Acquired Resistance to Endocrine Treatments Is Associated with Tumor-Specific Molecular Changes in Patient-Derived Luminal Breast Cancer Xenografts

Paul Cottu1,3, Ivan Bièche2, Franck Assayag2, Rania El Botty3, Sophie Chateau-Joubert9, Aurélie Thuleau3, Thomas Bagarre3, Benoît Albaud3, Audrey Rapinat4, David Gentien5, Pierre de la Grange5, Vonick Sibut6, Sophie Vacher2, Rana Hatem2, Jean-Luc Servely7,9, Jean-Jacques Fontaine9, Didier Decaudin1,3, Jean-Yves Pierga1, Sergio Roman-Roman8, and Elisabetta Marangoni3

Abstract

Purpose: Patients with luminal breast cancer (LBC) often become endocrine resistant over time. We investigated the molecular changes associated with acquired hormonoresistances in patient-derived xenografts of LBC.

Experimental Design: Two LBC xenografts (HBCx22 and HBCx34) were treated with different endocrine treatments (ET) to obtain xenografts with acquired resistances to tamoxifen (TamR) and ovariectomy (OvaR). PI3K pathway activation was analyzed by Western blot analysis and IHC and responses to ET combined to everolimus were investigated in vivo. Gene expression analyses were performed by RT-PCR and Affymetrix arrays.

Results: HBCx22 TamR xenograft was cross-resistant to several hormonotherapies, whereas HBCx22 OvaR and HBCx34 TamR exhibited a treatment-specific resistance profile. PI3K pathway was similarly activated in parental and resistant xenografts but the addition of everolimus did not restore the response to tamoxifen in TamR xenografts. In contrast, the combination of fulvestrant and everolimus induced tumor regression in vivo in HBCx34 TamR, where we found a cross-talk between the estrogen receptor (ER) and PI3K pathways. Expression of several ER-controlled genes and ER coregulators was significantly changed in both TamR and OvaR tumors, indicating impaired ER transcriptional activity. Expression changes associated with hormonoresistance were both tumor and treatment specific and were enriched for genes involved in cell growth, cell death, and cell survival.

Conclusions: PDX models of LBC with acquired resistance to endocrine therapies show a great diversity of resistance phenotype, associated with specific deregulations of ER-mediated gene transcription. These models offer a tool for developing anticancer therapies and to investigate the dynamics of resistance emerging during pharmacologic interventions.

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Introduction

At least 70% of breast cancers are classified as estrogen receptor positive (ER+), commonly called luminal breast cancers (LBC). Interfering with the ER pathway with antiestrogens (e.g., tamoxifen) or estrogen deprivation (e.g., aromatase inhibitors or ovariectomy), decreases mortality from ER+ breast cancer. However, endocrine treatments (ET) efficacy is limited by intrinsic and acquired resistance (1). The main mechanisms of intrinsic resistance to tamoxifen are lack of expression of ERα and failure to convert tamoxifen to its active metabolite, while acquired resistance to ET has been associated with several mechanisms (2). These include deregulation of ER-associated transcription factors and coactivators, activation of receptor tyrosine kinase signaling, aberrant expression of cell-cycle regulators, increased binding with the activating protein-1 (AP-1) transcription complex, and activation of the stress-activated protein kinase/JNK pathway (1, 2).

The majority of the information on these potential mechanisms has been derived from breast cancer cell lines selected for adaptation to exposure to antiestrogens...
Translational Relevance

Acquired resistance to endocrine therapy occurs with high frequency in patients with luminal breast cancer (LBC). We report here the establishment of four new patient-derived xenografts of LBC with acquired resistance in vivo to tamoxifen and estrogen deprivation. Gene expression profiling and in vivo drug-response studies showed a great diversity of endocrine resistance phenotypes, associated with tumor-specific deregulations of estrogen receptor (ER)-mediated gene transcription. One tamoxifen-resistant xenograft showed a cross-talk between the ER and PI3 kinase pathways and a high response to the fulvestrant-everolimus combination. These models offer a clinically relevant tool to evaluate anticancer therapies in the context of endocrine resistance and to investigate the dynamics of acquired resistances.

or long-term estrogen deprivation. However, such models identify mechanisms that can induce tamoxifen resistance in vitro rather than those that actually mediate resistance in patients with breast cancer, and data obtained from well-controlled experimental conditions may widely differ from what happens in a real tumor (3). In vivo models using cell line-derived xenografts have added some value to the biologic analyses of ET resistance, and have been of great help in testing and developing new drugs in this particular setting. It is nonetheless well known that cell line-derived xenografts do not adequately reflect breast cancer heterogeneity or morphology in vivo and have poor predictive value with regard to the clinical setting (4). Within this context, our group has established patient-derived xenografts (PDX) models of primary breast cancer of all subtypes of and we have shown, as well as other groups, that breast cancer PDX faithfully recapitulate the morphologic and biologic features of the parental tumors (5, 6).

The analysis of dynamic changes associated with tumor relapse in paired samples before and after resistance would be of great interest from a therapeutic point of view. However, paired primary and metastatic samples are not easily obtained in the current clinical practice, and biopsies from metastasis often yield poor tumor contents. In this report, we describe the establishment of four PDX models with acquired resistance to different ET, derived from two previously described ER+ breast cancer PDXs. We show that acquired resistance was not associated with ER loss or ESR1 mutations and that the PI3K pathway activation status was similarly activated in sensitive and resistant models. We evaluated the everolimus efficacy in hormone resistant xenografts in combination with various ET modalities and found that each xenograft displayed a specific pattern of response to these agents. Gene expression analyses showed transcriptomic reprogramming which was tumor specific and treatment specific.

Expression changes associated with hormonoresistance were both tumor and treatment specific and were enriched for genes involved in cell growth, cell death, and cell survival.

Materials and Methods

Establishment of xenografts models resistant to ET

HBCx22 and HBCx34 PDX models have been established from untreated early-stage LBC as previously described (7). Both tumor models responded to ET (7). Luminal B status has been established on both patients' tumors and derived xenografts, and assessed on the basis of low PR/high Ki67 expression (7). To establish hormonoresistant models from these xenografts, tumor-bearing mice were treated during 6 to 8 months with different ET, including tamoxifen, fulvestrant, and ovariectomy and letrozole combination. At tumor escape, resistant tumors were re-engrafted in Swiss nude mice for three serial passages and treated with the therapy under which resistance had emerged (Fig. 1A). Resistant xenografts were established when tumors had successfully undergone these three passages, and exhibited a resistance phenotype defined by a tumor growth pattern similar between the control group and the treated group.

In vivo efficacy studies

In vivo efficacy studies with ET were performed in female Swiss nude mice as previously described (7) in accordance with the French Ethical Committee. Everolimus was provided by Novartis Pharma and was administered orally at a dose of 2.5 mg/kg 3 × week. Optimal tumor growth inhibition (TGI) of treated tumors versus controls was calculated as the ratio of the mean relative tumor volume (RTV) in treated group to the mean RTV in the control group at the same time. Statistical significance of TGI was calculated by the paired Student t test by comparing the individual RTVs in the treated and control groups. Kaplan-Meier survival analysis and log-rank tests were used to determine and compare the progression-free survival probability between the different treatments arms for the HBCx34 TamR xenograft.

Morphologic and IHC analyses of tumors

Xenografted tumors were fixed in 10% neutral buffered formalin, paraffin embedded, and hematoxylin-eosin-saffron (H&E) stained. Outgrowths were analyzed by IHC for expression of biomarkers: ER, Progesterone Receptor (PR), and Ki67 rabbit monoclonal antibodies were purchased from Clinisciences. Phospho-S6, P-mTOR, P-AKT, insulin-like growth factor-I receptor (IGF-IR), and PTEN rabbit antibodies were purchased from Cell Signaling Technology (Ozyme). Tissue microarrays (TMA) were built from the in vivo efficacy studies as previously described (8). Three xenografts from each treatment group and two tissue cores per tumor were included in the TMA. Tumor sampling from treated xenografts was performed 24 hours after the last
treatment. For Ki67 quantification, 10 × pictures were automatically analyzed with the help of AxioVision 4.6.3 Zeiss software measurement tool (Carl Zeiss S.A. S.). The percentage of Ki67-positive cells was quantified by the ratio of Ki67-positive nuclei on total number of nuclei in one field. The Dunnett’s multiple comparisons test was used for multiple comparisons. Phosphorylated S6 IHC scores were defined as follow: ++ more than 50% of P-S6 + cells, + between 10% and 50% of P-S6 + cells, ± between 1 and 10%, and – less than 1%.

Western blot analysis
Proteins were extracted as described previously (9). Lysates were resolved on 4% to 12% TGX gels (Bio-Rad), transferred into nitrocellulose membranes (Bio-Rad) and immunoblotted with rabbit antibodies against GAPDH, AKT, P-AKT, S6, or P-S6 (Cell Signaling Technology). After washes, membranes were incubated with the appropriate secondary antibodies horseradish peroxidase-conjugated affinity-purified goat anti–rabbit (Jackson ImmunoResearch Laboratories, Inc., Interchim).

Real-time RT-PCR
RNA extraction and qRT-PCR were performed as previously described (10, 11). For gene normalization, we used the human TATA box-binding protein (TBP, Genbank accession NM_003194). Detailed protocols for cDNA synthesis, PCR amplifications, and normalizations have been described elsewhere (12).

Mutation screening
Mutations of PIK3CA (exons 9 and 20), PIK3R1 (exons 11–15), AKT1 (exon 4), and ER81 (exons 5, 7, and 8 encoding the recently underlined somatic mutations E380Q, Y392L, S463P, P535H, L536R, Y537C/N/S, D538G and R555C; refs. 13) were detected by sequencing.
analyzed the phosphorylation of AKT, mTOR, and S6 and described as a mechanism of resistance to ET (16, 17), we not shown). As the PI3K/AKT pathway activation has been maintained (Fig. 1B). In addition, we sequenced the tumors from both HBCx22 and HBCx34 xenografts (data not shown). The IHC and Western blot analyses of the HBCx34 xenografts showed PTEN expression and absence of AKT phosphorylation. S6 and mTOR proteins where phosphorylated at similar levels in both parental and resistant tumors (Fig. 1C and D). The status of PIK3CA, PIK3R1, and AKT1 genes was found to be wild-type in both parental and resistant xenografts. Overall, these results show that, in these models, hormonal resistance is not associated with ER loss, ERS1 hotspots mutations, or changes in the activation profile of the PI3K pathway.

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of cDNA fragments obtained by RT-PCR amplification. Details of the primers and PCR conditions are available on request. The amplified products were sequenced with the BigDye Terminator Kit on an ABI Prism 3130 automatic DNA sequencer (Applied Biosystems) with detection sensitivity of 5% mutated cells, and the sequences were compared with the corresponding cDNA reference sequences (PIK3CA NM_006218, PIK3R1 NM_181523, AKT1 NM_005163, ESR1 NM_000125).

Microarray data analysis
GeneChip Human 1.1 ST arrays were hybridized according to Affymetrix recommendations using the Ambion WT Expression Kit protocol (Life Technologies) and Affymetrix labeling and hybridization kits. Arrays were normalized according to the GC-RMA normalization procedure (14). Analyses of array datasets were made using EASANA (GenoSplice technology), normalization, background corrections, and gene annotations were performed as previously described (15). Only genes expressed in at least one compared condition were analyzed. We performed an unpaired Student t test to compare gene intensities in the different biologic replicates. Genes were considered significantly regulated when fold-change was ≥1.5 and P ≤ 0.05. The DAVID (Database for Annotation, Visualization and Integrated Discovery) Gene Ontology website was used to test the significance of enrichment in specific gene ontology annotations. The identification of biologic functions and the upstream regulator analyses associated with gene expression datasets were conducted by Ingenuity Pathway Analysis (Ingenuity Systems) according to their standard procedures. Raw data files are available from Gene Expression Omnibus under accession number GSE55561.

Results
ER and PI3K pathway status is conserved between sensitive and resistant models
To generate in vivo models of endocrine resistance, we used two previously described PDX models of hormono sensitive ER þ breast cancer, HBCx22 and HBCx34 (7). From each model, we established a tamoxifen-resistant and ovariectomy-resistant xenograft (named HBCx22 TamR, HBCx22 OvaR, HBCx34 TamR, and HBCx34 OvaR), as described in the Materials and Methods section (two examples are shown in Fig. 1A). To compare tumor morphology and ERþ expression between sensitive and resistant tumors, we performed H&E and IHC analyses. Tumor morphology was conserved between parental and resistant tumors (Fig. 1B) and IHC analysis showed ER expression was maintained (Fig. 1B). In addition, we sequenced the ESR1 gene hot spots recently found to be mutated in metastases from patients with advanced ER þ breast cancer (13). ESR1 status was found to be wild-type in resistant and responder tumors from both HBCx22 and HBCx34 xenografts (data not shown). As the PI3K/AKT pathway activation has been described as a mechanism of resistance to ET (16, 17), we analyzed the phosphorylation of AKT, mTOR, and S6 and the expression of the tumor suppressor PTEN by IHC and Western blot analysis. The profile of the three HBCx22 tumors was similar, showing no PTEN expression and a similar level of expression of P-AKT, P-mTOR, and P-S6 in the sensitive and in OvaR and TamR tumors (Fig. 1C and D). We also assessed the mutational profile of PIK3CA, PIK3R1 and AKT1 genes by sequencing analysis. The HBCx22 tumors presented a 24-bp in-frame deletion in the exon 13 of PIK3R1 (c.1704_1727del), previously shown to activate the PI3K pathway (ref. 18; data not shown). The IHC and Western blot analyses of the HBCx34 xenografts showed PTEN expression and absence of AKT phosphorylation. S6 and mTOR proteins where phosphorylated at similar levels in both parental and resistant tumors (Fig. 1C and D). The status of PIK3CA, PIK3R1, and AKT1 genes was found to be wild-type in both parental and resistant xenografts. Overall, these results show that, in these models, hormonal resistance is not associated with ER loss, ESR1 hotspots mutations, or changes in the activation profile of the PI3K pathway.

Resistance profile is tumor and treatment specific and in vivo mTOR targeting by everolimus alone arrests tumor growth in endocrine-resistant tumors
The addition of the mTOR inhibitor everolimus to ET has been successful in the treatment of advanced ER þ breast cancer (19), but it is unclear whether this combination therapy is synergistic. To further refine the resistance profile of the resistant xenografts, we analyzed the response to ET and everolimus, alone and in combination, in the HBCx22 TamR and OvaR models and the HBCx34 TamR model (Fig. 2A–C, respectively). HBCx22 TamR displayed a lack of response to all ET tested (Fig. 2A), whereas HBCx22 OvaR showed a moderate response to tamoxifen (TGI = 46%; Fig. 2B). The effect of everolimus, alone or combined with ET, in HBCx22 TamR and OvaR was identical, with durable tumor stabilization (TGI = 90%). In contrast, in HBCx34 TamR, resistance to tamoxifen was not associated with resistance to other ET treatments (Fig. 2C). Treatment by everolimus alone or combined to tamoxifen resulted in long-term tumor stabilization, whereas combination of everolimus with fulvestrant resulted in tumor regressions (Fig. 2C). Statistical significances of TGI are provided in Supplementary Table S1.

A Kaplan–Meier analysis of tumor progression demonstrated that control and everolimus arms had a significantly shorter progression-free survival than the fulvestrant and fulvestrant-everolimus arms (log-rank test, P = 0.0002; Fig. 2D). Moreover, the fulvestrant-everolimus combination yielded a significantly higher rate of complete responses than fulvestrant alone (55.5% vs. 0, P = 0.034 by the Fisher exact test) and this benefit remained significant at 127 days (log-rank test, P = 0.024). Although synergism cannot be readily confirmed without dose response in vivo studies, these results strongly underline the high potency of the fulvestrant-everolimus combination in this particular setting.
To analyze protein expression changes in treated tumors, tumors were included in TMA and IHC analyses were performed with the following antibodies: ER, PR, Ki67, P-AKT, P-mTOR, and P-S6. Representative sections from tumors corresponding to experiment shown in Fig. 2A (HBCx22 TamR) are illustrated in Fig. 3A. ER expression was decreased in the fulvestrant-treated groups, consistent with fulvestrant mode of action inducing ER degradation (20). P-AKT and P-mTOR were not affected by treatments, whereas the phosphorylation of S6 was strongly decreased in the everolimus-treated groups (Fig. 3A). Representative sections from tumors corresponding to experiment shown in Fig. 2C (HBCx34 TamR) are illustrated in Fig. 3B. Interestingly, in this tumor, P-S6 and P-mTOR were inhibited by fulvestrant alone. P-S6 expression was also decreased in the everolimus-treated group and completely abolished in tumors treated by fulvestrant and everolimus. Representative sections of tumors corresponding to HBCx22 OvaR are illustrated in the Supplementary Fig. S1. Phospho-S6 intensity scores are provided in Supplementary Table S2.

Quantifications of Ki67 expression are shown in Fig. 3C. Overall, there was a good correlation between Ki67 expression and tumor growth in vivo, with the exception of the tamoxifen-treated group in the HBCx34 TamR xenograft, which did not respond to treatment in terms of tumor growth, but showed a significant decrease in the number of Ki67-positive cells.

In summary, these results show that each tumor displayed a specific profile in terms of hormonoresistance and response to treatment combinations. The fulvestrant–everolimus combination induced tumor regressions in one tamoxifen-resistant tumor.

To investigate the transcriptional activity of ER, we performed RT-PCR and IHC analyses of IGF-1R and PR genes whose expression is regulated by ER (Fig. 4A). IGF-1R mRNA expression was decreased in both the HBCx22 OvaR and TamR models when compared with HBCx22 parental.
tumor. This pattern was also observed to a lesser extent in the HBCx34 OvaR tumor, but not in the HBCx34 TamR tumor. The PR gene mRNA expression was significantly decreased in the HBCx22 TamR model and in both HBCx34 OvaR and TamR xenografts. IHC analyses confirmed very low levels of PR protein expression and a decrease in the IGF-IR protein expression in the HBCx22 resistant tumors but not in the HBCx34 models, where IGF-IR expression was higher. We also observed a decreased expression of MYB in the four resistant models (data not shown). We next analyzed the gene expression level of the ER coregulators FOXA1, GATA3, PBX1, and GREB1 (Fig. 4B). In the HBCx22 model, FOXA1 and PBX1 genes were repressed in the OvaR but not in the TamR tumor. GATA3 showed no significant variations. In contrast, the HBCx34 xenograft showed an expression increase of these three genes in the TamR tumors. Finally, the expression of GREB1, an ER regulatory factor, was strongly decreased in HBCx22 TamR, HBCx34 TamR, and HBCx34 OvaR (Fig. 4C).

Overall, these results suggest that ER transcriptional activity might be impaired in hormone resistant tumors.

Acquired resistance to hormonotherapies is associated with specific gene expression changes involving multiple biologic processes

To identify dynamic gene expression changes associated with endocrine resistance, sensitive and resistant tumors were profiled with gene expression array. The complete lists of genes differentially expressed in HBCx22 TamR, OvaR and HBCx34 TamR, OvaR tumors compared with parental xenografts are reported in Supplementary Tables S3–S6, respectively. The Venn diagrams generated with the 4-gene
lists showed that genes deregulated in TamR and OvaR tumors were only partially overlapping in both HBCx22 and HBCx34 xenografts, indicating treatment-specific gene deregulations (Fig. 4D). In addition, TamR and OvaR signatures were tumor specific, with only 210 and 107 genes commonly deregulated, respectively (Fig. 4D).

The biologic processes (BP) corresponding to genes specifically deregulated in TamR and OvaR tumors are represented in Supplementary Tables S3–S6. In HBCx22 TamR tumors, the top biologic processes were response to wounding and response to hormone stimulus, including estrogen and steroid hormones, and processes associated with wound healing and inflammation, regulation of cell proliferation, and response to TGF-β signaling. In HBCx34 TamR, the top biologic processes were related to vitamin and nucleotide biosynthesis and regulation of phosphate metabolism (Supplementary Table S5). The top biologic processes of HBCx22 OvaR were nitrogen compound and nucleotide metabolism and antigen processing, while the HBCx34 OvaR top biologic processes included cell adhesion, response to vitamin, and intracellular signaling cascades (Supplementary Table S6). To identify upstream regulators of the gene expression changes, we performed an Upstream Regulator Analysis with the Ingenuity Software. Several transcription factors were predicted to be activated in the HBCx22 Tam-R tumors, including NFκB1, ETS1, Ap1, SP1, STAT3, and CEBPB (Supplementary Table S7). In the HBCx34 TamR
gene dataset, the upstream regulators BACH1 and FOXA1 were predicted to be activated.

Overall, these results indicate that ET resistance is associated with tumor-specific and treatment-specific gene expression signatures, involving multiple biologic processes.

**Hormonoresistance is associated with expression modifications of genes related to cell cycle, apoptosis, and ERBB receptors**

We next analyzed the molecular and cellular functions of the gene signatures with the Ingenuity Software. The lists of top five molecular and cellular functions with their respective scores are shown in Table 1. "Cellular growth and proliferation" were in the top five lists of altered function in both TamR and OvaR tumors and "cell death and survival" were the most altered functions of OvaR tumors. To further validate these findings, we performed an RT-PCR expression analysis of several genes included in these categories and significantly modulated in at least one gene expression dataset. The expression of ERBB4 gene was upregulated in three out of four tumors, and the HBCx34 TamR showed a concomitant upregulation of ERBB2, ERBB3, and ERBB4 genes (Fig. 5). Several genes associated with cellular proliferation were changed, the gene expression of MKI67, WEE1, and CDC25B were increased in the HBCx34 TamR. Finally, we found expression changes in genes associated with cell death and cell survival functions, such as BCLXL, BCL2, and BBC3 (PUMA). In summary, the molecular changes occurring in hormono resistant impair the expression of several genes associated with cell proliferation, cell death and survival, including some targetable genes such as WEE1 and ERBB3.

**Discussion**

We report here the analysis of four original hormono resistant models, obtained from two ER" breast cancer PDX, which have been rendered resistant to multiple modalities of ET, thus mimicking common clinical settings (21). Multiple resistances emerged from a single tumor, when challenged with different treatments (tamoxifen and estrogen deprivation). The resistance phenotype was specific of both the original tumor and the treatment modality under which resistance appeared. HBCx22 TamR tumor exhibited a general resistance to ET, thus mimicking common clinical settings (21). Conversely, resistance to tamoxifen was not associated with a universal endocrine resistant phenotype, as suggested by the different drug–response profiles of HBCx22 TamR and HBCx34 TamR. This is consistent with clinical observations where
patients resistant to a given class of drug may still benefit from alternate ET (21, 22), while ER expression is generally highly conserved in distant metastases (23, 24). Li and colleagues recently identified \textit{ESR1} mutations in metastases of advanced ER$^+$ breast cancer (13). The fact that we did not find such mutations in TamR and OvaR tumors, suggests the existence of other mechanisms of resistance in these PDX. A potential mechanism of endocrine resistance is aberrant signaling through the PI3K signaling pathway (17, 25). Preclinical studies have shown that breast cancer cells with upregulated AKT signaling are resistant to ET, but sensitivity may be restored by treatment with mTOR inhibitors (26, 27). However, no clinical study has demonstrated that secondary resistance to ET is associated with increased PI3K signaling in patients, and synergy between ET and everolimus has not been demonstrated in patient’s tumors. The recent clinical studies showing that everolimus is beneficial to patients displaying acquired ET resistance (19) did not evaluate the efficacy of everolimus alone, which could spare the patient’s additional ET-related side effects. The finding that phosphorylation of mTOR and S6 was constitutively high in both parental and hormone resistant tumors, suggests that activation of the AKT/mTOR pathway is not the predominant mechanism of endocrine resistance in these tumors. This is in line with recent genomic studies showing that tumors harboring \textit{PI3KCA} mutations were not resistant to tamoxifen and had a good clinical prognosis (28, 29). Our results show that mTOR

Figure 5. RT-PCR analysis of selected genes related to ERBB receptors, cell growth, and cell death functions. $n=5$, mean $\pm$ SD. Statistical significance is measured by the Student $t$ test. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$. 

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targeting has antiproliferation effect per se without restoring tamoxifen sensitivity in TamR tumors. A lack of synergy between tamoxifen and everolimus has been also reported in the MCF-7 cell line (30). However, HBCx34 TamR tumor still responded to fulvestrant and combination with everolimus resulted in a marked tumor regression. Interestingly, the IHC analysis of treated tumors showed that fulvestrant alone induced a marked decrease of P-S6. As fulvestrant directly induces ER degradation (20), decrease of P-S6 in fulvestrant-treated tumors indicates that there is a cross-talk between the ER and PI3K/AKT pathways in this tumor. Preclinical in vitro data also suggest a synergism between fulvestrant and the PI3K pathway inhibition in cells with an activated PI3K pathway which has also retained ER expression (17). Results obtained with the HBCx34 TamR xenograft confirm these observations. Fulvestrant has also been used in advanced ET-resistant patients with some efficacy (31). Combinations studies are only beginning in the clinic and our data bring a further preclinical rationale.

The expression analysis of ER-dependent genes indicated that ER transcriptional activity is impaired in TamR and OvR tumors, despite persistence of ER expression. Loss of PR and other ER target genes expression has been consistently associated with loss of sensitivity to tamoxifen in clinical cohorts (32). Disrupting ER signaling in resistant tumors has also been found in vitro and associated with an increase in promoter DNA-methylation of these genes (33). Epigenetic silencing of ER-responsive genes is conserved on the long term, and most probably involves negative regulators of proliferation. Epigenetic modifications and alterations of chromatin may contribute to ET resistance by altering ER expression and inducing a switch from classical ER signaling to other signaling pathways through estrogen-responsive elements (34). In this process, several pioneer factors can dynamically modulate chromatin openness in breast cancer cells (35). Among the known pioneer factors contributing to the estrogen response, FOXA1, GATA3, and PBX1 seem to play a role in ET resistance (36–38). In the HBCx34 TamR tumor, FOXA1 and GATA3 expression was increased, supporting the possibility of an epigenetic origin of the generalized ET resistance in this tumor. It has been demonstrated that FOXA1 actively contributes to ER binding on the chromatin in metastatic breast tumors, suggesting that FOXA1 may promote ET resistance in breast cancer (39). PBX1 expression was increased in both HBCx34 TamR and OvR tumors in comparison with sensitive counterparts. PBX1 expression has been shown to be higher in ET-resistant breast cancer that performs poorly over time and its target genes define an expression signature predictive of resistance to endocrine therapies (36). In contrast with FOXA1 and PBX1, GREB1 expression was strongly decreased in both HBCx22 TamR and HBCx34 TamR tumors. GREB1 is a chromatin-bound ER coactivator, essential for ER-mediated transcription and expressed in tamoxifen-responsive tumors (40). In the HBCx22 tumor, ET resistance was not associated with increased expression of FOXA1, PBX1, and GATA3. The analysis of HBCx22 TamR gene expression dataset suggests activation of several transcription factors, including NFKB1, ETS1, AP-1, SP1, STAT3, and CEBPB. Interestingly all of them have been associated with endocrine resistance. AP-1, SP1, or NFKB can interact with ER to activate transcription of additional genes lacking estrogen response elements (41). NF-kB activation has been associated with tamoxifen resistance in the clinic (42) and ETS transcription factors recruit nuclear receptor coactivators to estrogen-responsive genes, thus leading to hormone-independent growth and resistance to hormone-based therapies (43). Differentially expressed genes were found to be very partially overlapping between tumors and treatments, suggesting that transcriptional reprogramming is both tumor and treatment specific. These observations are consistent with a previous work showing the existence of gene expression signatures specific to tamoxifen resistance and letrozole resistance in MCF-7 cells, mainly involving ER-responsive genes (44). These data have been confirmed on MCF7 xenografts studies which also suggested specific gene expression profiles according to the resistance context, involving ER target genes as well as FOX family genes (3). Gene expression data obtained from clinical series of tamoxifen-treated patients confirmed a potential poor predictive value of a high expression of CXCL or SERPIN genes from which, we also identified in the TamR tumors (45). A parallel dynamic assessment was also performed in a small series of 15 letrozole-resistant patients, again pointing out the role of ER-responsive and proliferation genes in ET resistance, with striking individual variations (46). Among the genes differentially regulated in hormone resistant tumors, we found an increased expression of ERBB3 and ERBB4, whose expression has been associated with hormonoresistance (47). Additional in vivo experiments will be necessary to determine whether targeting ERBB receptors could restore tamoxifen response or increase the antitumor activity of fulvestrant in the HBCx34 TamR xenograft. A number of genes involved in cell growth, cell death, and cell-cycle control were modulated in hormone resistant tumors; these biologic functions have been frequently associated with endocrine resistance both in vitro and in vivo (2, 48, 49). In patients, cell cycle, cell growth, and cell survival signatures are independent predictors of outcome in tamoxifen-treated patients (50). Our data provide further evidence that acquisition of endocrine resistance is associated with deregulations of cell survival and cell death functions in patient-derived samples.

In summary, our data indicate that molecular changes associated with acquired ET resistance are both tumor and treatment specific, and that alternative therapeutic modalities in hormone resistant patient should be considered on an individual clinical and biologic basis. As the data obtained here have been generated from only two tumors, further validations using more PDX and clinical cohorts are needed to validate our results. We believe that these models will help enriching the biologic data associated with hormonoresistance in human breast cancer, and may serve as predictive tools to evaluate the efficacy of new drugs and drug combinations in the context of endocrine resistance.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: P. Cottu, F. Assayag, S. Roman-Roman, E. Marangoni Development of methodology: P. Cottu, I. Bieche, F. Assayag, R. El Botty, A. Thuleau, T. Bagarre, S. Vacher, R. Hatem, E. Marangoni Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P. Cottu, I. Bieche, F. Assayag, S. Chateau-Joubert, A. Thuleau, T. Bagarre, B. Albaud, A. Rapinat, D. Gentien, S. Vacher, R. Hatem, I. L. Servely, J. F. Fontaine, D. Decaudin Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P. Cottu, I. Bieche, F. Assayag, S. Chateau-Joubert, A. Thuleau, T. Bagarre, D. de la Grange, V. Sibut, S. Vacher, R. Hatem, I. L. Servely, S. Roman-Roman, E. Marangoni Writing, review, and/or revision of the manuscript: P. Cottu, I. Bieche, F. Assayag, R. El Botty, S. Chateau-Joubert, J. J. Fontaine, D. Decaudin, S. Roman-Roman, E. Marangoni

References

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P. Cottu, F. Assayag, A. Thuleau, T. Bagarre, D. Decaudin, J.-Y. Pierga
Study supervision: P. Cottu, F. Assayag, J.-Y. Pierga, S. Roman-Roman, E. Marangoni

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