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Estrogen withdrawal, increased breast cancer risk and the KRAS-variant

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The *KRAS*-variant is a biologically functional, microRNA binding site variant, which predicts increased cancer risk especially for women. Because external exposures, such as chemotherapy, differentially impact the effect of this mutation, we evaluated the association of estrogen exposures, breast cancer (BC) risk and tumor biology in women with the *KRAS*-variant. Women with BC ($n = 1712$), the subset with the *KRAS*-variant ($n = 286$) and *KRAS*-variant unaffected controls ($n = 80$) were evaluated, and hormonal exposures, *KRAS*-variant status, and pathology were compared. The impact of estrogen withdrawal on transformation of isogenic normal breast cell lines with or without the *KRAS*-variant was studied. Finally, the association and presentation characteristics of the *KRAS*-variant and multiple primary breast cancer (MPBC) were evaluated. *KRAS*-variant BC patients were more likely to have ovarian removal pre-BC diagnosis than non-variant BC patients ($p = 0.033$). In addition, *KRAS*-variant BC patients also appeared to have a lower estrogen state than *KRAS*-variant unaffected controls, with a lower BMI ($P < 0.001$). Finally, hormone replacement therapy (HRT) discontinuation in *KRAS*-variant patients was associated with a diagnosis of triple negative BC ($P < 0.001$). Biologically confirming our clinical findings, acute estrogen withdrawal led to oncogenic transformation in *KRAS*-variant positive isogenic cell lines. Finally, *KRAS*-variant BC patients had greater than an 11-fold increased risk of presenting with MPBC compared to non-variant patients (45.39% vs 6.78%, OR 11.44 [3.42–37.87], $P < 0.001$). Thus, estrogen withdrawal and a low estrogen state appear to increase BC risk and to predict aggressive tumor biology in women with the *KRAS*-variant, who are also significantly more likely to present with multiple primary breast cancer.

Introduction

MicroRNA (miRNA) binding site variants in the 3′ untranslated region (3′UTR) of important growth and survival genes are a recently discovered, novel class of functional, biologically active, germ-line mutations that are powerful biomarkers of cancer risk and treatment response.[1] One of the first mutations discovered in this class is the *KRAS*-variant, a *let-7* binding site mutation in the 3′UTR of the *KRAS* oncogene.[2] This mutation predicts an increased risk of several cancers, including non-small cell lung cancer,[2] triple negative breast cancer (TNBC) in premenopausal women[3] and ovarian cancer.[4-6] The *KRAS*-variant also predicts unique tumor biology, with tumors in *KRAS*-variant patients exhibiting a *KRAS*-addicted signature, as well as an estrogen negative, basal-like gene expression pattern.[3,5] Perhaps most powerful is the extensive evidence that the *KRAS*-variant is biologically functional, as exemplified by its role as a strong biomarker of response to cancer therapy. *KRAS*-variant patients with ovarian cancer or head and neck cancer are cisplatin resistant,[5,7] those with colon cancer or head and neck cancer exhibit cetuximab sensitivity,[7,8] and those with non-small cell lung cancer (NSCLC) are resistant to erlotinib but sensitive to sorafenib.[9] Cell line data further supports the unique response of the *KRAS*-variant to chemotherapy exposure.[8]

Women with the *KRAS*-variant are also at a significantly increased risk of developing multiple primary cancers, including breast and ovarian cancer, as well as a third independent cancer in their lifetime.[6] Multiple primary cancer, although difficult to predict, is not rare, as one in 8 cancer patients will be diagnosed with a new primary cancer after their first cancer diagnosis

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(metachronous cancer), and one in 40 patients will be diagnosed with 2 cancers at the same time (synchronous cancer).[10] While it is hypothesized that some metachronous cancers are caused by primary cancer treatment, it is also thought that genetics may play a significant role in the development of both synchronous and metachronous cancers.[11] Multiple primary breast cancer (MPBC) is one of the most common forms of multiple primary cancer.[12] Currently known risk factors for MPBC include young age at first diagnosis;[13-15] first BC of lobular histology;[12,16,17] high BMI (>30) in pre-menopausal patients with a hormone-receptor negative first primary;[18] positive family history of BC;[19] and mutations in *BRCA1, BRCA2*[20] or *CHEK2*.[21] Most recently, the *KRAS*-variant was found to also be associated with MPBC in a small case series, as it was found in 57.1% (4/7) of patients who developed bilateral BC and ovarian cancer who were uninformative (BRCA negative).[6] Factors thought to decrease MPBC risk have also been identified, and include menarche after age 13, multiparity,[17] treatment with anti-hormonal agents or chemotherapy[22,23] and prophylactic surgical intervention.[24] These findings suggest that multiple primary BC risk can be impacted by estrogen alterations either before or after the first BC diagnosis.

In general, evidence suggests that elevated estrogen increases primary BC risk. Such evidence includes increased BC risk in women experiencing early menarche, late menopause, obesity (in post-menopausal patients), nulliparity, or advanced maternal age at first birth.[25] In addition, *in vitro* studies using the breast epithelial line MCF10A show that excess estrogen and its metabolites can lead to increased transformation, i.e. BC initiation.[26,27] However, estrogen is not a risk for BC for all women, as has become clear through clinical studies of HRT use.[28,29] Initially, the Million Women Study and Women's Health Initiative reported that current and/or prolonged use of HRT correlated with an increased risk of BC. Because these tumors tended to be lower grade, with over-representation of lobular or tubular subtypes compared to other ductal cancers,[30] it was hypothesized that HRT was causing cancers that otherwise would not have arisen. However, a follow-up WHI report found that there was actually no increased BC risk for patients assigned to estrogen-only preparations compared to placebo. In fact, after a median follow up of 11.8 years (IQR 9.1–12.9), post-menopausal use of estrogen alone was associated with a lower BC incidence than placebo[31] (HR 0.77 (CI 0.62–0.95, p 0.02). These findings suggest that there may be a group of women for whom estrogen is actually protective against BC development.

For women with the *KRAS*-variant, there is growing evidence that estrogen may differentially impact their overall cancer risk and tumor biology. This evidence includes: a higher risk of non-small cell lung cancer in women versus men with the *KRAS*-variant (unpublished data); ovarian cancer almost exclusively post-menopausally for women with the *KRAS*-variant;[6] an increased risk of estrogen receptor (ER) negative tumor development in *KRAS*-variant patients (TNBC and type II uterine cancer,[32]) and; the finding that post-menopausal *KRAS*-variant BC patients with a history of HRT use are more likely to develop biologically aggressive BC.[33] Although it may seem unusual that an external exposure, such as estrogen, could impact the function of an inherited 3′UTR variant like the *KRAS*-variant, there is in fact strong evidence that such miRNA binding site mutations are "influenced" by external exposures.[34] This is believed to be through alterations in miRNAs, which are immediate responders to cellular stress, and which directly act through the 3′UTR sites affected by these mutations.[34]

Based on the association of the *KRAS*-variant with cancer in women and the biological impact of external exposures on this type of mutation, we hypothesized that estrogen alterations could impact cancer risk and tumor biology for individuals with the *KRAS*-variant. Here, we test this hypothesis, with a large cohort of BC survivors and unaffected *KRAS*-variant controls. We further biologically confirm our findings by utilizing an isogenically matched normal breast epithelial cell line with, or without the *KRAS*-variant. Finally, we define the association of the *KRAS*-variant with MPBC in these patients. We show here for the first time that estrogen withdrawal appears to increase both BC risk and to predict aggressive tumor biology for women with the *KRAS*-variant. We furthermore find that *KRAS*-variant BC patients are at a significantly elevated risk for both synchronous and metachronous BC development, which is not explained by other known risk factors.

Methods

Study groups

A cohort of BC patients were invited (through the Susan Love Foundation) to join a study called "The *KRAS*-variant and hormones" (http://www.armyofwomen.org/current/view?grant_id=438). 1906 women responded to the invitation and completed questionaires regarding age at diagnosis; anthropomorphic measurements including weight and height; reproductive history including parity, age at first birth, use of contraceptives and hormone replacement therapy; and personal and familial cancer history. Participants signed a consent approved through the Yale University Human Investigation Committee (HIC), and were mailed a cheek swab or saliva kit (Oragene) for DNA testing, and requested to supply pathology reports for their BC(s). 1712 patients supplied DNA samples. Pathology reports were used in all cases of second primary BCs, where synchronous second primary BCs were either in the contralateral breast, or if in the same breast were classified as multi-centric on the pathology reports, with different pathologies. Metachronous BC was of different pathology if in the same breast and classified as a new primary, or in the contralateral breast.

Control samples were provided by the Human Genetics Sample Bank at the Ohio State University Medical Center (OSUMC). All controls were women who had the *KRAS*-variant but were unaffected by cancer at the time of testing (n = 80). The Columbus Area Controls Sample Bank is a collection of control samples for use in human genetics research that includes both donor's anonymized biological specimens and linked phenotypic data. The data and samples are collected under the protocol "Collection and Storage of Controls for Genetics Research Studies," which is approved

by the Biomedical Sciences Institutional Review Board at OSUMC. Recruitment takes place in OSUMC primary care and internal medicine clinics. If individuals agree to participate, they provide written informed consent, complete a questionnaire that includes demographic, medical and family history information, and donate a blood sample, which is used for genomic DNA extraction and the establishment of an EBV-transformed lymphoblastoid cell culture, cell pellet in Trizol, and plasma.

KRAS-variant testing

For all participants, DNA was extracted from buccal swabs or saliva according to the manufacturer's protocol (Oragene). Coded patient samples were genotyped for the *KRAS*-variant using a Taqman-based assay as previously described,[2] in the MiraDx CLIA certified laboratory, through MiraKind, a non-profit organization.

Isogenic cell line creation

We generated isogenic MCF10A lines with and without the *KRAS*-variant using the CompoZr^{TM} custom designed zinc-finger nuclease (ZFN) targeted genome editing technology (Sigma-Aldrich),[35] per manufacturer's instructions. MCF10a cells are an immortalized, non-transformed mammary epithelial cell line derived from human fibrocystic mammary tissue and have a lack of tumorigenicity in nude mice and lack of anchorage independent growth.[36] A ZFN pair was designed and constructed to specifically target the *KRAS* 3′UTR. The donor construct containing the homology arms on either side of the *KRAS*-variant was generated by PCR amplifying a 2087 base pair region containing the *KRAS*-variant from genomic DNA with forward primer 5′ AGGACTCTGATTTTGAGGACATC 3′ and reverse primer 5′ AACATGCCCCACAAAGTTTC 3′ and cloning into the pGEM-T (Promega) cloning vector. The ZFN plasmids (500 ng) and the donor plasmid (2 ug) were transfected into 2×10^5 MCF10A cells by nucleofection, program T-024, according to manufacturer's instructions (Amaxa), in media containing 100 uM chloroquine. The media was changed after 4 hrs and the cells were incubated overnight and re-seeded as single cells into 24-well plates. After passage and DNA collection, clones were assessed for the presence of the *KRAS*-variant using an allele-specific primer and a PCR-based TaqMan assay using.[2] Secondary validation was carried out by allele-specific sequencing of TOPO TA® cloned, PCR amplified genomic DNA using forward primer 5′ AAGGCATACTAGTACAAGTGGTAATTT 3′ and reverse primer 5′ TAGGAGTAGTACAGTTCATGACAAAAA 3′, which hybridize to the *KRAS* locus outside of the region corresponding to the donor plasmid recombination site. In addition, 2 positive clones were authenticated using bi-allelic short tandem repeat (STR) analysis at 16 different genomic loci, yielding 32 diagnostic markers for confirmation (Genetica DNA Laboratories, Inc.). STR analysis confirmed that the MCF10A$^{KRAS\text{-variant}-/-}$ (Parental, WT) and the 2 MCF10A$^{KRAS\text{-variant}+/-}$ (MT) cell lines were (a) identical to the ATCCs STR profile and (b) identical to each other, except for the presence or absence of the *KRAS*-variant.

Cell line and anchorage independent growth assays

MCF10A (WT) and (MT) cells were cultured in regular DMEM/F12 medium (Invitrogen) as per the Brugge lab protocol.[37] Anchorage independent growth was assessed as described previously.[38] After thawing and growing cells until confluence in EGF supplemented media (20 ng/ml), cells were plated into conditions of study for 2 passages. For estrogen depletion experiments phenol red free DMEM/F12 medium (Invitrogen) and 5% charcoal-stripped horse serum (Thermo Fisher), were used, and Tamoxifen or estrogen was added to a final concentration of 1uM after the first passage, as appropriate. To plate, 100 μl of MCF10A (WT) or (MT) cells at a density of 400,000 cells/ml were mixed with 2ml of media for the condition under study containing 2 ml 0.7% noble agar (USB). 1ml of the cell mixture was added to 1ml of 1.0% noble agar in a well of a 6-well dish. Cells were fed twice weekly by layering on a 50:50 mixture of media with 0.7% agar for 2 weeks, followed by only media for 2–3 additional weeks. The number of colonies present in each of 10 microscope fields per well from a total of 3 wells per experiment was counted and is reported as an average of the 2 separate MT lines.

Statistics

Data was analyzed using the R environment for statistical computing and graphics. Continuous data was assessed for normality using Shapiro-Wilk test and parametric or non-parametric tests applied as appropriate. Student t tests were used to compare continuous variables that were normally distributed and Mann Whitney U test for non-normally distributed data. Categorical data was analyzed using 2×2 contingency tables (chi-square). In order to assess association between the likelihood of being diagnosed with a second primary BC and KRAS variants, we used logistic regression and quantified differential risk through odds ratios (OR). A similar analysis was replicated to associate the time from primary diagnosis to diagnosis with a second primary BC through the Cox proportional hazard model. Differential timing of second primary cancers was compared through hazard ratios (HR). In both modeling frameworks, when adjusting for potential confounders, we selected order and scope of interaction effects through the Bayesian Information Criterion (BIC). In the Cox proportional hazard model, the assumption of proportionality was assessed both visually by inspection of Kaplan-Meier survival curves and formally, through the analysis of Schoenfeld residuals ($P > 0.10$).

Results

KRAS-variant BC patients vs non-variant BC patients

We first evaluated history of estrogen exposure in BC patients with and without the *KRAS*-variant. Of the 1712 patients who supplied DNA samples, 17.4% ($n = 298$) had the *KRAS*-variant, and 70 (4.0%) had other known genetic mutations associated with increased BC risk, including *BRCA1, BRCA2* and *PTEN*. In the 1642 women without other mutations, 286 (17.42%) had the *KRAS*-variant, and 1356 (82.58%) did not. We evaluated the

association of self-reported estrogen exposures in these BC patients to determine if there were any differences for BC patients with vs. without the *KRAS*-variant. By univariate analysis, *KRAS*-variant BC patients were significantly more likely to have had an oophorectomy before their BC diagnosis (15.5% vs 10.7%, p = 0.024) and to be on HRT when diagnosed with BC (66.3% vs 54.4%, p = 0.034) than non-variant BC patients (**Table S1**). By multivariate analysis, *KRAS*-variant BC patients continued to be significantly more likely to have a history of ovarian removal (oophorectomy) pre-diagnosis (OR = 1.42, CI 1.03–1.42, p = 0.033) (**Table 1**). In addition, although *KRAS*-variant patients were not significantly more likely to have a family history of breast or ovarian cancer than non-variant BC patients (62.66% vs 64.01%, NS), they were significantly more likely to have a family history of a relative with multiple primary cancers than non-variant BC patients (4.98% vs 0.92%, $P < 0.0001$), in agreement with our prior findings of increased multiple primary cancer risk.[6]

The association of HRT with BC subtype and grade

We next evaluated the association of hormone replacement therapy (HRT) use and tumor biology in women with the *KRAS*-variant. We grouped post-menopausally diagnosed BC patients into 3 HRT use groups based on their HRT use at the time of their diagnosis. These groups comprised "never users," "current users" (women on HRT at the time of their BC diagnosis), or "past users" (women with a history of HRT preceding their BC diagnosis by at least 6 months). We then compared histologic BC tumor subtypes (ER/PR+, HER2+, or ER/PR/HER2- [triple negative]) and grade with these categories of HRT use for *KRAS*-variant (n = 133) vs non-variant BC patients (n = 612) with complete histologic tumor documentation.

Overall, there was no difference in tumor grade between *KRAS*-variant versus non-variant BC patients, but the TNBC tumor subtype was significantly more common in post-menopausal women with the *KRAS*-variant (13.9% vs 7.7%, p = 0.029). For non-variant BC patients, there were no differences in the proportion of women with each tumor subtype between the never, current or past HRT user groups. However, as reported previously[30] there was a trend for current or past HRT users to have lower grade breast tumors than never users, but this difference was not statistically significant in our study cohort. For *KRAS*-variant BC patients, past HRT users were significantly more likely to be diagnosed with TNBC than *KRAS*-variant never or current HRT users (35.5% [n = 11/31] vs 6.6% [6/91], $P < 0.0001$). In addition, compared to non-variant past HRT users, *KRAS*-variant past HRT users were significantly more likely to be diagnosed with TNBC (35.5% vs 7.3% [n = 11/151] $P < 0.0001$, **Table 2**), and also to have significantly higher-grade tumors (2.33 vs 1.98, p = 0.029). In contrast, there were no statistically significant differences in tumor subtype or grade between *KRAS*-variant never or current HRT users.

Table 1. *KRAS*-variant BC cases compared to non-variant BC cases. By a logistic regression model, with predictors included in the model assuming a linear additive structure, BC patients with the *KRAS*-variant were more likely to have had an oophorectomy compared to non-variant breast cancer patients

BC Patient Characteristics with versus without the *KRAS*-Variant	OR (95% C.I.)[p.val]
Baseline	Prob = 15.88%
Lobular	0.82 (0.53, 1.26) [0.365]
ER positive	0.76 (0.52, 1.11) [0.154]
Ovaries removed	1.42 (1.03, 1.96) [0.033]
BMI	0.98 (0.96, 1.01) [0.277]
BCP	1.23 (0.78, 1.94) [0.364]
Personal Cancer History	0.80 (0.54, 1.20) [0.278]
Age at Diagnosis	1.00 (0.98, 1.03) [0.705]
Menopause at Diagnosis	1.27 (0.83, 1.96) [0.266]
Ever pregnant	0.93 (0.65, 1.32) [0.686]

KRAS-variant BC patients vs unaffected KRAS-variant controls

We then evaluated if differences in hormonal exposures might impact BC risk in women with the *KRAS*-variant, by comparing hormonal exposures in *KRAS*-variant BC patients (n = 286) with a cohort of *KRAS*-variant cancer free unaffected controls (n = 80). In univariate analysis we found numerous significant differences, including factors associated with HRT use, pregnancy, OCP use and BMI (**Table S2**). By multivariate analysis we confirmed that *KRAS*-variant BC patients remained significantly more likely to have a lower BMI, and have fewer live births than *KRAS*-variant cancer free controls (**Table 3**). Of note, we found no difference in age of diagnosis vs age of enrollment between the BC patients and the controls.

KRAS-variant MCF10A cell lines and Transformation

To biologically confirm our clinical findings, that a low estrogen state and/or estrogen withdrawal may be associated with increased BC risk for women with the *KRAS*-variant, we created an isogenic MCF10a line, with (MCF10a$^{KRASv+/-}$, MT1 and MT2) vs. without (MCF10a$^{KRASv-/-}$, WT) the *KRAS*-variant. We found that *KRAS* mRNA was lower in the MT cells, but *KRAS* protein was fairly equivalent or slightly elevated (**Fig. S1**), consistent with prior reports in *KRAS*-variant-associated tissues.[39]

WT and MT lines were plated in soft agar to test for transformation, as measured by anchorage independent growth. There was no colony formation seen in the presence of Epidermal Growth Factor (EGF) during the course of the experiment for either the WT or MT lines, indicating that neither line, at baseline, was transformed (**Fig. S2**). However, when the cell lines were grown without EGF, as is standard to promote transformation, the MT lines exhibited low levels of colony formation by the fifth soft agar plating (10 +/− 2.24, $P < 0.001$). We next evaluated if estrogen withdrawal would enhance transformation, consistent with our clinical findings, by growing the cells in charcoal stripped serum, tamoxifen, or a combination of the two. We found for both MT lines a 2-fold increased colony formation rate when cells were grown in Tamoxifen (p = 0.002), a 6.2 fold increased colony formation in charcoal stripped media ($P < 0.001$), and a 7.9 fold increased colony formation with the

Table 2. Histologic breast cancer subtype and history of hormone replacement therapy use. Tumor grade between all *KRAS*-variant vs. non-*KRAS*-variant BC patients was non-significant. *KRAS*-variant patients were significantly more likely to have triple negative breast cancers as a group (13.9% vs 7.7%, p = 0.029). *KRAS*-variant patients with a history of past HRT use were significantly more likely to have TNBC. There were no differences in cancer subtype by HRT use for non-variant patients

		***KRAS*-variant**	**Non-*KRAS*-variant**	**P value**
Never on HRT	ER+	77.1% (27/35)	85.2% (127/149)	NS
	HER2+	22.9% (8/28)	19.9% (28/141)	NS
	TN	11.4% (4/35)	9.3% (14/150)	NS
	Grade	2.24	2.16	NS
On HRT when diagnosed (current)	ER+	85.5% (47/55)	84.8% (156/184)	NS
	HER2+	16.3% (7/43)	12.0% (17/142)	NS
	TN	3.6% (2/56)	6.6% (12/182)	NS
	Grade	2.02	2.01	NS
Stopped HRT >6 months before diagnosis (past)	ER+	53.1% (17/32)	89.7% (139/155)	<0.0001
	HER2+	6.9% (2/29)	11.2% (15/134)	NS
	TN	35.5% (11/31)	7.3% (11/151)	<0.0001
	Grade	2.33	1.98	**p=0.029**

combination ($P < 0.001$, **Fig. 1A**). Supporting that the impact of charcoal stripping on transformation was due to estrogen depletion, return of estrogen to the media resulted in decreased colonies for the MT cell lines (p = 0.018, **Fig. 1B**). These findings biologically confirm wide spread transformation in normal breast epithelium with acute estrogen withdrawal in breast cells with the *KRAS*-variant.

Multiple primary BC Risk in KRAS-variant BC patients

Based on our findings that estrogen withdrawal appears to increase wide spread breast cell transformation in *KRAS*-variant breast epithelial cells, we evaluated the association of the *KRAS*-variant with MPBC. We found that overall women with the *KRAS*-variant (GT or GG) did exhibit a 2.04-fold increase risk of having a second primary BC, compared to women without the variant (12.93% vs 6.78% with MPBC, $P < 0.001$). In addition, we found a genetic dose effect of the *KRAS*-variant, with women heterozygous (GT) for the variant exhibiting a 1.81-fold increased risk of having a second primary BC (11.64% with MPBC, p = 0.006), and women homozygous (GG) for the *KRAS*-variant having an 11.64-fold increase risk of having a second primary BC (45.39% with MPBC, $P < 0.001$) compared to non-variant BC patients (**Table 4**).

We next investigated if second BC for *KRAS*-variant patients primarily occurred at the same time as their first diagnosis (synchronous MPBC), or after their first diagnosis (metachronous MPBC). We found that women with the *KRAS*-variant had a 2.63-fold increased risk of being diagnosed with a synchronous second primary BC compared to non-variant BC patients (6.79% vs 2.70% with synchronous MPBC, p = 0.001). This was again most pronounced for women homozygous for the *KRAS*-variant, who had a 12.03-fold increased risk of having a synchronous second primary BC (25.02% with synchronous MPBC, p = 0.003) compared to non-variant patients. However, women with the *KRAS*-variant also continued to be at an elevated risk for a metachronous BC, with a 1.72-fold increased risk of developing a metachronous second primary tumor when compared to non-variant patients (8.05% vs 4.84% with metachronous MBPC, p = 0.05). This difference was again primarily explained by the large increased risk of metachronous BC for the homozygous *KRAS*-variant group, who had a 14.72-fold increased risk of developing a second primary BC after their first BC diagnosis (42.80% with metachronous MPBC, $P < 0.001$, **Table 4**, **Fig. S3**).

Table 3. *KRAS*-variant BC cases compared to KRAS-variant controls. Women with breast cancer with the *KRAS*-variant by a binary logistic model were significantly more likely to have fewer live births, and to have a lower Body Mass Index (BMI)

Age at diagnosis/enrollment		
	Odds Ratio (95% CI)	p-value
Age at diagnosis	0.95 (0.89–1.03)	0.211
Use of HRT[1]	2.73 (0.91–8.18)	0.07
Duration of HRT use	1.00 (0.99–1.00)	0.62
Number of live births	0.62 (0.39–0.98)	0.04
Age at first birth	1.11 (0.99–1.24)	0.06
BMI	0.93 (0.87–1.00)	0.04
OCP use[2]	2.15 (0.63–7.42)	0.22
Duration of OCP	1.01 (0.94–1.09)	0.77
Oophorectomy before diagnosis/enrollment[3]	1.3 (0.44–3.89)	0.63

[1]Compared to no HRT use.
[2]Compared to no OCP use.
[3]Compared to no ovarian procedure.

Multiple primary breast cancer risk and other risk factors

We next evaluated MPBC risk controlling for the extent of surgery and time of follow up. Controlling for extent of primary surgery, women with the *KRAS*-variant who had a lumpectomy or a unilateral mastectomy were significantly more likely to have a synchronous second primary tumor than non-variant patients (lumpectomy OR = 4.32, CI 1.15–16.40, p = 0.03; unilateral mastectomy OR = 18.42, CI 3.88–87.82, $P < 0.001$, **Table S3A**). In addition, controlling for number of years at risk, women with the *KRAS*-variant treated with a lumpectomy were significantly more likely to develop a second, metachronous primary BC (OR = 1.84, CI-1.03–3.27, p = 0.04) when compared to non-variant patients treated in the same manner (**Table S3B**). This was confirmed using a time to event analysis (p = 0.05). Of

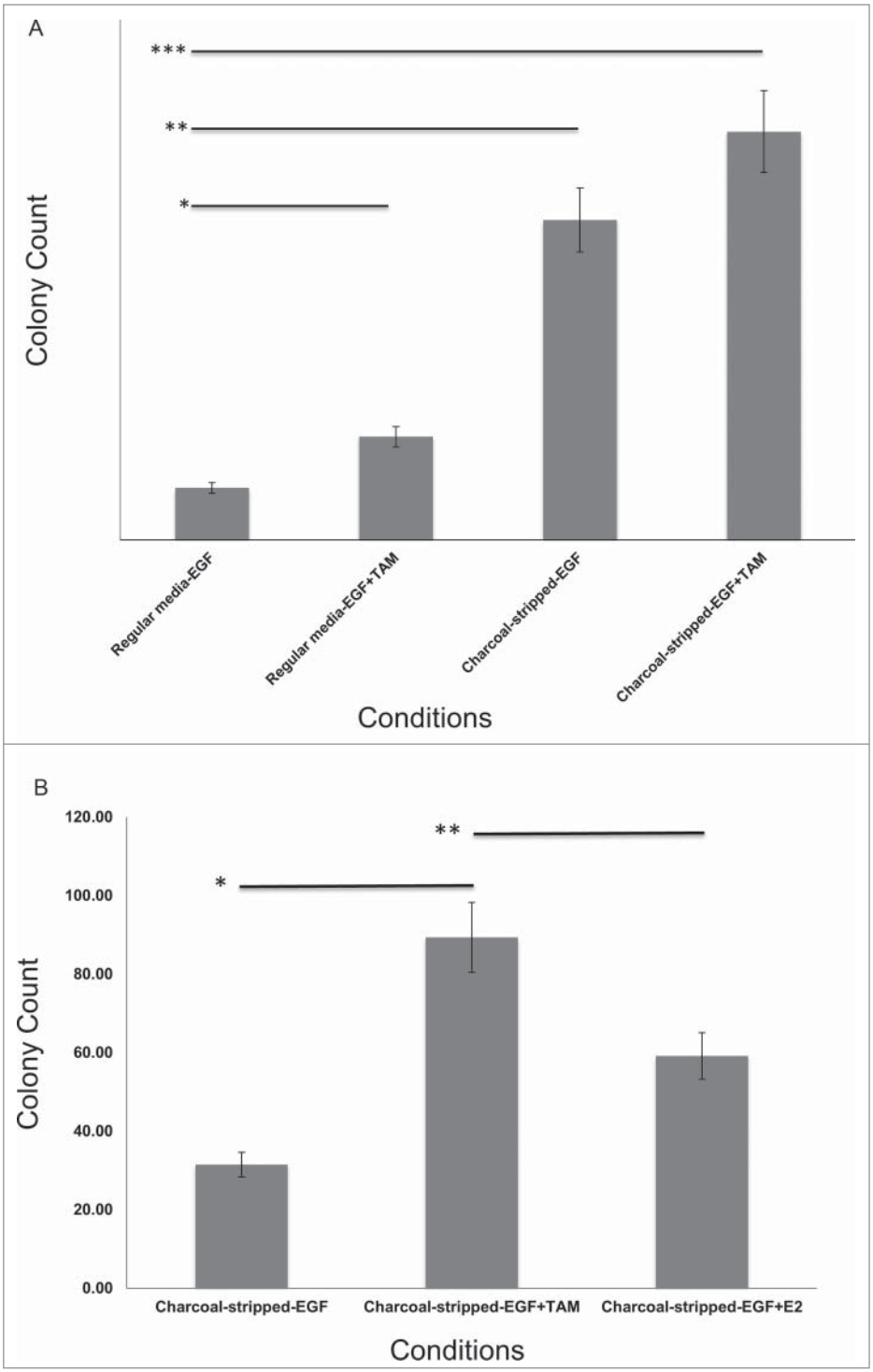

Figure 1. Transformation in MCF10A$^{KRAS+/-}$(MT1 and MT2) epithelial breast cell lines. (**A**) Under EGF and estrogen withdrawal conditions, MT cells become transformed and develop colonies in an anchorage independent growth assay. Each sample represents 10 counted 10 × fields from 3 different experimental replicates, and is the average of the MT1 and MT2 lines. Experiments were repeated 3 times. Data is from Passage 2 into soft agar. Error bars represent SEM. TAM = tamoxifen, 10 ug/ml. *p = 0.002, **$P < 0.001$, ****$P < 0.001$ (**B**) MCF10A MT lines form colonies which are reduced when estrogen is returned to the media. Each sample represents 10 counted 10 × fields from 3 different experimental replicates, and is the average of the MT1 and MT2 lines. Experiments were repeated 3 times. Data is from Passage 3 into soft agar. Error bars represent SEM. TAM = tamoxifen, final concentration 1 uM. *$P < 0.001$, **p = 0.018

note, we found that *KRAS*-variant and non-variant patients did not significantly differ in their choice of lumpectomy, unilateral mastectomy or bilateral mastectomy at the time of diagnosis (**Table S4A**). As expected, women with either a unilateral or a bilateral mastectomy were more likely to have a diagnosis of a synchronous MPBC (**Table S4B**).

We next evaluated the association of lobular histology with the *KRAS*-variant and second BC risk. We found that the *KRAS*-variant was not associated with lobular histology, although in agreement with prior reports, in our cohort lobular histology alone was associated with increased rates of second primary BC, both synchronous and metachronous (**Table S5A and B**).

Finally, we controlled for lobular histology, extent of surgery and number of years at risk and evaluated the association of the *KRAS*-variant with MPBC. We found that women with the *KRAS*-variant treated with unilateral mastectomy were significantly more likely to have a synchronous second primary tumor regardless of lobular histology (OR = 40.75, CI = 4.98–339.72, $P < 0.01$). In addition, women with the *KRAS*-variant treated with lumpectomy with non-lobular histology continued to be significantly more likely to develop a second, metachronous primary BC (OR = 2.01, CI = 1.05–3.86, p = 0.04). Similar conclusions were found using a time to event analysis (HR = 2.011, p = 0.03)(**Table 5**).

To confirm that having the *KRAS*-variant was an independent predictor of MPBC, we performed a multivariate analysis using a logistic regression model, assuming that the predictors included in the model had a linear additive structure. We confirmed using this model that the *KRAS*-variant was an independent predictor of MPBC risk considering all other risk factors (OR = 2.26, CI 1.44–2.26, $P < 0.001$, **Table S6**).

Discussion

In this study we show for the first time that estrogen withdrawal increases

Table 4. Second breast cancer risk in *KRAS*-variant breast cancer patients. Women with the *KRAS*-variant are significantly more likely to be diagnosed with multiple primary breast cancer, including synchronous and metachronous second primary breast cancer. This is especially true for *KRAS*-variant homozygous (GG) patients

Second Primary Tumor Risk

KRAS-Variant Genotype	No.	% Second Primary BC (95% C.I.)	OR (95% C.I.) [p.val]	
TT	1357	6.78% (5.56%–8.25%)	1.00 (Baseline)	
TG or GG	286	12.93% (9.52%–17.32%)	2.04 (1.36–3.06) [<0.001]	
TG	275	11.64% (8.35%–15.99%)	1.81 (1.18–2.77) [0.006]	
GG	11	45.39% (20.25%–73.04%)	11.44 (3.42–37.87) [<0.001]	
Synchronous Second Primary Tumors				
		Second Primary BC (95% C.I.) {OR} [p.val]		
KRAS Variants	No.	Combined	Unilateral	Contralateral
TT	1561	2.70% (1.95%–3.73%){1.00-baseline}	0.23% (0.07%–0.72%){1.00-baseline}	2.62% (1.87%–3.64%){1.00-baseline}
TG or GG	1296	6.79% (4.31%–10.50%){2.63} [0.001]	4.49% (2.58%–7.71%){20.29} [<0.001]	3.00% (1.51%–5.90%){1.15} [0.73]
TG	257	6.23% (3.85%–9.87%){2.39}[0.005]	3.85% (2.09%–6.98%){17.22}[<0.001]	3.09% (1.55%–6.04%){1.19} [0.67]
GG	8	25.02% (6.27%–62.24%) {12.03}[0.003]	30.03% (10.05%–62.01%){184} [<0.001]	10.02% (1.39%–46.57%){4.15}[0.18]
Metachronous Second Primary Tumors (Excluding double mastectomy cases)				
		% Second Primary BC (95% C.I.) OR [p.val]		
KRAS Variants	No.	Combined	Unilateral	Contralateral
TT	1393	4.84% (3.74%–6.23%){1.00-baseline}	0.52% (0.23%–1.14%){1.00-baseline}	4.40% (3.36%–5.76%{1.00-baseline}
TG or GG	236	8.05% (5.19%–12.33%){1.72}[0.04]	2.10% (0.88%–4.97%){4.12} [0.02]	
TG	229	6.98% (4.32%–11.09%){1.48}[0.16]	1.73% (0.65%–4.52%){3.38} [0.06]	6.73% (4.16%–10.73%){1.57} [0.13]
GG	7	42.80% (14.32%–76.88%){14.72}[<0.001]	22.23% (5.57%–57.88%){54.8} [<0.001]	

breast cancer risk in women with the *KRAS*-variant, who are also significantly more likely to present with and develop multiple primary breast cancers. This finding was confirmed biologically in cell lines with the *KRAS*-variant compared to isogenic controls. BC risk appears to be increased by a low estrogen state in general, and abrupt estrogen withdrawal, as found with oophorectomy, discontinuation of HRT, or in our cell line assays, enhances transformation and appears to increase the risk of aggressive breast tumor biology. We find that women with the *KRAS*-variant are at greatest risk of presenting with multiple primary synchronous breast cancer, although also continue to be at risk of metachronous breast cancer development. These findings further highlight the unique paradigm of 3′UTR mutations, as well as give new insight into how this mutation could meaningfully subgroup patients to develop the best preventive approaches for breast cancer.

The role of estrogen withdrawal on BC risk for women with the *KRAS*-variant could be due to a relationship between the *KRAS*-variant, its downstream pathways and estrogen signaling, as there are known interaction between estrogen signaling and

Table 5. Second breast cancer risk in *KRAS*-variant breast cancer patients controlling for lobular histology, extent of surgery and time. Women with the *KRAS*-variant continue to be at a significantly increased risk of synchronous and metachronous breast cancer when controlling for lobular histology, extent of surgery and time

A. Frequencies of second primary BC by Extent of Surgery, Histology and Time

Synchronous Tumors				
		No.	KRAS TT% Second Primary BC (95% C.I.)	KRAS TG/GGOR (95% C.I.) [p.val]
Lumpectomy	Non-Lobular	748	0.51% (0.19%–1.36%)	4.43 (0.97–20.38) [>0.5]
	Lobular	97	0.94% (0.29%–2.95%)	2.88 (0.40–20.99) [>0.5]
Unilateral	Non-Lobular	247	0.38% (0.05%–2.67%)	40.75 (4.98–339.72) [<0.01]
	Lobular	45	0.70% (0.09%–5.20%)	26.55 (2.42–295.05) [<0.01]
Bilateral	Non-Lobular	166	13.62% (8.96%–20.15%)	6.44 (0.98–41.97) [>0.5]
	Lobular	54	22.57% (12.93%–36.29%)	0.95 (0.24–3.75) [>0.5]
Metachronous Second Primary Tumors (Adjusted by no. years at risk)				
		No.	KRAS TT% Second Primary BC (95% C.I.)	KRAS TG/GGOR (95% C.I.) [p.val]
Lumpectomy	Non-Lobular	792	4.89% (3.49%–6.80%)	2.01 (1.05–3.86) [0.04]
	Lobular	110	12.08% (7.23%–19.46%)	1.08 (0.21–5.38) [>0.5]
Unilateral	Non-Lobular	245	1.77% (0.76%–4.06%)	1.54 (0.29–8.27) [>0.5]
	Lobular	47	4.59% (1.86%–10.82%)	0.83 (0.10–6.92) [>0.5]
Time to Second Primary Tumor Development				
		No.	KRAS TTHR (95% C.I.) [p.val]	KRAS TG/GGHR (95% C.I.) [p.val]
Lumpectomy	Non-Lobular	792	1.00-Baseline	2.01 (1.08–3.77) [0.03]
	Lobular	110	2.39 (1.31–4.36) [<0.001]	1.38 (0.30–6.21) [>0.5]
Unilateral	Non-Lobular	245	0.34 (0.15–0.81) [<0.001]	1.31 (0.26–6.69) [>0.5]
	Lobular	47	0.82 (0.30–2.29) [>0.5]	0.89 (0.11–7.23) [>0.5]

Odds Ratios (OR) and Hazard Rations (HR) refer to a comparison of *KRAS*-variants within extent of surgery category and lobular status.

the RAS pathway. Alternatively, the relationship between estrogen and the *KRAS*-variant may instead be due to alterations in miRNA expression or regulation caused by this powerful hormone. In support of the later, we have previously shown that TNBC tumors from women with the *KRAS*-variant have significantly higher aromatase expression and ER Beta expression. Both of these genes are regulated by the miRNA *let-7*, which is known to be low in *KRAS*-variant associated tissues and tumors. One could speculate that sudden estrogen withdrawal disrupts these biological interactions in *KRAS*-variant tissues, ultimately leading to escape, independent signaling and growth, and oncogenesis. Extensive cell line work is ongoing to define the relationship with estrogen and the series of mechanistic events leading to cancer in individuals with the *KRAS*-variant. Regardless, our cell line findings confirm that breast cells with the *KRAS*-variant are transformed by estrogen withdrawal. In addition, our clinical findings that BC patients with the *KRAS*-variant are more likely to have an oophorectomy than non-variant patients, have a lower BMI, and thus lower circulating estrogen than controls, and that HRT discontinuation leads to aggressive tumor biology, supports the hypothesis that acute estrogen withdrawal alters breast cell biology for *KRAS*-variant individuals.

A genetic marker of increased risk of synchronous MPBC has not been previously identified. Other BC associated genetic mutations are generally considered to predict an increased risk of second, metachronous BC, likely due to the continued DNA damage-prone state of the tissues in these individuals. For women with the *KRAS*-variant, our findings here suggest instead a scenario where an "event" promotes cancer initiation, globally impacting their breast tissue. Based on our results, we hypothesize that the event could be some form of acute estrogen withdrawal, a hypothesis requiring further confirmation. As treatment for BC general involves acute estrogen withdrawal, through chemotherapy and/or anti-estrogen therapy, it seems possible that the continued risk of metachronous breast cancer in *KRAS*-variant patients may be partly a result of treatment for their first BC. Studies are currently on-going for women with the *KRAS*-variant to both better understand the first potential "causative event," as well as to define the most efficacious, and safest, treatment strategies to avoid metachronous breast cancer.

Limitations of our clinical studies include self-reported lifestyle factors for our BC patients, which are prone to recall bias. However, our most critical findings, regarding tumor biology post-HRT, and second BC risk, were all confirmed with pathologic documentation. Another limitation of our study is that our population was not prospectively collected, allowing survivor bias for metachronous BC development. However, our cohorts in this study have identical length of follow up, and we controlled for time in our metachronous BC analysis. Also, as women with the *KRAS*-variant are significantly more likely to be diagnosed with premenopausal TNBC, which is the most deadly form of breast cancer, if anything, this bias should have decreased our ability to identify an association between metachronous BC and the *KRAS*-variant.

Perhaps most importantly, the findings from this study further highlight the critical importance of studying biologically functional 3′UTR miRNA binding site mutations in the appropriate cohorts. Unlike previously discovered mutations that impact DNA repair, 3′UTR mutations instead alter the appropriate cellular response to external factors. Since both lifestyle and environmental exposures will differ across populations, and represent external factors, increasing subject numbers as is standard by large consortia by combining patients of numerous ethnic backgrounds and cultures should be avoided in the study of 3′UTR mutations. Since such consortia have begun to study 3′UTR mutations, it should be recognized that their findings, or lack of findings, will be biased against finding the mutations that are perhaps the most important – those that could be managed by lifestyle modifications. Utilizing the correct cohorts to define the factors that can modify cancer risk in biologically functional 3′UTR mutations should be an extremely high priority in cancer prevention studies at this time.

Although the best estrogen management strategies for women with the *KRAS*-variant are yet to be defined, our findings do suggest that sudden estrogen withdrawal, such as that caused by oophorectomy or abrupt discontinuation of HRT, may increase breast cancer risk for these women. It also appears that women with the *KRAS*-variant are significantly at increased risk of MPBC, and at the time of their first BC diagnosis should be carefully evaluated for other synchronous primaries. While those at highest risk are women homozygous for the *KRAS*-variant, a relatively rare genotype (~3% of the healthy population), it is important to note that the prevalence of homozygote *KRAS*-variant patients is still >10 fold higher than *BRCA* mutant individuals in the healthy population (~0.25%). While the best way to integrate these findings into current BC management is an active area of discussion between both physicians and BC patients, this marker is a potentially vital additional tool to help guide both estrogen tailoring and BC management for women with the *KRAS*-variant, who comprise one in 5 newly diagnosed BC patients.

Disclosure of Potential Conflicts of Interest

FJS and JBW declare that they have financial interests in Mira Dx, a company that has licensed the *KRAS*-variant from Yale University.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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