Published in final edited form as:

Ann Surg Oncol. 2015 December ; 22(13): 4287–4294. doi:10.1245/s10434-015-4597-x.

Receptor Tyrosine Kinase Expression Predicts Response to Sunitinib in Breast Cancer

Philip M. Spanheimer, MD, Allison W. Lorenzen, MD, James P. De Andrade, MD, Mikhail V. Kulak, PhD, Jennifer C. Carr, MD, George W. Woodfield, MS, Sonia L. Sugg, MD, and Ronald J. Weigel, MD, PhD

Department of Surgery, University of Iowa, Iowa City, IA

Ronald J. Weigel: ronald-Weigel@uiowa.edu

Abstract

Background—Preliminary data indicate that tyrosine kinase inhibitors (TKIs) function through rearranged during transfection (RET) in breast cancer. However, TKIs are not specific and can block several receptor tyrosine kinases (RTKs). This study used cell lines and primary breast cancer specimens to determine factors associated with TKI response.

Methods—Proliferation was assessed after short interfering RNA knockdown with or without sunitinib in breast cancer cell lines by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Breast cancer tissue and matched normal breast was obtained from 30 women with invasive breast carcinoma. Gene expression was assessed by reverse transcriptase-polymerase chain reaction. Fresh tissue was treated in vitro with sunitinib or control media for 30 min, and response was assessed by phosphorylation-specific western blot.

Results—The RTKs including epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR1-3), platelet-derived growth factor receptor (PDGFRa/b), and Kit were overexpressed in triple-negative breast tumors relative to HER2- and estrogen receptor-alpha (ERa)-positive tumors and normal breast tissue. Knockdown of EGFR reduced in vitro proliferation in MCF-7 and MDA-MB-231 but not in SKBR-3 or ZR-75-1 breast cancer cells. With the exception of RET, response to sunitinib was independent of RTK expression in all four cell lines. Both ERa-positive and low-EGFR-expressing tumors had an increased in vitro sunitinib response, as determined by alteration of Erk activation. Expression of other RTKs and additional clinical factors were not associated with response.

Conclusion—Triple-negative breast cancers overexpress RTKs but have decreased in vitro response to the TKI sunitinib. In addition to RET, TKIs that block EGFR may increase the therapeutic efficacy of TKIs in breast cancer.

Breast cancer is the most common cancer and the second most common cause of cancer-related death in women.¹ The expression of estrogen receptor-alpha (ERa), progesterone

Presented orally at the Society of Surgical Oncology 67th Annual Cancer Symposium, Phoenix, AZ, March 2014.

Electronic supplementary material The online version of this article (doi:10.1245/s10434-015-4597-x) contains supplementary material, which is available to authorized users.

DISCLOSURE None of the authors have any conflict of interest to declare related to this work.

receptor (PR), and human epidermal growth factor receptor 2 (HER2) has been used clinically to classify breast cancers into established molecular subtypes. Each subtype has a functionally distinct biology characterized by different patterns of gene expression.² Furthermore, each subtype has distinct clinical behavior including response to treatment and patterns of recurrence and survival.^{3–5} Based on the success of identifying biologically distinct tumors through subtype classification and using that classification to stratify patients for treatment, additional markers of tumor biology and response to treatment are being sought.

The rearranged during transfection (RET) proto-oncogene, a negative prognostic indicator in breast cancer,⁶ is expressed in association with ER α .^{7–9} Previously we have shown that disruption of RET-signaling pathways leads to increased apoptosis and decreased growth of luminal breast cancer cells both in vitro in a xenograft model.^{10,11} Additionally, in primary breast cancer specimens, in vitro treatment with the RET inhibitor sunitinib leads to decreased phosphorylation of the RET downstream mediator Erk, with a significantly greater effect in RET-expressing tumors.¹¹ However, sunitinib is a nonselective tyrosine kinase inhibitor (TKI) with anti-RET activity and additional activity against other receptor tyrosine kinases (RTKs) including vascular endothelial growth factor receptor (VEGFR1-3), platelet-derived growth factor receptor (PDGFR)a, PDGFRb, and Kit, with relatively poor sensitivity to EGFR.¹²

This study sought to determine the expression pattern and physiologic effects of RTK expression in breast cancer. Furthermore, it sought to determine the relative contributions of RTKs in mediating the antitumor activity of sunitinib in order to understand the mechanisms of action and to identify targets for therapy and markers of response that could be used to select breast cancer patients for TKI treatment.

METHODS

Cell Lines

Cell lines MCF-7, SKBR-3, MDA-MB-231, and ZR-75-1 were obtained from the American Type Culture Collection (ATCC) and grown as follows: MCF-7 using Dulbecco modified Eagle medium (DMEM), SKBR-3 using McCoy's 5A medium, MDA-MB-231 using Leibovitz's L-15 medium, and ZR-75-1 using Roswell Park Memorial Institute (RPMI) medium. All media were supplemented with 10 % fetal bovine serum (FBS), 100 U/ml penicillin, 100 μ g/ml streptomycin, and 1 % GlutaMAX (Ambion, Calsbad, CA, USA).

Tissue Samples

Fresh tumor samples and corresponding normal breast tissue were collected from 30 patients with invasive breast cancer from the University of Iowa Breast Molecular Epidemiologic Resource (BMER), an institutional review board-approved tumor bank, as previously described.¹¹ De-identified patient information including age, receptor status, nodal status, and clinical and pathologic staging was obtained through the BMER. Tissue was obtained in the surgical pathology suite within 15 min after resection and transported immediately to the laboratory.

Tissue specimens were minced sharply into approximately 1-mm pieces and aliquoted in equal distribution for RNA or protein analysis. Samples for RNA were immediately placed in RNeasy lysis buffer (Qiagen) and frozen at -80°C . Samples for protein analysis were placed immediately in minimal media containing 500 nmol sunitinib (Sigma Aldrich, St Louis, MO) or control media. Samples were incubated at 37°C for 30 min and placed on ice for protein extraction as described in the following sections.

RNA Extraction and Real-Time Polymerase Chain Reaction

Primary tumor RNA was extracted using the Trizol method. The RNA was quantified using TaqMan primer/probes for RET, epidermal growth factor receptor (EGFR), VEGFR1, VEGFR2, VEGFR3, PDGFRa, PDGFRb, Kit, Erk, and dual specificity phosphatase 4 (DUSP4) (Life Technologies, Grand Island, NY), with 18S (Life Technologies, Grand Island, NY) as the endogenous control. Transcriptase-polymerase chain reaction (RT-PCR) was performed in technical triplicate for each sample.

MTT Proliferation Assay

Cell lines in antibiotic-free media were treated with small interfering RNA (siRNA) to RET, EGFR, VEGFR1, VEGFR2, VEGFR3, PDGFRa, PDGFRb, or Kit (Ambion) for 72 h, and after knockout, cells were plated at 5000 cells per well in technical triplicate. Cells were allowed to adhere overnight, and medium containing the appropriate drug or control media was added. Samples were allowed to grow for 72 h, after which they were incubated with MTT (0.5 mg/ml) for 3 h at 37°C . Crystals solubilized in 10 % sodium dodecyl sulfate (SDS) in 0.01 mol/L HCl for 3 h at 37°C and read on an Infinite 200 Pro plate reader (Tecan, Switzerland) at an absorbance wavelength of 570 nm. Samples were averaged over at least three biologic replicates.

Western Blot

Total protein was isolated from the aforementioned samples using radioimmunoprecipitation assay (RIPA) with Halt protease inhibitor cocktail (Thermo Scientific, Rockford, IL, USA) and PhosStop phosphatase inhibitor (Roche, Indianapolis, IN, USA). The antibodies used for Western blot were RET (Cell Signaling Technologies, Danvers, MA, USA), Erk (Cell Signaling), p-Erk (Cell Signaling), AKT (Cell Signaling), p-AKT (Cell Signaling), GREB-1 (Abcam, Cambridge, MA, USA), and GAPDH (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Erk activation was determined by quantifying protein levels using ImageJ (<http://rsb.info.nih.gov/ij/>) and calculating the fraction of phosphorylated Erk relative to nonphosphorylated Erk. The response to sunitinib was determined by comparing this fraction between drug and control treated samples.

Statistical Analysis

Statistical analysis was performed using the two-sided Student's *t* test for continuous variables, with the paired test applied where appropriate. Frequency association of categorical variables was performed using Fisher's exact test for comparisons between two groups. All statistical calculations were performed using R (www.r-project.org). Statistical significance was defined as a *p* value lower than 0.05.

RESULTS

Patient Demographics

Tissue samples of tumor and normal breast tissue were obtained from 30 women with stages 1 and 2 invasive breast cancer. Patient demographics showed no significant difference in patient age, stage, or rate of positive nodes between the tumor subtype groups (Table 1).

Receptor Tyrosine Kinase Genes are Overexpressed in Triple-Negative Breast Cancer

Previous studies demonstrated the association of RET expression and the ER α -positive breast cancer subtype.⁷⁻⁹ To assess the expression levels of other RTKs, RT-PCR was performed on tumor and normal breast samples, and tumor sample results were stratified by breast cancer subtype (Fig. 1). The findings showed EGFR expression to be significantly higher in triple-negative tumors (mean expression, 100 %) compared with HER2 overexpression (7 %; $p = 0.03$), ER α -positive tumors (12 %; $p = 0.01$), and normal breast tissue (5 %, $p = 0.004$). In triple-negative tumors, VEGFR1, VEGFR2, PDGFR α , PDGFR β , and Kit were similarly overexpressed relative to HER2-over-expressing and ER α -positive tumors as well as normal breast tissue. Expression of VEGFR3 was higher in triple-negative and HER2 subtype tumors than in ER α -positive tumors and normal breast tissue.

EGFR Knockout Reduces Proliferation in MCF-7 and MDA-MB-231 Breast Cancer Cells

Targeted expression knockdown of RTKs was performed to determine the effect on proliferation in luminal, HER2, and triple-negative breast cancer cells. The use of RT-PCR demonstrated knockdown of the specific RTK with siRNA transfection (Supplemental Fig. 1). In MCF-7 ER α -positive luminal breast cancer cells, knockdown of RET resulted in a 10 % decrease in cell viability after 72 h compared with nontargeting (NT) siRNA transfection (1.0 vs 0.90; $p < 0.01$) (Fig. 2). Even more significant, knockdown of EGFR resulted in 34 % reduction in cell viability (1.0 vs 0.66; $p = 0.02$). Knockdown of VEGFR1 (1.03; $p = 0.92$), VEGFR2 (1.06; $p = 0.84$), VEGFR3 (1.03; $p = 0.91$), PDGFR α (1.02; $p = 0.64$), PDGFR β (1.03; $p = 0.79$), or Kit (1.03; $p = 0.55$) did not result in a significant difference in proliferation compared with nontargeting transfection.

In ZR-75-1, another ER α -positive luminal breast cancer line, knockdown of RET did not decrease viability compared with nontargeting siRNA (1.0 vs 0.95; $p = 0.40$). Knockout of VEGFR3 (1.19; $p = 0.03$), PDGFR β (1.20; $p = 0.04$), and Kit (1.20; $p = 0.04$) resulted in a slight increase in cell viability. In SKBR-3 HER2 overexpressing breast cancer cells, knockdown of RET (0.92; $p = 0.41$), EGFR (0.97; $p = 0.48$), VEGFR1 (0.99; $p = 0.98$), VEGFR2 (0.98; $p = 0.89$), VEGFR3 (1.00; $p = 0.88$), PDGFR α (0.97; $p = 0.74$), PDGFR β (0.99; $p = 0.92$), or Kit (1.00; $p = 0.96$) did not alter cell viability (Fig. 2).

In MDA-MB-231 triple-negative breast cancer cells, knockdown of EGFR resulted in a significant reduction in proliferation with 41 % viability compared with nontargeting transfection (Fig. 2) ($p = 0.01$). In comparison, knockdown of RET (0.96; $p = 0.87$) VEGFR1 (0.82; $p = 0.35$), VEGFR2 (0.77; $p = 0.68$), VEGFR3 (0.87; $p = 0.52$), PDGFR α (0.85; $p = 0.63$), PDGFR β (0.77; $p = 0.74$), or Kit (1.07; $p = 0.69$) did not significantly alter viability. The results suggest an important role for EGFR in contributing to cell proliferation

in ER α -positive and triple-negative breast cancer, whereas the other RTKs tested, except for RET, did not appear to play an important role in cell growth in any of the cell lines.

Sunitinib Sensitivity is Not Mediated by RTKs in Breast Cancer Cell Lines

Previously, we showed that sunitinib caused a significant reduction in luminal breast cancer growth in vitro and in a xenograft model and that these effects are significantly reduced in the absence of functional signaling of the RET kinase.¹¹ Sunitinib has activity against multiple kinases, so we performed knockdown of RTKs in luminal, HER2, and basal breast cancer cell lines to determine which receptors affected sunitinib response as measured by in vitro changes in proliferation.

We calculated the response to sunitinib by comparing the viability of cells treated with sunitinib or vehicle after siRNA transfection, which we report as a percentage change in viability with sunitinib treatment. Treatment in vitro with sunitinib after NT siRNA transfection in MCF-7 cells resulted in a 32 % reduction in cell viability compared with cells grown in control (sunitinib-free) media ($p = 0.02$) (Fig. 3). As a positive control, the data confirmed that knockdown of RET abrogated response to sunitinib in MCF-7 cells (+2 %; $p = 0.004$) (Fig. 3). The response to sunitinib in MCF-7 cells was not significantly affected by knockdown of EGFR (−29 %; $p = 0.44$), VEGFR1 (−27 %; $p = 0.68$), VEGFR2 (−24 %; $p = 0.25$), VEGFR3 (−27 %; $p = 0.66$), PDGFR α (−24 %; $p = 0.23$), PDGFR β (−21 %; $p = 0.28$), or Kit (−23 %; $p = 0.69$) compared with the 32 % reduction with sunitinib treatment in NT siRNA-treated cells.

Similarly, in ZR-75-1, sunitinib resulted in a significant reduction in viability (−19 %; $p = 0.04$), and the response to sunitinib was not significantly affected by knockdown of EGFR (−15 %; $p = 0.09$), VEGFR1 (−26 %; $p = 0.12$), VEGFR2 (−21 %; $p = 0.29$), VEGFR3 (−22 %; $p = 0.43$), PDGFR α (−18 %; $p = 0.64$), PDGFR β (−17 %; $p = 0.93$), or Kit (−15 %; $p = 0.83$). Neither SKBR-3 nor MDA-MB-231 cells express RET and, as expected, knockdown of RET did not alter growth (Fig. 2). However, the effect of each of the other RTKs in response to sunitinib was assessed.

In SKBR-3 breast cancer cells, treatment with sunitinib did not alter in vitro proliferation during 72 h of treatment (5 % reduction in viability with NT transfection; $p = 0.98$) (Fig. 3). Similarly, knockdown of EGFR (−7 %; $p = 0.92$), VEGFR1 (−6 %; $p = 0.86$), VEGFR2 (−8 %; $p = 0.34$), VEGFR3 (−5 %; $p = 0.92$), PDGFR α (−2 %; $p = 0.48$), PDGFR β (+1 %; $p = 0.89$), or Kit (+2 %; $p = 0.75$) did not significantly alter the effect of sunitinib treatment compared with the effect in NT siRNA-treated cells.

Finally, in MDA-MB-231 basal breast cancer cells, sunitinib did not affect proliferation in NT siRNA-treated cells (10 % reduction in viability; $p = 0.85$). Similar to SKBR-3, knockdown of EGFR (+4 %; $p = 0.74$), VEGFR1 (+5 %; $p = 0.86$), VEGFR2 (+4 %; $p = 0.66$), VEGFR3 (+4 %; $p = 0.96$), PDGFR α (−2 %; $p = 0.37$), PDGFR β (+5 %; $p = 0.81$), or Kit (−7 %; $p = 0.63$) did not affect sunitinib response (Fig. 3). The findings suggest that the panel of RTKs examined do not mediate sunitinib sensitivity.

ER α -Positive and EGFR-Negative Primary Breast Tumors Demonstrate Increased Sunitinib Response

Previously we showed that in vitro response of primary breast cancers to sunitinib treatment correlated with expression of RET.¹¹ To investigate other markers of response to sunitinib, we analyzed the effect of sunitinib treatment in primary breast cancer tissue measured by reduction in Erk activation and attempted to correlate the response with clinical factors and expression of RTKs. The findings showed that ER α -positive tumors had a larger reduction in Erk activation with sunitinib treatment (36 %) than ER α -negative tumors (14 %; $p = 0.04$) (Fig. 4). Nodal positivity and tumor stage did not correlate with response to sunitinib. Similarly, the relative expression of Erk or the Erk phosphatase DUSP4 did not correlate with response to sunitinib treatment. Stratification by expression status for VEGFR1, VEGFR2, VEGFR3, PDGFR α , PDGFR β , and Kit showed no significant difference in reduction of Erk activation with sunitinib treatment for high- and low-expressing tumors (Fig. 4). Interestingly, tumors that expressed low levels of EGFR had a greater reduction in Erk activation with sunitinib treatment than high-EGFR-expressing tumors (mean reduction, 42 vs 25 %; $p = 0.01$).

DISCUSSION

Inhibition of RET signaling with sunitinib or vandetanib resulted in decreased proliferation in vitro and in a xenograft model in breast cancer.¹¹ Additionally, response to sunitinib was greatly blunted by knockdown of RET in luminal breast cancer cell lines, and RET expression was associated with increased in vitro response to sunitinib in primary breast cancer tumor tissue.¹¹ However, the role of RTKs targeted by sunitinib remained uncharacterized in breast cancer. One study investigated sunitinib response and found that RET, PDGFR β , and VEGFR2 were correlated with response to sunitinib, with different effects of each receptor in breast, liver, and renal cancer xenografts.¹³

In the current study, we investigated the expression of additional RTKs according to breast cancer subtype and characterized the relative effects of each receptor on response to sunitinib in breast cancer cell lines and in primary tumor samples. Within the panel of RTKs examined, triple-negative breast cancers consistently demonstrated overexpression of all RTKs relative to other molecular subtypes, suggesting the potential therapeutic approach of using specific TKIs in the treatment of triple-negative breast cancer. Although EGFR contributed to proliferation of ER α -positive and triple-negative breast cancer cells, sunitinib response did not correlate with EGFR expression, and the response actually was greater in EGFR-negative tumors. These results are consistent with the relatively low sensitivity of EGFR to sunitinib.^{14,15}

Trials conducted with sunitinib and vandetanib used as monotherapy and in combination with existing chemotherapy have not demonstrated efficacy of TKIs in breast cancer.^{16–19} However, these trials have been uniformly underpowered and have not enrolled patients based on markers of response. Additionally, expression of RTKs in breast cancer has not previously been characterized and was not used to select patients for treatment or to inform the selection of TKI according to affinity for expressed receptors.

Laboratory studies continue to show the promise of TKIs in breast cancer. Therefore, a better understanding concerning the physiologic effects of RTK expression in breast cancer and how these effects are modulated by TKIs is imperative to the selection of patients with tumor biology most likely to respond to a specific treatment.

The current study findings continue to support RET as the therapeutic target and most predictive marker of response to sunitinib treatment in breast cancer. Interestingly, knockdown of EGFR resulted in reduced proliferation in both MCF-7 ER α -positive luminal breast cancer cells and MDA-MB-231 triple-negative breast cancer cells. Sunitinib has a low affinity for EGFR, confirmed by the lack of an effect in response to sunitinib treatment with EGFR knockout. Although EGFR is expressed at a lower level in luminal breast cancer than in triple-negative breast cancer, it is expressed at a level that contributes to cell growth. Additional study is needed to determine what constitutes physiologically relevant levels of EGFR expression in luminal breast cancer. However, selection of a TKI with activity against RET and EGFR, such as vandetanib, could be more efficacious for patients with luminal breast cancer who express sufficient levels of EGFR. Hence, using a TKI that targets both receptors may increase the therapeutic efficacy of TKI therapy.

High EGFR expression was negatively correlated with response to sunitinib measured by relative Erk activation in primary breast cancer tumor samples. This was most likely observed for two reasons. First, sunitinib has a relatively low affinity for EGFR. Additionally, we observed a strong correlation between high RET expression and low EGFR expression, such that high EGFR tumors were likely to have a poor in vitro sunitinib response due to low expression of RET. However, even low levels of EGFR expression can influence proliferation, as shown by reduced proliferation in MCF-7 after knockout of EGFR. This finding implies that our designation of “high” EGFR expression could have excluded tumors that have a relatively low expression of EGFR but do have physiologically relevant levels that are contributing to cellular growth characteristics.

In this study we used in vitro proliferation and relative Erk activation as markers of activity and response to sunitinib treatment. This approach was selected so that results could be compared for each receptor. The PDGF family receptors are expressed primarily in the stroma rather than on tumor cells,²⁰ and the VEGF family receptors are expressed and create physiologic effects via the endothelial cells.²¹ Additionally, the role of Erk in mediating signaling effects is variable for each receptor. For these reasons, the results of this study could either overemphasize or minimize the clinical role of specific RTKs expressed by the tumor cell and could have a direct impact on proliferation by the use of Erk as a mediator.

Clinical trials of TKIs in breast cancer have not demonstrated antitumor efficacy. However, in vitro and animal model data continue to show promising results for TKIs as potential therapy for breast cancer. In this trial, we sought to characterize factors associated with increased response to sunitinib in breast cancer cell lines and primary breast tumors in order to identify patients with tumor biology most likely to respond to TKI treatment. The results of this study continue to support the RET proto-oncogene as both the therapeutic target and the most predictive marker of response to sunitinib in breast cancer. Based on these findings,

further clinical trials of TKIs in breast cancer should consider the inclusion of RET and potentially EGFR as a marker for patient selection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by National Institutes of Health grants R01CA109294 (PI: R. J. Weigel), R01CA183702 (PI: R. J. Weigel), and T32CA148062 (PI: R. J. Weigel) and by a generous gift from the Kristen Olewine Milke Breast Cancer Research Fund. Philip M. Spanheimer, James P. De Andrade, Allison W. Lorenzen, and Jennifer C. Carr were supported by NIH grant T32CA148062.

References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. *CA Cancer J Clin*. 2013; 63:11–30. [PubMed: 23335087]
2. Sorlie T, Perou CM, Tibshirani R, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci USA*. 2001; 98:10869–74. [PubMed: 11553815]
3. Spanheimer PM, Carr JC, Thomas A, et al. The response to neoadjuvant chemotherapy predicts clinical outcome and increases breast conservation in advanced breast cancer. *Am J Surg*. 2013; 206:2–7. [PubMed: 23375759]
4. Rouzier R, Perou CM, Symmans WF, et al. Breast cancer molecular subtypes respond differently to preoperative chemotherapy. *Clin Cancer Res*. 2005; 11:5678–85. [PubMed: 16115903]
5. Meyers MO, Klauber-Demore N, Ollila DW, et al. Impact of breast cancer molecular subtypes on locoregional recurrence in patients treated with neoadjuvant chemotherapy for locally advanced breast cancer. *Ann Surg Oncol*. 2011; 18:2851–7. [PubMed: 21442348]
6. Wang C, Mayer JA, Mazumdar A, Brown PH. The rearranged during transfection/papillary thyroid carcinoma tyrosine kinase is an estrogen-dependent gene required for the growth of estrogen receptor positive breast cancer cells. *Breast Cancer Res Treat*. 2012; 133:487–500. [PubMed: 21947652]
7. Spanheimer PM, Woodfield GW, Cyr AR, et al. Expression of the RET proto-oncogene is regulated by TFAP2C in breast cancer independent of the estrogen receptor. *Ann Surg Oncol*. 2013; 20:2204–12. [PubMed: 22878616]
8. Boulay A, Breuleux M, Stephan C, et al. The RET receptor tyrosine kinase pathway functionally interacts with the ER-alpha pathway in breast cancer. *Cancer Res*. 2008; 68:3743–51. [PubMed: 18483257]
9. Stine ZE, McGaughey DM, Bessling SL, Li S, McCallion AS. Steroid hormone modulation of RET through two estrogen responsive enhancers in breast cancer. *Hum Mol Genet*. 2011; 20:3746–56. [PubMed: 21737465]
10. Cyr AR, Kulak MV, Park JM, et al. TFAP2C governs the luminal epithelial phenotype in mammary development and carcinogenesis. *Oncogene*. 2015; 34(4):436–44. [PubMed: 24469049]
11. Spanheimer PM, Park JM, Askeland RW, et al. Inhibition of RET Increases the efficacy of antiestrogen and is a novel treatment strategy for luminal breast cancer. *Clin Cancer Res*. 2014; 20:2115–25. [PubMed: 24526731]
12. Morandi A, Plaza-Menacho I, Isacke CM. RET in breast cancer: functional and therapeutic implications. *Trends Mol Med*. 2011; 17:149–57. [PubMed: 21251878]
13. Bousquet G, Varna M, Ferreira I, et al. Differential regulation of sunitinib targets predicts its tumor-type-specific effect on endothelial and/or tumor cell apoptosis. *Cancer Chemother Pharmacol*. 2013; 72:1183–93. [PubMed: 24061866]
14. Aparicio-Gallego G, Blanco M, Figueroa A, et al. New insights into molecular mechanisms of sunitinib-associated side effects. *Mol Cancer Ther*. 2011; 10(12):2215–23. [PubMed: 22161785]

15. Kudo M. Current status of molecularly targeted therapy for hepatocellular carcinoma: clinical practice. *Int J Clin Oncol*. 2010; 15:242–55. [PubMed: 20509038]
16. Yardley DA, Dees EC, Myers SD, et al. Phase II open-label study of sunitinib in patients with advanced breast cancer. *Breast Cancer Res Treat*. 2012; 136:759–67. [PubMed: 23073758]
17. Bergh J, Bondarenko IM, Lichinitser MR, et al. First-line treatment of advanced breast cancer with sunitinib in combination with docetaxel versus docetaxel alone: results of a prospective, randomized phase III study. *J Clin Oncol*. 2012; 30:921–9. [PubMed: 22331954]
18. Mayer EL, Isakoff SJ, Klement G, et al. Combination antiangiogenic therapy in advanced breast cancer: a phase I trial of vandetanib, a VEGFR inhibitor, and metronomic chemotherapy, with correlative platelet proteomics. *Breast Cancer Res Treat*. 2012; 136:169–78. [PubMed: 23001754]
19. Boer K, Lang I, Llombart-Cussac A, et al. Vandetanib with docetaxel as second-line treatment for advanced breast cancer: a double-blind, placebo-controlled, randomized phase II study. *Investigat New Drugs*. 2012; 30:681–7.
20. Frings O, Augsten M, Tobin NP, et al. Prognostic significance in breast cancer of a gene signature capturing stromal PDGF signaling. *Am J Pathol*. 2013; 182:2037–47. [PubMed: 23583284]
21. Imoukhuede PI, Popel AS. Quantitative fluorescent profiling of VEGFRs reveals tumor cell and endothelial cell heterogeneity in breast cancer xenografts. *Cancer Med*. 2014; 3:225–44. [PubMed: 24449499]

FIG. 1.

Receptor tyrosine kinase (RTK) expression in breast cancer subtypes and normal breast tissue. Primary tumor samples and corresponding normal breast tissue were analyzed by real-time polymerase chain reaction (PCR) for expression of RTKs. Mean expression levels are shown with standard deviation. The expression level for each subtype is normalized to the triple-negative expression level

FIG. 2.

The effect of receptor tyrosine kinase (RTK) knockdown on proliferation in breast cancer cell lines. The MCF-7, ZR-75-1, SKBR-3, and MDA-MB-231 cell lines were treated with targeted small interfering RNA (siRNA) to RTKs. Cell viability was measured using MTT assay. Knockdown of RET and epidermal growth factor receptor (EGFR) significantly reduced proliferation in MCF-7 ER α -positive luminal breast cancer cells, and knockdown of EGFR significantly reduced proliferation in MDA-MB-231 triple-negative breast cancer cells but not in ZR-75-1 estrogen receptor-alpha (ER α)-positive luminal breast cancer cells or SKBR-3 HER2 overexpressing breast cancer cells. * $p < 0.05$

FIG. 3.

Sunitinib sensitivity with knockdown of receptor tyrosine kinases (RTKs) in breast cancer cell lines. **a** Knockdown of RET in MCF-7 cells abrogated response to sunitinib, with a representative growth curve shown. In MCF-7, ZR-75-1 (**b**), SKBR-3 (**c**), and MDA-MB-231 (**d**), knockdown of the epidermal growth factor receptor (EGFR), VEGFR1-3, PDGFRa/b, or Kit cell lines did not alter the response to sunitinib in breast cancer cell lines. $*p < 0.05$ vs siNT; # p = not significant compared with siRET control

FIG. 4.

Correlation of sunitinib response with clinical and molecular profile in primary breast tumors. **a** Western blots from a representative tumor showing phosphorylated and unphosphorylated Erk and GAPDH. **b** Estrogen receptor (ER)-positive (ER+) tumors had increased response to sunitinib compared with ER- tumors. The response to sunitinib was not affected by nodal status or stage. A paired *t* test was used to calculate *p* values. **c** Relative expression levels of Erk and the Erk phosphatase DUSP4 were not associated with response to sunitinib. **d** Tumors with relatively low epidermal growth factor receptor (EGFR) expression had increased response to sunitinib, with no relation to expression of the remaining receptor tyrosine kinases (RTKs) demonstrated

TABLE 1

Clinical characteristics of breast tumor samples by subtype

	ER α -positive	HER2+	Triple negative	<i>p</i> value
Number	20	4	8	n/a
Mean age (years)	59.7	48.3	57.5	0.35
Stage 1: <i>n</i> (%)	15 (75)	3 (75)	4 (50)	0.37
Stage 2: <i>n</i> (%)	5 (25)	1 (25)	4 (50)	0.37
Node positive: <i>n</i> (%)	4 (25)	1 (25)	3 (25)	1.0