Targeting Fibroblast Growth Factor Receptors Blocks PI3K/AKT Signaling, Induces Apoptosis, and Impairs Mammary Tumor Outgrowth and Metastasis

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Abstract

Members of the fibroblast growth factor receptor (FGFR) family have essential roles in normal physiology and in cancer where they control diverse processes. FGFRs have been associated with breast cancer development. Thus, models to study the role of FGFR in breast cancer and their targeting potential are important. We present an in vitro and in vivo analysis of FGFRs in the breast cancer model cell lines 67NR and 4T1. We show that both tumor cell lines coexpress FGFRs and ligands and display autocrine FGFR signaling activity. Fibroblast growth factor receptor substrate 2 (FRS2), a downstream mediator of FGFR, is constitutively tyrosine phosphorylated and multiple signaling pathways are active. Treatment of 67NR and 4T1 cultures with TKI258, an FGFR tyrosine kinase inhibitor (TKI), caused a rapid decrease in FRS2 phosphorylation; decreased the activity of extracellular signal–regulated kinase 1/2 (ERK1/2), AKT, and phospholipase Cγ; and blocked proliferation of both tumor lines. Furthermore, TKI258 induced 4T1 apoptotic cell death via blockade of the phosphoinositide 3-kinase/AKT pathway. In vivo, one dose of TKI258 rapidly lowered FRS2 phosphorylation and ERK1/2 and AKT activity in mammary tumors. Long-term TKI258 treatment of 4T1 tumor–bearing mice had a significant effect on primary tumor outgrowth and 4T1 tumor–induced lung metastases. A microarray analysis was carried out to identify targets with roles in TKI258 antitumor activity and potential prognostic markers in human breast tumors. Of interest are the downregulated matrix metalloproteases (MMP), in particular MMP9, which is essential for metastatic spread of 4T1 tumors. Cancer Res; 70(10); 4151–62. ©2010 AACR.

Introduction

Deregulated activity of receptor tyrosine kinases (RTK) has been implicated in breast cancer development. ErbB2 overexpression has been intensely studied and has been successfully targeted in the clinic with antibodies and small molecular tyrosine kinase inhibitors (TKI; ref. 1). Considering that only 25% of patients are eligible for ErbB2-directed treatments, it is essential to uncover additional therapeutic targets. The association between fibroblast growth factors (FGF) and mammary cancer was first established in mouse mammary tumor virus–induced tumors (2) and elevated FGF8 levels have been found in human breast tumors (3). FGF receptor (FGFR) amplification has been reported in subtypes of breast cancer (4, 5) and FGFR1 levels have been linked to poor survival rates (6). Intriguingly, genome-wide screens aimed at uncovering breast cancer–associated genes identified single nucleotide polymorphisms in FGFR2 (7, 8). Based on the increasing evidence supporting the relevance of FGFRs, we have explored the role of this receptor in breast cancer models. We show here that 4T1 and 67N mammary tumor cells (9) coexpress multiple FGFRs and ligands and display autocrine FGFR activity. Accordingly, many targets of FGFR signaling, the docking protein fibroblast growth factor receptor substrate 2 (FRS2), Src, phospholipase Cγ (PLCγ), Shp-2, signal transducer and activator of transcription 3 (STAT3), and phosphoinositide 3-kinase (PI3K)/AKT (10–12), are active in both tumor cell lines.

We investigated the effects of FGFR inhibition using TKI258, an FGFR TKI (13). Treatment of 4T1 and 67N cultures with TKI258 decreased the activity of numerous signaling proteins and blocked cell proliferation. Treatment of tumor-bearing mice with TKI258 led to a strong reduction of mammary tumor growth and, for the aggressive 4T1 model, a decrease in lung metastasis. Moreover, we provide evidence that FGFR blockade downregulates key players involved in the metastatic process, in particular matrix metalloprotease-9 (MMP9) and the transcription factor Twist, which have been shown to be major regulators of lung metastasis (14, 15).
Materials and Methods

Kinase inhibitors. TKI258 (13) was provided by Drs. D. Graus-Porta and C. Garcia-Echeverria (Novartis Institutes for Biomedical Research, Basel, Switzerland); NVP-BEZ235 was provided by Dr. S-M. Maira (Novartis Institutes for Biomedical Research, Basel, Switzerland); STI571 and PTK787 were from Novartis Institutes for Biomedical Research. All inhibitors were prepared as 10 mmol/L DMSO stocks.

Proliferation, migration, and apoptosis assays. The anti-proliferative effects of TKI258 were evaluated in 96-well plates over 24 to 48 hours using a bromodeoxyuridine (BrdUrd) ELISA kit (GE Healthcare; ref. 16). The cytotoxic effects of TKI258, U0126, PD173074, and NVP-BEZ235 were evaluated in 96-well plates 24 to 48 hours after treatment by measuring total cell number and cell death using the YO-PRO assay (Invitrogen; ref. 17). Cell migration using Transwell assays was performed as described (ref. 18; see Supplementary Methods for a complete description).

In vivo treatments and analysis of tumor and metastasis formation. Animal experiments were done according to the Swiss guideline governing animal experimentation and approved by the Swiss veterinary authorities. 4T1 and 67NR cells (5 x 10^5 or 5 x 10^6) were injected in the fourth mammary fat pad of 10-week-old BALB/c mice (RCC, Basel, Switzerland). Once palpable, tumors were measured daily and volume was calculated using the following formula: height x [(diameter/2)^2 x π]. Mice were randomly distributed into treated or control groups when tumors reached 50 to 100 mm^3. For experimental metastasis, 2.5 x 10^3 4T1 cells were injected into tail veins; 5 days later, mice were randomized and treated with vehicle or TKI258 (p.o., once daily at 20, 40, or 50 mg/kg), or vehicle (polyethylene glycol 300) and PTK787 (p.o., once daily at 25 or 50 mg/kg) for the indicated times. For experimental metastasis, 2.5 x 10^3 4T1 cells were injected into tail veins; 5 days later, mice were randomized and treated with water or TKI258 (p.o., once daily, 9 days at 40 mg/kg). At the end point, mice were sacrificed and tumors and lungs were dissected. Lungs were placed in Bouin’s solution to visualize and count metastases. Pictures of the lungs were taken with a Leica MacroFluo Z6 and the number of nodules and the surface occupied by the metastasis were quantified using image access software.

Gene expression analysis. RNA from TKI258- or DMSO-treated 4T1 cells (triplicate experiments) was amplified and labeled using the Ambion MesageAMP III RNA Amplification Kit (Applied Biosystems). The same protocol was applied on RNA from three tumors from mice treated 14 days with TKI258 or water. Biotinylated, fragmented cRNA was hybridized to Affymetrix Mouse Gene 1.0 ST Array (Affymetrix). Data analysis and gene filtering were done using R/Bioconductor (19). Signal condensation was done using only the RMA algorithms as described in the GEO database. Affymetrix probe sets were mapped to in vitro and in vivo regulated genes using the NetAffx web site. Hierarchical clustering was done with cluster 3.0 (21, 22) on log 2 median centered data using uncentered correlation and average linkage clustering algorithms. Trees were displayed by Java Tree View v1.1.1. Kaplan-Meier analysis was done using JMP IN 5.1 (SAS Institute, Inc.) and relative P values were calculated with log-rank test. P values of the overlaps were calculated using Fisher’s exact test. Functional classification analysis was done using Ingenuity Pathway Analysis (Ingenuity Systems, Inc.). Gene-set enrichment analysis was done with GSEA (23) using default settings by collapsing probe sets to unique genes and taking the probe set median expression value. Significance of the enrichment was estimated by 1,000 random gene-set permutations.

Results

Constitutive FGFR signaling in 4T1 and 67NR tumor cells. BALB/c mice develop mammary tumors following injection of 67NR and 4T1 tumor cell lines; 4T1 tumors are more aggressive, forming distant lung metastases (9). Both cell lines were examined for FGFR and FGFR expression by reverse transcription-PCR (RT-PCR). 4T1 cells express FGFR1, FGFR2, and FGFR3 (Fig. 1A), with FGFR2 expression being the highest. 67NR cells express FGFR2 and FGFR3. FGF1, which activates all receptors, was found in both lines (Fig. 1A). A Western blot analysis confirmed that FGFR2 and FGFR3 are expressed (Supplementary Fig. S1A). As coexpression of FGFRs and FGF1 was observed, we tested the hypothesis that these tumor cell lines have autocrine FGFR activity.

The activity of signaling proteins downstream of FGFR was measured in lysates of cells grown in full medium or serum starved (Fig. 1B). The adaptor protein FRS2 links FGFRs to various pathways (11, 12). Probing of FRS2 immunoprecipitates (IP) from 4T1 cells revealed high levels of phospho-tyrosine (Ptyr); the level of Ptyr196-FRS2, a docking site for growth factor receptor binding protein 2 (Grb2; ref. 11), was also elevated in both cell lines (Fig. 1B). Furthermore, Ptyr and Ptyr783 were detectable in PLCγ, and the levels of phosphorylated extracellular signal-regulated kinase 1/2 (P-ERK1/2), P-AKT, P-STAT3, and P-Src were also high (Fig. 1B). With the exception of P-AKT, which was slightly lower in serum-starved cells, there was little or no change in activity of the other signaling proteins following serum deprivation (Fig. 1B). Taken together, these results provide strong evidence supporting the hypothesis that 4T1 and 67NR cells possess autocrine FGFR activity.
Figure 1. FGFR signaling in 4T1 and 67NR cells. A, semiquantitative RT-PCR for FGFRs and FGF1, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as control. B, lysates from cells in serum or serum starved were immunoblotted with the indicated antibodies. FRS2 and PLCγ IPs were probed for P Tyr content. C, lysates from serum grown 4T1 cultures were treated for 1 h with 1 μmol/L TKI258, then IPs of FGFR3 and Gab1 were probed for P Tyr and IPs of Gab1 were probed for Shp-2 and Grb2. Whole-cell lysates (WCL) and IPs were reprobed as indicated. D, serum-starved cultures were pretreated for 1 h with 1 μmol/L TKI258 or DMSO before FGF2 stimulation. Whole-cell lysates were prepared and immunoblot analyses done with the indicated antibodies. FRS2 and PLCγ IPs were probed for P Tyr content.
TKI258 lowers FGFR activity and blocks signaling pathways. To gain more insight into the intracellular pathways controlled by FGFR, we used TKI258, an FGFR TKI (13, 24). Initially, a global phospho-proteomic screen was undertaken to identify proteins undergoing changes in P tyr in response to TKI258 treatment of 4T1 cells. Lysates made from controls and TKI258-treated cultures were subjected to tryptic digestion before IP with P tyr antibodies. Pulled down peptides were subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) for detection and quantification of P tyr changes. Supplementary Table S1 lists peptides that were significantly changed in two independent analyses. One FGFR2-specific P tyr peptide from the kinase domain was 1.67-fold decreased, showing that TKI258 blocked FGFR signaling. FRS2-, Mapk3-, and Gab1-specific P tyr peptides were also strongly decreased following FGFR inhibition. FGFR3-specific P tyr peptides were not detected in this analysis; however, IPs of FGFR3 from lysates of treated cells revealed a loss of P tyr (Fig. 1C, left). FGFR1 levels are low in 4T1 cells; however, manual inspection of the LC-MS/MS data revealed lower levels of one FGFR1-specific P tyr peptide. These results suggest that all the FGFRs in 4T1 cells are active and blocked by TKI258.

Gab1 and FRS2 IPs [Fig. 1C (middle) and D] from lysates of inhibitor-treated cells revealed lower P tyr content compared with controls; P tyr196-FRS2 levels were also strongly decreased in TKI258-treated 4T1 and 67NR cells (Fig. 1D). Moreover, decreased levels of Shp-2 and Grb2 were complexed with FRS2 in TKI258-treated 4T1 cells (Fig. 1C, right) and FRS2 IPs revealed a dramatic shift [Fig. 1C (right) and D], reflecting lower activity of mitogen-activated protein kinase, which phosphorylates multiple threonine residues on FRS2 (25). P-AKT and P tyr-PLC γ levels were also decreased in inhibitor-treated cells; no effect on constitutive STAT3 or Src activity was seen (Supplementary Fig. S1B). Taken together, these results confirm the phospho-proteomic analysis and show that TKI258 has a strong effect on FGFR-mediated signaling.

TKI258 treatment blocks proliferation and migration. Effects of FGFR inhibition on proliferation were examined using BrdUrd incorporation assays. We observed a dose-dependent decrease of cells in S phase, with an 80% decrease in 4T1 cells treated with 1 µmol/L TKI258 (Fig. 2A), and a shift in the cell cycle distribution toward G1 (Fig. 2B). Chemokinetic and chemotactic response of 4T1 cells to serum was measured in Transwell assays in DMSO or TKI258. *, P < 0.001 for chemokinesis (one-way ANOVA); **, P < 0.001 for chemotaxis (t test). D, cultures were treated for 48 h with different concentrations of TKI258; cell death was determined with the YO-PRO assay and plotted relative to vehicle-treated cells.

Figure 2. Cellular effects of TKI258. A, cultures were treated for 48 h with the indicated concentrations of TKI258; BrdUrd was added 2 h before end of experiment. Percentage of incorporated BrdUrd relative to controls is plotted (left). Cultures were treated for 24 h with 1 µmol/L TKI258 and flow cytometry was done after propidium iodide staining. Percent G1 cells is indicated (right). B, lysates from cells treated with 1 µmol/L TKI258 for different times were immunoblotted with the indicated antibodies; tubulin served as control. C, chemokinetic and chemotactic response of 4T1 cells to serum was measured in Transwell assays in DMSO or TKI258. *, P < 0.001 for chemokinesis (one-way ANOVA); **, P < 0.001 for chemotaxis (t test). D, cultures were treated for 48 h with different concentrations of TKI258; cell death was determined with the YO-PRO assay and plotted relative to vehicle-treated cells.
decrease at the highest dose (Fig. 2A, left). Furthermore, flow cytometry of propidium iodide–stained cells revealed a strong G1 accumulation (Fig. 2A, right). Cyclin D1 protein (Fig. 2B) and RNA (data not shown) decreased rapidly following TKI258 treatment, and in 4T1 cells there was an increase in p27 (Fig. 2B). Another inhibitor that blocks FGFR, PD173074 (26), also lowered proliferation of 4T1 and 67NR cells (Supplementary Fig. S1C, left). Next, we examined the effect of FGFR inhibition on the directional (chemotactic) and random (chemokinetic) migration in Transwell assay chambers. Serum was added to the lower chamber to determine chemotaxis and to both chambers for chemokinesis. Both types of migration were significantly blocked in the presence of TKI258 (Fig. 2C).

TKI258 induces apoptosis in 4T1 cells that is rescued by expression of Myr-AKT. There was also an increase in dying cells in TKI258-treated 4T1, but not 67NR, cultures (Fig. 2D). The same results were seen with PD173074 (Supplementary Fig. S1C, right). The process is due to apoptosis because increased levels of cleaved caspase-3 and cleaved poly(ADP-ribose) polymerase were detected in the cells (Fig. 2B). The differential response of the cell lines might be due, in part, to the ability of TKI258 to decrease P-AKT levels in 4T1 cells, but not in 67NR cells, growing in full serum (Supplementary Fig. S1D, panels 4 and 5 versus 7 and 9). This might also explain the lack of p27 induction in the 67NR cells because it was recently shown that the induction kinetics and final p27 level were dependent on the duration
Figure 4. In vivo effects of TKI258. A, 4T1 tumor–bearing mice were treated once with TKI258 or vehicle. Tumors were collected at various times, and lysates prepared and immunoblotted as indicated. B, 4T1 tumor–bearing mice were treated daily with TKI258 (40 or 20 mg/kg) or water for 14 d. *, P < 0.005 (one-way ANOVA). C, 67NR tumor–bearing mice were treated with TKI258 (40 mg/kg) or water for 5 d, followed by 2 d off treatment. *, P < 0.05 (Mann-Whitney test). D, 4T1 tumors from 14-d–treated TKI258 or control group were harvested; frozen sections were stained for CD31, cleaved caspase-3, or phospho-histone H3. CD31-positive area was measured and plotted as percent of tumor area. Cleaved caspase-3–positive and total cells were counted and plotted as percentages. Phospho-histone H3–positive and total cells were counted and plotted as percentages. **, P < 0.01; *, P < 0.05 (t test).
of AKT inhibition when a panel of PI3K inhibitors were tested (27).

Next, we analyzed if the ERK and PI3K/AKT pathways were responsible for TKI258 activity on proliferation and apoptosis. The mitogen-activated protein/ERK kinase inhibitor UO126 and the PI3K inhibitor NVP-BEZ235 (28) were used to target the respective pathways. In UO126-treated 4T1 cultures, proliferation was 40% decreased at the maximal dose (Fig. 3A), with no evidence of apoptosis (data not shown). NVP-BEZ235 blocked proliferation by 50% and induced cell death (Fig. 3B). These results suggest that active FGFR signaling maintains high PI3K/AKT pathway activity, which is essential for 4T1 cell survival. To explore this, a vector expressing active AKT (Myr-AKT) and a control vector (pBN) were introduced into 4T1 cells and pools were examined for TKI258 sensitivity. Myr-AKT–expressing cells were 2-fold less sensitive than controls to TKI258 treatment (Fig. 3C) and maintained high levels of P-Myr-AKT in the presence of TKI258 in conditions of serum depletion or full serum (Fig. 3D). Taken together, these results show the importance of the PI3K/AKT pathway for 4T1 cell survival.

Decreased tumor growth in TKI258-treated mice. Next, the in vivo effects of TKI258 were examined. First, a single 50 mg/kg dose was administered to 4T1 tumor–bearing mice, then tumors were collected 2, 8, and 24 hours after dosing and lysates prepared from three tumor-bearing mice per
time point were analyzed. Vehicle-treated mice had high levels of Ptyr196-FRS2 and active ERK1/2, AKT, and PLCγ (Fig. 4A). Importantly, within 2 hours of TKI258 administration, there was a significant decrease in Ptyr196-FRS2 and P-ERK1/2 levels in the tumors; P-AKT and P-PLCγ levels decreased to a lesser extent (Fig. 4A). Ptyr196-FRS2 levels remained low for 24 hours, whereas active ERK1/2, AKT, and PLCγ started to increase by 8 hours (Fig. 4A). These results show that TKI258 rapidly blocks the FGFR pathway \textit{in vivo} in the tumors.

Next, we tested the long-term effects of TKI258 treatment. Mice bearing 4T1- and 67NR-induced tumors were randomly distributed into treated or control groups. 4T1 tumor-bearing mice were dosed daily at 20 and 40mg/kg for 14 days; 67NR tumor-bearing mice were dosed as indicated with 40 mg/kg for 16 days. Nonsignificant changes in body weight were observed in TKI258-treated animals (Supplementary Fig. S2A). Importantly, there was a significant reduction in tumor outgrowth and in tumor weight in the TKI258-treated groups of 4T1 tumor- and 67NR tumor-bearing mice (Fig. 4B and C; Supplementary Fig. S2B), showing that blockade of FGFR has strong antitumor activity.

To determine the mechanisms underlying TKI258 activity, 4T1-induced tumors collected on day 24 were examined for vessel density, proliferation, and apoptosis. Quantification of CD31-stained and cleaved caspase-3-stained sections revealed a significant decrease in vessel density and a significant increase in cell death in TKI258-treated animals (Fig. 4D, top and middle). There was no significant change in the mitotic marker phosphohistone H3 (Fig. 4D, bottom). Taken together, these results suggest that TKI258 inhibits tumor outgrowth mainly by impairing cell survival, which might result from decreased P-AKT (Fig. 4A) and/or decreased vessel density. The effects of TKI258 are rapid, showing significant differences in tumor volume in the treated groups within 3 days; however, consistent long-term tumor shrinkage was not observed (Fig. 4B and C).

Figure 6. Meta-analysis of TKI258-regulated genes in human breast tumors. A, unsupervised hierarchical clustering of TKI258-regulated genes identified \textit{in vitro} in 4T1 cells (1,648 genes) in the TRANSBIG and ERASMUS cohorts. Red tree and box indicate cluster 1A patients and relative overexpressed genes in the cluster, respectively. B, unsupervised hierarchical clustering of genes regulated by TKI258 in 4T1 tumors (254 genes) in the TRANSBIG and ERASMUS cohorts. Yellow tree and box indicate cluster 2B and cluster 1B patients and relative overexpressed genes in the clusters, respectively. C, Kaplan-Meier plots of cluster 1A patients (red line) compared with the others (gray line) and plots considering only patients with basal-like tumors. P values were computed using log-rank \( t \) test. D, Kaplan-Meier plots of patients in cluster 1B (green line) and cluster 2B (orange line) compared with others (gray line) and by considering only patients with basal-like tumors. P values were computed using log-rank \( t \) test.
TKI258-treated animals have fewer lung metastases. To analyze the effects of TKI258 on metastasis, lungs of 4T1 tumor-bearing mice sacrificed on day 24 were removed and stained with Bouin’s fixative, then metastatic nodules were quantified. In vehicle-treated animals, multiple large nodules were evident, whereas the extent of lung metastasis was dramatically reduced in TKI258-treated mice (Fig. 5A, top). Quantification of foci number and percent covered by metastases revealed a significant decrease in both parameters (Fig. 5A, bottom). To assess the effects of TKI258 directly in the lungs, 4T1 cells were injected through the tail vein and, 5 days later, TKI258 was administered for 9 days (Fig. 5B). TKI258 had a slight, nonsignificant effect on foci number (Fig. 5B, top), whereas the percent covered by metastases was significantly decreased (Fig. 5B, bottom). Thus, FGFR blockade also impairs the ability of 4T1 cells to grow in the lungs following tail vein injection.

Array data on TKI258-treated 4T1 cells and tumor-bearing mice. To find genes changed by TKI258, a genome-wide transcriptome analysis was done and differentially regulated genes were identified. From triplicates of 16-hour TKI258- or vehicle-treated 4T1 cells, 2,064 significantly changed probe sets (1,648 annotated genes) were identified. The same analysis performed on triplicate tumors from 14-day TKI258-treated versus vehicle-treated mice led to the identification of 543 genes (254 annotated genes); 65 genes overlapped between the data sets and 61 showed the same trend in vitro and in vivo (Supplementary Table S2). Consistent with a reduction in lung metastasis, genes related to cell motility and invasion were identified. In particular, several MMPs (MMP1, MMP3, MMP9, MMP10, and MMP13) involved in extracellular matrix degradation (29, 30) were downregulated by TKI258. A quantitative RT-PCR analysis showed that within 8 hours of TKI258 addition, there was >80% decrease in MMP1, MMP3, MMP9, and MMP10 levels (Fig. 5C); MMP9 protein was almost undetectable after 24 hours of treatment (Fig. 5D). Interestingly, all these MMPs have activator protein-1 binding sites in their promoter (31), connecting TKI258-mediated ERK inhibition to their decreased expression.

TKI258-regulated genes identify clusters of breast tumors with increased metastatic potential. Based on the ability of TKI258 to reduce metastases and perturb genes involved in cell motility and invasion, we tested whether TKI258-regulated genes might be enriched for prognostic markers in human tumors. Two cohorts of breast cancer patients available in the GEO database were used: TRANSBIG (GSE7390) with 198 patients and ERASMUS (GSE2034) with 286 patients. Analyzed were 1,648 human orthologous genes affected by in vitro, and 254 genes affected by in vivo, TKI258 treatment. Unsupervised hierarchical clustering analysis in both cohorts identified a group of patients characterized by overexpression of the same subset of 64 in vitro TKI258-regulated genes (cluster 1A, P = 4.2 × 10−70, Fisher’s exact test); 99.4% of these genes are negatively regulated by TKI258 (Fig. 6A; Supplementary Table S3A). The clustering analysis was also performed with genes affected by in vivo treatment, which identified two patient groups overexpressing distinct gene sets (cluster 1B, 62 merged genes; cluster 2B, 42 merged genes; Fig. 6B) that overlapped in both cohorts (cluster 1B, 14 overlapping genes, P = 4.06 × 10−70, Fisher’s exact test; cluster 2B, 15 overlapping genes, P = 4.14 × 10−11, Fisher’s exact test; Supplementary Table S3B).

Kaplan-Meier analysis revealed that in both cohorts, cluster 1A and cluster 2B patients, had a worse prognosis compared with other patients (Fig. 6C and D, top). The difference in metastasis-free survival was even stronger in the subgroup of basal-like patients (Fig. 6C and D, bottom). Conversely, patients in cluster 1B had a better prognosis compared with others (Fig. 6D, green line). Of note is the fact that the majority of cluster 1B genes (79%; Supplementary Table S3) were upregulated by the inhibitor, whereas the majority of cluster 1A and cluster 2B genes (99.4% and 65%, respectively; Supplementary Table S3A and B) were negatively regulated by TKI258. Cluster 1A and cluster 2B cohorts contain almost the same patients (Supplementary Fig. S3A and B), whereas the overlap between the subsets of overexpressed genes characterizing these clusters is very low (three common genes; Supplementary Fig. S3C). This suggests that cluster 1A and cluster 2B genes might identify distinct pathways and mechanisms that contribute to tumor progression. Of note, Ingenuity Pathway Functional Analysis revealed that cell cycle and DNA replication functions are more enriched in cluster 1A genes, whereas cellular movement and inflammatory response functions are more enriched in cluster 2B genes (P < 0.05, Fisher’s exact test; Supplementary Fig. S4). In summary, our analysis of genes regulated by TKI258 led to the identification of breast cancer patients in which a fraction of TKI258-downregulated genes are highly expressed and are prognostic. Indeed, these patients tend to have a poor prognosis, in particular in the basal-like group. This cluster of highly expressed genes might reflect activation of signaling pathways that we identified in our analysis of FGFR in 4T1 tumors.

Discussion

Despite recent advances in breast cancer treatment, there are patients for whom no targeted therapies are available (32). Based on evidence implicating FGFRs as breast cancer risk factors (7, 8, 33) and the identification of FGFR amplification and overexpression in specific subgroups (4, 5, 34), further studies on the potential of targeting this receptor family are warranted. In the work presented here, we show that 4T1 and 67NR breast cancer models display autocrine FGFR activity due to coexpression of receptors and ligands, and both are sensitive to FGFR inhibition. TKI258 blocks multiple signaling pathways activated by FGFRs (35), inhibits proliferation, and causes a strong reduction in mammary tumor outgrowth and, in the 4T1 model, lung metastasis formation. In addition to FGFR, TKI258 inhibits vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR; ref. 13), prompting us to test the VEGFR inhibitor PTK787 (36) and the PDGFR inhibitor STI571 (37). Neither inhibitor affected the proliferation or survival of 4T1 cultures (Supplementary Fig. S5A and B), nor did PTK787 affect tumor outgrowth (Supplementary Fig. S5C), suggesting that the major effects of TKI258 are...
related to FGFR blockade. Taken together, our results suggest that in breast tumors with active FGFR signaling, anti-FGFR therapeutics including TKIs or antibodies might show clinical efficacy.

The question arises about what induces the high levels of cleaved caspase-3 in the tumors from TKI258-treated mice. A transient loss of P-AKT, P-ERK, and P-PLCγ was seen in the tumors, and in vitro, we found that the AKT pathway has an important role in protecting 4T1 cells from TKI258-induced cell death. In addition to AKT, TKI258 treatment also lowered tumor-induced angiogenesis, which could also contribute to tumor cell death. TKI258 has the potential to block the proangiogenic FGFR in the vessels themselves, and in the tumor cells, we show that TKI258 decreases the levels of MMP9, a metalloprotease with an angiogenesis-promoting role (Supplementary Tables S4 and S5). Both activities could contribute to lower vessel density (38). Despite the significant increase in apoptosis, TKI258 only slowed tumor outgrowth; no regression was observed. Because there was no significant decrease in proliferation markers in the tumors, this suggests that the proapoptotic activity of TKI258 was insufficient to overcome growth-promoting signals, likely due to factors supplied by the tumor environment. Future work will test if FGFR inhibition in combination with other anti-tumor agents causes tumor regression.

The metastic process is complex, and tumor cells need to overcome many barriers to reach and grow in distant organs (39). We recently uncovered a novel pathway essential for 4T1 lung metastasis (14). The serpin protease nexin-1 (PN-1), via binding its receptor LRP-1, controls MMP9 expression, and PN-1–silenced 4T1 cells have decreased MMP9 levels and impaired metastatic potential (14). Interestingly, TKI258 decreased PN-1 levels (Supplementary Tables S4 and S5). The microarray analysis also revealed many other TKI258-regulated genes that are known to be involved in metastasis, including integrins, extracellular matrix proteins, and transcription factors. For example, collagen type IV α5, which was reported to be downregulated in invasive cancers (40), was increased by TKI258 treatment. Moreover, two mediators of epithelial-mesenchymal transition (EMT), E-cadherin and the Twist transcription factor, were also identified (Supplementary Table S4 and Supplementary Fig. S6A and B). During development, FGFR induces EMT (41), an event characterized by loss of epithelial properties and an increased mesenchymal phenotype (42, 43). Interestingly, Twist-knockdown 4T1 cells have been shown to form primary mammary tumors with impaired metastatic potential (15). In summary, FGFR uses multiple pathways to control proliferation, survival, and metastatic spread of this breast cancer model.

By using a “biased” approach, we had the possibility of linking cancer gene expression signatures to a molecular alteration, being TKI258-mediated blockade of FGFR signaling in this work. Interestingly, cluster 2B, from the in vivo experiment, was enriched for genes encoding proteins involved in inflammatory response and cellular movement (Supplementary Fig. S4), suggesting that blocking signaling pathways downstream of FGFR exerts effects not only in the cancer cells but also on the tumor microenvironment. To test this, cluster 2B genes were compared with genes uncovered in a study of an invasive cancer model in which gene expression in the tumor epithelium and the stroma was monitored (ref. 44; GSE15299). Remarkably, when a gene-set enrichment analysis was performed (GSEA), cluster 2B genes were top-scoring in terms of enrichment and significance in the stromal component of the invasive cancer (normalized enrichment score, 2.06; P < 0.001; Supplementary Table S6). On the other hand, cluster 1A genes, which were selected after in vitro TKI258 treatment, were top-scoring in the epithelial component of the tumor (normalized enrichment score, 2.19; P < 0.001; Supplementary Table S6). Taken together, these results suggest that in human tumors, inhibition of an RTK, such as FGFR, might result in a concomitant transcriptional reprogramming of genes that are important for tumor-stroma interaction and cancer progression.

Gene expression signatures have become important tools not only to define cancer subtypes and prognosis but also for defining combined oncogenic pathway activity in tumors (45, 46). It has also been shown that biased approaches relying on experimental models are powerful in identifying cancer signatures because they are less prone to genetic heterogeneity (47). Therefore, we took advantage of the “TKI258 gene signature” to study its expression in breast cancer patient cohorts and identified subgroups overexpressing clusters of genes downregulated by TKI258 (clusters 1A and 2B), whose members have a high probability of metastatic disease. The two identified genetic signatures are very stable because their prognostic significance was confirmed in both independent cohorts. The patients overexpressing cluster 1A or 2B genes are roughly the same (Supplementary Fig. S3), and it is tempting to speculate that there might be active FGFR signaling in these tumors. This information was not available in the database; however, our own analysis for FGF/FGFR genes scored FGFR4 as being significantly regulated in the two cohorts of patients (Supplementary Table S7). Future work will be directed to testing this signature in more defined groups of breast tumors. In summary, FGFRs seem to be a valuable target for treatment of subgroups of breast cancer patients. We show here that many biological aspects of the tumor, from cell proliferation to invasion and metastasis, are dependent on FGFR signaling, and blockade of the receptor has a strong influence on tumor growth and metastasis formation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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