women with reduced CYP2D6 metabolism: a multicenter study. *J. Clin. Oncol.* 29(24), 3232–3239 (2011).

10 Broly F, Gaedigk A, Heim M, Eichelbaum M, Morike K, Meyer UA. Debrisoquine/sparteine hydroxylation genotype and phenotype: analysis of common mutations and alleles of *CYP2D6* in a European population. *DNA Cell Biol.* 10(8), 545–558 (1991).

11 Madlensky L, Natarajan L, Tchu S *et al.* Tamoxifen metabolite concentrations, CYP2D6 genotype, and breast cancer outcomes. *Clin. Pharmacol. Ther.* 89(5), 718–725 (2011).

12 Kiyotani K, Mushiroda T, Sasa M *et al.* Impact of *CYP2D6*10* on recurrence-free survival in breast cancer patients receiving adjuvant tamoxifen therapy. *Cancer Sci.* 99(5), 995–999 (2008).

13 Hosono N, Kato M, Kiyotani K *et al. CYP2D6* genotyping for functional-gene dosage analysis by allele copy number detection. *Clin. Chem.* 55(8), 1546–1554 (2009).

14 Kiyotani K, Mushiroda T, Imamura CK *et al.* Significant effect of polymorphisms in *CYP2D6* and *ABCC2* on clinical outcomes of adjuvant tamoxifen therapy for breast cancer patients. *J. Clin. Oncol.* 28(8), 1287–1293 (2010).

15 Goetz MP, Knox SK, Suman VJ *et al.* The impact of cytochrome P450 2D6 metabolism in women receiving adjuvant tamoxifen. *Breast Cancer Res. Treat.* 101(1), 113–121 (2007).

16 Schroth W, Goetz MP, Hamann U *et al.* Association between *CYP2D6* polymorphisms and outcomes among women with early stage breast cancer treated with tamoxifen. *JAMA* 302(13), 1429–1436 (2009).

17 Province MA, Goetz MP, Brauch H *et al. CYP2D6* genotype and adjuvant tamoxifen: meta-analysis of heterogeneous study populations. *Clin. Pharmacol. Ther.* 95(2), 216–227 (2014).

18 Lim HS, Ju Lee H, Seok Lee K, Sook Lee E, Jang IJ, Ro J. Clinical implications of CYP2D6 genotypes predictive of tamoxifen pharmacokinetics in metastatic breast cancer. *J. Clin. Oncol.* 25(25), 3837–3845 (2007).

19 Kiyotani K, Mushiroda T, Imamura CK *et al.* Dose-adjustment study of tamoxifen based on *CYP2D6* genotypes in Japanese breast cancer patients. *Breast Cancer Res. Treat.* 131(1), 137–145 (2012).

20 Martinez De Duenas E, Ochoa Aranda E, Blancas Lopez-Barajas I *et al.* Adjusting the dose of tamoxifen in patients with early breast cancer and CYP2D6 poor metabolizer phenotype. *Breast* 23(4), 400–406 (2014).

21 Regan MM, Leyland-Jones B, Bouzyk M *et al. CYP2D6* genotype and tamoxifen response in postmenopausal women with endocrine-responsive breast cancer: the breast international group 1–98 trial. *J. Natl Cancer Inst.* 104(6), 441–451 (2012).

22 Nowell SA, Ahn J, Rae JM *et al.* Association of genetic variation in tamoxifen-metabolizing enzymes with overall survival and recurrence of disease in breast cancer patients. *Breast Cancer Res. Treat.* 91(3), 249–258 (2005).

23 Wegman P, Elingarami S, Carstensen J, Stal O, Nordenskjold B, Wingren S. Genetic variants of *CYP3A5*, *CYP2D6*, *SULT1A1*, *UGT2B15* and tamoxifen response in postmenopausal patients with breast cancer. *Breast Cancer Res.* 9(1), R7 (2007).

24 Hertz DI, Mcleod HI, Irvin WJ Jr. Tamoxifen and CYP2D6: a contradiction of data. *Oncologist* 17(5), 620–630 (2012).

25 Ingle JN, Suman VJ, Johnson PA *et al.* Evaluation of tamoxifen plus letrozole with assessment of pharmacokinetic interaction in postmenopausal women with metastatic breast cancer. *Clin Cancer Res.* 5(7), 1642–1649 (1999).

26 Stearns V, Johnson Md, Rae JM *et al.* Active tamoxifen metabolite plasma concentrations after coadministration of tamoxifen and the selective serotonin reuptake inhibitor paroxetine. *J. Natl Cancer Inst.* 95(23), 1758–1764 (2003).

27 Jin Y, Desta Z, Stearns V *et al. CYP2D6* genotype, antidepressant use, and tamoxifen metabolism during adjuvant breast cancer treatment. *J. Natl Cancer Inst.* 97(1), 30–39 (2005).

28 Clarke R, Liu MC, Bouker KB *et al.* Antiestrogen resistance in breast cancer and the role of estrogen receptor signaling. *Oncogene* 22(47), 7316–7339 (2003).

29 Lien EA, Solheim E, Kvinnsland S, Ueland PM. Identification of 4-hydroxy-N-desmethyltamoxifen as a metabolite of tamoxifen in human bile. *Cancer Res.* 48(8), 2304–2308 (1988).

30 Desta Z, Ward BA, Soukhova NV, Flockhart DA. Comprehensive evaluation of tamoxifen sequential biotransformation by the human cytochrome P450 system *in vitro*: prominent roles for CYP3A and CYP2D6. *J. Pharmacol. Exp. Ther.* 310(3), 1062–1075 (2004).

31 Gjerde J, Hauglid M, Breilid H *et al.* Effects of *CYP2D6* and *SULT1A1* genotypes including *SULT1A1* gene copy number on tamoxifen metabolism. *Ann. Oncol.* 19(1), 56–61 (2008).

32 Falany JL, Pilloff DE, Leyh TS, Falany CN. Sulfation of raloxifene and 4-hydroxytamoxifen by human cytosolic sulfotransferases. *Drug Metab. Dispos.* 34(3), 361–368 (2006).

33 Nishiyama T, Ogura K, Nakano H *et al.* Reverse geometrical selectivity in glucuronidation and sulfation of cis- and trans-4-hydroxytamoxifens by human liver UDP-glucuronosyltransferases and sulfotransferases. *Biochem. Pharmacol.* 63(10), 1817–1830 (2002).

34 Ogura K, Ishikawa Y, Kaku T *et al.* Quaternary ammonium-linked glucuronidation of trans-4-hydroxytamoxifen, an active metabolite of tamoxifen, by human liver microsomes and UDP-glucuronosyltransferase 1A4. *Biochem. Pharmacol.* 71(9), 1358–1369 (2006).

35 Sun D, Sharma AK, Dellinger RW *et al.* Glucuronidation of active tamoxifen metabolites by the human UDP glucuronosyltransferases. *Drug Metab. Dispos.* 35(11), 2006–2014 (2007).

36 Ingelman-Sundberg M. Genetic polymorphisms of cytochrome P450 2D6 (CYP2D6): clinical consequences, evolutionary aspects and functional diversity. *Pharmacogenomics J.* 5(1), 6–13 (2005).

37 Gross AS, Kroemer Hk, Eichelbaum M. Genetic polymorphism of drug metabolism in humans. *Adv. Exp. Med. Biol.* 283, 627–640 (1991).

38 Sistonen J, Sajantila A, Lao O, Corander J, Barbujani G, Fuselli S. *CYP2D6* worldwide genetic variation shows high frequency of altered activity variants and no continental structure. *Pharmacogenet. Genomics* 17(2), 93–101 (2007).

39 Sideras K, Ingle JN, Ames MM *et al.* Coprescription of tamoxifen and medications that inhibit CYP2D6. *J. Clin. Oncol.* 28(16), 2768–2776 (2010).

40 Borges S, Desta Z, Li L *et al.* Quantitative effect of *CYP2D6* genotype and inhibitors on tamoxifen metabolism: implication for optimization of breast cancer treatment. *Clin. Pharmacol. Ther.* 80(1), 61–74 (2006).

41 Loprinzi CL, Kugler JW, Sloan JA *et al.* Venlafaxine in management of hot flashes in survivors of breast cancer: a randomised controlled trial. *Lancet* 356(9247), 2059–2063 (2000).

42 Loprinzi CL, Sloan JA, Perez EA *et al.* Phase III evaluation of fluoxetine for treatment of hot flashes. *J. Clin. Oncol.* 20(6), 1578–1583 (2002).

43 Stearns V, Beebe KI, Iyengar M, Dube E. Paroxetine controlled release in the treatment of menopausal hot flashes: a randomized controlled trial. *JAMA* 289(21), 2827–2834 (2003).

44 Orleans RJ, Li L, Kim MJ *et al.* FDA approval of paroxetine for menopausal hot flushes. *N. Engl. J. Med.* 370(19), 1777–1779 (2014).

45 Zembutsu H, Sasa M, Kiyotani K, Mushiroda T, Nakamura Y. Should CYP2D6 inhibitors be administered in conjunction with tamoxifen? *Expert Rev. Anticancer Ther.* 11(2), 185–193 (2011).

46 Ahern TP, Pedersen L, Cronin-Fenton DP, Sorensen HT, Lash TL. No increase in breast cancer recurrence with concurrent use of tamoxifen and some CYP2D6-inhibiting medications. *Cancer Epidemiol. Biomarkers Prev.* 18(9), 2562–2564 (2009).

47 Kelly CM, Juurlink DN, Gomes T *et al.* Selective serotonin reuptake inhibitors and breast cancer mortality in women receiving tamoxifen: a population based cohort study. *BMJ* 2010(8), 340 (2010).

48 Siegelmann-Danieli N, Kurnik D, Lomnicky Y *et al.* Potent CYP2D6 inhibiting drugs do not increase relapse rate in early breast cancer patients treated with adjuvant tamoxifen. *Breast Cancer Res. Treat.* 125(2), 505–510 (2011).

49 Desmarais JE, Looper KJ. Managing menopausal symptoms and depression in tamoxifen users: implications of drug and medicinal interactions. *Maturitas* 67(4), 296–308 (2010).

50 Dezentje VO, Van Blijderveen NJ, Gelderblom H *et al.* Effect of concomitant CYP2D6 inhibitor use and tamoxifen adherence on breast cancer recurrence in early-stage breast cancer. *J. Clin. Oncol.* 28(14), 2423–2429 (2010).

51 Dusetzina SB, Alexander GC, Freedman RA, Huskamp HA, Keating NL. Trends in co-prescribing of antidepressants and tamoxifen among women with breast cancer, 2004–2010. *Breast Cancer Res. Treat.* 137(1), 285–296 (2013).

52 Higgins Mj, Stearns V. *CYP2D6* polymorphisms and tamoxifen metabolism: clinical relevance. *Curr. Oncol. Rep.* 12(1), 7–15 (2010).

53 Wegman P, Vainikka L, Stal O *et al.* Genotype of metabolic enzymes and the benefit of tamoxifen in postmenopausal breast cancer patients. *Breast Cancer Res.* 7(3), R284–R290 (2005).

54 Xu Y, Sun Y, Yao L *et al.* Association between *CYP2D6 *10* genotype and survival of breast cancer patients receiving tamoxifen treatment. *Ann. Oncol.* 19(8), 1423–1429 (2008).

55 Ramon Y, Cajal T, Altes A, Pare L *et al.* Impact of *CYP2D6* polymorphisms in tamoxifen adjuvant breast cancer treatment. *Breast Cancer Res. Treat.* 119(1), 33–38 (2010).

56 Abraham JE, Maranian MJ, Driver KE *et al.* *CYP2D6* gene variants: association with breast cancer specific survival in a cohort of breast cancer patients from the United Kingdom treated with adjuvant tamoxifen. *Breast Cancer Res.* 12(4), R64 (2010).

57 Goetz MP, Suman VJ, Hoskin TL *et al.* CYP2D6 metabolism and patient outcome in the Austrian Breast and Colorectal Cancer Study Group trial (ABCSG) 8. *Clin. Cancer Res.* 19(2), 500–507 (2013).

58 Newman WG, Hadfield KD, Latif A *et al.* Impaired tamoxifen metabolism reduces survival in familial breast cancer patients. *Clin. Cancer Res.* 14(18), 5913–5918 (2008).

59 Teh LK, Mohamed NI, Salleh MZ *et al.* The risk of recurrence in breast cancer patients treated with tamoxifen: polymorphisms of *CYP2D6* and *ABCB1*. *AAPS J.* 14(1), 52–59 (2012).

60 Sirachainan E, Jaruhathai S, Trachu N *et al.* *CYP2D6* polymorphisms influence the efficacy of adjuvant tamoxifen in Thai breast cancer patients. *Pharmgenomics Pers. Med.* 5, 149–153 (2012).

61 Damodaran SE, Pradhan SC, Umamaheswaran G, Kadambari D, Reddy KS, Adithan C. Genetic polymorphisms of *CYP2D6* increase the risk for recurrence of breast cancer in patients receiving tamoxifen as an adjuvant therapy. *Cancer Chemother. Pharmacol.* 70(1), 75–81 (2012).

62 Okishiro M, Taguchi T, Jin Kim S, Shimazu K, Tamaki Y, Noguchi S. Genetic polymorphisms of *CYP2D6 10* and *CYP2C19 2, 3* are not associated with prognosis, endometrial thickness, or bone mineral density in Japanese breast cancer patients treated with adjuvant tamoxifen. *Cancer* 115(5), 952–961 (2009).

63 Stingl JC, Parmar S, Huber-Wechselberger A *et al.* Impact of *CYP2D6*4* genotype on progression free survival in tamoxifen breast cancer treatment. *Curr. Med. Res. Opin.* 26(11), 2535–2542 (2010).

64 Kiyotani K, Mushiroda T, Hosono N *et al.* Lessons for pharmacogenomics studies: association study between *CYP2D6* genotype and tamoxifen response. *Pharmacogenet. Genomics* 20(9), 565–568 (2010).

65 Lash TL, Cronin-Fenton D, Ahern TP *et al.* CYP2D6 inhibition and breast cancer recurrence in a population-based study in Denmark. *J. Natl Cancer Inst.* 103(6), 489–500 (2011).

66 Park Ih, Ro J, Park S *et al.* Lack of any association between functionally significant *CYP2D6* polymorphisms and clinical outcomes in early breast cancer patients receiving adjuvant tamoxifen treatment. *Breast Cancer Res. Treat.* 131(2), 455–461 (2012).

67 Park HS, Choi JY, Lee MJ *et al.* Association between genetic polymorphisms of *CYP2D6* and outcomes in breast cancer patients with tamoxifen treatment. *J. Korean Med. Sci.* 26(8), 1007–1013 (2011).

68 Thompson AM, Johnson A, Quinlan P *et al.* Comprehensive *CYP2D6* genotype and adherence affect outcome in breast cancer patients treated with tamoxifen monotherapy. *Breast Cancer Res. Treat.* 125(1), 279–287 (2011).

69 Rae JM, Drury S, Hayes DF *et al. CYP2D6* and *UGT2B7* genotype and risk of recurrence in tamoxifen-treated breast cancer patients. *J. Natl Cancer Inst.* 104(6), 452–460 (2012).

70 Kiyotani K, Mushiroda T, Zembutsu H, Nakamura Y. Important and critical scientific aspects in pharmacogenomics analysis: lessons from controversial results of tamoxifen and CYP2D6 studies. *J. Hum. Genet.* 58(6), 327–333 (2013).

71 Nakamura Y, Ratain MJ, Cox NJ, McLeod HL, Kroetz DL, Flockhart DA. Re: *CYP2D6* genotype and tamoxifen response in postmenopausal women with endocrine-responsive breast cancer: the Breast International Group 1–98 trial. *J. Natl Cancer Inst.* 104(16), 1264; author reply 1266–1268 (2012).

72 Hoskins JM, Carey LA, Mcleod HL. CYP2D6 and tamoxifen: DNA matters in breast cancer. *Nat. Rev. Cancer* 9(8), 576–586. (2009).

73 Lash TL, Rosenberg CL. Evidence and practice regarding the role for CYP2D6 inhibition in decisions about tamoxifen therapy. *J. Clin. Oncol.* 28(8), 1273–1275 (2010).

74 Goetz MP, Rae JM, Suman VJ *et al.* Pharmacogenetics of tamoxifen biotransformation is associated with clinical outcomes of efficacy and hot flashes. *J. Clin. Oncol.* 23(36), 9312–9318 (2005).

75 Schroth W, Antoniadou L, Fritz P *et al.* Breast cancer treatment outcome with adjuvant tamoxifen relative to patient *CYP2D6* and *CYP2C19* genotypes. *J. Clin. Oncol.* 25(33), 5187–5193 (2007).

76 Tucker AN, Tkaczuk KA, Lewis LM, Tomic D, Lim CK, Flaws JA. Polymorphisms in cytochrome P4503A5 (CYP3A5) may be associated with race and tumor characteristics, but not metabolism and side effects of tamoxifen in breast cancer patients. *Cancer Lett.* 217(1), 61–72 (2005).

77 Rudberg I, Mohebi B, Hermann M, Refsum H, Molden E. Impact of the ultrarapid *CYP2C19*17* allele on serum concentration of escitalopram in psychiatric patients. *Clin. Pharmacol. Ther.* 83(2), 322–327 (2008).

78 Sim SC, Risinger C, Dahl ML *et al.* A common novel *CYP2C19* gene variant causes ultrarapid drug metabolism relevant for the drug response to proton pump inhibitors and antidepressants. *Clin. Pharmacol. Ther.* 79(1), 103–113 (2006).

79 Kiyotani K, Mushiroda T, Tsunoda T *et al.* A genome-wide association study identifies locus at 10q22 associated with clinical outcomes of adjuvant tamoxifen therapy for breast cancer patients in Japanese. *Hum. Mol. Genet.* 21(7), 1665–1672 (2012).

80 Grabinski JL, Smith LS, Chisholm GB *et al.* Genotypic and allelic frequencies of *SULT1A1* polymorphisms in women receiving adjuvant tamoxifen therapy. *Breast Cancer Res. Treat.* 95(1), 13–16 (2006).

81 Kuehl P, Zhang J, Lin Y *et al.* Sequence diversity in CYP3A promoters and characterization of the genetic basis of polymorphic CYP3A5 expression. *Nat. Genet.* 27(4), 383–391 (2001).

82 Murdter TE, Schroth W, Bacchus-Gerybadze L *et al.* Activity levels of tamoxifen metabolites at the estrogen receptor and the impact of genetic polymorphisms of phase I and II enzymes on their concentration levels in plasma. *Clin. Pharmacol. Ther.* 89(5), 708–717 (2011).

83 Gjerde J, Geisler J, Lundgren S *et al.* Associations between tamoxifen, estrogens, and FSH serum levels during steady state tamoxifen treatment of postmenopausal women with breast cancer. *BMC Cancer* 10, 313 (2010).

84 Suzuki H, Sugiyama Y. Single nucleotide polymorphisms in multidrug resistance associated protein 2 (MRP2/ABCC2): its impact on drug disposition. *Adv. Drug Deliv. Rev.* 54(10), 1311–1331 (2002).

85 Horikawa M, Kato Y, Tyson CA, Sugiyama Y. The potential for an interaction between MRP2 (ABCC2) and various therapeutic agents: probenecid as a candidate inhibitor of the biliary excretion of irinotecan metabolites. *Drug Metab. Pharmacokinet.* 17(1), 23–33 (2002).

86 Kisanga ER, Mellgren G, Lien EA. Excretion of hydroxylated metabolites of tamoxifen in human bile and urine. *Anticancer Res.* 25(6C), 4487–4492 (2005).

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

* Precision medicine for cancer and pharmacogenomics.

** Hitoshi ZEMBUTSU, M.D., Ph.D.: 国立がん研究センター研究所遺伝医学研究分野〔〒104-0045 東京都中央区築地5-1-1〕; Division of Genetics, National Cancer Center Research Institute, Tokyo 104-0045, JAPAN

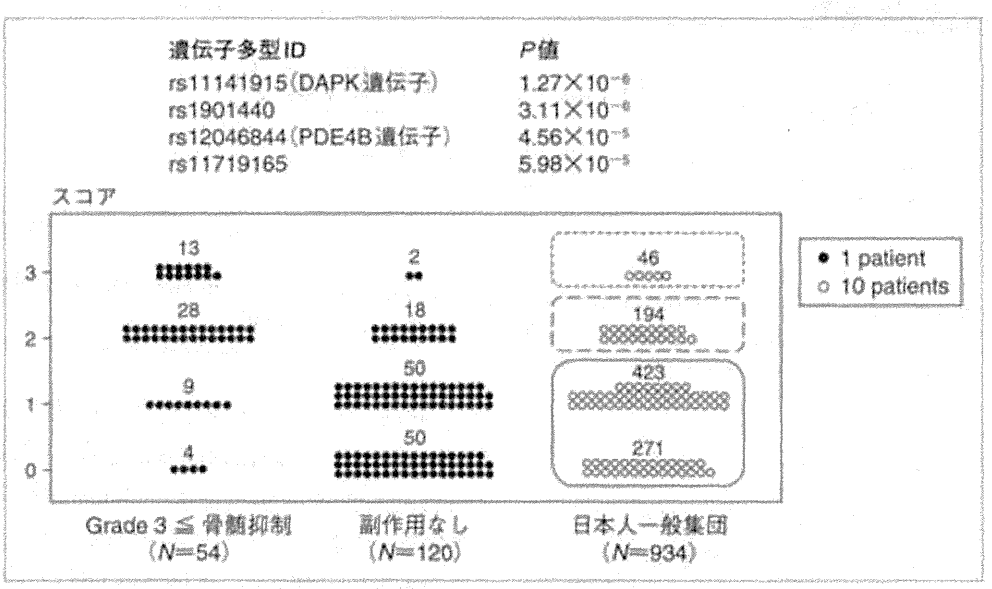

図1　4 SNPの遺伝子型に基づくゲムシタビン骨髄抑制予測システム
4つのSNPを用いてスコアリングを行うことで，grade 3以上の重篤な骨髄抑制をきたした症例では高スコア，副作用を認めなかった症例では低スコアを示した．一番右の群は日本人一般集団において予測されるスコアの分布．ゲムシタビン投与前にハイリスク(高スコア)であることが予測できれば，他剤による治療を優先して患者に提供することが可能になるものと考えられる．

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～

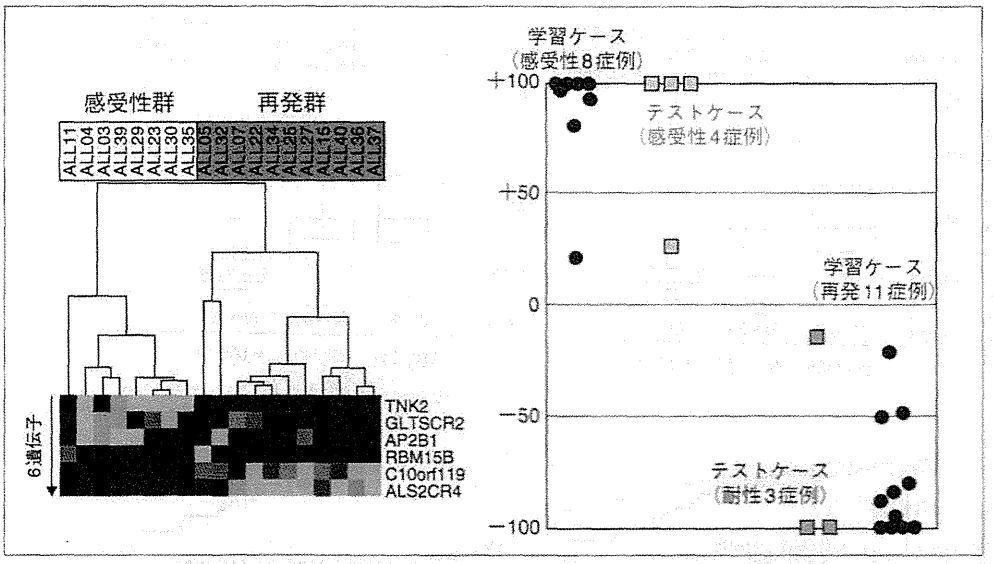

図2　Ph⁺ALL再発予測6遺伝子を用いたスコアリング

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

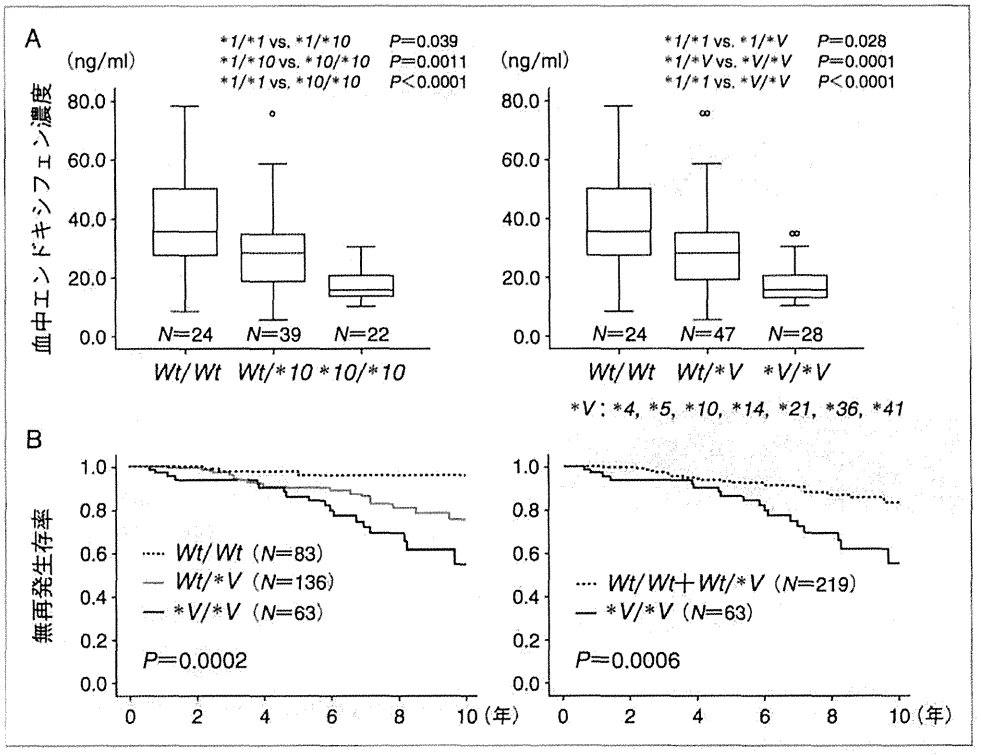

図3　*CYP2D6*遺伝子型とタモキシフェン活性本体（エンドキシフェン）の血中濃度と治療効果の関係
A：左は日本人に最も多い*CYP2D6*遺伝子多型（*10；活性減弱型）と血中エンドキシフェン濃度の関係．右は酵素活性低下または消失型のアレルを*Vと分類した場合の結果．酵素活性が弱いまたは消失するアレルを持つ症例では，血中エンドキシフェン濃度が有意に低下することがわかる．B：遺伝子型の違いと，タモキシフェン治療後の無再発生存期間の関係を示す．最も酵素活性の低い*CYP2D6* *V/*Vの症例群が最も再発率が高い結果となった．

「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．

文 献

1) Innocenti F, Undevia SD, Iyer L, et al. Genetic variants in the UDP-glucuronosyltransferase 1A1 gene predict the risk of severe neutropenia of irinotecan. J Clin Oncol 2004 ; 22 : 1382.
2) Sugiyama E, Kaniwa N, Kim SR, et al. Pharmacokinetics of gemcitabine in Japanese cancer patients : the impact of a cytidine deaminase polymorphism. J Clin Oncol 2007 ; 25 : 32.
3) Kiyotani K, Uno S, Mushiroda T, et al. A genome-wide association study identifies four genetic markers for hematological toxicities in cancer patients receiving gemcitabine therapy. Pharmacogenet Genomics 2012 ; 22 : 229.
4) Zembutsu H, Yanada M, Hishida A, et al. Prediction of risk of disease recurrence by genome-wide cDNA microarray analysis in patients with Philadelphia chromosome-positive acute lymphoblastic leukemia treated with imatinib-combined chemotherapy. Int J Oncol 2007 ; 31 : 313.
5) Stearns V, Johnson MD, Rae JM, et al. Active tamoxifen metabolite plasma concentrations after coadministration of tamoxifen and the selective serotonin reuptake inhibitor paroxetine. J Natl Cancer Inst 2003 ; 95 : 1758.
6) Jin Y, Desta Z, Stearns V, et al. CYP2D6 genotype, antidepressant use, and tamoxifen metabolism during adjuvant breast cancer treatment. J Natl Cancer Inst 2005 ; 97 : 30.
7) Kiyotani K, Mushiroda T, Imamura CK, et al. Dose-adjustment study of tamoxifen based on CYP2D6 genotypes in Japanese breast cancer patients. Breast Cancer Res Treat 2012 ; 131 : 137.
8) Kiyotani K, Mushiroda T, Imamura CK, et al. Significant effect of polymorphisms in CYP2D6 and ABCC2 on clinical outcomes of adjuvant tamoxifen therapy for breast cancer patients. J Clin Oncol 2010 ; 28 : 1287.
9) Goetz MP, Knox SK, Suman VJ, et al. The impact of cytochrome P450 2D6 metabolism in women receiving adjuvant tamoxifen. Breast Cancer Res Treat 2007 ; 101 : 113.
10) Goetz MP, Suman VJ, Hoskin TL, et al. CYP2D6 metabolism and patient outcome in the Austrian Breast and Colorectal Cancer Study Group trial (ABCSG) 8. Clin Cancer Res 2013 ; 19 : 500.
11) Thompson AM, Johnson A, Quinlan P, et al. Comprehensive CYP2D6 genotype and adherence affect outcome in breast cancer patients treated with tamoxifen monotherapy. Breast Cancer Res Treat 2011 ; 125 : 279.
12) Rae JM, Drury S, Hayes DF, et al. CYP2D6 and UGT2B7 genotype and risk of recurrence in tamoxifen-treated breast cancer patients. J Natl Cancer Inst 2012 ; 104 : 452.
13) Regan MM, Leyland-Jones B, Bouzyk M, et al. CYP2D6 genotype and tamoxifen response in postmenopausal women with endocrine-responsive breast cancer : the breast international group 1-98 trial. J Natl Cancer Inst 2012 ; 104 : 441.
14) Brauch HB, Schroth W, Ingle JN, Goetz MP. CYP2D6 and tamoxifen : awaiting the denouement. J Clin Oncol 2011 ; 29 : 4589 ; author reply 4590.
15) Nakamura Y, Ratain MJ, Cox NJ, et al. Re : CYP2D6 genotype and tamoxifen response in postmenopausal women with endocrine-responsive breast cancer : the Breast International Group 1-98 trial. J Natl Cancer Inst 2012 ; 104 : 1264 ; author reply 1266.
16) Ratain MJ, Nakamura Y, Cox NJ. CYP2D6 genotype and tamoxifen activity : understanding interstudy variability in methodological quality. Clin Pharmacol Ther 2013 ; 94 : 185.
17) Goetz MP, Sun JX, Suman VJ, et al. Loss of Heterozygosity at the CYP2D6 Locus in Breast Cancer : implications for germline pharmacogenetic studies. J Natl Cancer Inst 2014 ; 107.
18) Kiyotani K, Mushiroda T, Zembutsu H, Nakamura Y. Important and critical scientific aspects in pharmacogenomics analysis : lessons from controversial results of tamoxifen and CYP2D6 studies. J Hum Genet 2013 ; 58 : 327.
19) Province MA, Goetz MP, Brauch H, et al. CYP2D6 genotype and adjuvant tamoxifen : meta-analysis of heterogeneous study populations. Clin Pharmacol Ther 2014 ; 95 : 216.

* * *

Breast Cancer RESEARCH

RESEARCH ARTICLE Open Access

VAV3 mediates resistance to breast cancer endocrine therapy

Helena Aguilar[1], Ander Urruticoechea[1,26], Pasi Halonen[2], Kazuma Kiyotani[3], Taisei Mushiroda[3], Xavier Barril[4,5], Jordi Serra-Musach[1,6], Abul Islam[7], Livia Caizzi[8,9], Luciano Di Croce[5,8,9], Ekaterina Nevedomskaya[10], Wilbert Zwart[10], Josefine Bostner[11], Elin Karlsson[11], Gizeh Pérez Tenorio[11], Tommy Fornander[12], Dennis C Sgroi[13], Rafael Garcia-Mata[14], Maurice PHM Jansen[15], Nadia García[16], Núria Bonifaci[1], Fina Climent[17], María Teresa Soler[17], Alejo Rodríguez-Vida[18], Miguel Gil[18], Joan Brunet[6], Griselda Martrat[1], Laia Gómez-Baldó[1], Ana I Extremera[1], Agnes Figueras[16], Josep Balart[16], Robert Clarke[19], Kerry L Burnstein[20], Kathryn E Carlson[21], John A Katzenellenbogen[21], Miguel Vizoso[22], Manel Esteller[5,22,23], Alberto Villanueva[16], Ana B Rodríguez-Peña[24], Xosé R Bustelo[24], Yusuke Nakamura[3,25], Hitoshi Zembutsu[25], Olle Stål[11], Roderick L Beijersbergen[2] and Miguel Angel Pujana[1*]

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.

* Correspondence: miguelangel.pujana@gmail.com
[1]Breast Cancer and Systems Biology Unit, Translational Research Laboratory, Catalan Institute of Oncology (ICO), Bellvitge Institute for Biomedical Research (IDIBELL), Avda. Gran via 199, L'Hospitalet del Llobregat, Barcelona 08908, Catalonia, Spain
Full list of author information is available at the end of the article

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).

The MYC-Vav3 wild-type and oncogenic expression constructs we used have been described previously [30,31].

Short hairpin RNA assays

For the *ESR1* and *VAV3* expression depletion assays, we used MISSION shRNA (Sigma-Aldrich). The lentiviral packaging, envelope, control and green fluorescent protein (GFP) expression plasmids (psPAX2, pMD2.G, non-hairpin-pLKO.1, scrambled-pLKO.1 and pWPT-GFP) were purchased from Addgene (Cambridge, MA, USA). Production and collection of lentiviral particles were carried out according to a modified Addgene protocol. Initial viral titers $>5 \times 10^5$/ml were confirmed by Lenti-X GoStix lentivirus testing (Clontech Laboratories, Mountain View, CA, USA), and supernatants were then concentrated by ultracentrifugation or with the Lenti-X Concentrator (Clontech Laboratories) and stored at −80°C. Concentrated viral supernatants were titrated for optimal inhibition of the target.

Genetic association study

The Institutional Review Board of the Institute of Medical Science (The University of Tokyo) approved the study, and written informed consent was obtained from all patients. A total of 240 patients with primary breast cancer, recruited by the Shikoku-*10 collaborative group (Tokushima Breast Care Clinic, Yamakawa Breast Clinic, Shikoku Cancer Center, Kochi University Hospital and Itoh Surgery and Breast Clinic), Kansai Rosai Hospital, Sapporo Breast Surgical Clinic and Sapporo Medical University Hospital from September 2007 to September 2008, were included in the genome-wide association study (GWAS), and 105 patients recruited by the same centers from October 2008 to January 2010 were included in the replication study. All patients were Japanese women pathologically diagnosed with ERα-positive invasive breast cancer. They received adjuvant tamoxifen monotherapy between 1986 and 2008. The data on primary breast cancer diagnoses or recurrences were confirmed by extraction from the patients' medical records. Patients without recurrence were censored at the date of the last clinical evaluation. Recurrence-free survival time was defined as the time from surgical treatment to the diagnosis of breast cancer recurrence (locoregional, distant metastasis or contralateral breast events) or death. Patients received tamoxifen 20 mg/day for 5 years. Treatment was stopped at the time of recurrence. Genomic DNA was extracted from peripheral blood or frozen breast tissue using the QIAGEN DNA Extraction Kit. In the GWAS, 240 patients were genotyped using the Illumina Human610-Quad BeadChip array (Illumina, San Diego, CA, USA). Quality control was assured by excluding single-nucleotide polymorphisms (SNPs) with low call rates (<99%) and those with a Hardy–Weinberg equilibrium *P*-value $<1.0 \times 10^{-6}$. SNPs with a minor allele frequency <0.01 were also excluded from the analyses. The multiplex PCR-based Invader assay (Third Wave Technologies, Madison, WI, USA) on ABI PRISM 7900HT (Applied Biosystems, Foster City, CA, USA) was used in the replication study. For statistical analysis, recurrence-free survival curves were estimated using the Kaplan–Meier method. The statistical significance of relationships between clinical outcomes and genetic variations was assessed using a logrank test.

Tumor series and immunohistochemistry

For the Bellvitge Institute for Biomedical Research (IDIBELL, Barcelona, Spain) cohort, the IDIBELL Ethics Committee approved the study and written informed consent was obtained from all patients. Twenty-nine patients treated with primary endocrine therapy before surgical excision of breast tumors were chosen from the clinical database activity of the Catalan Institute of Oncology (ICO) Breast Cancer Unit. All patients were postmenopausal and diagnosed with ERα -positive and HER2-negative breast cancer. The patients received treatment with either an ERα antagonist (tamoxifen or toremifene) or an aromatase inhibitor (letrozole or exemestane). Patients received therapy until a maximum response was achieved (range, 4 to 27 months), unless tumor progression was observed during a twice-monthly radiological and clinical assessment. After endocrine therapy was completed, full tumor excision was performed by either lumpectomy or radical mastectomy. Response was defined as the percentage of fibrosis and other patterns of pathological response attributable to tumor reduction at surgery. Tissue was obtained at surgery or biopsy, fixed in buffered formalin and processed for use in paraffin-embedded sections. A Stockholm cohort was analyzed in the Swedish study, which consisted of postmenopausal breast cancer patients enrolled in a randomized adjuvant trial between November 1976 and April 1990. The study design and long-term follow-up data were previously reported in detail [32]. Ethical approval for the Swedish study was obtained from the Karolinska Institute Ethics Council. Immunohistochemistry was performed using the heat-mediated antigen retrieval method with citrate buffer. The VAV3 polyclonal antibody used for immunohistochemistry has been described previously [30]. Scoring of the immunohistochemical results was performed in a blind and independent manner by two pathologists.

Results

A chemical compound screen identifies YC-1 as reducing viability of cellular models of acquired resistance

Acquired resistance to aromatase inhibitors in postmenopausal women can be modeled in MCF7-LTED cells [17]. Using this model, we carried out a cell-based chemical compound screen out to identify potential therapeutic

strategies that could prevent and/or overcome resistance. More than 1,200 compounds were assessed for their differential effect on the viability of MCF7-LTED cells (as defined by MTT-based assays) relative to the parental MCF7 cells. Thirteen compounds showed higher relative inhibition in MCF7-LTED cells (Figure 1A and Additional file 1: Table S1). Subsequent validation using independent cell cultures and compound solutions identified YC-1 (3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole) as being the most effective, with a 27-fold difference in the half-maximal inhibitory concentration (IC_{50}) was revealed between MCF7-LTED and MCF7 cells (4.9 μM and 131 μM, respectively) (Figure 1B).

YC-1 is a direct activator of soluble guanylyl cyclase (sGC) Thus, increased levels of cGMP were observed in cell cultures exposed to this compound (Figure 1C). Next, the effect of YC-1 on a collection of breast cancer cell lines was examined. IC_{50} values <10 μM were obtained for several cell lines (Additional file 2: Table S2), including MCF7-LCC9 and MCF7-LY2, which correspond to models of acquired resistance to fulvestrant and to the raloxifene analogue LY-117018, respectively. These cell lines also showed cross-resistance to tamoxifen [33,34].

Intriguingly, an activator of sGC derived from the structural development of YC-1, BAY 41-2272, displayed a lower differential inhibitory effect (Additional file 3: Figure S1A). In addition, assessment of another sGC activator, A-350619, and complementary evaluation of an inhibitor of phosphodiesterase activity did not reveal the expected differences (Additional file 3: Figure S1B). Although YC-1 has been used extensively in cancer research, including preclinical studies in breast cancer [35], it is unclear whether a direct target beyond sGC exists.

YC-1 binds to estrogen receptor α

To investigate novel molecular targets of YC-1, the chemical structure of YC-1 was used to query the ChEMBL [36] and BindingDB [37] databases for similar compounds with reported biological activity. Strikingly, WAY-169916, which has been shown to bind ERα [38], and a series of

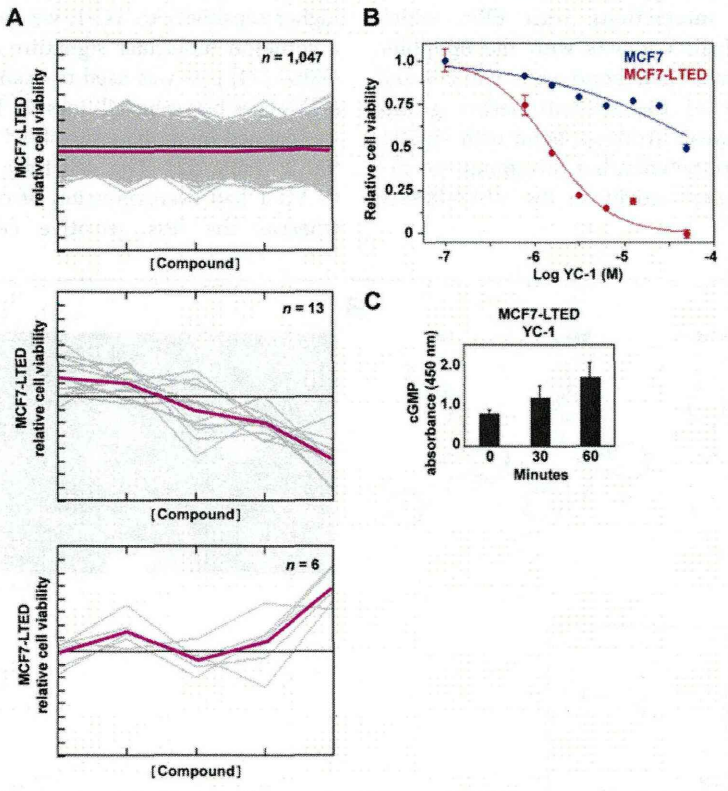

Figure 1 A chemical compound screen identifies an activator of soluble guanylyl cyclase as reducing the viability of long-term estrogen-deprived MCF7 cells. (A) Compounds with no differential effect (top panel), with an inhibitory effect on the long-term estrogen deprivation (LTED) of MCF7 cells (MCF7-LTED cells) relative to MCF7 cells (middle panel) and with an inhibitory effect on MCF7 relative to MCF7-LTED cells (bottom panel). The *y*-axis indicates relative viability of MCF7-LTED cells, and the *x*-axis indicates increasing concentrations of the compounds. Colored lines indicate average values. **(B)** Corroboration of the inhibitory effect of YC-1 on MCF7-LTED cells. **(C)** Time-dependent increase of cGMP in MCF-LTED cells exposed to YC-1.

related compounds [23,39] were retrieved at a 60% similarity cutoff value. WAY-169916 is an unusual ERα ligand: It is able to bind ERα, leading to its constrained or unconstrained conformation (responsible for partial agonist activity, binding mode 1, or for an antagonist effect, binding mode 4, respectively) [23]. The relative preferences for these ERα conformations explain the graded activities across the compound series [23]. Thus, compound 6 (hereinafter referred to as WAY6) was the WAY-169916 analogue most similar to YC-1 (Figure 2A), which was found to lead preferentially to the unconstrained conformation [23].

Molecular docking was used to examine the potential binding mode of YC-1 to ERα. The predicted mode was very similar to binding mode 1 of WAY6 when docked in both the partially constrained (Figure 2B) and unconstrained (Additional file 4: Figure S2A) conformations. Although a binding mode similar to binding mode 4 was also found to be possible in the latter conformation (Additional file 4: Figure S2B), it had a lower score. As shown in Figure 2B, the binding mode of YC-1 was almost perfectly aligned with WAY6 and maintained the main molecular interactions with ERα, which comprised van der Waals contacts with the lipophilic cavity and a double hydrogen bond with Glu353 and Arg394. The absence of the trifluoromethyl group, which is engaged in a weak hydrogen bond with His524, could cause some loss of potency, but this group was not essential for the biological activity in the WAY-169916 series [38].

The MCF7-LTED model was previously shown to be less sensitive to fulvestrant than the parental MCF7 [17], and this difference appeared to be coherent with the described differential ERα binding mode of fulvestrant relative to WAY-169916 [23]. Next, to validate the binding prediction between YC-1 and ERα, we performed an agonist fluoligand assay, which showed the competition with fluorescein-labeled estradiol. The results of this assay revealed YC-1 IC_{50} and K_i values of 33 μM and 26 μM, respectively (Figure 2C), which are in agreement with the inhibitory effects observed in the cell lines (Additional file 2: Table S2). Intriguingly, two of the cell lines that showed relative inhibition by YC-1 (AU565 and SKBR3) are generally considered ERα-negative [18]. Thus, the combined targeting of at least sGC and ERα would make it difficult to interpret the phenotypic consequences of therapy based on YC-1. Consequently, the specific molecular perturbations mediated by YC-1 should be identified.

Molecular perturbations mediated by YC-1

Having defined breast cancer cell lines with relatively higher sensitivity to YC-1, we evaluated the existence of a common molecular signature among these lines. The GSEA [24] tool was used to examine gene set expression differences between cell lines of "high" and "low" sensitivity (defined by an IC_{50} threshold of 10 μM) (Additional file 2: Table S2). The cell lines with higher sensitivity to YC-1 had overexpression of cell cycle pathway genes, whereas the less sensitive cell lines cells showed

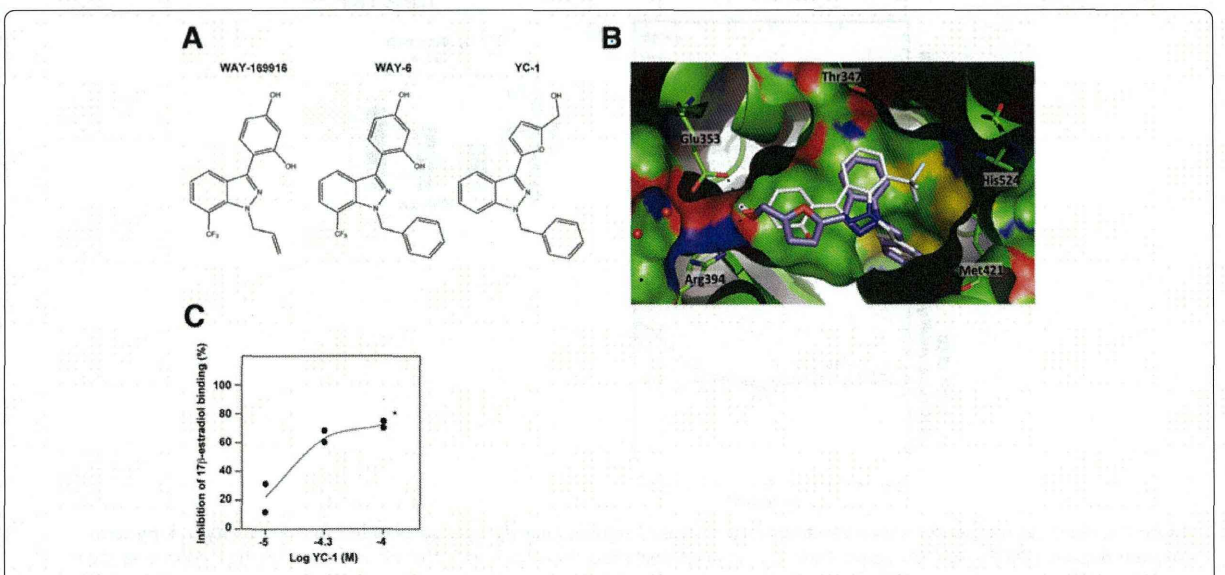

Figure 2 YC-1 binds to estrogen receptor α. (A) Chemical structures of WAY-169916, WAY6 and YC-1. **(B)** Predicted binding mode of YC-1 (purple) in the partially constrained conformation of estrogen receptor α (ERα) (chain A, Protein Data Bank code 3OS8 [Swiss-Prot:P03372]). The binding mode of WAY6 (white sticks) is shown as a reference. **(C)** The results of the ERα agonist fluoligand assay using YC-1 are shown, along with the concentration–inhibition curve with duplicates. *YC-1 was not completely soluble at concentrations >100 μM.

overexpression of ribosome pathway genes, among others (Additional file 5: Figure S3 and Additional file 6: Table S3). These results are consistent with the increased dependence of the cell cycle and proliferation highlighted in endocrine therapy resistance in previous studies [40].

To examine the potentially selective YC-1 mechanism of action in models of acquired resistance, the levels and subcellular localization of ERα were examined. Although both were altered by YC-1 treatment, no substantial differences were observed between MCF7 and MCF7-LTED cells (Additional file 7: Figure S4). Subsequently, whole-genome expression data were obtained for both cell lines in basal and YC-1 exposure conditions. Consistent with the results described above, expression of the ribosome pathway was clearly differentiated between MCF7 and MCF7-LTED cells in basal conditions and with exposure to YC-1 (Additional file 8: Figures S5A and S5B and Additional file 9: Table S4). Exposure to YC-1 led to a significant alteration of the cell cycle pathway in both settings (Additional file 8: Figure S5B). Accordingly, targets of a central positive regulator of the cell cycle, E2F1, were revealed to be significantly underexpressed with exposure to YC-1 (Additional file 10: Table S5). Protein analysis revealed a larger relative decrease in the expression of this transcription factor in MCF7-LTED cells exposed to YC-1 (Additional file 8: Figure S5C). Together, these results indicate that YC-1 may reduce the potential of cell proliferation in such a way that MCF7-LTED cells are relatively more sensitive.

Having observed pathway differences, we aimed to identify the largest gene expression differences between MCF7 and MCF7-LTED cells exposed to YC-1. Thus, we defined a twofold or greater change in MCF7-LTED cells (between basal and YC-1 conditions), and a 1.5-fold or greater expression change in MCF7 cells. In this analysis, we identified 19 and 8 genes, respectively, that were down- and upregulated in MCF7-LTED cells exposed to YC-1 (Figure 3A). Consistent with the binding of YC-1 to ERα, many of these perturbed genes corresponded to loci that are differentially regulated by ERα in endocrine therapy resistance. Analysis of ChIP data of responsive and nonresponsive breast tumors [7] revealed significant differential ERα binding at several of these loci, with 10 of 27 showing increased binding in the nonresponsive setting (Figure 3B). From among this set, *VAV3* was further included in a 271-gene list associated with poor clinical outcome [7]. Following on from these observations, we performed ERα ChIP assays using extracts of MCF7 and MCF7-LTED cells in basal (DMSO) or YC-1-exposed conditions. By this method, we found two *VAV3* sites with significant binding of ERα relative to the nonspecific immunoglobulin control (Figure 3C). In addition, both sites showed ERα sensitivity (that is, lower binding) with exposure to YC-1, and one site (binding site 1) had significantly more binding (2.4-fold) in MCF7-LTED cells than in MCF7 cells (Figure 3C). Similarly, specific analysis of these sites in the original breast cancer data set [7] showed substantial ERα binding in nonresponder and metastasis cases (Figure 3D). Consistent with these observations, and among the potential ERα downstream effectors identified above, *VAV3* showed the highest expression associated with *ESR1* in breast tumors [25] (mutual information = 0.23, $P < 0.001$). Moreover, shRNA-mediated depletion of ERα revealed a decrease of VAV3 in MCF7-LTED cells, but not in parental MCF7 cells (Figure 3E). Collectively, these results indicate that VAV3 function may be critical in endocrine therapy resistance governed by ERα transcriptional regulatory plasticity.

VAV3 is perturbed by YC-1 and determines acquired resistance

Consistent with the observations described above, total and pY173 VAV3 (whose phosphorylation regulates activity) [31,41] decreased in MCF7-LTED cells, but not in MCF7 cells, exposed to YC-1 (Figure 4A). According to the position of VAV3 in its canonical signaling pathway, EGFR levels were decreased in both MCF7 and MCF7-LTED cells exposed to YC-1, but ERK1/2 phosphorylation was decreased only in MCF7-LTED cells exposed to YC-1 (Figure 4A). In addition, PAK1 and RAC1 levels were not altered under these conditions (Figure 4A). Similarly to MCF7-LTED, MCF7-LCC9 cells exposed to YC-1 showed loss of expression of VAV3, but not of PAK1 or RAC1 (Figure 4B). Nonetheless, depletion of VAV3 reduced RAC1 activity in both MCF7 and MCF7-LTED cells, and this alteration was recovered through reconstitution using a shRNA-resistant MYC-Vav3 expression construct (Additional file 11: Figure S6).

Next, lentivirus-mediated transduction of shRNAs directed against expression of *VAV3* significantly reduced the viability of MCF7-LTED and MCF7-LCC9 cells relative to MCF7 cells ($P < 0.05$) (Figure 4C). A clonogenic assay also indicated relative loss of viability of MCF7-LTED cells, and, to a lesser extent, MCF7-LCC9 cells, with shRNA-mediated depletion of VAV3. Differences relative to MCF7 cells were <0.8-fold (Figure 4D). Reconstitution with MYC-Vav3 significantly recovered proliferation in MCF7-LTED cells, although not to the level of the shRNA control assay (Figure 4E), which might have been due to Vav3 overexpression (Additional file 11: Figure S6) and/or to specific roles of splicing variants. Reconstitution with Vav1 or Vav2 could not be assessed, as the overexpression of the murine counterparts caused cell death (data not shown). Analysis of poly(ADP-ribose) cleavage did not reveal substantial differences among the cell lines (Figure 4F), which further indicates that YC-1 primarily inhibits cell proliferation. Thus, a reduction in E2F1 was observed in MCF7-LTED cells exposed to 2 μM YC-1

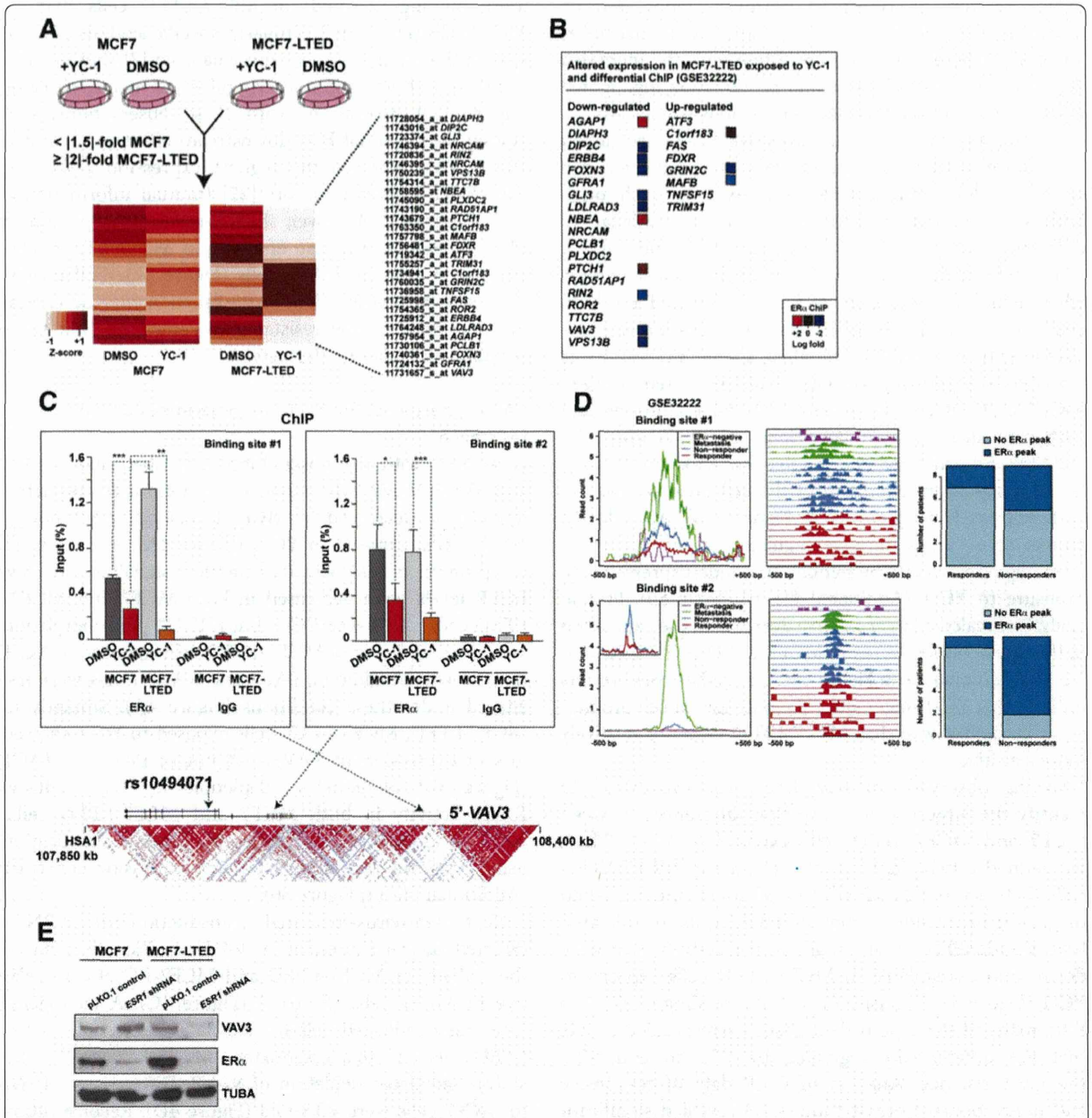

Figure 3 Genes specifically perturbed by YC-1 in long-term estrogen deprivation of MC7 cells and their link to the response to endocrine therapy. (A) Genes whose expression change differentiates the effect of YC-1 between long-term estrogen deprivation of MCF7 (MCF7-LTED) cells and MCF7 cells. The bottom heatmap shows the normalized expression differences for the probes and genes that passed the defined thresholds. DMSO, Dimethyl sulfoxide. **(B)** Logarithmic fold changes between the responder and nonresponder breast tumors for the genes (Gene Expression Omnibus data set [GSE:32222]) shown in **(A)** determined by chromatin immunoprecipitation (ChIP) assay. **(C)** ChIP assay results for estrogen receptor α (ERα) and immunoglobulin G (IgG) at two sites in the *VAV3* locus, both for MCF7 and MCF7-LTED cells with or without exposure to YC-1 (significant differences are indicated by asterisks: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). The bottom graph shows the genomic locus with the linkage disequilibrium structure in Japanese individuals found in HapMap and the relative position of the variant rs10494071 (presented below). **(D)** Detailed analysis of the Gene Expression Omnibus [GSE:32222] data set for the two sites depicted above. Left panels show the normalized average intensity of ERα binding ±500 bp around the sites in different sample sets as depicted in the insets. Middle panels show relative ERα binding in the above sites across 23 breast cancer samples. Right panels are graphs showing the number of cases with or without an ERα binding event (peak) in nonresponders and responders. Top right panels show that 44% of the nonresponders had ERα binding, whereas only two (22%) of nine responders had binding. Bottom right panels show that 78% of the nonresponders had ERα binding, whereas only one (11%) of nine responders had binding. **(E)** Short hairpin RNA (shRNA)–mediated depletion of ERα led to a decrease in VAV3 levels in MCF7-LTED cells, but not in MCF7 cells.

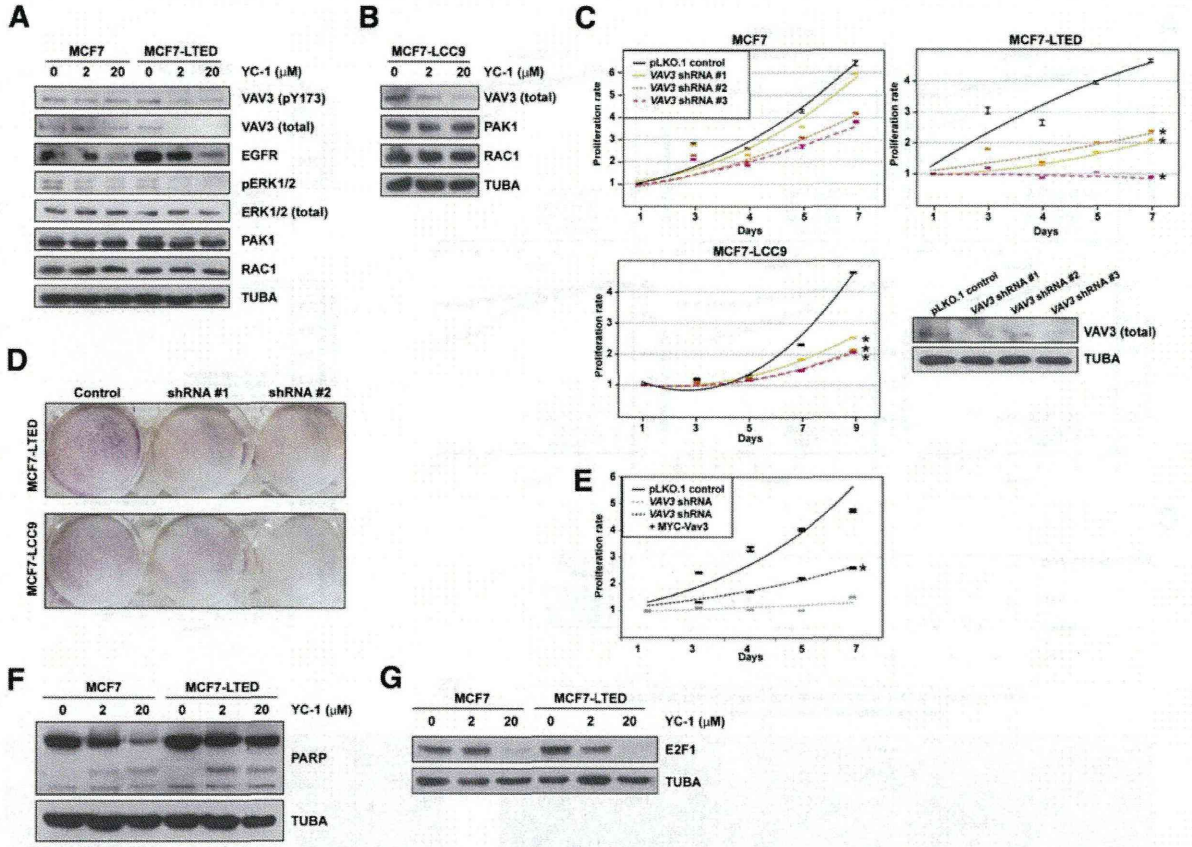

Figure 4 Study of VAV3 in models of acquired resistance to endocrine therapies. (A) Western blot analysis results for VAV3 (pT173 and total, top panels), signaling components and control tubulin α (TUBA) from MCF7 cells and long-term estrogen deprivation of MCF7 (MCF7-LTED) cells in basal and YC-1 exposure conditions. pERK, phosphorylated extracellular signal-regulated protein kinase. **(B)** Western blot analysis results for VAV3, PAK1 and RAC1, as well as control TUBA, in MCF7-LCC9 cell extracts from basal and YC-1 exposure conditions. **(C)** Short hairpin RNA (shRNA)–mediated depletion of VAV3 reduces the viability, in methylthiazol tetrazolium (MTT)–based assays) of MCF-LTED and MCF7-LCC9 cells relative to parental MCF7. The asterisks correspond to significant differences ($P < 0.05$) in the viability rate (slope of the trends (shown), including three replicas, and relative to the control pLKO.1). The bottom right panel shows the results of short hairpin RNA (shRNA)–mediated depletion of VAV3 relative to the negative control assay. **(D)** shRNA-mediated depletion of VAV3 reduces the viability (clonogenic assays) of MCF-LTED and MCF7-LCC9 cells. **(E)** Reconstitution with MYC-Vav3 partially recovers viability of MCF-LTED cells. The asterisk corresponds to a significant difference ($P < 0.05$) relative to shRNA-mediated depletion of VAV3. **(F)** No substantial differences in poly(ADP-ribose) (PARP) cleavage were observed between MCF7 and MCF7-LTED cells exposed to YC-1. **(G)** Top panel, reduction of E2F1 expression in MCF7 and MCF7-LTED cells exposed to YC-1; MCF7-LTED, but not the parental MCF7, show a reduction at 2 μM YC-1. Bottom panel, control TUBA.

(Figure 4G). These results are also consistent with those of VAV3 depletion in prostate cancer cells [42].

VAV3/VAV3 association with clinical outcome

Having identified VAV3 as a determinant of acquired resistance in cellular models, we next assessed its relevance in the clinical scenario. By examining the results of a Japanese GWAS regarding response to tamoxifen [43], we identified 20 SNPs in *VAV3* that are associated with clinical outcomes (logrank *P*-values <0.05) (Additional file 12: Table S6). In a subsequent assessment of an independent patient series, the associations in several SNPs were replicated. Of the variants analyzed, rs10494071 showed the strongest association in the combined analysis ($P = 8.4 \times 10^{-4}$) (Figure 5A). The rs10494071 variant is located within *VAV3* intron 19 (Figure 3C) and may represent an expression quantitative trait locus. In a study of monocytes [44], the minor allele was associated with lower expression levels of *VAV3* ($P = 2.2 \times 10^{-11}$).

An association between the rs10494071 minor allele, which in turn was associated with a better tamoxifen response (Figure 5A), and lower germline expression of *VAV3* seemed to be consistent with mediation of resistance by this signaling component. Next, we analyzed an expression data set from ERα-positive breast cancer patients treated with tamoxifen [45]. The results of this

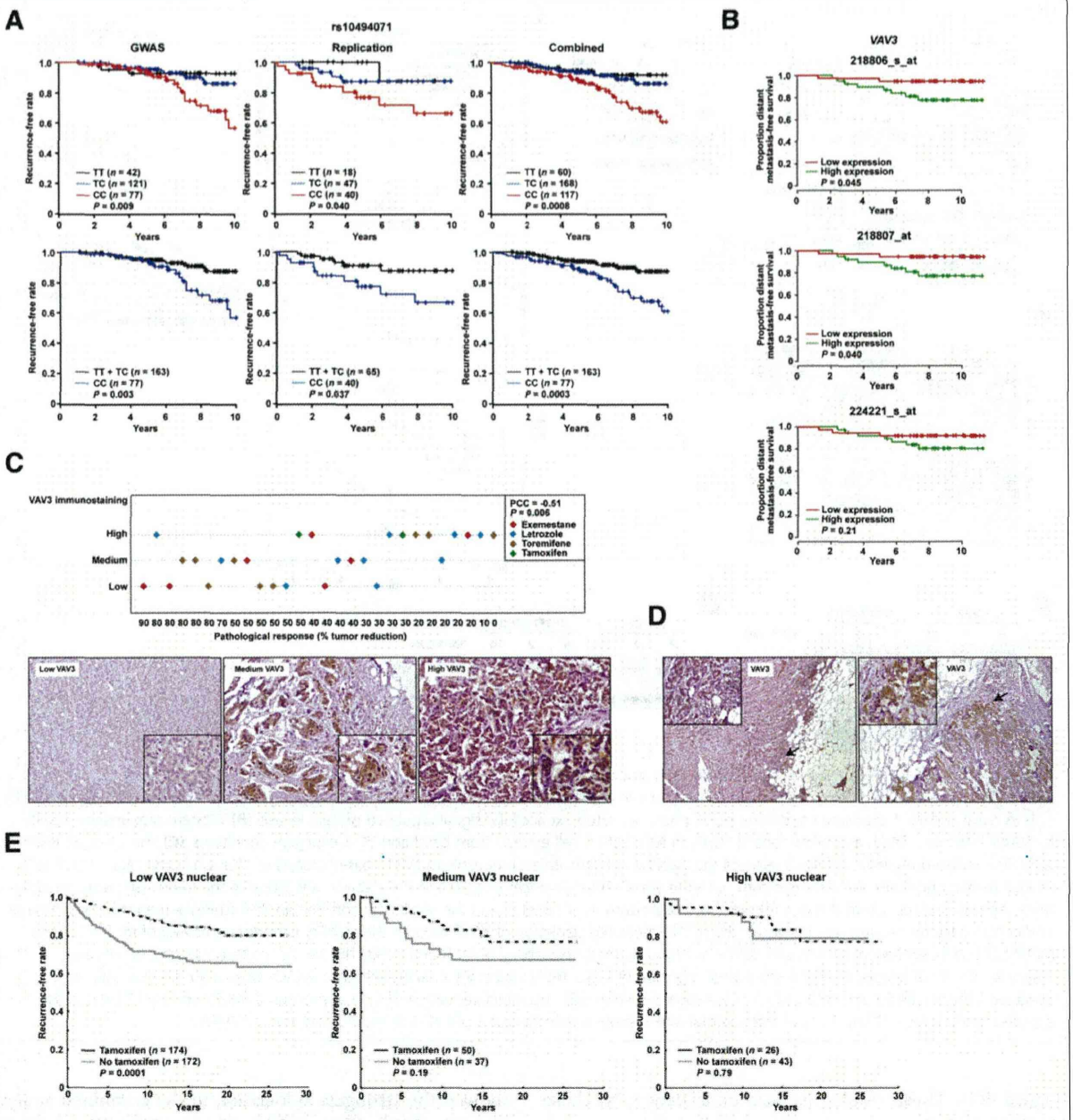

Figure 5 Association between *VAV3*/VAV3 and clinical response to endocrine therapies. (A) Association between rs10494071 and the response to tamoxifen in Japanese patients. The Kaplan–Meier curves show the recurrence-free survival rate over time (years) and between patients stratified according to the three possible rs10494071 genotypes (TT, TC and CC). The logrank test *P*-values are shown. **(B)** Association between *VAV3* tumor expression and response to tamoxifen (Gene Expression Omnibus data set [GSE:9195]). Graphs show the proportion of patients with metastasis-free survival over time (years) and stratified according to high (above the median) or low (below the median) *VAV3* expression in breast tumors. The results shown are for three *VAV3* microarray probes. Logrank *P*-values are shown. **(C)** Association between VAV3 tumor expression and the pathological response to endocrine therapies. Top panel: Graph depicting the correlation between VAV3 immunostaining score and pathological response (percentage of tumor reduction posttreatment). Bottom panels: Representative examples of the three immunostaining scores. Insets: Cells with nuclear and cytoplasmic positivity. PCC, Pearson's correlation coefficient. **(D)** Examples of increased VAV3 staining at the invasive tumor front. **(E)** Graphs showing the Kaplan–Meier curves for patients who did or did not receive tamoxifen in the Swedish study. Panels from left to right show the results for patients whose tumors revealed low, medium or high nuclear VAV staining. The logrank test *P*-values are shown.

analysis also suggest that low *VAV3* expression might be associated with better outcomes (logrank *P*-values <0.05 for two probes) (Figure 5B).

Complementarily to the germline association study, we assessed a series of 29 breast tumors, which had been collected by biopsy after endocrine therapy, for VAV3 expression by immunohistochemistry. A negative correlation (Pearson's correlation coefficient = −0.51, $P = 0.006$) was revealed between the scores of VAV3 staining (low, medium or high) and the pathological response to therapy (that is, tumor reduction) (Figure 5C). These 29 cases included a variety of endocrine therapies, but no bias with respect to therapy type was apparent. Moreover, consistent with the role of VAV3 in promoting breast cancer progression [30], comparatively higher staining was observed at the tumor fronts (Figure 5D). In addition, higher staining scores could be linked to nuclear positivity (insets in Figures 5C and 5D), and, intriguingly, this localization has previously been shown to be necessary for the function of the androgen receptor in prostate cancer [46].

To further assess the above-described immunohistochemical association, we performed an independent tumor tissue microarray analysis with detailed molecular, histopathological and clinical information [32,47-49]. The results of this study revealed a significant association between the benefit of tamoxifen therapy and low nuclear VAV3 staining. Conversely, high nuclear VAV3 was not associated with tamoxifen benefit (Figure 5E). In addition, nuclear VAV3 was found to be positively correlated with markers of poor therapy response, particularly phospho-Ser305 ERα and nuclear phospho-Ser473 AKT (*P*-values <0.01) (Table 1) [48]. These correlations, and those between cytoplasmic VAV3 and tumor size and grade, as well as ERα/PR status, were analogous to those previously observed for nuclear and cytoplasmic PAK1 [49] (Table 1). The interpretation of the negative and positive correlations, respectively, of phospho-Ser65 4EBP1 and nuclear S6K2 [47] with nuclear VAV3 may be more complex; indeed, we observed a modest correlation between cytoplasmic VAV3 and phospho-Ser2448 mammalian target of rapamycin (mTOR) ($P = 0.034$). Together, these data reinforce the link between the VAV3 signaling axis and resistance to endocrine therapy.

Therapeutic strategy based on VAV3 evidence

Therapy based on YC-1 should be discouraged because of its multiple targets. In addition, to date, no compounds that specifically target VAV proteins have been identified. Having identified a critical role for VAV3, we hypothesized that compounds whose IC_{50} value is inversely correlated with *VAV3* expression might represent promising therapeutic strategies for the endocrine therapy–resistant setting. To test this hypothesis, we analyzed data from the Genomics of Drug Sensitivity in Cancer project [50]. In this analysis, we found that the strongest positive and negative IC_{50} correlations with *VAV3* expression across all cancer cell lines were for thapsigargin and erlotinib, respectively (Figure 6A). These correlations appeared robust in the analysis of breast cancer only (Figure 6A, insets). Notably, the finding that *VAV3* expression opposes the effect of thapsigargin is congruent with those of previous studies of VAV proteins [51,52]. Conversely, erlotinib inhibits EGFR, which has been extensively linked to endocrine therapy resistance [1,53]. Importantly, VAV3 functions downstream of receptor protein tyrosine kinases, which include EGFR [54]. In accordance with these

Table 1 VAV3 nuclear and cytoplasmic expression in relation to other tumor markers assessed by the Spearman's rank correlation[a]

	Nuclear VAV3, *n* (%)				Cytoplasmic VAV3, *n* (%)			
Score	**–**	**1+**	**2+**	**3+**	**–**	**1+**	**2+**	**3+**
All tumors	607 (85.9)	3 (0.4)	43 (6.1)	54 (7.6)	229 (32.4)	154 (21.8)	215 (30.4)	109 (15.4)
Tumor size (>20 mm vs. ≤20 mm)	$R_s = -0.04$, $P = 0.30$				$R_s = 0.13$, $P = 0.0009$			
Tumor grade (1, 2 or 3)	$R_s = -0.09$, $P = 0.026$				$R_s = 0.16$, $P = 0.00007$			
ERα (>10% vs. ≤10%)	$R_s = 0.05$, $P = 0.20$				$R_s = -0.12$, $P = 0.002$			
PR (>10% vs. ≤10%)	$R_s = 0.06$, $P = 0.14$				$R_s = -0.15$, $P = 0.0002$			
HER2 status (positive vs. negative)	$R_s = 0.00$, $P = 0.99$				$R_s = 0.05$, $P = 0.16$			
Phospho-Ser167 ERα (%)	$R_s = 0.12$, $P = 0.002$				$R_s = -0.11$, $P = 0.003$			
Phospho-Ser305 ERα (%)	$R_s = 0.11$, $P = 0.006$				$R_s = -0.09$, $P = 0.016$			
PAK1 (cytoplasm 0 to 3 positivity)	$R_s = -0.07$, $P = 0.077$				$R_s = 0.12$, $P = 0.003$			
Phospho-Ser473 AKT (nuclear %)	$R_s = 0.18$, $P < 0.00001$				$R_s = -0.20$, $P < 0.00001$			
Phospho-Ser2448 mTOR (high vs. low)	$R_s = 0.06$, $P = 0.11$				$R_s = -0.08$, $P = 0.034$			
Phospho-Ser65 4EBP1 (cytoplasm 0 to 2 positivity)	$R_s = -0.15$, $P = 0.0001$				$R_s = 0.19$, $P < 0.00001$			
S6K2 (nuclear %)	$R_s = 0.21$, $P < 0.00001$				$R_s = -0.26$, $P < 0.00001$			

[a]ERα, Estrogen receptor α; mTOR, mammalian target of rapamycin; PR, Progesterone receptor. $P < 0.05$ values are statistically significant.