Spotlight on Clinical Response

Discordant Cellular Response to Presurgical Letrozole in Bilateral Synchronous ER+ Breast Cancers with a KRAS Mutation or FGFR1 Gene Amplification

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Abstract
We describe herein a patient presenting with bilateral estrogen-receptor–positive (ER+) breast tumors who was enrolled in a clinical trial exploring molecular aberrations associated with hormone-refractory tumor cell proliferation. Short-term (two week) hormonal therapy with the aromatase inhibitor letrozole substantially reduced proliferation as measured by Ki67 immunohistochemistry in one tumor, whereas the second was essentially unchanged. Extensive molecular and genetic work-up of the two tumors yielded divergent lesions in the two tumors: an activating KRAS mutation in the responsive tumor and an amplification of the fibroblast growth factor receptor-1 (FGFR1) locus in the treatment-refractory tumor. These findings provide an insight to possible mechanisms of resistance to antiestrogen therapy in ER+ breast cancers. First, they illustrate the necessity of clinically approved assays to identify FGFR1 gene amplification, which occur in approximately 5% of breast tumors and have been linked to antiestrogen resistance. It is quite possible that the addition of FGFR inhibitors to ER-targeted therapy will yield a superior antitumor effect and improved patient outcome. Second, they suggest that the role of activating mutations in RAS, although rare in breast cancer, may need to be explored in the context of ER+ breast tumors.

Introduction
A 60-year-old female presented to the Vanderbilt Breast Center (Nashville, TN) with an abnormal mammogram showing scattered microcalcifications and upper-quadrant nodularity in her left breast. A diagnostic mammogram and ultrasound confirmed these findings. A core biopsy revealed the lesion to be an estrogen-receptor–positive (ER+), progesterone-receptor–positive (PR+), HER2-negative, and low-grade invasive mammary carcinoma. The patient also had a palpable abnormality in the right breast, which upon ultrasound was identified as a hypoechoic lesion suspicious for malignancy. A core biopsy revealed it to be a low-grade, ER+ HER2-negative invasive mammary carcinoma.

Before definitive breast surgery, the patient was consented and enrolled in a presurgical trial of the aromatase inhibitor letrozole (Fig. 1A; Vanderbilt University, Nashville, TN, NCT00651976). This trial examines the short-term cellular and molecular response to estrogen deprivation with letrozole (2.5 mg QD for 10–21 days) in stage I and II operable ER+/HER2-negative breast cancer. Molecular correlates include gene expression, proteomic, and mutational screening to identify biomarkers and effectors of resistance to estrogen deprivation in cancers that do not exhibit a change in tumor cell proliferation and/or that retain a high proliferation as measured by Ki67 immunohistochemistry (IHC). The trial design is based in large part on the results of the Immediate Preoperative Anastrozole, Tamoxifen, or Combined with Tamoxifen (IMPACT) study, in which tumor cell proliferation (measured by Ki67 IHC) after 2 weeks of antiestrogen therapy was a surrogate for long-term patient disease-free survival following adjuvant endocrine therapy (1–3).

The patient received 16 days of letrozole followed by bilateral mastectomy with sentinel lymph node biopsy the day after the last dose. Her final pathology was consistent with a right invasive mammary carcinoma, stage II (T1bN1; 0.9 cm, 2 involved lymph nodes), low-histologic grade, ER+, PR+, HER2-negative, and a left multicentric invasive mammary carcinoma, stage I (T1bN0; 0.7 cm, 0.5 cm), low-histologic grade, ER+, PR+, HER2-negative tumor. A follow-up right axillary dissection revealed that none of the 15 lymph nodes removed were involved by cancer. She subsequently
underwent adjuvant chemotherapy (anthracycline/taxane) followed by 5 years of endocrine therapy. The patient continues to do well without evidence of recurrence since her original diagnosis on November 2008.

**Materials and Methods**

Formalin-fixed, paraffin-embedded (FFPE) pretreatment core biopsies and posttreatment surgical specimens were used to assess the status of >400 known somatic oncogenic mutations in 33 genes as described (4). Sanger sequencing was conducted to verify mutation status. IHC was conducted in both the pretreatment biopsy and in the posttreatment surgical biopsy of both tumors for Ki67 (Dako #M7240), ER-α (Santa Cruz #sc542), p-ER-α S118 (Cell Signaling #2511), PR (Dako #M3569), and p-ERK1/2 (Cell Signaling, #9101). IHC for ER and PR was conducted according to the methods reported elsewhere (5). FFPE tumor sections were scanned at ×100 magnification, and the area containing the highest number of positive cells was selected. Positive and negative tumor cells were manually counted at ×400; the percentage of positive cells was calculated with at least 700 viable cells. Ki67 IHC was scored by 3 independent pathologists to ensure precision and estimate the standard deviation in Ki67 scoring. FISH for fibroblast growth factor receptor-1 (FGFR1) was conducted using the standard manufacturer’s protocol (Zytovision). Affymetrix U133 + 2.0 gene expression arrays were used to capture gene expression patterns in both posttreatment surgical specimens (deposited as GSE39387). Recurrence score was estimated from the microarray data signal intensity values according to the methods of Paik and colleagues (6), with rescaling in the presence of data from the additional patients in the trial.

**Results**

An activating KRAS mutation (G12D) was identified in the left breast tumor, whereas no screened mutations were identified in the tumor from the right breast. Of note, however, the total incidence of PIK3CA mutations identified in the initial analysis was approximately 40% (9 of 20 patients), which is similar to the reported frequency on the Catalogue of Somatic Mutations in Cancer database (7). The status of the G12 codon was confirmed by Sanger sequencing. Figure 1B shows the c.35G>A transition resulting in the change from glycine to aspartic acid at codon 12. The mutation was confirmed in the left tumor, but not the right tumor, in both the forward and reverse directions by PCR [primer sequence and conditions for KRAS exon 1 were previously described (8)]. The same tumor block used for IHC was used for DNA extraction and sequencing to eliminate the potential for left-/right-sided errors.
The Ki67 score in the KRAS-G12D tumor decreased from 14 ± 2.5% (pretreatment) to 1 ± 1% after letrozole, whereas Ki67 in the KRAS-WT tumor was essentially unchanged (4.5 ± 1.2% to 2.5 ± 1.2%; Fig. 1C and D). The KRAS-G12D tumor had a substantially higher Ki67 score at baseline than the KRAS-WT tumor (14% vs. 4.5%, respectively). We calculated Allred scores (5; noted in Fig. 2A) for ER and PR in the pre- and postletrozole samples. ER scores were equivalent in both tumors, suggesting that the difference in proliferative response was not due to variations in ER expression. The PR score was higher in the KRAS-G12D tumor than in the KRAS-WT tumor (Allred scores of 6 and 3, respectively). Consistent with estrogen deprivation, PR expression decreased significantly in both tumors in response to letrozole.

ER-α is phosphorylated at serine-118 by ERK1/2, which lies downstream of KRAS, resulting in estrogen-independent transcriptional activity (9). Therefore, we hypothesized that the KRAS-G12D tumor may have higher phosphorylation of ERK1/2T202/Y204 and ER-αS118. IHC for the phosphorylated epitopes of both proteins was conducted. However, we did not observe any differences in P-ERαS118 signal between the tumors (data not shown). P-ERK1/2T202/Y204 signal was high in both pretreatment biopsies, but essentially absent in the posttreatment specimens, possibly reflecting sample-fixation issues known to induce rapid loss of phospho-ERK1/2 immunoreactivity (10). As ERαS118 is also phosphorylated by estradiol-induced CDK7 (11), it is also plausible that letrozole-induced estrogen deprivation resulted in loss of extracellular signal-regulated kinase (ERK) phosphorylation regardless of KRAS genotype. In addition, this could be a downstream effect of loss of PI3K pathway activity, which is downregulated in breast tumors following

Figure 2. FGFR1 amplification in a breast tumor showing molecular endocrine resistance. A, representative IHC and Allred scores for ER-α (ER) and PR in the bilateral tumors, before and after 2 weeks of letrozole therapy. PR staining was substantially decreased in both tumors with letrozole therapy. B, Heat map of the genes with the highest fold change between the left and right tumors. Among these, FGFR1 was substantially upregulated in the right tumor. C, representative FISH for FGFR1. Percentage of nuclei showing >5 FGFR1 gene cluster signals (green) per centromere 8 signal (red) reported in each panel.
letrozole treatment (12). Although, the tumor harboring KRAS-G12D exhibited a strong antiproliferative response to estrogen deprivation, we questioned whether KRAS activation in this tumor may abrogate apoptotic pathways. Therefore, we also stained all 4 samples for the apoptotic marker cleaved caspase-3. Little or no cleaved caspase-3 staining was detected in any of the samples (data not shown), supporting a cytostatic but not an apoptotic effect of the aromatase inhibitor.

In examining gene expression data from the patient, we identified genes with the greatest differential expression between the KRAS-G12D and KRAS-WT tumor (Fig. 2B). A microarray approximation of the “Recurrence Score (6)” for the 2 tumors revealed that the KRAS-G12D tumor had a “High” recurrence score, whereas the recurrence score in the KRAS-WT tumor was “Low.” This suggests KRAS mutations in breast cancer may contribute to a more invasive phenotype with higher likelihood of recurrence. Notably, genes most highly expressed in the KRAS-G12D tumor included a number of chemokine ligands, including CXCL9, 10, 11, and 13, possibly representing a strong immune response in this tumor. CXCL9, 10, and 11 are all CXCR3 ligand-chemokines, which can be transcriptionally activated by TNF-α stimulation of mesenchymal stem cells, thereby promoting invasive phenotypes in breast cancer cells through paracrine activation of CXCR3 (13).

In contrast, the KRAS-WT tumor highly expressed the mRNA for the tyrosine kinase FGFR1. FISH analysis for FGFR1 confirmed genomic amplification of the 8p12 locus in the KRAS-WT, but not the KRAS-mutant tumor (Fig. 2C), in both pre- and posttreatment specimens. Genomic amplification of FGFR1 has been directly linked with endocrine resistance (14). We speculate that this finding may offer an explanation for the lack of change in Ki67 in this tumor.

Discussion

Activating mutations in KRAS are common in diverse cancer types, such as melanoma, colorectal, pancreatic, and nonsmall cell lung cancers. In lung and colorectal cancer, KRAS mutations have been associated with resistance to EGFR (EGFR)-targeted therapies, primarily due to decoupling of the Ras pathway from EGFR. Activating KRAS mutations are rare in breast cancer, and no data exist on whether KRAS mutations can induce resistance to antiestrogen therapy. Correlative studies have identified an association between Ras pathway activation as defined by high levels of p-ERK1/2 and P-RAF and resistance to tamoxifen in patients with ER+ breast cancer (15). However, mutational status of the pathway was not assessed in this study. Ras activation coupled with estrogenic stimuli can cooperatively enhance invasive phenotypes (16). ER+ MCF-7 human breast cancer cells engineered to overexpress v-Ha-Ras were found to be less responsive to estrogen-induced proliferation and tamoxifen-induced inhibition of proliferation (17). It is intuitive that by conferring a gain of function, KRAS mutations should confer estrogen-independent growth and survival in an ER+ tumor. However, on the basis of the cellular markers assessed in this case, the evidence does not support the hypothesis that KRAS mutations confer resistance to estrogen deprivation in ER+ breast cancer. However, it is noteworthy that the KRAS-mutant tumor showed higher Ki67 staining, and thus may be a poor prognostic marker per se as is observed in other cancers.

The contribution of FGFR1 amplification to endocrine resistance in breast cancer is clearer. Recent work showed that FGFR1 amplification occurs in approximately 10% of breast cancers (predominantly ER+ and HER2-negative), is a poor prognostic factor, and can directly compensate for loss of estrogen signaling through estrogen-dependent or -independent PI3K and mitogen-activated protein kinase activation (14, 18, 19). Interestingly, Turner and colleagues also found that FGFR1 signaling repressed PR expression, which was lower in the FGFR1-amplified breast tumor in our patient at baseline (Fig. 2A). Up to now, clinical data linking FGFR1 amplification with endocrine resistance are only correlative. With the inclusion of a matched FGFR1 gene-nonamplified control, we provide evidence supporting a role for FGFR1 in resistance to endocrine therapy. These findings support further exploration of the utility of FGFR1-targeted agents for the treatment of endocrine-resistant breast cancer.

Disclosure of Potential Conflicts of Interest

N. Wagle has ownership interest (including patents) in Foundation Medicine and is a consultant/advisory board member of Foundation Medicine. L.A. Garraway has a commercial research grant from Novartis and has ownership interest (including patents) in Foundation Medicine. No potential conflicts of interest were disclosed by the other authors.

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