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ORIGINAL REPORT

Dofequidar Fumarate (MS-209) in Combination With Cyclophosphamide, Doxorubicin, and Fluorouracil for Patients With Advanced or Recurrent Breast Cancer

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ABSTRACT

Purpose
To evaluate the efficacy and tolerability of dofequidar plus cyclophosphamide, doxorubicin, and fluorouracil (CAF) therapy in comparison with CAF alone, in patients with advanced or recurrent breast cancer. Dofequidar is a novel, orally active quinoline derivative that reverses multidrug resistance.

Patients and Methods
In this randomized, double-blind, placebo-controlled trial, patients were treated with six cycles of CAF therapy: 28 days/cycle, with doxorubicin (25 mg/m^2) and fluorouracil (500 mg/m^2) administered on days 1 and 8 and cyclophosphamide (100 mg orally [PO]) administered on day 1 through 14. Patients received dofequidar (900 mg PO) 30 minutes before each dose of doxorubicin. Primary end point was overall response rate (ORR; partial or complete response). In total, 221 patients were assessable.

Results
ORR was 42.6% for CAF compared with 53.1% for dofequidar + CAF, a 24.6% relative improvement and 10.5% absolute increase (P = .077). There was a trend for prolonged progression-free survival (PFS; median 241 days for CAF *v* 366 days for dofequidar + CAF; P = .145). In retrospectively defined subgroups, significant improvement in PFS in favor of dofequidar was observed in patients who were premenopausal, had no prior therapy, and were stage IV at diagnosis with an intact primary tumor. Except for neutropenia and leukopenia, there was no statistically significant excess of grade 3/4 adverse events compared with CAF. Treatment with dofequidar did not affect the plasma concentration of doxorubicin.

Conclusion
Dofequidar + CAF was well tolerated and is suggested to have efficacy in patients who had not received prior therapy.

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Despite the advances in chemotherapeutic intervention, many cancers are either inherently resistant or develop resistance to chemotherapy.[1,2] Consequently, multidrug resistance (MDR) remains a major obstacle to the successful treatment of cancer.[1,3,4] One mechanism by which MDR operates is via the increased cellular efflux of cytotoxic compounds due to increased expression of membrane transport proteins such as P-glycoprotein (P-gp) and MDR-associated protein (MRP).[1,4,5] MDR affects many structurally and functionally unrelated agents including cytotoxic drugs that are hydrophobic, natural products, such as taxanes, vinca alkaloids, anthracyclines, epipodophyllotoxins, topotecan, dactinomycin, and mitomycin.[1,6,7] These represent some of the most commonly used chemotherapeutic agents.

In tumors with low levels of P-gp expression at baseline or diagnosis, P-gp expression increases after exposure to chemotherapy agents, thus leading to the development of MDR. In breast cancer patients who had received prior chemotherapy, P-gp expression has been shown to increase from 11% in untreated patients to 30% after chemotherapy.[8] Furthermore, compared with P-gp–negative tumors, a significant increase in resistance to paclitaxel and doxorubicin was reported in P-gp positive breast cancer tissue, irrespective of prior therapy.

The degree of P-gp expression also strongly correlated with the degree of drug resistance observed.[8]

Chemotherapy remains the treatment of choice for women with hormone receptor–negative and hormone-refractory breast cancer disease.[9-11] However, many tumors that are initially responsive to chemotherapy frequently relapse and develop resistance to the broad spectrum of cytotoxic drugs currently employed.[8,12,13] Consequently, MDR remains a major reason for treatment failure in patients with metastatic breast cancer and highlights the urgent need for MDR modifiers in breast cancer chemotherapy.

Since the discovery of verapamil as an MDR-reversing agent,[14] many compounds have been investigated as MDR inhibitors.[14-16] Dofequidar fumarate (Fig 1), is a novel, orally active, quinoline-derived inhibitor of MDR.[17] In preclinical studies, dofequidar reversed MDR in P-gp– and MRP-1–expressing cancer cells in vitro (1 to 3 μmol/L), as well as enhancing the antitumor effects of doxorubicin in MDR tumor–bearing mice.[17-19] A phase I trial in healthy volunteers showed dofequidar to be well tolerated (10 to 1,200 mg) with no dose-limiting toxicities and an effective plasma concentration was maintained for 8 hours at 900 mg (data on file, Schering AG, Berlin, Germany). In a phase II combination trial in patients with recurrent breast cancer, dofequidar potentiated the antitumor effects of CAF (cyclophosphamide, doxorubicin, and fluorouracil) therapy; patients who had not responded to treatment with three cycles of CAF responded to subsequent treatment with dofequidar plus CAF. The numbers of patients with an objective response were two of seven at 600 mg and two of six at 900 mg dofequidar, though dose escalation was stopped at 1,200 mg due to increased hematologic toxicity (data on file, Schering AG). On the basis of this result, this phase III study was conducted to compare the efficacy and safety of dofequidar plus CAF with placebo plus CAF in patients with advanced or recurrent breast cancer.

Study Design

This was a randomized, multicenter, double-blind, placebo-controlled trial conducted at 46 centers across Japan, comparing the efficacy and safety of dofequidar plus CAF with placebo plus CAF. Female patients (age 20 to 70 years) with advanced (stage IV at diagnosis with an intact primary tumor) or recurrent breast cancer were enrolled onto the study. Other inclusion criteria included a histologically defined, measurable or assessable primary lesion; two or fewer regimens of prior chemotherapy in both neo/adjuvant and metastatic settings, (excluding prior endocrine or single-agent fluorouracil therapy); 180 mg/m^2 anthracyclines (doxorubicin equivalent) or less previously; a performance status of 0 to 2; and adequate bone marrow, renal, hepatic and cardiac functions. Patients who progressed or had a recurrence in less than 6 months with anthracycline-containing chemotherapy, and those who had a history of major cardiac disease, uncontrolled hypertension, symptomatic brain metastasis, or simultaneous malignancy were excluded. The trial was approved by the institutional review board and was conducted in accordance with the Declaration of Helsinki (1996). All patients provided written informed consent before study entry.

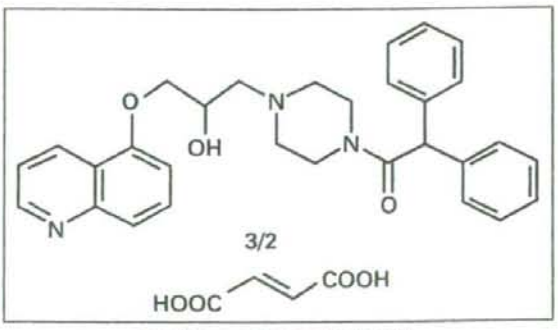

Fig 1. Structure of dofequidar (MS-209).

Dosing and Dose Modification for Toxicity

Patients were treated with six cycles of CAF therapy with dofequidar or placebo, and each treatment cycle lasted for 28 days; drugs were administered as follows: days 1 and 8, doxorubicin (25 mg/m^2) and fluorouracil (500 mg/m^2), each infused over 15 minutes; days 1 through 14, cyclophosphamide (100 mg orally [PO]); dofequidar (900 mg/d; 3 × 300 mg tablets) or placebo administered 30 minutes before each doxorubicin dose to ensure adequate blood concentration of dofequidar. The doses of doxorubicin and fluorouracil were reduced to 20 mg/m^2 and 400 mg/m^2, respectively, if any of the following criteria were met: grade 3 nonhematologic toxicity (except nausea and vomiting); grade 3 or worse neutropenia (< 1,000/mm^3) maintained for at least 5 days with an episode of fever of 38.5°C or higher; grade 3 or worse thrombocytopenia (< 50,000/mm^3); and grade 4 neutropenia (< 500/mm^3). The next cycle was postponed for 3 weeks unless the patient had a WBC count of at least 4,000/mm^3, or a neutrophil count of at least 2,000/mm^3 and a platelet count of at least 100,000/mm^3. Patients were followed up for 3 months after completion or discontinuation of treatment.

Treatment Assignment

Patients were randomly assigned to their treatment by the Trial Register Center. Treatment assignment was securely stored and coded until completion of the study. Investigators were also blinded to the assigned treatment. Patients were stratified by the number of prior chemotherapy regimens, including adjuvant chemotherapy, by a history of prior use of anthracyclines, and by the presence of liver metastases.

Efficacy

The primary study end point was the overall response rate (ORR) in the full analysis set (FAS; all patients who received treatment at least once and met all inclusion/exclusion criteria). Efficacy assessment by lesion and ORR assessment were made at each treatment cycle (every 4 weeks) and at treatment completion. Objective responses were assessed through blinded reading of radiographs by an independent expert panel. The secondary study end points included complete response rate (CR), time to treatment failure (TTF), time to progression (TTP), and progression-free survival (PFS).

Subgroup analyses were conducted to assess PFS within specific patient subpopulations, including premenopausal women, patients who had no prior therapy, and patients who had advanced primary breast cancer.

Safety and Tolerability

Adverse events (AEs) were recorded at the end of each treatment cycle and at the end of the study period using data from the safety population (all patients who received treatment at least once in the study). AEs were categorized according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC) Version 2. The incidence of significant decreases in left ventricular ejection fraction (LVEF) and serious AEs were recorded. The CBC was evaluated weekly. Serum chemistries and urinalysis were evaluated every 2 weeks. The minimum hematology values and LVEF in each treatment cycle were also recorded and analyzed in the per-protocol set (PPS; all patients who received treatment at least once and had no protocol deviations).

Pharmacokinetics

To assess the effect of concomitant dofequidar use on the pharmacokinetics of doxorubicin, the plasma doxorubicin concentration on day 1 of cycle 1 was compared between treatment groups. Blood samples were taken at baseline and at 15 minutes, 30 minutes, and 1, 2, 4, and 6 hours after the start of doxorubicin administration. Plasma doxorubicin concentrations were determined by reversed-phase high-performance liquid chromatography. Area

under the plasma concentration-time curve (AUC) was calculated using the linear trapezoidal rule.

Statistical Analyses

The primary end point was analyzed using the Fisher's exact test at a significance level of 2.5% in a one-sided test. A difference in response rates of 20% between the two treatment groups was used as the basis for a statistically significant difference. CR, TTF, TTP and PFS were analyzed by the log-rank test at a significance level of 5% in a two-sided test. The CR, TTF, TTP and PFS were analyzed in the FAS, and the pharmacokinetic data analyzed in the PPS.

Patient Characteristics

A total of 227 patients were recruited onto the study (Fig A1, online only), of which 225 patients were included in the safety analysis (n = 113 for the dofequidar group; n = 112 for the placebo group); two patients did not receive the study treatment and were thus excluded. Four patients did not meet the inclusion/exclusion criteria; therefore, the FAS consisted of 221 patients (n = 113 for the dofequidar group; n = 108 for the placebo group). The PPS consisted of 199 patients (n = 100 for the dofequidar group; n = 99 for the placebo group). There were 22 patients excluded from the PPS analysis due to protocol deviations. Baseline patient characteristics were well balanced between the two treatment arms (Table 1). Most patients had predominantly recurrent disease and had received prior chemotherapy plus endocrine therapy. Also, many patients who had advanced primary breast cancer had received no prior therapy.

Table 1. Patient Demographics (full analysis set)

Characteristic	Dofequidar + CAF (n = 113) No.	%	Placebo + CAF (n = 108) No.	%
Age, years				
Mean	54.4		52.4	
SD	7.69		8.97	
Medical history known	65	57.5	60	55.6
Weight, kg				
Mean	56.2		54.1	
SD	7.52		7.73	
Height, cm				
Mean	154.7		154.7	
SD	5.71		5.61	
Body surface area, m^2				
Mean	1.5		1.5	
SD	0.11		0.11	
Disease state				
Recurrent	81	71.7	80	74.1
Advanced	32	28.3	28	25.9
Prior therapy				
Radiotherapy + chemotherapy + endocrine therapy	32	22.1	32	29.6
Chemotherapy + endocrine therapy	55	48.7	54	50.0
Radiotherapy	1	0.9	1	0.9
No prior therapy	25	22.1	21	19.4
Menopausal status				
Premenopausal	24	21.2	26	24.1
Postmenopausal	88	77.9	79	73.1

Abbreviations: CAF, cyclophosphamide, doxorubicin, and fluorouracil; SD, standard deviation.

Efficacy

The ORR, rated as CR or partial response rate, was 42.6% for CAF plus placebo versus 53.1% for dofequidar plus CAF (Table 2). Although this represents a 24.6% relative improvement and a 10.5% absolute increase in response rate for patients receiving dofequidar plus CAF compared with those receiving CAF plus placebo, this response was not statistically significant ($P = .077$). A higher value was observed in the dofequidar treatment group for all secondary end points compared with placebo, though these results were not statistically significant. Among them, Figure 2 shows a trend for prolonged PFS (median, 241 days for CAF plus placebo *v* 366 days for dofequidar plus CAF; $P = .145$).

Dofequidar plus CAF significantly improved PFS in several patient subgroups, including patients who were premenopausal ($P = .046$; Fig 3A), patients who had not received prior therapy ($P = .0007$; Fig 3B), and patients who had advanced primary breast cancer ($P = .017$; Fig 3C). An extended follow-up showed that dofequidar plus CAF also significantly improved overall survival ($P = .0034$; Fig 3D) in patients who had no prior therapy.

Safety and Tolerability

A similar number of patients completed six treatment cycles in both groups (n = 53 for the dofequidar group; n = 51 for the placebo group). The mean number of treatment cycles was 4.5 in the dofequidar group and 4.3 in the placebo group. More than half of patients in both groups included in each cycle from cycle 2 onward had a delay in treatment, mostly due to prolonged hematologic toxicities.

Dofequidar plus CAF was well tolerated throughout the study. No statistically significant excess of grade 3/4 AEs, except for neutropenia ($P = .006$) and leukopenia ($P = .005$), was found in the dofequidar group compared with placebo (Table A1, online only). Importantly, there was no marked difference in the incidence of neutropenia-related morbidity, such as febrile neutropenia or infection, between the two treatment groups. No significant differences in the incidence of cardiac AEs were found between the two treatment groups. In addition, dose intensities of chemotherapeutic agents were similar in both treatment arms. No significant difference in the incidence of serious AEs (SAEs) was observed between either group. However, there was a trend for a higher incidence of SAEs from leukopenia in the dofequidar group than in the placebo group ($P = .060$; Fisher's exact test); five leukopenia cases were reported for dofequidar, whereas no such case was reported for placebo.

A total of 124 patients discontinued the study (n = 61 for the dofequidar group; n = 63 for the placebo group). The major reasons for discontinuation were progressive disease (n = 23 for the dofequidar group; n = 28 for the placebo group), grade 4 hematologic toxicity (n = 20 for the dofequidar group; n = 6 for the placebo group), failure to meet treatment continuation criteria (n = 6 for the dofequidar group; n = 8 for the placebo group), and consent withdrawal (n = 6 for the dofequidar group; n = 12 for the placebo group). Of the 225 patients who received treatment in the study, 14 patients died during the treatment period (n = 3), the follow-up period (n = 2), or the follow-up period after study termination (n = 9). There were 49 other serious AEs in 32 patients during the study and follow-up period.

Pharmacokinetics

The mean plasma concentrations of doxorubicin in the dofequidar- and placebo-treatment groups at 15 minutes postadministration reached 0.997 μg/mL and 1.259 μg/mL, respectively, followed by biphasic elimination in both treatment groups. Mean plasma concentrations in

Table 2. Response Rates for Patients Treated With Dofequidar Plus CAF (n = 113) or Placebo Plus CAF (n = 108)

Treatment Group	Parameter (No. of patients)					Overall Response Rate (%)	95% CI
	Complete Response	Partial Response	No Change (stable disease)	Progressive Disease	Not Assessable		
Dofequidar	5	55	40	10	3	53.1	43.5 to 62.5
Placebo	4	42	41	14	7	42.6	33.1 to 52.5

NOTE. Odds ratio = 1.53 (range, 0.87-2.69); $P = .077$ for dofequidar *v* placebo.
Abbreviation: CAF, cyclophosphamide, doxorubicin, and fluorouracil.

the dofequidar and placebo groups remained similar at 1, 2, 4, and 6 hours after the start of doxorubicin administration. Thus the elimination pattern for the first 6 hours after the start of administration was similar in both groups. The plasma concentrations of doxorubicin in the terminal phase (4 and 6 hours postadministration) were slightly higher in the dofequidar group compared with placebo (1.2- to 1.3-fold). However, AUC (0 to 6 hours) values showed no statistically significant difference between the dofequidar and placebo groups (mean, 0.480 $\mu g \cdot h/mL$; standard deviation [SD], 0.324; range, 0.237-1.692; and mean, 0.407 $\mu g \cdot h/mL$; SD, 0.062; and range, 0.289-0.500, respectively). Therefore, treatment with dofequidar did not affect the plasma concentrations of doxorubicin in patients (Fig 4).

Chemotherapy remains the preferred adjuvant treatment for patients with hormone receptor–negative disease and for patients with more aggressive, hormone receptor–positive tumors.[11,20] However, despite the use of conventional adjuvant chemotherapy regimens, a significant proportion of patients with breast cancer still experience disease recurrence because of inherent or acquired drug resistance.[12] In this randomized phase III trial, the efficacy and safety of the multidrug resistance inhibitor dofequidar plus CAF was compared with CAF plus placebo in patients with recurrent or advanced breast cancer. Although, there was an observed relative improvement and absolute increase in response rate for patients who received dofequidar plus CAF, these results did not reach statistical significance. This improvement in response rate may have been reflected in the observation that there was a trend for prolonged PFS, which favored patients in the dofequidar plus CAF group.

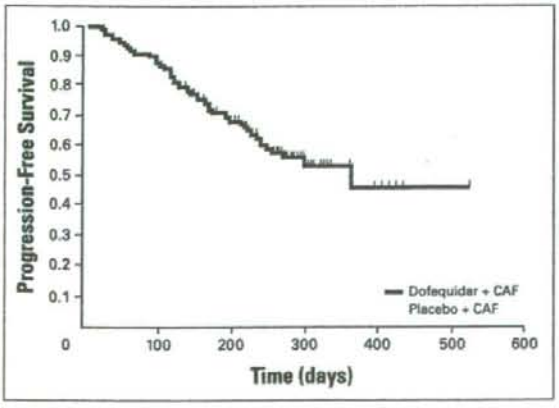

Fig 2. Progression-free survival in patients treated with dofequidar plus cyclophosphamide, doxorubicin, and fluorouracil (CAF) and placebo plus CAF ($P = .145$).

To date, only two randomized trials have examined the efficacy of a P-gp inhibitor in combination with chemotherapy in breast cancer patients. Wishart et al[21] examined quinidine combined with epirubicin in patients with advanced breast cancer, but failed to show any significant difference in overall survival or PFS compared with placebo. In a more recent prospective study of patients with anthracycline-resistant metastatic breast cancer (n = 99), verapamil combined with vindesine and fluorouracil resulted in a significantly longer overall survival and a higher response rate compared with patients who did not receive the P-gp inhibitor (median survival, 323 *v* 209 days; $P = .036$, respectively; ORR, 27% *v* 11%; $P = .04$, respectively).[22]

In the subgroup analyses, dofequidar in combination with CAF displayed a significantly increased PFS in patients who had not received prior therapy, who had advanced primary breast cancer or who were premenopausal. In addition, dofequidar also significantly improved overall survival in the patient group who had no prior therapy. Although the patient numbers in these analyses were small, the results remain important within these clinically significant patient populations. Both preclinical and clinical data have indicated that newer-generation MDR modulators can prevent the development of resistance.[23,24] A phase I/II trial in patients with acute myeloid leukemia showed that dosing with cyclosporine before and in combination with daunorubicin prevented chemotherapy resistance, while also resulting in a decrease in MDR-1 RNA expression.[24] Our results may highlight one potential treatment approach to MDR tumors that has not yet been fully exploited in the clinical environment, specifically the prevention of the emergence of resistance through the early use of P-gp inhibitors.[1-3] It seems reasonable that agents such as dofequidar may be useful in the adjuvant or even neoadjuvant setting with the goal of preventing or delaying the induction of MDR associated with chemotherapy.

The potential clinical significance of P-gp and MRP expression in breast cancer is supported by the results from a number of studies. For example in a study of primary breast cancer patients (n = 259), MRP expression was associated with an increased risk of treatment failure in patients with small tumors (T1) and node-positive patients who received adjuvant cyclophosphamide, methotrexate, and fluorouracil (CMF) chemotherapy but not in node-negative patients.[25] Burger et al[12] reported that the expression of MDR1 mRNA in primary breast tumors was inversely correlated with the efficacy of first-line chemotherapy. Additionally, the high level of MDR1 expression was suggested to be a significant predictor of poor prognosis in patients

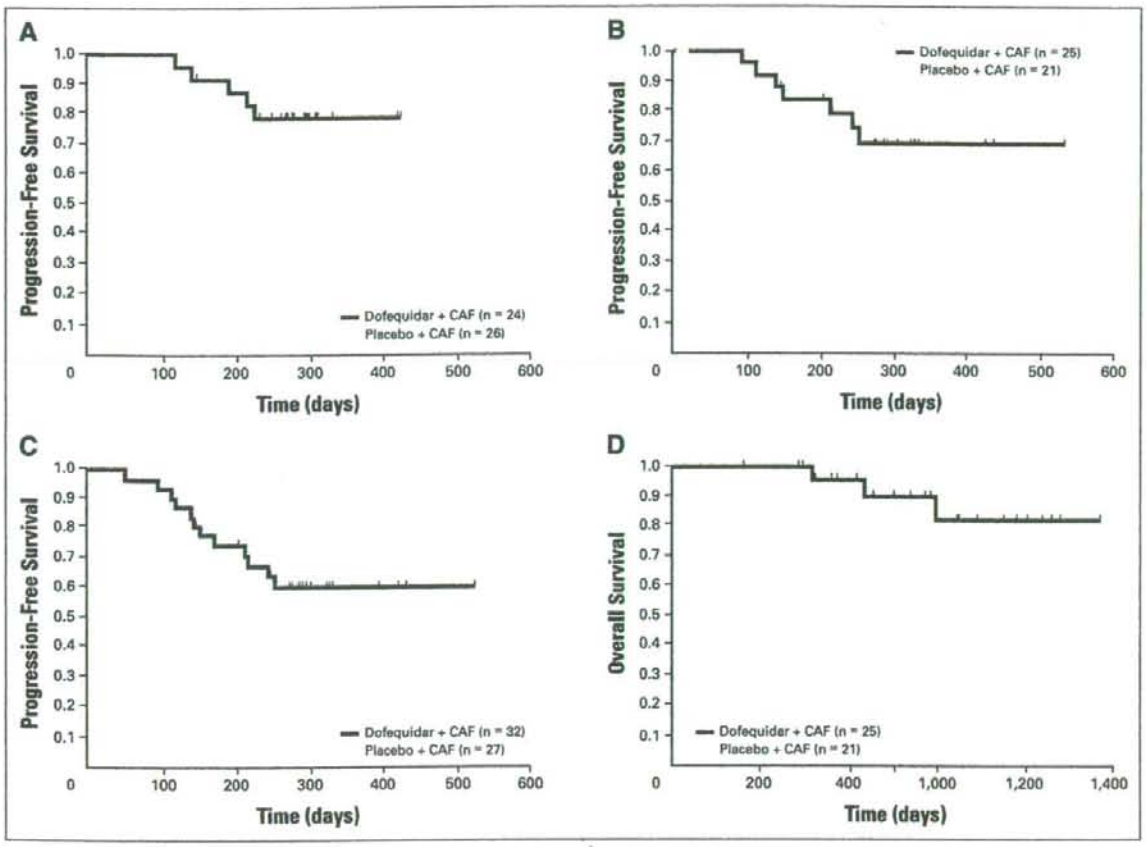

Fig 3. Subgroup analyses. (A) Progression-free survival in premenopausal patients (P = .046); (B) progression-free survival in patients who had no prior therapy (P = .0007); (C) progression-free survival in patients who were stage IV at diagnosis with an intact primary tumor (P = .017); and (D) overall survival in patients who had no prior therapy (P = .0034).

with advanced disease.[12] Significantly increased expression of P-gp and MRP-1 has also been reported in an immunohistochemical study of patients treated with preoperative chemotherapy, whereas pretreatment expression of MRP-1 was associated with significantly shorter PFS in patients.[26] In a more recent study, MRP-1 expression was shown to be an independent predictor for shorter relapse-free survival and overall survival, after adjuvant CMF treatment, in premenopausal, hormone receptor–positive patients.[27] However, MRP-1 expression did not affect patients' response to adjuvant tamoxifen plus goserelin treatment.[27]

These findings and our results support the view of Leonard et al,[3] who indicate that future patients will need to be carefully selected for the identification and development of effective drug-resistance modulators. Patient populations who may derive maximal benefit from MDR inhibition, for example, the no-prior-therapy, advanced-disease, or premenopausal patient group in the present study, could quite easily be overlooked or lost within a large, heterogeneous trial population.[3] Furthermore, by refining future clinical trials to incorporate specific disease and patient characteristics, a clearer picture of drug resistance in cancer will be obtained and the most effective MDR inhibitor/chemotherapeutic agent(s) selected.

Many MDR inhibitors have required high serum concentrations for MDR reversal, which resulted in unacceptable toxicity, thereby limiting their clinical impact.[7,28-32] Although more recent agents have shown improved tolerability profiles, this has been countered by unpredictable pharmacokinetic interactions with other transporter molecules (eg, cytochrome P450–mediated drug metabolism and excretion, necessitating dose reductions in chemotherapy agents and leading to inconsistent chemotherapy dosing among patients).[1-5] Similarly, the addition of the MDR-modulating agent valspodar (PSC 833) to chemotherapy agents did not improve treatment outcome.[33,34] Toxicity was increased in the valspodar-treated group compared with chemotherapy agents alone, despite the reduction of chemotherapy doses in the valspodar-containing regimen. In our study, dofequidar was well tolerated, with no indication of the unacceptable toxicity associated with early MDR inhibitors. Importantly, dofequidar did not affect the plasma concentrations of doxorubicin in patients during the study and displayed an acceptable pharmacokinetic profile.

In conclusion, this study suggests that treatment with dofequidar resulted in possible clinical benefit for patients who had not received prior therapy, who were premenopausal, or who were stage IV at diagnosis with an intact primary tumor. Dofequidar was also well

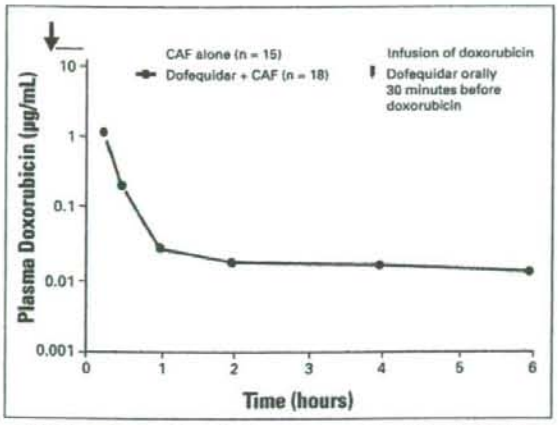

Fig 4. Plasma levels of doxorubicin in patients receiving dofequidar or placebo. CAF, cyclophosphamide, doxorubicin, and fluorouracil.

tolerated in the clinical setting and had no impact on doxorubicin pharmacokinetics. Further studies are merited to assess the effect of dofequidar in specific patient populations with breast cancer.

Although all authors completed the disclosure declaration, the following author or immediate family members indicated a financial interest. No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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Appendix

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

Relationship between the signal ratios of HER-2/CEP17 and c-MYC/CEP17 and the pathological response of neoadjuvant therapy using docetaxel and trastuzumab in breast cancer

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Abstract. The purpose of this study was to assess the efficacy and predictive biomarkers of combination docetaxel-trastuzumab in a neoadjuvant setting by means of a phase II trial. Women with histologically-confirmed advanced invasive breast cancer whose tumours overexpressed HER-2 received 4 cycles of docetaxel (70 mg/m^2 every 3 weeks) and trastuzumab (4 mg/kg loading dose, 2 mg/kg weekly thereafter). Twenty-one patients were enrolled, and all completed 4 cycles of treatment. Two patients were later found to be inoperable, and neither pathological nor clinical response was assessed. The pathological complete response rate was 21% (4/19; 95% CI, 6-46%) and the overall clinical response rate 89% (17/19; 95% CI, 67-99%). The relationship between the expression of biomarkers (HER-2, c-MYC, BRCA1 and Ki-67) and pathological response was assessed. The results suggested the possibility that tumours showing a high signal ratio of HER-2/CEP17 or c-MYC/CEP17 might be more sensitive to this combination therapy. Based on these results, it can be speculated that approximately 30% pCR might be obtained in cases with a high signal ratio of HER2/CEP17 or c-MYC/CEP17. Further trials are needed.

Introduction

Neoadjuvant (also known as primary or pre-operative) therapy is a major development in the management of breast cancer. It increases the possibilities for breast-conserving surgery by downstaging the primary tumour and lymph node metastases (1). It also offers early systemic treatment for micrometastasis (2). Following the reports of the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-18 trial (3,4), interest in neoadjuvant chemotherapy has rapidly developed and its use has become widespread in the treatment of patients with locally-advanced breast cancer.

Docetaxel is a semi-synthetic taxoid derived from the European yew tree, *Taxus baccata* (5). It is one of the most active chemotherapeutic agents in the treatment of patients with breast cancer. Docetaxel is active in the neoadjuvant setting, both as a single agent and in combination with anthracycline-containing regimens (6-8). There is a growing amount of information on neoadjuvant docetaxel therapy, but its activity combined with a molecular target agent has not been fully clarified. Trastuzumab is a humanised monoclonal antibody directed against the human epidermal growth factor receptor-2 (HER-2) protein. HER-2 gene amplification, which leads to protein overexpression, is associated with short survival in breast cancer (9,10); consequently, trastuzumab is used to treat such patients. Several clinical trials show that trastuzumab-containing regimens yield high rates of clinical and pathological complete response (pCR) in women with locally-advanced HER2-overexpressing breast cancer (11). Based on these findings, the incorporation of the docetaxel-trastuzumab combination into neoadjuvant therapy would appear to be promising. In the neoadjuvant setting, pCR rate is considered to be correlated with disease-free and overall survival (4,12).

Some studies, investigating the relationship between the signal ratio of HER-2/chromosome 17 centromere (CEP17) and response rate to trastuzumab monotherapy in breast cancer, reported a higher response rate in tumours with high signal ratio. c-MYC is a proto-oncogene that has been implicated in the control of cellular growth, proliferation and cell survival, and plays pivotal roles in proliferation, differentiation and apoptosis. Results from reports on the prognostic value of the overexpression of c-MYC mRNA or protein are conflicting (13), and should be interpreted with caution. Kim *et al* (14) demonstrated that high c-MYC gene copy number tumours are more responsive to chemotherapy using trastuzumab and taxanes. However, the relationship between HER-2 or c-MYC gene copy number and the pathological response to neo-

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Key words: biological markers, breast neoplasms, clinical trial, docetaxel, neoadjuvant therapy, phase II, trastuzumab

Table I. Classification of pathological responses according to the Japanese Breast Cancer Society.

Response	Description of pathological findings
Grade 3 (Complete response)	Necrosis or disappearance of all tumour cells. Replacement of all cancer cells by granuloma-like and/or fibrous tissue. In the case of complete disappearance of cancer cells, pretreatment pathological evidence of the presence of cancer is necessary
Grade 2 (Marked response)	Marked changes in ≥2/3 of tumour cells
Grade 1 (Slight response)	1a) Mild response: mild change in cancer cells regardless of the area, or marked changes in <1/3 of cancer cells 1b) Moderate response: marked changes in ≥1/3 but <2/3 of tumour cells
Grade 0 (No response)	Almost no change in cancer cells

adjuvant therapy with a docetaxel/trastuzumab-containing regimen has not been reported. In addition, the major role of the gene BRCA1 is to respond to DNA damage by participating in the cellular pathways for DNA repair, mRNA transcription, cell cycle regulation and protein ubiquitination (15). The Ki-67 protein is a proliferation marker expressed only in cycling cells, and correlates with S-phase fraction (16). Several *in vitro* and *in vivo* studies have demonstrated that the immunohistochemical expression of BRCA1 and Ki-67 in breast cancer cells might be a useful predictive factor for chemotherapy using taxanes.

In this study, we conducted an open-label multicentre phase II trial in patients with operable HER-2-overexpressing breast cancer, and reported the efficacy and safety of triweekly (i.e., once every 3 weeks) docetaxel combined with weekly trastuzumab as neoadjuvant chemotherapy (17). We then investigated the relationship between the signal ratios of HER-2/CEP17 and c-MYC/CEP17 estimated by FISH, the immunohistochemical expression of BRCA1 and Ki-67, and the pathological complete response rate of cancer cells undergoing neoadjuvant chemotherapy using docetaxel and trastuzumab. We further evaluated the usefulness of investigating these factors.

Materials and methods

Study design and ethics. This was a multicentre open-label single-arm phase II trial, conducted in accordance with the Declaration of Helsinki. The protocol was reviewed and approved by the institutional review board of each participating centre. All patients gave their written informed consent.

Patients. Women with histologically-confirmed locally-advanced breast cancer whose tumours overexpressed HER-2 were eligible for the study. HER-2 status was confirmed by immunohistochemistry (IHC), and patients with tumours graded with an IHC score of 3+ were enrolled. Other inclusion criteria were a tumour diameter ≥3 cm, a node-positive tumour or both, an Eastern Cooperative Oncology Group (ECOG) performance status of 0-1, age of 20-75 years, measurable disease, haemoglobin >9 g/dl, a white blood cell count between 4000/mm^3 and 12000/mm^3, neutrophils >2000/mm^3, platelets >100000/mm^3, serum bilirubin within normal range, aspartate aminotransferase and alanine aminotransferase <100 IU/l and serum creatinine ≤1.5 times the upper normal limit. Prior chemo-, radio-, or immunotherapy, or prior endocrine therapy, were not allowed. Pregnant women or women who might be pregnant were excluded from the study. Other exclusion criteria included contralateral breast cancer, uncontrolled concomitant disease, active concomitant malignancy (disease-free period <5 years), a history of myocardial infraction or clinically important cardiovascular disease, a left ventricular ejection fraction <50% or below the upper limit of normal, a New York Heart Association functional classification of II-IV, suspected infection with fever, motor paralysis or peripheral neuropathy, pleural or pericardial effusion requiring treatment, symptomatic brain metastasis, oedema of grade 2 or higher, interstitial pneumonia or lung fibrosis or an allergy to polysorbate 80.

Treatment procedure. Patients received docetaxel every 3 weeks and trastuzumab every week. In each cycle, 70 mg/m^2 docetaxel was administered intravenously (i.v.) over more than 60 min. Trastuzumab (2 mg/kg) was administered i.v. over 90 min, with the exception of the first treatment (day 1 of the first cycle) in which a loading dose of trastuzumab 4 mg/kg was administered i.v. over 90-min. For the first cycle, docetaxel was administered on day 2, and trastuzumab on days 1, 8 and 15. After the first cycle, docetaxel was administered on day 1 and trastuzumab on days 1, 8 and 15 of each cycle. Patients received 4 cycles of combination treatment, unless disease progression or unacceptable toxicity was observed.

Outcome measures. The primary endpoints were pathological response and clinical tumour response. Pathological response was assessed at the time of breast surgery according to the 'General Rules for Clinical and Pathological Recording of Breast Cancer: Histopathological Criteria for Assessment of Therapeutic Response in Breast Cancer' developed by the Japanese Breast Cancer Society (18). Hematoxylin and eosin (H&E) stained slides from the primary tumour were obtained. Slides were prepared by each institution as 5 mm interval gross tissue sections. A central review committee, consisting

Table II. Baseline characteristics of 21 women with HER-2-overexpressing breast cancer treated with a combination of docetaxel and trastuzumab.

Characteristic	Patients (n=21)
Median (range)	(33-69)
Age (years)	54
ECOG performance status, no. (%)	
0	18 (86)
1	3 (14)
Median (range) tumour size, cm	5.4 (1.3-15)
Clinical lymph nodes status, no. (%)	
N0	8 (38)
N1	12 (57)
N3	1 (5)
Pathological characteristics, no. (%)	
Ductal invasive carcinoma	20 (95)
Unknown	1 (5)
Postmenopausal, no. (%)	13 (62)
Receptor status, no. (%)	
Estrogen receptor positive	3 (14)
Progesterone receptor positive	0 (0)
Proposed surgery, no. (%)	
Mastectomy	17 (81)
Lumpectomy	4 (19)

of two pathologists working independently of local pathologists, assessed the pathological response to the therapy. The criteria are shown in Table I.

Evaluation of biological markers. All specimens obtained by core needle biopsy pre-treatment were fixed with 10% formalin-buffered solution and embedded in paraffin, and thin sections were used for FISH evaluation and immunohistochemistry.

FISH examination of HER-2 was performed using FISH kits for the evaluation of HER-2 gene status (Vysis Ltd., USA) according to protocol. The nuclei of 20 carcinoma cells in invasive lesions were identified, the numbers of fluorescent signal of both HER2 and CEP17 were counted and their signal ratios were calculated. We divided cases into high (>6.0) and low (≤6.0) groups according to signal ratio (19). FISH examination of c-MYC was also performed using c-MYC FISH kits (Dako Ltd., Denmark) According to the protocol, the numbers of c-MYC and CEP17 signals were counted and their signal ratios calculated. The cutoff line for the high and low groups was defined as 2.5 according to single color cut-off 5.0 (14). Immunohistochemistry for BRCA1 (Ab-1, Oncogene, USA) and Ki-67 (MIB1, Dako) was performed using specimens from core needle biopsy. The cutoff for IHC was defined ≥10% cells stained positive.

Table III. Pathological response of 19 women with HER-2-overexpressing breast cancer treated with a combination of docetaxel and trastuzumab.

Response[a]	No. of patients (%)
Grade 3	4 (21)[b]
Grade 2	7 (37)
Grade 1	8 (42)
Grade 0	0 (0)

[a]Classified according to the criteria of the Japanese Breast Cancer Society. [b]95% confidence interval, 6-46%.

Statistical consideration. The primary endpoint of this study was pCR response rate. The sample size was 20 patients, calculated based on binominal distribution (with a type I error of 5% and a study power of 80%). The correlation between pCR and each biomarker was assessed for significance in all analysis.

Results

Between July 2004 and March 2005, 21 women were enrolled. Table II summarises the baseline characteristics of all 21 patients. The median age was 54 years (range 33-69). Median pre-treatment tumour size was 5.4 cm (range 1.3-15 cm). Clinically-positive lymph nodes were observed in 13 patients (N1=12, N2=1). Tumours with invasive ductal carcinoma were present in 20 patients (95%) and mastectomy was recommended for 17 patients (81%).

All patients completed 4 cycles of combination treatment. No patients required docetaxel dose reduction. Two patients were later found to be inoperable owing to liver metastasis, and were therefore excluded from the efficacy analysis.

The overall clinical tumour response rate was 89% (95% CI, 67-99%) with complete response in 5 patients (26%), partial response in 12 (63%) and stable disease in 2 (11%). Eleven patients (52%) underwent breast-conserving surgery.

Table III shows the results of pathological response. Four of 19 cases (21%) were grade 3 (pCR), 7 (37%) were grade 2 and 8 (42%) grade 1. The pCR rate was 21%.

Table IV shows the relationship between the pCR rate and the biomarkers (HER-2, c-MYC, BRCA1 and Ki-67). Four cases (4/13, 29%) with high HER-2 expression achieved pCR, whereas none with low expression did. Three cases (3/10, 30%) with high c-MYC expression achieved pCR, whereas only one case (1/9, 11%) with low c-MYC did. Patients with high c-MYC expression also seemed to have a high pCR rate compared to patients with low expression. Three cases (3/14, 21%) were positive for Ki-67 and 1 (1/5, 20.0%) was negative. Three cases (3/13, 23%) were positive for BRCA1 and one (1/6, 16%) was negative. No significant difference in pCR rate was shown based on the predefined cutoff of all the biological markers.

Table IV. Association between pathological response and the expression of biological markers in 19 women with HER-2-overexpressing breast cancer treated with a combination of docetaxel and trastuzumab.

Biological marker	No. of patients	No. who achieved pCR (%)
HER2		
High (>6)	14	4 (29)
Low (<6)	5	0 (0)
c-MYC		
High (>2.5)	10	3 (30)
Low (<2.5)	9	1 (11)
BRCA1		
Positive (≥10%)	13	3 (23)
Negative (<10%)	6	1 (17)
Ki-67		
Positive (≥10%)	14	3 (21)
Negative (<10%)	5	1 (20)

pCR, pathological complete response.

Discussion

Previous studies have indicated the efficacy and safety of the docetaxel/trastuzumab combination in patients with HER-2-overexpressing metastatic breast cancer (20-23). In our trial, the results showed that this pairing is promising as neoadjuvant chemotherapy with a pCR rate of 21% and an overall clinical tumour response rate of 89%.

The pCR and overall clinical tumour response rates in our trial are within the ranges of those achieved by docetaxel- or trastuzumab-containing regimens in this setting. Trudeau *et al* reviewed the published results of randomised controlled trials of neoadjuvant taxane chemotherapy, and reported the pCR and overall clinical tumour response rates of docetaxel-containing regimens as ranges of 5-31 and 25-91%, respectively (24). Although participants in these trials were not patients with HER-2-overexpressing cancer, these results in part support the efficacy of our regimen. Montemurro and Aglietta reported that the pCR and overall clinical tumour response rates of trastuzumab-containing neoadjuvant therapy ranged between 12 and 65% and 60 and 93%, respectively (11). The pCR rate in our trial is not low considering that relatively large tumours (median, 5.4; range, 1.3-15 cm) were included.

Trastuzumab is a humanised monoclonal antibody directed against HER-2 protein. Thus, it is reasonable to consider that HER-2 would predict response to a trastuzumab-containing regimen. It has been reported that trastuzumab is more effective in cases with a high signal ratio of HER-2/CEP17. Response rates in a Genentech H0649 clinical trial of trastuzumab were as follows: the response rate was 0% in cases with <2.0 signal ratio, 13% in cases with 2.0-6.0 and 25% in cases with >6.0. In this study, 4 cases (4/13, 29%) with high HER-2 expression achieved pCR, whereas no cases with low expression did (14).

Overexpression of c-MYC may be correlated with better treatment outcome, considering that proliferating cells are usually more sensitive to chemotherapy. A high signal ratio of c-MYC/CEP17 is known to be more sensitive to trastuzumab therapy. c-MYC plays two conflicting roles in both apoptosis and cell proliferation. Under HER-2 overexpression, the survival signals suppress only the apoptotic role of c-MYC, resulting in its cell proliferation role dominating. Trastuzumab, however, blocks the survival signals of HER-2 so that c-MYC can induce apoptosis (13). In the present study, 3 cases (3/10, 30%) with high c-MYC expression achieved pCR, whereas only one case (1/9, 11%) with low c-MYC did. Patients with high c-MYC expression also had a higher pCR rate than did patients with low expression.

Based on these results, we can suggest that the signal ratios of HER2/CEP17 and c-MYC/CEP17 might be useful factors in predicting sensitivity to docetaxel/trastuzumab-combination therapy. If patients with a high signal ratio of HER2/CEP17 or c-MYC/CEP17 were treated with this regimen, a higher rate of pathological complete response could be expected.

We found few patients bearing tumours negative for Ki-67 or BRCA1. This sample size is too small to be considered for its correlation with pCR. Pre-clinical study results suggested that BRCA1 might be required for the response to spindle poisons (25), and a recent retrospective study showed that increased BRCA1 expression is correlated with a longer time-to-progression in patients with metastatic breast cancer treated with taxane-containing chemotherapy (26). However, another clinical trial showed no significant correlation between the expression of BRCA1 and response to docetaxel (27). The role of BRCA1 in predicting response to taxane therefore remains to be clarified.

This was a small sample size single arm phase II trial. In the absence of a control group, we cannot draw any definite conclusions from the results. Although pCR has been shown to predict disease-free and overall survival (4,12), the effect of combination docetaxel and trastuzuman on survival should be confirmed by a clinical trial with long-term follow-up. In addition, the predictive biomarkers of response to this combination should be confirmed by a large-scale randomised controlled trial. Considering that no reliable predictive bio-marker of response to chemotherapy in early-stage breast cancer has been found to date, a clinical trial prospectively designed to investigate the association between biomarker expression and chemotherapeutic response will be needed.

Despite these limitations, we conclude that combination treatment with tri-weekly docetaxel and weekly trastuzumab is a promising regimen in patients with HER-2-overexpressing operable breast cancer. Further study is warranted.

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CLINICAL INVESTIGATION **Breast**

HAPLOTYPE-BASED ANALYSIS OF GENES ASSOCIATED WITH RISK OF ADVERSE SKIN REACTIONS AFTER RADIOTHERAPY IN BREAST CANCER PATIENTS

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Purpose: To identify haplotypes of single nucleotide polymorphism markers associated with the risk of early adverse skin reactions (EASRs) after radiotherapy in breast cancer patients.

Methods and Materials: DNA was sampled from 399 Japanese breast cancer patients who qualified for breast-conserving radiotherapy. Using the National Cancer Institute-Common Toxicity Criteria scoring system, version 2, the patients were grouped according to EASRs, defined as those occurring within 3 months of starting radiotherapy (Grade 1 or less, $n = 290$; Grade 2 or greater, $n = 109$). A total of 999 single nucleotide polymorphisms from 137 candidate genes for radiation susceptibility were genotyped, and the haplotype associations between groups were assessed.

Results: The global haplotype association analysis ($p < 0.05$ and false discovery rate < 0.05) indicated that estimated haplotypes in six loci were associated with EASR risk. A comparison of the risk haplotype with the most frequent haplotype in each locus showed haplotype GGTT in *CD44* (odds ratio [OR] = 2.17; 95% confidence interval [CI], 1.07–4.43) resulted in a significantly greater EASR risk. Five haplotypes, CG in *MAD2L2* (OR = 0.55; 95% CI, 0.35–0.87), GTTG in *PTTG1* (OR = 0.48; 95% CI, 0.24–0.96), TCC (OR = 0.48; 95% CI, 0.26–0.89) and CCG (OR = 0.50; 95% CI, 0.27–0.92) in *RAD9A*, and GCT in *LIG3* (OR = 0.46; 95% CI, 0.22–0.93) were associated with a reduced EASR risk. No significant risk haplotype was observed in *REV3L*.

Conclusion: Individual radiosensitivity can be partly determined by these haplotypes in multiple loci. Our findings may lead to a better understanding of the mechanisms underlying the genetic variation in radiation sensitivity and resistance among breast cancer patients. © 2007 Elsevier Inc.

Radiosensitivity, Single nucleotide polymorphism, SNP, Haplotype, Early adverse skin reaction.

INTRODUCTION

Breast cancer is the most frequently diagnosed female malignancy worldwide, and the number of cases has been increasing. Breast-conserving surgery followed by radiotherapy (RT) is the most common form of primary breast cancer treatment for patients with early-stage breast cancer (1, 2). However, RT for breast cancer patients occasionally induces adverse effects such as a poor cosmetic outcome (3), fibrosis or thickening of the dermis (4), and radiation pneumonitis (5), but the risk factors remain poorly understood (6–8).

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The adverse reactions to RT are complex, and the heterogeneity in normal tissue reactions can result from the combined effect of several different genetic alterations (see Andreassen *et al.* [9], Fernet and Hall [10], and Gatti [11] for review). Approximately 60% of the first-degree relatives of radiosensitive breast cancer patients are themselves radiosensitive (12). About 90% of the variability in radioresponsiveness in the right-sided field can be explained by the radioresponsiveness in the left-sided field, as shown by analysis of the occurrence of telangiectasia of the skin in patients treated with bilateral internal mammary fields (13). Furthermore, *in vitro* assays for the radiosensitivity of peripheral blood lymphocytes have suggested that breast cancer patients as a group are more radiosensitive than healthy controls (14–16). This could indicate that some patients have genes that affect, at least in part, both the susceptibility to breast cancer and radiosensitivity.

Radiation effects can be categorized as early or late phase. Early damage results from the death of a large number of cells (*e.g.*, in the epidermal layer of the skin) and is usually repaired rapidly. In contrast, late damage is more likely to result from a combination of vascular damage and loss of parenchymal cells. The late injury might improve, but repair tends to be incomplete (17). Lopez *et al.* (18) found no evidence of a relationship between early (or acute) and late normal tissue reactions assessed in the same patients. However, some common genes might contribute to both early and late morbidity, as suggested by the model of Andreassen *et al.* (9).

As yet, few studies have examined the prognostic markers on genes with biologic functions putatively related to adverse skin reactions. An association between polymorphisms in DNA repair genes (*XRCC1* and *APEX1*) and acute reactions was found in one study (19). In addition to *XRCC1*, polymorphisms in *ATM*, *TGFB1*, *XRCC3*, and *SOD2* genes were associated with late reactions in other studies (20–25). Recently, one report (26) showed no association between the single nucleotide polymorphisms (SNPs) in these genes and the risk of radiation-induced subcutaneous fibrosis. Therefore, it is not clear whether the SNPs of these genes alone could account for the variation in individual adverse reactions after RT. Additional investigations with large numbers of genes for each adverse effect would be required to establish the effects of such genetic variation (9).

In the present study, to analyze the effects of individual genetic variation on adverse skin reactions we focused on early skin reactions during and immediately after RT, as defined by the National Cancer Institute Common Toxicity Criteria scoring system. We considered *(1)* how candidate genes for genotyping should be selected, *(2)* how cancer patients for genetic analyses should be chosen, and *(3)* which genetic analytical methods should be applied. First, in previous studies to systematically select candidate genes for effective genotyping we examined the skin reactions among the mouse strains with heterogeneity in response to ionizing radiation (27, 28), and the association between the expressed genes and interstrain variation was assessed using comprehensive high-density microarrays (27–30). We also used human cell culture lines with highly variable *in vitro* radiosensitivity to search for genes that contribute to the variation (31, 32). In addition, we have searched for potential radiation susceptibility genes by systematic *in vitro* screening with siRNA (33). In the present study, we also analyzed other genes that may be related to radiosensitivity, including some in the DNA repair pathway (9, 10, 34–37).

Second, to evaluate the extrinsic risk factors for adverse skin reactions, we studied a group of 284 breast cancer patients who had undergone breast-conserving surgery and RT (38). The analysis tested for associations among 45 clinical factors. Several extrinsic risk factors were identified and subsequently used in the present study to exclude patients who were ineligible for genetic analysis.

Third, in the present study we applied haplotype analysis instead of sole SNP analysis. Population genetic principles describe how variation is structured into haplotypes and indicate that the statistical power of association tests using phased data is likely to increase with reduction in dimension (39–41). Genetic analysis of haplotype frequencies enables the detection of predisposing haplotypes, even without typing the true functional SNP, by using SNPs for which single-locus analysis shows no association (42). In the present study we used a genetic haplotype approach to investigate polymorphisms in 137 genes that were candidates for affecting the risk of early adverse skin reactions (EASRs) after RT.

METHODS AND MATERIALS

Subjects

A total of 399 breast cancer patients were enrolled from nine collaborating institutions in Japan: the Research Center Hospital for Charged Particle Therapy of the National Institute of Radiological Sciences; Chiba Cancer Center; St. Luke's International Hospital; Shiga University of Medical Science Hospital; Kanazawa University Hospital; Toyama University Hospital; Nagoya City University Hospital; Tohoku University Hospital; and Yokohama City University Hospital. All patients underwent RT after breast-conserving surgery between 2001 and 2005 and were followed for >8 months. All the patients and 227 healthy donors provided written informed consent to participate in the study, which was approved by the Ethical Committee at the National Institute of Radiological Sciences and by each collaborating institution. All identifying information was managed at the Medical Information Processing Office of the Research Center for Charged Particle Therapy, National Institute of Radiological Sciences.

A multi-institutional study of nongenetic risk factors for adverse skin reactions to RT among breast cancer patients showed that the institution, operative procedure, and magnitude of photon energy were associated with the development of adverse skin reactions, despite variable selection procedures (38). In the present study patients who underwent mastectomy were excluded, and the collaborating institutions were all equipped with appropriate treatment modalities and performed breast-conserving RT with linear-accelerated electron facilities. As a result, 154 of the 284 patients described in the previous report (38) were eligible for the present genetic investigation and were included, along with an additional 245 new patients who were enrolled after the previous analysis.

The National Cancer Institute Common Toxicity Criteria scoring system, version 2 (http://ctep.info.nih.gov), was used to grade

radiation dermatitis developing within 3 months of starting RT. The distribution of patient EASRs was Grade 0 in 22, Grade 1 in 268, Grade 2 in 105, and Grade 3 in 4 patients. The patients were dichotomized into a low-grade (LG) group (Grade 1 or less, $n = 290$) and a high-grade (HG) group (Grade 2 or greater, $n = 109$) for genetic analysis. The clinical features of the groups are shown in Table 1. Except for the age distribution, no significant differences were found between the two groups in any of the features, including the photon energy used for RT (Table 1). The patients were not stratified any further for this genetic analysis.

Candidate genes and SNPs

The selection of candidate genes for SNP typing was determined from our previous comprehensive gene expression analyses (29–33) and the published data (see Appendix 1 for supplementary references). A total of 137 candidate genes were chosen (see Appendix 2, Supplementary Table). Information on SNPs for the candidate genes was obtained from the Japanese SNP database (jSNP, http://snp.ims.u-tokyo.ac.jp) (43) and the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/) (44).

To test the experimental conditions for the mass extension assays (45) with human genomic DNA and to examine the allele frequencies of the selected SNPs in the Japanese population, typing of the SNP markers was performed using blood samples from the 227 healthy subjects in the control group. Furthermore, several loci were typed to confirm the allele frequencies in the Japanese population using DNA samples from other healthy subjects provided by the Health Science Research Resources Bank, Japan Health Science Foundation (Osaka, Japan) (46).

Table 1. Clinical patient features

Characteristic	LG ($n = 290$)	HG ($n = 109$)	Difference (p)
Age at RT (y)			
Mean ± SD	54 ± 10	50 ± 11	0.032 (CA)
Range	26-88	30-77	
Family history of cancer	157 (54.1)	52 (47.7)	0.26 (FE)
TNM stage classification*			0.58 (FE)
0	13 (4.5)	5 (4.6)	
I	179 (61.7)	61 (56.0)	
II	94 (32.4)	42 (38.5)	
III	3 (1.0)	0 (0.0)	
Unknown	1 (0.3)	1 (0.9)	
Drug therapy			0.32 (FE)
Chemotherapy	50 (17.2)	12 (11.0)	
Hormonal therapy	136 (46.9)	47 (43.1)	
Both	35 (12.1)	14 (12.8)	
None	69 (23.8)	33 (30.3)	
Unknown	0 (0.0)	3 (2.8)	
Radiotherapy			
Photon energy level*			0.46 (FE)
4-MV	176 (60.7)	60 (55.0)	
6-MV	111 (38.3)	47 (43.1)	
Both	3 (1.0)	2 (1.8)	
Dose (Gy)†			
Mean	49.97	49.87	
Range	46.0-60.0	46.0-50.0	
Multileaf collimator	244 (84.1)	96 (88.1)	0.35 (FE)
Wedge filter	285 (98.3)	106 (97.2)	0.46 (FE)
Boost	86 (29.7)	26 (23.9)	0.26 (FE)

Abbreviations: LG = low grade; HG = high grade; RT = radiotherapy; CA = Cochran-Armitage test; FE = Fisher exact test.

* Due to rounding not all percentages add up to 100%.

† Fractionation size was 2 Gy, 5 times per week.

SNP typing

Extraction of genomic DNA from whole blood was performed with an automatic nucleic acids isolator, NA3000S (Kurabo, Osaka, Japan) or with the QIAamp DNA blood kit (Qiagen, Hilden, Germany). The DNA concentration was measured using PicoGreen reagent (47).

Single nucleotide polymorphism typing was performed using the MassARRAY system (Sequenom, San Diego, CA) according to the manufacturer's instructions. In brief, polymerase chain reactions were performed in 5-μL reactions with 2.5 ng of DNA template and final concentrations of 1 mmol/L $MgCl_2$, 200 μmol/L diethylnitrophenyl thiophosphates, 0.1 U of HotStarTaq Polymerase (Qiagen), and a primer concentration of 200 nmol/L under the following conditions: 55 cycles at 95°C for 20 sec, 56°C for 30 sec, and 72°C for 1 minute. The mass extension reaction was performed using the MassEXTEND enzymes-thermosequenase, hME termination mixes, and hME extension primers; 55 cycles were performed for 5 sec at 94°C, 5 sec at 52°C, and 5 sec at 72°C. After desalting, the reaction products were loaded into the SpectroCHIP and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The primer sequences are available on request.

Statistical analysis

Allele and genotype frequencies for each polymorphism were calculated, and the Hardy-Weinberg equilibrium was evaluated using the chi-square test among healthy donors and among the breast cancer patient group. Statistical significance and the strength of the associations between the grade of EASR of the breast cancer patients and each of the SNPs or haplotypes were assessed using the two-tailed Fisher exact test and odds ratio (ORs), respectively. The calculation of 95% confidence intervals (CIs) of the ORs was performed by evaluating the statistics for a random sampling of 10,000 iterated permutations at fixing the total numbers of both cases and controls. These statistical analyses were performed using SNPAlyze software, version 6.0 (http://www.dynacom.co.jp/e/products/package/snpalyze/index.html; DYNACOM, Chiba, Japan). Pairwise linkage disequilibrium (LD) analysis and haplotype analysis (expectation-maximization algorithm) were also performed using the SNPAlyze software, UCSC Genome Browser Gateway (http://genome.ucsc.edu/cgi-bin/hgGateway), haplo.stats (http://cran.r-project.org/src/contrib/Descriptions/haplo.stats.html) (48), and Haploview, version 3.32 (http://www.broad.mit.edu/mpg/haploview/) (49). Because testing multiple loci could have led to false-positive associations owing to multiple testing, we estimated the false-discovery rate (FDR) (http://faculty.washington.edu/~jstorey/qvalue/) (50, 51) and used FDR <0.05 as a criterion for additional analysis of loci associated with radiosensitivity.

RESULTS

SNP markers for candidate genes

The ontologic classification of candidate genes for the association study is shown in Fig. 1. Approximately one-half of the gene set was categorized into DNA repair, transcription, cell death, or cell cycle control.

The SNP sites of the candidate genes were selected using position and allele frequency information obtained from the jSNP and dbSNP databases. First, 1,025 SNP sites were

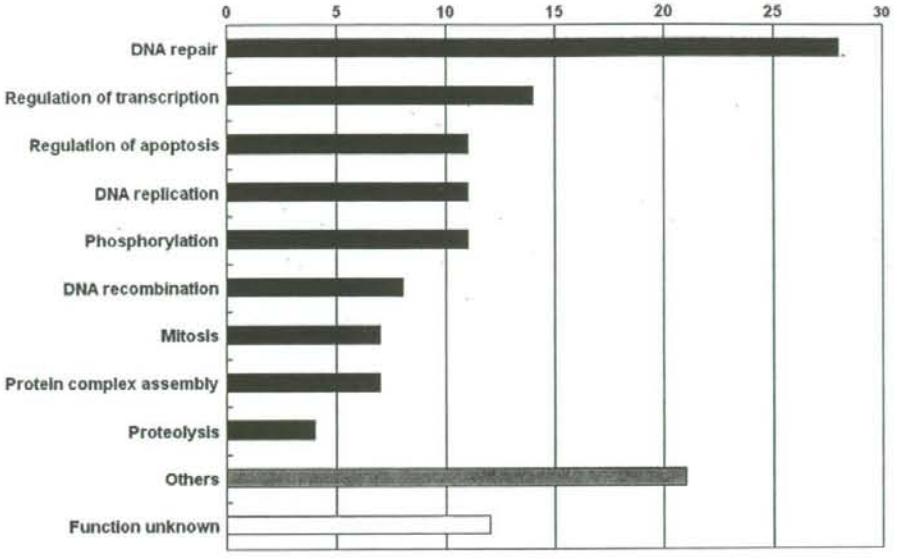

Fig. 1. Ontologic classification of candidate genes for typing. Total of 137 candidate genes assigned to biologic categories by Gene Ontology database at hierarchy level 7 (59). The axis indicates the number of genes. Some genes were categorized into multiple classes. Genes indicated as "function unknown" were not categorized, although our quantitative analyses of gene expression using comprehensive microarrays suggested that differences in their expression might contribute to variations in radiosensitivity of mouse strains or human cell lines (29–32).

chosen from the 137 candidate genes. The mean value of the SNP sites per gene was approximately 7. Of the 1,025 SNP sites, 26 (2.5%) showed a low sensitivity for distinguishing alleles in our typing system and were removed from further analysis.

Of the 999 SNPs (Appendix 2), 359 sites were excluded because they were not polymorphic in our breast cancer patient group. In addition, 104 sites were excluded from the following association study because of their low allele frequency (minor allele frequency, <5%). Two tri-allelic sites were not analyzed further. Two other SNP sites were excluded because of disjunction to the Hardy-Weinberg equilibrium ($p < 0.001$). Also, 22 SNPs were removed from additional analysis because the genotypes of these SNPs were identical to those of the respective contiguous SNPs. Finally, 510 SNP sites on 123 genes were subjected to testing. The positional properties of the SNPs are shown in Table 2.

Table 2. Position of SNPs

SNP position	No. of SNPs (%)
5′ Flanking	124 (24.3)
Exon	
5′ UTR	6 (1.2)
Synonym	33 (6.5)
Nonsynonym	43 (8.4)
3′ UTR	23 (4.5)
Intron	186 (36.5)
3′ Flanking	95 (18.6)
Total	510 (100)

Abbreviations: SNP = single nucleotide polymorphism; UTR = untranslated region.

Typing accuracy was estimated to 99.95% by retyping of 34,206 reactions for 761 individuals, including healthy donors and breast cancer patients.

Allele and genotype frequencies

To select the loci for haplotype analysis, the allele and genotype distributions for each SNP site in the HG group of patients were compared with those in the LG group. To avoid false-negative findings, we set the significance level at 0.05. Of 510 SNPs, 14 sites for 10 genes showed association with the EASR grade in allele frequency (Table 3). In genotype frequency, 21 SNP sites for 17 genes showed an association with the EASR grade according to either a dominant or a recessive model (Table 3). Nineteen genes containing 25 SNPs were subjected to LD mapping and haplotype analysis.

Association between haplotypes and EASRs after RT

Linkage disequilibriums were measured by D' among the SNPs on each of the 19 loci, using the allele frequency data of the breast cancer patients, and LD maps were constructed (data not shown). Haplotype tag SNPs were first selected using the Haploview program. Then the overall differences in the haplotype distribution between the HG and LG groups were assessed using the haplo.stats program, and sets of tagging SNPs showing the lowest p value were selected for 19 loci. Haplotype differences with $p < 0.05$ and an FDR < 0.05 were revealed in *RAD9A*, *PTTG1*, *LIG3*, *REV3L*, *CD44*, and *MAD2L2* genes (Table 4).

Haplotypes with a possible risk and the effect of each haplotype are presented in Table 5. ORs are presented for comparison of the risk haplotypes to the most frequent haplotype

Table 3. Association of SNPs between high- and low-grade groups

				Allele (M/m)				Genotype (MM/Mm/mm)		Dominant model		Recessive model	
rsSNP ID	Gene	Chr	Mm	HG (n = 218)	LG (n = 580)	p	OR (95% CI)	HG (n = 109)	LG (n = 290)	p	OR (95% CI)	p	OR (95% CI)
rs2294638	MAD2L2	1	GC	141/77	318/262	0.013	1.51 (1.10–2.09)	48/45/16	86/146/58	0.25	1.45 (0.83–2.90)	0.0087	1.87 (1.18–2.97)
rs913060	TGFBR3	1	GA	166/52	480/100	0.043	0.67 (0.45–0.98)	65/36/8	203/74/13	0.31	0.59 (0.23–1.74)	0.056	0.63 (0.40–1.01)
rs1926261	TGFBR3	1	CT	116/102	361/219	0.023	0.69 (0.51–0.95)	35/46/28	120/121/49	0.063	0.59 (0.35–1.03)	0.11	0.67 (0.41–1.06)
rs1806649	NFE2L2	2	CT	199/19	552/28	0.043	0.53 (0.29–1.01)	90/19/0	262/28/0	NC	NC	0.037	0.51 (0.27–0.99)
rs2075747	OGG1	3	GA	155/63	388/190	0.31	1.20 (0.86–1.71)	51/53/5	137/114/38	0.017	3.15 (1.38–13.92)	1.0	0.98 (0.62–1.52)
rs3805169	NEIL3	4	TC	170/48	473/107	0.27	0.80 (0.55–1.19)	70/30/9	188/97/5	0.0036	0.19 (0.04–0.59)	0.91	0.97 (0.62–1.56)
rs3756402	RAD17	5	TC	199/19	500/80	0.054	1.68 (1.01–3.05)	91/17/1	213/74/3	1.0	1.13 (0.18–2.28)	0.036	1.83 (1.07–3.51)
rs3811999	PTTG1	5	CT	195/23	476/104	0.012	1.85 (1.20–3.12)	88/19/2	199/78/13	0.37	2.51 (0.74–7.15)	0.018	1.92 (1.15–3.46)
rs2961950	PTTG1	5	AG	159/59	380/200	0.051	1.42 (1.02–2.00)	59/41/9	122/136/32	0.47	1.38 (0.67–3.60)	0.033	1.62 (1.04–2.54)
rs2961952	PTTG1	5	GA	126/92	383/197	0.032	0.70 (0.51–0.98)	35/56/18	127/129/34	0.24	0.67 (0.36–1.31)	0.040	0.61 (0.37–0.95)
rs3757244	MAP3K7	6	CT	211/7	539/41	0.045	2.29 (1.13–7.36)	102/7/0	249/41/0	NC	NC	0.038	2.40 (1.15–7.54)
rs190246	REV3L	6	GT	113/105	353/227	0.024	0.69 (0.50–0.94)	31/51/27	104/145/41	0.016	0.50 (0.29–0.88)	0.19	0.71 (0.43–1.14)
rs240962	REV3L	6	GT	104/114	335/245	0.013	0.67 (0.49–0.91)	24/56/29	97/141/52	0.069	0.60 (0.36–1.03)	0.028	0.56 (0.32–0.92)
rs818707	ALAD	9	CT	208/10	528/52	0.039	2.05 (1.11–4.94)	100/8/1	242/44/4	1.0	1.51 (0.19–3.06)	0.037	2.20 (1.14–5.74)
rs2282367	MAT1A	10	GA	205/13	525/55	0.12	1.65 (0.95–3.55)	98/9/2	236/53/1	0.21	0.208 (0.08–1.26)	0.047	2.04 (1.09–4.67)
rs8193	CD44	11	CT	118/100	369/211	0.018	0.67 (0.49–0.92)	27/64/18	118/133/39	0.43	0.79 (0.44–1.54)	0.0034	0.48 (0.28–0.76)
rs2286620	RAD9A	11	TC	183/35	432/148	0.0045	1.79 (1.22–2.77)	76/31/2	161/110/19	0.077	3.75 (1.13–10.19)	0.012	1.85 (1.17–3.04)
rs917570	RAD9A	11	CG	196/22	488/92	0.041	1.68 (1.06–2.93)	87/22/0	203/82/5	0.33	NC	0.058	1.69 (1.03–3.03)
rs2268622	TGFB3	14	TC	113/105	347/233	0.045	0.72 (0.52–0.99)	29/55/25	108/131/51	0.25	0.72 (0.42–1.29)	0.058	0.61 (0.36–0.98)
rs3744355	LIG3	17	GC	144/74	435/145	0.013	0.65 (0.46–0.92)	49/46/14	158/119/13	0.0060	0.32 (0.13–0.73)	0.093	0.68 (0.43–1.06)
rs73234	SH3GL1	19	CG	131/79	390/178	0.10	0.76 (0.54–1.07)	39/53/13	141/108/35	1.0	0.99 (0.52–2.12)	0.030	0.60 (0.37–0.95)
rs243336	SH3GL1	19	GC	126/92	377/203	0.070	0.74 (0.54–1.02)	34/58/17	130/117/43	0.88	0.94 (0.52–1.86)	0.016	0.56 (0.34–0.89)
rs25487	XRCC1	19	CT	154/64	443/137	0.10	0.74 (0.53–1.06)	59/36/14	168/107/5	0.015	0.37 (0.17–0.84)	0.50	0.86 (0.54–1.34)
rs918546	BAX	19	GT	126/92	362/216	0.22	0.82 (0.59–1.13)	30/66/13	117/128/44	0.52	1.33 (0.70–2.84)	0.020	0.56 (0.33–0.89)
rs3087869	COMT	22	AG	154/64	390/190	0.39	1.17 (0.85–1.66)	52/50/7	142/106/42	0.039	2.47 (1.18–7.82)	0.91	0.95 (0.61–1.49)

Abbreviations: rsSNP = reference single nucleotide polymorphism; ID = identifier; LG = low grade; HG = high grade; Chr = chromosome; M = major allele; m = minor allele; OR = odds ratio; CI = confidence interval; NC = insufficient sample size to perform calculation.

SNPAlyze software used for Fisher exact test and calculation of OR and its 95% CI (bootstrap method).

Table 4. Haplotype association and FDR

Gene	SNPs for haplotype	p*	FDR
RAD9A	rs2255990, rs2286620, rs917570	0.015	0.033
PTTG1	rs2910190, rs3811999, rs1862391, rs2961951	0.016	0.033
LIG3	rs3744355, rs2074518, rs3744357	0.017	0.033
REV3L	rs190246, rs240962	0.023	0.033
CD44	rs187116, rs3794116, rs3794107, rs8193	0.026	0.033
MAD2L2	rs2294638, rs746218	0.040	0.041
NFE2L2	rs1806649, rs2364724	0.067	0.053
TGFBR3	rs1926261, rs2296620	0.068	0.053
ALAD	rs818707, rs1805312	0.091	0.055
TGFB3	rs2268622, rs3917145	0.10	0.055
SH3GL1	rs2705, rs243387	0.11	0.055
RAD17	rs3756402, rs299081	0.11	0.055
OGG1	rs1801129, rs2075747	0.11	0.055
XRCC1	rs25487, rs2682585	0.17	0.075
NEIL3	rs3805169, rs13112358	0.22	0.092
MAT1A	rs2993763, rs2282367	0.28	0.11
MAP3K7	rs1144158, rs157692, rs205343, rs3757244, rs282065	0.38	0.14
BAX	rs918546, rs3745693	0.43	0.15
COMT	rs2020917, rs3087869	0.63	0.21

Abbreviations: SNP = single nucleotide polymorphism; FDR = false-discovery rate.

Estimates of FDR based on q values (http://faculty.washington.edu/~jstorey/qvalue/); tuning parameter, $\lambda = 0.2$.

* p value for global statistic corresponds to test for overall association between haplotypes and risk of adverse skin reactions.

in each locus. In the *CD44* gene, the haplotype GGTT significantly increased the risk of EASRs compared with the most common haplotype GGTC (OR = 2.17; 95% CI, 1.07–4.43). The overall difference in the haplotype distribution was assessed in *REV3L* (simulation-based $p = 0.023$), but no stratum with a significant risk haplotype was observed. The haplotypes CG in *MAD2L2* (OR = 0.55; 95% CI, 0.35–0.87), GTTG in *PTTG1* (OR = 0.48; 95% CI, 0.24–0.96), TCC (OR = 0.48; 95% CI, 0.26–0.89), and CCG (OR = 0.50; 95% CI, 0.27–0.92) in *RAD9A*, and GCT in *LIG3* (OR = 0.46; 95% CI, 0.22–0.93) were associated with a reduced risk of EASRs compared with the most common haplotype in each locus. These results have suggested that the group of breast cancer patients in this study could be stratified by specific haplotypes and that the individuals with the haplotype GGTT in *CD44* were at a significantly greater risk of EASRs compared with those with haplotypes CG in *MAD2L2*, GTTG in *PTTG1*, TCC or CCG in RAD9A, and GCT in *LIG3*.

DISCUSSION

The ultimate goals of our ongoing research are to find genetic variations that are associated with radiosensitivity and to use this information to identify genetic markers. In this report we analyzed the haplotypes of genes that were candidates for affecting the risk of EASRs after RT. Variations in the candidate genes were considered as haplotypes because the statistical power of the association tests using phased data is likely to increase (39–42). A total of 123 genes covering 510 SNPs were subjected to the first screening. From the LD maps for the 19 genes selected by the screening, the haplo-tag SNPs were selected for each locus. Global haplotype analysis ($p < 0.05$) and consideration of FDR ($q < 0.05$) showed that *CD44*, *MAD2L2*, *PTTG1*, *RAD9A*, *LIG3*, and *REV3L* loci were associated with EASR risk. We found haplotypes associated with an increased risk of EASRs in *CD44* and other haplotypes associated with a reduced risk of EASRs in *MAD2L2*, *PTTG1*, *RAD9A*, and *LIG3*. No significant risk was observed for any haplotype in *REV3L*.

Combinations of these haplotypes in multiple loci might determine the complexity of an individual's radiosensitivity. Andreassen *et al.* (9) suggested a model consisting of multiple genes with different effects on clinical radiosensitivity to explain patient-to-patient variability in normal tissue reactions after RT. The identification in the present study of five genes with different contributions to clinical radiosensitivity seems to support their model. Because a haplotype of one gene increased the risk of EASRs but other haplotypes of other genes reduced the risk of EASRs, the combined effect of haplotype contribution should be considered patient by patient. The present haplotypes, however, could only be estimated statistically. Therefore, the real haplotypes of these genes must be determined experimentally. This would provide an understanding of the mechanisms underlying the genetic variation in radiation sensitivity or resistance among the population and would enable the prediction of the risk of EASRs before RT. It might be possible to perform the required experiments using a recently reported new method for haplotype determination (52).

Five of the six genes shown in Table 5 (*REV3L*, *MAD2L2*, *PTTG1*, *RAD9A*, and *LIG3*) encode for proteins that act in the nucleus. The functions of three of them (*MAD2L2*, *REV3L*, and *PTTG1*) are related to chromosome maintenance, involving sister chromatid separation and the mitotic spindle checkpoint (see Nasmyth [37] for review). This suggests that functional variation might cause the malfunction of cell cycle regulation and lead to genome instability, including aneuploidy. The functions attributed to the identified genes appear consistent with the early damage that results from the death of a large number of cells in the epidermal layer of the skin (17). CD44 is a transmembrane adhesion receptor that is the major cell surface receptor for the nonsulfated glycosaminoglycan hyaluronan and is reported to be involved in lymphocyte extravasation (53). Appropriate repair after radiation injury and inflammation requires a resolution of the inflammatory response and removal of extracellular matrix breakdown products. We have previously analyzed interstrain variations in irradiated murine lung and found an increase in the number of CD44-positive cells in radioresistant mice (29). Therefore, the finding that this haplotype is associated with patients who developed EASRs was of great interest.

Our previous *in vitro* study showed that genes in the base-excision repair system, such as *LIG1* (data not shown) and *PCNA*, were likely candidates for genotyping (31). This base-excision repair system has been suggested to play a role in repairing DNA damaged by ultraviolet light and

Table 5. Estimated frequency of haplotypes and association with risk of EASRs

Gene	Haplotype*	Estimated frequency†			Effect	
		Pool (n = 798)	LG (n = 580)	HG (n = 218)	OR (95% CI)‡	p§
CD44	GGTC	0.40	0.42	0.33	1.0 (Reference)	
	GGTT	0.25	0.22	0.30	2.17 (1.07–4.43)	0.010
	AGTC	0.13	0.12	0.14	1.79 (0.82–3.90)	0.18
	AGTT	0.068	0.058	0.093	2.09 (0.93–4.67)	0.040
	AGAC	0.049	0.054	0.034	1.07 (0.36–3.18)	0.68
	AGAT	0.048	0.052	0.037	0.80 (0.27–2.40)	1.0
	AATC	0.038	0.041	0.030	0.88 (0.26–3.01)	0.82
	AATT	0.023	0.026	0.014	0.84 (0.12–5.83)	0.77
REV3L	GC	0.54	0.56	0.48	1.0 (Reference)	
	TT	0.41	0.38	0.48	1.47 (0.90–2.39)	0.014
	GT	0.044	0.045	0.041	0.96 (0.43–2.14)	0.84
	TC	0.010	0.014	NA	NA	NA
MAD2L2	GG	0.57	0.54	0.64	1.0 (Reference)	
	CG	0.31	0.34	0.24	0.55 (0.35–0.87)	0.0044
	CA	0.11	0.12	0.11	0.81 (0.46–1.42)	0.45
PTTG1	GCTG	0.24	0.23	0.27	1.0 (Reference)	
	ACTG	0.21	0.22	0.19	0.94 (0.51–1.71)	0.29
	ACTT	0.20	0.19	0.23	1.29 (0.73–2.27)	0.82
	GTTG	0.13	0.15	0.079	0.48 (0.24–0.96)	0.0081
	GCGG	0.090	0.094	0.076	0.86 (0.41–1.82)	0.29
	ACGG	0.064	0.064	0.061	0.86 (0.39–1.90)	0.60
	GCTT	0.036	0.025	0.059	2.17 (0.78–6.03)	0.13
	GTTT	0.016	0.022	NA	NA	NA
RAD9A	CTC	0.74	0.71	0.82	1.0 (Reference)	
	TCC	0.11	0.13	0.073	0.48 (0.26–0.89)	0.017
	CCG	0.11	0.12	0.073	0.50 (0.27–0.92)	0.022
	CTG	0.033	0.036	0.023	0.51 (0.18–1.39)	0.28
LIG3	GCC	0.42	0.41	0.45	1.0 (Reference)	
	CCC	0.25	0.24	0.29	1.14 (0.68–1.90)	0.63
	GTC	0.20	0.21	0.15	0.76 (0.45–1.29)	0.10
	GCT	0.11	0.13	0.041	0.46 (0.22–0.93)	0.0004
	CCT	0.015	0.007	0.033	3.73 (0.66–21.05)	0.021

Abbreviations: EASRs = early adverse skin reactions; NA = not applicable; other abbreviations as in Table 3.

* Haplotypes observed with >1% frequency in pool.

† Haplotype frequency was estimated using haplo.cc function of the haplo.stats.

‡ Odds ratio obtained using recursively the estimated posterior probabilities of pairs of haplotypes per subjects as weights in the logistic model (haplo.cc function of the haplo.stats).

§ The p-value based on the score statistics corresponds to the test for association between the specific haplotype and the risk of ASRs.

ionizing radiation (54, 55). The SNPs on the members of this pathway, *NEIL3*, *APEX1*, *POLB*, *POLD1*, *POLE*, *XRCC1*, *LIG1*, *LIG3*, *PARP1*, *PNKP*, *PCNA*, and *FEN1*, were subjected to genotyping analysis. Allelic and/or genotypic associations were observed between the SNPs on *NEIL3*, *LIG3*, and *XRCC1* and EASR risk, but an association between the haplotypes of these genes and EASR risk was suggested only for the *LIG3* locus (Tables 3–5).

RAD9A was selected because it was reported to act as a damage sensor in the DNA damage checkpoint response (56). This gene product is a subunit of the heterotrimeric RAD9-RAD1-HUS1 complex. RAD9A also interacts with the anti-apoptotic bcl-2 family of proteins (BCL-2/BCL-x_L) suggesting a role for RAD9A in regulating apoptosis after DNA damage (57). We propose that if cells contained unusual activity of these gene products, the cell cycle would not be completed after RT. The increased number of defective cells could activate the immune system, but not the apoptotic system, and cause inflammation.

A total of 27 cSNPs for *CD44*, *LIG3*, and *REV3L* and none for *MAD2L2*, *PTTG1*, and *RAD9A* genes were recorded in the jSNP or dbSNP database when this research began. However, because of a lower allele frequency (not polymorphic or <5%) in our patient group, these cSNPs in *CD44*, *LIG3*, and *REV3L* were not used for the haplotype analysis. At present, we cannot explain how the specific haplotypes with a greater risk affected the function of the gene products. It is possible that SNPs in regulatory regions (rSNPs) or in untranslated regions of mRNA contribute to functional differences in genes. With respect to *PTTG1*, the SNPs in the estimated haplotype (rs3811999 and rs1862391) were located within 2 kb from the transcriptional start site, suggesting that they might affect transcription of the *PTTG1* gene. Regarding *CD44*, rs8193 was located in the 3′ untranslated region of its mRNA,

indicating that this SNP might cause stability of *CD44* mRNA. Searching functional SNPs in the genes identified in the present study would facilitate the understanding of mechanisms contributing to variations in radiation susceptibility.

The rs25487 (Arg399Gln) polymorphism on *XRCC1* reportedly associates with the risk of early skin reactions after RT in breast cancer patients (19). Our findings suggest an association between the Arg399Gln genotype in *XRCC1* and the risk of EASRs (dominant model in Table 3). However, no overall significant difference in the haplotype distribution in the *XRCC1* gene was detected between the HG and LG groups (Table 4). Associations between the markers C-509T (rs1800469) in *TGFB1* (20, 22, 23) and Val16Ala (rs4880) in *SOD2* (20) and late adverse effects in breast cancer patients have been reported. To test whether these polymorphisms were also associated with early skin reactions, we examined these polymorphisms in our subjects. However, no association between these SNPs and EASRs was detected.

We also analyzed in detail the SNPs within and surrounding the *ATM* gene. The marker Asp1853Asn (rs1801516), which is associated with late reactions (24, 26), was not polymorphic in our 399 breast cancer patients and 115 healthy Japanese women. The marker IVS22-77 T>C (rs664677), which has also been reported to associate with late reactions (21), was not associated with EASRs in our study. Furthermore, we analyzed this region spanning approximately 200 kb with 53 SNPs selected from the jSNP and dbSNP databases; 34 SNPs were not polymorphic and minor allele frequency of 6 SNPs were <0.05. The remaining 13 SNPs were not associated with the risk of EASRs. Because the *ATM* gene has been reported to lie within a large single LD block according to the HapMap data (D' >0.8) with HAN Chinese and Japanese populations (58), these markers seemed to cover most of the *ATM* gene. The distinctive features of our study, which could have influenced the results, were the following: *(1)* that we analyzed EASRs that occurred within 3 months of starting RT; *(2)* that we tested the reported SNPs that were able to be typed using our matrix-assisted laser desorption/ionization time-of-flight mass spectrometry-based technique; and *(3)* the Japanese ethnicity of our subjects, which might have had some bearing on their radiosensitivity.

The use of selected genes imposes some limitations on our findings. The candidate genes for genotyping were selected using a limited number of gene expression analyses, in addition to the published genes we were interested in. Because the HapMap data has only been available recently, it might be able to perform a genome-wide association study for radiosensitivity in cancer patients. Furthermore, we emphasize the importance of a subsequent association study with a large number of patients and/or a meta-analysis of multiple populations.

CONCLUSION

We identified six novel haplotypes associated with the risk for EASRs after RT using a large-scale candidate-genes approach. Focused investigations of functions related to the haplotypes in these genes will improve our understanding further of how genetic factors contribute to individual radiosensitivity.

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