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Award Number: DAMD17-02-1-0666

TITLE: Biological Basis for Chemoprevention of Ovarian Cancer

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REPORT DATE: October 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 01-10-2005		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 1 Oct 2004 – 30 Sep 2005	
4. TITLE AND SUBTITLE Biological Basis for Chemoprevention of Ovarian Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER DAMD17-02-1-0666	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Andrew Berchuck, M.D. E-mail: berch001@mc.duke.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Duke University Medical Center Durham, NC 27710				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The rationale for ovarian cancer prevention is highlighted by the observations that pregnancy and birth control pill use are strongly protective. To achieve a better understanding of the etiology of ovarian cancer, which can then be translated into effective prevention strategies, we have initiated a case-control study that considers genetic susceptibility, epidemiologic risk factors and acquired genetic alterations. Subjects are interviewed in their homes and about 800 cases and 850 controls have been accrued thus far. Blood and cancer samples have been collected and molecular analyses of genetic polymorphisms (BRCA1/2, progesterone receptor, vitamin D receptor, transforming growth factor-beta receptor, BRAF) have been performed. In addition to analyses of polymorphism data within the North Carolina Study, we are performing joint analyses with other groups to validate positive associations. An initial ovarian cancer chemoprevention trial with levoneorel in chickens demonstrated a protective effect and we have shown that progestin mediated apoptosis in the ovarian epithelium is mediated by transforming growth factor-beta. In vitro data has suggested that vitamin D analogues may also represent appealing chemopreventives. A chemoprevention trial incorporates both progestins and vitamin D analogues is being initiated. These studies have the potential to increase our ability to identify high-risk women and to lead to the development of chemoprevention strategies that might decrease mortality from this disease.					
15. SUBJECT TERMS Ovarian Cancer					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 46	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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Introduction

Ovarian cancer is the fourth leading cause of cancer deaths among women in the United States. There are three potential approaches to decreasing ovarian cancer mortality: screening and early detection, more effective treatment and prevention. All of these avenues should be explored, but we believe that prevention represents the most feasible approach. The rationale for prevention is derived from epidemiologic studies that have examined the relationship between reproductive history, hormone use and ovarian cancer. It has been convincingly demonstrated that reproductive events which reduce lifetime ovulatory cycles are protective. Although most women are unaware of this protective effect, those who use oral contraceptive pills for more than 5 years or have 3 children decrease their risk of ovarian cancer by greater than 50%. The biological mechanisms that underlie the association between ovulation and ovarian cancer are poorly understood, however.

Our multidisciplinary ovarian cancer research group has been actively involved in studies that seek to elucidate the etiology of ovarian cancer and to translate this knowledge into effective preventive strategies. Joint consideration of genetic susceptibility, reproductive/hormonal and other exposures, acquired alterations in oncogenes and tumor suppressor genes and protective mechanisms such as apoptosis is required to accomplish this goal. We have initiated a molecular epidemiologic study of ovarian cancer in North Carolina that focuses on the identification of genetic polymorphisms that affect susceptibility to ovarian cancer. Over 1,600 subjects have been accrued thus far in this case-control study. We have examined several polymorphisms and also have forged a collaboration with a group in Australia that is also conducting a DOD funded case-control study of ovarian cancer. This will facilitate progress by allowing us to confirm positive results. In addition, we will pool polymorphism data to increase statistical power to examine relationships with less common histologic types (eg. borderline and non-serous) and gene-gene and gene-environment interactions.

We also are actively involved in development of chemopreventive strategies. We have performed a study in primates that suggests that the oral contraceptive has a potent apoptotic effect on the ovarian epithelium, mediated by the progestin component. In addition, in subsequent studies performed *in vitro*, we have induced apoptosis in epithelial cells treated with the progestin levonorgestrel. Progestin mediated apoptotic effects may be a major mechanism underlying the protection against ovarian cancer afforded by OCP use. This forms the basis for an investigation of the progestin class of drugs as chemopreventive agents for epithelial ovarian cancer. Initial studies to test the progestin levonorgestrel in an avian model of ovarian cancer have been undertaken and demonstrated a striking protective effect. In the present study, we are exploring the potential use of vitamin D compounds to enhance the apoptotic effect of progestins on the ovarian epithelium and to enhance the protection against ovarian cancer in the avian model. In addition, we are exploring the molecular pathways (most notably the TGF-beta pathway) that mediate progestin/vitamin D induced apoptosis in the ovarian epithelium. Finally, in an “idea project” we are exploring new pharmacologic approaches to targeting the progesterone receptor for ovarian chemoprevention.

Over the past seven years with support from the DOD Ovarian Cancer Research Program we have made considerable progress. This report focuses on the most recent progress in the past 12 months.

Body

Epidemiology and Tissue Core and Project 1: Genetic susceptibility to ovarian cancer

With the support of the Department of Defense Ovarian Cancer Research Program we initiated a molecular epidemiologic study of ovarian cancer to work towards the goal of a better understanding of the etiology of ovarian cancer. Drs. Andrew Berchuck (Gynecologic Oncologist) and Joellen Schildkraut (Epidemiologist) are working together to lead this study. Our initial plan was to accrue frozen tumor tissue and blood from 500 epithelial ovarian cancer cases treated at Duke University, the University of North Carolina at Chapel Hill and East Carolina University. In addition, 500 age and race-matched control subjects were to be accrued and both cases and controls were to be interviewed by telephone regarding known risk factors for ovarian cancer. After funding to support this project was received from the Department of Defense in 1998 with Dr Berchuck as PI, additional funding was received to support this project in the form of an RO1 grant from the NCI with Dr Schildkraut as PI. The additional funding has allowed us to increase the scope of the study such that nurse interviewers are visiting the homes of all the cases and controls to administer the study questionnaire. Research subjects are now accrued from hospitals in a 48 county region of central and eastern North Carolina using a rapid case ascertainment mechanism established through the state tumor registry. Prior to initiating the study, we had to go through the process of IRB approval in each of the various hospitals involved. The second DOD Ovarian Cancer Program Project which began in 2002 provides funding to increase our accrual to 820 ovarian cancer cases and an equal number of controls. Thus far about 800 women with ovarian cancer and 800 age and race-matched controls have been entered in the study and interviewed. The investigators have project meetings every month with all the research staff to review progress and address ongoing issues and at this point we are pleased with the accrual rate and other procedural aspects of the study. We continue to obtain blood specimens on over 99% of our study subjects. All clinical, epidemiologic and molecular data are stored as they are obtained in a computerized database. Paraffin blocks of tumor tissue are also obtained and these tissues are being used to assess alterations in cancer causing genes such as p53, cyclin E and HER-2/*neu*. We are continuing to test the hypothesis proposed in the first DOD program project grant that alterations in specific genes may represent molecular signatures that characterize distinct molecular epidemiological pathways of causation of ovarian cancer.

During the study interview a thorough history of the menstrual cycle and reproductive experiences of the study participants is obtained from each subject assisted by the use a life-time calendar method. In addition, information on oral contraceptives and hormone replacement therapy is obtained. Data on the family history of cancer, other risk factors, and potential confounders is also collected. The interview takes 60-90 minutes to complete. The interactions between the nurses and subjects has been uniformly positive. The women with ovarian cancer are highly motivated to talk about their history and have a high level of interest in supporting a study aimed at increasing our understanding of the causes of ovarian cancer. They greatly appreciate the opportunity to talk with a nurse who is truly interested in hearing all the details of their life experience.

Although most of the genes responsible for dominant hereditary ovarian cancer syndromes (BRCA1/2, MSH2/MLH1) likely have been discovered, there is evidence to suggest that polymorphisms in other genes may also affect cancer susceptibility in a more weakly penetrant fashion. In project 1, we are examining the role of genetic susceptibility in the development of ovarian cancer. These studies focus on genes involved in pathways implicated in the development of ovarian cancer. Since the effect of cancer susceptibility genes may be modified by other genes and exposures, he also will determine whether gene-gene and gene-environment interactions affect ovarian cancer susceptibility. Because of the low

incidence of ovarian cancer, the ability to identify “high risk” subsets of women is critical if we hope to translate our emerging understanding of the etiology of ovarian cancer into effective prevention strategies.

Demographic and clinical features of ovarian cancer cases and controls in the North Carolina Ovarian Cancer Study

	Cases (N=789)	Controls (N=823)	
Age in years			
mean (s.d)	55. 0 (12.0)	54. 4 (12.2)	
median (range)	56 (19 - 83)	55 (20 - 75)	
	n (%)	n (%)	p-value
Race			
Caucasian	670 (85)	678 (82)	0.237
African-American	100 (13)	128 (16)	
Other	19 (2)	17 (2)	
Menopause status			
Pre/Peri	284 (36)	325 (39)	0.148
Post	505 (64)	498 (61)	
Tubal ligation			
No	596 (76)	530 (64)	<0.001
Yes	193 (24)	293 (36)	
Oral contraceptive use (months)			
None	283 (34)	241 (29)	0.001
≤ 12	148 (18)	136 (17)	
> 12	381 (46)	432 (52)	
User of unknown duration	17 (2)	14 (2)	
Livebirths			
0	161 (20)	106 (13)	<0.001
1	147 (19)	136 (17)	
>1	481 (61)	581 (71)	
Family History of Ovarian Cancer (1st degree)			
No	756 (96)	796 (97)	0.221
Yes	33 (4)	25 (3)	
Family History of Ovarian Cancer (1st or 2nd degree)			
No	721 (91)	773 (94)	0.032
Yes	68 (9)	48 (6)	
Tumor Behavior			
Borderline	133 (23)		
Invasive	454 (77)		
About 60% of cancers are serous and 60% stage III/IV.			

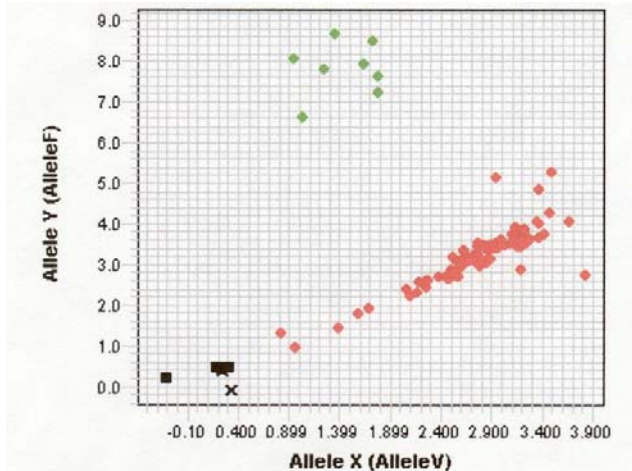
BRCA1/2: Since inherited BRCA1 or BRCA2 mutations strikingly increase ovarian cancer risk, polymorphisms in these genes could represent low penetrance susceptibility alleles. Prior studies of the BRCA2 N372H polymorphism suggested that HH homozygotes have a modestly increased risk of both breast and ovarian cancer. We have examined whether BRCA2 N372H or common amino acid-changing polymorphisms in BRCA1 predispose to ovarian cancer in the North Carolina ovarian cancer study. Cases included 312 women with ovarian cancer (76% invasive, 24% borderline) and 401 age- and race-matched controls. Blood DNA from subjects was genotyped for BRCA2 N372H and BRCA1 Q356R and P871L. There was no association between BRCA2 N372H and risk of borderline or invasive epithelial ovarian cancer. The overall odds ratio for HH homozygotes was 0.8 (95% CI = 0.4-1.5) and was similar in all subsets including invasive serous cases. In addition, neither the BRCA1 Q356R (OR = 0.9, 95% CI 0.5-1.4) nor P871L (OR = 0.9, 95% CI 0.6-1.9) polymorphisms were associated with ovarian cancer risk. There was a significant racial difference in allele frequencies of the P871L polymorphism ($P = 0.64$ in Caucasians, $L = 0.76$ in African Americans, $p < 0.0001$). In this population-based, case-control study, common amino acid changing BRCA1 and 2 polymorphisms were not found to affect the risk of developing ovarian cancer. These results were published in *Clinical Cancer Research* in 2003.

Progesterone receptor: In view of the protective effect of a progestin dominant hormonal milieu (OC use, pregnancy), progesterone receptor variants with altered biological activity might affect ovarian cancer susceptibility. A German group reported that an intronic insertion polymorphism in the progesterone receptor was associated with a 2.1-fold increased ovarian cancer risk. It subsequently was shown that this *Alu* insertion is in linkage disequilibrium with SNPs in exons 4 and 5. However, several subsequent studies by our group and others failed to confirm an association between these polymorphisms and ovarian cancer. In addition, there is little evidence that this complex of polymorphisms, termed PROGINS, alters progesterone receptor function.

More recently, sequencing of the progesterone receptor gene has revealed several additional polymorphisms, including one in the promoter region (+331G/A). The +331A allele creates a unique transcriptional start site that favors production of the progesterone receptor B (PR-B) isoform over progesterone receptor A (PR-A). The PR-A and PR-B isoforms are ligand-dependent members of the nuclear receptor family that are structurally identical except for an additional 164 amino acids at the N-terminus of PR-B, but their actions are distinct. The full length PR-B functions as a transcriptional activator and in the tissues where it is expressed it is a mediator of various responses, including the proliferative response to estrogen or the combination of estrogen and progesterone. PR-A is a transcriptionally inactive dominant-negative repressor of steroid hormone transcription activity that is thought to oppose estrogen-induced proliferation. An association has been reported between the +331A allele of the progesterone receptor promoter polymorphism and increased susceptibility to endometrial and breast cancers. It was postulated that upregulation of PR-B in carriers of the +331A allele might enhance formation of these cancers due to an increased proliferative response.

The +331G/A polymorphism in the progesterone receptor promoter was examined in cases and controls from the North Carolina Ovarian Cancer Study. A second, independent, case-control study from Australia (Dr. Chenevix-Trench) that is also funded by the DOD was examined to confirm associations seen in the North Carolina study. Data from the two studies was then pooled to increase statistical power. The +331G/A single nucleotide polymorphism in the promoter of the progesterone receptor was genotyped using a TaqMan assay. Allelic discrimination was performed using the MGB primer/probe TaqMan assay on the ABI Prism 7700 system. Some samples were sequenced using the ABI 3100 system to confirm the accuracy of the Taqman assay. The +331A allele was found in 59/504 (11.7%) Caucasian controls and the distribution of genotypes was in Hardy-Weinberg Equilibrium ($\chi^2 = 0.391$, $p = 0.53$). Only 1/81 (1.2%) African American controls and none of 67 African American women with ovarian cancer carried the +331A allele. In view of the rarity of the +331A allele in African Americans,

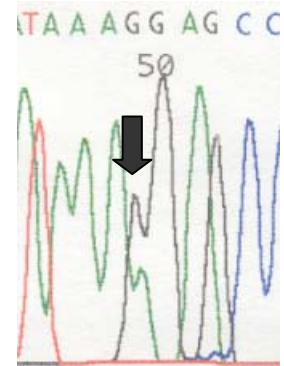
these subjects were excluded from further analyses. The +331AA homozygotes were combined with heterozygotes in calculating odds ratios. The +331A allele was associated with a modest reduction in risk of ovarian cancer. Analysis by histologic type revealed that there was a slight trend towards protection against the common serous histologic type (OR = 0.80, 95% CI 0.49–1.29) but there was a more striking protection against endometrioid and clear cell cancers (OR = 0.30, 95% CI 0.09–0.97).



PR promoter polymorphism

(left) TaqMan assay (green = GA heterozygotes, red = GG homozygotes)

(right) GA heterozygote



Relationship between PR promoter polymorphism and ovarian cancer risk in histologic types of ovarian cancer

	PR +331 G/A Genotype					OR	(95% CI)	
	GG	AG	AA	AG/AA				
Controls	445	58	1	59 (11.7%)		1.00	Reference	
Serous	244	26	0	26 (9.6%)		0.81	(0.50 -	1.32)
Mucinous	44	5	0	5 (10.2%)		0.80	(0.30 -	2.14)
Endometrioid	53	3	0	3 (5.4%)		0.43	(0.13 -	1.40)
Clear cell	23	0	0	0 (0.0%)		**		
Endometrioid/ clear cell	76	3	0	3 (3.8%)		0.30	(0.09 -	0.97)

In view of the potential for false-positive results in genetic association studies, confirmation was sought using an independent study population from Australia. The frequency of the +331A allele among Caucasian controls varied by less than 1% between the Australian and North Carolina studies. The Australian study was not a population-based case-control study and fewer data were available regarding risk factors. Nevertheless, the results of the Australian study were similar to those of the North Carolina study, with a modest overall protective effect that was most pronounced for endometrioid cancers (OR = 0.51, 95% CI = 0.17–1.53). The Breslow-Day chi-square test was used to assess homogeneity of the results from the two study populations. Analyses involving the combined data set showed a significant association between the +331A allele and decreased risk of endometrioid/clear cell cases. In combining the two studies there was a significant risk reduction (OR = 0.46, 95% CI = 0.23–0.92) ($P = 0.027$). These types represent 21% of invasive ovarian cancer cases. Endometriosis is known to increase risk of endometrioid and clear cell ovarian cancers, many of which may arise in ovarian deposits of endometriosis. In this study, endometriosis was associated with an increased risk of endometrioid/clear

cell cancers (OR = 3.87, 95% CI = 2.09-7.17. The +331A allele appeared to be strongly protective against endometriosis (OR = 0.19, 95% CI 0.03 – 1.38), but this study was under powered to prove this conclusively.

The literature is fraught with false-positive association studies of genetic susceptibility polymorphisms, but several features mitigate the likelihood of this in the present study. First, the known protective benefit of progestins against ovarian cancer provides a preexisting biologic plausibility for the observed association. In addition, the finding that the +331A allele is protective against both endometrioid/clear cell cancers and their precursor lesion (endometriosis) also is supportive. Confirmation of the positive association obtained in North Carolina study by the Australian study also represents an additional critical validation step. Finally, unlike many polymorphisms that lack known functional significance, the +331A allele increases transcription of PR-B *in vitro*. This study provides evidence for the existence of low penetrance ovarian cancer susceptibility polymorphisms. If multiple polymorphisms are identified that either increase or decrease the risk of various histologic types of ovarian cancer, this might be used in the future for risk stratification that would facilitate screening and prevention strategies. The paper describing the relationship between the progesterone receptor promoter polymorphism and ovarian cancer was published in the December 2004 issue of Cancer, Epidemiology, Biomarkers and Prevention (see appendix).

Meta-analysis of the progesterone receptor promoter polymorphism (+331 G/A) confirms its protective effect against endometrioid/clear cell ovarian cancers

Because of the potential for false-discovery in genetic association studies we have conducted a meta-analysis of several ongoing case-control studies to confirm this association. The +331G/A PR polymorphism was genotyped in blood DNA of 4,614 Caucasian subjects from population-based, case-control studies in the North Carolina Ovarian Cancer Study, Australia (Dr Trench), Massachusetts (Dr Daniel Cramer at Harvard) and Southern California (Dr. Leigh Pearce at USC). There were 2,269 subjects with invasive or borderline ovarian cancer (1,430 serous, 538 endometrioid/clear cell, 301 mucinous) and 2,345 controls. We conducted a meta-analysis using a fixed effects model to produce summary Mantel-Hanzel odds ratios (OR) for the four studies. The +331A allele (AA or GA) was present overall in 10.6% (151/1,430) of serous cases, 5.4% (34/538) of endometrioid/clear cell cases, 10.3% (31/301) of mucinous cases and 10.7% (251/2,345) of controls. The distribution of alleles in the controls conformed to Hardy-Weinberg equilibrium. There was no relationship between the +331A allele and serous or mucinous ovarian cancers in any of the individual studies or in the meta-analysis (serous OR = 0.98, 95% CI 0.79 - 1.22, mucinous OR = 0.91, 95% CI 0.59 - 1.38). In contrast, a protective effect against endometrioid/clear cell cancers was noted in each study (North Carolina OR = 0.45, Australia OR = 0.66, Massachusetts OR = 0.69 and Southern California OR = 0.30) and in the meta-analysis of all four studies (OR = 0.56, 95% CI 0.39 - 0.82) ($p < 0.003$). These findings provide further evidence that the A allele of the +331G/A PR promoter polymorphism is carried by about 11% of Caucasians and is protective against endometrioid and clear cell ovarian cancers. Efforts to identify other common ovarian cancer susceptibility polymorphisms are ongoing, and if successful could allow screening and prevention strategies to be focused on populations at increased risk.

TGF- β receptor 1: Progestin induced apoptosis in the ovarian epithelium may be mediated by the TGF- β pathway, and this pathway is the target for chemopreventive efforts in Project 2. In project 1, we are investigating the possibility that TGF- β receptors are appealing candidate ovarian cancer susceptibility genes. A polymorphism in the TGF- β I receptor has been described that involves deletion of 3 alanines from a 9 alanine tract (T β R1(6A)). IT has been suggested that the 6A allele might predispose to the development of ovarian cancer and other cancer types. In addition, there is some evidence that the

TβR1(6A) variant may be functionally significant and may confer an impaired ability to mediate TGF-β anti-proliferative effects.

In view of the evidence that the TGFβR1 polyalanine polymorphism may affect ovarian cancer risk, this polymorphism was genotyped in 588 ovarian cancer cases and 614 controls from the North Carolina study (see tables below). Significant racial differences in the frequency of the 6A allele were observed between Caucasian (10.7%) and African American (2.4%) controls ($p < 0.001$). One or two copies of the 6A allele of the TGFβR1 polyalanine polymorphism were carried by 18% of all controls and 19% of cases, and there was no association with ovarian cancer risk (OR = 1.07, 95% CI 0.80 – 1.44). The odds ratio for 6A homozygotes was 1.81 (95% CI 0.65 – 5.06), but these comprised only 0.98% of controls and 1.70% of cases. The 6A allele of the TGFβR1 polyalanine polymorphism does not appear to increase ovarian cancer risk. Larger studies are needed to exclude the possibility that the small fraction of individuals who are 6A homozygotes have an increased risk of ovarian or other cancers. Polymorphisms in other members of the TGF-β family of ligands, receptors and downstream effectors also are appealing candidates. This data was communicated as an oral presentation at the 2004 meeting of the International Gynecologic Cancer Society in Scotland and was published in the journal *Gynecologic Oncology* in 2005 (see appendix).

Vitamin D Receptor pathway: High circulating levels of vitamin D may protect against ovarian cancer, since mortality rates are higher in northern latitudes where there is less sunlight. The most biologically active form of vitamin D, 1,25 (OH)₂D₃, is produced in the skin through sunlight exposure and vitamin D exhibits significant antineoplastic properties. Several factors, both dietary and genetic regulate the production of 1,25 (OH)₂D₃ from its precursor. A recent study suggested that about 22% of the variation may be accounted for by a putative major gene effect. Highly polymorphic loci involved in the metabolism and function of vitamin D include the vitamin D binding protein and vitamin D receptor genes. It has been suggested that a polymorphism in the vitamin D receptor gene involving a shared haplotype that includes a change in the 3' untranslated region that alters transcriptional activity may be associated with increased prostate cancer risk. This has not been a uniform finding in all studies, however.

Vitamin D receptor polymorphisms are being examined in the North Carolina Ovarian Cancer Study to test the hypothesis that vitamin D biosynthesis in the skin can protect susceptible individuals from developing ovarian cancer and that genetic variation in the vitamin D pathway may modify this protective effect. Seven haplotype tagging SNPs that include three functional variants have been genotyped and analyses are being performed to examine the relationship between genetic variation, sunlight exposure and ovarian cancer risk.

BRAF polymorphisms

Mutations in the BRAF gene, which is part of the RAS pathway, occur in some borderline serous ovarian tumors. In view of this, polymorphisms in the BRAF gene are appealing candidates that might affect susceptibility to borderline ovarian cancer. Dr Chenevix-Trench organized a multicenter collaborative study of BRAF polymorphisms with each center contributing their borderline cases and matched controls. These polymorphisms were not found to affect susceptibility to borderline serous tumors and this data was published in the journal *Gynecologic Oncology* in 2005 (see appendix).

Illumina array

In the last few years since our grant was funded, high throughput techniques for SNP genotyping have been developed. Presently, we are designing an Illumina array experiment that will allow us to genotype 1,536 SNPs in candidate genes in all 1,600 of our samples. We will include haplotype tagging SNPs as

well as nonsynonymous SNPs that result in amino acid changes. This experiment will focus on the hormonal pathway genes as well as DNA repair and inflammation pathway genes. The advent of this high throughput technology will allow us to generate vastly more genotype data in the next year than we have generated in the past years combined.

Ovarian Cancer Association Consortium

Although case-control studies of some polymorphisms have reported positive associations, these generally have not been confirmed in subsequent studies. Groups from the US, UK and Australia met in at Cambridge University in April 2005 to review results of various ongoing ovarian cancer association studies. There was a consensus that many of the challenges inherent in this field can best be addressed by collaborative efforts. In view of this, the group elected to establish an ovarian cancer association consortium (OCAC). Dr. Berchuck successfully applied to the Ovarian Cancer Research Fund for a \$900,000 grant to fund the first three years of biannual meetings and other activities, and he will serve as the head of the steering committee. Dr. Georgia Chenevix-Trench, who also is funded by the DOD Ovarian Cancer Research Program also is a leading member of the organizing group. The aims of the consortium are an outgrowth of the North Carolina and Australian DOD funded studies and reflect the successful translation of the DOD funding into a continued and expanded effort. The second meeting of the ovarian cancer association consortium took place in Salt Lake City in late October in concert with the American Society of Human Genetics annual meeting.

Aim #1 - To develop an ovarian cancer association consortium (OCAC) that is dedicated to working together to identify and validate common low penetrance ovarian cancer susceptibility polymorphisms. The OCAC will meet each fall in concert with the American Society of Human Genetics meeting, and an annual spring meeting will be hosted by an OCAC member institution. This will provide the opportunity for face-to-face interactions that are critically important in sustaining the momentum of the OCAC.

Aim #2 – To perform a comprehensive review of the existing ovarian cancer susceptibility polymorphism literature. This effort will produce a review article and will serve as a marker of the state of the field as the OCAC begins its work.

Aim #3 – To determine whether polymorphisms in the progesterone receptor affect ovarian cancer risk. Polymorphisms in the progesterone receptor (PR) gene have been the most frequently examined. Several studies have suggested that polymorphisms in this gene affect risk, but not all studies have confirmed these findings. The OCAC members will genotype PR polymorphisms in several thousand cases and controls and the data will be analyzed centrally to resolve the issue of whether PR variants affect ovarian cancer risk.

Aim #4 – To examine associations between other promising candidate genetic variants and risk of ovarian cancer. In keeping with the goal of the OCAC to provide definitive evidence of genetic associations, the most promising candidate variants being studied by OCAC members will be genotyped in a collaborative manner as described above for the progesterone receptor.

Aim #5 – To assign groups to write additional grant proposals that focus either on specific molecular pathways using a comprehensive approach or methodological issues for association studies. The groups in the ovarian cancer association consortium are funded to study specific genes and/or gene pathways. This includes various steroid hormone, DNA repair and inflammation related pathways as well as others. The goal will be to assign groups to seek additional funding to study these pathways in the OCAC. In addition, the group will be uniquely positioned to study methodological issues related to genetic association studies and the statistical geneticists in the group will have the opportunity to apply for funding to use OCAC data for this purpose.

Aim #6 – To examine the interaction between major epidemiological risk factors and genetic polymorphisms. Because of the moderate size of most ovarian cancer association studies it has not been possible to perform analyses of gene-environment interactions. The OCAC will establish a common data

sheet that includes basic information relating to major epidemiological risk factors. This will focus mainly on family history and reproductive risk factors. Central analyses will be performed to examine interactions between factors such as OC use, genetic polymorphisms and ovarian cancer risk.

Relevance: Presently, ovarian cancer risk stratification is not used to guide clinical surveillance or interventions in the vast majority of women, other than in rare individuals with BRCA/HNPCC mutations. This must change in the future if we are to decrease ovarian cancer incidence and mortality. The long term goal of the OCAC is to identify a panel of ovarian cancer susceptibility polymorphisms that can be used in combination with known epidemiological risk factors such as parity and OC use to better stratify ovarian cancer risk. This would greatly facilitate implementation of screening and prevention strategies by allowing these to be focused on higher-risk populations. The newly formed ovarian cancer association consortium includes essentially all of the leading groups in this field. We are eminently well positioned to achieve this goal. The OCRF can make a major impact in our ability to stratify ovarian cancer risk and to reduce mortality from the disease by providing support for the first three years of OCAC activities.

Project 2: Chemoprevention of Ovarian Cancer

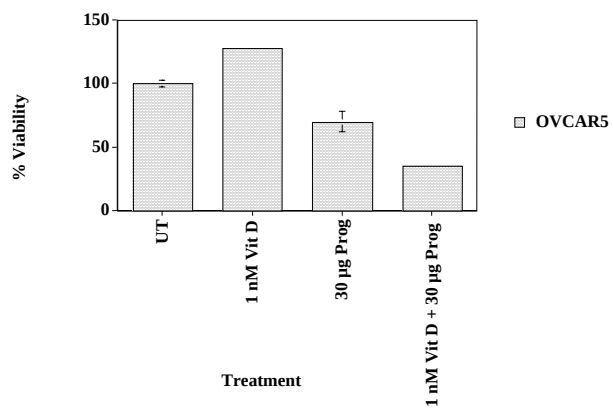
Project 2 is under the direction of Gustavo Rodriguez, M.D. (Gynecologic Oncologist). The prevention strategy outlined in our proposal focuses on the potential use of a combined approach incorporating both progestins and Vitamin D for the chemoprevention of ovarian cancer. The studies outlined in our prevention grant are designed to add further support to the notion that progestins and Vitamin D are potent apoptotic agents on human ovarian epithelial cells and to directly test the hypothesis in an animal model that these agents confer preventive effects against ovarian cancer. The aims in the grant are: (1) to evaluate the apoptotic effect of progestins and vitamin D analogues on the human ovarian epithelium *in vitro*, (2) elucidate the molecular mechanisms by which they induce apoptosis in ovarian epithelial cells, and (3) to directly test the hypothesis that progestins/vitamin D analogues confer preventive effects against ovarian cancer in a chemoprevention trial in the chicken, the only animal species with a high incidence of ovarian cancer.

There is significant potential to decrease ovarian cancer incidence and mortality through prevention. Epidemiological evidence has shown that routine use of the combination estrogen–progestin oral contraceptive pill (OCP) confers a 30-50% reduction in the risk of developing subsequent epithelial ovarian cancer, suggesting that an effective ovarian cancer preventive approach using hormones is possible. Investigations by our group have elucidated a mechanism that we believe is responsible for the ovarian cancer preventive effects of the OCP. Specifically, we have discovered that the progestin component of the OCP is functioning as a classic chemopreventive agent, by activating potent molecular pathways known to be associated with cancer prevention in the ovarian surface epithelium. We have discovered that progestins markedly induce programmed cell death (apoptosis) and differentially regulate expression of Transforming Growth Factor Beta (TGF- β) in the ovarian epithelium. These two molecular events have been strongly implicated in cancer prevention *in vivo*, and are believed to underlie the protective effects of other well-known chemopreventive agents such as the retinoids and Tamoxifen. Our laboratory and animal research findings are supported by human data demonstrating that progestin-potent OCPs confer twice the ovarian cancer protection as newer weak-progestin OCPs. These human data provide proof of principle that progestins are effective chemopreventive agents for ovarian cancer, and suggest that a regimen that has enhanced chemopreventive biologic potency in the ovarian epithelium will be more effective than a lower potency regimen for ovarian cancer prevention.

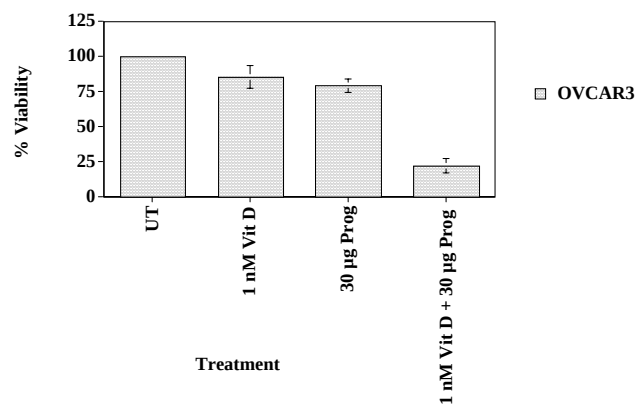
The finding that progestins activate these molecular pathways in the ovarian epithelium opens the door toward a further investigation of progestins as chemopreventive agents for ovarian cancer, and raises the possibility that other agents that similarly activate cancer preventive pathways in ovarian epithelial cells may be attractive ovarian cancer preventives. Among the non-progestins, there is environmental, epidemiologic, laboratory and animal evidence in support of Vitamin D as a potent ovarian cancer preventive. In addition, results from a prevention trial that we have performed in the chicken ovarian cancer animal model suggest an additive ovarian cancer protective effect of Vitamin D when added to progestin.

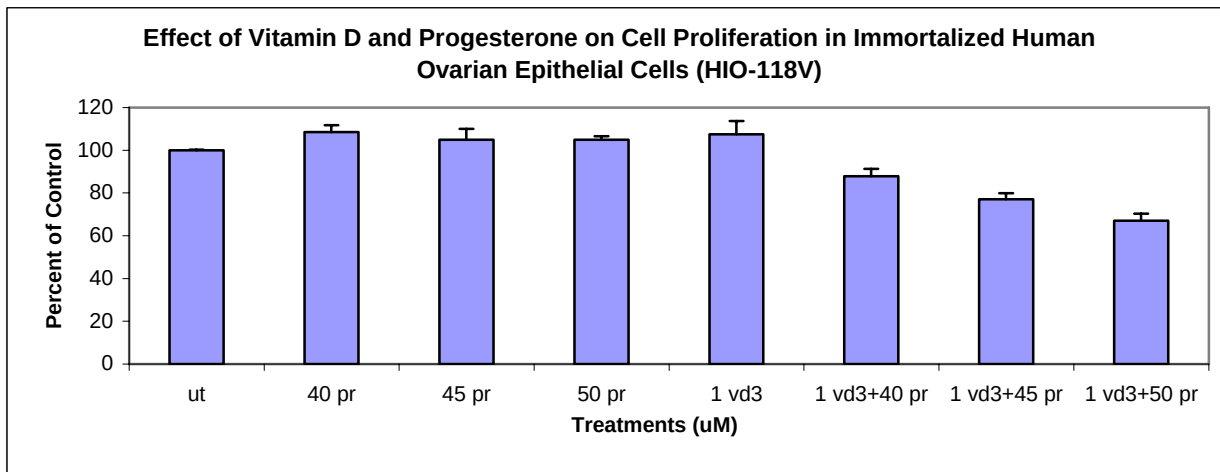
In our last annual summary, we presented evidence showing that the combination of a progestin and Vitamin D had a more potent biologic effect on cells derived from the human ovarian epithelium than either agent alone. We have expanded our studies to include immortalized cells derived from the normal human ovarian epithelium. Both drug classes markedly inhibited cell viability in a dose response fashion. The figures below demonstrate a marked impact on cell viability when the two agents are combined, and administered at a dosage that has a marginal impact for each agent given alone.

**Effect of Vitamin D and Progesterone
on Cell Proliferation (OVCAR5 Cells)**



**Effect of Vitamin D and Progesterone
on Cell Proliferation (OVCAR3 Cells)**





The data have been analyzed isobolographically to determine if the drug combinations are acting additively or synergistically. For these analyses we are using the CalcuSyn software (Biosoft). Raw data for each drug or drug combination dose are entered singly to generate a median effect plot. From this plot, the combination index (CI) equation is generated to determine whether the drug effects were additive, synergistic or antagonistic. CI values of <1 , $=1$ or >1 indicate synergy, additivity or antagonism, respectively. The data demonstrate that the combination of a progestin and Vitamin D act synergistically to inhibit cell viability. This novel finding has never previously been described, and has great potential for translating into a pharmacologic chemopreventive approach that has both enhanced efficacy and decreased toxicity.

We hypothesize that progestins and Vitamin D target the early steps of carcinogenesis in the ovarian epithelium, by activating pathways leading to apoptosis and thereby decreasing dysplastic ovarian epithelial cells, resulting in effective cancer prevention. In addition, we hypothesize that the combination of two preventive agents such as progestin plus Vitamin D will be a more potent ovarian cancer preventive than either agent used alone, making it possible to lessen the dose of each in order to achieve optimal chemoprevention, while minimizing side effects.

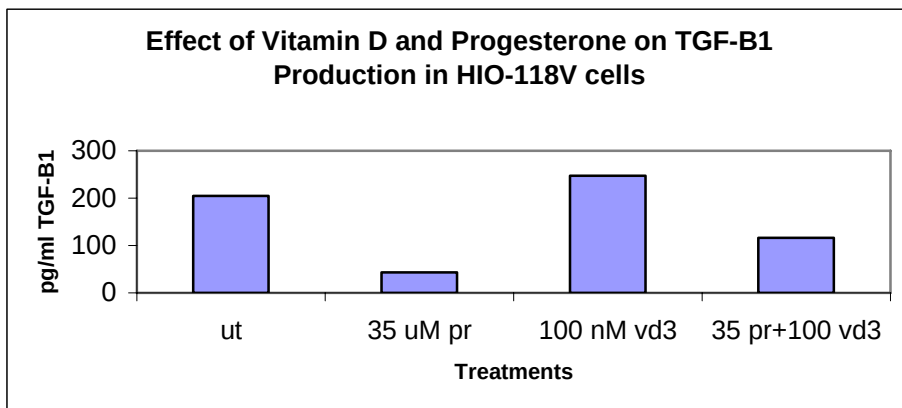
Molecular Mechanisms Underlying the Biologic Effects of Progestins and Vitamin D on the ovarian epithelium: We are performing experiments aimed toward elucidating the complex signaling events underlying the synergistic effects observed when combining progestins and Vitamin D. A better understanding of the biologic effects underlying the combination of these two agents will open the door toward promising pharmacologic strategies for ovarian cancer prevention that can then be explored in clinical trials.

We have been exploring whether activation of TGF-beta signaling events may underlie the chemopreventive biologic effects of progestins and Vitamin D on the ovarian epithelium. Previously, we have shown in a primate model that progestins differentially regulate expression of TGF-beta in the ovarian epithelium, by decreasing expression of the TGF-beta-1 isoform while at the same time increasing expression of the TGF-beta2/3 isoforms. In experiments performed *in vitro* in immortalized cells derived from normal human ovarian epithelium (HIO-118V), we have observed that progestin decreases production of TGF-beta-1, similar to what we have observed in primates *in vivo*. In contrast, Vitamin D increases production of TGF-beta-1 in the OVCAR 3 ovarian cancer cell line. When combining progestin and Vitamin D, the effect on TGF-beta-1 production is intermediate between that of each agent administered individually, despite the synergistic impact of the combination on cell viability. (See below)

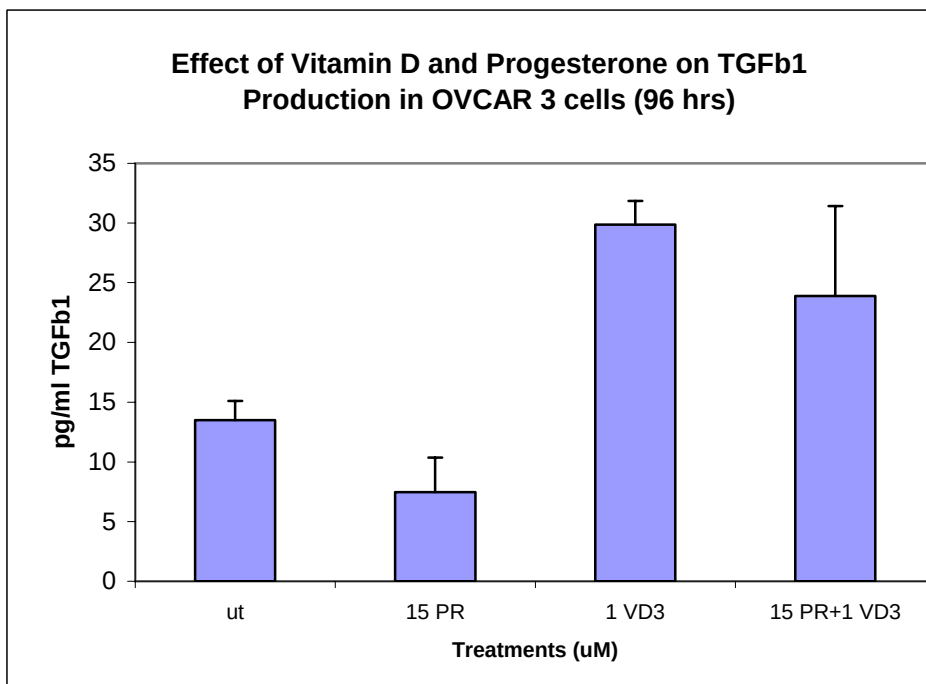
Experiments are underway to evaluate the effect of progestins and Vitamin D on the other TGF-beta isoforms, and also on downstream signaling effects within the TGF-beta pathway.

Effects of Hormone Treatments on TGF-beta-1 Production

Cells were incubated in low serum conditions with the hormonal interventions labeled below. The supernatant was collected and examined for production of TGF-beta using a TGF-beta ELISA. In the HIO-118V immortalized ovarian epithelial cell line, results demonstrate down-regulation of TGF-b1 secretion in response to progestin, with VitD3 having a minimal effect and abrogating the progestin effect. Results have been normalized using MTS assay results, thereby correcting for cell number.



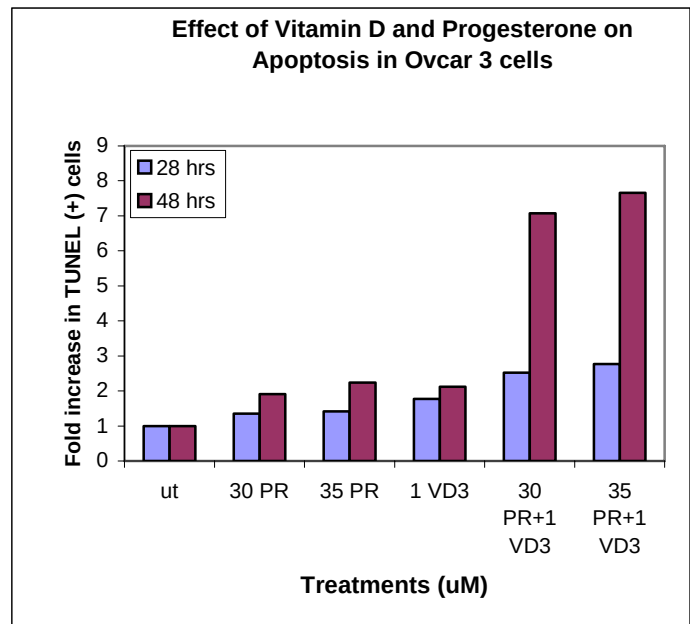
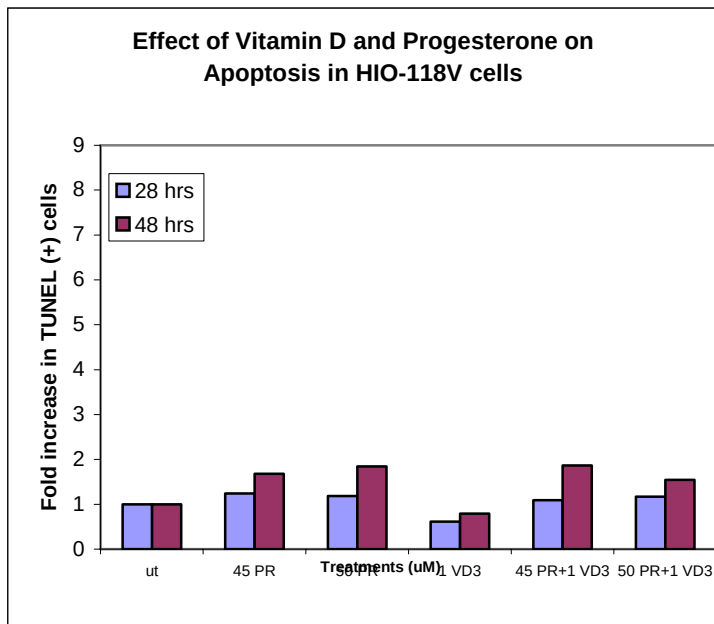
Similar trends are observed in the OVCAR-3 ovarian cancer cell line; however, Vitamin D up regulates TGF-beta production.



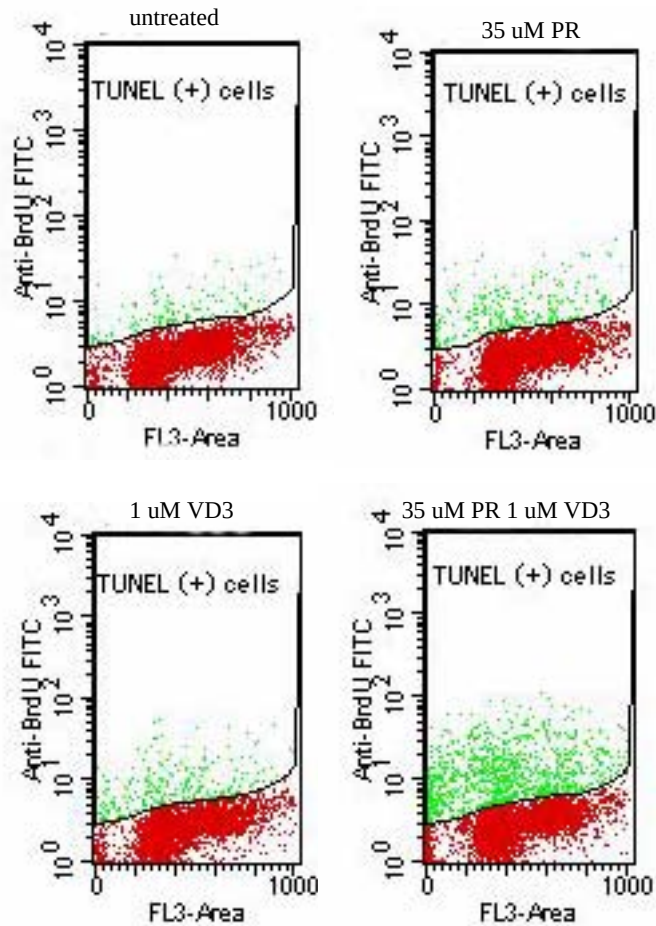
Apoptosis and Cell Cycle

Cells were incubated for 28 and 48 hours in the hormonal treatments as indicated below and assessed for TUNEL reactivity and cell cycle. In these experiments, Apoptosis (TUNEL) data and cell cycle data shown are analyzed from the same experiment; in each cell line's respective medium. MTS data are also in each cell line's respective media.

OVCAR 3 cells undergo a 7-fold increase in apoptosis at 48 hrs when treated with a combination of progesterone and vitamin D. HIO-118V cells show modest 1.7-fold increase in apoptosis with 45 uM progesterone alone and a 1.9-fold increase with the combination of 1 uM Vitamin D and 45 uM progesterone.



Effect of Vitamin D and Progesterone on Apoptosis (OVCAR 3)



The cell cycle results confirm the apoptosis results. In the OVCAR 3, there is a modest reduction in S-phase due to progesterone and Vitamin D administered alone and a more robust reduction in S-phase with the two agents combined. In the HIO-118V, there is a progesterone-induced block of cells going from S-phase to G2M at 28 hours; however, the effect from Vitamin D is inconclusive.

Effect of Vitamin D and Progesterone on Cell Cycle (OVCAR 3)

Treatments (uM)	G0-G1		S		G2M	
	28 hr	48 hr	28 hr	48 hr	28 hr	48 hr
ut	46	49	54	51	0	0
35 PR	60	60	40	40	0	0
1 VD3	52	57	42	41	6	2
35 PR + 1 VD3	69	67	31	34	0	0

Effect of Vitamin D and Progesterone on Cell Cycle (HIO-118V)

Treatments (uM)	G0-G1		S		G2M	
	28 hr	48 hr	28 hr	48 hr	28 hr	48 hr
ut	38	40	47	60	15	4
50 PR	41	45	52	55	7	0
1 VD3	43	41	42	51	16	8
50 PR + 1 VD3	41	48	55	52	4	1

Evaluation of Progestin and Vitamin D for Ovarian Cancer Chemoprevention in the Chicken

The planned chemoprevention trial evaluating progestins and Vitamin D as ovarian cancer preventives is well underway. Over 3600 birds were randomized into 6 groups, including

- 1) Control (contains baseline recommended allowance of Vitamin D)
- 2) High dose Vitamin D (5x the amount of D in group one)
- 3) High dose pulsed progestin
- 4) Low dose continuous progestin
- 5) High dose pulsed progestin plus High dose D
- 6) Low dose continuous progestin plus High dose D

The Vitamin D formulation we are using is 1 α , 25 dihydroxyvitamin D₃. The baseline vitamin D requirement is satisfied at 0.03125 mg/lb of feed. This is reflected in the diets that are formulated for groups 1,3and 4. Groups 2,5and 6 are receiving a 5x dose, or 0.156 mg/lb of feed. The low progestin dose group is receiving 0.05 mg/day Levonorgestrel equivalent (same as first chicken trial demonstrating a chemopreventive effect), and the high progestin dose group is receive a pulsed dose of 0.5mg/day quarterly.

The study is designed with sufficient sample size for adequate power to detect the subtle differences between the treatments and accounting for expected mortality during the trial, based on our experience with a similar flock and conditions in our first trial. The experimental design is a factorial and is properly balanced and easily analyzed. We hope to demonstrate dose response effects and this is the rationale for the low and high dose D and progestin design. Also the design will allow us to look for synergistic effects, especially with the low D and low P groups. Finally, the pulsed progestin arms will allow us to directly test the hypothesis that periodic administration of an agent that induces apoptosis in the ovarian surface epithelium will effectively clear premalignant cells, leading to significant cancer prevention. If this hypothesis is validated, it will open the door toward consideration of chemopreventive strategies involving periodic administration of preventive agents, thus decreasing the potential toxicity associated with chemoprevention.

The trial is progressing well. Bird welfare is excellent. We look forward to analysis of the data next year when the trial is complete.

Key research accomplishments

- 1) We have accrued over 1,600 subjects to a prospective, population-based, case-control study of ovarian cancer in North Carolina. Blood and tissue samples and epidemiologic data have been accrued as well. Analyses of genetic susceptibility polymorphisms and molecular epidemiologic signatures are ongoing.
- 2) The +331G/A polymorphism in the progesterone receptor is protective against endometrioid/clear cell ovarian cancers and this has been confirmed in a meta-analysis.
- 3) We have shown that progestins markedly activate TGF- β signaling pathways in the ovarian epithelium in primates, and that these effects are highly associated with apoptosis. We are now performing studies *in vitro* designed to characterize the complex biologic effects of progestins and vitamin D analogues on apoptotic and TGF- β signaling pathways in ovarian epithelial cells. These findings will provide guidance in conducting a chemopreventive trial in chickens with these agents.

Reportable outcomes

- 1) The +331G/A polymorphism appears to be protective against endometrioid and clear cell ovarian cancers.
- 2) Combinations of progestins and vitamin D may act in an additive fashion to decrease growth of ovarian cancer cells.

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- 12) Schildkraut JM, Moorman P, Halabi S, Calingaert B, Marks JR, Havrilesky L, Berchuck A. Analgesic drug use is associated with a decreased risk of ovarian cancer. *Epidemiology* (in press).

Conclusions

The studies initiated by our program will enable us to define more homogeneous subsets of ovarian cancer based on epidemiologic and molecular characteristics, to identify women who are at increased risk for this disease and to develop chemopreventive strategies designed to decrease ovarian cancer incidence and mortality. We anticipate that much of our data will grow to maturity in the coming few years with continued support from the DOD Ovarian Cancer Research Program.

Appendices

Progesterone Receptor Promoter +331A Polymorphism is Associated with a Reduced Risk of Endometrioid and Clear Cell Ovarian Cancers

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Abstract

Objective: The progestagenic milieu of pregnancy and oral contraceptive use is protective against epithelial ovarian cancer. A functional single nucleotide polymorphism in the promoter of the progesterone receptor (+331A) alters the relative abundance of the A and B isoforms and has been associated with an increased risk of endometrial and breast cancer. In this study, we sought to determine whether this polymorphism affects ovarian cancer risk.

Methods: The +331G/A polymorphism was genotyped in a population-based, case-control study from North Carolina that included 942 Caucasian subjects (438 cases, 504 controls) and in a confirmatory group from Australia (535 cases, 298 controls). Logistic regression analysis was used to calculate age-adjusted odds ratios (OR).

Results: There was a suggestion of a protective effect of the +331A allele (AA or GA) against ovarian cancer in the North Carolina study [OR, 0.72; 95% confidence interval (95% CI), 0.47-1.10]. Examination of genotype frequencies by histologic type revealed that this was

due to a decreased risk of endometrioid and clear cell cancers (OR, 0.30; 95% CI, 0.09-0.97). Similarly, in the Australian study, there was a nonsignificant decrease in the risk of ovarian cancer among those with the +331A allele (OR, 0.83; 95% CI, 0.51-1.35) that was strongest in the endometrioid/clear cell group (OR, 0.60; 95% CI, 0.24-1.44). In the combined U.S.-Australian data that included 174 endometrioid/clear cell cases (166 invasive, 8 borderline), the +331A allele was significantly associated with protection against this subset of ovarian cancers (OR, 0.46; 95% CI, 0.23-0.92). Preliminary evidence of a protective effect of the +331A allele against endometriosis was also noted in control subjects (OR, 0.19; 95% CI, 0.03-1.38).

Conclusions: These findings suggest that the +331G/A progesterone receptor promoter polymorphism may modify the molecular epidemiologic pathway that encompasses both the development of endometriosis and its subsequent transformation into endometrioid/clear cell ovarian cancer. (Cancer Epidemiol Biomarkers Prev 2004;13(12):2141-7)

Introduction

Epidemiologic studies have shown that both pregnancy and use of oral contraceptives dramatically reduce ovarian cancer incidence (1). Reduction in numbers of lifetime ovulations due to pregnancy or oral contraceptive use may decrease risk by reducing gonadotropin levels, oxidative stress, DNA replication errors, and inclusion cyst formation in the ovarian epithelium. In addition, whereas estrogens and androgens have been shown to increase ovarian cancer risk, both pregnancy and oral contraceptive use are characterized by a

protective progestagenic hormonal milieu (1, 2). We have previously reported that oral contraceptives with high progestin potency were associated with a greater ovarian cancer risk reduction than those with low progestin potency (3). In addition, we have shown that progestins may reduce ovarian cancer risk by stimulating the apoptosis of genetically damaged ovarian epithelial cells that otherwise might eventually evolve a fully transformed phenotype (4, 5). This may account for the observation that the protective effect of pregnancy and oral contraceptives is far greater than the extent to which lifetime ovulatory cycles are reduced (1).

In view of the protective effect of progestins against ovarian cancer, progesterone receptor variants with altered biological activity may affect ovarian cancer susceptibility. A German group reported that an insertion polymorphism in intron G of the progesterone receptor was associated with a 2.1-fold increased ovarian cancer risk (6, 7). It was subsequently shown that this intronic *AluI* insertion is in linkage disequilibrium with

Received 2/3/04; revised 6/29/04; accepted 7/6/04.

Grant support: NIH grant 1-R01-CA76016, Department of Defense grant DAMD17-02-1-0666, Society for Gynecologic Investigation, and National Health and Medical Research Council of Australia.

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polymorphisms in exons 4 and 5. However, several subsequent studies have failed to confirm an association between these polymorphisms and ovarian cancer risk (8-12). In addition, there is little published evidence that this complex of polymorphisms, termed PROGENS, alters progesterone receptor function.

More recently, sequencing of the progesterone receptor gene has revealed several additional polymorphisms, including one in the promoter region (+331G/A; ref. 13). The +331A allele creates a unique transcriptional start site that favors the production of progesterone receptor B (PR-B) isoform over progesterone receptor A (PR-A; ref. 13). The PR-A and PR-B isoforms are ligand-dependent members of the nuclear receptor family that are structurally identical, except for an additional 164 amino acids at the NH₂ terminus of PR-B, but their actions are distinct (14, 15). The full-length PR-B functions as a transcriptional activator, and in the tissues where it is expressed, it is a mediator of various responses, including the proliferative response to estrogen or the combination of estrogen and progesterone (16). PR-A is a transcriptionally inactive dominant-negative repressor of steroid hormone transcription activity that is thought to oppose estrogen-induced proliferation. An association has been reported between the +331A allele and increased susceptibility to endometrial (13) and breast cancers (17). It was postulated that up-regulation of PR-B in carriers of the +331A allele might enhance formation of these cancers due to an increased proliferative response.

We used a case-control study design to explore whether the +331G/A polymorphism in the progesterone receptor promoter affects susceptibility to various histologic types of ovarian cancer in North Carolina. A second, independent, case-control study from Australia was examined to confirm associations seen in the North Carolina study.

Materials and Methods

Subjects

North Carolina Ovarian Cancer Study. Primary ovarian cancer cases enrolled in the study were identified through the North Carolina Central Cancer Registry, a statewide, population-based tumor registry, using rapid case ascertainment. Eligibility criteria for ovarian cancer cases include diagnosis since January 1, 1999, ages 20 to 74 years at diagnosis, no prior history of ovarian cancer, and residence in a 48-county area of North Carolina. Physician permission was obtained before an eligible case was contacted. The diagnosis of epithelial ovarian cancer (borderline or invasive) was confirmed by the study pathologist. The response rate among eligible cases was 82%. Nonresponders were classified as patient refusal (6.7%), inability to locate the patient (4.0%), physician refusal (3.5%), death (2.6%), or debilitating illness (1.6%). Population-based controls were identified from the same 48-county region as the cases and were frequency matched to the ovarian cancer cases based on race (Black and non-Black) and age (5-year age categories) using list-assisted random digit dialing. Potential controls were screened for eligibility and were required to have at least one intact ovary and no prior diagnosis of ovarian cancer. Seventy-three percent of controls identified by random digit dialing who passed the eligibility

screening agreed to be contacted and were sent additional study information. Among those sent additional study information, the response rate was 68%. The study protocol was approved by the Duke University Medical Center Institutional Review Board and the human subjects committees at the North Carolina Central Cancer Registry and each of the hospitals where cases were identified. Trained nurse interviewers obtained written informed consent from study subjects at the time of the interview, usually in the home of the study subject. A 90-minute questionnaire was given to obtain information on known and suspected ovarian cancer risk factors including family history of cancer in first- and second-degree relatives, menstrual characteristics, pregnancy and breast-feeding history, hormone use, and lifestyle characteristics such as smoking, alcohol consumption, physical activity, and occupational history. A life events calendar, including marriage and education, was used to improve recall. Additionally, anthropometric descriptors (height, weight, waist, and hip circumference) were measured and blood samples (30 mL) were collected. Germ line DNA was extracted using PureGene DNA isolation reagents according to the manufacturer's instructions (Gentra Systems, Minneapolis, MN). Analysis of data from the North Carolina study was limited to Whites. Data from 81 African American controls and 67 cases were excluded because of the low frequency of the polymorphism. Data were collected from 16 non-Black, non-Caucasian cases and 10 controls but were excluded because of the significant racial diversity and small size of this group.

Australian Study. Details of cases and controls included in the Australian study have been described previously (18). Briefly, the case sample consisted of 553 women with primary epithelial ovarian cancer ascertained as incident case subjects as part of a large population-based, case-control study from major gynecologic-oncology treatment centers in New South Wales, Victoria, and Queensland from 1990 to 1993 ($n = 363$) and from the Royal Brisbane Hospital, Queensland from 1985 to 1996 ($n = 190$). Histopathologic information regarding tumor behavior (low malignant potential or invasive), histology, stage, and grade was available for all women; information on potential or known ovarian cancer risk factors was ascertained by detailed questionnaire for the subset of cases in the population-based study and included age, ethnicity, country of birth, parity, oral contraceptive use, tubal ligation, hysterectomy, and age at menarche. Limited information ascertained from hospital records was also available for the Royal Brisbane Hospital patients and included age, ethnicity, and country of birth. Because blood samples were not collected from controls who participated in the ovarian cancer case-control study, an additional group of women, selected based on date-of-birth distribution to best match cases, were included in the analyses. The control sample consisted of 300 adult female unrelated monozygotic twins (one per pair), ages 30 to 90 years, recruited through the volunteer Australian Twin Registry for the Semistructured Assessment for the Genetics of Alcoholism study. This study reported participation rates of ~70% for monozygotic female twins and recruited individuals nationally from major cities in the eastern states of Australia. Limited information

ascertained by detailed questionnaires as part of the Semi-structured Assessment for the Genetics of Alcoholism study was available for these women to assess confounding and included age, ethnicity, country of birth, parity, and age at menarche. More than 90% of case and control subject groups were of northern European descent, and all subjects were from major cities in the eastern Australian states. Approvals were obtained from the ethics committees of the University of Melbourne, New South Wales Cancer Council, Anti-Cancer Council of Victoria, and Queensland Institute of Medical Research in Australia. Written informed consent was obtained from each participant. DNA isolation methods have been detailed elsewhere (18). Fourteen Australian cases ages <30 years were excluded from this analysis because no controls were ages <30 years. Additionally, four cases and two controls were excluded because they did not have +331G/A polymorphism results. Thus, the Australian sample used for this analysis consisted of 535 cases and 298 controls.

Genotyping of +331G/A Polymorphism. Allelic discrimination was done using the MGB primer/probe Taqman assay on the ABI Prism 7700 system. Details of the methods are described in the following sections.

North Carolina Study. Each 20 μ L PCR reaction contained 18 pmol of forward primer 5'-CACGAGTTT-GATGCCAGAGAAA-3', 18 pmol of reverse primer 5'-GCGACGGCAATTTAGTGACA-3', 4 pmol of G-allele probe (VIC)-CGGCTCtTTTATC-(MGBNFQ)-3', 4 pmol of A-allele probe (FAM)-CGGCTCtTTTATCTC-(MGBNFQ)-3' (200 nmol/L), 10 μ L of 2 $\times$ Taqman Universal Master Mix without AmpErase UNG (Applied Biosystems, Foster City, CA), and 25 ng of extracted leukocyte DNA. Cycling conditions were 95°C for 10 minutes followed by 40 cycles of 92°C for 15 seconds and 60°C for 60 seconds. Allelic discrimination was done in 96-plate format in the ABI Prism 7700 and analyzed using the ABI Prism 7700 software. Some samples in the North Carolina ovarian cancer study were subjected to sequencing to confirm results obtained using the Taqman assay. To do this, a 50 μ L PCR reaction was done using forward primer 5'-AACTCAGCGAGGGACTGAGA-3' and reverse primer 5'-GAGGACTGGAGACGCAGAGT-3', 0.5 ng/ μ L genomic DNA, 0.5 nmol/L forward primer, 0.5 nmol/L reverse primer, 0.2 mmol/L deoxynucleotide triphosphate, 1.5 mmol/L MgCl₂ (Applied Biosystems), 1 $\times$ Applied Biosystems PCR buffer, and 0.025 units/ μ L AmpliTaq Gold DNA polymerase (Applied Biosystems). PCR conditions consisted of an initial denaturing step at 95°C for 12 minutes, 32 cycles of 94°C for 60 seconds, 55.0°C for 60 seconds, and 72°C for 3 minutes, and an extension step at 72°C for 10 minutes. Samples were held at 4.0°C until they were purified using QIAquick 96 vacuum filter plates (Qiagen, Germantown, MD) and finally eluted in 150 μ L of 10 mmol Tris-HCl (pH 8.5). A sequencing reaction was done using 1 μ L of purified product and 4.4 pmol of unlabeled forward primer in a BigDye Terminator Cycle Sequencing Reaction as described by the supplier (Applied Biosystems). Samples were analyzed on the ABI 3100 system and sequences determined using GeneScan software (Applied Biosystems).

Australian Study. Genotyping was done with Taqman methodology using identical probes as the North Carolina study. For detection and sequence confirmation

of positive controls, a 381-bp product was amplified using the forward primer 5'-GTACGGAGCCAGCA-GAAGTC-3' and reverse primer 5'-ATCCTGTCGT-CAGGGGAACT-3'. Denaturing high-performance liquid chromatography (Helix System, Varian Chromatography Systems, Walnut Creek, CA) was used to identify heterozygous GA individuals at 62°C recommended by the MELT program (<http://insertion.stanford.edu/melt.html>). Genotypes were confirmed by sequencing. Heterozygous GA PCR product was subcloned using the pGEM-T system to obtain G and A clones to use as control standards for the SDS allelic discrimination assay. The 15 μ L PCR reaction contained 900 nmol/L of forward primer 5'-GCGACGGCAATTTAGTGACA-3', 900 nmol/L of reverse primer 5'-TGCACGAGTTTATGCCAGA-3' (giving a 68-bp product), 150 nmol/L of A-allele probe, 200 nmol/L of G-allele probe, 1 $\times$ Platinum Quantitative PCR SuperMix UDG (including passive reference ROX dye, Invitrogen, Melbourne, Victoria, Australia), and 15 ng of genomic or control sample that had been dried in 96-well plates. PCR was done using the ABI 7700 SDS PCR machine for 2 minutes at 50°C and 2 minutes at 95°C followed by 45 two-step cycles of 15 seconds at 92°C and 1 minute at 60°C.

Statistical Analysis. The genotype data were tested for Hardy-Weinberg equilibrium using the χ^2 goodness-of-fit test. Multivariate unconditional logistic regression models, adjusted for age, were used to estimate odds ratio (OR) and 95% confidence interval (95% CI) for the association between polymorphism and epithelial ovarian cancer for all cases as well as for various disease categories. Potential confounders including menopausal status, tubal ligation, oral contraceptive use, body mass index, family history of breast or ovarian cancer in first- and second-degree relatives, and parity were individually adjusted for in the North Carolina data to determine if they changed the crude OR by $\geq 10\%$. Analysis stratified by each of these factors was also conducted to assess potential effect modification. We found no evidence of confounding by these factors and therefore felt it appropriate to combine the Australian and North Carolina data despite limited epidemiologic data in the Australian sample. The Breslow-Day χ^2 test was used to assess homogeneity of the results from the two study populations. Analyses involving the combined data set were based on a reanalysis of the raw data and were adjusted for study as well as age. All calculations were done using SAS 8.0 (SAS Institute, Inc., Cary, NC).

Results

The demographic features, epidemiologic risk factors, and pathologic characteristics of cases and controls in the North Carolina (Caucasians only) and Australian studies are shown in Table 1. Of note, the median ages of the cases and controls in both North Carolina and Australian studies are similar. Caucasian women with ovarian cancer in North Carolina were more likely to have used oral contraceptives compared with Australian women with ovarian cancer (67% and 49%, respectively). Invasive ovarian cancer cases comprised 77% of the North Carolina cases compared with 84% of the Australian cases. The +331G/A single nucleotide polymorphism in the promoter of the progesterone receptor initially was genotyped in samples from the North

Table 1. Demographics and pathologic characteristics of cases and controls

	North Carolina Study		Australian Study	
	Cases (<i>n</i> = 438), <i>n</i> (%)	Controls (<i>n</i> = 504), <i>n</i> (%)	Cases (<i>n</i> = 535), <i>n</i> (%)	Controls (<i>n</i> = 298), <i>n</i> (%)
Age*				
Median (range)	55 (20-74)	53 (20-75)	59 (30-95)	50 (30-94)
Menopause status				
Premenopausal/perimenopausal	166 (38)	204 (40)		
Postmenopausal	272 (62)	300 (60)		
Parity*				
0	93 (21)	68 (13)	71 (20)	38 (13)
1	73 (17)	72 (14)	51 (15)	20 (7)
2	146 (33)	210 (42)	103 (30)	75 (25)
≥3	126 (29)	154 (31)	123 (35)	160 (55)
Oral contraceptive use*				
Yes	294 (67)	349 (69)	169 (49)	
No	144 (33)	155 (31)	179 (51)	
Tumor behavior				
Borderline	102 (23)		87 (16)	
Invasive	336 (77)		448 (84)	
Tumor stage †				
1	160 (37)		166 (31)	
2	33 (8)		42 (8)	
3	224 (52)		276 (52)	
4	14 (3)		43 (8)	
Tumor histology				
Serous	270 (62)		318 (59)	
Endometrioid	56 (13)		63 (12)	
Mucinous	49 (11)		61 (11)	
Mixed cell	1 (0)		36 (7)	
Clear cell	23 (5)		32 (6)	
Other	39 (9)		25 (5)	

NOTE: Fourteen Australian cases ages <30 years were excluded from the entire analysis because no controls were ages <30 years.

*Parity use not known for 187 Australian cases and 298 Australian controls. Oral contraceptive use not known for 187 Australian cases and 5 Australian controls.

†Stage not known for eight Australian and seven NC cases.

Carolina Ovarian Cancer study using a Taqman assay. In 91 samples in which there was some ambiguity regarding the genotype using the Taqman assay, DNA sequencing was done for confirmation, and in all cases, the original genotypes were confirmed. The +331A allele was found in 59 of 504 (11.7%) Caucasian controls and the distribution of genotypes was in Hardy-Weinberg equilibrium ($P = 0.53$). Among individuals who reported

their race to be African American, only 1 of 81 (1.2%) controls and 0 of 67 with ovarian cancer carried the +331A allele. In view of the rarity of the +331A allele in African Americans, these subjects were excluded from analyses of the association with ovarian cancer risk.

There were very few +331A homozygotes and these were combined with GA heterozygotes in calculating crude and age-adjusted ORs (Table 2). In the North

Table 2. Association between +331G/A polymorphism and risk of invasive and borderline epithelial ovarian tumors

Genotype	Borderline and invasive cases, <i>n</i> (%)	Controls, <i>n</i> (%)	OR* 95% CI	Invasive cases, <i>n</i> (%)	Controls, <i>n</i> (%)	OR* 95% CI
North Carolina study						
	<i>n</i> = 438	<i>n</i> = 504		<i>n</i> = 336	<i>n</i> = 504	
GG	400 (91.3)	445 (88.3)	1.00 (reference)	307 (91.4)	445 (88.3)	1.00 (reference)
AG	37 (8.4)	58 (11.5)		28 (8.3)	58 (11.5)	
AA	1 (0.2)	1 (0.2)		1 (0.3)	1 (0.2)	
AG/AA	38 (8.7)	59 (11.7)	0.72 (0.47-1.10)	29 (8.6)	59 (11.7)	0.72 (0.45-1.15)
Australian study						
	<i>n</i> = 535	<i>n</i> = 298		<i>n</i> = 448	<i>n</i> = 298	
GG	483 (90.3)	266 (89.3)	1.00 (reference)	407 (90.8)	266 (89.3)	1.00 (reference)
AG	48 (9.0)	30 (10.1)		37 (8.3)	30 (10.1)	
AA	4 (0.7)	2 (0.7)		4 (0.9)	2 (0.7)	
AG/AA	52 (9.7)	32 (10.7)	0.83 (0.51-1.35)	41 (9.2)	32 (10.7)	0.76 (0.46-1.27)

NOTE: Fourteen Australian cases ages <30 years were excluded from the entire analysis because no controls were ages <30 years.

*ORs adjusted for age. For combined data, ORs are adjusted for the study as well.

Carolina sample, there was a suggestion that the +331A allele was associated with a modest reduction in risk of both borderline tumors and invasive ovarian cancers (OR, 0.72; 95% CI, 0.47-1.10). Samples from the Australian study were genotyped independently and 10.7% of controls were found to carry the +331A allele. The distribution of genotypes in controls was found to be in Hardy-Weinberg equilibrium ($P = 0.27$). Although not statistically significant, a similar inverse association with invasive ovarian cancer risk was observed (OR, 0.83; 95% CI, 0.51-1.35; Table 2). Excluding the borderline ovarian cancers revealed little change in the point estimates of the association between the +331A allele and ovarian cancer for either North Carolina or Australian comparisons (Table 2).

Analyses by histologic subtype for the North Carolina and Australian studies are presented in Table 3. A modest, nonsignificant decreased risk was observed in the North Carolina study among carriers of the +331A allele for the common serous histologic type (OR, 0.80; 95% CI, 0.49-1.29), but there was a striking decreased risk of endometrioid cancers (OR, 0.43; 95% CI, 0.13-1.40). Because endometrioid and clear cell ovarian tumors are thought to have a common etiology due to their association with endometriosis (19), these cases were combined to examine the overall association with the +331A allele of the progesterone receptor promoter polymorphism (OR, 0.30; 95% CI, 0.09-0.97). No consistent effect was observed between the +331A allele and mucinous ovarian cancers. These relationships according to histologic subtype were not modified by age, parity, history of oral contraceptive use, body mass index, or family history of breast/ovarian cancer.

In the Australian data, the protective effect of the +331A allele was most pronounced in endometrioid cancers (OR, 0.51; 95% CI, 0.17-1.53). The OR (95% CI) for

the combined endometrioid and clear cell group was 0.60 (0.25-1.44). The Breslow-Day χ^2 test was indicative of homogeneity between the North Carolina and the Australian studies with respect to the association between the +331A allele and risk of ovarian cancer overall ($P = 0.58$) as well as endometrioid and clear cell ovarian cancer ($P = 0.24$). Pooling data from both North Carolina and Australian studies and controlling for study site, the age-adjusted OR (95% CI) for the association between the +331A allele and endometrioid/clear cell cancers combined ($n = 174$; 166 invasive, 8 borderline) was 0.46 (0.23-0.92).

Associations between the +331A allele and endometriosis were examined in the North Carolina study because endometriosis is known to increase the risk of endometrioid and clear cell ovarian cancers (19). The rate of self-reported endometriosis was 12.6% in cases and 7.5% in controls, similar to other reports in the literature (19). Endometriosis was associated with an increased risk of ovarian cancer (OR, 1.76; 95% CI, 1.14-2.72). This was mostly attributable to an increased risk of endometrioid/clear cell cases (OR, 3.87; 95% CI, 2.09-7.17; non-endometrioid/clear cell cases OR, 1.36; 95% CI, 0.84-2.20). Preliminary evidence of a protective effect of the +331A allele of the progesterone receptor polymorphism against endometriosis was also noted in control subjects (OR, 0.19; 95% CI, 0.03-1.38).

Discussion

Epidemiologic studies have long suggested that heredity plays a role in ovarian cancer predisposition (20). Two high-penetrance ovarian cancer susceptibility genes, *BRCA1* and *BRCA2*, have been identified, defects that increase ovarian cancer risk dramatically (21, 22). It is estimated that up to ~10% of ovarian cancers are attributable to inherited mutations in *BRCA1* and *BRCA2* (22), but <0.5% of individuals in most populations carry these mutations. Although other high-penetrance genes may exist, low-penetrance polymorphisms are likely to contribute to the burden of ovarian cancers classified as sporadic. The PROGENS polymorphism in the progesterone receptor was initially reported to increase ovarian cancer risk (6, 7), but this finding was not confirmed by subsequent studies, including the North Carolina Ovarian Cancer study (8-12). The potential for false-positive results in association studies is now widely accepted, and confirmation in independent populations is now deemed critical prior to concluding that a true association exists (23).

A functional polymorphism in the progesterone receptor promoter (+331A) that favors production of PR-B is carried by ~11% of the Caucasian population (13). The group that described this polymorphism has reported associations between the +331A allele and increased risks of endometrial cancer (OR, 1.9; 95% CI, 1.10-3.29; ref. 13) and breast cancer (OR, 1.33; 95% CI, 1.01-1.74; ref. 17). The most striking increased risks were observed in obese women (endometrial cancer OR, 4.71; breast cancer OR, 2.30), suggesting an interaction between the polymorphism and the endogenous hormonal milieu. Because there were few rare allele homozygotes, these associations were based on a model in which heterozygotes were pooled with rare allele

Table 3. Association between progesterone receptor polymorphism and risk of invasive and borderline epithelial ovarian tumors by histologic type and study

	GG	AG	AA	AG/AA (%)	OR* (95% CI)
North Carolina study					
Controls	445	58	1	59 (11.7)	1.00 (reference)
Serous	244	26	0	26 (9.6)	0.81 (0.50-1.32)
Mucinous	44	5	0	5 (10.2)	0.80 (0.30-2.14)
Endometrioid	53	3	0	3 (5.4)	0.43 (0.13-1.40)
Clear cell	23	0	0	0 (0.0) [†]	
Endometrioid/ clear cell	76	3	0	3 (3.8)	0.30 (0.09-0.97)
Mixed	1	0	0	0 (0.0) [†]	
Other	35	3	1	4 (10.3)	0.86 (0.29-2.51)
Australian study					
Controls	266	30	2	32 (10.7)	1.00 (reference)
Serous	285	31	2	33 (10.4)	0.89 (0.52-1.52)
Mucinous	55	6	0	6 (9.8)	0.91 (0.36-2.27)
Endometrioid	59	3	1	4 (6.3)	0.51 (0.17-1.53)
Clear cell	29	3	0	3 (9.4)	0.83 (0.24-2.92)
Endometrioid/ clear cell	88	6	1	7 (7.4)	0.60 (0.25-1.44)
Mixed	32	3	1	4 (11.1)	1.01 (0.32-3.17)
Other	23	2	0	2 (8.0)	0.73 (0.15-3.44)

*ORs are according to genotype (AG/GG) compared with the reference group genotype (GG) and are adjusted for age and corresponding study.

[†]Sample size too small to calculate.

homozygotes. It was postulated that the rare allele of this polymorphism may increase endometrial and breast cancer risks by enhancing PR-B-mediated proliferation in response to estrogen.

In the population-based North Carolina Ovarian Cancer Study, risk analyses were confined to Caucasian subjects because of the rarity of +331A allele in African American women. Among Caucasian women, we observed a weak protective effect of the +331A allele against ovarian cancer (borderline and invasive). Histologic subtype analysis revealed that there was a weak, nonsignificant decrease in risk of serous cancers, which are the most common subtype, whereas a stronger decreased risk for endometrioid cancers was observed. This association became even stronger and statistically significant after combining endometrioid and clear cell cancers, with about a two-thirds reduction in risk (OR, 0.30; 95% CI, 0.09-0.97) in carriers of the +331A allele, although the 95% CIs are wide suggesting the instability of the estimate. In view of the potential for false-positive results in association studies of genetic polymorphisms, we sought to confirm our findings in the Australian study. The frequency of the +331A allele among Caucasian controls varied by <1% between Australian and North Carolina studies and controls reported in the Nurses' Health Study (13, 17). The Australian study was not a population-based, case-control study and fewer data were available regarding risk factors. Nevertheless, the results of the Australian study were similar to those of the North Carolina study, with a modest overall protective effect that was most pronounced for endometrioid cancers (OR, 0.51; 95% CI, 0.17-1.53). Age was not associated with genotype and adjusting for age had minimal effect on the ORs reported in this article.

Serous and endometrioid/clear cell ovarian cancers share many of the same risk factors, such as parity and oral contraceptive use, but there is evidence to suggest that differences exist in their etiology, molecular pathogenesis, and clinical behavior. For example, there are differences between these histologic subtypes with respect to behavior (borderline versus invasive) and stage that likely reflect etiologic heterogeneity. In addition, mutations in *BRCA1* and *BRCA2* predispose primarily to serous cancers (24), which arise from epithelial cells that line the ovarian surface or underlying inclusion cysts. In contrast, it is thought that some, if not all, endometrioid and clear cell cancers arise from deposits of ovarian endometriosis (19). Coexistent endometriosis is commonly noted in women with ovarian endometrioid/clear cell cancers, and a strong association between endometriosis and these cancers has been reported in epidemiologic studies. Because endometriosis is likely to be underdiagnosed, the relationship between endometriosis and clear cell/endometrioid ovarian cancers may be stronger than noted in case-control studies.

The finding that the +331A allele was associated with a decreased risk of endometrioid and clear cell ovarian cancers was somewhat unexpected in view of prior reports of an increased risk of endometrial and breast cancers in carriers of the +331A allele (13, 17). However, these three diseases differ with respect to associated risk factors and predisposing hormonal milieu. Endometriosis is associated with endometrioid

and clear cell ovarian cancers (19) but does not increase endometrial or breast cancer risk. In contrast, oral contraceptives are protective against all histologic types of epithelial ovarian cancer as well as endometrial cancers (1) but may increase breast cancer risk (25). In view of these significant differences in etiology, it is not surprising that predisposition to these cancers is affected differentially by the progesterone receptor promoter polymorphism.

PR-A and PR-B are both expressed in the ovarian (26), endometrial (27), and breast epithelium (28), and the relative expression of the isoforms is frequently altered during malignant transformation. In the present study, the +331A allele of the progesterone receptor promoter polymorphism was protective against endometrioid and clear cell ovarian cancers. We also observed preliminary evidence that this polymorphism may protect against endometriosis, the precursor of many of these cancers. Endometriotic implants have been shown to express only the PR-A isoform (27), and it has been suggested that the absence of PR-B may account for the lack of appropriate cycling of these glands. In normal cycling endometrium, PR-A expression is predominant during the proliferative phase, whereas a shift toward PR-B occurs with differentiation in the early secretory phase (29). Because the +331A allele of the progesterone receptor promoter polymorphism favors production of the PR-B isoform, it is possible that this might prevent the PR-A/PR-B imbalance in endometriotic implants and protect against the growth and spread of endometriosis to the extent that it becomes clinically apparent. The reduced risk of endometrioid and clear cell cancers in women with the +331A allele might be attributable to a lower likelihood of carriers developing more extensive endometriosis, which serves as a precursor for these cancers. In contrast to the pathogenic model proposed for endometriosis in which the +331A allele counters an abnormal imbalance in the PR-A/PR-B ratio in normal breast and endometrial tissues, the polymorphism may create an imbalance that enhances both the proliferative response to estrogen and cancer risk.

The literature is fraught with false-positive association studies of genetic susceptibility polymorphisms, but several features mitigate the likelihood of this in the present study. First, the known protective benefit of progestins against ovarian cancer provides a preexisting biological plausibility for the observed association. In addition, the finding that the +331A allele is protective against both endometrioid/clear cell cancers and their precursor lesion (endometriosis) is also supportive. Confirmation of the positive association obtained in the North Carolina study by the Australian study also represents an additional critical validation step. Finally, unlike many polymorphisms that lack known functional significance, the +331A allele is known to increase transcription of PR-B *in vitro* (13).

Despite the agreement between North Carolina and Australian data, the 95% CIs of the latter study are relatively wide. Furthermore, the control subjects in the Australian study were not collected in the context of an ovarian cancer study. However, allele frequencies in the Australian controls were similar to those seen in Caucasian controls in the North Carolina study. Another limitation of this study is that the number of cases of the less common histologic types was relatively modest,

limiting the power to draw definitive conclusions. Additional studies are needed to confirm the protective effect of the +331A allele against endometrioid and clear cell ovarian cancers.

In summary, the +331A allele of the progesterone receptor promoter polymorphism is carried by about one in nine Caucasian women and is associated with a decrease in risk of endometrioid and clear cell ovarian cancers. We also obtained preliminary evidence in support of a protective effect against endometriosis. These findings suggest that the +331G/A progesterone receptor promoter polymorphism may modify the molecular epidemiologic pathway that encompasses both the growth of endometriosis and its subsequent transformation into endometrioid/clear cell cancers. This study provides evidence for the existence of low-penetrance ovarian cancer susceptibility polymorphisms. If multiple polymorphisms are identified that either increase or decrease the risk of various histologic types of ovarian cancer, this might be used in the future for risk stratification that would facilitate screening and prevention strategies.

Acknowledgments

We thank the North Carolina Central Tumor Registry; the staff of the North Carolina Ovarian Cancer Study; Regina Whitaker for expert technical assistance; Rex C. Bentley, MD, for review of the pathology; and Adele Green for access to ovarian case-subject DNA and Nicholas Martin for access to control-subject DNA (Australian study).

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

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Received 28 October 2004

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.

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.

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.

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.

Acknowledgments

The study at Irvine was supported by research grants from the National Cancer Institute (CA-058860 and CA-78134), at Stanford and at Roswell Park Cancer Institute by grants from the Roswell Park Alliance and the National Cancer Institute (CA71966 and Core Grant CA16056), at DKFZ by the Federal Ministry of Education and Research of Germany, Programme of Clinical Biomedical Research grant 01 GB 9401, in North Carolina by the Department of Defence Program Grant (17-02-1-0666) and a National Institute of Health grant (1-RO1-CA76016), and at QIMR by grants from the National Health and Medical Research Council. GCT and DD are NHMRC Research Fellows and ABS is funded by an NHMRC Career Development Award. IC was supported in part by a grant from the Association for International Cancer Research, St. Andrews, UK (AICR).

We thank Adele Green for DNA and data from the Survey of Women's Health, Nick Martin for access to twin control DNA, the many gynecologists and oncologists for allowing us to contact their patients; Ursula Eilber, Anna Felberg for competent data management and programming, Nancy Eby, Andrea Busche-Bässler, Renate Birr, Ann-Sybil Krapfl-Gast, Eva Lederer, Susanne Siegrist, Silke Spahn, Beate Tilch, and Meredith Mills for data collection. We are particularly grateful to the many women who participated in this research.

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Transforming growth factor β receptor I polyalanine repeat polymorphism does not increase ovarian cancer risk

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Received 16 November 2004

Available online 22 March 2005

Abstract

Objectives. It has been suggested that the 6A allele of the type I TGF β receptor (*TGF β RI*) polyalanine repeat tract polymorphism may increase susceptibility to various types of cancer including ovarian cancer.

Methods. The *TGF β RI* polyalanine polymorphism was genotyped in 588 ovarian cancer cases and 614 controls from a population-based case-control study in North Carolina.

Results. Significant racial differences in the frequency of the 6A allele were observed between Caucasian (10.7%) and African-American (2.4%) controls ($P < 0.001$). One or two copies of the 6A allele of the *TGF β RI* polyalanine polymorphism was carried by 18% of all controls and 19% of cases, and there was no association with ovarian cancer risk (OR = 1.07, 95% CI 0.80–1.44). The odds ratio for 6A homozygotes was 1.81 (95% CI 0.655–0.66), but these comprised only 0.98% of controls and 1.70% of cases.

Conclusions. The 6A allele of the *TGF β RI* polyalanine polymorphism does not appear to increase ovarian cancer risk. Larger studies would be needed to exclude the possibility that the small fraction of individuals who are 6A homozygotes have an increased risk of ovarian or other cancers.

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Keywords: *TGF β RI*; Ovarian cancer; 6A allele; Case-control study

Introduction

The transforming growth factor β (TGF β) family of peptide growth factors have pleomorphic effects on cellular signaling, growth, differentiation and apoptosis and are potent negative regulators of cell growth [1–3]. Decreased TGF β activity due to dysregulation of elements of its associated signal transduction pathways facilitates unrestrained proliferation and a propensity for malignant transformation. Conversely, the TGF β pathway is upregulated by some agents such as anti-estrogens and retinoids that decrease cancer risk [4]. Thus, inherited or acquired alterations in

members of the TGF β pathway could affect cancer susceptibility and the process of malignant transformation.

TGF β signaling is initiated by three cell surface receptors, the type I and II serine/threonine kinase receptors [1,5] and the type III betaglycan receptor [6,7]. A repetitive microsatellite sequence in the coding region of the type II receptor is the target for inactivating mutations in some cancers with defective DNA mismatch repair [5]. The type I TGF β receptor (*TGF β RI*) is not a target of microsatellite instability [5]; however, it has been suggested that a polyalanine repeat polymorphism in *TGF β RI* increases susceptibility to colorectal and other cancers [8–10]. The most common allele of this polymorphism encodes 9 alanine (9A) amino acid residues. The next most common allele is nine base pairs shorter producing a *TGF β RI* with 6 alanines (6A). Other

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polymorphic alleles with 5, 7, 8, 10, 11 and 12 alanine encoding GCC repeats have been described as well [8,11]. Most prior studies of the *TGF β R1* polyalanine repeat polymorphism have not focused specifically on ovarian cancer, but a meta-analysis of published case-control studies has suggested a protective effect [10]. We sought to confirm this finding in a large population-based case-control study of ovarian cancer performed in North Carolina.

Materials and methods

Case-control study design

Epithelial ovarian cancer cases and controls were enrolled in a continuing population-based case-control study approved by the Duke University IRB. The 588 cases were identified from the North Carolina Central Cancer Registry and included women aged 20–74 with newly diagnosed epithelial ovarian cancer residing in a 48 county region of North Carolina. The ovarian cancer diagnosis was confirmed by the study pathologist. The 614 controls were identified by either random digit dialing or Health Care Financing Administration phone lists. Controls were matched for 5 year age intervals and race (black or non-black) from the same 48 county area of North Carolina. All controls were required to have at least one intact ovary. Both cases and controls participated in an extensive in-home interview conducted by study nurses. Epidemiologic data related to known and suspected ovarian cancer risk factors were collected. Laboratory investigators were blinded to the identity of cases and controls.

DNA extraction

A 30 ml peripheral blood sample was drawn from each woman at the time of the nurse interview. Genomic DNA was extracted from leukocytes using a Puregene DNA Isolation kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's protocol. An aliquot of the stock DNA was diluted to a PCR concentration of 30 ng/ μ l and the remainder of the stock frozen at -70°C . PCR DNA dilutions were arranged in 96 well microtiter plates.

PCR

Genomic DNA was amplified using the Advantage Genomic PCR kit (BD Biosciences) and previously published primer sequences FOR: 5' CCACAGGCGGTG-GCGGCGGGACCATG3' and REV: 5' [12]. 60 ng of DNA was amplified with 1 $\times$ PCR buffer, 4 $\times$ dNTPs, 1 μ M GC-Melt, 0.5 $\times$ Advantage Taq polymerase mix and 10 μ M each forward and reverse primer. The forward primer was 5' labeled with FAM (Sigma) and HPLC purified. Amplification conditions were as follows: 95 $^{\circ}$, 4 min; 94 $^{\circ}$, 30 s, 70 $^{\circ}$, 8 min times 30 cycles.

Genotyping

Fluorescent fragment analysis was performed by the Duke University DNA Analysis core facility. Fluorescently labeled PCR products were diluted 250-fold in sterile water and then run on an Applied Biosystems 3100 Genetic Analyzer with a 50 cm capillary array, POP6 polymer and ROX400HD size standards. Allele peak sizes were assigned to each sample after comparison with sequence verified *TGF β R1* 6A and 9A standards. All samples with 6A alleles

Table 1

Demographic and clinical features of ovarian cancer cases and controls in the North Carolina Ovarian Cancer Study

	Cases (N = 588)		Controls (N = 614)		
Age in years					
Mean (SD)	54.1	(11.5)	54.8	(12.3)	
Median (range)	54	(20–74)	54	(20–75)	
	<i>n</i>	(%)	<i>n</i>	(%)	<i>P</i> value
Race					
Caucasian	495	(84)	520	(85)	
African-American	77	(13)	83	(14)	
Other	16	(3)	11	(2)	
Menopause status					
Pre/Peri	226	(39)	248	(40)	0.55
Post	361	(61)	366	(60)	
Tubal ligation					
No	443	(75)	403	(66)	<0.001
Yes	144	(25)	211	(34)	
Oral contraceptive use (months)					
None	208	(35)	196	(32)	0.09
≤ 12	101	(17)	100	(16)	
>12	265	(45)	309	(50)	
User of unknown duration	13	(2)	9	(1)	
Livebirths					
0	123	(21)	81	(13)	<0.001
1	105	(18)	94	(15)	
>1	359	(61)	439	(71)	
Family history of ovarian cancer					
No	562	(96)	596	(97)	0.04
Yes	25	(4)	17	(3)	
Tumor behavior					
Borderline	133	(23)			
Invasive	454	(77)			
Histologic subtype					
Serous	353	(60)			
Endometrioid	71	(12)			
Mucinous	70	(12)			
Clear Cell	37	(6)			
Other	57	(10)			
Stage					
I	208	(35)			
II	42	(7)			
III	310	(53)			
IV	19	(3)			
Unknown	9	(2)			

Odds ratios are age and race adjusted.

1 missing tumor behavior and 5 missing stage.

were verified in a second independent PCR amplification. Further confirmation of allele size was provided by directly digesting the PCR products with BssSI (New England Biolabs). Digested PCR products were resolved on 6% nondenaturing polyacrylamide gels and visualized with ethidium bromide staining. The digest yields a common 66 bp fragment and 44 bp (6A), 47 bp (7A), 50 bp (8A), 53 bp (9A) and 56 bp (10A) variable fragments. Two ovarian cancer cell lines in which the polymorphism had been sequenced were used as positive controls for the 9A/9A (OVCA 433) and 6A/6A (OVCA432) genotypes.

Statistical analysis

The genotype data were tested for Hardy Weinberg Equilibrium using the Chi-square goodness of fit test. Multivariate unconditional logistic regression models, adjusted for age, were used to estimate odds ratios (ORs)

and 95% confidence intervals (CIs) for the association between the *TGF β RI* polymorphism and ovarian cancer for all cases as well as for various disease subsets. All calculations were performed using SAS 8.0 (SAS Institute Inc., Cary, NC). The sample size of the combined study provided 80% power to detect an OR of 1.5 or greater with a two-sided type 1 error level of 0.05.

Results

The demographic features, epidemiologic risk factors and pathological characteristics of cases and controls in the North Carolina ovarian cancer study are shown in Table 1. Eighty-four percent of cases and 85% of controls were Caucasian, while 13% of the cases and 14% of controls were African-American. The median age was also similar in each group. The known relationships between various

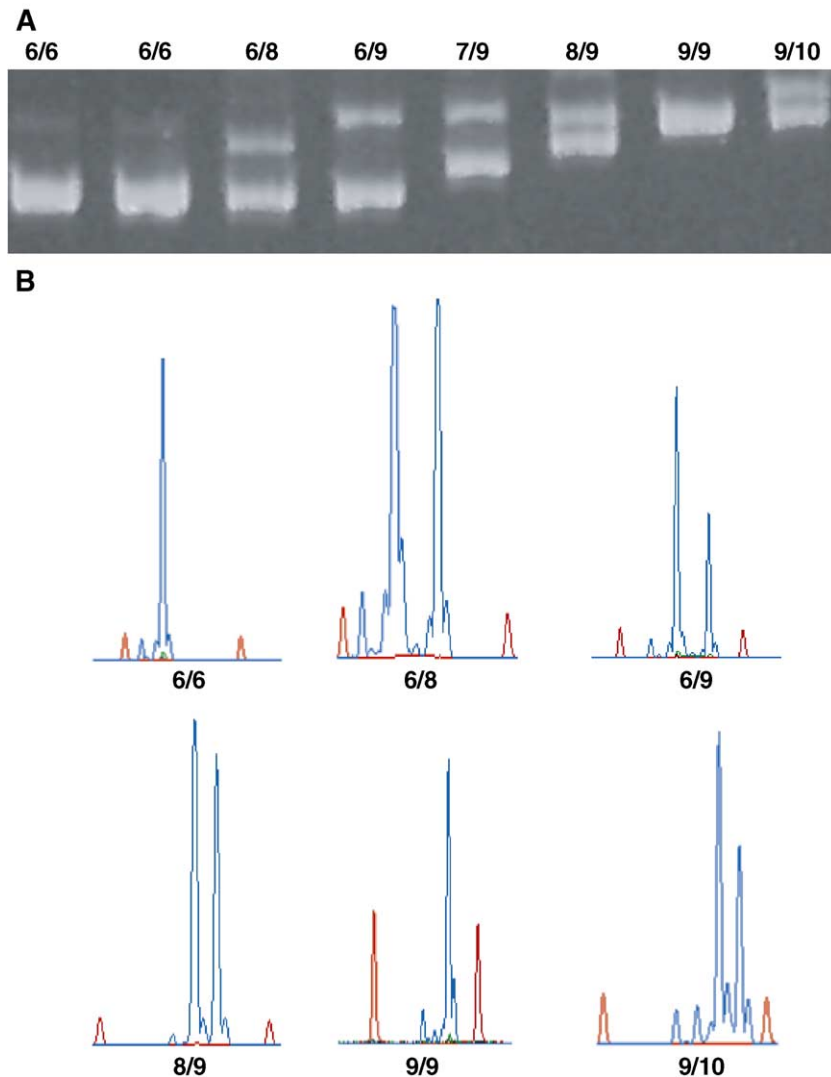


Fig. 1. *TGF β RI* polyaniline repeat polymorphism. (A) Restriction fragment analysis: Lane 1 demonstrates a 6A homozygote positive control (ovarian cancer cell line OVCA 432). Lanes 2–8 represent various alleles seen in North Carolina Ovarian Cancer study subjects. (B) Fluorescent fragment analysis: Homozygous samples (6A/6A and 9A/9A) have single peaks and heterozygous samples two peaks. Small peaks bracketing the sample peaks represent internal size standards.

Table 3
Relationship between *TGFβRI* polymorphism and risk of histologic subtypes of ovarian cancer

Genotype	Controls		Serous		OR ^a	95% CI	Endometrioid/clear cell		OR ^a	95% CI	Mucinous		OR ^a	95% CI
	n	(%)	n	(%)			n	(%)			n	(%)		
9A/9A	497	(81%)	277	(78%)	1.00	reference	88	(81%)	1.00	reference	54	(77%)	1.00	reference
6A/6A	6	(1%)	6	(2%)	1.92	(0.61–6.08)	1	(1%)	Too few to calculate		1	(1%)	Too few to calculate	
6A/9A	104	(17%)	61	(17%)	1.06	(0.75–1.51)	18	(17%)	0.98	(0.56–1.71)	15	(21%)	1.32	(0.70–2.49)
6A/6A or 6A/9A	110	(18%)	67	(19%)	1.11	(0.79–1.56)	19	(18%)	0.97	(0.56–1.68)	16	(23%)	1.32	(0.71–2.45)
Other	7	(1%)	9	(3%)	2.45	(0.86–6.93)	1	(1%)	Too few to calculate		0	(0%)	Too few to calculate	

^a Age and race adjusted.

with 6A/9A heterozygotes. There was no suggestion of an association with endometrioid or clear cell cancers. Although there was a small increased risk of the mucinous subtype (OR = 1.32, 95% CI 0.71–2.45), the confidence intervals were wide and include one. Finally, there was no relationship between the polyalanine polymorphism and stage (I/II vs. III/IV) or tumor behavior (borderline vs. invasive) or stage (Table 4).

Discussion

The polyalanine repeat polymorphism in the type I *TGFβ* receptor was identified in 1998 in the context of mapping the gene to chromosome 9q22 [12]. A variant allele was described with an inframe deletion of 3 alanine residues from a nine residue stretch. Although cells with the 6A variant were shown to retain sensitivity to the growth inhibitory effects of *TGFβ*, an increased frequency of the 6A allele was noted in a group of patients with cancer compared to controls. In the ensuing years, several case-control studies have been performed to determine whether the *TGFβRI* 6A allele predisposes to various types of cancers. A recent meta-analysis that pooled data from various studies concluded that the 6A allele increases risk of breast, ovarian and colorectal cancer [10]. However, most of the reported studies suggesting an association with cancer risk have not been comprised of carefully matched cases and controls. Population admixture and other potential confounders in such studies may lead to false-positive associations. In this regard, Lai pointed out that a meta-analysis of the various studies is problematic because of differences in patient characteristics, the way in which cases and controls were sampled and the types of cancers studied [13]. Formal analysis demonstrated a lack of homogeneity in the studies, which would preclude pooling the data.

Prior studies of the *TGFβRI* polymorphism in ovarian cancer have been inconsistent. The 6A allele was first associated with ovarian cancer susceptibility in the context of a study that predominantly focused on colon cancer risk [8,9]. This study included only 48 ovarian cancer cases and 8 (17%) carried the 6A allele, compared to 12% of controls. Baxter et al. examined the relationship between the polyalanine polymorphism and ovarian cancer risk in 304 cases

and 248 controls from the United Kingdom [9]. All subjects were Caucasian and 16.5% of controls were found to carry one or two copies of the 6A allele. There was no overall association of the 6A allele with ovarian cancer susceptibility. However, subgroup analysis revealed an increased risk of endometrioid and clear cell ovarian cancers (OR = 2.1, 95% CI 1.2–3.6). The control group in this study was comprised of staff volunteers and patients attending an obstetric clinic; and controls had a mean age of 39 compared to 62 in cases. The considerably younger age among controls in this study compared to cases is not ideal and could contribute to spurious results as endometrioid/clear cell cancer ovarian cancers and endometriosis both increase with aging.

The most recent meta-analysis of the *TGFβRI* polyalanine polymorphism by Pasche included 409 ovarian cancers [14]. There were 304 cases from the United Kingdom study and an additional 105 hospital-based ovarian cancers from the United States. Those either heterozygous or homozygous for the 6A allele had an increased risk of ovarian cancer (OR = 1.4, 95% CI 1.02–1.95) when compared to the large group of controls that were pooled in the meta-analysis. Based on less than 10 ovarian cancer cases who were 6A homozygotes, it was also concluded that the risk of ovarian cancer was more pronounced in this group (OR = 2.69, 95% CI 1.08–6.71).

The North Carolina Ovarian Cancer study is a population-based case-control study that is being conducted in the eastern and central areas of the state. Ovarian cancer cases are age and race matched to controls. In addition, controls must be at risk for ovarian cancer by virtue of having at least one ovary. Additional strengths include rapid case ascertainment, central pathology review and the availability of data regarding risk factors known to affect ovarian cancer susceptibility. The study population has also been previously analyzed for other polymorphisms including those in *BRCA1* and 2 and the progesterone receptor [15,16]. A polymorphism in the promoter of the progesterone receptor was found to be associated with increased risk of endometrioid/clear cell ovarian cancers, and this finding was confirmed in a second case-control study conducted by collaborators in Australia [17].

Because a significant fraction of subjects in the North Carolina ovarian cancer study are African-American, we were able to examine racial differences in allele frequencies

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North Carolina Ovarian Cancer Study

PURPOSE OF STUDY:

To identify the environmental, reproductive, and genetic factors that contribute to the development of ovarian cancer.

STUDY PERIOD:
1999-2008

STUDY ELIGIBILITY CRITERIA:

Diagnosis of primary epithelial ovarian cancer (including borderline) or primary peritoneal cancer;

Woman must be between the ages of 20 and 74 at diagnosis;

Woman must reside within 48-county study region (see map on p.3).

Also In This Issue...

- * Study Progress Report
- * Borderline Ovarian Cancer & Peritoneal Cancer Reporting
- * Study Participation--How it Works
- * And More!

Ovarian Cancer Screening in High-risk Women

The ROCA Ovarian cancer screening study is a national research study that aims to determine if use the CA125 blood test and trans-vaginal ultrasound will detect ovarian cancer earlier than standard care in women who may be at higher risk for the disease. For those women without ovaries, the study aims to determine if the blood test for CA125 can improve early detection of peritoneal cancer.

This study is designed for healthy women who have not developed ovarian cancer. Women with ovaries are eligible provided they are aged 30 or older and have not had ovarian cancer. Women over 30 without ovaries were eligible for the first three years of the pilot study only. Additionally, eligible participants must:

- have tested positive for the BRCA1 or BRCA2 genetic marker or have a close relative who has tested positive, **OR**
- have a family history of two or more blood relatives with breast or ovarian cancers, **OR**
- be of Ashkenazi Jewish ethnicity with a family history of one or more blood relatives with breast or ovarian cancers

If a woman is eligible and interested, she will:

- complete a questionnaire and have her blood drawn every three months for up to four years
- be referred for a trans-vaginal ultrasound and possibly a consultation with a gynecologic oncologist if her CA125 blood levels indicate a significant risk (for those women with ovaries)
- be referred for a consultation with a gynecologic oncologist if her CA125 blood levels indicate a significant risk (for those women without ovaries)

This national study is funded by the National Cancer Institute and is part of a larger project called the Cancer Genetics Network. The study is being conducted at eight regional centers across the United States and Duke University (as part of the Carolina Georgia Center) is one of those centers. Women may come to Duke or they may choose to use their own clinic or doctor's office. **Currently, the study is conducting only follow-up research on those women who have ovaries and who are already enrolled. However, study investigators are applying for NIH funding that would allow for another five years of accrual. If this application is funded, enrollment into the study could reopen as early as June, 2006. For more information, please contact the Study Coordinator, Stacy Murray, at 919-681-4556 or toll free at 1-866-292-7546.**

Focus on Rare Cancers:

Dr. Schildkraut attended a National Cancer Institute (NCI) sponsored symposium held in Boston, Massachusetts in September that was entitled 'Understudied Rare Cancers'. Ovarian cancer, as well as other cancer sites such as brain and ocular cancers, endometrial cancer, and esophageal cancer, is considered relatively rare and understudied. The NCI is very interested in having investigators around the country who are conducting epidemiologic research on rare cancers collaborate and form consortia. This is because it is difficult for any single research group to have a large enough study to address all the important research questions. Drs. Schildkraut and Berchuck at Duke have already become involved in creating such an ovarian cancer consortium that includes both national and international investigators.



Andrew Berchuck

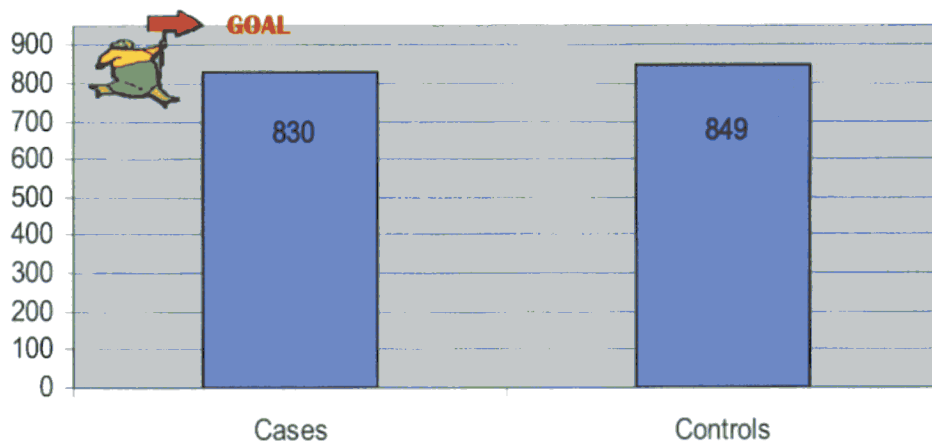


Joellen Schildkraut

NCOCS Enrollment (October 2005)

Women with ovarian cancer continue to participate in the North Carolina Ovarian Cancer Study at impressively high rates. Eighty-five percent of the women who we have contacted have agreed to participate. This fantastic response rate is due largely to the dedication of all the Gynecologic Oncologists and Cancer Registrars in our study area who have been participating in the rapid-case ascertainment of newly diagnosed ovarian cancer patients. The study team appreciates the efforts of both the Cancer Registrars and the rapid-case ascertainment team at the Central Cancer Registry!

Study Case/Control Accrual



Since April 1999, 47 hospitals across our 48-county study region have sent the North Carolina Central Cancer Registry contact information for women recently diagnosed with ovarian cancer. In September, 2005 we completed our 830th case interview which in addition to our 849 controls makes a total of over 1,600 completed interviews. While the response rate among these women is very encouraging, we still have a lot of work to do to achieve our overall goal of completing interviews with 950 affected women by the study's end. We will need the continued help of all participating hospitals in our study region if we are to reach this goal. If you have any questions or ideas, or if you are in our 48-county region and would like your hospital to participate in our study, contact Christine Lankevich at 1-888-246-1250.

Borderline Ovarian Cancer and Peritoneal Cancer Reporting

As you may know, there has been some question concerning whether or not borderline ovarian tumors and primary peritoneal cancer cases are eligible for the North Carolina Ovarian Cancer Study. In fact, borderline ovarian cancer is often not considered to be malignant and in some years has not been a reportable diagnosis to the North Carolina Central Cancer Registry (CCR.) **However, for rapid-case ascertainment purposes, the CCR has asked hospitals to include borderline ovarian cancer cases in their reporting.** The North Carolina Ovarian Cancer Study has always included both borderline ovarian cancer and primary peritoneal cancer cases as eligible diagnoses, and we appreciate the extra effort cancer registrars have taken to send in these cases. Thank you for your continued cooperation!

Visit One of the Following Websites to Learn More
About Ovarian Cancer :



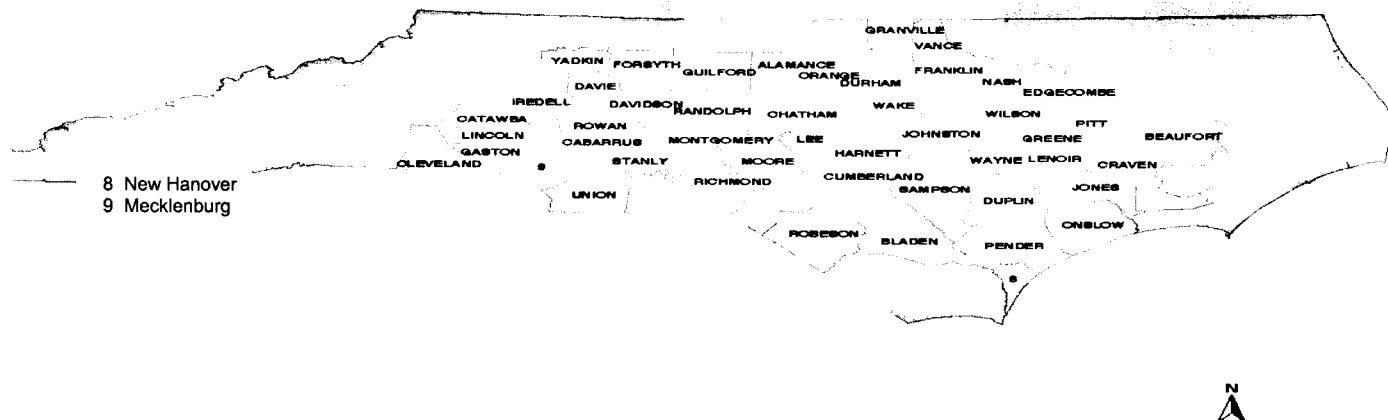
Gynecological Cancer Foundation: www.wcn.org/gcf
National Ovarian Cancer Coalition: www.ovarian.org
Women's Cancer Network: www.wcn.org
OncoLink: Ovarian Cancer: www.oncolink.upenn.edu
Ovarian Cancer National Alliance: www.ovariancancer.org
National Ovarian Cancer Coalition Triangle Chapter (TriNOCC): www.trinocc.org

List of Publications Generated from the North Carolina Ovarian Cancer Study

1. Moorman PG, Schildkraut JM, Calingaert B, Vine M, Halabi S, Berchuck A. Ovulation and ovarian cancer: A comparison of two methods for calculating lifetime ovulatory cycles. *Cancer Causes and Control* 13: 807-811; 2002.
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12. Hoyo C, Berchuck A, Halabi S, Bentley RC, Moorman P, Calingaert B, Schildkraut JM. Anthropometric Measurements and Epithelial Ovarian Cancer Risk in African American and White Women. *Cancer Causes and Control* (in press)
13. Schildkraut JM, Moorman PG, Halabi S, Calingaert B, Marks JR, Berchuck A. Analgesic drug use is associated with a decreased risk of ovarian cancer. *Epidemiology* (in press)

48-County Study Area

If you are in the study area and would like your hospital to participate call Christine Lankevich,



The NCOCS staff would like to thank all the hospital cancer registrars who have helped with study enrollment by using the Central Cancer Registry's Rapid Case Ascertainment System!

Alamance Regional Medical Center	Davis Regional Medical Center	Lexington Memorial Hospital	Presbyterian Healthcare
Beaufort County Hospital	Duke University Medical Center	Lincoln Medical Center	Raleigh Community Hospital
Betsy Johnson Memorial Hospital	Duplin General Hospital	Maria Parham Hospital	Rex Health Care
Cape Fear Valley Medical Center	Durham Regional Hospital	Medical Park Hospital	Rowan Memorial Hospital
Carolinas Medical Center	ECU-Pitt County Memorial Hospital	Moore Regional Hospital	Sampson Regional Medical Center
Carteret General Hospital	Forsyth Memorial Hospital	Moses H Cone Memorial Hospital	Stanly Memorial Hospital
Catawba Memorial Hospital	Gaston Memorial Hospital	Nash General Hospital	University of North Carolina Hosp.
Central Carolinas Medical Center	Heritage Hospital	New Hanover Regional Med. Ctr.	Union Regional Medical Center
Chatham Hospital	High Point Regional Hospital	Northeast Medical Center	Wake Forest U. Baptist Med. Ctr.
Cleveland Regional Medical Center	Iredell Memorial Hospital	Onslow Memorial Hospital	Wake Medical Center
Community General Hospital	Johnston Memorial Hospital	Pinehurst Surgical Clinic-Women's	Wayne Memorial Hospital
Craven Regional Medical Center	Lenoir Memorial Hospital	Care Center	Wilson Memorial Hospital

STUDY CONTACT INFORMATION

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Ovarian Awareness Walk

In recognition of September as National Ovarian Cancer Awareness Month, Duke University Medical Center helped coordinate several important events. Throughout the course of the month, three free educational forums lead by Duke physicians and researchers were offered to participants of the North Carolina Ovarian Cancer Study and their families. The last of these forums, "It Whispers...So Listen" took place at the **3rd annual Gail Parkins Memorial Ovarian Awareness Walk** on September 24, 2005 in Raleigh, NC. The Ovarian Awareness Walk, which was started by Melanie Bachelor in 2003 after her mother, Gail Parkins, lost her battle with ovarian cancer, honors the many women who are battling the disease. The event is an opportunity for ovarian cancer survivors, their friends, and families to walk together to raise money and spread awareness about ovarian cancer. Over 750 people registered for the event this year! At the completion of the 2 mile memorial walk there was a ceremony to honor all ovarian cancer survivors in attendance.



Study Participation—How It Works

The hospital Cancer Registrar sends monthly information on newly diagnosed ovarian cancer cases to the North Carolina Central Cancer Registry. (If needed, a representative from the Central Cancer Registry can assist with this task).

The Central Cancer Registry forwards potentially eligible cases to the study project manager for determination of study eligibility.

A consent form is sent to the attending physician

requesting permission to contact their patient. When physician consent is received, a letter and brochure describing the study are sent to the patient.

Shortly thereafter, a nurse-interviewer telephones the patient to discuss the study, determine eligibility, and, if eligible, invite her to participate.

Hospitals are paid \$10 for every eligible case reported to the NC Central Cancer Registry.