

腋窩リンパ節転移陰性乳がんに対する  
術後補助化学療法としての Docetaxel と  
Cyclophosphamide の忍容性および安全性  
—JECBC04 試験—

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[*Jpn J Cancer Chemother* 37(1): 57-63, January, 2010]

Tolerability and Safety of Docetaxel Plus Cyclophosphamide as Adjuvant Chemotherapy for Axillary Lymph Node-Negative Breast Cancer—JECBC04 Trial: Naohito Yamamoto<sup>\*1,2</sup>, Toshio Tabei<sup>\*2</sup>, Kenichi Inoue<sup>\*2</sup>, Hiroyuki Takei<sup>\*2</sup>, Nobuaki Sato<sup>\*2</sup>, Yasuhiro Yanagita<sup>\*2</sup>, Tomomi Fujisawa<sup>\*2</sup>, Hirofumi Fujii<sup>\*2</sup>, Toshiaki Saeki<sup>\*2</sup> and Masafumi Kurosumi<sup>\*2</sup> (<sup>\*1</sup>Chiba Cancer Center, Division of Breast Surgery, <sup>\*2</sup>Japan East Cancer Center Breast Cancer Consortium)

**Summary**

A recent foreign clinical trial showed that the combination of docetaxel plus cyclophosphamide (TC) is associated with a superior disease-free survival compared with doxorubicin plus cyclophosphamide as adjuvant chemotherapy for breast cancer. To assess the tolerability and safety of TC in a Japanese patient population, we conducted a multicenter, open-labeled clinical trial. Eligible patients were women who had axillary lymph node-negative breast cancer with surgical excision of the primary tumor. Patients were treated with 4 courses of TC (75 and 600 mg/m<sup>2</sup>, respectively), administered intravenously every 3 weeks. The primary endpoint was feasibility, which was defined as the proportion of patients who completed 4 courses of the chemotherapy. From October 2006 to November 2007, 39 patients were enrolled and 32 were evaluable. Seven patients were excluded because of the inadequate treatment schedule. Feasibility was 96.9% (31/32). One patient did not complete treatment because of the hypersensitivity. The mean administered dose was 73.2 mg/m<sup>2</sup> for docetaxel and 588.3 mg/m<sup>2</sup> for cyclophosphamide, respectively. The mean relative dose intensity was 96.1% and 95.7%, respectively. The grade 3/4 toxicity including leukopenia, neutropenia, and febrile neutropenia was manageable. From these results, we consider that TC might become a standard non-anthracycline adjuvant regimen for operable breast cancer. Key words: Breast cancer, Docetaxel, Cyclophosphamide, Adjuvant therapy (Received Apr. 6, 2009/Accepted Jun. 8, 2009)

**要旨** 海外の臨床試験成績から、docetaxel+cyclophosphamide (TC) の4コース投与は乳がんの術後補助化学療法として有効と考えられるが、国内での使用経験は十分ではない。このためTCの忍容性および安全性を評価する目的で多施設共同臨床試験を実施した。対象は原発乳がんの根治術が施行され、腋窩リンパ節転移陰性の女性とした。docetaxelおよびcyclophosphamideの用量はそれぞれ75および600 mg/m<sup>2</sup> (いずれも点滴静注)とし、3週間隔で4コース投与した。主要評価項目は試験治療を4コース完遂した患者の割合とした。2006年10月～2007年11月にかけて本試験には39名の患者が組み入れられ32名を評価した。7名は試験治療が手順どおりに投与されなかったため除外した。試験治療の完遂割合は96.9% (31/32)で、1名は過敏性反応のために試験治療を中止した。実投与量の平均値はdocetaxelが73.2 mg/m<sup>2</sup>, cyclophosphamideが588.3 mg/m<sup>2</sup>で、計画した投与量のそれぞれ96.1, 95.7%であった。grade 3/4の有害事象は白血球、好中球/顆粒球、発熱性好中球減少などで、投与中止を必要としたものはなかった。以上から、non-anthracyclineレジメンのTCは乳がんの術後標準治療の一つになり得ると考えられた。

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## はじめに

原発乳がんの手術後薬物療法は、腋窩リンパ節転移個数、腫瘍径、ホルモン感受性の有無、HER2発現の有無、年齢といった因子に基づいて選択される<sup>1)</sup>。これまで手術後の補助化学療法としては、cyclophosphamide+methotrexate+fluorouracil (CMF)、doxorubicin+cyclophosphamide (AC)などが標準的に使用されてきた<sup>2-4)</sup>が、その後、taxane系薬剤がレジメンに取り入れられるようになった<sup>5-7)</sup>。最近では長期生存者の増加に伴いanthracycline系薬剤による心毒性が懸念されるようになり、non-anthracyclineレジメンの開発が期待されている。

このような観点からUS Oncology 9735試験では、I・II期および手術可能なIII期の原発乳がんを対象として、ACの4コース投与とdocetaxel+cyclophosphamide (TC)の4コース投与を比較した結果、TC群の無病生存期間はAC群よりも有意に長いことが確認された<sup>8)</sup>。年齢、ホルモン感受性の有無、腋窩リンパ節転移の有無といった因子によるサブグループ解析でもこの傾向は一貫していたことから、TCは早期乳がんの術後補助化学療法の標準治療になり得ると結論された。しかし、この試験は海外の患者を対象としたもので、国内の患者集団でのTCの忍容性および安全性は十分に評価されていない。

こうしたことから、US Oncology 9735試験と同一の用法・用量で国内の乳がん患者にTCを投与した時の忍容性および安全性を評価する目的で、多施設共同オープン試験を実施した。

### I. 対象および試験方法

本臨床試験は2006年10月～2007年11月にかけて国内の6施設（新潟県立がんセンター、群馬県立がんセンター、埼玉県立がんセンター、神奈川県立がんセンター、栃木県立がんセンター、千葉県がんセンター）で実施された。試験の実施に際してはヘルシンキ宣言<sup>9)</sup>および臨床研究に関する倫理指針<sup>10)</sup>を遵守し、各施設の倫理審査委員会で事前に試験実施の承認を得るとともに、試験に参加した全患者から文書による同意を取得した。

#### 1. 対象集団

本試験の対象は、組織学的に診断された原発乳がん女性で、根治術が施行され、腋窩リンパ節郭清またはセンチネルリンパ節生検によって腋窩リンパ節転移が陰性であることが確認された患者とした。またSt. Gallen分類の中等度リスクに相当するよう年齢は20歳以上34歳以下であるか、あるいは35歳以上70歳以下で、「原発巣

の腫瘍最大径>2cm」「分化度がgrade 2または3」「腫瘍組織周囲への脈管侵襲」「human epidermal growth factor receptor-2 (HER2)の過剰発現 (immunohistochemistryが3+, またはfluorescence *in situ* hybridization (FISH)が陽性)」のいずれかを満たすこととした。

その他、①Eastern Cooperative Oncology Groupのperformance status (PS):0または1、②臓器機能が良好（ヘモグロビン:9 g/dL以上、白血球数:4,000/mm<sup>3</sup>以上12,000/mm<sup>3</sup>以下、好中球数:2,000/mm<sup>3</sup>以上、血小板数:10×10<sup>4</sup>/mm<sup>3</sup>以上、血清総ビリルビン値:施設基準値の上限以下、ASTおよびALT:100 IU/L未満、血清クレアチニン値:施設基準値上限の1.5倍以下)などを選択基準とした。

また、①術前、術後補助療法として放射線療法、内分泌療法、化学療法の施行歴がある、②感染症など試験治療に支障を来す可能性がある患者は本試験の対象から除外した。

#### 2. 試験治療

試験治療はdocetaxel (タキソテール, サノフィ・アベンティス株式会社, 東京)+cyclophosphamide (注射用エンドキサン, 塩野義製薬株式会社, 大阪)の併用療法とし、これを3週間隔で合計4コース投与することとした。docetaxelの用量は75 mg/m<sup>2</sup>、cyclophosphamideの用量は600 mg/m<sup>2</sup>とし、両薬剤とも各コースの第1日目に投与した。docetaxelの副作用であるアレルギーおよび浮腫を予防する目的でのステロイドを使用するかどうかは各施設の判断に任せた。各コース第1日目の標準的な投与手順を以下に示す。

手順1:生理食塩液100 mL+dexamethasoneまたはbetamethasone 8 mgを急速点滴静注

手順2:生理食塩液250 mL+docetaxelを1時間かけて点滴静注

手順3:生理食塩液250 mL+cyclophosphamideを30分かけて点滴静注

手順4:生理食塩液100 mLを急速点滴静注

ただし、以下の条件に1項目でも抵触する場合は、すべての項目が回復するまで投与を延期することとした。投与の延期は投与予定日から最大3週までとした。  
①白血球数<3,000/mm<sup>3</sup>、かつ好中球数<1,500/mm<sup>3</sup>、  
②grade 2以上の神経障害、③grade 2以上の浮腫、  
④grade 2以上の肝・腎機能障害、⑤その他、担当医師が必要と判断した場合。

また、以下に示す有害事象が認められた場合には以降の投与量を1段階減量し (docetaxelは75 mg/m<sup>2</sup>から60 mg/m<sup>2</sup>、cyclophosphamideは600 mg/m<sup>2</sup>から500

Table 1 Baseline characteristics of 32 evaluable patients with operable breast cancer

Characteristic		Category	n	%
Age		30-34	1	3.1
		35-70	31	96.9
Performance status		0	32	100.0
		1	0	0.0
Post menopause		Yes	11	34.4
		No	21	65.6
Hormone receptor	ER	+	8	25.0
		-	24	75.0
	PgR	+	6	18.8
		-	26	81.3
HER2	IHC	3+	5	15.6
		2+	7	21.9
		1+	14	43.8
		-	6	18.8
	FISH	+*	2	6.3
		-	2	6.3
	Not tested	13	40.6	
	Unknown	15	46.9	
Primary lesion		Right breast	15	46.9
		Left breast	17	53.1
TNM	T	Tis, T0, T1	16	50.0
		T2, T3, T4	16	50.0
	N	N0	32	100.0
	M	M0	32	100.0
Stage		I	16	50.0
		II a, II b, III b	16	50.0
Histological type	Invasive	Papillo tubular Ca.	6	18.8
		Solid tubular Ca.	11	34.4
		Scirrhus Ca.	11	34.4
		Other	3	9.4
		Unknown	1	3.1
Surgery		Lumpectomy	24	75.0
		Mastectomy	8	25.0
Allergy predisposition		Yes	30	93.8
		No	2	6.3
Anamnesis of hypersensitive drug reaction		No	32	100.0
		Yes	0	0.0
Complications		No	26	81.3
		Yes	6	18.8

\*: IHC=2+

mg/m<sup>2</sup>), 有害事象が回復しても投与量をもとに戻さないこととした。① grade 3 以上の非血液学的毒性 (悪心・嘔吐, 全身倦怠感を除く), ② grade 4 以上の血液学的毒性 (白血球減少, 好中球減少を除く), ③ grade 3 以上の発熱性好中球減少, ④ grade 4 の好中球減少, 白血球減少が 7 日間以上継続。なお, 1 段階を超える減量が必要な場合は試験を中止することとし, さらなる減量は認めなかった。

### 3. 併用療法に関する規定

試験治療の評価に影響を及ぼすと考えられる化学療法, ホルモン療法, biologic response modifier, 放射線療法, 手術療法の併用は禁止した。granulocyte colony-stimulating factor (G-CSF) 製剤は grade 4 または 38°C 以上の発熱があり, かつ grade 3 以上の好中球減少または白血球減少が認められた場合に投与してよいこととし, 予防目的での投与は禁止した。G-CSF 製剤を投与

Table 2 Reasons of dose reduction and dose delay

Reasons of dose reduction	Course			Total
	2	3	4	
Non-hematological toxicity greater than grade 3*	0	0	0	0
Hematological toxicity greater than grade 4†	0	0	0	0
Febrile neutropenia greater than grade 3	2	1	0	3
Grade 4 leukocytes and neutrophils for 7 days or more	0	0	0	0
Judgement of investigator	2‡	0	1§	3

\*: Except nausea, vomiting and fatigue

†: Except leukocytes and neutrophils

‡: Two were due to grade 4 leukocytes and neutrophils for 6 days or less.

§: One was due to rash grade 2 and the patient's wish.

Reasons of dose delay	Course			Total
	2	3	4	
Leukocytes <3,000/mm <sup>3</sup> and neutrophils <1,500/mm <sup>3</sup>	0	1	0	1
Neuropathy greater than grade 2	0	0	0	0
Edema greater than grade 2	0	0	0	0
Liver or renal disorders greater than grade 2	0	0	0	0
Judgement of investigator	1 <sup>¶</sup>	0	1 <sup>¶</sup>	2
Social factor	2	1	2	5

¶: One was due to the dental treatment.

¶: One was due to rash grade 2 and the patient's wish.

した場合は、白血球数が10,000/mm<sup>3</sup>以上または好中球数が5,000/mm<sup>3</sup>以上に回復した時点で投与を中止することとした。

#### 4. 評価項目

主要評価項目は試験治療を4コース完遂した患者の割合とした。副次評価項目は安全性, dose intensity (1コース当たりの実際の投与量), relative dose intensity (1コース当たりの実際の投与量/計画した投与量)とした。有害事象はNational Cancer Institute Common Terminology Criteria for Adverse Events version 3.0の日本語訳<sup>11)</sup>に基づいて評価した。

#### 5. 目標とする患者数

目標とする患者数は4コースの完遂割合の推定値に基づいて設定した。具体的には完遂割合の期待値を80%, 閾値を60%とし, 有意水準片側5%, 検出力90%で, 十分な完遂割合が達成されたかどうかを検証できる患者数を正規分布近似によって計算すると必要な患者数は44名となり, これに脱落などの影響を考慮し50名とした。

## II. 結 果

本試験には2006年10月~2007年11月の間に39名の患者が組み入れられた。予定症例集積期間1年間に対し予定された50名に満たなかったが, 効果安全性評価委員および運営委員にて協議した結果, 32名を評価に採用し忍容性の評価は可能と考え, 症例集積を終了した。

残る7名は試験治療が手順どおりに投与されなかったため評価から除外した。

評価可能であった32名の患者背景をTable 1に示す。35歳未満の患者は1名のみで, 残る患者の年齢はすべて35~70歳であった。PSは良好で全患者が0に分類された。閉経後の患者は11名(34.4%)で, エストロゲンおよびプロゲステロン受容体に感受性のある患者はそれぞれ8名(25.0%), 6名(18.8%)であった。

4コースの試験治療を完了したのは32名中31名で完遂割合は96.9%であった。1名はgrade 2の過敏性反応が出現し, 処置後に試験治療を再開したが再度同様の症状が出現したため試験治療を中止した。1コース当たりの実際の投与量(dose intensity)の平均値はdocetaxelが73.2 (SD 3.9) mg/m<sup>2</sup>, cyclophosphamideが588.3 (SD 26.2) mg/m<sup>2</sup>で, relative dose intensityはdocetaxelが96.1%, cyclophosphamideが95.7%であった。コースごとの減量および投与延期の内訳をTable 2に示す。減量理由で多かったものは, 「発熱性好中球減少」および「担当医師の判断」であった。

非血液毒性の有害事象をTable 3に示す。発現の多かった有害事象は悪心, 食欲不振, 脱毛, 粘膜炎(咽頭), 味覚変化, 便秘, 爪の変化, 疲労, 浮腫, 皮疹/落屑, 色素沈着, 神経障害, 筋肉痛および関節痛であった。ほとんどの有害事象はgrade 1または2で, grade 3に該当したのは疲労, 筋肉痛, 関節痛, 腹部痛が各1名(3.1%),

Table 3 Non-hematological toxicity

Adverse event	grade					
	1		2		3	
	n	%	n	%	n	%
Fatigue	19	59.4	8	25.0	1	3.1
Pain-muscle	8	25.0	5	15.6	1	3.1
Pain-joint	9	28.1	7	21.9	1	3.1
Pain-abdomen	0	0.0	0	0.0	1	3.1
Alopecia	6	18.8	26	81.3	0	0.0
Rash/desquamation	4	12.5	17	53.1	0	0.0
Nail changes	20	62.5	3	9.4	0	0.0
Hyperpigmentation	15	46.9	1	3.1	0	0.0
Mucositis-pharynx	18	56.3	4	12.5	0	0.0
Taste alteration	21	65.6	1	3.1	0	0.0
Nausea	18	56.3	1	3.1	0	0.0
Anorexia	16	50.0	2	6.3	0	0.0
Constipation	15	46.9	2	6.3	0	0.0
Diarrhea	8	25.0	3	9.4	0	0.0
Vomiting	4	12.5	0	0.0	0	0.0
Edema: limb	10	31.3	5	15.6	0	0.0
Edema: head and neck	1	3.1	1	3.1	0	0.0
Watery eye	7	21.9	1	3.1	0	0.0
Neuropathy-sensory	15	46.9	3	9.4	0	0.0
Neuropathy-motor	2	6.3	0	0.0	0	0.0
Allergic reaction/hypersensitivity	2	6.3	2	6.3	0	0.0
Cystitis	1	3.1	2	6.3	0	0.0
Phlebitis	0	0.0	1	3.1	0	0.0
Albumin, serum-low	9	28.1	0	0.0	0	0.0
AST, SGOT	9	28.1	0	0.0	0	0.0
ALT, SGPT	9	28.1	0	0.0	0	0.0
Alkaline phosphatase	3	9.4	0	0.0	0	0.0
Bilirubin	1	3.1	0	0.0	0	0.0
Creatinine	4	12.5	0	0.0	0	0.0
Calcium, serum-low	15	46.9	1	3.1	0	0.0
Sodium, serum-high	3	9.4	0	0.0	0	0.0
Potassium, serum-high	1	3.1	0	0.0	0	0.0
Potassium, serum-low	1	3.1	0	0.0	0	0.0
Others	9	28.1	4	12.5	0	0.0

grade 4/5 adverse events were not observed.

Table 4 Hematological toxicity

Laboratory event	grade							
	1		2		3		4	
	n	%	n	%	n	%	n	%
Leukocytes	1	3.1	1	3.1	15	46.9	7	21.9
Neutrophils/granulocytes	1	3.1	1	3.1	1	3.1	19	59.4
Febrile neutropenia	0	0.0	0	0.0	5	15.6	0	0.0
Fever	1	3.1	1	3.1	0	0.0	0	0.0
Hemoglobin	17	53.1	6	18.8	0	0.0	0	0.0
Platelets	5	15.6	0	0.0	0	0.0	0	0.0

grade 5 adverse events were not observed.

発熱性好中球減少が5名(15.6%)であった。grade 4以上に該当する有害事象はなかった。試験治療の中止を必要としたのは先に記載した過敏性反応の1名のみで、

1段階の減量を必要としたのは発熱性好中球減少の3名であった。

血液毒性に関する有害事象を Table 4 に示す。発現が

多かったのはヘモグロビン、白血球、好中球/顆粒球で、このうち白血球減少は grade 3~4 が 22 名 (68.8%)、好中球減少は grade 3~4 が 20 名 (62.5%) であった。投与延期を必要としたのは白血球および好中球が減少した 1 名であった。なお、grade 4 の好中球減少症 19 例中 7 例と grade 3 の発熱性好中球減少症 5 例中 2 例に G-CSF が使用された。

### Ⅲ. 考 察

腋窩リンパ節転移陰性の乳がん術後補助化学療法としては、これまで CMF や AC が使用されてきた<sup>3,4)</sup>。その後、報告されるようになった taxane 系薬剤<sup>5-7)</sup>のうち、docetaxel は cyclophosphamide との併用ではヒト腫瘍移植マウスでの研究で相乗的な効果を示すことが報告されていた<sup>12)</sup>。この組み合わせを用いた TC は近年、AC よりも再発抑制効果が優れることが示された<sup>8)</sup>。TC の再発抑制効果は部分集団でも検討され、年齢、ホルモン受容体あるいはリンパ節転移の有無の状況にかかわらず、TC が AC より優れる傾向が示された。

一方、国内での TC の報告は、US Oncology 9735 試験における投与量とは異なり、その使用経験は十分とはいえない<sup>13-15)</sup>。このためわれわれは、特に腋窩リンパ節転移陰性の中間リスク例に対する有用性に着目し、国内の患者に TC が投与可能かどうかを評価する目的で本試験を実施した。docetaxel と cyclophosphamide の投与順序に関しては US Oncology 9735 試験<sup>8)</sup>において特別の記載がなかったため、本試験実施開始時には docetaxel と cyclophosphamide の投与手順は厳密に規定しなかった。しかし、初期登録例のうち 7 例で docetaxel の前に cyclophosphamide が投与され、その 7 例中 5 例に grade 2 以上のアレルギー反応が起こったため効果安全評価委員会の判断で投与手順を docetaxel, cyclophosphamide の順と規定し、それ以外の投与手順で投与された 7 例は評価から除外した。その後、規定どおり投与された 32 例を評価可能症例とした。評価可能であった 32 名中 31 名 (96.9%) が 4 コースの投与を完了することができ、TC の忍容性は良好であった。また、relative dose intensity は両薬剤とも約 96% で、減量を必要とした患者は少なかった。US Oncology 9735 試験での TC 群の完遂割合は 93% で、2 剤の relative dose intensity は 99.8% であり<sup>8)</sup>、欧米と日本ではほぼ同等の忍容性であると考えられた。

本試験でみられた有害事象のうち grade 3~4 に該当したのは白血球、好中球減少、発熱性好中球減少などであった。US Oncology 9735 試験では grade 3~4 として 61% の好中球減少、8% 未満の感染症および 5% の発熱を

報告されている<sup>8)</sup>。今回の試験では grade 3~4 の好中球減少は同程度 (62.5%) であり、感染症は認めなかった。これらの多くでは試験治療の継続が可能であり、投与延期を必要としたのは白血球および好中球減少の 1 名のみであった。しかし 5 名 (15.6%) の発熱性好中球減少が出現しており、うち 3 名が 1 段階の減量を必要とした。9 名では試験中に G-CSF が投与された。白血球数や好中球数の変化を監視しながら必要に応じて減量、投与延期または G-CSF 投与で対処することによって TC の安全性は確保できるものと考えられた。また grade 1~2 ではあるものの、US Oncology 9735 試験では言及されていない皮疹/落屑 (65.6%) や過敏反応 (12.5%) も認められたこと、中止の 1 例は過敏反応によるものであったことを念頭に置き治療を行うことも重要である。

以上から、TC は海外と同一の用法・用量で国内の患者に投与可能で、白血球や好中球が減少した場合でも減量、投与延期または G-CSF 投与によって対処可能と考えられた。現在、AC は乳がんの術後補助化学療法として広く使用されているが、doxorubicin を含む anthracycline 系薬剤には心毒性があり、心筋障害、心不全などの重大な副作用が報告されている<sup>16-18)</sup>。このため、特に長期生存が期待される早期乳がんの術後補助化学療法では、anthracycline 系薬剤を含まないレジメンを確立することは重要な課題である。

これらを考慮すると、TC は術後補助化学療法の標準治療になり得るものと期待することができる。US Oncology では、さらに TC と docetaxel+doxorubicin+cyclophosphamide (TAC) を比較する臨床試験が進行中である (NCT00493870: Clinical Trial.gov)。今後はさらに使用経験を積んで TC の安全性プロファイルを確認するとともに、有効性についても評価することが望ましい。

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# 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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Received November 7, 2007; Accepted December 4, 2007

**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<sup>2</sup> 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 $\geq 2/3$ 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 $\geq 1/3$ 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 tri-weekly (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  $\geq 3$  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<sup>3</sup> and 12000/mm<sup>3</sup>, neutrophils

$> 2000/\text{mm}^3$ , platelets  $> 100000/\text{mm}^3$ , serum bilirubin within normal range, aspartate aminotransferase and alanine aminotransferase  $< 100$  IU/l and serum creatinine  $\leq 1.5$  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 infarction 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<sup>2</sup> 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 ( $\leq$ 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  $\geq$ 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 <sup>a</sup>	No. of patients (%)
Grade 3	4 (21) <sup>b</sup>
Grade 2	7 (37)
Grade 1	8 (42)
Grade 0	0 (0)

<sup>a</sup>Classified according to the criteria of the Japanese Breast Cancer Society. <sup>b</sup>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 ( $\geq 10\%$ )	13	3 (23)
Negative (<10%)	6	1 (17)
Ki-67		
Positive ( $\geq 10\%$ )	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 trastuzumab 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.

## Acknowledgments

We wish to thank the patients who participated in the JECBC 02 clinical trial. We also thank Drs Toshiaki Saeki, Hirofumi Fujii and Shinji Ohno for their helpful advice as members of the efficacy and safety evaluating committee. We are also

grateful to Yoshiko Nakagawa, Tomoko Kawamoto and Daisuke Nozaki for their scientific advice. This study was sponsored by the Advanced Clinical Research Organization.

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## 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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Conflict of interest: none.

**Acknowledgments**—We are grateful to all the DNA donors and the collaborating clinicians for participation in this study. A part of the DNA samples from healthy volunteers was provided by the Health

Science Research Resources Bank, Japan Health Science Foundation. We express our gratitude to Dr. Ken-ichi Ishikawa, Dr. Kaori Oota, Ms. Mutsuko Kitawaki, Ms. Tomoko Matsumoto, Mr. Kuniyoshi Matsumoto, Ms. Azusa Ohno, and Ms. Masayo Terada for managing the data and editing the manuscript.

Supplementary material may be found at [www.redjournal.org](http://www.redjournal.org) and <http://www2.nirs.go.jp/radgenomics/supplementary>

Received March 24, 2007, and in revised form June 5, 2007. Accepted for publication June 5, 2007.

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 popula-

tion using DNA samples from other healthy subjects provided by the Health Science Research Resources Bank, Japan Health Science Foundation (Osaka, Japan) (46).

### 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- $\mu$ L reactions with 2.5 ng of DNA template and final concentrations of 1 mmol/L MgCl<sub>2</sub>, 200  $\mu$ mol/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 SNPAlize software, version 6.0 (<http://www.dynacom.co.jp/e/products/package/snpalize/index.html>; DYNACOM, Chiba, Japan). Pairwise linkage disequilibrium (LD) analysis and haplotype analysis (expectation-maximization algorithm) were also performed using the SNPAlize 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

Table 1. Clinical patient features

Characteristic	LG ( $n = 290$ )	HG ( $n = 109$ )	Difference ( $p$ )
Age at RT (y)			
Mean $\pm$ SD	54 $\pm$ 10	50 $\pm$ 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) <sup>†</sup>			
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%.

<sup>†</sup> Fractionation size was 2 Gy, 5 times per week.

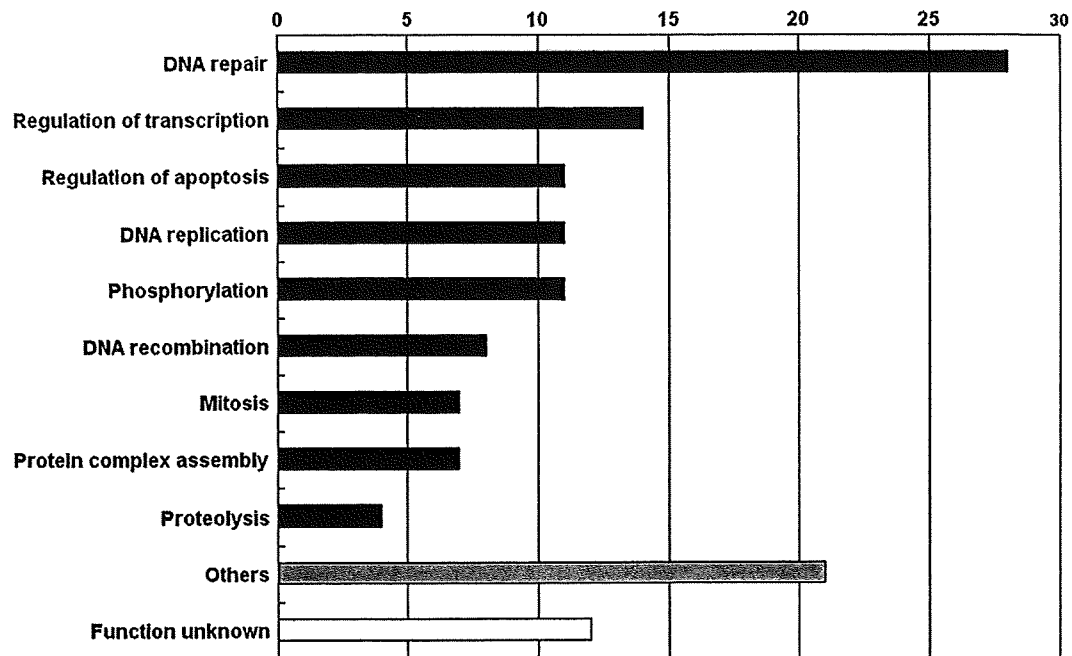


Fig. 1. Ontologic classification of candidate genes for typing. Total of 137 candidate genes assigned to biologic categories by Gene Ontology database at hierarchy level 7 (59). The axis indicates the number of genes. Some genes were categorized into multiple classes. Genes indicated as "function unknown" 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

rsSNP ID	Gene	Chr	Mm	Allele (M/m)				Genotype (MM/Mm/mm)				Dominant model		Recessive model	
				HG (n = 218)	LG (n = 580)	p	OR (95% CI)	HG (n = 109)	LG (n = 290)	p	OR (95% CI)	p	OR (95% CI)		
				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	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	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	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	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	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	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	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	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	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	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	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	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	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	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	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	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	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	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	TGFBR3	14	TC	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	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	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	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	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	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	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. SNPAnalyze 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	<i>p</i> *	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 <sup>†</sup>			Effect	
		Pool ( <i>n</i> = 798)	LG ( <i>n</i> = 580)	HG ( <i>n</i> = 218)	OR (95% CI) <sup>‡</sup>	<i>p</i> <sup>§</sup>
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.

<sup>†</sup> Haplotype frequency was estimated using haplo.cc function of the haplo.stats.

<sup>‡</sup> 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).

<sup>§</sup> 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<sub>L</sub>) 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 defec-

tive 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 *TGFBI* (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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