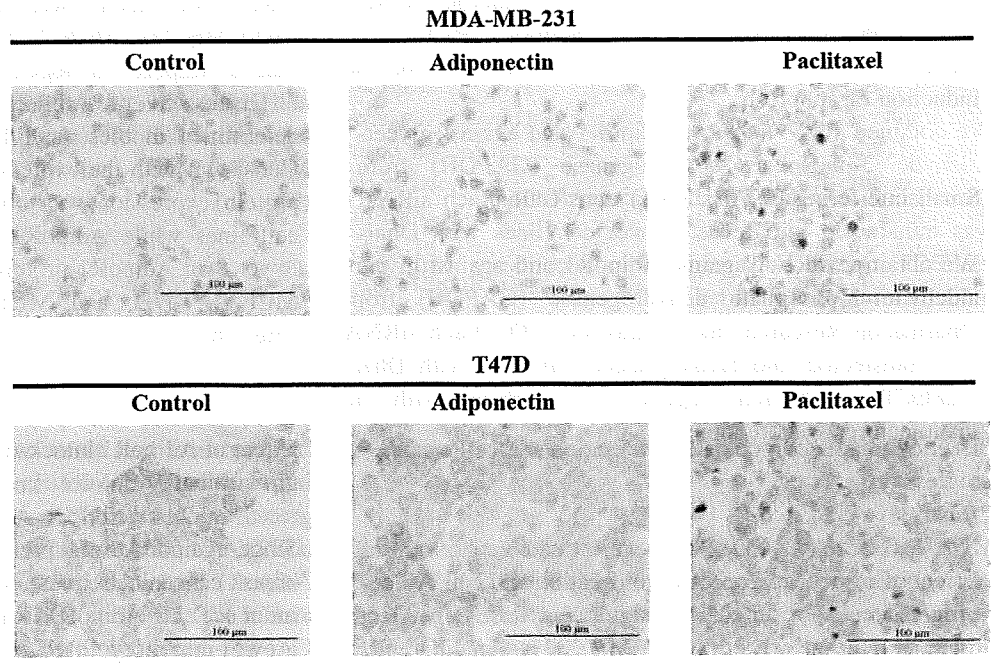

Fig. 2 Immunocytochemical staining of human breast cancer cells treated with adiponectin. Cells were treated with or without 10 μg/ml adiponectin for 48 h and fixed in ethanol. Cells undergoing apoptosis were detected using the DeadEnd TUNEL System as described in Materials and Methods. Paclitaxel (PTX) treatment (20 nM) was used as a positive control for the induction of apoptosis