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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®).

腫瘍内科，1(6)：599-605，2007

話　題

乳腺腫瘍（分子標的薬剤と乳癌化学療法）*

青儀健二郎**,***

Key Words：molecular target drug, trastuzumab, bevacizumab, lapatinib

はじめに

原発性乳癌の治療は，一般的には全身療法である薬物療法と，局所療法である手術および放射線療法の組合わせで行われる．また，再発した症例に対しては薬物療法が主たる治療となる．薬物療法としては，有効性が高いアンスラサイクリン系薬剤とタキサン系薬剤の2剤を中心とした抗癌剤療法と，タモキシフェン，アロマターゼ阻害剤を中心とする内分泌療法があり，さらに新規薬剤の開発も日々進んでいる．そのような状況の中，分子標的薬剤は，癌に対する選択性が高く，副作用が重篤なものが少ないことから，薬剤療法の第3のカテゴリーとして，近年注目されている．まずヒト上皮増殖因子受容体2型(human epidermal growth factor receptor 2：HER2)受容体のマウス由来モノクローナル抗体であるトラスツズマブ(ハーセプチン®)が2001年HER2蛋白陽性の進行再発乳癌に対して，本邦で初めて承認された．さらにトラスツズマブは，術後補助療法における有効性を示す報告もなされている．さらに進行再発乳癌に対する新規分子標的薬剤として抗血管内皮増殖因子(vascular endotherial growth factor：VEGF)モノクローナル抗体であるベバシツマブ，ヒト上皮増殖因子受容体1型(epidermal growth factor receptor 1：EGFR1)受容体およびHER2受容体のチロシンキナーゼ阻害剤であるラパチニブの臨床試験の結果も続々報告されている．これらの報告から，種々の分子標的薬剤の最近のエビデンスについて述べてみたい．

再発乳癌に対する分子標的薬剤のエビデンス

1．トラスツズマブ

トラスツズマブは増殖因子受容体ファミリーの一つであるHER2受容体のマウス由来モノクローナル抗体である．Slamonら[1]は，HER2受容体の過剰発現のある転移性乳癌患者に対して，化学療法単独(アンスラサイクリン＋サイクロフォスファミド，パクリタキセル)とトラスツズマブ＋化学療法とを比較する第III相臨床試験において，time to progression(TTP)，overall servival(OS)の延長，奏効率の改善が認められたと報告している．ただし，アンスラサイクリン系薬剤との併用群においては，心毒性の発生率が，パクリタキセルとの併用群，トラスツズマブ単独群と比較し，高いとされた．他の臨床試験でもパクリタキセルとの併用は奏効率52～67％と報告された[2][3]．またドセタキセルとトラスツズマブとの併用においても，ドセタキセル単独群に比べ，TTP，OS，奏効率において優れていた[4]．さらに，タキサン以外に，ナベルビンなどとの併用において，68～75％と高い奏効率が報告されてお

* Biological therapy for breast cancer.

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表1　乳癌領域における分子標的薬剤開発の現状

	一般名	製品名	開発状況	開発企業
抗HER2抗体	Trastuzumab	Herceptin®	米国，日本，その他の国で承認済	Chugai/Genentech/Rosche
	Pertuzumab	Omnitarg®	Phase II	Chugai/Genentech/Rosche
	Ertumaxomab	Rexomun®	Phase II	Trion Pharma
VEGFRチロシンキナーゼ阻害薬	Sorafenib	Nexavar®	米国で承認(腎細胞癌)，(P II)	Bayer/Onyx
	Sunitinib	Sutent®	米国で承認(腎細胞癌，消化管間質腫瘍)，(P II)	Pfizer
抗VEGF抗体	Bevacizumab	Avastin®	米国，日本，その他の国で承認(大腸癌)，(P III)	Chugai/Genentech/Rosche
EGFRチロシンキナーゼ阻害薬	Gefitinib	Iressa®	米国，日本，その他の国で承認(非小細胞肺癌)，(P II)	AstraZeneca
	Erlotinib	Tarceva®	米国で承認(非小細胞肺癌)，(P II)	Chugai/Roche/Genentech/OSI
	Lapatinib	Tykerb®	Phase III	GlaxoSmithKline
その他の細胞内シグナル伝達阻害薬				
ファルネシルトランスフェラーゼ阻害薬	Lonafamib	Sarasar®	Phase II	Schering-Plough
mTOR阻害薬	Everolimus	Certican®	Phase II	Novartis
MEK阻害剤	PD-0325901	−	Phase I, II	Pfizer

(http://clinicaltrials.gov/ct/gui, J Clin Oncol 2005；23：5386-403より引用)

り[5][6]，これらの薬剤は一般臨床でもトラスツズマブと併用で用いられている．しかし，どの組合わせがもっとも有効であるかを検証した第III相試験は行われていない．

現在，新規抗HER2抗体薬剤としてトラスツズマブ耐性症例に対するトラスツズマブとpertuzumabの併用や，抗HER2抗体と同時に抗CD抗体でもあるertumaxomabの第II相試験が進行中である(表1)．

2．ベバシツマブ

腫瘍細胞の増殖・進展に重要な役割を果たす血管新生を促進する因子の一つにVEGFがある．このうち一つのサブタイプであるVEGF-Aを認識するヒト化モノクローナル抗体であるベバシツマブは転移性大腸癌において有効性が証明されている[7]．そのベバシツマブの再発乳癌における有効性を示したのは，E2100試験[8]である．転移性乳癌患者に対し，パクリタキセル単独治療(90mg/m², d1, d8, d15/ 28日間)群(339例)と，パクリタキセル＋ベバシツマブ(10mg/kg, d1, d15)併用治療群(341例)の2群を比較する第III相試験となっている．プライマリーエンドポイントであるProgression-free survival(PFS)は，パクリタキセル単独群に比べ，パクリタキセル＋ベバシツマブ併用群は，ハザード比で0.51(0.43-0.62, $P<0.0001$)と有意に良好であった(図1)．奏効割合は全患者で，パクリタキセル単独群13.8％であったのに対し，併用群で29.9％，計測可能病変をもつ患者では，単独群で16％，併用群で37.7％と，有意に良好であった($P<0.0001$)．しかしOSでは2群間に有意差はなく(ハザード比0.84, $P=0.12$)，有害事象においてGrade 3以上の全身倦怠感($P=0.05$)，高血圧($P<0.0001$)が併用群で認められている．今後OS，有害事象に関して長期的な経過観察が必要である．現在，本邦においても同様のデザインで第II相試験が行われている．

またMillerら[9]は，アンスラサイクリンおよびタキサン既治療の転移性乳癌患者において，カペシタビン単独療法群(2500mg/m², d1-14q3wks)(230例)とベバシツマブ(15mg/kg, d1)＋カペシタビン併用療法群(232例)の2群比較を行った第III相比較試験の結果を報告している．ベバシツ

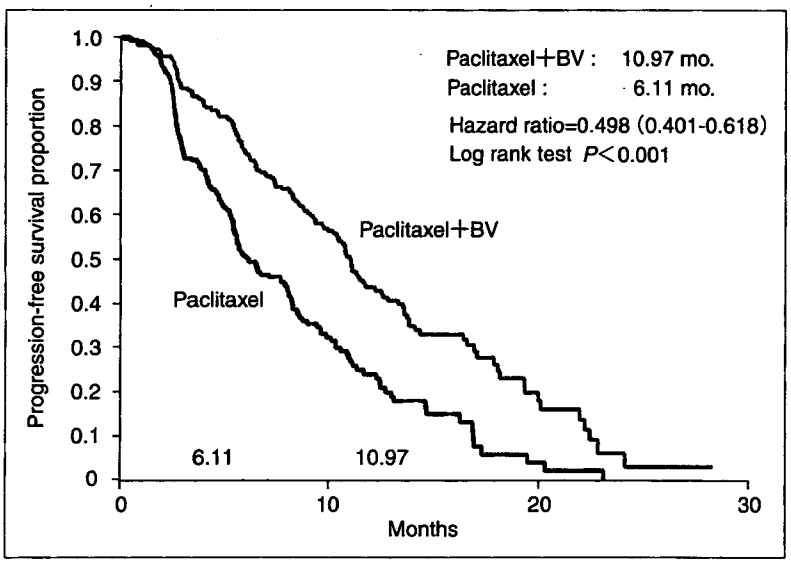

図1 E2100試験
BV：ベバシツマブ

マブ併用により奏効割合は増加したものの(19.8 % vs 9.1%, P=0.001), PFS(プライマリーエンドポイント)(4.86月 vs 4.17月, ハザード比0.98), OS(15.1月 vs 14.5 月)において差は認められなかった. 本邦においてもベバシツマブとパクリタキセル併用の第II相試験が進行中である.

3. ラパチニブ

最近, EGFR1およびHER2受容体のチロシンキナーゼ阻害剤である低分子化合物ラパチニブの有効性がGeyerら[10]により報告されている. これは, アンスラサイクリン, タキサン, トラスツズマブ治療後のHER2陽性転移性乳癌症例に対して, カペシタビンとの併用群(ラパチニブ; 1250mg/日連日投与, カペシタビン; 2000mg/m², d1-14q3wks, 163例)がカペシタビン単独群(2500mg/m²/日, d1-14q3wks, 161例)に比べ, 無増悪期間(time to progression; プライマリーエンドポイント)のハザード比で0.49(中央値8.4月 vs 4.4月, P<0.001)(図2), PFSはハザード比で0.59(P=0.002)と有意に併用群が良好な結果であったが, OSはハザード比で0.92(P=0.72)と有意な差は認められなかった. ラパチニブは少ない心毒性, 血液脳関門を通過しての脳転移巣への効果の可能性など, トラスツズマブにはない有用性で注目されている.

術後補助療法における分子標的薬剤療法のエビデンス

1. トラスツズマブ

HER2陽性乳癌患者に対するトラスツズマブの術後補助療法における有用性を示す臨床試験の解析結果, すなわちNational Surgical Adjuvant Breast and Bowel Project(NSABP) B-31とIntergroup Trial N 9831の共同解析[11], Herceptin Adjuvant(HERA) Trialの解析[12][13], またBreast Cancer International Research Group(BCIRG)006の解析結果が報告されている[14](図3).

NSABP B-31はリンパ節転移陽性(stageIIB-IIIA)のHER2陽性乳癌患者で, AC(anthracycline+cyclophosphamide, 60/600mg/m², 4回) followed by パクリタキセル(175mg/m² 3週ごと投与, 4回)群とAC followed by パクリタキセル+トラスツズマブ(毎週投与, 初回4 mg/kgで2回目以降は2 mg/kg, 計51回投与)群の2群間で比較しており, Intergroup Trial N 9831は同様の対象で, AC followed by パクリタキセル群, AC followed by パクリタキセル(80mg/m² 毎週投与, 12回)+トラスツズマブ追加投与群, AC followed by パクリタキセル+トラスツズマブ同時併用および追加投与群の3群比較になっている.

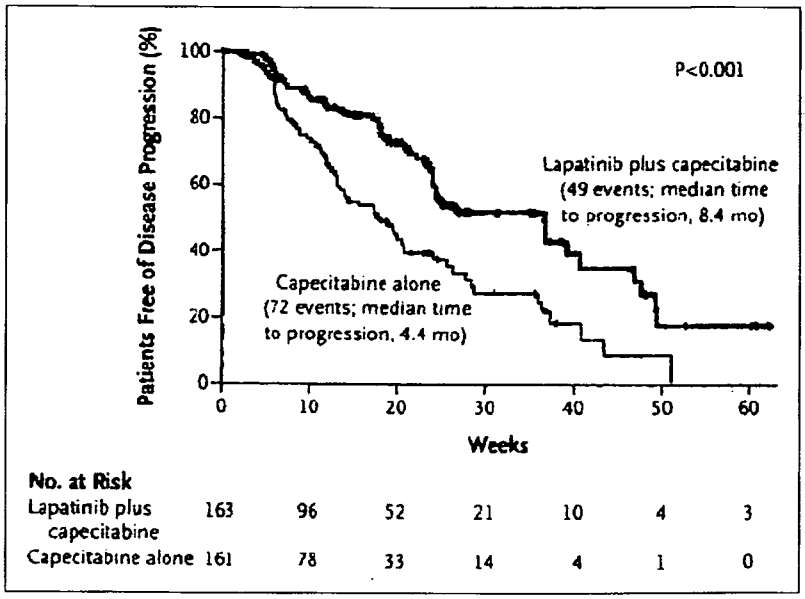

図2　ラパチニブ＋カペシタビン併用試験

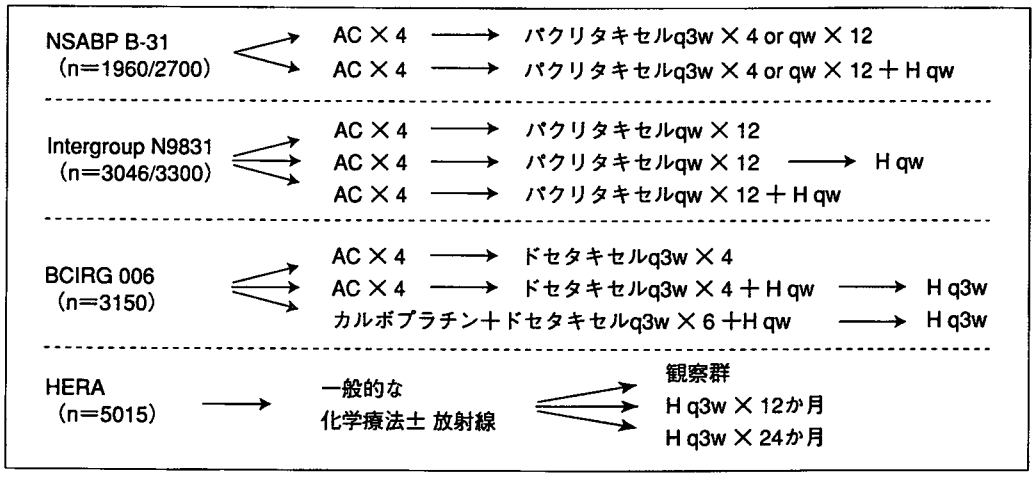

図3　ハーセプチンを用いた術後補助療法の臨床試験
H：ハーセプチン

今回2つの試験で同じ治療群をAC followed by パクリタキセル群(1,679人)対AC followed by パクリタキセル＋トラスツズマブ群(1,672人)の2群として統合解析した．プライマリーエンドポイントはdisease-free survival(DFS)，セカンダリーはOS, distant disease-free survival(DDFS)，などである．その結果，治療後3年のDFSでHR＝0.48, 2P＝3×10^{-12}，OSでHR＝0.67, 2P＝0.015, 無遠隔再発期間でHR＝0.47, $P<0.0001$，と有意にトラスツズマブを加えた群が良好であった．有害事象においては，トラスツズマブを加えた群におけるクラスIII, IVのうっ血性心不全の発現率が，B-31において4.1%，N 9831において2.9%であった．

HERA Trialは，通常の補助療法終了後，無治療群，トラスツズマブ1年治療群，トラスツズマブ2年治療群の3群比較で，5,090人が登録されたglobalな大規模試験である．トラスツズマブ

は初回8 mg/m²，2回目以降は6 mg/m²で3週ごと投与を行っている．2005年に報告されたデータ[13]では，中央観察期間1年で無治療群(1,693人)とトラスツズマブ1年治療群(1,694人)の2群間について解析されている．プライマリーエンドポイントはDFS，セカンダリーは，relapse-free survival(RFS)，DDFS，OS，有害事象である．2年目のDFSにおいて，トラスツズマブ1年治療群は無治療群に比べ，ハザード比0.54(0.43-0.67)(P<0.0001)と良好であった．RFSはハザード比0.50(0.40-0.63)(P<0.0001)，DDFSはハザード比0.51(0.40-0.66)(P<0.0001)であったが，OSにおいては有意差を認めなかった〔ハザード比0.76(0.47-1.23)(P<0.26)〕．有害事象においてトラスツズマブ1年治療群には心臓死はないものの，EF値減少が10 points以上でかつ左室駆出率50%未満であった症例が7.1%と無治療群が2.2%であったのに比べ高かった．OSに関しては，2007年の2年間追跡の報告では有意差が認められた[14]．

BCIRG006は，HER2陽性乳癌でリンパ節転移陽性，もしくは陰性であるが高リスクの術後患者に対して，AC(60/600mg/m²，4回)followed by ドセタキセル(100mg/m²，4回)治療(ACT)群(1,073人)，AC followed by ドセタキセル＋トラスツズマブ1年間併用群(ACTH)(1,074人)，ドセタキセル(75mg/m²)＋カルボプラチン(AUC6)(6回)＋トラスツズマブ1年間併用(TCH)群(1,075人)の3群比較を行っている．プライマリーエンドポイントはDFS，セカンダリーはOS，安全性，病理学的および分子学的マーカー変化である．中間解析では，DFSにおいてACTH群はACT群に比べハザード比で0.49(0.37-0.65)(P<0.0001)，TCH群はACT群に比べハザード比で0.61(0.47-0.79)(P=0.0002)と，2つのトラスツズマブ併用群において有意に良好な結果が示されたが，2つのトラスツズマブ併用群の間には差は認められなかった．OSは解析されておらず，有害事象においてはGrade 3以上の心血管系の事象(左室機能不全，虚血性心疾患，不整脈)の発生頻度は，ACT群0.95%，ACTH群2.34%，TCH群1.33%で，ACT群とACTH群の間では有意差が認められた(P=0.016)．

これらの試験では，各種event-free survivalにおいてトラスツズマブの有効性が示されているものの，トラスツズマブ併用による心臓関連有害事象の長期的な評価は必須である．また今後HERA studyにおいて，トラスツズマブ1年治療群と2年治療群の比較解析が2008年に行われるが，トラスツズマブの至適投与期間を探るため，その解析結果は重要である．また，トラスツズマブの至適投与法(抗癌剤との併用か，追加投与か)も明らかになると思われる．

2．ラパチニブ

ラパチニブの術後補助療法としての意義の検討は次の課題である．現在グローバル試験として行われているのは，ALTTO試験(Adjuvant lapatinib and/or trastuzumab treatment optimization trial)と呼ばれるものである．これは，乳癌術後に1年間，ラパチニブ単独投与，トラスツズマブ単独投与，両剤逐次投与，および両剤併用投与の4群を比較する第III相試験である．ラパチニブの術後補助療法における意義を明らかにする試験として期待されている．

術前化学療法における分子標的薬剤療法

Buzdarら[15]により，HER2陽性手術可能な乳癌患者に対して，術前化学療法(FEC＋Paclitaxel)にトラスツズマブを加えることにより，pCRが化学療法のみに比べて，大幅に改善した(25%から66.7%)ことが報告されている．心毒性の明らかな増加は報告されていないが，アンスラサイクリン系薬剤との併用が含まれているため，細心の注意が必要であろう．トラスツズマブの術前投与における適応はないが，本邦におけるHER2陽性手術可能乳癌症例に対するトラスツズマブを用いた術前化学療法(術後も含む)の臨床試験として，厚生労働省科学研究費研究班(安藤班)による医師主導治験があり，平成19年4月から登録を開始した．また，ラパチニブを使った術前療法(Neo-ALTTO試験)では，HER2陽性の局所進行乳癌症例に対して，パクリタキセルとラパチニブ，トラスツズマブ，および両剤の併用を組合わせる3群を比較する第III相試験があり，新たな知見が期待できる．

分子標的薬剤療法の今後と問題点

さまざまな作用機序をもった分子標的薬剤の臨床試験の結果について述べてきたが，分子標的薬剤の効果はpromisingなものであろう．今後，本邦の乳癌領域において，米国において進行性腎細胞癌やイマチニブを使用できない消化管間質腫瘍において承認されている，血小板由来増殖因子受容体および血管内皮増殖因子受容体からのシグナル伝達系阻害剤スニチニブ開発のため第III相試験なども計画されている．

しかし，今後は長期の観察期間において，分子標的薬剤の有害事象を的確にフォローアップし，また，これらの薬剤同士の併用や，適切な化学療法剤，ホルモン剤との組合わせを検討していく必要がある．また分子標的薬剤は高価であり，今後，効果規定・予測因子を評価しながら治療法を選択するなど，不適正な使用を監視する必要もあると思われる．

おわりに

分子標的薬剤の開発の現状について概略を述べた．乳癌に苦しむ患者のためには福音となるが，高価な薬剤でもあり，安全性も含め，適切な使用法を追及する努力が必要であろう．

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3. 転移性乳癌の治療

(1) ホルモン療法，化学療法，トラスツズマブ；治療法選択のための基本原則

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転移性乳癌の治療においては，Hortobagyiの提唱したアルゴリズムが，広く支持されている[1]。ホルモン感受性があり，生命を脅かす(life-threatening)ものでない転移巣をもつ症例は，まずホルモン療法から治療を開始し，それが無効である場合に，化学療法に移行するというものである。これは転移性乳癌が治癒困難であり，その治療が症状緩和や延命を目指すためである。しかし，新規抗癌剤，分子標的薬剤が出てきている昨今では，転移性乳癌の治療成績の向上が見込めるため，現状にそぐわない感がある。実際，トラスツズマブ(ハーセプチン®)を取り込んだアルゴリズムも作成されており，これに従いながら治療すべきである(図1)。

ホルモン療法

転移性乳癌に対するホルモン療法は，奏効率は化学療法に比べて低いものの，奏効期間が長く，有害事象も少ない[2]。奏効率は，エストロゲンレセプター，プロゲステロンレセプターともに陽性の場合は約60%，どちらかが陽性なら約30%，ともに陰性なら10%以下であるとされる[3]。ただし，更年期障害様の症状，心血管系，骨，子宮内膜への影響など，ホルモン製剤特有の有害事象には十分留意すべきである。具体的なホルモン療法としては，閉経前患者には，LH-RHアナログ＋タモキシフェン，合成黄体ホルモン薬(メドロキシプロゲステロン)の順に，閉経後患者には，タモキシフェンもしくはアロマターゼ阻害薬(アナストロゾール，レトロゾール，エキセメスタン)，メドロキシプロゲステロンの順に試みる。アロマターゼ阻害薬3剤の使用順に関しては，決定的なエビデンスはなく，大規模臨床試験による今後の検討を待ちたい。

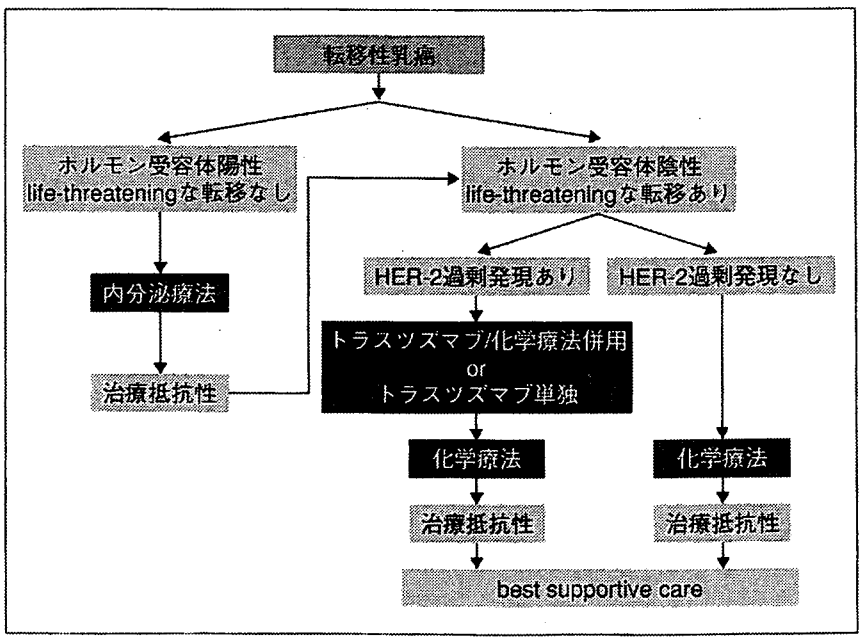

図1　転移性乳癌の治療アルゴリズム

化学療法

転移性乳癌に対する化学療法であるが，一次，二次治療としては，アンスラサイクリン系およびタキサン系薬剤を用いることでコンセンサスは得られており，わが国の乳癌診療ガイドラインにおいても推奨グレードAとされる[4]。転移性乳癌に対する一次治療としての奏効率は，ドキソルビシン：40～80%，エピルビシン：30～70%，パクリタキセル：19～67%，ドセタキセル：25～53%である[3)5)]。しかし，三次治療以降は決まったものはないために，新規抗

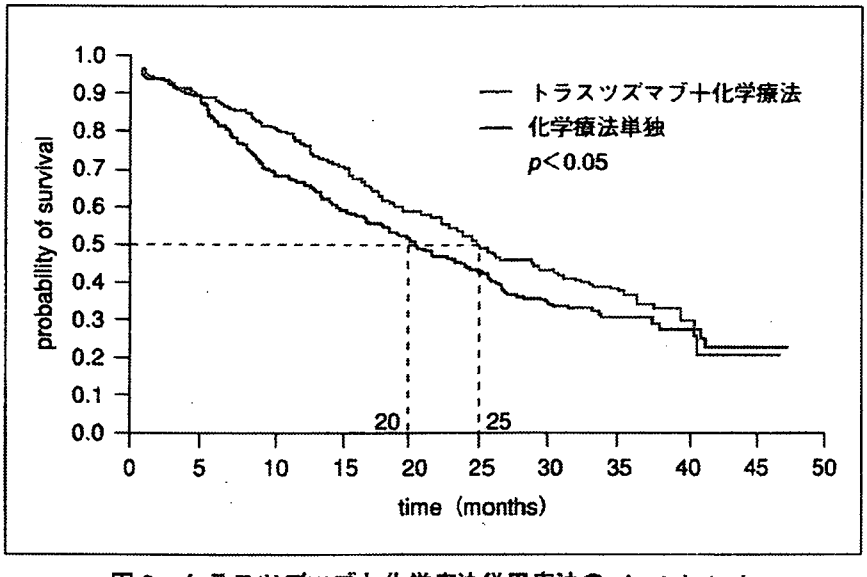

図2 トラスツズマブと化学療法併用療法の pivotal study

✓HER-2(+)転移性乳癌：
パクリタキセル±カルボプラチン
ドセタキセル±カルボプラチン } +トラスツズマブ
ビノレルビン

図3 NCCN Practice Guidelines in Oncology (v.2.2006)

癌剤を含めた治療体系の確立が模索されている。経口フッ化ピリミジン系抗癌剤(カペシタビン，S-1，UFT，5'-DFURなど)，ナベルビン，タキサン系の未使用薬剤が現在広く使われている。経口フッ化ピリミジン系抗癌剤についてはエビデンスが十分でないものが多いが，三次治療としての奏効率は15～30%程度であり，海外でのアンスラサイクリン系およびタキサン系薬剤治療例に対する第3相試験の標準治療群として設定されることが多い。

分子標的薬剤

分子標的薬剤として日常診療においてもHER-2受容体陽性(ハーセプテスト3+，もしくはFISH陽性)の進行再発乳癌症例に対して用いられるトラスツズマブは，化学療法との併用においてoverall survivalを延長する結果が報告されている[6](図2)。有害事象も心毒性，infusion reactionなどがあるが，患者のquality of lifeを維持できる治療である。その適応は，HER-2受容体蛋白を評価する免疫組織学的検査(immunohistochemistry；IHC)と，受容体をDNAレベルで評価するfluorescence *in situ* hybridization (FISH)を用いて決定する。高価な薬剤でもあり，適正に治療を決める必要がある。

今後の展開

転移性乳癌治療の3本柱であるホルモン療法，化学療法，分子標的薬剤療法の間のinteractionについて種々の検討がされている。HER-2陽性乳癌においてはアロマターゼ阻害薬の有効性が示唆されている。またトラスツズマブと抗癌剤の有効な組み合わせがガイドライン[7]において提示されている(図3)。この分野の研究の発展が期待される。

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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.

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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Tomo Suga and Atsuko Ishikawa contributed equally to this work.

Conflict of interest: none.

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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

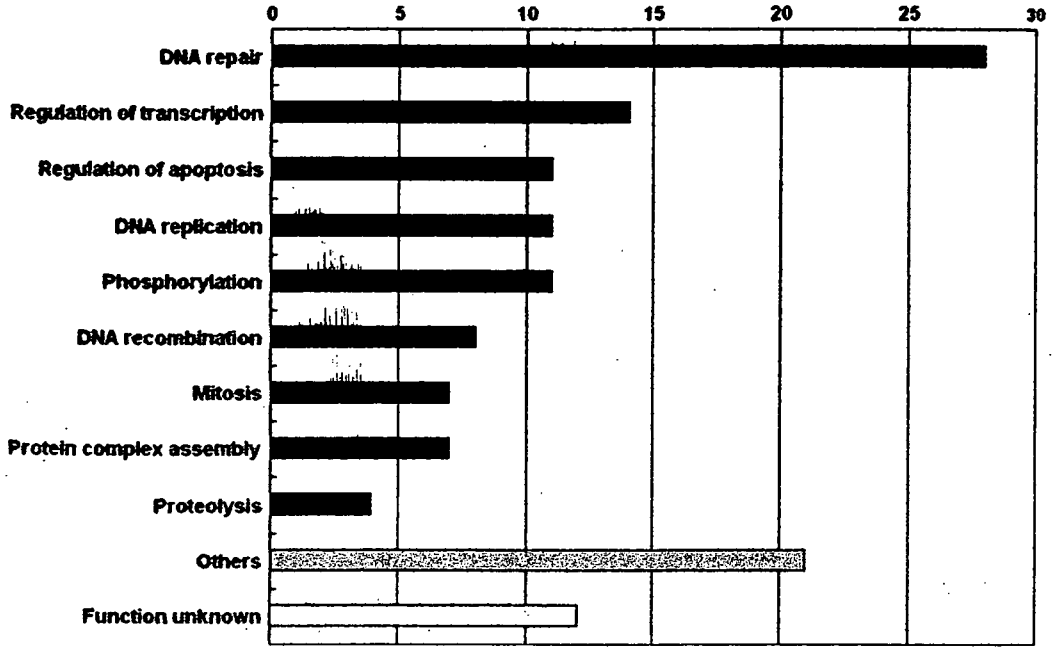

Fig. 1. Ontologic classification of candidate genes for typing. Total of 137 candidate genes assigned to biologic categories by Gene Ontology database at hierarchy level 7 (59). The axis indicates the number of genes. Some genes were categorized into multiple classes. Genes indicated as "function unknown" 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		Recessiv	
ıe	Chr	Mm	HG (n = 218)	LG (n = 580)	p	OR (95% CI)	HG (n = 109)	LG (n = 290)	p	OR (95% CI)	p	OI
2L2	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
3R3	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
3R3	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
L2	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
1	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
3	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
17	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
i1	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
i1	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
i1	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
3K7	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
iL	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
iL	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
)	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
1A	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
	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
)A	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
)A	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
3	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
	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
iL1	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
iL1	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
21	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
	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
T	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

SNP = reference single nucleotide polymorphism; ID = identifier; LG = low grade; HG = high grade; Chr = chromosome; M = major allele; m = minor allele; OR erval; NC = insufficient sample size to perform calculation.
are 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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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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A B S T R A C T

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.