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Cancer Research

A *KRAS*-Variant in Ovarian Cancer Acts as a Genetic Marker of Cancer Risk

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Abstract

Ovarian cancer (OC) is the single most deadly form of women's cancer, typically presenting as an advanced disease at diagnosis in part due to a lack of known risk factors or genetic markers of risk. The *KRAS* oncogene and altered levels of the microRNA (miRNA) *let-7* are associated with an increased risk of developing solid tumors. In this study, we investigated a hypothesized association between an increased risk of OC and a variant allele of *KRAS* at *rs61764370*, referred to as the *KRAS*-variant, which disrupts a *let-7* miRNA binding site in this oncogene. Specimens obtained were tested for the presence of the *KRAS*-variant from nonselected OC patients in three independent cohorts, two independent ovarian case-control studies, and OC patients with hereditary breast and ovarian cancer syndrome (HBOC) as well as their family members. Our results indicate that the *KRAS*-variant is associated with more than 25% of nonselected OC cases. Further, we found that it is a marker for a significant increased risk of developing OC, as confirmed by two independent case-control analyses. Lastly, we determined that the *KRAS*-variant was present in 61% of HBOC patients without *BRCA1* or *BRCA2* mutations, previously considered uninformative, as well as in their family members with cancer. Our findings strongly support the hypothesis that the *KRAS*-variant is a genetic marker for increased risk of developing OC, and they suggest that the *KRAS*-variant may be a new genetic marker of cancer risk for HBOC families without other known genetic abnormalities. *Cancer Res; 70(16); OF1–7. ©2010 AACR.*

Introduction

Ovarian cancer (OC) is the second most common gynecologic malignancy, but the most common cause of death from gynecologic malignancies (1). More than 90% of OCs are epithelial OCs, which are believed to arise from the epithelial component of the ovary or from the fimbriated end of the fallopian tubes (2). Epithelial OC is the fifth leading cause of cancer death in females in the United States, with more than 15,000 women dying yearly of this disease (1).

There are few identified risk factors for epithelial OC, except for aging. More than 90% of cases arise after menopause, with half of all OC cases diagnosed after age 63 (3). An inherited risk component exists for developing OC, as first-degree relatives of OC patients more frequently develop the disease than the general population, whose risk is 1/71 (4). Known familial cases make up approximately 10% of all OC cases, with the rest considered sporadic.

One of the best-studied hereditary syndromes linked to familial OC is hereditary breast and ovarian cancer syndrome (HBOC). HBOC is associated with mutations in the tumor suppressor genes *BRCA1* and *BRCA2*. Although the discovery of *BRCA1* and *BRCA2* represented a significant advance in understanding familial OC predisposition, these genes account for less than half of familial excess risk of OC (5). HBOC patients who test negative for *BRCA1* and *BRCA2* mutations are referred to as "mutation negative" or "uninformative." Additional gene alterations explaining any significant proportion of the remaining familial risk for OC have not been identified, and it has been hypothesized that the remaining genetic risks for familial OC are due to combinations among many loci of alleles that confer low-penetrance susceptibility (6).

Although common cellular tumor suppressors and oncogenes such as *p53, AKT, RAS, c-MYC*, and their associated pathways are thought to cooperate to lead to the development of OC (7), there have been no known germline mutations in these

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genes previously associated with OC. A study using a tagging single-nucleotide polymorphism (SNP) approach to identify moderate/low-risk susceptibility alleles of the proto-oncogenes *BRAF, ERBB2, KRAS, NMI*, and *PIK3CA* (8) found no evidence of OC association with these SNPs. Only when stratified by histologic subtype did one common variant allele have borderline evidence of association with epithelial OC (8). A recent genome-wide association study (GWAS) of tagging SNPs in thousands of OC patients and controls identified a region of the genome associated with OC; however, the studied variant alleles in this region were not within known genes, and these alleles predicted a decrease in OC risk (9).

The lack of the identification of powerful biomarkers of OC risk in these studies using the tagging SNP approach likely reflects inherent limitations of this approach; the SNPs studied are not representative of all SNPs, do not capture less common variants, and are rarely functional, but rather are SNPs that act as markers identifying common haplotypes. The tagging SNPs associated with disease risk thus far have been found to have only modest predictive power and account for a small fraction of heritability. Many believe that the study of functional and less heterozygous SNPs through candidate gene studies or more focused analyses is necessary to find important, less common variants with greater predictive power (10–12).

MicroRNAs (miRNA) are a class of ~22-nucleotide noncoding RNAs that are evolutionarily conserved and function by negatively regulating gene expression by binding to partially complementary sites in the 3′ untranslated regions (3′UTR) of target mRNAs. MiRNAs are aberrantly expressed in virtually all cancers, where they function as a novel class of oncogenes or tumor suppressors (13). Because miRNAs are global gene regulators, even small aberrations in miRNA levels or their target sites can lead to important cellular changes. In support of this concept, emerging evidence shows that SNPs within miRNAs or miRNA binding sites can be functional and act as powerful biomarkers of cancer risk when one allele alters miRNA function or binding characteristics (14–16).

The *let-7* family of miRNAs has been shown to play an important role in many cancers (17–19). *let-7* regulates multiple OC oncogenes, including *KRAS* and *c-MYC* (20). We previously identified a germline SNP in the 3′UTR of the *KRAS* oncogene (*rs61764370*). The variant, derived allele, referred to as the *KRAS*-variant, is relatively uncommon: It is almost absent in East Asians and Native Americans, is uncommon in Africans, and has a minor allele frequency of about 7% in populations of European descent, based on the genotyping of more than 2,400 samples representing 46 geographic populations (14). The *KRAS*-variant is functional and was previously shown by us to disrupt binding of *let-7* to *KRAS*, leading to increased *KRAS* levels in *in vitro* assays (14). The *KRAS*-variant was initially found to be a genetic marker of increased susceptibility to non–small-cell lung cancer (NSCLC) in two U.S. case-control studies, where it was identified in 18% to 20% of NSCLC patients versus 12% to 14% of noncancerous U.S. ethnicity-matched control populations (14). Of note, in people the *KRAS*-variant is almost always heterozygous in both normal and tumor tissues. In addition, lung tumors with the *KRAS*-variant were found to have lower *let-7* levels, which has previously been shown to be a biomarker of poor outcome (21). The *KRAS*-variant was subsequently shown to be a genetic marker of poor outcome in head and neck cancer (22).

Because of the importance of both *let-7* and *KRAS* in human solid tumors, we evaluated the frequency of the *KRAS*-variant in patients with other solid tumor types. The *KRAS*-variant was present in fewer than 18% of all patients with other solid tumor types tested and in control populations matched for ethnicity (n > 17,000 individuals tested).[9] In contrast, the *KRAS*-variant is present in more than 25% of patients with epithelial OC. Through case-control analysis, we here show that the *KRAS*-variant seems to be a genetic marker of risk for developing OC for the general female population (P < 0.020). Also, the *KRAS*-variant is strongly associated with uninformative HBOC families, being present in 61% of those tested (n = 31), and segregates with their family members with cancer (P < 0.001). These findings suggest that the *KRAS*-variant represents a new genetic marker of OC risk and may account for genetic risk in a number of HBOC families previously considered uninformative.

Materials and Methods

Samples from New Haven, Connecticut

Samples from patients with OC at Yale/New Haven Hospital were recruited and collected from fresh frozen tissue (n = 12), DNA isolated from paraffin-embedded formalin-fixed tissue (n = 23), blood (n = 71), or saliva (n = 51) between 2007 and 2009 (total n = 157; Supplementary Table S2). Because we have previously extensively validated that the *KRAS*-variant is not somatic but germline (identical in patient's normal and tumor tissues; ref. 14), we collected primarily germline DNA for these studies from either blood or saliva. Patient data were collected including age, ethnicity, and family history of cancer. OC subtype was established by pathologic classification, with only epithelial OC cases included in this study.

OC patients from HBOC families were recruited through the Yale Cancer Center Department of Genetics, and one individual was included from each family as the index case for statistical analysis.

Controls (all female) were recruited from Yale/New Haven Hospital beginning in 2008 from healthy friends and associates of patients; none were genetically related to the patient. All control DNA samples were derived from saliva. None of the controls had any prior diagnosis of cancer (other than nonmelanoma skin cancer). Information on age, ethnicity, and family history was recorded.

Samples from Turin, Italy

Between October 1991 and February 2000, there were 264 patients who underwent surgery for ovarian tumors at the Department of Gynecology, Gynecologic Oncology Unit, at

[9] F. Slack and J. Weidhaas, unpublished data.

the University of Turin in Italy, and tissue was collected after Institutional Review Board (IRB) approval. All patients were Italian of European ancestry. Of these patients, 23 were diagnosed with metastatic cancer, 19 with benign tumors, 6 with OC of nonepithelial origin, and 1 with endometriosis. Epithelial ovarian tumors from the remaining 215 patients were included in this study. Additional details on these samples are available (17). DNA from these samples was supplied for these studies.

Samples from Brescia, Italy

Tumor samples for DNA extraction were collected from 100 patients with epithelial OC at the Division of Gynecologic Oncology at University of Brescia, Italy, between September 2001 and December 2008 after IRB approval. All patients were of European ancestry. Clinical data were collected from medical records and were available for all patients. Fifty-nine patients were followed from the date of first surgery until death or May 5, 2009. Patients who received neoadjuvant chemotherapy were excluded from nonstatic parameters such as debulking, residual disease, and progression-free survival (PFS). DNA from these samples was supplied for these studies.

Case-control analysis

The Yale cases and controls were selected from those with complete information from Yale/New Haven Hospital (n = 100 and 101, respectively). All were women and were matched for age and ethnicity. For the controls that had their ovaries removed for benign reasons, their age at ovarian removal was recorded as their age of testing for this study.

The Connecticut case-control study was approved by the Connecticut Department of Public Health and all 32 hospitals that participated. Potential cases were English-speaking women from Connecticut, diagnosed at 35 to 79 years of age with OC between September 1, 1998 and February 28, 2003, with new primary invasive epithelial ovarian tumors. Controls were a representative sample of the general population of the study area and identified by list-based random digit dialing methods. Cases and controls were matched for age and ethnicity. Cases and controls with prior cancer were excluded from the analysis. Further details are available (23). DNA samples used in this study included 320 cases and 328 controls.

Statistical methods

For numerical variables (such as age), linear models were used to compare the differences between case and control groups. χ^2 and exact methods were performed to determine the distribution of ethnicity in cases and controls. Hardy-Weinberg testing was analyzed using the ALLELE procedure. Survival analyses were performed using Cox proportional hazards regression model. The association of the *KRAS*-variant with OC was determined using logistic regression modeling. All statistical analyses were performed using SAS version 9.1.2 (SAS Institute).

Detection of the *KRAS*-variant

DNA was collected using standard isolation methods from tissue, blood, buccal cell samples, or saliva. Only the Connecticut case-control underwent DNA amplification before testing. The *KRAS*-variant was assayed using an allele-specific primer and a PCR-based TaqMan assay using standard techniques. Validation of this assay through duplicate testing and sequencing was previously performed and reported (14). As previously shown, the *KRAS*-variant is almost always in the heterozygous state in its carriers, with less than 3% to 5% of any population containing the variant in the homozygous form (14). We thus combined the two genotypes in this work together and referred to both as people "carrying" the *KRAS*-variant.

Calculation of positive predictive value

The positive predictive value (PPV) is calculated by comparing the percentages of *KRAS*-variant–carrying and noncarrying patients with OC and without OC and multiplying by a lifetime risk of 1.4% of developing OC to determine the difference in lifetime cancer risk. Control frequency is based on the Yale controls. PPV is then the lifetime cancer risk of *KRAS*-variant–carrying patients (with OC) over the total *KRAS*-variant–carrying people.

Results

The *KRAS*-variant and ovarian cancer risk

We tested women diagnosed with epithelial OC who presented at Yale/New Haven Hospital for surgery (n = 157) for the *KRAS*-variant and discovered that more than 27% carried this variant allele. Because this was a significantly higher frequency than previously shown in any normal or cancerous population [18% (refs. 14, 22) and >9,000 additional people tested),[10] we validated this finding in two additional, independent cohorts of epithelial OC patients. The first was from a University Hospital in Northern Italy at the University of Turin (n = 215), and 26% of patients carried the *KRAS*-variant in this cohort. The second was from Brescia, Italy (n = 100), and again 25% of these OC patients carried the *KRAS*-variant. The frequency of the *KRAS*-variant was thus significantly higher in these OC cohorts than in any group previously studied, including noncancerous controls collected at Yale/New Haven Hospital (Fig. 1).

To investigate if the *KRAS*-variant predicts an increased risk of developing OC for nonselected female populations, we performed case-control analyses. The Yale case-control contained 100 cases and 101 controls and showed a significantly increased risk of developing OC for *KRAS*-variant carriers by multivariate analysis [odds ratio (OR), 2.46; 95% confidence interval (95% CI), 1.14–5.29; P = 0.020]. These findings were validated in a second independent case-control: The Connecticut OC case-control consists of 320 patients and 328 controls and also showed a significant increased risk of developing OC for the *KRAS*-variant carriers by multivariate analysis (OR, 1.7; 95% CI, 1.11–2.63, P = 0.016; Table 1). These findings suggest that the *KRAS*-variant may be a

[10] J. Weidhaas, unpublished data.

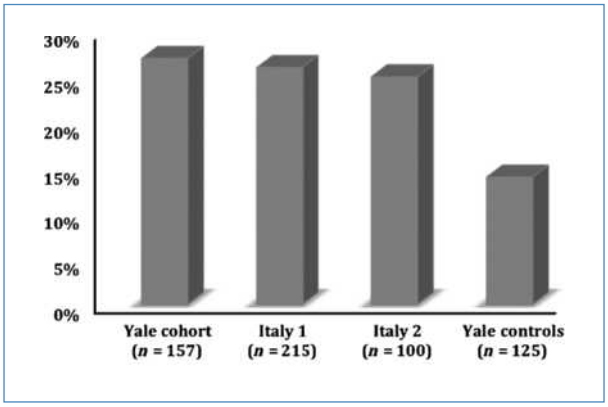

Figure 1. The *KRAS*-variant is found frequently in patients with OC compared with controls. Patients from three separate cohorts were tested for the presence of the *KRAS*-variant and frequency of carriers depicted with the number tested in parentheses.

genetic marker of an increased risk of developing OC in nonselected women.

Ovarian cancer variables and the *KRAS*-variant

We evaluated the distribution of the *KRAS*-variant in the different subtypes of epithelial OC. We found that the prevalence of the *KRAS*-variant varied between subtypes, being highest in nonmucinous cancers and rarely found in patients with mucinous OCs ($P < 0.05$; Table 2).

We studied a range of variables to see if there were specific characteristics segregating OC patients carrying the *KRAS*-variant versus those without. We found that there was not a significant difference in patient age at first surgery, tumor grade, residual tumor size, debulking, stage of OC presentation, response to platinum-based chemotherapy, or PFS (hazard ratio, 1.12; 95% CI, 0.71–1.76; Supplementary Table S1A–E). The trend toward worse PFS for OC patients carrying the *KRAS*-variant may suggest an effect of the *KRAS*-variant on OC outcome and may warrant further study.

Because the *KRAS*-variant is located in the 3′UTR of the *KRAS* oncogene, we tested available tumor samples for *KRAS* codon mutations ($n = 6$ non–*KRAS*-variant-carrying patients, $n = 10$ *KRAS*-variant–carrying patients). Not surprisingly, as nonmucinous OC rarely has activated *KRAS*, none of the ovarian tumors tested had the common *KRAS*-activating mutations. These observations agree with prior findings in cancer samples by us (14) as well as others (24) that the *KRAS*-variant is not enriched in tumors with other tumor-acquired *KRAS* mutations.

Association of the *KRAS*-variant with HBOC

As the *KRAS*-variant seemed to be associated with OC risk for sporadic OC, to further validate its role as a genetic marker of OC, we next examined OC patients considered to be at high risk for having a familial genetic abnormality with a family history consistent with HBOC. These patients had either personal and/or family histories (first- or second-degree relatives) of at least one additional case of OC and/or breast cancer, all were of European ancestry, and all had undergone *BRCA* mutation analysis. Sixty-seven patients fit these parameters: 23 were positive for *BRCA1* mutations, 13 were positive for *BRCA2* mutations, and 31 were uninformative (*BRCA1* and *BRCA2* mutation negative). Overall, 8 of 36 (22%) of *BRCA* mutation carriers also were carriers of the *KRAS*-variant: 7 of 23 (30%) of *BRCA1*-mutant carriers and 1

Table 1. Case-control results indicate that the *KRAS*-variant may be a genetic marker for an increased risk of developing OC

Yale case-control				
KRAS	**Univariate OR (95% CI)**	**Multivariate* OR (95% CI)**	**Case**	**Control**
Nonvariant (T/T)	1.00	1.00	74 (74.0)	88 (87.3)
Variant (G/G and G/T)†	2.38 (1.16–5.09)	2.46 (1.14–5.29)	26 (26.0)	13 (12.7)
P	0.02	0.02		
Connecticut case-control				
KRAS	**Univariate OR (95% CI)**	**Multivariate* OR (95% CI)**	**Case**	**Control**
Nonvariant (T/T)	1.00	1.00	225 (73.1)	272 (84.5)
Variant (G/G and G/T)†	2.01 (1.36–2.99)	1.7 (1.11–2.63)	83 (26.9)	50 (15.5)
P	0.0005	0.016		

*Adjusted for age and race.
†The G/G genotype is found in less than 5% of cases and controls and is thus combined with the G/T genotype for the variant category.

Table 2. Prevalence of the *KRAS*-variant in OC patients by subtype

Subtypes	*n*	Non–*KRAS*-variant patients, T/T (%)	*KRAS*-variant patients, G/T and G/G (%)
CC	22	14 (63.6)	8 (36.4)
MU	15	10 (66.7)	5 (33.3)
EN	52	37 (71.2)	15 (28.9)
SP	167	127 (76.1)	40 (23.9)
UN	37	30 (81.1)	7 (18.9)
MC	22	20 (90.9)	2 (9.1)

NOTE: Patients from the Yale and two Italian studies where subtype was documented.
Abbreviations: CC, clear cell; MU, malignant mixed Mullerian; EN, endometrioid; SP, serous papillary; UN, undifferentiated; MC, mucinous.

of 13 (8%) of *BRCA2* mutant carriers. The differential association of the *KRAS*-variant with *BRCA1* and *BRCA2* may represent a biological modification of *BRCA* penetrance by the *KRAS*-variant, a hypothesis that requires additional study.

Of the 31 uninformative HBOC patients with OC, 19 of 31 (61%) carried the *KRAS*-variant, a frequency significantly higher than documented rates for either the healthy population (14) or other OC patients ($P < 0.0001$, compared with control). For a *KRAS*-variant–carrying uninformative HBOC family member, this results in a PPV for developing OC of 6.78% (95% CI, 5.78–7.76). In contrast, the negative predictive value for a negative *KRAS*-variant test in an uninformative HBOC family member is 99.37% (95% CI, 99.22–99.53).

Families segregating the *KRAS*-variant

We tested at least two additional family members with known cancer status in two of the uninformative HBOC families whose proband carried the *KRAS*-variant and was negative for *BRCA1* and *BRCA2* mutations. In each of these families, at least two relatives diagnosed with cancer also carried the *KRAS*-variant (Fig. 2; Supplementary Fig. S1).

Finally, we compared the pedigrees of HBOC families with a *BRCA1* mutation ($n = 11$), a *BRCA2* mutation ($n = 8$), or the *KRAS*-variant ($n = 13$) and recorded the demographics and cancer types in their family members. We found that there are unique familial profiles for each of these groups, which differ by ethnicity, cancer type, and age of cancer onset, with

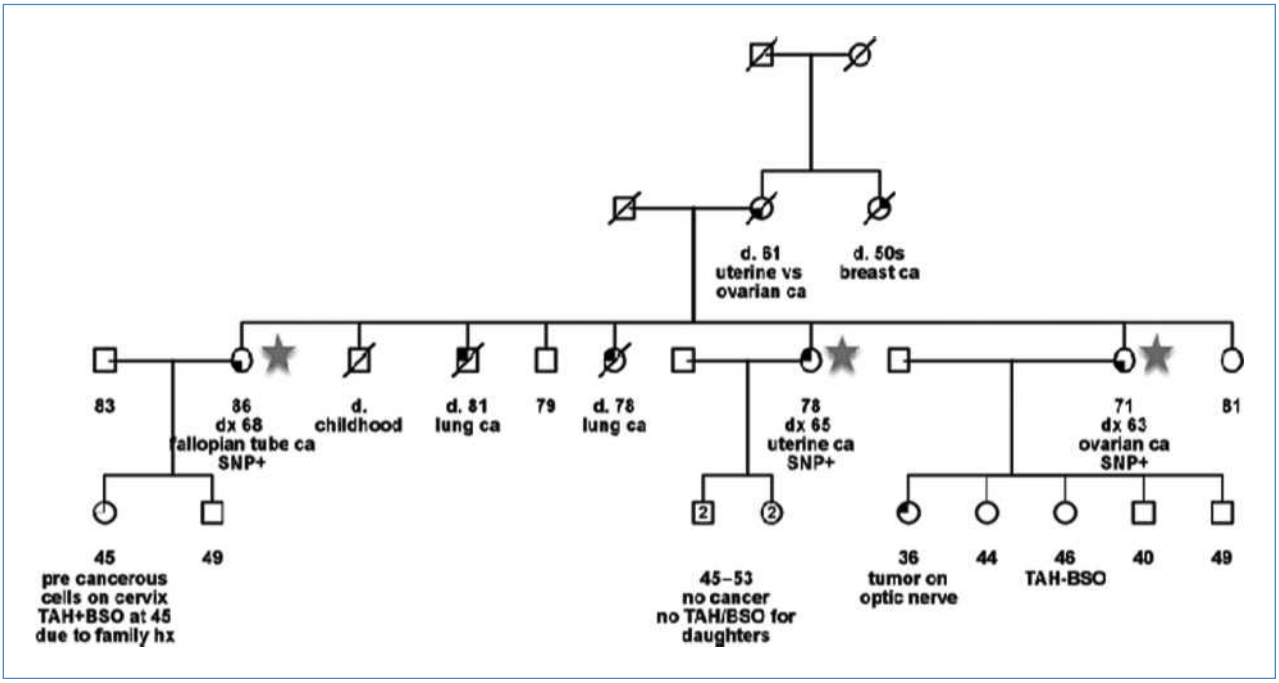

Figure 2. An HBOC family with the *KRAS*-variant. Circles are women, squares are men. A black area within the symbol indicates the person had cancer. A line through the symbol indicates the person has died. "d." means dead, with the age indicated. "dx" means diagnosed with cancer, with the age indicated. Star indicates tested for the *KRAS*-variant and a carrier. hx, history; ca, cancer.

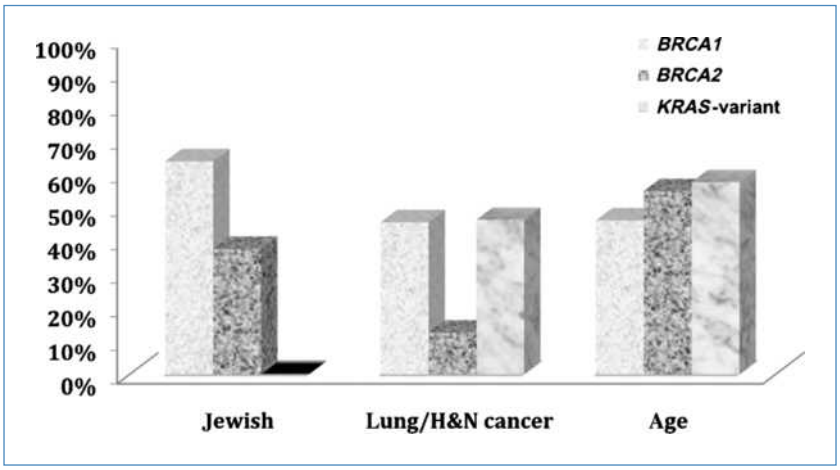

Figure 3. *KRAS*-variant–carrying families differ from *BRCA* families. Cancer types and basic demographics in *KRAS*-variant–carrying (marbled) versus *BRCA1*- and *BRCA2*-carrying HBOC families. The *KRAS*-variant families are less likely to be Jewish (Ashkenazi status not known) than *BRCA1*-mutant families ($P = 0.003$) and more likely to have a family history of lung cancer as compared with *BRCA2*-mutant families ($P = 0.02$); OC patients carrying the *KRAS*-variant are significantly older at the time of diagnosis than *BRCA1*-mutant patients with OC ($P = 0.006$).

KRAS-variant ovarian families being significantly more likely to be non-Jewish, have lung cancer in the family, and be older at the time of their OC diagnosis than *BRCA*-mutant OC patients (Fig. 3).

Discussion

Our results reveal that the variant allele at a polymorphism in the *KRAS* 3′UTR, the *KRAS*-variant, is associated with the risk of developing epithelial OC (OR, 2.46), is identified in more than 25% of nonselected OC patients, and is found in 61% of OC patients from HBOC families previously considered uninformative for gene mutations. These findings support the hypothesis that the *KRAS*-variant is a new genetic marker of an increased risk of developing OC and also suggest that this allele of *KRAS* may be a new HBOC locus.

Although it may seem surprising that a single-nucleotide variant could have such predictive power for disease risk, the *KRAS*-variant represents an entirely different entity than the tagging SNPs studied and used in GWAS. The *KRAS*-variant was identified through a candidate-gene search. It is functional and disrupts a *let-7* miRNA binding site that regulates the important human oncogene, *KRAS* (14). Perhaps most importantly, the *KRAS*-variant has not been included in prior GWAS platforms and has a minor allele frequency of <7%, whereas surrounding *KRAS* SNPs studied in the GWAS platforms have a minor allele frequency of ≥20%.[11] Thus, even if the *KRAS*-variant is in linkage disequilibrium with previously studied SNPs in this region, it was not possible to determine the association of the *KRAS*-variant with OC risk through these prior studies.

The OC in *KRAS*-variant carriers has a similar phenotype to the majority of epithelial OC and occurs primarily in postmenopausal women. This is unlike the OC associated with previously identified inherited genetic markers of OC risk, such as *BRCA* mutations, which disrupt DNA repair pathway genes and are associated with early-onset cancer. This suggests that the *KRAS*-variant may not act through altered DNA repair, but perhaps instead creates an environment where alterations that occur normally with aging allow aberrant cell growth and oncogenesis. In support of this hypothesis, we previously reported that the *KRAS*-variant is associated with increased *KRAS* levels in the background of lower *let-7* levels (14), and others have shown that *let-7* levels decrease with age (25). Although *KRAS* mutations have not been associated with nonmucinous epithelial OC, the *KRAS*-variant may represent a novel form of *KRAS* activation and overexpression or lead to disruption of the epidermal growth factor receptor signaling pathway, a pathway frequently misregulated in OC. Because the *KRAS*-variant, like disruption of other miRNA binding sites in the 3′UTR of *KRAS* (20), does not alter *KRAS* mRNA levels, and because we did not have access to unprocessed tumor tissue for our studies, these hypotheses require further validation in other patient cohorts.

Although our study is somewhat limited by the small number of uninformative HBOC patients, the frequent association of the *KRAS*-variant with these patients and their family members with cancer further strengthens the hypothesis that the *KRAS*-variant is a genetic marker of OC risk. Identification of new such markers of OC risk is critical for these uninformative families, as those who test positive in these families will have a confirmed increased inherited risk, whereas those who test negative will in fact be at a decreased risk of developing OC compared with the general female population, information that will be equally valuable to them.

Genetic risk factors for cancer have been historically very difficult to identify, and those that are known are found in very few patients and make up a small minority of cancer cases (4). Because the 3′UTR of a gene is a critical regulatory region, we have proposed that this region is likely to harbor variants, such as the *KRAS*-variant, that will be associated with a large proportion of cancer cases and can be as powerful as gene-coding mutations in shaping disease risk (14). Our findings support this hypothesis and reinforce that the role of such 3′UTR variants in familial cancer syndromes should be intensively studied.

[11] K. Kidd, personal communication.

Disclosure of Potential Conflicts of Interest

F.J. Slack and J.B. Weidhaas: ownership interest, MiraDx. The other authors disclosed no potential conflicts of interest.

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