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## Discordant cellular response to pre-surgical 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 ER+ breast tumors who was enrolled in a clinical trial exploring molecular aberrations associated with hormone-refractory tumor cell proliferation. Short-term (2-week) hormonal therapy with the aromatase inhibitor letrozole substantially reduced proliferation as measured by Ki67 immunohistochemistry in one tumor, while the second was essentially unchanged. Extensive molecular and genetic workup of the two tumors yielded divergent lesions in the two tumors: an activating *KRAS* mutation in the responsive tumor, and an amplification of the *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 ~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

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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 with an abnormal mammogram showing scattered micro-calcifications and upper-quadrant nodularity in her left breast. A diagnostic mammogram and ultrasound (US) confirmed these findings. A core biopsy revealed the lesion to be an estrogen-receptor-positive (ER+), progesterone receptor-positive (PR+), HER2-negative, low grade invasive mammary carcinoma. The patient also had a palpable abnormality in the right breast, which upon US 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.

Prior to definitive breast surgery, the patient was consented and enrolled in a pre-surgical trial of with the aromatase inhibitor letrozole (Figure 1A; Vanderbilt University 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 IMPACT study, where tumor cell proliferation (measured by Ki67 IHC) after two 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 histological grade, ER+, PR+, HER2-negative, and a left multicentric invasive mammary carcinoma, stage I (T1bN0; 0.7 cm, 0.5 cm), low histological 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) pre-treatment core biopsies and post-treatment surgical specimens were utilized to assess the status of >400 known somatic oncogenic mutations in 33 genes as described (4). Sanger sequencing was carried out to verify mutation status. Immunohistochemistry (IHC) was performed in both the pre-treatment biopsy and in the post-treatment surgical biopsy of both tumors for Ki67 (Dako #M7240), ER $\alpha$  (Santa Cruz #sc542), p-ER $\alpha$ <sup>S118</sup> (Cell Signaling #2511), PR (Dako #M3569) and p-ERK1/2 (Cell Signaling, #9101). IHC for ER and PR was carried out according to the methods reported elsewhere (5). FFPE tumor sections were scanned at 100 $\times$  magnification and the area containing the highest number of positive cells was selected. Positive and negative tumor cells were manually counted at 400 $\times$ ; 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 FGFR1 was carried

out using the standard manufacturer's protocol (Zytovision; Bremerhaven, Germany). Affymetrix U133+2.0 gene expression arrays were used to capture gene expression patterns in both post-treatment surgical specimens (deposited as GSE39387). Recurrence Score was estimated from the microarray data signal intensity values according to the methods of Paik et al (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, while 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 ~40% (9/20 patients), which is similar to the reported frequency on the Catalogue of Somatic Mutations in Cancer (COSMIC) database (7). The status of the G12 codon was confirmed by Sanger sequencing. Figure 1A demonstrates 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 pre-treatment core biopsy and surgical specimen 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 utilized 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  $14 \pm 2.5\%$  (pre-treatment) to  $1 \pm 1\%$  after letrozole, whereas Ki67 in the *KRAS*-WT tumor was essentially unchanged ( $4.5 \pm 1.2\%$  to  $2.5 \pm 1.2\%$ ; Figure 1B–C). The *KRAS*-G12D tumor had a substantially higher Ki67 score at baseline than the *KRAS*-WT tumor (14% versus 4.5%, respectively). We calculated Allred scores (5) (noted in Figure 2A) for ER and PR in the pre- and post-letrazole 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 $\alpha$  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 ERK1/2<sup>T202/Y204</sup> and ER $\alpha$ <sup>S118</sup>. IHC for the phosphorylated epitopes of both proteins was performed. However, we did not observe any differences in P-ER $\alpha$ <sup>S118</sup> signal between the tumors (data not shown). P-ERK1/2<sup>T202/Y204</sup> signal was high in both pre-treatment biopsies, but essentially absent in the post-treatment specimens, possibly reflecting sample-fixation issues known to induce rapid loss of phospho-ERK1/2 immunoreactivity (10). As ER<sup>S118</sup> is also phosphorylated by estradiol-induced CDK7 (11) it is also plausible that letrozole-induced estrogen deprivation resulted in loss of ERK phosphorylation regardless of *KRAS* genotype. Additionally, this could be a downstream effect of loss of PI3K pathway activity, which is down regulated in breast tumors following letrozole treatment (12). Although the tumor harboring *KRAS*-G12D exhibited a strong anti-proliferative 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 (Figure 2B). A microarray approximation of the 'Recurrence Score (6)' for the two tumors revealed that the *KRAS*-G12D tumor had a 'High' recurrence score (RS) whereas the RS 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- $\alpha$  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 fibroblast growth factor receptor-1 (*FGFR1*). FISH analysis for *FGFR1* confirmed genomic amplification of the 8p12 locus in the *KRAS*-WT, but not the *KRAS*-mutant tumor (Figure 2C), in both pre- and post-treatment 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 non-small cell lung cancers. In lung and colorectal cancer, *KRAS* mutations have been associated with resistance to 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 anti-estrogen therapy. Correlative studies have identified an association between Ras pathway activation as defined by high levels of P-ERK1/2<sup>Y202/T204</sup> and P-RAF<sup>S338</sup> 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, based on 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 demonstrated 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 MAPK activation (14, 18, 19). Interestingly, Turner *et al.* also found that FGFR1 signaling repressed PR expression, which was lower in the *FGFR1*-amplified breast tumor in our patient at baseline (Figure 2A). Up to now, clinical data linking *FGFR1* amplification with endocrine resistance are only correlative. With the inclusion of a matched *FGFR1* gene-non-amplified 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.

## Acknowledgments

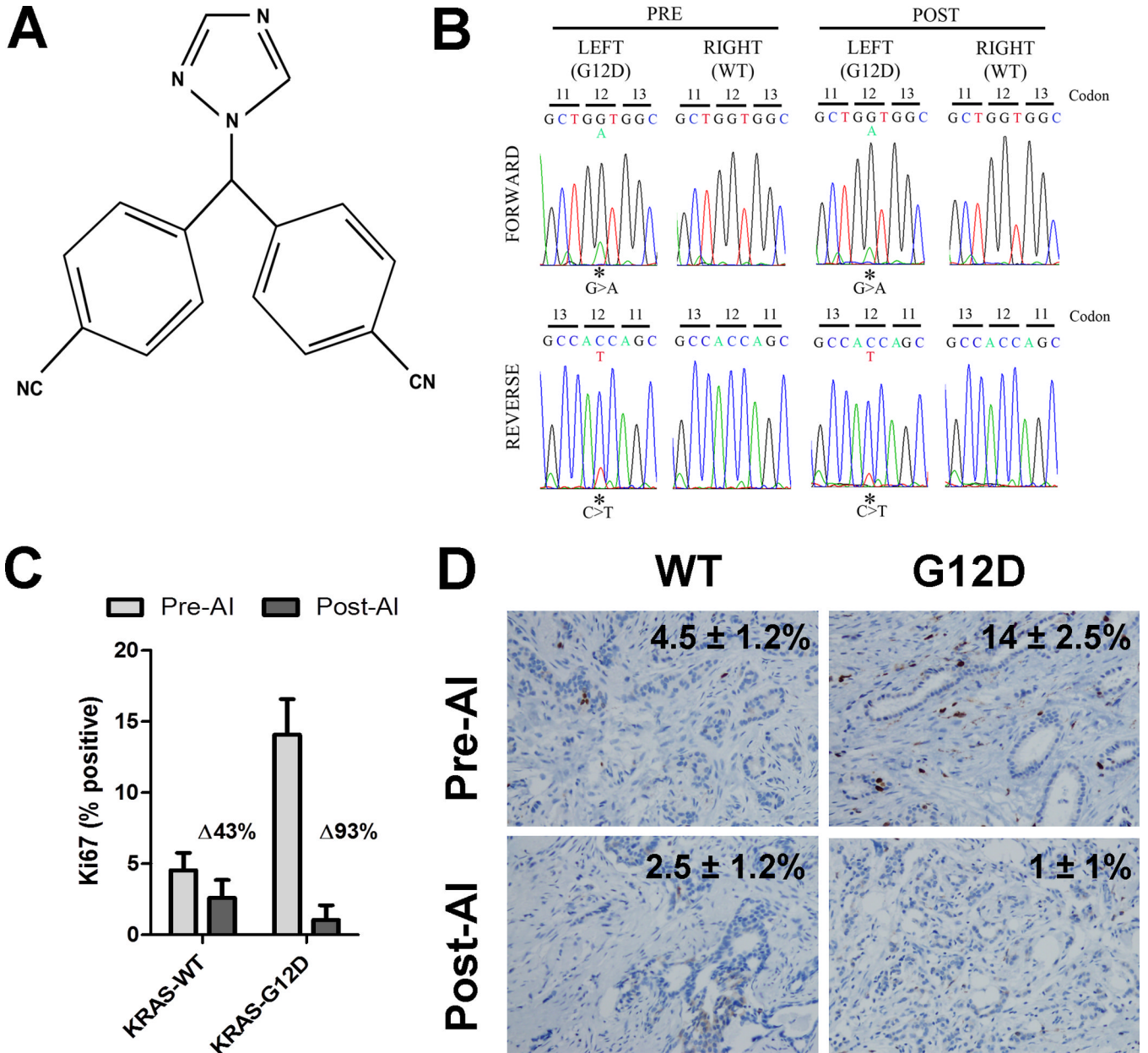
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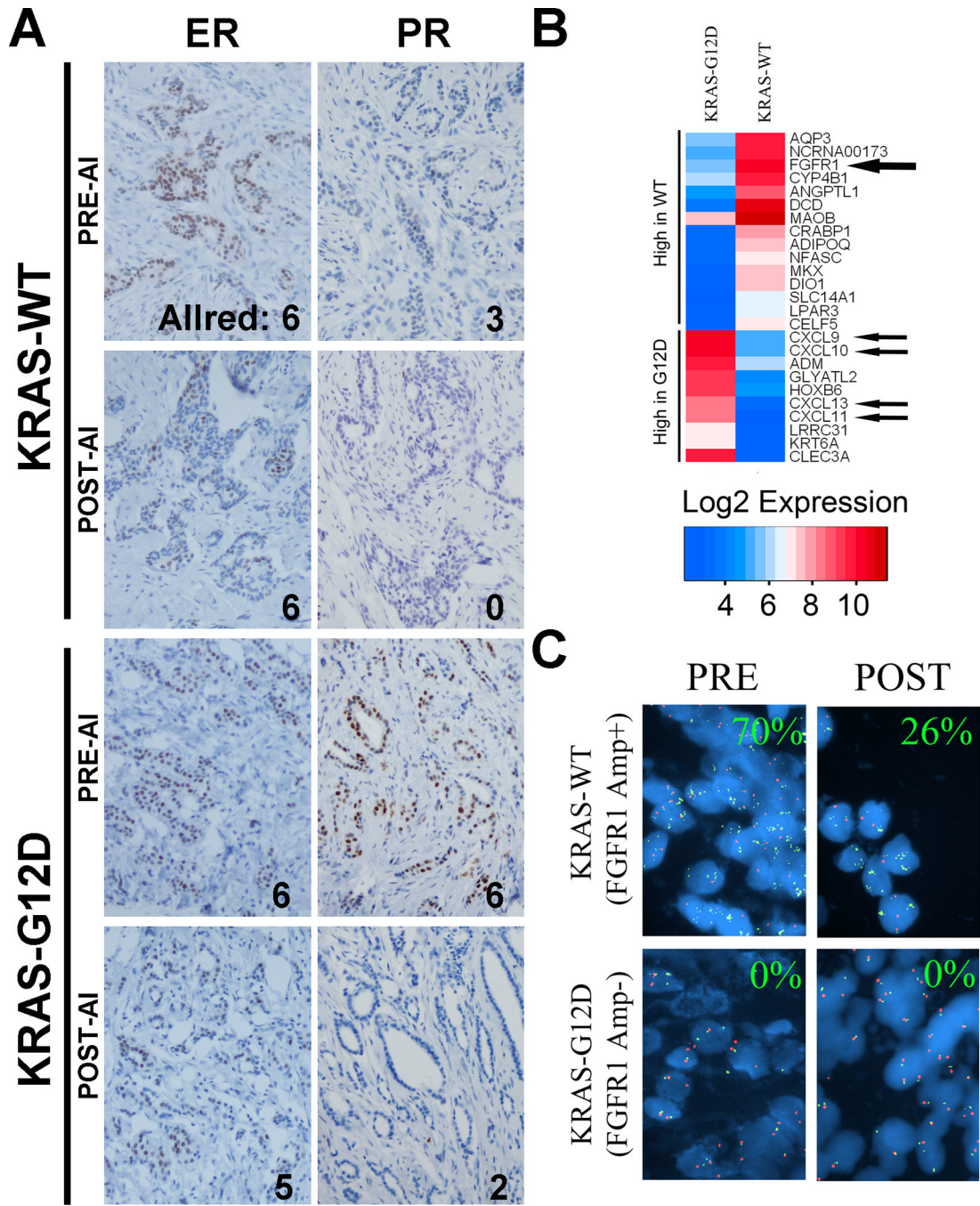
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**Figure 1. A breast tumor harboring mutant KRAS does not demonstrate molecular endocrine resistance**

A) Chemical structure of letrozole B) Sanger sequencing traces of KRAS codon 12 in the left and right bilateral tumors, before and after 2 weeks of letrozole therapy. C) Plot of Ki67 scores in left and right breast tumors before and after 2 weeks of letrozole therapy. Ki67 IHC was scored by 3 blinded independent pathologists to account for variability in scoring. Bars represent mean ± s.d. D) Representative IHC of Ki67 in left and right breast tumors before and after 2 weeks of letrozole therapy.



**Figure 2. FGFR1 amplification in a breast tumor demonstrating molecular endocrine resistance**  
 A) Representative IHC and ALLRED scores for ER $\alpha$  (ER) and PR in the bilateral tumors, before and after 2 weeks of letrozole therapy. PR staining was substantially decrease in both tumors with letrozole therapy. B) Heatmap 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. Percent nuclei demonstrating >5 FGFR1 gene cluster signals [green] per centromere 8 signal [red] reported in each panel.