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BRAF polymorphisms and the risk of ovarian cancer of low malignant potential

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Abstract

Objective. The object of this study was to test the hypothesis that *BRAF* is a low-risk susceptibility gene for low malignant potential (LMP) ovarian cancer. A recent study of the relationship between *BRAF* polymorphisms and malignant melanoma identified strong linkage disequilibrium across the *BRAF* gene with one of the three most common haplotypes (haplotype C) having a population attributable risk of approximately 1.6%. We therefore hypothesized that the same *BRAF* haplotype may confer an increased risk of serous ovarian tumors of low malignant potential.

Methods. We genotyped 383 cases of LMP ovarian cancer, including 234 of serous histology, and 987 controls for seven SNPs, representative of the most common *BRAF* gene haplotypes, using MALDI-TOF mass spectrometry.

Results. Haplotype information was obtained for 369 LMP ovarian cancer cases and 983 healthy controls. None of the haplotypes were found to be associated with risk of LMP ovarian cancer (OR for haplotype C 0.81, 95% CI = 0.54–1.22), or with the risk of serous LMP ovarian cancer (OR for haplotype C 0.90, 95% CI = 0.56–1.45).

Conclusion. We found no evidence to suggest that *BRAF* is a low-risk LMP ovarian cancer susceptibility gene.

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Keywords: *BRAF*; Susceptibility; Polymorphism; Ovarian cancer; Low malignant potential; LMP

Introduction

The pathogenesis of ovarian cancer is poorly understood, as is the relationship between borderline (low malignant potential) and invasive ovarian adenocarcinoma. There is evidence to suggest that serous ovarian cancers of low malignant potential (LMP) will not progress to high-grade ovarian cancer but, in contrast, mucinous LMP tumors share

specific somatic mutations with their benign and invasive counterparts and may be part of a continuum [1–4].

The *RAF* family of genes (including *BRAF*) encode cytoplasmic serine–threonine kinases that bind to Ras, mediating a cellular response to growth signals. Somatic missense mutations in the kinase domain of *BRAF* have been identified in common moles [5] and malignant melanomas [6,7], as well as in other types of cancer [8–11], including serous ovarian tumors of low malignant potential [12,13]. However, *BRAF* gene mutations are rare in invasive and in non-serous tumors [12,13]. Therefore,

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further knowledge of *BRAF* gene involvement in ovarian tumorigenesis may help to gain a better understanding of the etiology of LMP tumors and the nature of their relationship with their malignant counterparts.

The majority of ovarian cancer patients present with no remarkable family history [14,15], making it unlikely that high penetrance germline mutations, in *BRAF* or any other gene, play an important role in disease susceptibility. Instead, heritable genetic factors that may be involved in susceptibility to ovarian cancer are likely to be associated with small increases in risk, and could be conferred by relatively common variants. If they occur at a high frequency within the population, they may be important risk factors at the population level. Meyer et al. [16] found a suggestion for a possible relationship between *BRAF* polymorphisms and malignant melanoma. More recently, a study by James et al. [17] identified strong linkage disequilibrium across the *BRAF* gene in Caucasians from Australia, and found one of the three most common haplotypes (haplotype *C*) to have a population attributable risk of malignant melanoma of approximately 1.6%. No studies to date have examined the association between *BRAF* variants and the risk of LMP ovarian cancer.

We hypothesized that the *BRAF* haplotype *C*, identified by James et al. [17], may confer an increased risk for serous ovarian cancer of low malignant potential. We set out to test this hypothesis in a case-control study, comprising 383 cases, including 234 of serous histology (the largest collection of LMP ovarian cancer cases to genotyped date), and 987 healthy controls.

Materials and methods

Subjects

A case-control sample, drawn from six case-control studies conducted in three different countries (Table 1), comprised 383 ovarian cancer cases of low malignant potential, with no selection for family history, and 987 healthy controls. Of the 383 tumors, 234 were serous, and the remainder mucinous (128), endometrioid (7), clear cell

(2), and undetermined or mixed histologies (12). Information on potential or known ovarian cancer risk factors was available for most cases. Age was known for all but one of the cases (99.7%), tubal ligation and parity for 85%, and hysterectomy, oral contraceptive pill (OCP) use, and smoking for 82% of cases. The age range was 19–95 years. Questionnaire information regarding ethnicity was available for 48% of cases. 32% reported Caucasian ethnicity, while the remaining 16% were of mixed ethnicity.

Limited information on potential or known ovarian cancer risk factors was available for controls, including age (for 91%) tubal ligation (for 37%), hysterectomy, OCP use, parity, and smoking (for 35%). Ages ranged from 20 to 80 years. Ethnicity information was available for 78% of control subjects. 71% were of Caucasian ethnicity, while the remaining 9% were of mixed ethnicity.

Details of the six studies are as follows:

1. Familial Registry of Ovarian Cancer (FROC). Patients with epithelial ovarian cancer diagnosed between March 1, 1997 and July 31, 2001 were identified through the Greater Bay Area Cancer Registry operated by the Northern California Cancer Centre as part of the Surveillance, Epidemiology and End Results (SEER) Program of the National Cancer Institute. We used rapid case ascertainment to identify cases within 1 month of diagnosis. Eligible patients were those diagnosed with invasive or LMP epithelial ovarian cancer at ages 20 years to 64 years who resided in six Bay Area counties. Of the 579 women who provided epidemiologic data and a blood or mouthwash sample, 115 patients were diagnosed with LMP epithelial ovarian cancer. Control women were identified through random-digit dial and were frequency-matched to cases on race/ethnicity and 5-year age group. Full description of the study design and methods are available in McGuire et al. [18]. DNA was purified from peripheral blood leucocytes ($n = 218$) using the Puregene Kit (Gentra Systems, Minneapolis, MN) and from exfoliated cells in buccal mouthwash rinses ($n = 12$) as previously described [19]. DNA was quantified by spectrophotometry.

Table 1
Sources of ovarian cancer cases and controls

Source	Cases	(% Genotyped)	Case source location	Controls	(% Genotyped)	Control source location
FROC	115	(99)	San Francisco Bay Area	115	(100)	San Francisco Bay Area
QIMR	94	(88)	SWH and RBH, Australila	594	(100 ^a)	ATR
DUMC	76	(100)	North Carolina, USA	141	(100)	North Carolina, USA
IRV	43	(100)	Irvine, USA	53	(98)	Irvine, USA
DKFZ	29	(93)	Heidelberg and Freiburg, Germany	55	(96)	Heidelberg and Freiburg, Germany
PAH	26	(100)	Southampton, UK	29	(97)	Southampton, UK
Total	383			987		

ATR = Australian Twin Registry; DKFZ = Deutsches Krebsforschungszentrum for German Cancer Research Center; DUMC = Duke University Medical Center; FROC = Familial Registry of Ovarian Cancer; IRV = Population-based cancer registry of Orange County; PAH = Princess Anne Hospital; QIMR = Queensland Institute of Medical Research; RBH = Royal Brisbane Hospital; SWH = Survey of Women's Health.

^a Genotype drawn from Melanoma Study by James et al. [29].

2. Queensland Institute of Medical Research (QIMR). Incident cases of 94 epithelial ovarian adenocarcinoma of low malignant potential were ascertained from two sources: the Royal Brisbane Hospital, Queensland, Australia, during the period 1985–1996 ($n = 28$) and as part of the Survey of Women's Health, a large population-based case-control study, ascertained via major gynecological-oncology treatment centres in New South Wales, Victoria, and Queensland [20] ($n = 45$). A further 21 cases were ascertained from both of these sources. Germline DNA was obtained from the cases, either as blood samples (28 cases from the Royal Brisbane Hospital and 14 ascertained from both sources) or from archival paraffin blocks (45 cases from the population-based study and 7 ascertained from both sources). DNA was extracted from blood samples by the salt-precipitation method, as described in Chenevix-Trench et al. [21]. DNA was extracted from archival paraffin blocks by the method of Levi et al. [22]. DNA from blood was quantified by spectrophotometry, but those from paraffin blocks were used without quantification. Age information was available for all 94 cases and ethnicity was known for 88 (94%) of cases. Further information on potential or known ovarian cancer risk factors was collected at interview as part of the population-based case-control study, and was available for all 66 of these case subjects, but for none of the subjects ascertained at the Royal Brisbane Hospital. Controls were the mothers of twin 12-year-old children taking part in the Brisbane Adolescent Twin Study, a genetic study of normal development of a range of phenotypes. These families are volunteers from the Australian Twin Registry, and are believed to be representative of the general Australian population. Since the children are the main focus of that study, only limited information is available about the mothers, notably age and ancestry. The mean age of the mothers was 41.7 years [Inter Quartile Range (IQR) = 39–45].
3. Duke University Medical Center, USA. LMP ovarian cancer cases ($n = 76$) were identified through the North Carolina Central Cancer Registry (CCR), a statewide population-based tumor registry, using rapid case ascertainment. Eligible cases were women aged 20 to 74 years who were diagnosed with epithelial ovarian cancer of low malignant potential since January 1, 1999, had no prior history of ovarian cancer, and resided in a 48 county area of North Carolina. All cases underwent standardized pathologic and histologic review by the study pathologist to confirm diagnosis. Population-based controls ($n = 141$) were identified from the same region as the cases, and were frequency-matched to the ovarian cancer cases on the basis of race and age, using list-assisted random digit dialling. Genomic DNA was extracted from leukocytes using a Puregene DNA Isolation kit (Gentra Systems, Minneapolis, MN), according to the manufacturer's protocol and quantified by spectrophotometry.
4. University of California, Irvine. Patients with LMP ovarian cancer, diagnosed between March 1994 and April 1995, were identified through the Cancer Surveillance Program of Orange County, a population-based cancer registry, as part of the California Cancer Registry. We used rapid case ascertainment to identify cases within 1 month of diagnosis. Eligible patients were those diagnosed with LMP ovarian cancer at any age, who resided in Orange County California. Control women were identified through random-digit dialling and were frequency-matched to cases on race/ethnicity and 5-year age group. Samples from 43 LMP ovarian cancer cases and 53 controls were included in this study. Whole blood in an 8-ml ACD tube was extracted using a Qiagen Maxi column and eluted in Tris–EDTA. Extracted DNA was quantified by fluorimetry.
5. Deutsches Krebsforschungszentrum (DKFZ) for German Cancer Research Center, Germany. A population-based case-control study of ovarian cancer was conducted in two defined geographic areas around the towns of Heidelberg and Freiburg in southern Germany [23]. Incident cases of epithelial ovarian cancer or LMP tumor, between 1993 and 1996, were identified through frequent monitoring of admissions and surgery schedules of 26 hospitals in the study areas. All study subjects were asked to give a blood sample, and to complete a self-administered questionnaire on ethnicity, as well as known and suspected risk factors for ovarian tumors. Clinical data for the patients were extracted from hospital records, and pathology reports were requested from the pathology institutes serving these hospitals. A total of 29 patients with ovarian adenocarcinoma of low malignant potential was recruited and included in this analysis. Controls were randomly selected from lists of residents in the counties, provided by the population registries. For the purpose of this study, we included two population controls, individually matched by age and study area to each case. DNA was extracted from blood samples using the FlexiGen Kit (Qiagen) according to the manufacturer's instructions and quantified by spectrophotometry.
6. Princess Anne Hospital (PAH), Southampton, UK. Incident cases of ovarian tumors, including 26 with LMP tumors, were ascertained from women undergoing primary surgery at Hospitals in and around Southampton, UK, as part of a study of ovarian carcinogenesis, conducted at the Princess Anne Hospital, Southampton (PAH). The control subjects ($n = 29$) were white female out-patients for obstetric related, non-neoplastic disease conditions. While age information was available for all cases, further epidemiological data such as reproductive factors, oral contraceptive use, smoking, and obesity were not available for either cases or controls. However, both control and case groups were residents of the greater Southampton area, which has a predominantly Anglo-

Table 2
BRAF gene SNPs used to infer haplotype

dbSNP_ID	Exon/Intron ^a	Change (transcribed strand)
rs765373	Promoter	T > C
rs7810757	5'UTR	G > A
rs1267621	Intron-1	T > C
rs1267609	Intron-3	A > G
rs1267649	Intron-5	G > C
IVS12-48CT	Intron-12	A > G
rs1267639	Intron-13	A > G

^a Within the 18 exon transcript ENST00000288602.

Saxon population. Germline DNA was extracted from blood using a salt-chloroform method [24] and quantitated by spectrophotometry.

Genotyping

In order to infer *BRAF* haplotypes, genotypes were obtained for seven intronic/promoter SNPs (Table 2). SNP identity and type are given in Table 2; further information and full sequence can be found in the public databases (<http://www.ncbi.nlm.nih.gov/SNP/>), using the ‘rs’ accession number. PCR and extension primers were designed using the Sequenom MassARRAY assay-design software. Details are available from the authors upon request. PCR was carried out in three separate multiplex reactions which were subsequently pooled for genotyping. Genotyping was performed using a primer extension reaction, and MALDI-TOF mass spectrometry (MassARRAY, Sequenom Inc., San Diego) as detailed by Bansal et al. [25]. QIMR control genotypes were drawn from previously genotyped controls, included in the melanoma study by James et al. [17], and all other genotypes were generated specifically for this study.

Statistical analysis

All analyses were carried out in the R 1.9.1 statistical language [26]. Haplotype analysis used the R haplo.stats package [27,28], which estimates (posterior) haplotype probabilities for unphased genotypes, and performs logistic regression analysis, allowing for uncertainty in haplotype imputation. In the logistic regression analyses, all haplotypes with less than 1% frequency were pooled into a single “rare” group.

Table 3
Risk of LMP ovarian cancer associated with *BRAF* haplotypes

Haplotype		Controls	Cases	Serous	All cases		Serous cases	
		%	%	%	OR	(95% CI) ^a	OR	(95% CI) ^a
A	CACGCGG	84	83	82	1.00	Reference	1.00	Reference
B	TGTGCAG	7	7	6	1.01	(0.68–1.50)	1.19	(0.75–1.90)
C	TATAGGA	6	6	6	0.81	(0.54–1.22)	0.90	(0.56–1.45)
Rare	–	3	4	6	1.26	(0.76–2.10)	1.87	(0.78–2.42)

^a OR (95% CI) = odds ratio (95% confidence interval), adjusted for age.

Results

Genotypes were obtained for 93% or more cases from each study group, except from QIMR, which had a genotyping success rate of 88%. The QIMR group was comprised of DNA samples obtained from paraffin blocks (55%) and DNA extracted from blood lymphocytes (45%). All blood-lymphocyte DNA yielded successful *BRAF* genotypes, but only 79% of the paraffin-block DNA samples were amplified successfully. Thus, the large proportion of paraffin-block samples in the QIMR group accounts for the lower genotyping success rate, and is consistent with the fact that DNA from paraffin blocks are generally of poorer quality than that extracted from fresh blood. None of the genotypes showed significant deviation from Hardy–Weinberg equilibrium. A number of samples were genotyped more than once due to PCR failure for one or more SNPs in the multiplex. This yielded a set of independently replicated genotypes for the successful SNPs in that multiplex. 126 genotypes were repeated in this way, with a success rate of 99.2% and only 1 unresolved error. The sample which produced the unresolved error was removed from analysis.

After data cleaning, genotype information to infer haplotypes was available for 369 cases and 983 controls. The seven *BRAF* SNPs were in tight linkage disequilibrium, such that 98% of chromosomes were defined by the three most common haplotypes (Table 3). There was no association between the risk of LMP ovarian cancer and any of the minor *BRAF* haplotypes. Haplotype C, which had shown an association with melanoma in a previous study [17], was associated with an odds ratio of 0.81 (95% CI = 0.54–1.22) for total LMP cases and 0.90 (95% CI = 0.56–1.45) for cases of serous ovarian cancer of low malignant potential. Adjustment for hysterectomy, OCP use, and age did not alter the risk estimate. Sample size was too small for a meaningful statistical analysis of individual phased genotypes. However, individuals carrying phased genotype C were underrepresented in the cases compared to controls. Of the controls, 71% were homozygous for the A haplotype (A/A phased genotype), 12% were heterozygous for the C haplotype (C/A, C/B, C/rare phased genotypes), and 0.3% were homozygous for the C haplotype (C/C phased genotype). Of the cases, 70% were homozygous for the A haplotype, 11% were heterozygous for the C haplotype, and none were homozygous for the C haplotype.

Discussion

We genotyped 369 cases of epithelial ovarian cancer of low malignant potential, including 227 serous cases, and 983 healthy controls for 7 SNPs in the *BRAF* gene, representative of the most common *BRAF* haplotypes. Our results provide no support for the hypothesis that the *BRAF* gene is associated with serous LMP ovarian cancer risk. The absence of a significant association between *BRAF* haplotype and ovarian cancer of low malignant potential is unlikely to be due to the confounding effect of ovarian cancer risk factors. Although calculation of ORs with adjustment for known risk factors was not possible for the entire data set, adjustment for age, hysterectomy, and OCP use, for a limited data set, did not alter the risk estimate. Confounding due to differences in ethnicity, on the other hand, cannot be excluded as a factor contributing to the absence of a significant finding. Only 32% of cases were known to be of Caucasian ethnicity, compared with 71% of controls. Of the cases, 52% had no available information on ethnicity, while this number was only 22% in controls.

Our sample size may have been too small to detect a modest increase in risk associated with rare *BRAF* haplotypes. With a wildtype haplotype frequency of 84% and haplotype *C* frequency of 7%, our study had 80% power to detect an increase in risk of LMP ovarian cancer of 1.76-fold or greater, and an increase in risk of serous LMP ovarian cancer of 1.92-fold or greater, associated with the *C* haplotype. The upper confidence limit for an increase in risk of serous cancers was 1.45-fold for the *C* haplotype, and we had little power to detect small increased risks of this order of magnitude. However, even if this were a true estimate of risk associated with the *C* haplotype, it would account for at most 2.9% of sporadic ovarian cancers of low malignant potential in the population. In addition, it should be noted that our point estimate of risk for haplotype *C* was below 1, providing no evidence for an increased risk of LMP ovarian cancer associated with this haplotype.

James et al. [17] found a substantial increase in the risk of malignant melanoma for homozygous carriers of the *BRAF* *C* haplotype (OR 5.80, 95% CI = 1.40–39.07), although their sample size for this group was small. Our sample size was too small to carry out meaningful analyses for individual phased genotypes. While we had 80% power to detect an increase in risk of LMP ovarian cancer of 5.80-fold (equivalent to the OR reported in [17]) or greater, the power to detect an effect of 1.40-fold (equivalent to the lower CI reported in [17]) or lower, associated with the homozygous *C/C* phased genotype, was only 5%. Based on qualitative analysis of our groups of phased genotypes, however, there was no evidence for a genotypic effect of the *BRAF* *C/C* phased genotype on the risk of LMP ovarian cancer.

In conclusion, we found no evidence to suggest that the *BRAF* gene is acting as a low-risk predisposition gene in the development of serous ovarian cancer of low malignant

potential, and that germline variants in the gene can in anyway enhance or substitute for the effect of a somatic mutation in *BRAF* which occurs frequently in serous LMP ovarian cancers.

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