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14. ABSTRACT

To achieve a better understanding of the etiology of ovarian cancer, 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 950 cases and 950 controls have been accrued thus far. Blood and cancer samples have been collected and molecular analyses of polymorphisms in single genes, including most recently the androgen receptor, have been performed. We also have performed an Illumina array experiment with 1,536 haplotype tagging single nucleotide polymorphisms in about 150 candidate genes. This data presently is being analyzed. We also have played a leadership role in establishing an international consortium in which groups are working together to validate initial positive associations. An initial ovarian cancer chemoprevention trial with levoneorestrel 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 in chickens that incorporates both progestins and vitamin D analogues is near completion. 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.

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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 1,800 subjects have been accrued thus far in this case-control study. We have examined several polymorphisms and found that a polymorphism in the promoter of the progresterone receptor is associated with a decreased risk of endometrioid and clear cell ovarian cancers. We forged a collaboration relationship with Dr Georgia Chenevix-Trench in Australia, who also conducting a DOD funded case-control study of ovarian cancer. This collaboration was vitally important in allowing us to confirm these positive results prior to publication. This successful paradigm subsequently led to the establishment of an international ovarian cancer association consortium that includes 14 case-control studies. The investigators have met every six months for the past two years and have collaborated on several validation studies of polymorphisms, including those in cell cycle genes and this work has also been accepted for publication. 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 eight 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 in 1998 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 provided funding to increase our accrual to 820 ovarian cancer cases and an equal number of controls. We have exceeded this accrual and over 950 women with ovarian cancer and 950 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. We continue to obtain blood specimens from 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.

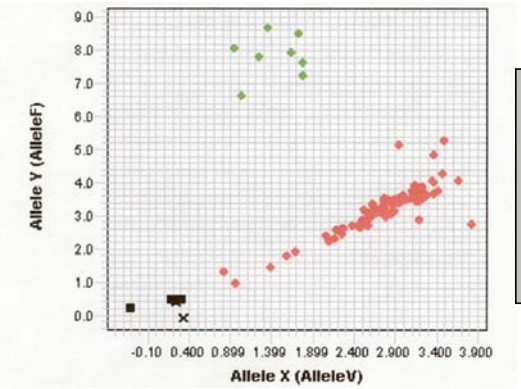
BRCA1/2: Since inherited BRCA1or BRCA2 mutations strikingly increase ovarian cancer risk, polymorphisms in these genes could represent low penetrance susceptibility alleles. Prior studies of the BRCA2 N372H polymorphism suggested that HH homozygotes have a modestly increased risk of both breast and ovarian 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 (χ^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,

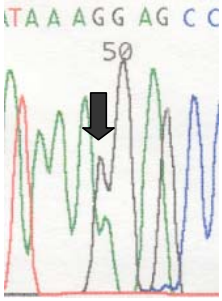
these subjects were excluded from further analyses. The +331AA homozygotes were combined with heterozygotes in calculating odds ratios. The +331A allele was associated with a modest reduction in risk of ovarian cancer. Analysis by histologic type revealed that there was a slight trend towards protection 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							
	GG	AG	AA	AG/AA		OR	(95% CI)	
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%)	**		
Endometriod/ 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).

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.

In the past year, an international ovarian cancer association consortium has been established to validate initial positive findings from individual studies. The first gene to be examined was the progesterone receptor and the relationship between the PR promoter polymorphism and decreased risk of endometrioid and clear cell cancers was again confirmed. Dr Berchuck gave an oral presentation of this work at the 2006 meeting of the International Gynecologic Cancer Society in Santa Monica.

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 (TβR1(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 $(OH)_2D_3$, 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 $(OH)_2D_3$ 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).

Androgen receptor

Androgens may play a role in the development of some ovarian cancers. Two trinucleotide repeat polymorphisms have been described in exon 1 of the androgen receptor *(AR)* gene that may affect its function. A highly polymorphic CAG repeat encodes a polyglutamine tract with alleles that vary from 5 – 34 repeats. A less polymorphic GGC repeat encodes a polyglycine tract and allele lengths vary from 6 - 20 repeats. Previous studies of ovarian cancer and *AR* repeat polymorphisms have been inconsistent. We analyzed CAG and GGC repeat length polymorphisms in the *AR* gene using data from a population-based case-control study of ovarian cancer that included 594 cases and 681 controls (see submitted manuscript in appendix). Repeat lengths for each individual were determined by fluorescent DNA fragment analysis using ABI GeneScan software. Change point models were used to determine appropriate repeat length cut points by race (African American vs. Caucasian). No relationship was observed between CAG repeat length and ovarian cancer among Caucasians. Among African Americans, a short CAG allele < 16 repeats was associated with a > 2-fold increase in ovarian cancer risk (age-adjusted OR = 2.8; 95% CI = 1.4 -5.9). No relationship with GGC polymorphism was observed among either race. These results suggest that the short CAG alleles (< 16 repeats) in *AR* increase ovarian cancer risk in African Americans. The failure to observe this relationship in Caucasians may be due to the rarity of such short CAG alleles in this population or could reflect racial differences in disease etiology.

Illumina array

In the last few years since our grant was funded, high throughput techniques for SNP genoyping 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 1,900 of our samples. We will include haplotype tagging SNPs for about 150 genes 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 allows us to generate vastly more genotype data in the next year than we have generated in the past years combined. At this point, the genotyping has been completed in the Duke Illumina genotyping facility and data is undergoing cleaning and analysis.

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 serves 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 member of the steering committee.

The aims of the consortiuim 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 October 2005 in concert with the American Society of Human Genetics annual meeting. All groups conducting ovarian cancer case-control studies of genetic polymorphisms were invited to join the consortium. Presently participants include, Duke, USC, Australia, Cambridge, London, Denmark, Poland/NCI, Harvard, Yale, Pittsburgh, Hawaii, Stanford, Mayo and Moffitt. In 2006 meetings were held in Durham at Duke University in April and in October in Dallas. The aims of the consortium are listed below.

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 not 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. (This work has been accomplished and was presented by Dr Berchuck as an oral presentation at the 2006 meeting of the International Gynecologic Cancer Society in Los Angeles (see appendix).)

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 <u>not</u> 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.

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. Of note, a compelling and growing body of published evidence over the past several years is demonstrating vitamin D deficiency in a significant proportion of the population in industrialized countries, and linking vitamin D deficiency to host of chronic diseases, including cancer. Thus, Project 2 which involves examining the role of vitamin D and the combination of vitamin D and progestin for ovarian cancer prevention is timely. Below follows a brief discussion of the vitamin D endocrine system, as well as data regarding vitamin D deficiency and epidemiologic evidence underlying the cancer protective effect of vitamin D. In addition, progress to date for Project 2 is summarized.

Vitamin D Endocrine System

The beneficial effects of vitamin D are due to the activity of its dihydroxylated metabolite, 1,25 $(OH)_2D_3$ ("calcitriol"), the active form of the molecule. A schematic representation[1] of 1,25 $(OH)_2D_3$ production and the variety of targets for beneficial vitamin D action are shown in Figure 1. As

1 Adapted from Holick M, Vitamin D: importance in the prevention of cancers, type 1 diabetes, heart disease, and osteoporosis. *Am J Clin Nutr* 2004;79:362–71.Source: American Cancer Society

summarized in Figure 1, the human body obtains vitamin D (specifically vitamin D_3 or "cholecalciferol") through synthesis by skin tissue exposed to sunlight or orally through the diet. Of these two sources, however, very little is supplied by the diet as few foods contain appreciable amounts of vitamin D. During exposure to sunlight, 7-dehydrocholesterol (7-DHC), which is present in abundance in the skin, is converted by ultraviolet B (UVB) radiation to previtamin D_3 ($preD_3$). Once formed, $preD_3$ undergoes thermally induced transformation to vitamin D_3. Both pre-vitamin D_3 and vitamin D_3 can be further converted by UVB radiation to a number of degradation products (*e.g.*, Upper right, Figure 1, Lumisterol and Suprasterol). This serves as a regulatory mechanism to limit the excess production of vitamin D_3 in skin. Although skin has the capacity to produce large amounts of vitamin D, daily production is capped at 10,000 IU or somewhat higher as production and degradation of vitamin D_3 and pre-vitamin D_3 reach equilibrium. There have been no reported cases of vitamin D intoxication related to excess sun exposure.

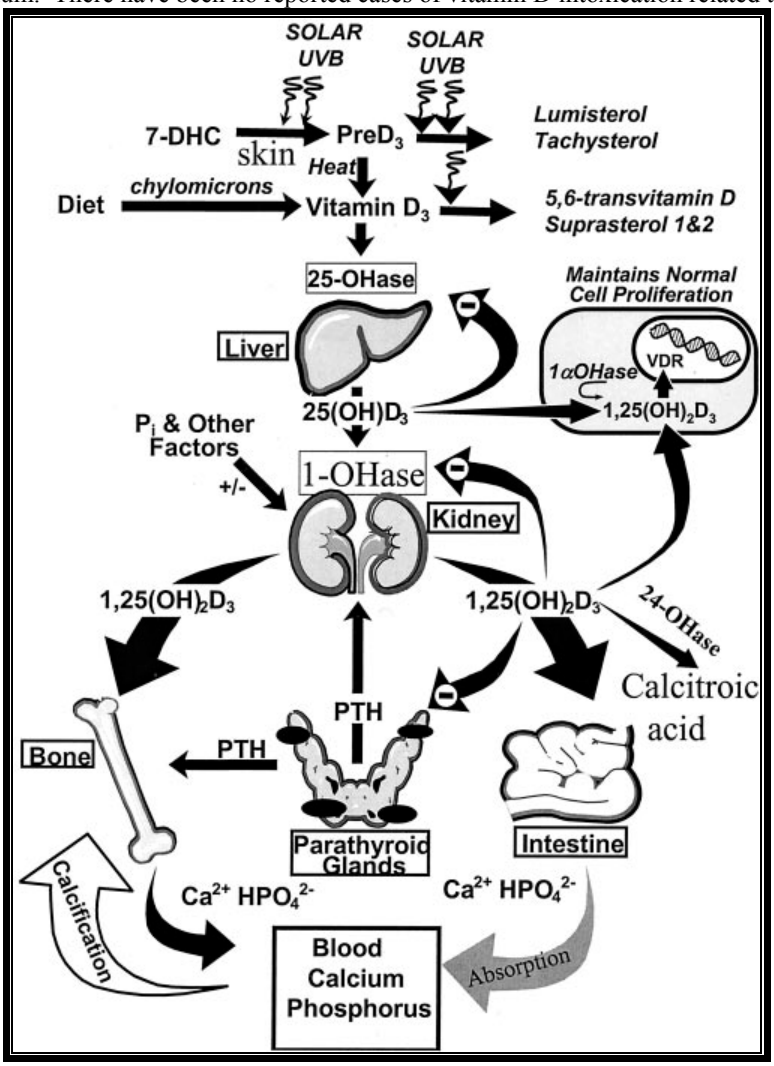


Figure 1. Synthesis and Targets of 1,25 (OH)D_3
(From Holick M, Am J Clin Nutr 2004;80(suppl):1678S– 88S)

Factors that limit the amount of UVB radiation to which skin is exposed will significantly curtail the amount of vitamin D_3 that is produced. This includes covering of the skin with sunscreens or clothes. In addition, the ozone layer absorbs UVB radiation, thereby decreasing the amount of UVB radiation available for the skin to produce previtamin D_3. Thus, during the winter months, when the angle of sunlight is tangential, sunlight passes through more ozone, severely limiting UVB radiation, and thus the cutaneous production of vitamin D_3. In northern latitudes such as mid North America or Europe, the skin is incapable of producing sufficient vitamin D_3 during the winter, even with adequate exposure to the sun. For example, sunlight exposure from November through February in Boston is insufficient to produce significant vitamin D synthesis in the skin.[2]

Vitamin D_3 from either dietary or skin sources enters the circulation and is metabolized in the liver by vitamin D_3-25-hydroxylase (25-OHase) to 25-hydroxyvitamin D_3 [$25(OH)D_3$]. $25(OH)D_3$ reenters the circulation and is converted in the kidney by 25-hydroxyvitamin D_3-1alpha-hydroxylase (1-OHase) to 1,25-dihydroxyvitaminD_3 [$1,25(OH)_2D_3$], the active hormone. A variety of factors, including serum phosphorus (Pi) and parathyroid hormone (PTH), calcium, and circulating levels of $1,25\ (OH)_2D_3$ regulate the renal 1-OHase production of $1,25(OH)_2D_3$.

Historically, it was assumed that the most important role of $1,25(OH)_2D_3$ is to regulate calcium metabolism and promote bone health through the interaction of the active vitamin with its major target tissues, the bone, kidney, intestine, and parathyroid gland. However, there is a growing body of compelling evidence that vitamin D is important not just for bone health, but also for overall health and well-being. Research over the past decade has demonstrated that the risk of a number of chronic diseases and cancer are increased in individuals who live in higher latitudes or have vitamin D deficiency. In addition, the vitamin D receptor has been shown to be expressed ubiquitously throughout most epithelia **including the ovarian surface epithelium** as well as in cells of the immune system. Via interaction with the vitamin D receptor (VDR) in these tissues, $1,25(OH)_2D_3$ is now known to confer potent biologic effects that include protection against diabetes, hypertension, autoimmune diseases and cancer. Moreover, it has been recently elucidated that these same tissues **including the ovarian surface epithelium** express the 1-alpha-hydroxylase enzyme, and thereby have the capacity to produce the active form of vitamin D_3 ($1,25(OH)_2\ D_3$) from $25(OH)D_3$. It is therefore possible that $1,25(OH)_2D_3$ produced locally in tissues via an autocrine effect has a profound local biologic effect, contributing to the non-skeletal benefits of vitamin D nutrition.[3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16]

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Vitamin D Deficiency

As noted above, the primary "natural source" source of vitamin D_3 in humans is skin tissue, which produces vitamin D_3 when exposed to sunlight.[17] The skin can produce up to 10,000 or somewhat more International Units ("IU") of vitamin D_3 daily from sunlight exposure, depending on the degree and directness of exposure to ultraviolet radiation from the sun. However, modern humans generally avoid sunlight by remaining mainly indoors and going outside only when fully clothed and/or protected by sun blocking products.[18] In addition, humans in Northern Industrialized Countries generally receive tangential UV radiation for much of the year, limiting endogenous production of vitamin D. As a result, it is now well accepted that vitamin D deficiency is widespread and chronic in most industrialized countries.[19, 20, 21, 22, 23]

Vitamin D deficiency is particularly notable in the elderly who are often confined indoors and who are especially susceptible to the diseases related to vitamin D deficiency such as osteoporosis and cancer.[24] The scope of vitamin D deficiency extends well beyond the elderly however, as widespread vitamin D deficiency is common in adolescents and young adults,[25] and is thought to predispose these individuals to a number of diseases later in life.[26]

The statistics in the U.S. suggest a vitamin D deficiency epidemic:[27]

- 32% of doctors and med school students are vitamin D deficient.
- 40% of the U.S. population is vitamin D deficient.
- 42% of African American women of childbearing age are deficient in vitamin D.
- 48% of young girls (9-11 years old) are vitamin D deficient.

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27 http://www.newstarget.com/003069.html

- Up to 60% of all hospital patients are vitamin D deficient.
- 81% of the children born to these mothers were deficient.
- Up to 80% of nursing home patients are vitamin D deficient.

Vitamin D and Cancer

It has been estimated by vitamin D experts that as many as 50,000-63,000 individuals in the United State and 19,000-25,000 in the UK die prematurely from cancer annually due to insufficient vitamin D, at a cost to society of 40-50 billion dollars.[28] Strong epidemiological evidence has linked vitamin D deficiency (either due to lower serum $25(OH)D_3$ levels or lower ultraviolet exposure) to an increased risk of a number of cancers including those especially relevant to women (such as breast, ovarian, and colon cancer), as well as to cancers of the prostate, other GI sites, and hematological system.[29, 30, 31, 32, 33]

There is a geographic distribution for most of these cancers that favors a higher risk in Northern than in Southern Latitudes in the U.S. For example, as shown in Figure 3, there is a geographic variation in incidence and mortality of ovarian cancer, with higher levels in the North. Patterns for breast, colon and prostate cancer all show a similar pattern essentially exhibiting higher rates in the Northern States.

Similarly, some of the highest cancer incidence rates in Europe occur in the extreme northern part of the continent where for several months each winter, the significant lack of ultraviolet light essentially confers a vitamin D "Holiday."

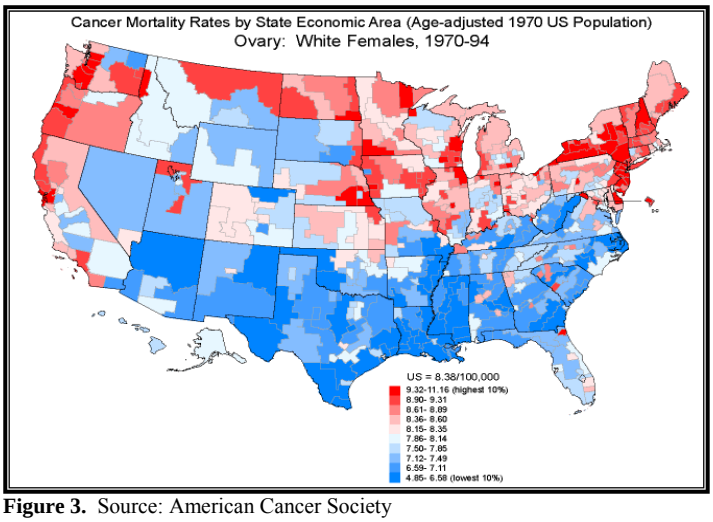

Figure 3. Source: American Cancer Society

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The mechanism underlying the cancer protective effect of vitamin D nutrition may involve the activation in tissues by $1,25(OH)_2D_3$ of a number of biologic effects related to cancer prevention, including programmed cell death, inhibition of proliferation and induction of differentiation. Induction of these cancer preventive biologic effects in healthy tissue causes genetically damaged cells to be efficiently eliminated rather than to persist to transform into cancers. [34, 35, 36, 37, 38] As described above, the source of $1,25\ (OH)_2\ D_3$ is likely to include production of the active hormone locally via conversion by 1-alpha hydroxylase of circulating $25(OH)D_3$.

With regard to ovarian cancer, several recent studies have provided strong human evidence to an ovarian cancer protective effect of vitamin D.[39 40 41 42] In addition, results from a prevention trial that we performed previously in the chicken ovarian cancer animal model suggested an additive ovarian cancer protective effect of vitamin D when added to progestin, and data that we present below suggests that vitamin D and progestins have synergistic effects in the ovarian epithelium. **Thus, we anticipate a great opportunity to develop a potent pharmacologic strategy for ovarian cancer prevention using these two agents.**

Progress to Date for Project Two

Progestins and Vitamin D Have Synergistic Effects on Ovarian Epithelial Cell Apoptosis:

In an earlier report, we presented evidence showing that both progestin and Vitamin D markedly inhibit cell viability in a dose response fashion. In experiments evaluating the combination, we show that combining the two confers a dramatically more potent biologic effect on cells derived from the human ovarian epithelium than either agent alone. As shown below, in both ovarian cancer cell lines as well as immortalized cell cultures derived from the normal human ovarian epithelium, there is 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.

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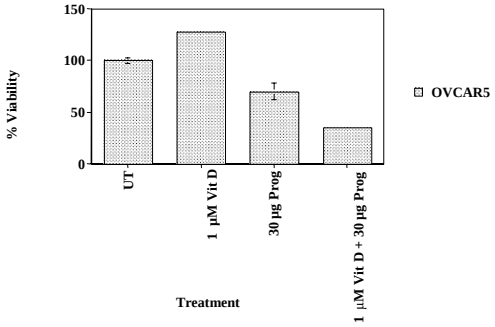
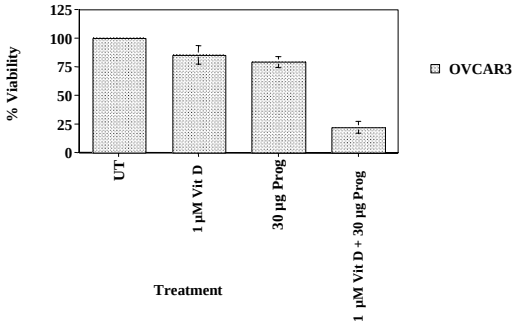
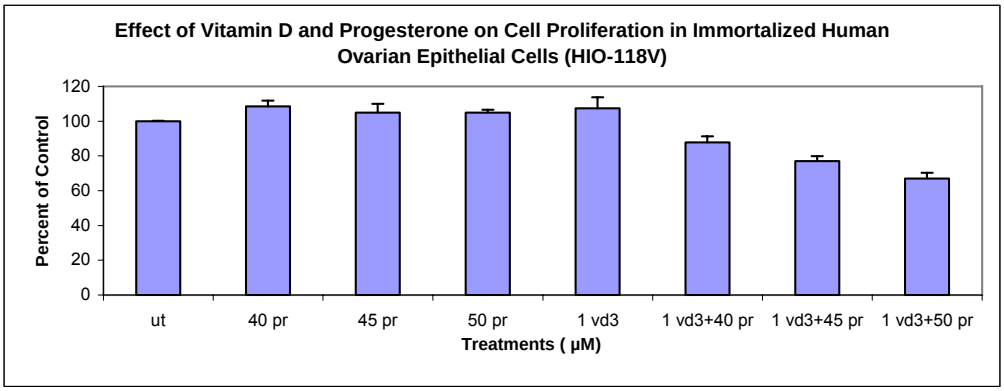
Effect of Vitamin D and Progesterone on Cell Proliferation (OVCAR5 Cells)



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.

Search for Molecular Mechanisms Underlying the Biologic Effects of Progestins and Vitamin D on the Ovarian epithelium Suggest an Impact of the Drugs on TGF-beta Signaling, Cell cycle, and Vitamin D 24- Hydroxylase

We have been 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 approaches for ovarian cancer prevention that can then be explored in clinical trials. Our strategy has involved several approaches, including

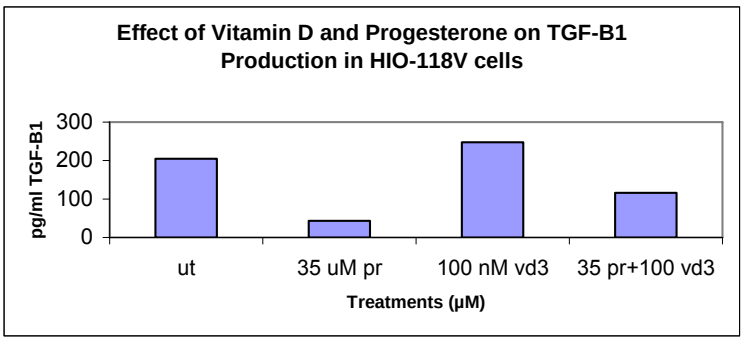
1) examining the effects of the progestin/Vitamin D combination on TGF-beta signaling events
2) examination of the effects of the two agents on apoptosis and the cell cycle

3) determining whether progestin might alter the actual pharmacology of Vitamin D by inhibiting its degradation.

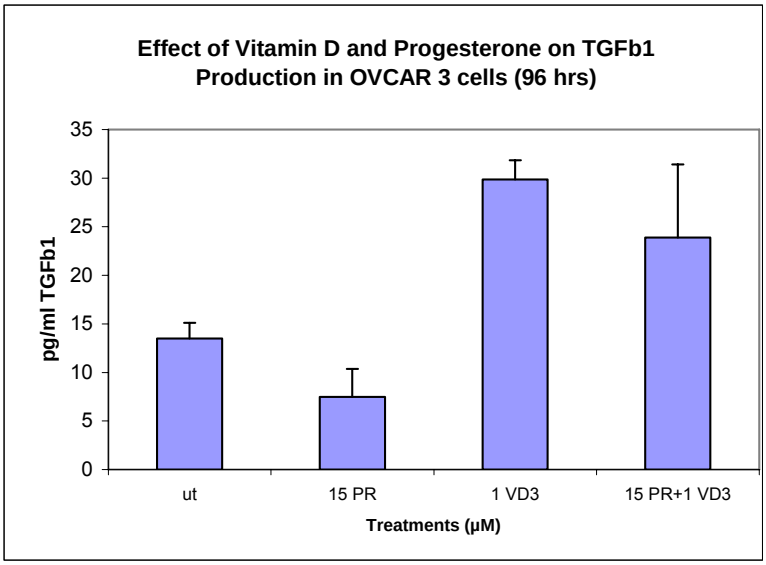
Activation of TGF-beta signaling events

Previously, we have shown in a primate model and in humans that the normal ovarian epithelium expresses the TGF-beta1. In a primate model, we demonstrated 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. Importantly, the isoform switch in TGF-beta expression is associated markedly with apoptosis. 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. Thus, the synergistic effects of the combination of progestin and Vitamin D are unlikely to be related solely to effects secondary to TGF-beta1. (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.

TGF-beta ELISA was performed as follows: 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.



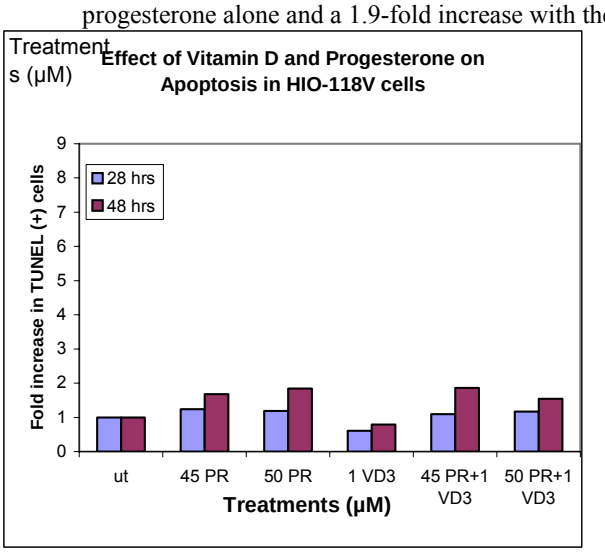
Similar trends are observed in the OVCAR-3 ovarian cancer cell line; however, Vitamin D up regulates TGF-beta production.

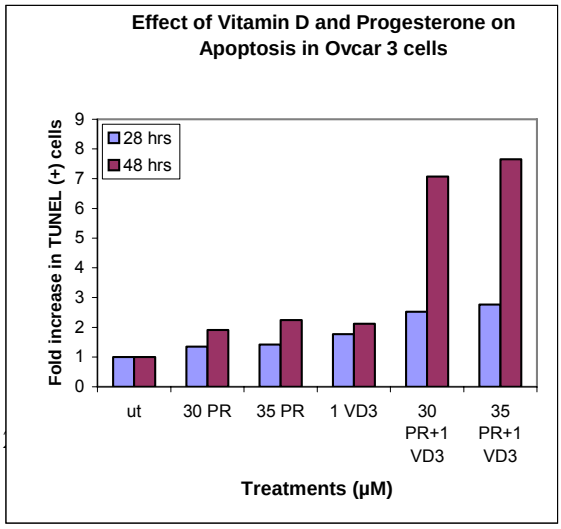


Apoptosis and Cell Cycle

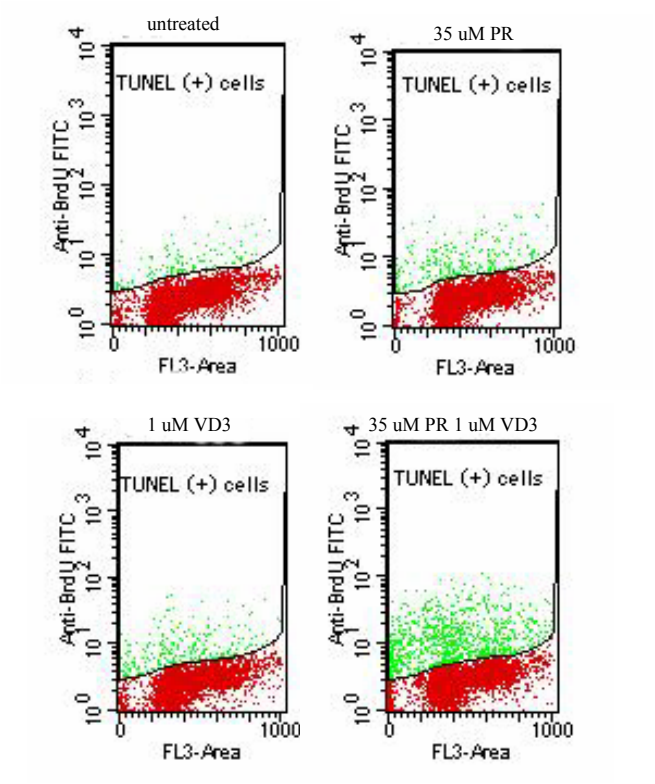
Cells were incubated for 28 and 48 hours in the hormonal treatments as indicated below and assessed for TUNEL reactivity and cell cycle. In these experiments, Apoptosis (TUNEL) data and cell cycle data shown are analyzed from the same experiment; in each cell line's respective medium, and in conditions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

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

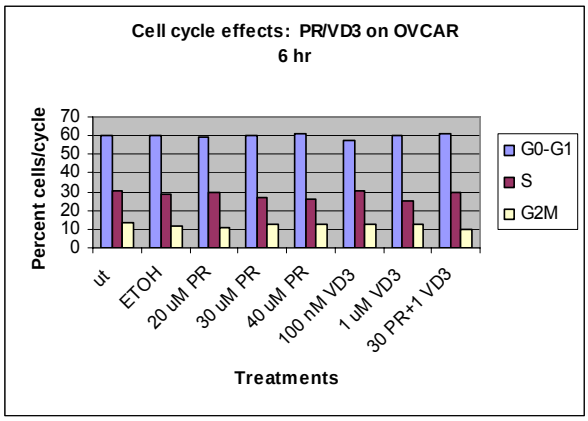


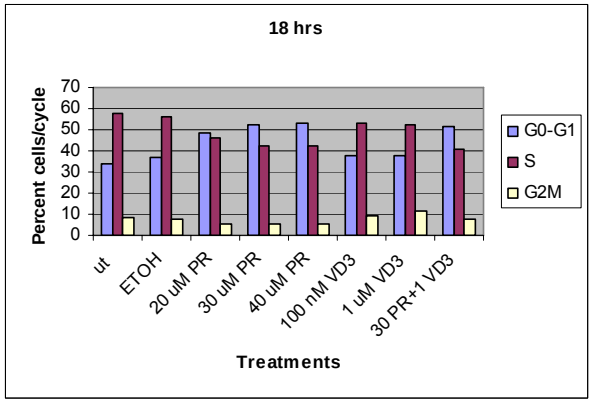


Effect of Vitamin D and Progesterone on Apoptosis via Tunnel in OVCAR 3



The following are detailed cell cycle experiments evaluating the impact of Vitamin D and progestin, alone or in combination. The data demonstrate an effect primarily from progestin, that is unaffected by the addition of Vitamin D. No cell cycle effect is seen at 6 hrs. At 18 hrs, there is a dose-dependent response to PR, indicating that cells appear to be blocked at the G1-S checkpoint. 1 uM VD3 causes no apparent effect on cell cycle but does not reverse progestin effects effects.



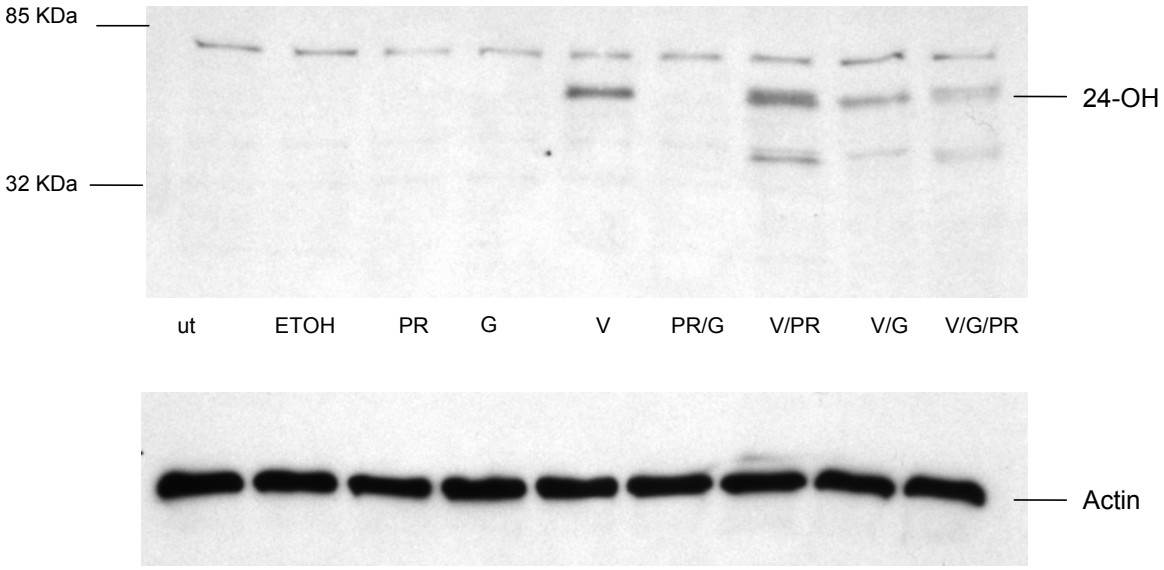


BCL-2/BAX

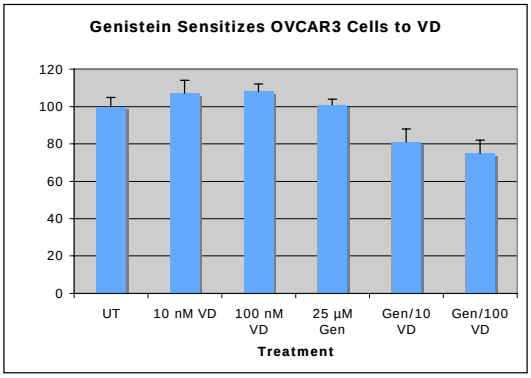
We are examining the intrinsic and extrinsic cell death pathways to elucidate the events underlying the synergistic effects of the progestin and Vitamin D combination. At dosages of progestin and Vitamin D shown above to have synergistic effects, we have observed a decrease in the anti-apoptotic protein BCL-2 via western blot, suggesting a pro-apoptotic effect via the intrinsic cell death pathway. We have experienced difficulty with our antibody to the pro-apoptotic protein BAX. Thus, BAX results are inconclusive at this time, but experiments are being repeated using different antibodies and or conditions.

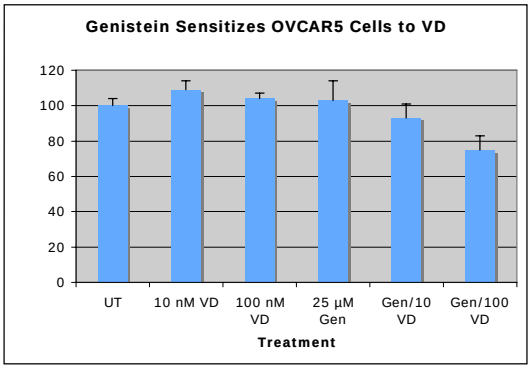
Effects of Progestin on Vitamin D 24 hydroxylase

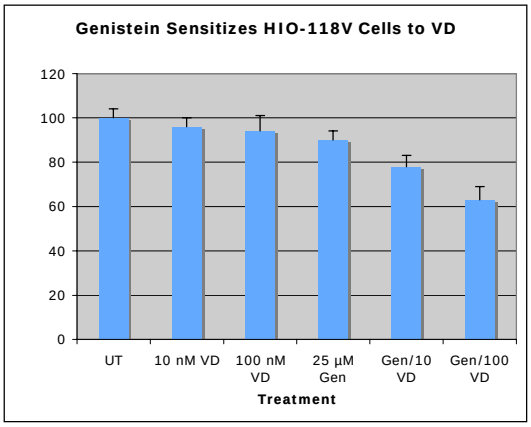
The Vitamin D metabolizing enzyme 24 hydroxylase (24-OH) converts the active form of Vitamin D (1,25 (OH)2 D) to an inactive form via 24 hydroxylation. Of note, many cancer cells over-express 24-OH, rendering them resistant to the effects of Vitamin D. Moreover, 24-OH is normally induced in cells in response to Vitamin D. This serves to inhibit unbridled Vitamin D effects and to turn off Vitamin D once it has achieved its biologic effect. Agents such as Genistein and ketoconazole are known to cause degradation of 24-OH. We examined the effect of progestin on 24-OH in cells derived from the ovarian epithelium. We demonstrate that progestin causes degradation of 24-OH. This has not been previously shown, but may explain in part the synergy associated with the progestin-Vitamin D combination. Namely, by inhibiting Vitamin D's degradation via inhibition of 24-OH, the active form of Vitamin D has a longer local biologic half life, and thus cellular effect. Below we show in OVCAR 3 cells that 24-OH is produced in response to Vitamin D by western blot. We have also shown that the addition of 25 μM progesterone (PR) and/or 25 μM Genistein (G) in combination with 100 nM Vitamin D (V) causes the degradation of 24-OH. In this experiment, cells were treated for 18 hours in OptiMem. Similar results were obtained when cells were grown in RPMI. Of note, resolution below is poor. The apparent band at the level of 24-OH in the V/PR lane is actually two bands.



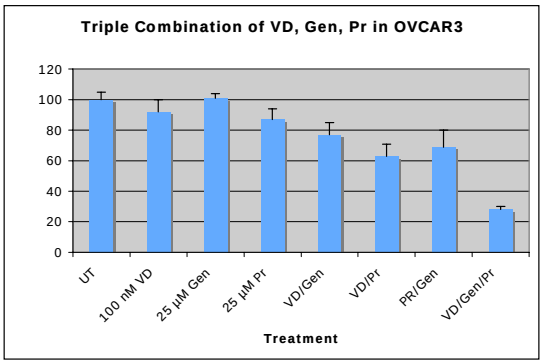
The treatment of ovarian cell lines with Vitamin D requires relatively large doses to see a negative impact on cell growth. It is likely due to high expression of 24-OH by these cells, and further induction of 24-OH in the presence of Vitamin D. We thus tested the hypothesis that inhibition of 24-OH would enhance Vitamin D's effect. As noted above, Genistein, a soy isoflavonoid, is a 24-OH inhibitor. When we treated cell lines for 24 hours with Genistein with Vitamin D and continued Vitamin D exposure for another 48 hours, we saw improved response of our cell lines to Vitamin D. The two bars on the right in the graphs below demonstrate the effect of Vitamin D in cells pretreated for 24 hours with Genistein.

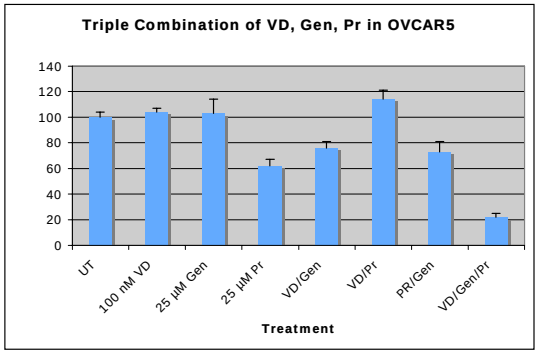


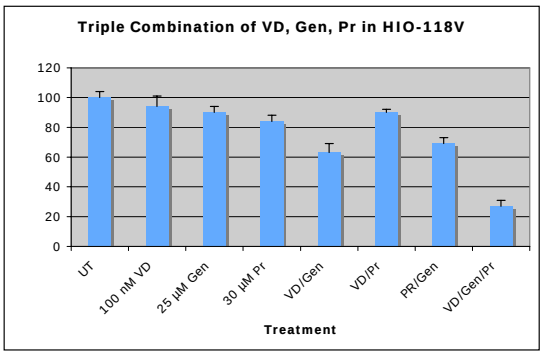




Next, we combined the 24 hour treatment with Genistein and the combination treatment of Vitamin D and progesterone. We see very pronounced killing of the ovarian cell lines with these lower doses of Vitamin D in combination with progesterone following pretreatment with Genistein. Of note, this is consistent with our western blot data, demonstrating marked reduction of 24-OH with the progestin and Genistein combination.







The viability of ovarian cells treated for 24 hours with Vitamin D, Genistein and progesterone, followed by an additional 48 hours of growth in the presence of Vitamin D and progesterone is 28 +/- 2% for OVCAR3 cells, 22 +/- 3 % for OVCAR5 cells and 27 +/- 4% for HIO-118V cells. In order to determine whether these conditions alter TGF-beta expression, we treated the ovarian cell lines with single agents and the combination of all 3 of them. Cells were treated for 48 hours in serum-free medium, supernatants were collected and acid activated, and the data were normalized by MTS cell proliferation assay. An ELISA assay for TGFbeta1 was performed. The data are expressed as percent untreated

control. The native supernatant (not acid activated) did not have measurable levels of TGFbeta1 in any sample.

TGFbeta1 Levels as Measured by ELISA:

OVCAR3	UT	100%
	100 nM VD	238%
	25 µM Genistein	116%
	20 µM Progesterone	200%
	VD/Gen/Pr	0%
OVCAR5	UT	100%
	100 nM VD	89%
	25 µM Genistein	72%
	10 µM Progesterone	108%
	VD/Gen/Pr	3%
HIO-118V	UT	100%
	100 nM VD	50%
	25 µM Genistein	86%
	30 µM Progesterone	86%
	VD/Gen/Pr	12%

These data show that TGFbeta1 is severely down regulated in samples that are treated with the drug combination that markedly lowers 24-OH, and causes significant cell kill. Future studies will determine whether TGFbeta2 and -3 levels are also altered in the course of this drug combination.

Summary of In Vitro Evidence

As shown above, we demonstrate synergistic activation of cell death in cells derived from the ovarian surface epithelium by the combination of progestin and Vitamin D. The mechanism underlying this effect appears to involve the intrinsic cell death pathway. In addition, progestin decreases the degradation of Vitamin D via the possible degradation of 24-OH, which metabolically inactivates the active form of Vitamin D. Apoptotic effects are associated with a marked decrease in production of TGF-beta-1. In the setting of further inhibition of 24-OH, we see massive cell death, and complete abrogation of TGF-beta-1. These findings are all novel, and worthy of further investigation. In the following 12 months, we will examine the effects on other TGF-beta isoforms and signaling molecules within the apopotic and TGF-beta pathways.

Our findings support our original hypotheses, and provide further evidence in favor of the combination of progestin and Vitamin D for the chemoprevention of ovarian cancer.

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 being completed November 2006. A large flock of birds was randomized into several groups, receiving:

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 1α, 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.

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 has progressed well, and bird welfare has been excellent. We look forward to analysis of the data shortly.

Key research accomplishments

1) We have accrued over 1,900 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) An international ovarian cancer association consortium (OCAC) has been created to work towards an understanding of the role of genetic polymorphisms in ovarian cancer susceptibility. Dr Berchuck serves as head of the OCAC steering committee.

3) The +331G/A polymorphism in the progesterone receptor is protective against endometrioid/clear cell ovarian cancers and this has been confirmed by the international ovarian cancer association consortium.

4) Short alleles of the Androgen receptor gene CAG repeat polymorphism increase risk of ovarian cancer in African American women in North Carolina.

5) 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.

6) In view of *in vitro* evidence suggesting that there may be synergy with respect to ovarian cancer chemoprevention between progestins vitamin D analogues, and this concept has been tested in the context of a chemoprevention trial in chickens that is being completed in November 2006.

Reportable outcomes

1) The +331G/A polymorphism appears to be protective against endometrioid and clear cell ovarian cancers.

2) An international ovarian cancer association consortium has been formed that will work together to validate associations between genetic polymorphisms and risk of the disease.

3) Combinations of progestins and vitamin D may act in an additive fashion to decrease growth of ovarian cancer cells and is being studied in the context of a chemoprevention trial in chickens.

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Conclusions

The studies initiated by our program have the potential to enable us to define a moderate risk population based on epidemiologic and molecular genetic risk factors and to develop chemopreventive strategies designed to decrease ovarian cancer incidence and mortality.

With regard to ovarian cancer risk stratification, currently this is not used currently used clinically in the general population. This must change in the future if we are to decrease ovarian cancer incidence and mortality. The studies of genetic polymorphisms and molecular epidemiology initiated by our group are melding with those of other groups in the formation of an ovarian cancer association consortium. The long term goal is to identify a panel of ovarian cancer susceptibility polymorphisms that can be used in combination with known epidemiological risk factors to better stratify ovarian cancer risk. This would greatly facilitate implementation of prevention strategies by allowing these to be focused on higher-risk populations.

There is reason to believe that chemoprevention of ovarian cancer can contribute to a decline in mortality. The investigations ongoing in our program that include both *in vitro* experiments and chemoprevention trials in chickens are paving the way towards implementation of progestins and vitamin D analogues in this context. This may represent the best approach to decreasing ovarian cancer deaths in the 21st century.

Appendices

Oral presentation: 2006 meeting of the International Gynecologic Cancer Society, Santa Monica CA

PROGESTERONE RECEPTOR POLYMORPHISMS AFFECT RISK OF ENDOMETRIOID AND CLEAR CELL OVARIAN CANCERS: VALIDATION BY AN INTERNATIONAL OVARIAN CANCER ASSOCIATION CONSORTIUM

CL Pearce, JM Schildkraut, AH Wu, MC Pike, A Berchuck for the Ovarian Cancer Association Consortium. University of Southern California, Keck School of Medicine, Los Angeles, CA, USA Duke University Medical Center, Durham, NC USA.

Background and Aims: Studies have suggested that a functional single nucleotide polymorphism (SNP) in the progesterone receptor (PR) promoter (+331G/A) decreases risk of endometrioid/clear cell ovarian cancers (OCs). In addition, the PROGINS complex of linked PR polymorphisms has been associated with an increased risk of OC. An International Ovarian Cancer Association Consortium has been formed to validate findings.

Methods: The +331G/A and PROGINS SNPs were genotyped in 8,562 Caucasian subjects from 10 case-control studies. There were 3,261 subjects with invasive OC (including 728 endometrioid/clear cell) and 5,301 controls. Single SNP and haplotype analyses were conducted using logistic regression.

Results: The minor allele frequencies in controls were 6% for the +331 SNP and 15% for PROGINS. There was no overall association between the +331 SNP and OC risk, but analyses restricted to endometrioid/clear cell cases confirmed the protective effect of the minor allele (OR=0.77, 95% CI 0.59-1.0). The PROGINS allele was associated with a nominally increased risk of OC among all cases (OR=1.10, 95% CI 1.01-1.19). This effect was stronger among endometrioid/clear cell cases (OR=1.19, 95% CI 1.03-1.37). Haplotype analysis of endometrioid/clear cell cases revealed that those with the haplotype containing the minor +331 allele were protected against OC regardless of their PROGINS haplotype.

Conclusions: These findings provide validation of prior reports that the +331 and PROGINS polymorphisms affect risk of endometrioid/clear cell OC. Efforts to identify and validate other relevant polymorphisms are ongoing, and if successful could allow screening and prevention strategies to be focused on populations at increased risk.

Trinucleotide repeat polymorphisms in the androgen receptor gene and risk of ovarian cancer

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Running title: Androgen receptor polymorphisms in ovarian cancer

Key Words: Ovarian Cancer, Genetic Polymorphisms, Androgen Receptor Gene

ABSTRACT

Introduction: Androgens may play a role in the development of ovarian cancers. Two trinucleotide repeat polymorphisms have been described in exon 1 of the androgen receptor (AR) gene that may affect its function. Previous studies of ovarian cancer and AR repeat polymorphisms have been inconsistent.

Methods: We analyzed CAG and GGC repeat length polymorphisms in the AR gene using data from a population-based case-control study of ovarian cancer that included 594 cases and 681 controls. Repeat lengths were determined by fluorescent DNA fragment analysis using ABI GeneScan software. Change point models were used to determine appropriate repeat length cut points by race (African American vs. Caucasian) for both the shorter and the longer CAG and GGC repeats.

Results: No relationship was observed between CAG repeat length and ovarian cancer among Caucasians. Among African Americans, having a short repeat length on either allele was associated with a 2-fold increase in ovarian cancer risk (age-adjusted odds ratio (OR) = 2.2; 95% confidence interval (CI) = 1.1-4.1). Having short CAG repeat lengths for both alleles was associated with a 5-fold increased risk for developing ovarian cancer (age-adjusted OR = 5.4; 95% CI = 1.4-1.7). No relationship with the GGC repeat length polymorphisms was observed.

Conclusion: These results suggest that having a short CAG repeat length in AR increases ovarian cancer risk in African Americans. The failure to observe this relationship in Caucasians may be due to the rarity of such short CAG alleles in this population or could reflect racial differences in disease etiology.

INTRODUCTION

It has been suggested that androgens may play a role in the development of ovarian cancer.(1, 2) Androgen is produced by ovarian theca lutein cells and androgen receptors (ARs) are found in the normal surface epithelium of the ovaries. Most ovarian cancers express AR and anti-androgens inhibit ovarian cancer growth.(3-7) Epidemiologic studies also support a role of androgen in ovarian cancer where increasing waist-to-hip ratio(8, 9) and polycystic ovarian syndrome,(9, 10)which may be correlated with elevated androgen levels in women, have been associated with increased risk of ovarian cancer. In one study, higher levels of serum androstenedione were reported among women diagnosed with ovarian cancer compared to controls.(11) Additionally, oral contraceptive use, which is inversely associated with ovarian cancer risk, suppresses testosterone production by 35-70 percent.(1, 2)

Two highly polymorphic trinucleotide repeat polymorphisms in exon 1 of the AR gene have been studied in relation to cancer risk.(12) The CAG trinucleotide repeat of *AR* encodes a polyglutamine tract,(13) the length of which has been shown to be inversely associated with the ability of the AR-ligand complex to transactivate androgen-responsive genes. Molecular analyses have demonstrated that the transactivational capacity of the AR decreases with increasing number of glutamines encoded by the CAG repeat tract.(14) Indeed, shorter AR CAG repeats lengths are associated with a higher risk of prostate cancer.(15) Racial differences in *AR* CAG repeat length have been noted, with African Americans having a lower mean CAG length as compared with Caucasians.(16, 17) A second *AR* GGC trinucleotide repeat polymorphism codes for a polyglycine tract of variable length(18) but its functional significance has not been extensively examined. Data from one study suggest that while AR transactivation activity may not be affected by GGC repeats, translation of *AR* mRNA may be inversely related to GGC repeats with increased AR protein produced from alleles with shorter GGC repeats.(19) This suggests that shorter GGC repeats may result in an increased capacity to respond to androgen exposure.

There are five published studies that have addressed the association between CAG repeat length and ovarian cancer. Two case-control studies reported an increased risk of ovarian cancer

associated with increasing CAG repeat length among Caucasian women.(20, 21) The data from both studies suggest that women who carry two alleles with $\geq$ 22 CAG repeats are more likely to develop ovarian cancer than those with two alleles with < 22 repeats (OR =1.31; 95% CI = 1.01-1.69). However, other published studies have not found evidence to support the association between longer CAG repeat length and ovarian cancer,(12, 22, 23) although there may have been little power to detect an association due to the small number of ovarian cancer cases in two of the studies.(12, 23) Kadouri et al.(12) also examined the relationship between GGC repeat length and 29 ovarian cancer cases and did not find evidence to support a relationship.

In view of the conflicting data concerning the relationship between *AR* repeat polymorphisms and ovarian cancer risk we examined this relationship in a large population-based, case-control study of ovarian cancer in North Carolina. In contrast to prior studies, change point statistical analysis was used to determine the appropriate threshold for dichotomizing repeat lengths. In addition, this is the first study to examine the relationship between AR repeat length polymorphisms and ovarian cancer risk in a relatively large group of African-American women. This is of interest because African Americans have shorter CAG repeat lengths relative to Caucasians, which might mediate an increased respond to androgen exposure.(16)

MATERIALS AND METHODS

Subjects

Study subjects were enrolled through the ongoing North Carolina Ovarian Cancer (NCOC) study, a population-based, case-control study of newly diagnosed epithelial ovarian cancer. Epithelial ovarian cancer cases were identified through the North Carolina Central Cancer Registry (CCR), a statewide population-based tumor registry, using rapid case ascertainment. Pathology reports for all ovarian cancer cases diagnosed in the study area were forwarded to the North Carolina CCR and then to the study office within two months of diagnosis. Eligibility criteria for ovarian cancer cases include: diagnosis since January 1, 1999, age 20 to 74 years at diagnosis, no prior history of ovarian cancer, and residence in a 48 county area of North Carolina. For data included in the current analyses the last diagnosis among Caucasians was November 2003. In

order to maximize the number of African American subjects, the last date of diagnosis was extended to October 2005. All participants were English speaking, mentally competent to complete an interview, and able to give informed consent. Physician permission was obtained before an eligible case was contacted. All cases underwent standardized pathologic and histologic review by the study pathologist to confirm diagnosis. Both invasive and borderline epithelial ovarian cancer cases were included. The response rate among eligible cases was 75%. Non-responders were classified as: patient refusal (7%), inability to locate the patient (9%), physician refusal (4%), death (4%) or debilitating illness (2%).

Population-based controls were identified from the same 48 county region as the cases and were frequency-matched to the ovarian cancer cases on the basis of race (African American and Caucasian) and age (5-year age categories) using list-assisted random digit dialing (RDD). As required for the cases, controls had to be English-speaking, mentally competent to complete an interview and able to give informed consent. 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 RDD who passed the eligibility screening agreed to be contacted and sent additional study information. Among those sent additional study information the response rate was 64%. Non-responders were classified as: refusal 27% and unable to contact 9%. The study protocol was approved by the Duke University Medical Center Institutional Review Board and the Human Subjects committees at the North Carolina CCR and each of the hospitals where cases were identified.

Questionnaire Data

Trained nurse interviewers obtained written informed consent from study subjects at the time of the interview, which was usually conducted in the home of the study subject. A 90-minute questionnaire was administered 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 breastfeeding history, infertility, hormone use, and lifestyle characteristics such as smoking, alcohol consumption, physical activity, and occupational history.

A life events calendar, which marked significant life events including marriage and education, was used to improve recall of reproductive and contraceptive history. Additionally, anthropometric descriptors (height, weight, waist and hip circumference) were measured and a blood sample (30 ml) was collected.

Laboratory Analyses

DNA Extraction. Germline DNA was extracted from peripheral blood lymphocytes using PureGene DNA isolation reagents, according to manufacturer's instructions (Gentra Systems, Minneapolis, MN).

***AR* trinucleotide repeat length analysis**. 30 ng of genomic DNA was used as template for PCR amplification of the region containing the CAG and GGC trinucleotide repeats in 25 µl reaction volumes. The CAG repeat was amplified using primers previously reported(12) with the exception that the forward primer was modified by the addition of a 5' fluorescent label (6-Carboxyfluorescein; 6-FAM). The GGC repeat was amplified using two rounds of PCR with primers as described.(24) For the GGC repeat analysis, the forward primer used in the second round of PCR was labeled with 6-FAM.

PCR for the CAG repeat was performed using Platinum Taq DNA polymerase (Invitrogen; Carlsbad, CA) with conditions as follows: 94ºC for 3 minutes, then 5 rounds (4 cycles each) of 94ºC for 30 seconds, 64ºC for 30 seconds for round 1, then decreased by 2ºC each round down to 56ºC, 72ºC for 30 seconds, followed by 29 cycles of 94ºC for 30 seconds, 54ºC for 30 seconds, and 72ºC for 30 seconds, followed by a final 5 minute extension at 72ºC. For the GGC repeat, Pfu DNA Polymerase (Stratagene; La Jolla, CA) was used with PCR conditions as follows: round 1, 98ºC for 45 seconds, then 17 cycles of 98ºC for 1 minute and 70ºC for 5 minutes, then a 10 minute extension at 70ºC. One µl of the first round PCR products was used as template for the second round of PCR under the same conditions except that 34 cycles of PCR were performed.

The PCR products for both repeats were diluted 1:100 in nuclease free water and these dilutions were run on an Applied Biosystems 3100 Automated Capillary Instrument followed by fragment analysis using GeneScan Analysis software (Applied Biosystems; Foster City, CA). In

order to independently validate the fragment length call, a subset of samples were also analyzed by nucleotide sequencing after purification from high resolution agarose gels of individual amplicons produced from each allele. Unlabeled forward primers were used for sequencing the amplicons using an ABI 3730 Prism capillary DNA sequencer for the CAG repeat (N = 6), or for the GGC repeat (N = 15), the Thermosequenase Radiolabeled Terminator Cycle Sequencing Kit (USB Corporation; Cleveland OH). The GGC sequencing reactions were resolved on denaturing 5% polyacrylamide sequencing gels followed by exposure at -80ºC to Kodak BioMax MR radiographic film with an intensifying screen. The length of the trinucleotide repeats by sequencing was found to be longer relative to the repeat length determined by fragment length call of the GeneScan software in all cases. Since the sequencing results provide direct visualization of the number of repeats present, we systematically adjusted the Gene Scan fragment lengths by the addition of 12.0 nucleotides (4 repeats) for the CAG repeat analysis and 8.4 nucleotides (2.8 repeats) for the GGC repeat analysis.

Statistical analysis

We performed a two staged analysis of the association between repeat length and ovarian cancer. In the first stage, we used Bayesian model selection and model averaging to determine the weight of evidence in the data for each possible cut-point in repeat length and to estimate an average (over threshold values) measure of association. This approach allows us to determine if the association is significant marginal to the choice of threshold and obviates the need for a multiple comparisons adjustment. In the second stage, we fit multivariate models of association fixing the repeat length threshold to its most probable value *a posteriori*. The purpose of these second stage analyses was to verify that the observed associations between repeat length and ovarian cancer were not confounded.

In stage 1, separate Bayesian change point models were fit to the self-reported African American and Caucasian short and long CAG repeat alleles, CAG_S and CAG_L, respectively and short and long GGC repeat alleles, GGC_S and GGC_L, respectively. The designation of CAG_S and CAG_L as well as the GGC_S and GGC-L reflect the comparison of the repeat length of the

two alleles within an individual. The change point model specifies that odds of disease is constant before and after a threshold value, but is different in the 2 regions. We used uniform prior probabilities over the possible discrete thresholds of the data and on whether or not there is a change point and used a Beta(2,2) prior over pre- and post-threshold probabilities of disease. The Beta(2,2) distribution has mean 0.5 and standard deviation 0.22. This is equivalent to adding two cases and two controls in each of the pre-and post-threshold samples. Under this model, we calculated (1) the probability there was a change point, and (2) given that there was, the probability that it occurred at each of the possible values. Calculation (1) was equivalent to a Bayesian hypothesis test of Ho: the case control fraction does not depend on a thresholded short CAG repeat length versus Ha: that it does. We report the posterior probability for association of disease to CAG_S length, the posterior probabilities of the thresholds given that the threshold model is the true model and the odds ratio (OR) for disease given a CAG_S repeat smaller than each probable threshold. In addition, we calculate estimates of ORs that account for uncertainty in the threshold's value. This was accomplished by summarizing the marginal (over threshold) posterior distribution on the OR for the association between repeat length and ovarian cancer. The resulting OR is a threshold independent measure of association. Parallel analyses were calculated to determine the relationship between the CAG_L , the GGC_S and the GGC_L repeat alleles in the AR gene and ovarian cancer risk.

The stage 1 change point models were fit under the assumption that there was no potential confounding by other covariates. In our stage 2 analysis, we checked this assumption by fitting multivariable unconditional logistic regression models controlling for potential confounders to determine whether confounding bias would explain any observed association between CAG and GGC repeat length and epithelial ovarian cancer. We examined the CAG_S and CAG_L repeat polymorphism variables using the cut points identified through the change point analysis as having the highest probabilities. Additionally, because of evidence from prior reports, we present the association between the number of CAG repeats $\geq$ 22 and ovarian cancer risk among Caucasian study participants. To control for confounding, we simultaneously adjusted for variables with known

associations with disease status. These variables included age at diagnosis/interview, tubal ligation (yes or no), months of oral contraceptive use, body mass index (kg/m^2) (BMI) one year prior to diagnosis/interview, waist-to-hip ratio, family history of breast or ovarian cancer in first degree relatives (yes or no), and total months pregnant. We report both age-adjusted ORs and 95% CIs as well as ORs adjusted for additional potential confounders.

An interim analysis after the first three years of data collection revealed a statistically significant association between the CAG repeat polymorphism, but no evidence of an association with the GGC polymorphism. We therefore discontinued the analysis of the GGC repeats in this dataset. Thus our final sample size for the CAG polymorphism is approximately twice as large as that for GGC. For much of our sample we had additional genotype data on ninety-nine unrelated single nucleotide polymorphismsn (SNPs) unlinked to disease status and selected from 22 chromosomes. Among the self-reported African-American subjects we had this genotype data on 77 of 99 cases and 88 of 141 controls. Among the self-reported Caucasian subjects we had this genotype data on 473 of 495 cases and all of the controls. In order to address the possibility of population-stratification the genotype data from these 99 SNPs along with the location of each SNP within its chromosome were input into the program Structure (version 2.0) to estimate the degree of racial admixture for each individual. Structure is a program that implements a model-based clustering method for inferring population structure.(25) It allows the user to select the number of parent populations represented in the sample. We set this parameter to two to allow for African and European ancestral populations. Structure estimated the admixture fractions for each individual. These admixture fractions were also used as an alternate to self reported race status performing race-specific change point analyses in stage 1. Change point analyses were performed using R [www.r-project.org]. All other analyses were performed using SAS 9.1 (SAS Institute Inc., Cary, NC)

RESULTS

The demographic features, epidemiologic risk factors, and pathological characteristics of cases and controls are shown in Table 1 stratified by self-reported race. Tubal ligation was the only risk factor that had a statistically significant association in both races, where an inverse relationship is observed. Waist-to-hip ratio was higher in cases than controls both in Caucasians ($p<0.001$) and among African Americans ($p=0.059$). Fewer months of pregnancy and months of oral contraceptive use are observed among cases compared to controls for both races although these differences are statistically significant among Caucasians only. Tumor behavior was invasive in 76% and borderline in 24% for both African-American and Caucasian cases. The distribution of histologic subtype was similar in both racial groups.

The CAG repeat length distributions in cases and controls by race are found in Table 2. No differences in the mean CAG repeat length in the AR gene were detected for either the short or long repeat alleles in Caucasian cases and controls. Among African Americans, the mean CAG_S and CAG_L repeat lengths were lower among cases compared to controls. The mean CAG_S length among cases was 16.8 (SD = 2.6) compared to 18.0 (SD = 2.7) among controls ($p = 0.001$) and the mean CAG_L length for cases was 20.7 (SD = 2.9) compared to 21.4 (SD = 2.5) for controls ($p = 0.044$). Both the mean CAG_S and CAG_L lengths were both significantly lower among African Americans compared to Caucasians ($p < 0.001$).

Parallel analyses to determine the association between GGC repeat and ovarian cancer risk were conducted in a subset of the population which included 186 and 213 self-reported Caucasian ovarian cancer cases and controls, respectively and 59 and 67 self-reported African American ovarian cancer cases and controls (see Table 2). There was no evidence of differences in the mean GGC_S or GGC_L allele length between cases and controls in either racial group.

Change point analysis was used to determine appropriate cut-points for CAG repeat length for both the short and long CAG repeats alleles, stratified by self-reported race, either African American or Caucasian. Cut-points were detected for both CAG_S and CAG_L repeat alleles among African Americans only. Tables 3 and 4 present model probabilities associated with the

relationship between ovarian cancer and CAG_S repeat length allele and the CAG_L repeat allele, respectively, conditional on thresholded CAG length among African Americans and Caucasians. The tables present estimates of posterior model probabilities for each cutpoint. OR estimates and 95% CIs for the association between CAG repeat length allele and ovarian cancer for each cutpoint are also shown. Among African Americans, the posterior probability of a change point association in the *AR* CAG_S repeat allele is about 72% and the most likely threshold is between 15 and 16 with a posterior probability of 31% given that the change point class of models is correct. The odds ratio for the association between CAG repeat length at the threshold between 15 and 16 is 2.77 (95% CI = 1.31 - 5.26). The posterior probability of a change point association in the *AR* CAG_L repeat allele is about 73% and the most likely threshold is between 18 and 19 with a posterior probability of 21%. In contrast, the data for Caucasian ovarian cancer cases and controls does not support a change point model; for the *A*R CAG_S repeat allele, the posterior probability of this class of change point models is 31% and no threshold has a posterior probability exceeding 14%. For the CAG_L allele it is 36% and no threshold has a posterior probability exceeding 13%.

We repeated the change point analysis in a subset of cases and controls defined as African Americans based on having admixture fraction less than 10% as determined from the Structure analysis. A total of 13 cases and 21 controls of the self-reported African Americans who had an admixture fraction $\leq$ 90% were omitted. An additional 22 cases and 52 controls of the self-reported African Americans were omitted from this analysis due to missing admixture information. In this analysis the relationship between CAG_S length and ovarian cancer became even stronger suggesting that admixture does not explain the observed association with ovarian cancer (data not shown). In this analysis, the posterior of a change point association was 0.871 and given the association the probability of a change point between 15 and 16 was 0.675. For the CAG_L there was no evidence of an admixture effect.

Change point analysis did not detect an association between ovarian cancer and GGC repeat length for either the GGC_S or GGC_L repeats among those whose self-reported race was African American, those whose self reported race was Caucasian or among those limited to having

an African American admixture fraction of more than 90%. In fact, the probability for a change point did not exceed 41% for either the GGC_S or GGC_L allele in any of these groups (data not shown). Given a prior probability of 50% in favor of such an association, this is evidence against association.

Additional multivariable analyses to determine whether confounding could explain the association between CAG repeat length and ovarian cancer among African Americans are presented in Table 5. The age-adjusted OR for the association between the CAG_S repeat length allele < 16 and ovarian cancer was 2.8 (95% CI = 1.4 – 5.9) in African Americans. A similar relationship was found between the CAG_L repeat length allele < 19 and ovarian cancer (age adjusted OR = 2.5; 95% CI = 2.5 (1.3 -4.8). Having both a CAG_S repeat < 16 and a CAG_L repeat < 19 was associated with a 5-fold increased risk of ovarian cancer (age-adjusted OR = 5.4; 95% CI = 1.6 – 17.9). Also shown in Table 5, simultaneously controlling for age, months pregnant, months of oral contraceptive use, BMI, family history of ovarian or breast cancers in a first degree relative, and tubal ligation did not substantially change the relationship between CAG repeat length and ovarian cancer detected in the age-adjusted analyses. Additional analyses limited to invasive ovarian cancers as well as histologic subtype (serous, endometrial and clear cell only) did not reveal any substantial differences in the relationship with CAG repeat length and ovarian cancers (data not shown). Although we did not detect evidence for a threshold in CAG repeat length in Caucasians, we calculated the age-adjusted OR for a CAG_S repeat length < 16 of 0.8 (95% CI = 0.4 – 1.5) and for the CAG_L repeat length < 19 of 0.6 (95% CI = 0.3 – 1.2) (data not shown).

We also conducted unconditional logistic regression analyses in Caucasian subjects using a cut-point of $\geq$ 22 CAG repeats to compare our data to those of previously published reports.(20, 21, 26) The age-adjusted ORs for the association between those who carry either one or two alleles with $\geq$ 22 CAG repeats versus those with two alleles with < 22 repeats were 1.2 (95% CI = 0.9 – 1.6) and 1.2 (95% CI 0.8 – 1.7) respectively (see Table 6).

DISCUSSION

The mean and median androgen receptor CAG lengths for both the CAG_S and alleles in Caucasian subjects in North Carolina Ovarian Cancer study population are similar to lengths reported in previous studies.(20, 21, 26) No relationship was found between CAG repeat length and ovarian cancer among the Caucasians in this study. To our knowledge, this is the first study to evaluate the association between CAG repeat length in *AR* and ovarian cancer risk in African-American women. We found an increase in ovarian cancer risk associated with both CAG_S and CAG_L repeat length alleles in African Americans. These differences were evidenced by both the shorter mean repeat length of the CAG_S and CAG_L alleles as well as the higher prevalence of the CAG_S repeat length < 16 and the CAG_L repeat length < 19 among African-American cases compared to controls .

The association between *AR* CAG repeat length and ovarian cancer risk in African Americans is further supported by an analysis of these data that omitted self-reported African American subjects with evidence of significant admixture of > 10% and found a similar association. Therefore, this result is not likely to be explained by confounding due to population stratification. Additionally, our results remained significant when simultaneously controlling for other potential confounders. Our results suggest that having one short CAG repeat length in African Americans, which are associated with higher levels of androgenic activity, more than doubles the risk of ovarian cancer. Additionally, those with both short CAG_S and short CAG_L repeats may have a 5-fold increased risk of ovarian cancer. This is also the largest study to evaluate the relationship between the *AR* GGC repeat length polymorphism and ovarian cancer. Similar to the finding of a small case-control study by Kadouri et al,(12) we did not detect a relationship in either racial group.

The observed association between CAG repeat length and ovarian cancer is biologically plausible in view of the inverse relationship between CAG length and transactivation activity on the receipt and binding affinity of androgens.(14) Short CAG repeat alleles may facilitate greater chronic androgen stimulation leading to increased proliferative activity. Shorter CAG repeat alleles have also been associated with other hyper-androgenic clinical conditions including risk of

baldness and having prostatic hyperplasia in men and hirsutism,(28) annovulation,(29) and acne in women.(30) Additionally, data from a nested case-control study by Helzlsouer et al. found that increased serum androgen levels were associated with an increased risk of ovarian cancer.(11)

Our findings are not consistent with two recent reports by Terry et al.(20) and Santarosa et al.(21) Both studies support an association between having two alleles with $\geq$ 22 CAG repeats and ovarian cancer risk in Caucasian subjects. The relationship in the study by Santarosa was stronger with an OR of 3.45 (95% CI = 1.42 – 8.34) compared to 1.31 (95% CI = 1.01 -1.59) in the study by Terry et al.(20) In our study we found an OR of 1.2 (95% CI = 0.8 – 1.7) for this association, and although this is in the same direction with similar precision and does not conflict with the findings of Terry et al., it is lower in magnitude and not statistically significant. As suggested by Terry et al., a possible explanation for the discrepancies between published reports and the current study include differences in the prevalence in the carriage of subjects having two AR CAG repeats $\geq$ 22. It is known that allele frequency varies according to ethnicity.(16, 31) In our study we found that the prevalence of two CAG repeats $\geq$ 22 differed markedly between Caucasian and African-American controls,16% and 9%, respectively. The prevalence of two AR CAG repeats $\geq$ 22 in our Caucasian subjects also differs from the prevalences among Caucasians in the studies by Terry (prevalence = 24%) and Spurdle et al. (prevalence = 26%), a positive and a negative study respectively, but is more similar to that of Santarosa et al. (prevalence = 18%) which was a positive study. Due to the known ethnic variation in CAG repeat length it is possible the results of the association with *AR* CAG length could be due to chance. However our analyses of admixture among the Caucasian subjects did not support that population stratification was a major concern; only 8% of cases and 11% of controls had evidence of significant (> 10%) admixture.

Strengths of this study include the fact that this is a large population-based study of both Caucasian and African-American women. Our approach using the change point analysis provided a more objective and thorough evaluation of a cut-point in the association between *AR* CAG repeat length and ovarian cancer risk, avoiding multiple comparisons at different thresholds. In addition,

we estimated the association between *AR* CAG repeat length while simultaneously controlling for other potential confounders, thus providing evidence that confounding bias is unlikely to account for the association. We were able to determine that it was unlikely that population stratification among African Americans biased our results. It is also unlikely that selection bias related to genotype would have occurred and influenced our results. Limitations of our study include a somewhat small sample of African-American subjects. We attempted to find an independent dataset that could be used for an validation of the association between *AR* CAG repeat length and ovarian cancer in African Americans, but were unsuccessful. Finally, we were not able to conclusively determine why the findings among African-American women and Caucasian women differed. The failure to observe the relationship in Caucasians may be due to the rarity of the short CAG alleles in this population or could reflect racial differences in disease etiology.

Similar to studies in ovarian caner, analyses of the relationship between the short *AR* CAG repeat length polymorphism and prostate cancer risk also have yielded conflicting results. Likewise, differences in the association with prostate have been noted between racial groups.(17, 32-35) Pettaway(32) has suggested racial differences in genetic variation in several genes in the androgen/androgen receptor pathway may be related to clinically observed differences in the biology of prostrate cancer among racial groups.(32) For example, in addition to CAG repeat length in *AR*, genetic variants in the 5 alpha-reductase type 2 also differ between African Americans and Caucasians. However, is it has also been suggested that racial differences and inconsistent findings in studies of prostate cancer may be due to linkage disequilibrium between *AR* CAG repeat length polymorphisms and another susceptibility locus on the X chromosome.(36) These possible explanations are also relevant to studies of ovarian cancer.

In summary, our finding of an association between short *AR* CAG repeat lengths and ovarian cancer among African Americans warrants replication in a larger dataset and further study is needed to more fully understand the complexities of this relationship. We did not detect a relationship between CAG repeat length and ovarian cancer among Caucasian women and were not able to confirm previous reports for such an association. Additionally we were unable to detect

a relationship between the GGC repeat polymorphism and ovarian cancer in either African-American or Caucasian women. However, we believe further study of the positive finding in African American women may provide insight into the etiology of ovarian cancer.

Table 1. Demographics and pathologic characteristics of ovarian cancer cases and controls from the North Carolina Ovarian Cancer Study, by self-reported race

	Caucasians					African Americans				
	Cases (N=495)		Controls (N=540)			Cases (N=99)		Controls (N=141)		
	n	(%)	n	(%)	p-value	n	(%)	n	(%)	p-value
Age in years										
20-49	160	(32)	191	(35)	0.664	39	(39)	50	(35)	0.720
50-64	222	(45)	196	(36)		44	(44)	65	(46)	
65-75	113	(23)	153	(28)		16	(16)	26	(18)	
Menopause Status										
Pre/peri	180	(36)	225	(42)	0.081	42	(42)	62	(44)	0.775
Post	315	(64)	315	(58)		57	(58)	78	(56)	
Months pregnant										
0	83	(17)	59	(11)	0.004	8	(8)	7	(5)	0.425
1-8	26	(5)	19	(4)		4	(4)	10	(7)	
9-18	182	(37)	206	(38)		36	(36)	48	(34)	
19-36	177	(36)	215	(40)		37	(37)	51	(36)	
>36	26	(5)	40	(7)		14	(14)	25	(18)	
OC use (months)										
None	165	(33)	161	(30)	0.050	40	(40)	60	(43)	0.134
<12	42	(8)	44	(8)		14	(14)	5	(4)	
12-36	107	(22)	120	(22)		23	(23)	35	(25)	
37-60	45	(9)	46	(9)		3	(3)	6	(4)	
>60	122	(25)	164	(30)		15	(15)	31	(22)	
user of unknown duration	14	(3)	5	(1)		4	(4)	4	(3)	
History of breast/ovarian cancer in 1st degree relative										
Yes	89	(18)	87	(16)	0.440	28	(28)	24	(17)	0.037
No	406	(82)	451	(84)		71	(72)	117	(83)	
Tubal ligation										
Yes	122	(25)	164	(30)	0.040	32	(32)	75	(53)	0.001
No	373	(75)	376	(70)		67	(68)	66	(47)	
Polycystic ovarian syndrome										
Yes	2	(0)	4	(1)	0.688	0	(0)	2	(1)	0.513
No	493	(100)	536	(99)		99	(100)	138	(99)	
BMI 1 yr prior to diagnosis/interview										
Quartile 1: <22.42	103	(21)	131	(25)	0.167	NA		NA		
Quartile 2: 22.42-25.739	133	(28)	133	(25)		NA		NA		
Quartile 3: 25.74-29.759	104	(22)	132	(25)		NA		NA		
Quartile 4: >29.76	143	(30)	133	(25)		NA		NA		

BMI 1 yr prior to diagnosis/interview									
Quartile 1: <27.341	NA		NA		23	(24)	34	(25)	0.368
Quartile 2: 27.341-30.33	NA		NA		17	(18)	33	(24)	
Quartile 3: 30.34-36.4	NA		NA		26	(27)	35	(26)	
Quartile 4: >36.4	NA		NA		31	(32)	34	(25)	
Waist/hip ratio at interview									
Quartile 1: <0.739	82	(17)	133	(25)	0.000	NA		NA	
Quartile 2: 0.739-<0.7871	103	(21)	134	(25)		NA		NA	
Quartile 3: 0.7871-<0.8351	152	(31)	134	(25)		NA		NA	
Quartile 4: >0.8351	150	(31)	134	(25)		NA		NA	
Waist/hip ratio at interview									
Quartile 1: <0.772	NA		NA		16	(16)	35	(25)	0.059
Quartile 2: 0.772-<0.828	NA		NA		25	(26)	35	(25)	
Quartile 3: 0.829-<0.876	NA		NA		20	(21)	35	(25)	
Quartile 4: >0.876	NA		NA		36	(37)	34	(24)	
Infertility, doctor diagnosed in female									
Yes	62	(13)	53	(10)	0.166	8	(8)	10	(7)	0.775
No	433	(87)	487	(90)		91	(92)	131	(93)	
Tumor behavior									
Borderline	117	(24)				24	(24)		
Invasive	378	(76)				75	(76)		
Tumor histology									
Serous	300	(61)				61	(62)		
Endometrioid	63	(13)				12	(12)		
Mucinous	48	(10)				11	(11)		
Clear Cell	37	(7)				2	(2)		
Other	46	(9)				13	(13)		

Table 2. Mean and median CAG and GGC trinucleotide repeat length polymorphism lengths in ovarian cancer cases and controls enrolled in the North Carolina Ovarian Cancer study by self-reported race*

	Caucasians					African Americans				
	Cases		Controls		p-value	Cases		Controls		p-value
CAG repeats	(N=484)		(N=522)			(N=99)		(N=140)		
CAG_St										
mean (SD)	19.4	(2.3)	19.3	(2.2)	0.685	16.8	(2.6)	18.0	(2.7)	0.001
Median (range)	19.0	(6 - 25)	19.0	(5 - 25)		17.0	(8 - 23)	17.0	(10 - 26)	
CAG_L										
mean (SD)	22.6	(2.6)	22.4	(2.5)	0.146	20.7	(2.9)	21.4	(2.5)	0.044
median (range)	23.0	(15 - 34)	22.0	(15 - 32)		21.0	(15 - 29)	22.0	(14 - 27)	
GGC repeats	(N=186)		(N=213)			(N=59)		(N=67)		
GGC_S										
mean (SD)	16.6	(1.53)	16.4	(1.86)	0.377	15.4	(1.74)	15.2	(1.77)	0.593
median (range)	17.0	(6 - 18)	17.0	(6 - 18)		16.0	(9 - 18)	16.0	(9 - 18)	
GGC_L										
mean (SD)	17.2	(0.89)	17.2	(1.04)	0.800	16.6	(0.83)	16.6	(0.97)	0.925
median (range)	17.0	(14 - 19)	17.0	(12 - 20)		17.0	(14 - 18)	17.0	(14 - 18)	

* p-values are from Student's t-Test

Table 3. Model probabilities conditional on thresholded CAG_S length and odds ratios for CAG_S repeat length less than versus greater than a threshold t by self-reported race.

	Caucasians (484 cases and 522 controls)			**African Americans (99 cases and 140 controls)**		
CAG_S Length	Posterior Pr(T=t /change)	Odds Ratio	(95% Interval)	Posterior Pr(T=t/change)	Odds Ratio	(95% Interval)
7.5	0.110	1.62	(0.19 - 6.30)	NA	NA	NA
9.5	NA	NA	NA	0.029	4.26	(0.34 - 19.54)
10.5	0.098	1.80	(0.35 - 5.80)	NA	NA	NA
11.5	0.099	1.91	(0.48 - 5.44)	0.029	2.91	(0.60 - 9.26)
12.5	0.106	1.96	(0.59 - 5.11)	0.067	3.98	(0.91 - 12.45)
13.5	0.134	2.03	(0.74 - 4.64)	0.072	3.05	(1.01 - 7.52)
14.5	0.053	1.26	(0.55 - 2.48)	0.029	2.12	(0.83 - 4.54)
15.5	0.048	0.88	(0.44 - 1.57)	0.306	2.77	(1.31 - 5.26)
16.5	0.039	0.91	(0.51 - 1.48)	0.053	1.84	(1.04 - 3.00)
17.5	0.030	0.89	(0.63 - 1.21)	0.045	1.79	(1.02 - 2.92)
18.5	0.017	1.02	(0.77 - 1.31)	0.156	2.15	(1.18 - 3.65)
19.5	0.019	0.94	(0.73 - 1.20)	0.049	2.02	(1.02 - 3.70)
20.5	0.025	0.90	(0.67 - 1.19)	0.030	2.09	(0.88 - 4.35)
21.5	0.025	0.93	(0.66 - 1.27)	0.025	2.28	(0.75 - 5.80)
22.5	0.047	0.82	(0.52 - 1.23)	0.041	3.74	(0.77 - 13.13)
23.5	0.070	1.50	(0.63 - 3.09)	0.024	3.68	(0.39 - 16.43)
24.5	0.081	1.40	(0.33 - 4.10)	0.024	3.66	(0.40 - 16.35)
25.5	NA	NA	NA	0.020	2.15	(0.17 - 10.03)
Overall	0.314			0.724		

Table 4. Model probabilities conditional on thresholded CAG_L length and odds ratios for CAG_L repeat length less than versus greater than a threshold t by self-reported race.

	Caucasians (484 cases and 522 controls)			**African Americans (99 cases and 140 controls)**		
CAG Length	Posterior Pr(T=t /change)	Odds Ratio	(95% Interval)	Posterior Pr(T=t/change)	Odds Ratio	(95% Interval)
14.5	NA	NA	NA	0.021	1.41	(0.10 - 5.93)
15.5	0.078	1.62	(0.18 - 6.28)	0.020	2.14	(0.24 - 8.35)
16.5	0.067	1.44	(0.24 - 4.80)	0.015	1.73	(0.43 - 4.79)
17.5	0.082	0.67	(0.17 - 1.71)	0.022	1.85	(0.81 - 3.69)
18.5	0.067	0.69	(0.33 - 1.26)	0.205	2.44	(1.24 - 4.41)
19.5	0.025	0.87	(0.57 - 1.29)	0.204	2.24	(1.22 - 3.80)
20.5	0.017	0.91	(0.68 - 1.20)	0.150	2.05	(1.18 - 3.34)
21.5	0.032	0.84	(0.65 - 1.08)	0.180	2.08	(1.20 - 3.39)
22.5	0.036	0.83	(0.65 - 1.06)	0.008	1.32	(0.72 - 2.23)
23.5	0.048	0.81	(0.62 - 1.04)	0.007	1.18	(0.61 - 2.12)
24.5	0.015	1.06	(0.77 - 1.43)	0.010	1.39	(0.56 - 2.99)
25.5	0.021	0.92	(0.61 - 1.34)	0.011	1.02	(0.34 - 2.45)
26.5	0.024	0.99	(0.58 - 1.58)	0.034	0.53	(0.08 - 1.74)
27.5	0.060	0.72	(0.36 - 1.27)	0.070	0.35	(0.03 - 1.27)
28.5	0.065	0.71	(0.27 - 1.49)	NA	NA	NA
29.5	0.053	1.30	(0.35 - 3.48)	NA	NA	NA
30.5	0.082	0.93	(0.13 - 3.24)	NA	NA	NA
32.5	0.127	0.62	(0.05 - 2.35)	NA	NA	NA
33.5	0.102	0.93	(0.07 - 3.84)	NA	NA	NA
Overall	0.362			0.734		

Table 5. Relationship between androgen receptor CAG repeat polymorphisms and ovarian cancer among African-American women enrolled in the North Carolina Ovarian Cancer Study.

	Cases		Controls					
	n	(%)	n	(%)	OR*	(95% CI)	OR**	(95% CI)
CAG_S repeat < 16								
No	76	(77)	126	(89)	1.0	(reference)	1.0	(reference)
Yes	23	(23)	15	(11)	2.8	(1.4 - 5.9)	2.5	(1.1 - 5.5)
CAG_L repeat < 19								
No	72	(73)	121	(86)	1.0	(reference)	1.0	(reference)
Yes	27	(27)	20	(14)	2.5	(1.3 - 4.8)	2.7	(1.3 - 5.8)
Number of repeats with CAG_S < 16 or CAG_L < 19								
None	60	(61)	111	(79)	1.0	(reference)	1.0	(reference)
1	28	(28)	25	(18)	2.2	(1.1 - 4.1)	2.1	(1.1 - 4.3)
2	11	(11)	5	(4)	5.4	(1.6 - 17.9)	4.8	(1.4 - 17.0)

* age adjusted

** adjusted for age, months pregnant, months of OC use, BMI, tubal ligation, family history of breast or ovarian caner in a first degree relative, waist-to-hip ratio

Table 6. Androgen Receptor CAG repeat polymorphisms ≥ 22 versus < 22 repeats among Caucasian cases and controls in the NCOC study.

	Cases		Controls					
	n	(%)	n	(%)	OR*	(95% CI)	OR**	(95% CI)
CAG repeat ≥ 22								
0 alleles	163	(34)	198	(38)	1.0	(reference)	1.0	(reference)
1 alleles	237	(49)	240	(46)	1.2	(0.9 - 1.6)	1.2	(0.9 - 1.6)
2 alleles	84	(17)	84	(16)	1.2	(0.8 - 1.7)	1.2	(0.8 - 1.8)
Either 1 or 2 alleles	321	(66)	324	(62)	1.2	(0.9 - 1.5)	1.2	(0.9 - 1.5)

* age adjusted

** adjusted for age, months pregnant, months of oral contraceptive use duration, BMI, tubal ligation, family history of breast or ovarian cancer in a first degree relative, waist-to-hip ratio

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