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Prevalence of the variant allele rs61764370 T>G in the 3′UTR of *KRAS* among Dutch *BRCA1*, *BRCA2* and non-*BRCA1*/*BRCA2* breast cancer families

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Conflict of interest Joanne B. Weidhaas has founded a company that has licensed IP discussed in this article.

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Abstract

Recently, a variant allele in the 3′UTR of the *KRAS* gene (rs61764370 T>G) was shown to be associated with an increased risk for developing non-small cell lung cancer, as well as ovarian cancer, and was most enriched in ovarian cancer patients from hereditary breast and ovarian cancer families. This functional variant has been shown to disrupt a *let-7* miRNA binding site leading to increased expression of KRAS in vitro. In the current study, we have genotyped this *KRAS*-variant in breast cancer index cases from 268 *BRCA1* families, 89 *BRCA2* families, 685 non-*BRCA1*/*BRCA2* families, and 797 geographically matched controls. The allele frequency of the *KRAS*-variant was found to be increased among patients with breast cancer from *BRCA1*, but not *BRCA2* or non-*BRCA1*/*BRCA2* families as compared to controls. As *BRCA1* carriers mostly develop ER-negative breast cancers, we also examined the variant allele frequency among indexes from non-*BRCA1*/*BRCA2* families with ER-negative breast cancer. The prevalence of the *KRAS*-variant was, however, not significantly increased as compared to controls, suggesting that the variant allele not just simply associates with ER-negative breast cancer. Subsequent expansion of the number of *BRCA1* carriers with breast cancer by including other family members in addition to the index cases resulted in loss of significance for the association between the variant allele and mutant *BRCA1* breast cancer. In this same cohort, the *KRAS*-variant did not appear to modify breast cancer risk for *BRCA1* carriers. Importantly, results from the current study suggest that *KRAS*-variant frequencies might be increased among *BRCA1* carriers, but solid proof requires confirmation in a larger cohort of *BRCA1* carriers.

Keywords

KRAS-variant; *Let-7*; Breast cancer susceptibility; Association; *BRCA1*

Introduction

Breast cancer is the most frequently diagnosed type of cancer in women from western countries and an appreciable fraction is attributable to genetic predisposition to the disease. Disease-causing mutations in the high-risk breast cancer susceptibility genes *BRCA1* and *BRCA2* together explain about 20% of the familial clustering of breast cancer. Population-based studies have estimated the cumulative risk of breast cancer to be 65 and 45% for

BRCA1 and *BRCA2* mutation carriers, respectively [1]. The penetrance of *BRCA1* and *BRCA2* mutations, however, is generally higher in women from families with multiple affected individuals, suggesting the presence of other risk-modifying factors in these families [2–4]. Indeed, it was shown that low-risk breast cancer susceptibility alleles exist that may act multiplicatively on the breast cancer risk for *BRCA1* and *BRCA2* mutation carriers [5, 6].

Besides *BRCA1* and *BRCA2*, three other high-risk breast cancer susceptibility genes have been recognized: *p53*, *PTEN*, and *STK11*. However, these account for only a small fraction (less than 1%) of the familial breast cancer risk and are rarely observed outside the context of the cancer syndromes they cause (e.g., Li-Fraumeni syndrome, Cowden syndrome, and Peutz-Jeghers syndrome, respectively). Another 5% of the familial breast cancer risk is explained by mutations in moderate-risk genes *ATM*, *BRIP1*, *CHEK2*, *NBS1*, and *PALB2* and low-risk alleles such as those in or near the *FGFR2*, *TNRC9/TOX3*, and *MAP3K1* genes [7, 8]. This means that so far three quarters of the familial clustering of breast cancer remains unexplained. Importantly, twin studies suggested that a significant proportion of the remaining familial breast cancer risk is most probably due to genetic factors [9, 10].

MicroRNAs (miRNAs) are a class of small non-coding RNA molecules that negatively regulate gene expression by binding partially complementary sites in the 3′ untranslated region (UTR) of their target mRNAs. The importance of miRNAs in cancer is stressed by their widespread deregulation in virtually all cancer types [11, 12]. Recently, a variant allele in the 3′UTR of the *KRAS* gene (rs61764370 T>G) was shown to disrupt a *let-7* miRNA binding site leading to increased expression of KRAS in vitro and lower *let-7* miRNA levels in vivo [13]. Consistent with the oncogenic nature of KRAS, the variant allele was shown to confer an increased risk for developing non-small cell lung cancer (NSCLC), as well as ovarian cancer ([13, 14]), although the results in NSCLC were not replicated [15]. In head and neck cancers, the *KRAS*-variant was shown to be a genetic marker of poor outcome as well [16]. As the frequency of the *KRAS*-variant was found to be increased among unselected ovarian cancer cases as well as within the context of hereditary breast and ovarian cancer (HBOC) families, we wondered whether the variant allele frequency might also be increased in breast cancer families. Therefore, we genotyped the *KRAS*-variant in breast cancer index cases from 268 *BRCA1* families, 89 *BRCA2* families, and 685 non-*BRCA1*/*BRCA2* families and compared its prevalence with 797 geographically matched controls. We also investigated the association of the *KRAS*-variant with specific characteristics of *BRCA1* families.

Materials and methods

Study population

Families with clustering of breast cancer were selected from the database of the Rotterdam Family Cancer Clinic at Erasmus University Medical Center—Daniel den Hoed Cancer Center in Rotterdam, representing the Southwestern part of the Netherlands. Selected families included at least two cases of female breast cancer or at least one case of female breast cancer and one case of ovarian cancer in first-or second-degree relatives. For each family, the youngest breast cancer patient who had been tested for *BRCA1* and *BRCA2* was assigned to be the index case. The control population was geographically matched and included 797 females from cystic fibrosis families who were either spouses of individuals at risk of being carrier of a *CFTR* mutation or individuals who were tested negative for a *CFTR* mutation. This unmatched case–control study was approved by the local ethical committee and all individuals gave full informed consent to search for susceptibility alleles.

Genotyping

DNA isolated from peripheral blood was amplified using a custom-made Taqman genotyping assay (Applied Biosystems, Foster City, CA) designed specifically to identify the T or variant G allele of the *KRAS*-variant (rs61764370). Forward primer: 5′-GCCAGGCTGGTCTCGAA-3′, reverse primer: 5′-CTGAATAAATGAGTTCTGCAAAACAGGT T-3′, VIC reporter probe: 5′-CTCAAGTGATTCACCCA C-3′, and FAM reporter probe: 5′-CAAGTGATTCACC-CAC-3′. Because of the low frequency of homozygotes for the variant allele, patient samples that were either heterozygous (TG) or homozygous (GG) for the variant allele were considered positive for the *KRAS*-variant.

Statistical analyses

Associations between the *KRAS*-variant and *BRCA1*-, *BRCA2*-, or non-*BRCA1*/*BRCA2*-related familial breast cancer, as well as associations between the *KRAS*-variant and specific characteristics of *BRCA1* families were tested for significance by a χ^2-test. Case–control odds ratios and their confidence intervals were calculated using Woolf approximations. The difference in mean age of onset between *BRCA1* index cases carrying the variant allele and *BRCA1* index cases not carrying the variant allele was determined by a *t*-test. The result of the Shapiro–Wilk test showed that the assumption of a normal distribution for the age of onset variable was valid. *KRAS*-variant frequencies among affected and unaffected *BRCA1* carriers were compared using a Cox proportional hazard model. For this purpose, *BRCA1* carriers were censored at either age of breast cancer diagnosis or age at last follow-up. Only *BRCA1* carriers that were censored at breast cancer diagnosis were considered to be affected. Hazard ratios for the TG and GG genotypes combined were estimated using TT homozygotes as a baseline. *P* values of 0.05 or smaller were considered statistically significant. All statistical analyses were performed with STATA statistical package, release 11.0 (STATA Corp, College Station, TX).

Results

To establish whether the *KRAS*-variant (rs61764370 T>G) is implicated in breast cancer susceptibility, we have evaluated the prevalence of this variant in patients with breast cancer from *BRCA1*, *BRCA2*, and non-*BRCA1*/*BRCA2* families as compared to controls. The variant allele was detected in 108 of 685 (15.8%) of indexes with breast cancer from non-*BRCA1*/*BRCA2* families and 12 of 89 (13.5%) indexes with breast cancer from *BRCA2* families. These frequencies were not significantly different from the 17.3% (138 of 797) prevalence among the control individuals. In contrast, the variant allele was present in 63 of 268 (23.5%) indexes with breast cancer from *BRCA1* families, which was significantly different from the prevalence in controls (Table 1; $P = 0.025$; OR= 1.47 (1.05–2.06)). However, after adjustment for multiple testing (Bonferroni correction for three tests) this difference was not significant anymore ($P = 0.073$).

As *BRCA1* carriers mostly develop ER-negative breast cancers and *BRCA2* carriers mostly develop ER-positive breast cancers, we wondered whether the *KRAS*-variant was associated with ER-negative breast cancer rather than mutant *BRCA1* breast cancer. We had information on ER-status of indexes from 483 non-*BRCA1*/*BRCA2* families of whom 127 were ER-negative and 356 were ER-positive. The variant allele was present in 18 (14.2%) indexes with ER-negative breast cancer and 63 (17.7%) indexes with ER-positive breast cancer which was not significantly different from the prevalence in the controls (Table 1). These results suggest that the *KRAS*-variant not just simply associates with ER-negative breast cancer.

To evaluate whether the variant allele associates with a particular phenotype in *BRCA1* families, we compared family characteristics of *BRCA1* families with a *KRAS*-variant positive index case to *BRCA1* families with a *KRAS*-variant negative index case (Table 2). There was no evidence for the variant to be associated with the presence of either one or more ovarian cancer cases or bilateral breast cancer cases in the family. We did find a higher frequency of the *KRAS*-variant in families with three or more breast cancer cases younger than 60 years compared with families with two or less breast cancer cases younger than 60 years (20.4 vs. 27.9%), however, this was not statistically significant ($P = 0.16$). Additionally, we found no difference in mean age of onset between *BRCA1* index cases carrying the variant allele and *BRCA1* index cases not carrying the variant allele (39.8 vs. 39.0 years, respectively; $P = 0.55$).

In order to gain more statistical power for the analysis of association between the *KRAS*-variant and mutant *BRCA1* breast cancer, we expanded this analysis by including other family members in addition to the index cases. In total, we had DNA from 390 *BRCA1* carriers with breast cancer available for genotyping among the 268 *BRCA1* families. However, the *KRAS*-variant was present in only 20.3% (79 of 390) of affected *BRCA1* carriers (Table 3), which is lower than the initial 23.5% we found among the index cases (Table 1). Consequently, the prevalence of the *KRAS*-variant among *BRCA1* carriers was not significantly different from the controls ($P = 0.22$; OR= 1.21 (0.89–1.65)), despite expansion of the number of *BRCA1* carriers with breast cancer.

To determine whether or not the *KRAS*-variant modifies *BRCA1* mutant breast cancer risk, all affected and unaffected *BRCA1* carriers among the 268 *BRCA1* families were genotyped for the variant allele. Mean age at censure was 41.0 years for affected *BRCA1* carriers and 45.5 years for unaffected *BRCA1* carriers. Similar to the frequency in the controls, the *KRAS*-variant was present in 17.3% (45 of 260) unaffected BRCA1 carriers (Table 3). Importantly, there was no evidence of association between the *KRAS*-variant and breast cancer risk among *BRCA1* carriers (Table 3; $P = 0.50$; HR= 1.07 (0.88–1.30)).

Discussion

In the current study we have found an increase in the frequency of the *KRAS*-variant (rs61764370 T>G) in breast cancer index cases from *BRCA1* families as compared to geographically matched controls, whereas the frequency of this variant allele was not significantly different among breast cancer index cases from *BRCA2* or non-*BRCA1*/*BRCA2* families. However, expansion of the number of *BRCA1* carriers with breast cancer by including other family members in addition to the index cases did not improve significance for the association between the variant allele and mutant *BRCA1* breast cancer. Additionally, the *KRAS*-variant did not appear to modify breast cancer risk for *BRCA1* carriers. Still the question remains whether the breast cancer risk conferred by the *KRAS*-variant is not too small to be detected in our case–control study. Considering the relatively high prevalence in the general population, the variant allele would not be expected to confer a dramatically increased breast cancer risk. Also, the breast cancer risk conferred by the *KRAS*-variant may be larger in GG homozygotes than TG heterozygotes. Therefore, we feel that this trend toward association between the *KRAS*-variant and mutant *BRCA1* breast cancer requires validation in a larger cohort of *BRCA1* carriers, providing more statistical power and enabling the analysis of TG heterozygotes and GG homozygotes separately.

Interestingly, stratification of breast cancer patients from non-*BRCA1*/*BRCA2* families according to their tumor's ER-status did not reveal an increased *KRAS*-variant frequency among ER-negative breast cancers, suggesting that the variant is not just simply associated with ER-negative breast cancer. This may seem unexpected as the *KRAS*-variant has been

shown to lead to increased KRAS expression in vitro and hyperactivation of the MAPK pathway has been shown to downregulate ERα expression in vitro [13, 17]. However, one may wonder whether the level of increased KRAS expression as a result of the *KRAS*-variant is similar to that of an oncogenic *RAS* or *RAF* mutation. This may thus be a matter of dosage and therefore MAPK pathway activation should be assessed in tumors from patients that carry the *KRAS*-variant. Unfortunately, due to missing ERBB2 status of the vast majority of non-*BRCA1*/*BRCA2* cases we have not been able to assess variant allele frequencies in index cases with triple (ER, PR, and ERBB2) negative breast cancers, preventing us to study whether the *KRAS*-variant frequency is increased specifically in *BRCA1* carriers or more generally in patients with triple negative or basal-like breast cancer.

If the *KRAS*-variant were to be specifically associated with mutant *BRCA1* breast cancer, this would suggest a biological interaction between BRCA1 and KRAS or between BRCA1 and *let-7* miRNA. Many human cancers, including breast cancers, display altered expression of the *let-7* miRNA family and *let-7* has been shown to act as a tumor suppressor regulating the expression of *RAS*, *HMGA2*, *LIN28*, and *PEBP1* [11, 18, 19]. Recently, *let-7* was shown to regulate stem cell-like properties in breast tumor initiating cells as downregulation of *let-7* increased proliferation and self-renewal, whereas expression of *let-7* enhanced differentiation [20]. As the *KRAS*-variant allele was associated with lower *let-7* levels in NSCLC patients as compared to patients without the variant allele, the *KRAS*-variant may therefore act in tumorigenesis through maintaining a proliferative cell state [13]. Interestingly, knockdown of *BRCA1* in primary breast epithelial cells was shown to increase the number of cells displaying a stem/progenitor phenotype [21]. The presence of the *KRAS*-variant in the germline of a *BRCA1* carrier may thus further facilitate this stem cell phenotype imposed by *BRCA1*. If the *KRAS*-variant would eventually be conclusively associated with mutant *BRCA1* breast cancer, it might be interesting to study this hypothesis in future research.

miRNAs make up one of the most abundant classes of regulatory genes, with 30% of the human genes harboring miRNA target sites [22, 23]. Thus far, many miRNAs have already been implicated in cancer as either oncogenes or tumor suppressor genes [11, 18]. This makes both the miRNA genes themselves as well as the miRNA binding sites in the 3′UTR of well-known cancer genes attractive candidate breast cancer susceptibility alleles. Although several key targets of many miRNAs have been identified, also many target genes remain elusive. A significant portion of these miRNA target genes include well-known breast cancer oncogenes and tumor suppressor genes, such as *ERBB2*, *CCND1*, *MYC*, and *PTEN*, but also breast cancer susceptibility genes such as *ATM* [18, 24]. The *KRAS*-variant was the first single nucleotide variant in a miRNA binding site shown to be implicated in cancer risk, but many more are likely to be discovered. Screening of miRNA genes and their target sequences in the 3′UTR of genes known to be implicated in breast cancer may thus provide a productive strategy for defining part of the excess familial breast cancer risk.

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Table 1

KRAS 3′UTR SNP genotype frequencies among indexes from *BRCA1*, *BRCA2*, and non-*BRCA1*/*BRCA2* families

Population	Genotype	Frequency	OR	95% CI	*P* value
Controls	GG+TG	138/797 (17.3%)	1.00		
	TT	659/797 (82.7%)			
BRCA1 carriers	GG+TG	63/268 (23.5%)	1.47	1.05–2.06	0.025 *
	TT	205/268 (76.5%)			
BRCA2 carriers	GG+TG	12/89 (13.5%)	0.74	0.39–1.40	0.36
	TT	77/89 (86.5%)			
Non-*BRCA1*/*BRCA2* carriers	GG+TG	108/685 (15.8%)	0.89	0.68–1.18	0.42
	TT	577/685 (84.2%)			
ER-positive	GG+TG	63/356 (17.7%)	1.03	0.74–1.43	0.87
	TT	293/356 (82.3%)			
ER-negative	GG+TG	18/127 (14.2%)	0.79	0.46–1.34	0.38
	TT	109/127 (85.8%)			

Differences in *KRAS*-variant allele frequencies between cases and controls were tested for significance by a χ^2-test. Case–control odds ratios and their confidence intervals were calculated using Woolf approximations

* After adjustment for multiple testing (Bonferroni correction for three tests) the *P*-value between *BRCA1* carriers and controls was not statistically significant anymore ($P = 0.073$)

Table 2

Association of the *KRAS*-variant with specific characteristics of *BRCA1* families

Family characteristic	Frequency of the GG+TG genotype	OR	95% CI	*P* value
No OvC in family	38/152 (25.0%)			
One or more OvC in family	22/96 (22.9%)	0.89	0.49–1.63	0.71
No Bil BrC in family	36/147 (24.5%)			
One or more Bil BrC in family	24/106 (22.6%)	0.90	0.50–1.63	0.73
Less than 3 BrC < 60 years in family	29/142 (20.4%)			
Three or more BrC < 60 years in family	31/111 (27.9%)	1.51	0.84–2.70	0.16

Association of the *KRAS*-variant with specific characteristics of *BRCA1* families was tested for significance by a χ^2-test. Case–control odds ratios and their confidence intervals were calculated using Woolf approximations

OvC ovarian cancer, *Bil* bilateral, *BrC* breast cancer

Table 3

KRAS 3′UTR SNP genotype frequencies among affected and unaffected *BRCA1* carriers

Genotype	Frequency in unaffected carriers	Frequency in carriers with breast cancer	HR	95% CI	*P* value
TT	215/260 (82.7%)	311/390 (79.7%)	1.00		
GG+TG	45/260 (17.3%)	79/390 (20.3%)	1.07	0.88–1.30	0.50

Differences in *KRAS*-variant allele frequencies between affected and unaffected *BRCA1* carriers were tested for significance by a Cox proportional hazards model. Mean age at censure was 41.0 years for affected *BRCA1* carriers and 45.5 years for unaffected *BRCA1* carriers. Hazard ratios for the TG+GG genotype were estimated using TT homozygotes as a baseline