Tumor and Stem Cell Biology

GDNF–RETSignaling in ER-Positive Breast Cancers Is a Key Determinant of Response and Resistance to Aromatase Inhibitors

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Abstract

Most breast cancers at diagnosis are estrogen receptor-positive (ER+) and depend on estrogen for growth and survival. Blocking estrogen biosynthesis by aromatase inhibitors has therefore become a first-line endocrine therapy for postmenopausal women with ER+ breast cancers. Despite providing substantial improvements in patient outcome, aromatase inhibitor resistance remains a major clinical challenge. The receptor tyrosine kinase, RET, and its coreceptor, GFRalpha1, are upregulated in a subset of ER+ breast cancers, and the RET ligand, glial-derived neurotrophic factor (GDNF) is upregulated by inflammatory cytokines. Here, we report the findings of a multidisciplinary strategy to address the impact of GDNF–RET signaling in the response to aromatase inhibitor treatment. In breast cancer cells in two-dimensional and three-dimensional culture, GDNF-mediated RET signaling is enhanced in a model of aromatase inhibitor resistance. Furthermore, GDNF–RET signaling promoted the survival of aromatase inhibitor-resistant cells and elicited resistance in aromatase inhibitor-sensitive cells. Both these effects were selectively reverted by the RET kinase inhibitor, NVP-BBT594. Gene expression profiling in ER+ cancers defined a proliferation-independent GDNF response signature that prognosed poor patient outcome and, more importantly, predicted poor response to aromatase inhibitor treatment with the development of resistance. We validated these findings by showing increased RET protein expression levels in an independent cohort of aromatase inhibitor-resistant patient specimens. Together, our results establish GDNF–RET signaling as a rational therapeutic target to combat or delay the onset of aromatase inhibitor resistance in breast cancer.

Cancer Res; 73(12); 1–13. ©2013 AACR.

Introduction

Approximately 70% of the breast tumors are positive for estrogen receptor (ER)-α (called hereafter ER) expression, and the majority of these rely upon estrogen (E2)-mediated ER signaling for their growth. Endocrine therapy is the most common and effective treatment for this subset of breast cancers; targeting ER function by antagonizing binding of estrogens to the ER (selective ER modulators, e.g., tamoxifen), promoting ER degradation (selective ER downregulators, e.g., fulvestrant, also known as ICI182,780), or blocking estrogen biosynthesis (aromatase inhibitors; ref. 1). Aromatase inhibitors have become the first-line treatment choice for postmenopausal women with ER+ breast cancers (2). However, de novo or acquired aromatase inhibitor resistance still limits their benefit for many patients. Several molecular mechanisms have been proposed to contribute to aromatase inhibitor resistance. First, tumor cells can become hypersensitive to residual E2 and remain dependent on ER signaling for their growth (3). Of relevance for the current study, some ER+ breast cancer cell lines cultured long-term under E2 deprivation (LTED) display ER hypersensitivity to E2, thus modeling breast cancers that have developed resistance to aromatase inhibitor treatment (4, 5). Second, tumor cells may escape the inhibitory effects of aromatase inhibitors by increasing ER activity independently of E2. This can result from EGFR receptor (EGFR), HER2, or insulin-like growth factor-1 receptor (IGF-IR) overexpression (4, 6) leading to the activation of signaling cascades including the mitogen-activated protein kinase and PI3K/AKT pathways that promote ER phosphorylation, cell proliferation, and cell survival (7).

These findings highlight the concept that combining aromatase inhibitors with therapies targeting signaling pathways...
that interact with ER is a strategy to enhance aromatase inhibitor therapy response and prevent resistance, and have led to a number of combination therapy clinical trials. For example, targeting of HER2 with trastuzumab or lapatinib in combination with the nonsteroidal aromatase inhibitors, anastrozole or letrozole, respectively, has shown clinical benefit and improved outcome for patients with metastatic breast cancer compared with treatment with aromatase inhibitors alone (8, 9). Furthermore, the Breast Cancer Trials of Oral Everolimus-2 (BOLOERO-2) study reported recently that the mTOR inhibitor, everolimus, combined with the aromatase inhibitor, exemestane, improved progression-free survival compared with exemestane alone in patients with ER+ advanced breast cancer previously treated with the aromatase inhibitors, letrozole or anastrozole (10). However, despite the positive outcome of such trials, many patients fail to benefit from these combined therapeutic approaches. As a consequence, there remains an urgent need to better understand the mechanisms of aromatase inhibitor resistance, and to find and develop appropriate and more efficient therapeutic strategies.

Expression of the receptor tyrosine kinase, REarranged during Transfection (RET), and its coreceptor, GFRα1 [glycosyl phosphatidylinositol-anchored glial-derived neurotrophic factor (GDNF) family α-receptor-1] is low in normal breast but upregulated in a subset of ER+ breast cancers (11–13). Moreover, we have previously shown that the RET ligand GDNF is upregulated by inflammatory cytokines and is expressed on infiltrating stromal fibroblasts and to a lesser extent by tumor cells in xenograft models (11). In RET+ ER+ breast cancer cells, GDNF stimulation results in an E2-independent increase in ER phosphorylation and transcriptional activity (13). However, little is known about the transcriptional program associated with GDNF–RET signaling in breast cancer cells or the relevance of this pathway to human disease. In particular, a role for GDNF–RET signaling in response and resistance to aromatase inhibitor treatment has yet to be explored. In this study, we have identified a GDNF response gene set (RGS) with prognostic and predictive value in breast cancer, and have shown the use of targeting GDNF–RET signaling in the context of aromatase inhibitor treatment.

Materials and Methods

Cell lines and assays

All cell lines were short tandem repeat (STR) profiled in December 2012 by DNA Diagnostic Centre (DCC). MCF7 cells used in the microarray experiments were maintained long term in phenol red-free RPMI-1640 medium plus 10% dextran charcoal-treated (DCC) FBS, 1 nmol/L E2 (Sigma), 2 mmol/L L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin. LTED cells were generated as previously described (4) by culturing cells in phenol red-free RPMI-1640 plus 10% DCC for a minimum of 20 weeks. MCF7, T47D, and ZR75-1 cells were cultured over the same period in phenol red-free RPMI-1640 supplemented with 10% FBS, 10 μg/mL insulin, and 1 nmol/L E2. MCF7 cells expressing full-length human aromatase (MCF7-2A) at clinically relevant levels or transfected the pBabeneo backbone (MCF7-neo) have been previously described (14). MCF7-2A and MCF7-neo cells were maintained in RPMI-1640 containing 10% FBS, 2 mmol/L L-glutamine, and 1 mg/mL Genitcine/G418 (Invitrogen). For functional analysis, cells were E2 deprived for 3 days by culturing in phenol red-free RPMI-1640 supplemented with 10% DCC.

Cell-based assays, siRNA transfection, immunohistochemistry, antibodies, and quantitative real-time PCR (qRT-PCR) analysis were as described previously (11, 13) except the TaqMan probe sets (Applied Biosystem): RET (Hs00240887_m1), GFRA1 (Hs00237133_m1), ESR1 (Hs00174860_m1), PGR (Hs01556702_m1) and the following antibodies (Cell Signaling Technology): phospho-RET-Tyr905 (#3221), phospho-c-Jun (#2361), and c-Jun (#9165).

Generation of the GDNF-RGS

MCF7 cells were E2 deprived for 3 days and then serum-starved overnight in the presence or absence of 100 nmol/L ICI182,780 (Tocris Bioscience). The following day, cells were treated with GDNF (20 ng/mL) for 0, 4, 8, 24, and 48 hours in the presence or absence of ICI182,780. Triplicate samples from 3 independent experiments were hybridized onto whole-genome HumanHT12_v3 Expression BeadChips (Illumina) by the Genomics Services Group, Wellcome Trust Centre for Human Genetics. Data were extracted using BeadStudio (Illumina) software and were transformed and normalized using variance-stabilizing transformation and robust spline normalization method in the Lumi (2.6.0) package in R (http://www.bioconductor.org). Probes were discarded if they were not detected in any of the samples (detection P > 0.01). Microarray data have been submitted to ArrayExpress database (E-MEXP-3662). To identify genes significantly regulated by GDNF treatment, a confidence score (15, 16) was calculated for each gene at each time point of GDNF treatment. Confidence score was defined as the sum of individual scores given for fold change, P value, expression level, and present calls.

Analysis of clinical datasets

Breast cancer subtypes in the NKI295 and Pawitan datasets were as reported by the authors. In TransBig dataset, the subtypes were retrieved and classify using PAM50 from ROCK (17). An unscaled GDNF-RGS score that recapitulates the degree of similarity to MCF7 cells upon GDNF treatment was generated as described previously (18). Thus, a high tumor GDNF-RGS score corresponds to a signature highly concordant with GDNF activation in MCF7 cells.

Kaplan–Meier analysis and multivariate Cox proportional hazard regression analysis were carried out with the survival (2.36–12) and survplot (0.0.6) packages in R. GDNF-RGS–positive and -negative tumors were classified using the centroid Spearman correlation method with the nearest centroid more than 0.1 as described previously (19). The sample was not assigned if the correlation was 0.1 or less.

Correlation of GDNF-RGS with response to letrozole

The cohort of patients treated with neoadjuvant letrozole has been described previously (20). Briefly, core biopsies from ER+ tumors were collected pre and post-14 days letrozole treatment and subject to gene expression analysis. Response
was classified on the basis of tumor volume reduction after 3 months letrozole treatment as assessed by ultrasound. Patients with more than 50% reduction in tumor volume were considered responders. Follow-up data were available for 52 of the 58 patients. To examine the association between the GDNF-RGS, the log intensity (median centered) of the 53 out of 67 GDNF-RGS genes available in the dataset were extracted using the ROCK database. When multiple probe sets mapped to the same gene, the one with the highest variance in the dataset was selected.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism Software as reported in the figure legends and Results.

Results

GDNF–RET signaling is enhanced in an in vitro model of aromatase inhibitor resistance

MCF7-LTED provides a widely accepted model of breast cancer cells that have developed resistance to aromatase inhibitor treatment (21). To investigate GDNF–RET signaling in the context of aromatase inhibitor resistance, we monitored the time-dependent changes in RET, its coreceptor, GFRα1 and ESR1 mRNA expression in MCF-7 cells, and in 2 additional ER^{+} cell lines (T47D and ZR75-1) during E2 deprivation (Supplementary Fig. S1A). Compared with parental MCF7 cells, MCF7-LTED cells show a marked increase in RET and ER mRNA and protein expression (Fig. 1A). This increase in RET expression is comparable with that observed in an independent model of E2-deprived MCF7 cells (22). Compared with MCF7 cells, parental T47D cells express lower levels of RET (Fig. 1A) but retain responsiveness to GDNF/GFRα1 treatment (12). As previously reported (12), parental ZR75-1 have the lowest level of RET expression. In contrast to MCF7-LTED cells, T47D-LTED and ZR75-1-LTED cells have undetectable levels of ER, RET, and GFRα1 (Fig. 1A) and do not respond to GDNF/GFRα1 treatment (Supplementary Fig. S1B). RET expression can be regulated by ER activation (12, 23), and in the in vitro cell models used here, RET expression levels mirror the levels of ER expression (Fig. 1A). Moreover, when parental MCF7 cells are cultured in the presence of E2, RET expression is enhanced and this can be blocked by ICI182,780 treatment that targets ER for proteasomal degradation (Fig. 1B). MCF7-LTED cells are hypersensitive to residual E2 (4, 5), and in the absence of exogenously added E2, a higher level of RET expression is observed (Fig. 1A and B). E2-mediated ER activation in these cells again results in increased RET expression that can be blocked by ICI182,780 treatment (Fig. 1B).

As the majority of the breast tumors that display endocrine therapy resistance retain ER expression, we used MCF7-LTED cells to investigate GDNF–RET signaling in the context of aromatase inhibitor resistance. The increased RET expression in MCF7-LTED cells is mirrored by enhanced GDNF–RET downstream signaling including increased ER activation monitored by Ser167 and Ser118 phosphorylation (Fig. 1C; ref. 24) and transcriptional upregulation of E2-dependent genes, TFF1 and PGR (Fig. 1D). Notably, the upregulation of TFF1 and PGR, but not that of the ER-independent gene EGR1, is inhibited in the presence of ICI182,780. Consistent with their higher levels of RET expression and more sustained GDNF-induced RET signaling, MCF7-LTED cells show increased GDNF-induced transcriptional activation compared with MCF7 cells (Fig. 1D).

NVP-BBT594 impairs GDNF–RET signaling and GDNF-dependent growth of MCF7-LTED cells

Blocking RET with nmol/L concentrations of the RET inhibitor, NVP-BBT594 (Fig. 2A) or siRNA transfection, (Supplementary Fig. S1C) shows that GDNF signaling and ER phosphorylation are mediated solely via the RET receptor. Similarly, NVP-BBT594 blocks the GDNF-mediated enhancement of MCF7-LTED cell viability in two-dimensional (2D) culture (not shown) and three-dimensional (3D) colony formation (Fig. 2B). Compared with MCF7-LTED cells, parental MCF7 cells form smaller colonies in 3D culture but respond to GDNF and NVP-BBT594 treatment. The addition of 10 pmol/L E2, to mimic the E2 level in postmenopausal patients that have relapsed on aromatase inhibitor treatment and ceased aromatase inhibitor therapy, increases 3D colony formation of both MCF7 and MCF7-LTED cells, and this effect is efficiently reverted by NVP-BBT594 (Fig. 2B). Parental T47D cells cultured in the absence of E2 and parental ZR75-1 cells, with or without E2, do not form colonies when cultured on Matrigel (Fig. 2C and D and Supplementary Fig. S1D). However, as previously reported (12) when parental T47D cells are cultured in presence of low level E2, GFRα1/GDNF stimulation results in increased 3D colony formation, which is significantly reverted by NVP-BBT594 (Fig. 2C). Conversely, consistent with their low-level ER and RET expression (Fig. 1A and B), T47D-LTED and ZR75-1-LTED cells do not respond to GFRα1/GDNF stimulation and minimally respond to the presence of E2 (Fig. 2C and D and Supplementary Fig. S1C). Importantly, NVP-BBT594 has no significant impact on T47D-LTED and ZR75-1-LTED 3D colony formation showing that the effects observed in MCF7-LTED cells are due to selective RET inhibition by NVP-BBT594 rather than off-target toxicity.

GDNF-promoted aromatase inhibitor resistance can be reverted by RET inhibition

To assess further the effect of GDNF signaling in the response and adaptation to aromatase inhibitor treatment, we used MCF7 cells expressing aromatase enzyme (MCF7-2A) or the backbone vector (MCF7-neo). Treatment with androstenedione, which is converted into estrogens by aromatase, results in a concentration-dependent increase in MCF7-2A cell growth, but has no effect on MCF7-neo cells (Supplementary Fig. S2A). Conversely, increasing concentrations of letrozole, or the alternate aromatase inhibitors, exemestane and anastrozole, in the presence of androstenedione impair MCF7-2A, but not MCF7-neo, cell survival (Supplementary Fig. S2B). Consistent with the known E2-mediated modulation of RET expression (see Fig. 1B; refs. 12, 23), MCF7-2A cells show increased RET levels compared with MCF7-neo cells, and in both cell lines RET expression is reduced by E2 deprivation (Fig. 3A). Treatment with androstenedione restores basal RET mRNA and protein expression in the MCF7-2A cells but has no effect
in MCF7-neo cells (Fig. 3A). Importantly, letrozole impairs androstenedione-induced RET expression both at mRNA and protein level in MCF7-2A cells (Supplementary Fig. S2C).

GDNF stimulation of aromatase inhibitor sensitive MCF7-2A cells results in RET autophosphorylation, activation of extracellular signal-regulated kinase (ERK)-1/2 and AKT, and enhanced ER phosphorylation (Fig. 3B). The GDNF-induced ER phosphorylation is abrogated by the mTOR inhibitor everolimus, partially blocked by PI3K/AKT and c-Jun-NH2-kinase inhibition but unaffected by MAP–ERK kinase (MEK) inhibition (Supplementary Fig. S2D). MCF7-2A cells show a high hormone dependency as evidenced by their inability to grow when deprived of androstenedione (Supplementary Fig. S2A). Consequently for cell-based assays, MCF7-2A cells were cultured with 10 nmol/L androstenedione. GDNF administration significantly increases the resistance of MCF7-2A cells to letrozole (no GDNF, SF50 = 1.71 nmol/L; plus GDNF, SF50 = 802 nmol/L; Fig. 3C), and the RET inhibitor NVP-BBT594
impairs GDNF-mediated RET downstream signaling (Supplementary Fig. S2E) and significantly enhances the antiproliferative effects of letrozole (SF$_{50}$ = 2.9 nmol/L; Fig. 3C). Of note, the effect of GDNF on MCF7-2A cells is more pronounced when cells are cultured in 3D (Fig. 3D). In these experimental conditions that better mimic in vivo tumor growth, GDNF...
promotes colony formation both in the absence and presence of letrozole, whereas NVP-BBT594 completely abrogates this GDNF-induced resistance (Fig. 3D).

Identification of GDNF response genes in breast cancer cells

The preclinical in vitro models described here, together with our previous findings (13), suggest that increased RET expression and activation in ER+ breast cancers can promote resistance to endocrine therapy. However, the transcriptional program induced by GDNF–RET signaling in ER+ breast cancer cells and in particular the role of GDNF-induced ER-dependent versus ER-independent signaling in response to endocrine therapy is unknown. To address this, E2-deprived MCF7 cells were pretreated with or without ICI182,780 that targets ER for proteasomal degradation and thereby blocks expression of ER-dependent genes (Supplementary Fig. S3A). Cells were then GDNF stimulated for 0, 4, 8, 24, or 48 hours, and RNA from 3 independent experiments was subject to gene expression profiling. Hierarchical cluster analysis shows that the samples divide into ICI182,780-treated and -untreated groups and that within these 2 groups, the samples cluster according to early (4–8 hours) and late (24–48 hours) GDNF response (Fig. 4A). qRT-PCR of independent samples was used to validate the gene expression profiling and confirms that GDNF treatment induces transcriptional activation of
ER-dependent genes, TFF1 and TOP2A, in MCF7 cells and that this activation is blocked with pretreatment with ICI182,780. Conversely, the ER-independent genes, ISG15 and PARP9, are upregulated in response to GDNF treatment both in the presence and absence of ICI182,780 (Supplementary Fig. S3B).

Gene set enrichment analysis was applied to identify gene sets correlated with GDNF treatment in the presence or absence of ICI182,780 (Supplementary Table S1A). Many of the identified gene sets are related to response to serum, metabolic and apoptosis pathways, DNA damage, and immune response pathways. Importantly, no correlation was found...
between the GDNF-regulated genes in MCF7 cells and other growth factor response gene sets indicating that GDNF-regulated genes do not have a substantial overlap with other growth factor signaling pathways.

To detect genes significantly regulated by GDNF, a confidence score was calculated for each gene at each time point of GDNF treatment with a cut-off of ≥11.0, as reported previously (15, 16). Eighty-three genes, 50 upregulated and 33 downregulated, were identified (Fig. 4B and Supplementary Table S1B). Gene ontology analysis revealed that a significant fraction of the GDNF-regulated genes are functionally associated with immune system processes, apoptosis, and response to stimulus (chemical, oxidative stress, biotic; Supplementary Table S1C). A comparison of the gene expression profiles in the presence and absence of ICI182,780 revealed that 42 out of 50 (84%) and 18 out of 33 (54.5%) of GDNF-upregulated and -downregulated genes, respectively, were fully or partially dependent on ER (Fig. 4C). A comparison of GDNF-regulated genes with a comprehensive E2-regulated gene dataset (25) revealed that not all of the GDNF/ER-dependent genes are reported to be E2 dependent (Fig. 4C). This suggests that GDNF treatment can promote ER-mediated transcription of a subset of genes that are independent of the canonical E2 pathway involving estrogen response element sites. This is consistent with a previous study (26) that reported a subset of EGF-induced ER genomic targets that are distinct from those induced by E2 (see Discussion).

**A proliferation-independent GDNF-RGS positivity correlates with poor clinical outcome**

It is well established that proliferation-related genes can dominate gene expression signatures, which de facto identify highly proliferative tumors (27). Consequently, the 83 GDNF-dependent gene list was robustly filtered (Supplementary Fig. S4). First, all potential proliferation-related genes based on Gene ontology analysis were removed. Second, genes previously reported in 2 independent proliferation metagene signatures were removed (28, 29). Finally, the remaining 69 genes were correlated to Ki67 protein levels and natures were removed (28, 29). Finally, the remaining 69 genes were characterized by active GDNF signaling have a significant correlation with Ki67 expression in breast cancers (30). Genes that showed $r_s > 0.5$ or $r_s < -0.5$ were removed to generate the final GDNF-RGS comprising 67 proliferation-independent genes (Fig. 4B and Supplementary Table S1). The proliferation-independent GDNF-RGS is largely populated by GDNF late response genes and, as a result, the GDNF-RGS score is higher in the samples treated with GDNF for 24 to 48 hours (Fig. 4D). ICI182,780 has a significant impact on the GDNF-RGS score as 54 out of 67 of the GDNF-RGS genes are ER dependent (Fig. 4C). However, samples that were GDNF treated in presence of ICI182,780 for 24 to 48 hours show a higher GDNF-RGS score than untreated samples (without GDNF and without ICI182,780 pretreatment) indicating that GDNF-dependent ER-independent genes are accounted for within the GDNF-RGS score (Fig. 4D).

Breast cancers can be divided into molecular intrinsic subtypes that differ in their gene expression and clinical outcomes. Among the ER$^+$ breast cancers, 2 subtypes can be distinguished: luminal A and luminal B (31). The GDNF-RGS score differs significantly between subtypes (ANOVA: $P < 0.001$; Fig. 5A–C) with a Bonferroni-corrected analysis showing that GDNF-RGS score is higher in luminal B than in luminal A tumors in all 3 datasets analyzed (Fig. 5A, $P < 0.001$; Fig. 5B, $P < 0.03$; Fig. 5C, $P < 0.001$; refs. 32–34). Furthermore, analysis of 597 breast cancer samples from The Cancer Genome Atlas (http://cancergenome.nih.gov) using the ROCK database (17) shows that amongst the 5 intrinsic breast cancer subtypes, expression of the RET ligand GDNF is highest in the luminal B subtype [Significance Analysis of Microarrays (SAM) score = 0.007; $q$ value = 0.02]. Within ER$^+$ breast cancers, luminal B tumors are characterized by poorer prognosis when compared with luminal A tumors (35), indicating that the GDNF-RGS and expression of GDNF associate with a clinically relevant molecular subtype of breast cancer. Consistent with this, using the nearest centroid method (19), GDNF-RGS positivity in ER$^+$ cancers significantly associates with a decrease in distant metastases-free survival (DMFS; Fig. 5A, right) and in relapse-free survival (RFS; Fig. 5B and C, right). Equivalent results are obtained when patients are stratified accordingly to their GDNF-RGS score (Supplementary Fig. S5). A significant association of GDNF-RGS positivity with decreased overall survival, RFS, or DMFS is also found if all cases are analyzed independently of their hormonal status (Supplementary Fig. S6). Importantly, multivariate Cox analysis reveals that the GDNF-RGS has an independent prognostic value in all 3 datasets analyzed (Table 1 and Supplementary Fig. S6). Finally, these analyses indicate that patients bearing tumors characterized by active GDNF signaling have a significantly worse outcome either in the presence (NKI295, Pawitan) or absence (TransBig) of adjuvant treatment (Fig. 5; Table 1).

**GDNF-RGS correlates with response to aromatase inhibitor treatment**

We next evaluated whether the GDNF-RGS correlates with response to aromatase inhibitor treatment by retrieving gene expression data from biopsies of 52 ER$^+$ breast cancers taken before and after 2 weeks of neoadjuvant letrozole treatment (20). The patients were subsequently divided into responder and nonresponder groups defined by a more than 50% and less than 50% reduction, respectively, in tumor volume following a further 3 months of aromatase inhibitor treatment. Pairwise comparison shows a significant decrease in GDNF-RGS score after 2 weeks of letrozole treatment in the responder cohort ($P = 0.009$) but not in the nonresponder cohort ($P = 0.804$; Fig. 6A).

To validate these findings, we examined the effect of the nonsteroidal aromatase inhibitor, anastrozole, on the GDNF-RGS score using gene expression data and Ki67 staining available for 69 paired ER$^+$ tumors biopsies taken pre- and post 2 weeks of neoadjuvant anastrozole treatment (30). In such studies, a lack of response to aromatase inhibitor treatment as monitored by a decrease in Ki67 staining has been shown to predict poor long-term disease outcome (1). The GDNF-RGS score does not correlate with levels of Ki67 in the pretreatment samples (Supplementary Fig. S7A–S7C) consistent with the absence of proliferation-related genes within the GDNF-RGS. However, this pretreatment GDNF-RGS score shows a relatively weak but statistically significant correlation with the proportional 2-week change in Ki67 ($r_s = -0.24,$
Furthermore, the change in GDNF-RGS score in the pre- and posttreatment samples positively correlates with posttreatment Ki67 ($r_s = 0.32$, $P = 0.007$) and with the proportional 2-week change in Ki67 ($r_s = 0.35$, $P = 0.0035$; Supplementary Fig. S7B and S7C). These data support the concept that GDNF-RET signaling plays an important role in the response and adaptation of patients with breast cancer to aromatase inhibitor treatment. Conversely, GDNF-RGS did not stratify for outcome in patients with ER$^+$ breast cancer that had exclusively received tamoxifen as adjuvant therapy (Supplementary Fig. S7D and S7E). This suggests that the different mechanism of action of aromatase inhibitor and tamoxifen in breast cancer may influence the ER-dependent GDNF-mediated transcriptional profile.

RET expression increases in aromatase inhibitor-resistant breast cancers

GDNF exerts its function as a ligand for the RET receptor tyrosine kinase (36). We have previously reported that RET expression is enhanced in primary tumors from patients who subsequently developed invasive recurrence after adjuvant tamoxifen treatment (13). To assess changes in RET protein expression in response to aromatase inhibitor, we stained 52 paired samples of primary breast cancers and locally recurrent or metastatic tumors arising after adjuvant aromatase inhibitor treatment (Fig. 6B and C; ref. 37). RET expression is detected in 55.8% (29 out of 52) of the primary tumors. This percentage of RET-positive tumors is comparable with that reported for tamoxifen-resistant tumors (59.6%) and is significantly higher than found in a nonselected group of ER$^+$ invasive breast cancers (37 out of 126; 29.4%; ref. 13). This reflects the fact that the majority of patients in the aromatase inhibitor-treated cohort had previously received adjuvant tamoxifen treatment. After developing resistance to aromatase inhibitor treatment, RET expression is detected in 73.1% (38 out of 52) with a borderline statistical significance ($P = 0.065$) compared with the paired pretreatment samples. As expected, ER expression was retained in the majority of post-aromatase inhibitor-treated samples with no significant change in ER H-score (Fig. 6C and Supplementary Fig. S8).

Discussion

In this study, we highlight the GDNF-RET pathway as an important determinant of resistance to aromatase inhibitor treatment in ER$^+$ breast cancers. Crucially, direct inhibition of GDNF-RET signaling by the NVP-BBT594 compound in ER$^+$ breast cancer cells enhances the sensitivity to aromatase inhibitor treatment and reverts aromatase inhibitor resistance.
In addition, we have linked gene expression data derived from Morandi et al. to independent GDNF transcriptional profiles and show that this correlates with worse prognosis and poor response to aromatase inhibitor treatment in patients with breast cancer.

A major concern in the generation of growth factor gene response signatures is that the signature can be dominated by proliferation-related genes. As extensively reported, proliferation-related gene signatures correlate with higher proliferating and higher grade tumors, and consequently with poor prognosis (27). GDNF is a weak mitogen for breast cancer cells (11, 12) but interrogation of the gene expression data shows that the GDNF-induced transcriptional program included serum and other mitogen response pathways (Supplementary Tables S1A and S1C). As a consequence, the initial list of 83 genes significantly regulated by GDNF with a confidence score 11 or more was subject to robust filtering and higher grade tumors, and consequently with poor prognosis. Importantly, we show that the GDNF-RGS significantly correlated with a decrease in DMFS and in RFS (Fig. 5) in patients with breast cancer.

Although a demonstration of prognostic value is of interest, more importantly this study revealed that a GDNF-RGS score is predictive for response to aromatase inhibitor treatment in 2 independent studies (Fig. 6A and Supplementary Fig. S7A–A7C). Moreover, the correlation of the change in GDNF-RGS and the change in proliferation index of the tumors indicates that activation of GDNF signaling is also associated with the response to aromatase inhibitor treatment. Clues as to the mechanism by which GDNF–RET signaling may promote the response and adaptation of breast cancers to aromatase inhibitor treatment have come from taking a global approach to examine GDNF–RET signaling in breast cancer cells. First, this study has revealed that GDNF can promote both E2-independent activation of ER (Figs. 2 and 3) and a noncanonical ER transcriptional program (Fig. 4C). Promotion of a noncanonical ER cistrome has been reported in breast cancer cells following EGF stimulation in a process dependent on the transcription factor AP-1 (26). It is of note that GDNF stimulation of MCF7-2A cells activates c-Jun (Fig. 3B), a key component of the AP-1 complex. Second, the GDNF-RGS is enriched with genes related to immune response pathways, in particular STAT1 target genes (Fig. 4B and Supplementary Table S1). STAT1 mediates the inflammatory response of IFN.

Despite the ability to identify breast cancer subsets, predict disease outcome, and/or response to therapies (45, 46), there is still a lack of well-defined targets causally associated with therapy and increased resistance to chemotherapy (31, 38). The data presented here suggest that the GDNF-mediated RET signaling in breast cancer cells triggers a transcriptional program associated with a more aggressive tumor phenotype independently of promitogenic effects. Importantly, we show that the GDNF-RGS significantly correlated with a decrease in DMFS and in RFS (Fig. 5) in patients with breast cancer.

Table 1. Multivariate Cox proportional hazard regression analyses of outcome according to the GDNF-RGS

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR (95% CI)</th>
<th>P</th>
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<tbody>
<tr>
<td>Van de Vijver and colleagues 2002 dataset (226 ER+ patients) DMFS (34)</td>
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<tr>
<td>GDNF-RGS</td>
<td>1.88 (1.02–3.47)</td>
<td>0.042</td>
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<tr>
<td>Tumour size (&gt;20 vs. &lt;20 mm)</td>
<td>2.12 (1.17–3.83)</td>
<td>0.013</td>
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<tr>
<td>Lymph node status</td>
<td>0.64 (0.35–1.15)</td>
<td>0.135</td>
</tr>
<tr>
<td>Age (&gt;50 vs. ≤50 y)</td>
<td>0.63 (0.26–1.49)</td>
<td>0.291</td>
</tr>
</tbody>
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| Grade (3 vs. 2/1) | 1.36 (0.54–3.46) | 0.514 |
| Tumour size (>20 vs. ≤20 mm) | 2.57 (0.96–6.85) | 0.060 |
| Lymph node status | 0.67 (0.25–1.80) | 0.431 |
| Age (>50 vs. ≤50 y) | 0.42 (0.17–1.07) | 0.060 |
| PgR status | 0.75 (0.24–2.36) | 0.485 |

| Pawitan and colleagues 2005 dataset (130 ER+ patients) RFS (33) | | |
| GDNF-RGS | 2.95 (1.07–8.06) | 0.035 |
| Grade (3 vs. 2/1) | 1.36 (0.54–3.46) | 0.514 |
| Tumour size (>20 vs. ≤20 mm) | 2.57 (0.96–6.85) | 0.060 |
| Lymph node status | 0.67 (0.25–1.80) | 0.431 |
| Age (>50 vs. ≤50 y) | 0.42 (0.17–1.07) | 0.060 |
| PgR status | 0.75 (0.24–2.36) | 0.485 |

| Desmedt and colleagues 2007 dataset (134 ER+ patients) RFS (32) | | |
| GDNF-RGS | 2.04 (1.07–3.88) | 0.030 |
| Grade (3 vs. 2/1) | 1.03 (0.50–2.10) | 0.939 |
| Tumour size (>20 vs. ≤20 mm) | 2.03 (1.10–3.73) | 0.023 |
| Age (>50 vs. ≤50 y) | 0.96 (0.49–1.90) | 0.909 |

*No patients received systemic adjuvant therapy.*
have been identified recently (13) and platelet-derived growth factor receptor pathways that can impact upon ER, it has been considered desirable to target common downstream pathways in ER+ breast cancers. mTOR inhibitors have shown a beneficial effect on progression-free survival when combined with aromatase inhibitor therapy (10). However, as shown here, GDNF activates multiple downstream pathways and, given the adaptability of tumor cells, targeting only one is likely to lead to compensatory upregulation of others. Such compensatory mechanisms have been well documented, for example, inhibition of mTOR in breast cancer cells results in enhanced IGF-IR signaling by abrogating a negative feedback loop (50). Similarly, we show that blockade of GDNF-induced MEK/ERK1/2 and PI3K signaling in MCF7-T2A cells results in increased AKT and c-Jun phosphorylation, respectively (Supplementary Fig. S2).

Here, we show using a multidisciplinary approach that GDNF–RET signaling is an important determinant of aromatase inhibitor therapy response and resistance in ER+ breast cancers. The priority now is to determine whether RET inhibition is achievable in the clinical setting to prolong the efficacy of aromatase inhibitors in recurrent and/or metastatic disease and whether targeting growth factor signaling pathways in combination with an aromatase inhibitor could prevent or delay the onset of aromatase inhibitor resistance.

Disclosure of Potential Conflicts of Interest
L.-A. Martin has commercial research grant from Pitzer and AstraZeneca. M. Dowsett has commercial research grant from Novartis and AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Morandi, Q. Gao, A. Mackay, M. Zvelebil, M. Dowsett
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Acknowledgments
The authors thank Marjan Iravani and Zara Ghazoui for array samples processing and guidance on the gene expression analysis, Paola Francica for setting up the MCF7-T2A model, Antony Fears for help with the qRT-PCR analysis, Christine Desmedt (Institut Jules Bordet, Brussels, Belgium) and Jorge Reis-Filho for the helpful discussion on the gene expression data, Miguel Angel Pujana (IDIBELL, Barcelona) for providing the MCF7 long-term estrogen deprivation gene expression data, and Novartis (Novartis Institutes for BioMedical Research) for supplying the NVP-BBT594 inhibitor.

Grant Support
This work was funded by the Association of International Cancer Research (grant 09-0533 to I. Plaza-Menacho and C.M. Isacke), by Breakthrough Breast Cancer (C.M. Isacke), and NHS funding to the NIHR Biomedical Research Centre.

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Received November 17, 2012; revised March 29, 2013; accepted April 4, 2013; published OnlineFirst May 6, 2013.
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Cancer Res  Published OnlineFirst May 6, 2013.

Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-12-4265

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