test until robust confirmatory data are available from adequately powered prospective trials [72,73].

Recently, as a result of meta-analysis on data from 4973 tamoxifen-treated patients, the International Tamoxifen Pharmacogenomics Consortium (12 globally distributed sites) reported that CYP2D6 poor metabolizer status was associated with poorer invasive DFS using strict inclusion criteria (IDFS: HR: 1.25; 95% CI: 1.06, 1.47; p = 0.009) [17]. The potential role of *CYP2D6* genotype assessment in determining if the patients with ER-positive breast cancer should receive tamoxifen is still controversial. Prospective studies are necessary to establish if genotype-guided personalized tamoxifen therapy improves clinical outcomes of the patients with ER-positive breast cancer [17].

Dose-adjustment study of tamoxifen based on *CYP2D6* genotypes

The breast cancer patients who are heterozygous and homozygous for decreased function and null alleles of *CYP2D6* are reported to show lower plasma concentrations of endoxifen and 4-hydroxytamoxifen compared with patients with homozygous wild-type allele [27], resulting in worse clinical outcome in tamoxifen therapy. Kiyotani *et al.* reported tamoxifen dose adjustment study using 98 Japanese breast cancer patients, who had been taking 20 mg of tamoxifen daily as adjuvant setting [19]. In their study, dosages of tamoxifen were increased to 30 and 40 mg/day for the patients who have one or no normal allele of *CYP2D6*, respectively. In the patients with *CYP2D6*1/*10* and *CYP2D6*10/*10*, the plasma endoxifen levels after dose increase were 1.4- and 1.7-fold higher, respectively, than those before the increase ($p < 0.001$) [19]. These plasma concentrations of endoxifen achieved similar level of those in the *CYP2D6* wild-type patients receiving 20 mg/day of tamoxifen. In addition, they showed that the incidence of adverse events was not significantly different between before and after dose adjustment, and concluded that their study provided the evidence that dose adjustment could be useful for the patients carrying *CYP2D6*10* allele to maintain the effective endoxifen level. Similar genotype-guided tamoxifen dosing study was reported [9,20]. Irvin *et al.* also showed the similar results, and the feasibility of genotype-driven tamoxifen dosing and demonstrates that doubling the tamoxifen dose can increase endoxifen concentrations in IM and PM patients [9].

Possible genetic markers for clinical response to tamoxifen

As shown in Figure 1, UGTs, SULTs and the other CYPs are involved in the metabolism of tamoxifen. Some reports suggest that genetic variations in these genes may affect the efficacy or toxicity of tamoxifen therapy [14,22,27,76–79]. Several genetic polymorphisms are reported in *SULT1A1*, and some investigations on *SULT1A1*2*, which causes decreased SULT1A1 activity, failed to find association with tamoxifen efficacy [23,80]. Genetic polymorphisms in the *CYP3A4* have been reported, however, their contribution to influence the tamoxifen metabolism might be small because of their low allelic frequencies. On the other hand, *CYP3A5*3* allele is known to influence to CYP3A5 expression level [81]. Several studies investigated the association of *CYP3A5*3* with tamoxifen metabolism or clinical outcome of tamoxifen therapy, however, none of them report their significant association [27,74–76,81–83]. *CYP2C19*2* and *CYP2C19*3* are known to be null allele, and *CYP2C19*17*, which is recently identified genetic variation and located in promoter region of this gene, is associated with increased CYP2C19 activity (UM phenotype) [77,78]. The significant association with clinical outcome after tamoxifen treatment was found in *CYP2C19*17* carriers, but not in *CYP2C19*2* nor **3* carriers [77,78]. ABCC2 plays an important role in the biliary excretion of conjugated drugs and xenobiotics [84,85]. Tamoxifen and its metabolites are excreted into the biliary tract in liver as glucuronides or sulfates [86]. In a recent study, an intronic SNP in *ABCC2* was found to be significantly associated with the clinical outcome of breast cancer patients treated with tamoxifen, however, this SNP was not associated with plasma concentration of endoxifen or other metabolites [14]. This suggests that the contribution of ABCC2 to biliary excretion of tamoxifen and its metabolites might be limited. A genome-wide association study for clinical outcome of the breast cancer patients treated with tamoxifen was reported [79]. In this study, 240 patients were analyzed by genome-wide genotyping, and 105 and 117 cases were used for replication studies as independent cohorts, respectively. Out of 15 SNPs which showed significant associations with recurrence-free survival in genome-wide association study stage, rs10509373 in *C10orf11* gene on 10q22 was significantly associated with tamoxifen efficacy in the two independent replication stages [79]. Although further validation studies and functional analysis would be required to verify their results, *C10orf11* could be a promising genetic marker to predict the clinical outcomes of patients receiving tamoxifen therapy [79].

Conclusion

There have been several reports on the association between *CYP2D6* genotype and clinical outcome or tamoxifen metabolism in breast cancer patients treated with tamoxifen. The results of the association studies

Table 2. Studies evaluating association of *CYP2D6* genotype with response to tamoxifen therapy.

Study findings	Studies	n	Ratio of monotherapy (%)	Tamoxifen dose and duration	Outcome[†]	HR (95% CI)	p-value	Ref.
Positive	Goetz *et al.*	190	–	20 mg/day for 5 years	DFS	2.44 (1.22–4.90)	0.012	[74]
	Goetz *et al.*	180	100	20 mg/day for 5 years	RFS	2.69 (1.34–5.37)	0.005	[15]
	Schroth *et al.*	206	100	–	RFS	2.24 (1.16–4.33)	0.02	[75]
	Newman *et al.*	115	63.5	20 mg/day, median duration >4 years	RFS	1.9 (0.8–4.8)	0.19	[58]
	Kiyotani *et al.*	58	100	20 mg/day for 5 years	RFS	8.67 (1.06–71.09)	0.044	[12]
	Xu *et al.*	152	100	–	DFS	4.7 (1.1–20.0)	0.04	[54]
	Schroth *et al.*	1325	100	For 5 years	RFS	2.12 (1.28–3.50)	0.003	[16]
	Kiyotani *et al.*	282	100	20 mg/day for 5 years	RFS	9.52 (2.79–32.45)	0.0032	[14]
	Ramon *et al.*	91	39.8	–	DFS		0.016	[55]
	Park *et al.*	110	21.80	20 mg/day, median duration 3.9 years	RFS	5.59 (0.93–33.5)	0.05	[67]
	Thompson *et al.*	542	100	20 mg/day for 5 years	RFS	1.52 (0.98–2.36)	0.06	[68]
	Teh *et al.*	95	–	20 mg/day	RFS	13.14 (1.54–109.9)	0.004	[59]
	Sirachainan *et al.*	39	100	–	DFS		0.036	[60]
	Damodaran *et al.*	132	6.80	For 5 years	RFS	7.15 (1.77–28.89)	0.006	[61]
	Goetz *et al.*	453	100	20 mg/day for 5 years	Disease event	2.45 (1.05–5.73)	0.04	[57]
	Province *et al.*	4973 (1996)	100	20 mg/day for 5 years	IDFS	1.25 (1.06–1.47)	0.009	[17]
Negative	Wegman *et al.*	76	50	40 mg/day for 2 years	RFS	<1.0[‡]	–	[53]
	Nowell *et al.*	160	14.2	Not reported	DFS	0.67 (0.33–1.35)	0.19	[22]
	Wegman *et al.*	103	–	40 mg/day for 2 years	RFS	0.87 (0.38–1.97)	0.74	[23]
		111	–	40 mg/day for 5 years	RFS	0.33 (0.08–1.43)	0.14	
	Okishiro *et al.*	173	42.2	20 mg/day, median 52 months	RFS	0.94 (0.34–2.60)	0.95	[62]
	Stingl *et al.*	493	58	20 mg/day	TTP	–	0.10	[63]
	Kiyotani *et al.*	167	0	20 mg/day for 5 years	RFS	0.64 (0.20–1.99)	0.44	[64]
	Abraham *et al.*	3155	48.4	20 mg/day	RFS	1.57 (0.64–3.84)	0.32	[56]
	Lash *et al.*	340	–	–	Disease event	1.3 (0.60–2.9)	0.88	[65]
	Park *et al.*	130	18.2	–	RFS	1.34 (0.42–4.28)	0.63	[66]
	Rae *et al.*	588	95.7	20 mg/day for 5 years	RFS	1.22 (0.76–1.96)	0.44	[69]
	Regan *et al.*	973	100	20 mg/day for 5 years	RFS	0.58 (0.28–1.21)	0.35	[21]

All reports were retrospective studies.
[†]RFS was defined as time from surgery or randomization to diagnosis of the recurrence of breast cancer (locoregional, distant metastasis and contralateral breast events). DFS was defined as time from surgery or randomization to diagnosis of the recurrence of breast cancer or death. IDFS specifically excludes all *in situ* cancer events (ipsilateral or contralateral DCIS, ipsilateral or contralateral LCIS and all *in situ* cancers of nonbreast sites).
[‡]Not calculated HR according to *CYP2D6* genotypes.
DFS: Disease-free survival; HR: Hazard ratio; IDFS: Invasive DFS; RFS: Recurrence-free survival.

of tamoxifen metabolism with *CYP2D6* genotype are consistent in most of the studies, however, the results of the association studies of tamoxifen efficacy with *CYP2D6* genotype are still controversial. Although there might be several reasons for these controversial results, well-designed prospective studies will clarify if *CYP2D6* genotype test could improve the outcomes of women with ER-positive breast cancer. Moreover, the combined genetic test of *CYP2D6* with a few predictive genetic markers may provide new insights into personalized selection of hormonal therapy for the patients with breast cancer. The potent CYP2D6 inhibitors including paroxetine should be avoided in the breast cancer patients receiving tamoxifen as alternative treatment should be available in most cases.

Future perspective

The dose-adjustment studies based on the *CYP2D6* genotypes showed that the increase of tamoxifen dose was able to increase the plasma endoxifen level, and expected to improve the prognosis of the tamoxifen-treated patients with reduced *CYP2D6* genotype [9,19]. A large-scale prospective study will clarify whether the dose-adjustment strategy could improve tamoxifen therapy in breast cancer patients. Although there are some discrepant reports questioning the association between *CYP2D6* genotype and clinical outcome after tamoxifen therapy, one of the largest meta-analysis performed by International Tamoxifen Pharmacogenomics Consortium reported that *CYP2D6* could be a strong predictor of invasive DFS using strict inclusion criteria (postmenopausal women with ER-positive breast cancer receiving 20 mg/day tamoxifen for 5 years). In either case, prospective studies are essential to finally conclude if genotype-guided selection of tamoxifen therapy improves clinical outcomes of women with ER-positive breast cancer. If the results will show the positive association of *CYP2D6* genotype with clinical outcome of tamoxifen-treated patients, US FDA may approve and recommend routine use of the *CYP2D6* genotype test for personalized tamoxifen therapy in adjuvant or metastatic breast cancer setting.

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Executive summary

- Tamoxifen treatment reduced the risk of breast cancer relapse for at least 15 years, particularly estrogen receptor positive invasive tumors in premenopausal women.
- CYP2D6 is known to be a key enzyme to generate one of the potent tamoxifen metabolites, endoxifen.
- Although there are some discrepant reports questioning the association between *CYP2D6* genotype and clinical outcome after tamoxifen therapy, the highest level of evidence to test the CYP2D6–tamoxifen hypothesis will come from larger scale prospective clinical trials.
- Combined analysis of newly identified genetic marker(s) with previously identified ones, *CYP2D6*, *ABCC2* and so on, might be useful to predict the clinical outcome of patients receiving tamoxifen therapy.

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Lecture

解　説

がんのオーダーメイド医療とファーマコゲノミクス*

前佛　均**

Key Words：genetic polymorphism, pharmacogenomics, precision medicine, Philadelphia-positive acute lymphoblastic leukemia(Ph⁺ALL), imatinib

はじめに

ゲノムとは「生命の設計図」であり，われわれの体はさまざまなタンパクによって健康が維持されているが，ゲノムの中にはこれらタンパクを作る情報の担い手として遺伝子が存在する．ある薬剤を服用した場合，必ずしも全員に効くわけでなく，しかも一部の患者に重い副作用が出現することがあるように，その効果や副作用には個人間で大きな違いがある．このような違いをこれまでは薬が効かない「体質」，薬に弱い「体質」などと理解してきたが，ゲノム研究が進み今やこの「体質」が遺伝暗号の個人差などで科学的に説明されつつある．個々の患者のゲノム情報を正確に理解した上で，効果的で副作用の少ない治療，つまり一人ひとりの体質に合わせた治療法「オーダーメイド医療」または「個別化医療」(英語ではpersonalized medicine, precision medicineなどと呼ばれる)が可能になってくるものと期待されている．

近年，ゲノム網羅的な遺伝子多型タイピングや全ゲノムシーケンス技術の急速な進歩により，薬の作用とゲノム情報を結びつけ特定の患者における薬剤反応性に関連する要因を見出し，一人ひとりにあった薬剤を適切に使い分けようとする“ファーマコゲノミクス研究”が世界的に推進されている．ファーマコゲノミクス研究成果に基づく抗がん剤の副作用予測の実用例としては，イリノテカンにおける白血球減少症の予測があり，これは，イリノテカンの主代謝酵素UGT1A1のプロモーター領域における2塩基の挿入多型(UGT1A1*28)を有する患者では代謝活性が低下した結果，白血球減少のリスクが高くなるというものである[1]．2005年，米国ではUGT1A1*28をホモ接合で有する患者では用量を少なくとも1レベル減量するよう添付文書に追記され，わが国でも2008年に添付文書が改訂された．このように，ゲノム情報を利用することで特定の患者における薬剤応答性に関連する要因を見出し，一人ひとりに最も適切な薬剤の選択が可能となり，より安全で適切な個別化がん治療が可能になるものと考えられる．

ファーマコゲノミクスによる最適ながん化学療法(ゲムシタビン)

現在多くの悪性腫瘍に対する治療薬として保険適用となっているゲムシタビンは骨髄抑制をはじめ，有害事象の発生頻度が決して少なくない薬剤であるが，その副作用発現を規定する遺伝的要因についてはいまだ十分に解明されていない．近年，ゲノム全体にわたる遺伝子多型をジェノタイピングする技術が進歩し，ゲノムワイド関連解析(genome-wide association study, GWAS；「ジーワス」と呼ばれる)という方法によりこれまで副作用との関連がまったく知られていなかった新たな副作用関連遺伝子を発見できるようになってきた．ゲムシタビンは細胞内でcytidine deaminase(CDA)などの酵素により代謝を受けることが知

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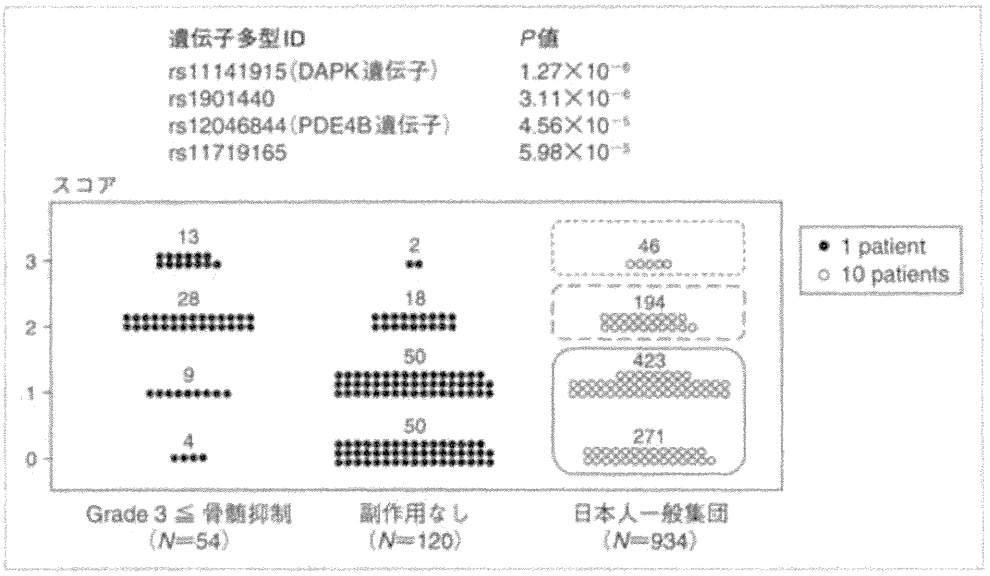

図1　4 SNPの遺伝子型に基づくゲムシタビン骨髄抑制予測システム
4つのSNPを用いてスコアリングを行うことで，grade 3以上の重篤な骨髄抑制をきたした症例では高スコア，副作用を認めなかった症例では低スコアを示した．一番右の群は日本人一般集団において予測されるスコアの分布．ゲムシタビン投与前にハイリスク(高スコア)であることが予測できれば，他剤による治療を優先して患者に提供することが可能になるものと考えられる．

られていることから，これらの既知遺伝子上の多型と副作用との関係を調べた報告は存在するものの，強い関連性を報告したものはない[2]．われわれはゲノム情報を用いた新たなゲムシタビン副作用予測診断法を開発することを目的にゲムシタビン単剤による抗腫瘍治療を受けた174症例を対象に解析を行った．174例中grade 3以上の白血球/好中球減少症をきたした54例をcase，副作用を示さなかった120例をcontrolとしcase-control studyを行った．解析は21例のcaseおよび58例のcontrolをGWAS(ゲノムワイド関連解析)に用い，33例のcaseおよび62例のcontrolをGWAS結果の再現性確認のためのreplication studyに用いた．

ゲノムワイド関連解析の結果をもとに，有意差上位100 SNPについて関連解析(replication study)を行ったところ，$P<0.05$を示す4 SNPが同定され，GWAS結果と組み合わせると，図1の上に示すように$P=1.27\times10^{-6}\sim5.98\times10^{-5}$という強い関連を示すことが明らかとなった．

さらにこの4 SNPを用いた骨髄抑制予測診断システムを構築するため，4つのSNPについて骨髄抑制リスクに働くと考えられるgenotypeをそれぞれ1点とし，各症例についてリスクgenotypeの合計点数別に骨髄抑制発現群(case)と副作用を認めなかった群(control)で分布を調べた結果が図1である．スコア0または1を示した113例のうち骨髄抑制群は11.5%，スコア2については60.9%，スコア3については86.7%を占めており，コントロール群に比べ有意に高いスコアを示すことが確認された(trend test $P=1.31\times10^{-11}$)．さらに日本人一般集団をこのスコアリングシステムにあてはめた際のスコア分布を検討した結果，図1の右側のような分布となることが明らかとなり，このスコアリングシステムをゲムシタビン治療開始前の患者に応用することで骨髄抑制の危険性を抑え，より安全かつ適切な治療選択に有用となる可能性が示された[3]．

フィラデルフィア染色体陽性ALLに対する個別化治療

成人急性リンパ性白血病は，一般的に予後不良であったが，その原因の一つが特に予後不良といわれている9番染色体と22番染色体の相互転座によって生じるPhiladelphia(Ph)染色体を有する急性リンパ性白血病がALL全体の1/3～

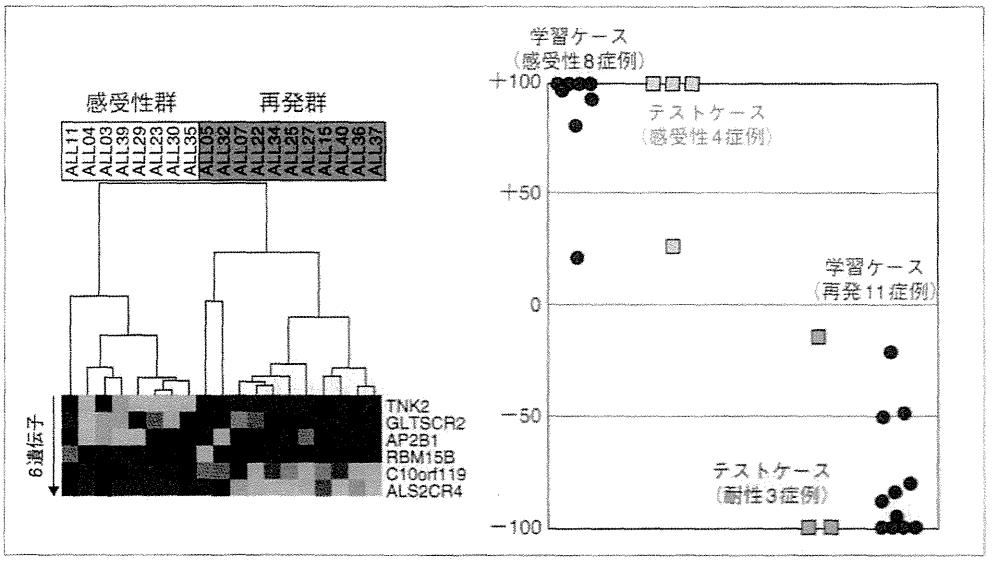

図2　Ph⁺ALL再発予測6遺伝子を用いたスコアリング

再発予測6遺伝子を用いたsupervised cluster解析の結果，感受性群・再発群が明瞭にクラスターされた(左)．また6遺伝子によるスコアリングの結果学習ケースでは，感受性の強い症例はプラスのスコア，再発をきたした症例はマイナスのスコアを示した．テストケースの7症例については，感受性を示した4症例は全例プラスのスコアを示し，耐性を示した3症例については，全例が再発群と同じマイナスのスコアを示した．

1/4を占めているためであった．われわれは，成人白血病の多施設共同研究グループJALSGとの共同研究として，Ph陽性ALL(Ph⁺ALL)に対して，イマチニブ併用化学療法(ALL202)を受けた症例を対象に網羅的な遺伝子発現情報解析により寛解導入後の血中bcr-abl再上昇を予測する診断システムの開発を検討した[4]．

解析対象症例の中で，寛解導入療法開始から63日までの間にbcr-abl値が検出限界以下になり，その後観察期間中再上昇しない8症例を「感受性群」，地固め療法までにbcr-abl値が検出限界以下となりその後再上昇した11症例を「再発群」，寛解導入不成功の3症例を「耐性群」と分類した．患者骨髄生検検体をサンプルとして単核球分画からTotal RNAを抽出し，マイクロアレイによる体系的遺伝子発現解析を行った．

網羅的遺伝子発現情報を用いてrandom permutation testを行い，感受性群と再発群の間で有意に発現量の異なる遺伝子をスクリーニングしたところ，$P<1\times10^{-3}$未満かつ片群で6割以上の症例で発現情報を有する16遺伝子を同定した．同定された16遺伝子を用いて，leave-one-out cross validation testを行ったところ，図2に示すように有意差上位6遺伝子を用いることで，治療感受性の強い症例はプラスのスコア，再発症例はマイナスのスコアを示し，スコアリング結果が最も明瞭に分離することが判明した．さらに，スコアが未知である7症例をこの6遺伝子を用いてスコア化すると，感受性を示した4症例はすべてプラスのスコアを示し，治療耐性を示した3症例については全例が再発群と同じようにマイナスのスコアを示すことが明らかとなった．これらの結果はReal Time RT-PCRでも再現性が確認されており，Ph⁺ALLに対するイマチニブ併用化学療法に対する感受性を診断するコンパニオン診断法としての有用性が期待されている[4]．

乳がん個別化内分泌治療を目指したファーマコゲノミクス研究

1．*CYP2D6*遺伝子多型とタモキシフェンの体内動態および治療効果

タモキシフェンは内服後，肝臓で代謝を受けることによってホルモンレセプター陽性乳がんに対し抗腫瘍効果を発揮することが以前より知られている[5]．タモキシフェンは肝臓で主にCytochrome P450 2D6(CYP2D6)により活性代謝物

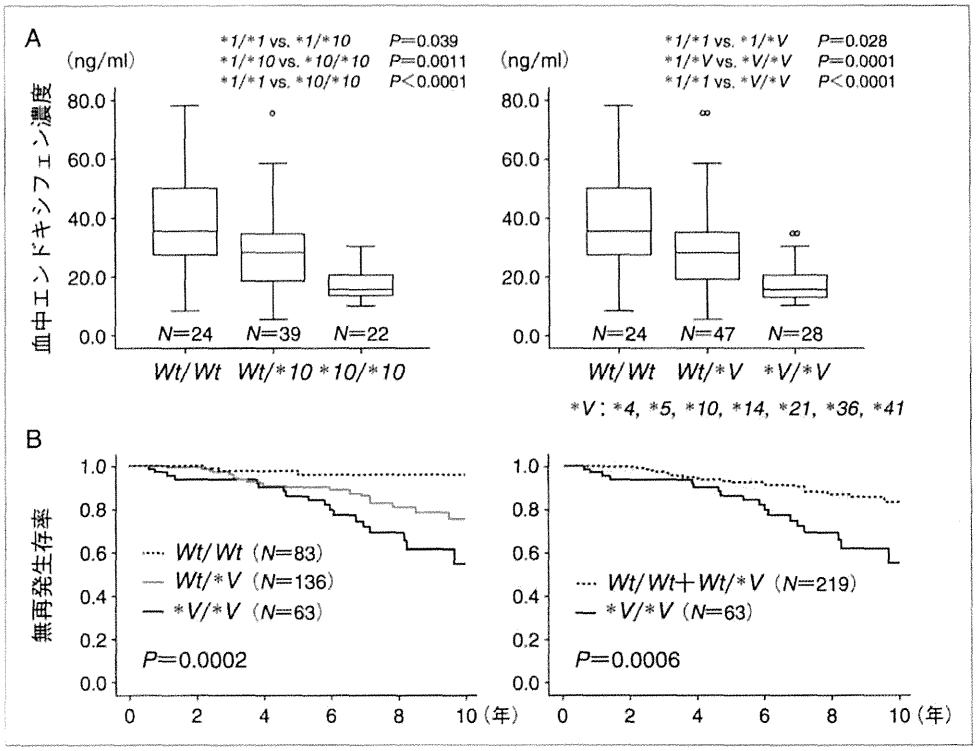

図3　*CYP2D6*遺伝子型とタモキシフェン活性本体（エンドキシフェン）の血中濃度と治療効果の関係
A：左は日本人に最も多い*CYP2D6*遺伝子多型（*10；活性減弱型）と血中エンドキシフェン濃度の関係．右は酵素活性低下または消失型のアレルを*Vと分類した場合の結果．酵素活性が弱いまたは消失するアレルを持つ症例では，血中エンドキシフェン濃度が有意に低下することがわかる．B：遺伝子型の違いと，タモキシフェン治療後の無再発生存期間の関係を示す．最も酵素活性の低い*CYP2D6* *V/*Vの症例群が最も再発率が高い結果となった．

「エンドキシフェン」に変換され乳がん細胞に対し抗腫瘍効果を発揮することがわかっている．つまり，このCYP2D6の酵素活性の強弱がタモキシフェンによる治療効果に影響を与える可能性があるのではないかと考えられてきた[6]．一方*CYP2D6*は酵素活性に変化を与える（活性を下げる，消失させるなど）遺伝子多型が多数存在することが知られている．われわれは，*CYP2D6**4, *5, *10などの酵素活性低下または消失型のアレルを"*V"と分類して，*CYP2D6*の遺伝子型とタモキシフェンのPK（薬物体内動態）の関係を検討した（図3-A）[7]．*CYP2D6*が正常型のホモ（Wt/Wt）や正常型と*10あるいは変異型のヘテロ（Wt/*10, Wt/*V）の症例に比べて，*10あるいは変異型のホモ（*10/*10, *V/*V）の症例においてタモキシフェン代謝産物であるエンドキシフェンの血中濃度が有意に低下していることがわかる．さらにわれわれは*CYP2D6*の遺伝子型がタモキシフェンによる治療効果に与える影響を検討した[8]．浸潤性乳がんに対し根治手術が行われ，術後にタモキシフェン単剤治療を5年間行った282症例を対象とし，*CYP2D6*の遺伝子型がタモキシフェン治療効果に与える影響を解析した結果が図3-Bである．正常型をホモで持つ症例（Wt/Wt）や正常型と変異型（活性低下または消失型）のヘテロ（Wt/*V）の症例に比べ，変異型をホモ（*V/*V）で持つ症例では無再発生存率が有意に低下していることが確認された（$P=0.0002$）．このように，*CYP2D6*の遺伝子多型がタモキシフェンによる治療効果に影響を与えているということが明らかとなりつつある[9]．

2．*CYP2D6*遺伝子-タモキシフェン研究の現状

2015年1月現在，CYP2D6-タモキシフェン研究に関する論文は370例以上にのぼるが，われわれと同様に関連を認めたとする報告と，認

めなかったとする報告がおよそ半々の状況である[10)~13)]．2010年のサンアントニオ乳がん会議において，関連を認めなかったとする報告やBIG 1-98やATACなどの比較的大きな規模の臨床試験に参加した症例を用いた検討において，関連を示さなかったことから[12)13)]，2010～2013年の間は“CYP2D6はタモキシフェン治療効果マーカーとして臨床応用することは難しい”という意見が広まった．

しかしその後，議論が分かれている原因を究明する報告が続出し[14)~18)]，CYP2D6-タモキシフェンに関連が認められない原因として，主に次の3要因があげられている．①患者の肝細胞におけるCYP2D6の機能を調べるためには，germline mutationを調べる必要があるにもかかわらず，多くの研究で乳がん細胞からDNAを抽出している．乳がん細胞では*CYP2D6*の存在する22番染色体のloss of heterozygosity（LOH；ヘテロ接合性の欠失）が高頻度（30～40%）で生じているため，患者さんの正常肝における*CYP2D6* genotypeを反映しているとはいえない．実際にいくつかの論文では，遺伝学的にあり得ないようなgenotype頻度データが掲載されており，misgenotypeの可能性が非常に高い結果が散見される[14)]．②エンドポイントとして多くの研究で術後タモキシフェン治療後の無再発生存を用いているが，タモキシフェン単剤治療症例だけではなく，治療予後に大きな影響を与える治療（強力な化学療法など）も併用されている症例が混在しているためCYP2D6のタモキシフェン治療効果に対する影響を検討することは困難である．③Germline mutationを正確に調べるためには，患者血液などから抽出した良質なゲノムDNAを用いて，必要かつ十分なCYP2D6多型を調べなくてはならないが，パラフィン包埋組織などから抽出した質の低下したDNAを用いているため，CYP2D6*5（遺伝子そのものが欠損）を中心に，調べなくてはならないCYP2D6多型を測定していない（できていない）報告が多い[18)]．

最近，日米独韓など12施設からなる国際タモキシフェン・ファーマコゲノミクス・コンソーシアム（ITPC）により集積された4,973例を用いたCYP2D6-タモキシフェンの詳細なメタ解析の結果，解析対象症例の閉経，エストロゲン受容体発現，投与量，投与期間を厳格に規定したところ，強い関連を認めたことが報告された[19)]．さらにわれわれが行っているCYP2D6-タモキシフェンの関係を解明するための多施設共同前向き研究（C-GENT study）の中間解析の結果，有意な関連を示す結果が得られている．公表されている論文のすべてが，適切な研究デザインのもと良質なデータを用いた解析結果を示しているとは限らず，わが国における診療ガイドライン作成・改訂にあたり，エビデンスとして論文を評価する際には，十分な専門的知識を有する基礎・臨床医による討議と慎重な検討が必要である．

これからのファーマコゲノミクス研究

高速SNPタイピング技術や次世代シーケンサーの技術開発に伴い，生殖細胞系列（germline）および体細胞系列（somatic）における網羅的ヒトゲノム情報が短時間で安価に入手可能となった現在，ゲノム情報を利用した研究が急速に進歩し，ファーマコゲノミクス研究基盤が世界的に整備されつつある．近い将来抗がん剤をはじめ薬剤の副作用による被害や無効な薬剤投与によるがんの再発・病状の悪化は“体質だから仕方ありません”では片付けられない問題になるものと考えられる．つまり薬剤に対する反応性を投与前に予測可能にし（コンパニオン診断），不幸を避ける方向へ医療を変えていく必要に迫られている．すでに米国では生殖細胞系列（血液）および体細胞系列（がん組織）におけるゲノム情報を医療に取り入れ，より適切な治療法の選択に用いる試みが急速に拡大し，日常診療の中の一つの判断基準として根付きつつある．わが国においても，薬剤を「より有効に，より安全に」という患者にとって当然の願いを早く実現するためには，多くの患者・医療関係者の協力，国家的な情報・資料収集のためのさらなる体制整備が不可欠であり，患者－医療従事者－研究者の密接な協力のもと，ファーマコゲノミクス研究成果を日常診療に取り入れ，オーダーメイド医療（個別化医療）を実現することで，がんなどの難治性疾患の治療成績をさらに向上させていく必要がある．

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Lymph node shape in computed tomography imaging as a predictor for axillary lymph node metastasis in patients with breast cancer

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Abstract. The aim of the present study was to evaluate whether preoperative computed tomography (CT) is a useful modality for the diagnosis of axillary lymph node metastasis. The axillary lymph node status was examined in patients with primary breast cancer who had undergone surgery. In total, 75 patients were analyzed with preoperative contrast CT images, following which the patients underwent an intraoperative sentinel lymph node biopsy to determine possible predictors of axillary lymph node metastasis. The lymph node shape was classified into three groups, which included fat-, clear-and obscure-types. Multivariate analysis revealed that clear-type lymph nodes in preoperative contrast CT imaging may be an independent predictor of lymph node metastasis (odds ratio, 15; P=0.003). Therefore, the results indicated that preoperative CT examination is useful to predict axillary lymph node metastasis.

Introduction

Axillary lymph node excision in breast cancer was previously the standard optimal surgical procedure for breast cancer. However, currently this procedure is not always essential since the status of axillary lymph node metastasis can be predicted by an intraoperative sentinel lymph node biopsy (SNB) (1). Despite this development, a number of institutions in Japan perform lymph node excision for cases demonstrated to be negative by intraoperative SNB. Thus, axillary lymph node dissection tends to be unnecessary, particularly in a number of patients with early stage breast cancer (2).

Axillary lymph node metastasis is a multifactorial event, and several clinicopathological factors have been reported as predictors of lymph node metastasis in breast cancer (3). However, since only a few methods exist for precisely predicting the axillary lymph node metastasis of an individual patient with breast cancer, a number of patients may not receive appropriate treatment for such metastasis.

The development of diagnostic imaging systems has facilitated the evaluation of axillary lymph node metastasis prior to surgery for breast cancer (4). Computed tomography (CT) is one of the representative modalities that can be used to evaluate the lymph node status, and is commonly used in hospitals due to its noninvasive and inexpensive characteristics. However, the number of studies investigating the clinical usefulness of CT in determining the axillary lymph node status is limited (5).

Therefore, the aim of the present retrospective study was to examine whether contrast CT imaging for the preoperative evaluation of the axillary lymph node status was a clinically useful modality.

Materials and methods

Patients. A total of 75 patients with primary breast cancer that had undergone surgical treatment at the First Department of Surgery of Sapporo Medical University (Sapporo, Japan) between 2009 and 2010 were recruited for the study. The clinical data from the Medical Records Department were retrospectively obtained. Written informed consent was required from all patients. All the patients were Japanese females that had been pathologically diagnosed with invasive ductal carcinoma without distant dissemination by whole body CT and bone scintigraphy. In this department, preoperative contrast CT is normally performed.

Data on clinical information were confirmed from the medical records of the patients and are shown in Table I. Tumor status was classified according to UCLA-integrated staging system classification with tumor, node and metastasis categories (6). The expression of the estrogen receptor or progesterone receptor was designated as positive when positive staining was observed and a total Allred score of ≥3 was achieved. Tumors that were immunohistochemically scored 2+ or 3+ and were fluorescence *in situ* hybridization-positive, were regarded as HER2-positive (7). Patients were classified into the following two groups: Group A consisted of patients

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Key words: breast cancer, computed tomography, lymph node shape

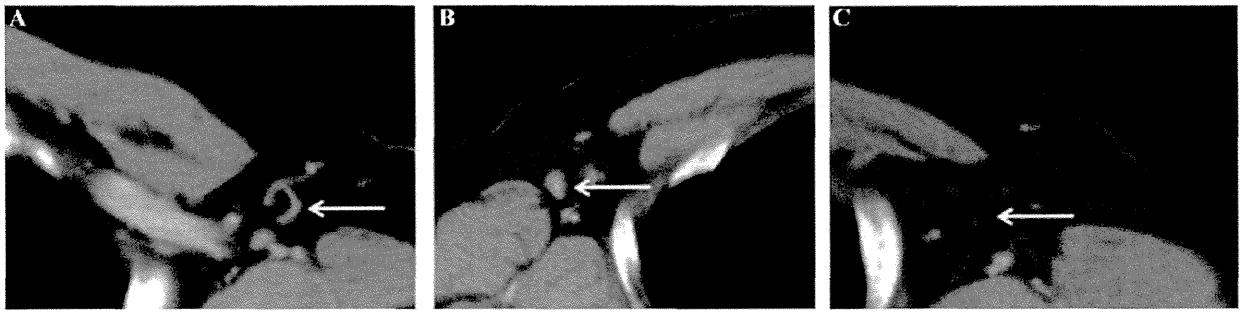

Figure 1. CT images showing (A) fat-, (B) clear-and (C) obscure-type axillary lymph nodes. CT, computed tomography.

who had been diagnosed as negative by SNB, while group B comprised patients who had been diagnosed as axillary lymph node metastasis-positive.

Evaluation of axillary lymph nodes by preoperative contrast CT. Although the axillary lymph nodes were not palpable in any patient, enhanced whole body CT (Aquilion 64; Toshiba, Tokyo, Japan) with contrast was preoperatively performed since this is the standard procedure in Japan. A helical CT unit (64-slice CT system; Light Speed VCT vision; GE Healthcare, Milwaukee, WI, USA) was used for the evaluation of the axillary lymph nodes. The patients were in a supine position and raised their arms during the CT examination. CT images of the axillary lymph nodes were obtained as 2-mm slices through the axilla. The most caudally located enhanced lymph nodes were considered to be the sentinel lymph nodes. Lymph node size and shape were evaluated, as well as the Hounsfield units (HU) of the axillary lymph nodes in the CT images. The average of the region of interest (ROI) was used to evaluate the HU as a CT score. Lymph node shapes were classified into three groups, according to a previous study (8). Nodes with an internal fat concentration were classified as the fat-type (Fig. 1A), those with a size of ≥10 mm that appeared as rounded nodes without any internal fat were classified as the clear-type (Fig. 1B), while the nodes with unclear borders were classified as the obscure-type (Fig. 1C).

SNB. Prior to the initiation of surgery, 3-5 ml indigo carmine was injected into the peritumor, as well as subcutaneous and intradermal portions of the areola. Sentinel lymph nodes were located following massaging the expected area for 2-3 min. All the sentinel lymph nodes identified were sliced into 2-mm sections and stained with hematoxylin and eosin. A surgeon conducted the SNB, while a pathologist evaluated the specimens during the surgery. Finally, SNB specimens were embedded in paraffin and evaluated.

Statistical analysis. Analysis of the continuous variables, including age, tumor size, lymph node size and the CT score, was conducted with the t-test, whereas the χ^2 test was applied for the categorical variables (Table I). For the logistic regression analysis, odds ratios and 95% confidence intervals (CIs) were calculated following adjustment for age. All the statistical analyses and corresponding P-values were two-sided, and $P<0.05$ was considered to indicate a statistically significant difference. All statistical calculations were performed using JMP version 9.0 software (SAS Institute, Cary, NC, USA).

Table I. Clinical characteristics of the 75 patients with breast cancer.

Characteristics	Patients
Mean age, years (range)	
Total (n=75)	56 (35-84)
Pre-menopause (n=28)	54 (32-60)
Post-menopause (n=47)	60 (40-82)
pT[a], n (%)	
pTis	14 (18.7)
pT1	23 (30.6)
pT2	38 (50.7)
HR status, n (%)	
ER(+), PgR(+)	40 (53.4)
ER(+), PgR(-)	19 (25.3)
ER(-), PgR(+)	7 (9.3)
ER(-), PgR(-)	9 (12.0)
HER2 status, n (%)	
Positive	11 (14.7)
Negative	64 (85.3)
pN[a], n (%)	
pN0	56 (74.7)
pN1	19 (25.3)
pN2	0 (0)
Surgery, n (%)	
Breast-conserving	28 (37.3)
Mastectomy	47 (62.7)

[a]UCLA-integrated staging system classification with tumor, node and metastasis categories (2002). HR, hormone receptor; ER, estrogen receptor; PgR, progesterone receptor.

Results

Characteristics of the patients. A total of 75 patients who had received adequate treatment for primary breast cancer were

Table II. Differences in the distributions of possible predictors for positive SNB.

Characteristics	Group A (n=56)	Group B (n=19)	P-value
Menopause (pre/post), n	17/39	11/08	0.034
Tumor size[b], cm	1.55±0.15	2.19±0.26	0.034
Axillary lymph node size[b], cm	0.56±0.05	0.92±0.09	0.0007
Axillary lymph node shape in contrast CT (fat/clear/obscure), n	17/08/31	2/14/3	<0.0001
CT score (ROI)[a,b]	0.16±21.6	31.4±31.9	<0.0001

[a]Average of the ROI. [b]Results are expressed as the mean ± standard deviation. SNB, sentinel lymph node biopsy; CT, computed tomography; ROI, region of interest.

Table III. Univariate and multivariate analyses of the predictors of SNB.

	Univariate analysis			Multivariate analysis		
Predictors	Odds ratio	95% CI	P-value	Odds ratio	95% CI	P-value
Tumor size (≥2 cm, <2 cm)	0.84	0.29-2.39	0.74	0.45	0.10-1.8	0.26
Lymph node size (≥0.5, <0.5)	0.12	0.0062-0.64	0.01	0.16	0.0071-1.6	0.12
Shape						
Obscure	0.15	0.040-0.58	0.006	0.30	0.056-1.6	0.15
Clear	17	4.7-60	<0.001	15	2.5-89	0.003
Fat	0.27	0.56-1.3	0.102	0.16	0.025-1.1	0.06
CT score (ROI[a]; ≥0, <0)	0.22	0.047-0.74	0.013	0.95	0.15-6.0	0.95

[a]Average of the ROI. Values in brackets are the optimal cut-off point defined using a receiver operating characteristic curve. CI, confidence interval; SNB, sentinel lymph node biopsy; CT, computed tomography; ROI, region of interest.

analyzed in the study (Table I). A mastectomy was performed for 61% of the population.

Patients were classified into the following two groups according to the histological diagnosis from the SNB. Group A (n=56) patients were diagnosed as axillary lymph node metastasis-negative by SNB, while group B (n=19) patients were diagnosed as axillary lymph node metastasis-positive.

Difference in the distributions of the possible predictors of axillary lymph node metastasis. Differences in the menopausal status, histological type, tumor size, axillary lymph node size, axillary lymph node shape in contrast CT and CT scores (the average of the ROI) were analyzed between groups A and B (Table II). The menopausal status, tumor size, axillary lymph node size, axillary lymph node shape and CT score exhibited statistically significant differences when comparing the two groups (Table II). In addition, the ratio of the premenopausal group was higher in group B compared with group A (P=0.034), and the primary tumor size, axillary lymph node size and CT score (ROI) were larger in group B compared with group A (P=0.034, P=0.0007 and P<0.0001, respectively). Furthermore, of the 56 patients in group A, fat-, clear- and obscure-type lymph nodes were observed in 17 (30.4%), 8 (14.3%) and 31 cases (55.3%), respectively. By contrast, fat-, clear- and obscure-type lymph nodes were identified in two (10.5%), 14 (73.7%) and three cases (15.8%) in group B, respectively, indicating that there were statistically significant differences (P<0.0001) in the distribution of the lymph node shapes in preoperative contrast CT between the two groups (Table II).

Identification of the predictors for axillary lymph node metastasis. To identify the risk factors for axillary lymph node metastasis, logistic regression analysis of the menopausal status, tumor size, axillary lymph node size, axillary lymph node shape and CT score was conducted since the aforementioned predictors significantly differed between the groups (Table III). In univariate analysis, the menopausal status, axillary lymph node size, obscure-type lymph nodes, clear-type lymph nodes and the CT score were demonstrated to be predictors of lymph node metastasis (P=0.036, P=0.01, P=0.006, P<0.001 and P=0.013, respectively, with 95% CIs of 0.11-0.93, 0.0062-0.64, 0.04-0.58, 4.7-60 and 0.15-6.0, respectively). In addition, with regard to the multivariate analysis, clear-type axillary lymph nodes were shown to be significantly associated with axillary lymph node metastasis following adjustment for the menopausal status, axillary lymph node size, obscure-type lymph nodes and the CT

score (P=0.003; 95% CI, 2.5-89; Table III), indicating that the axillary lymph node shape in preoperative contrast CT imaging was an independent indicator of axillary lymph node metastasis (SNB-positive).

Discussion

Lymph node metastasis is an important factor that affects the prognosis and management of patients with breast cancer (9). Although the axillary lymph nodes should be dissected for patients who are considered to be axillary lymph node-positive, lymph node dissection often causes complications, including arm edema, motor disturbance of the arm and axillary numbness (10-12). Therefore, axillary lymph node dissection should be performed only following consideration of whether the procedure is essential in each patient with breast cancer. In the present study, to identify preoperative predictors for axillary lymph node metastasis, the association of possible predictors and preoperative contrast CT observations were investigated with axillary lymph node metastasis. Axillary lymph node shape in preoperative contrast CT imaging was found to be an independent predictor of metastasis. As shown in Table III, multivariate analysis indicated that clear-type axillary lymph nodes in contrast CT were likely to be a predictor of metastasis (odds ratio, 15; P=0.003; 95% CI, 2.5-89). Although soybean-shaped lymph nodes have been reported to be significantly metastatic and 'C'-shaped and ring-like lymph nodes are more likely to be nonmetastatic in contrast-enhanced CT imaging (8), the clear- and fat-type lymph nodes defined in the present study were demonstrated to correspond to the former and latter, respectively. The pathological association between the lymph node shape in contrast CT and the localization of cancer cells in lymph nodes has not yet been established. Thus, further clinicopathological investigations may clarify how the localization of cancer cells in lymph nodes influences their imaging or shape in contrast CT.

Tumor size has been reported to be one of the main predictors of axillary lymph node metastasis in several studies (13-16). Although statistically significant differences were observed in the distribution of tumor size between groups A and B (Table II), tumor size was not found to be an independent predictor for axillary lymph node metastasis in the present study (Table III). However, future studies with larger sample sizes are required to validate the association between tumor size and lymph node metastasis, since 50% of the tumors in the present study were small (<20 mm). SNB has become a standard procedure, and preoperative evaluation of the axillary lymph nodes based on imaging modalities is considered to be important for selecting appropriate breast cancer treatment (16,17). Several diagnostic imaging modalities have been used for the preoperative diagnosis of the sentinel lymph node status. Ultrasonography, magnetic resonance imaging and multidetector CT have been reported to be useful imaging systems to preoperatively evaluate the lymph node status (18-20).

Lymph node size was also shown to be associated with lymph node metastasis through univariate analysis; however, lymph node size is unlikely to be an independent predictor according to the results from the multivariate analysis (Table III). In the present study, univariate analysis demonstrated that the CT score (ROI) was a predictor of lymph node metastasis, indicating that high contrast lymph nodes on CT images, which may be a consequence of vessel development in the lymph nodes, may be associated with metastasis (Table III). These observations indicate that the evaluation of the lymph node status by preoperative contrast CT may support the intraoperative diagnosis by SNB.

In Japan, CT examinations are indispensable for the preoperative metastatic search, and are conducted in all institutions. CT is also considered to be very important for preoperative sentinel lymph node examination. The results of the present study indicate that preoperative CT examinations are useful in predicting axillary lymph node metastasis, and can provide supportive information for intraoperative sentinel lymph node diagnosis. Although further large-scale studies are required to validate these results, the observations of the present study provide useful information for identifying predictors of axillary lymph node metastasis, and may aid surgeons to determine appropriate surgical strategies for individual patients with breast cancer.

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Breast Cancer RESEARCH

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VAV3 mediates resistance to breast cancer endocrine therapy

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Abstract

Introduction: Endocrine therapies targeting cell proliferation and survival mediated by estrogen receptor α (ERα) are among the most effective systemic treatments for ERα-positive breast cancer. However, most tumors initially responsive to these therapies acquire resistance through mechanisms that involve ERα transcriptional regulatory plasticity. Herein we identify VAV3 as a critical component in this process.

Methods: A cell-based chemical compound screen was carried out to identify therapeutic strategies against resistance to endocrine therapy. Binding to ERα was evaluated by molecular docking analyses, an agonist fluoligand assay and short hairpin (sh)RNA–mediated protein depletion. Microarray analyses were performed to identify altered gene expression. Western blot analysis of signaling and proliferation markers, and shRNA-mediated protein depletion in viability and clonogenic assays, were performed to delineate the role of VAV3. Genetic variation in *VAV3* was assessed for association with the response to tamoxifen. Immunohistochemical analyses of VAV3 were carried out to determine its association with therapeutic response and different tumor markers. An analysis of gene expression association with drug sensitivity was carried out to identify a potential therapeutic approach based on differential VAV3 expression.

Results: The compound YC-1 was found to comparatively reduce the viability of cell models of acquired resistance. This effect was probably not due to activation of its canonical target (soluble guanylyl cyclase), but instead was likely a result of binding to ERα. VAV3 was selectively reduced upon exposure to YC-1 or ERα depletion, and, accordingly, VAV3 depletion comparatively reduced the viability of cell models of acquired resistance. In the clinical scenario, germline variation in *VAV3* was associated with the response to tamoxifen in Japanese breast cancer patients (rs10494071 combined P value $= 8.4 \times 10^{-4}$). The allele association combined with gene expression analyses indicated that low VAV3 expression predicts better clinical outcome. Conversely, high nuclear VAV3 expression in tumor cells was associated with poorer endocrine therapy response. Based on *VAV3* expression levels and the response to erlotinib in cancer cell lines, targeting EGFR signaling may be a promising therapeutic strategy.

Conclusions: This study proposes VAV3 as a biomarker and a rationale for its use as a signaling target to prevent and/or overcome resistance to endocrine therapy in breast cancer.

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Introduction

Endocrine therapies are the cornerstone of the curative and palliative treatment of ERα-positive breast cancer. However, even patients who initially respond to these therapies may eventually develop resistance. Current knowledge of the molecular mechanisms of acquired resistance to endocrine therapies suggests a model in which crosstalk between ERα and growth factor signaling pathways plays an important role [1-3]. There may also be resistance mechanisms partially or totally independent of growth factor signaling, such as mutations in the *ESR1* gene, which encodes for ERα, that alter ligand and/or coactivator binding [4-6].

Beyond the alterations in growth factor signaling pathways identified to date, the binding plasticity of ERα to chromatin is central in therapeutic resistance and cancer progression [7]. This plasticity is mediated by the interaction of ERα with FOXA1 and, importantly, as a result, a rewired transcriptional program that endorses resistance [8]. In this scenario, however, it is not fully understood which transcriptional outputs—possibly those involved in growth factor signaling pathways—may be critical in the acquisition of the resistant phenotype.

In recent years, different breast cancer cell models have been generated in efforts to decipher the mechanisms of acquired resistance to endocrine therapies [3,9,10]. One popular model was based on the long-term estrogen deprivation (LTED) of the ERα-positive breast cancer cell line MCF7 [11-14]. This model was designed to recapitulate the effects of the therapeutic use of aromatase inhibitors (AIs) in postmenopausal breast cancer [15]. Relevant differences, but also similarities, have been described between the MCF7-LTED model and other cell models of acquired resistance [16,17]. Although this observation raises potential limitations, the results obtained with these models should be evaluated in the corresponding clinical settings. In our present study, in which we start with an analysis of the response of MCF7-LTED cells to different small compounds and then report our testing of predictions in different cohorts of breast cancer patients, we propose that *VAV3*/VAV3 is a key ERα-downstream determinant of the response to endocrine therapies.

Methods

Cell culture and viability assays

MCF-7 cells were routinely cultured and maintained in Roswell Park Memorial Institute medium containing 10% fetal bovine serum and 2 mM glutamine. MCF7-LTED cells were established in phenol red-free medium containing 10% dextran-coated, charcoal-stripped serum [17]. All other cell lines used in this study were cultured according to standard protocols [18]. The epidermal growth factor (EGF) (Sigma-Aldrich, St Louis, MO, USA) was used at 10 ng/ml for 5 minutes. Cellular viability was evaluated using standard methylthiazol tetrazolium (MTT)–based assays (Sigma-Aldrich). The results of these assays are expressed relative to vehicle-treated controls and to the original time point.

Chemical compound screen

MCF7 and MCF7-LTED cells were plated in 384-well microtiter plates, and five compound dilutions (1 nM to 10 μM final concentration) from the Library of Pharmacologically Active Compounds (LOPAC1280) (1,258 compounds; Sigma-Aldrich) were added to the cultures. Cell viability was assessed after 72 hours using MTT-based assays and the EnVision spectrofluorometer (PerkinElmer, Waltham, MA, USA). The screen was performed in triplicate. Data quality was assessed (Z'-factor > 0.5 for all screens), and data analysis was performed using the cellHTS2 module in the Screensaver database [19]. The data were normalized between 0 and 1 using positive (1 μM phenylarsene oxide) and negative (0.1% dimethyl sulfoxide (DMSO)) controls. For hit selection, the difference between the normalized percentage inhibition (NPI) in MCF7 and MCF7-LTED cells was calculated by subtraction (ΔNPI = NPI(MCF7-LTED) – NPI(MCF7)), and the differentials were clustered with the MeV software package [20] using the Cluster Affinity Search method with the Euclidean distance metric (threshold of 0.7). Based on the 18 clustered differential profiles, 83% of the compounds ($n = 1,047$) had no differential effect between the cell lines, 1% ($n = 13$) were more selective towards MCF7-LTED cells and 0.5% ($n = 6$) were more selective toward MCF7 cells. The YC-1 compound was purchased from Sigma-Aldrich and from Chemgen Pharma International (custom synthesis order; Calcutta, India), and erlotinib was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

cGMP, subcellular fractionation, and Western blotting

The cGMP levels were measured using the Amersham cGMP Direct Biotrak EIA system (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Fractionation was performed with a subcellular protein fraction kit (Thermo Fisher Scientific, Asheville, NC, USA). Cells were lysed in buffer containing 50 mM Tris-HCl pH 8, 0.5% Nonidet P-40, 100 mM NaCl and 0.1 mM ethylenediaminetetraacetic acid, supplemented with protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA) and 1 mM NaF. Lysates were clarified twice by centrifugation at 13,000 × *g*, and protein concentration was measured using the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA). Lysates were resolved in SDS-PAGE gels and transferred to Immobilon-P membrane (EMD Millipore, Billerica, MA, USA) or polyvinylidene fluoride membrane (Roche Molecular Biochemicals), and target

proteins were identified by detection of horseradish peroxidase–labeled antibody complexes with chemiluminescence using an Amersham ECL Western Blotting Detection Kit (GE Healthcare Life Sciences).

ERα structural analysis and binding assay

Chains A and C of the RCSB Protein Data Bank (PDB) structure 3OS8 [Swiss-Prot:P03372] were superimposed and used as representative structures of the partially constrained and unconstrained forms, respectively. Hydrogen atoms and protonation states were automatically assigned using the Protonate 3D function of the Molecular Operating Environment (Chemical Computing Group, Montreal, QC, Canada) [21], and the structures were saved in Mol2 file format, which was then used as input for docking analysis in rDock [22]. The cavity was defined as the available space 6 Å around the crystallized ligand. Both WAY6 and YC-1 were docked to each of the conformations in exhaustive sampling mode (100 genetic algorithm runs). The binding mode in chain A (binding mode 1, as previously described [23]) was considered to be responsible for the partial agonist activity, and the binding mode in chain C (binding mode 4, as previously described [23]) caused a shift in the conformation of helices 3 and 11, which displaced helix 12 and resulted in an inactive state. To test the performance of the docking program, WAY6 bound to chain C was cross-docked to chain A, and vice versa. The experimental binding mode of WAY6 was reproduced in both cases, although modes 1 and 4 scored very similarly in chain C, suggesting that these modes can coexist in the unconstrained (inactive) conformation. By contrast, binding mode 4 was clearly disfavored in chain A, indicating that this binding mode is incompatible with the partially constrained (active) conformation. The ERα agonist fluoligand assay was performed by Cerep (Paris, France) using YC-1 final concentrations from 10 to 250 μM.

Gene expression analyses

RNA samples were extracted using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) and the RNeasy kit (QIAGEN, Valencia, CA, USA), and quality was evaluated in the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNAs were amplified using the Ribo-SPIA system (NuGEN Technologies, San Carlos, CA, USA) and subsequently hybridized on the Human Genome U219 microarray platform (Affymetrix, Santa Clara, CA, USA). The data have been deposited in the Gene Expression Omnibus (GEO) [GSE:38829]. Publicly available whole-genome expression data for 51 breast cancer cell lines were analyzed using the preprocessed and normalized values [18]. The Gene Set Expression Analysis (GSEA) was run using default values for all parameters [24]. Preprocessed and normalized microarray data from breast tumors and tumor response to tamoxifen were taken from the corresponding repositories: the Stanford microarray repository (NKI-295 data set) [25] and the GEO record [GSE:9195], respectively. Cox proportional hazard regression analysis was used to evaluate differences in distant metastasis-free survival according to *VAV3* expression (three microarray probes were treated independently).

Chromatin immunoprecipitation data analysis

Chromatin immunoprecipitation (ChIP) data were downloaded from the GEO database [GSE:32222] [7] and analyzed using MACS version 2.0.9 software (macs2diff function) [26]. Significance was defined by a *Q*-value <0.01 and using default values for the remaining parameters. Differentially bound genomic regions were annotated to the closest ENSEMBL (hg19) annotated gene using the R-Bioconductor package ChIPpeakAnno [27]. Previously aligned reads were extracted from the sequence read archive [SRP:032421], and sequence counts were normalized to the library size. ERα and nonspecific immunoglobulin control (IgG) ChIP assays were performed as previously described [28,29]. Briefly, the DNA was purified using a phenol-chloroform extraction protocol, the antibodies used were anti-ERα (SC-543 and SC-7207; Santa Cruz Biotechnology) and anti-IgG (ab46540; Abcam, Cambridge, UK), and three independent biological replicates were obtained in all cases. The primers used were site 1: forward 5′-CACTTCCTTTCCTGGTTGGA-3′ and reverse 5′-AGTAAAAGGGGTGCCCTCTC-3′, and site 2: forward 5′- TGTGGTGTTTCCTGTTAGT GG-3′ and reverse 5′- TTGCCAATAACTTAAAGCGTA GG-3′.

Antibodies and RAC1 activity assay

The antibodies we used were anti-E2F1 (KH95; Santa Cruz Biotechnologies), anti–epidermal growth factor (anti-EGFR) (1005; Santa Cruz Biotechnologies), anti-ERα (SP-1; Abcam), antibody against phosphorylated extracellular signal-regulated protein kinases 1 and 2 (anti-phospho-ERK1/2) (D13.14.4E; Cell Signaling Technology, Danvers, MA, USA), anti-NUP62 (nucleoporin 62 kDa, clone 53; BD Transduction Laboratories, San Jose, CA, USA), anti-PAK1 (2602; Cell Signaling Technology), anti-RAC1 (05-389; EMD Millipore), anti-phospho-serine 235/236 ribosomal S6 (D57.2.2E; Cell Signaling Technology), anti-VAV3 (07-464, Millipore; and 2398, Cell Signaling Technology), anti-phospho-tyrosine 173 VAV3 (anti-pT173 VAV3, ab52938; Abcam) and anti–tubulin α (anti-TUBA) (DM1A + DM1B; Abcam). Secondary antibodies for used for immunofluorescence (Alexa Fluor) were obtained from Molecular Probes (Eugene, OR, USA). To measure RAC1 activity, we used the Rac1 G-LISA Activation Assay Biochem Kit (BK128; Cytoskeleton, Denver, CO, USA).