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Evaluating the ovarian cancer gonadotropin hypothesis: A candidate gene study

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HIGHLIGHTS

- We examine whether variation in gonadotropin signaling pathway genes is associated with ovarian cancer risk.
- We evaluate individual SNP effects and gene-level effects through burden testing using the admixture likelihood (AML) method.
- Four putative ovarian cancer susceptibility loci near *INHBB*, *LHCGR*, *GNRH*, and *FSHB* are identified.

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ABSTRACT

Objective. Ovarian cancer is a hormone-related disease with a strong genetic basis. However, none of its high-penetrance susceptibility genes and GWAS-identified variants to date are known to be involved in hormonal pathways. Given the hypothesized etiologic role of gonadotropins, an assessment of how variability in genes involved in the gonadotropin signaling pathway impacts disease risk is warranted.

Methods. Genetic data from 41 ovarian cancer study sites were pooled and unconditional logistic regression was used to evaluate whether any of the 2185 SNPs from 11 gonadotropin signaling pathway genes was associated with ovarian cancer risk. A burden test using the admixture likelihood (AML) method was also used to evaluate gene-level associations.

Results. We did not find any genome-wide significant associations between individual SNPs and ovarian cancer risk. However, there was some suggestion of gene-level associations for four gonadotropin signaling pathway genes: *INHBB* ($p = 0.045$, mucinous), *LHCGR* ($p = 0.046$, high-grade serous), *GNRH* ($p = 0.041$, high-grade serous), and *FSHB* ($p = 0.036$, overall invasive). There was also suggestive evidence for *INHA* ($p = 0.060$, overall invasive).

Conclusions. Ovarian cancer studies have limited sample numbers, thus fewer genome-wide susceptibility alleles, with only modest associations, have been identified relative to breast and prostate cancers. We have evaluated the majority of ovarian cancer studies with biological samples, to our knowledge, leaving no opportunity for replication. Using both our understanding of biology and powerful gene-level tests, we have identified four putative ovarian cancer loci near *INHBB*, *LHCGR*, *GNRH*, and *FSHB* that warrant a second look if larger sample sizes and denser genotype chips become available.

Introduction

Invasive epithelial ovarian cancer (ovarian cancer) is a hormone-related cancer with oral contraceptive (OC) use and parity as well-established protective factors [1]. Long-standing hormonal hypotheses include incessant ovulation, direct effects of estrogen and progesterone, and gonadotropin signaling [2–4]. The gonadotropin hypothesis suggests that ovarian cancer develops from excess stimulation of ovarian tissue by the pituitary gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), whose secretion is controlled by the gonadotropin releasing hormone (GnRH) [4].

Ovarian cancer also has a significant heritable component in which a first-degree family history of ovarian cancer is associated with an approximate two-fold increased risk [5]. This risk may be attributable to high-penetrance susceptibility genetic mutations as well as several common variants that have been identified through genome-wide association studies (GWAS) [6–13]. It was widely expected that genes involved in hormone signaling and action would be associated with ovarian cancer risk, but none of the GWAS-identified variants or major genes appears to be involved in hormonal pathways. Conversely, in other cancers affected by hormones, such as breast cancer, hormone-related genetic variation was associated with disease risk [14]. However, a substantial portion of ovarian cancer's excess familial risk still remains unexplained.

Detailed analyses of gene-level effects of genes involved in gonadotropin signaling have not been undertaken. Three population-based case–control studies have assessed the effects of genetic variants in the FSH receptor gene (*FSHR*), but with conflicting results [15–17]. Hence, we have carried out a comprehensive multi-center analysis to determine whether genetic variation in 11 genes involved in the gonadotropin signaling pathway is associated with ovarian cancer risk.

Methods

This study was approved by the ethics committees of each institution. Each subject provided written informed consent prior to inclusion in the study.

Study populations

In total, 41 study sites from the Ovarian Cancer Association Consortium (OCAC), which constituted 31 study sets, have been included in this analysis; study sites were grouped into sets based on the scope of genotyping data (genome-wide versus targeted approaches) as well as the geographic region. Briefly, 20 study sites were conducted in Europe, 19 in North America, and two in Australia. Nine study sites were case-only so their cases were pooled with case–control study sites from the same geographic region. In addition, the three Polish case–control study sites were combined into a single study set. An overview of each site's characteristics and number of cases and controls are presented in Supplementary Table S1. Our analyses excluded participants who were not of European ancestry ($n = 4605$), as determined by the program LAMP (Local Ancestry in Admixed Populations) (see "Statistical analysis"), or were missing ethnicity information ($n = 23$). In addition, only invasive epithelial ovarian cancer cases were considered. Hence, a combined total of 46,176 participants ($n =$ 15,361 cases and 30,815 controls) was used in our final analyses. Details regarding sample quality control have been published previously [9].

Imputation analyses

These analyses were based on genotype data from three GWAS and replication efforts [8,18] as well as the large-scale genotyping array by the Collaborative Oncological Gene-environment Study (COGS) [12]. Details of these large-scale genotyping efforts have been published previously.

To account for different marker sets and improve genome coverage, imputation of the entire scope of genetic variation in the genome was carried out by combining the available genotyped data as well as information from the March 2012 release of the 1000 Genomes Project using the program IMPUTE2 [19]. The data were pre-phased using SHAPEIT software to reduce computation time [20].

Gene and SNP selection

Eleven gonadotropin signaling pathway genes were evaluated in our analyses: *ACVR1*, *ACVR2*, *CGA*, *FSHB*, *FSHR*, *GNRH*, *GNRHR*, *INHA*, *INHBA*, *INHBB*, and *LHCGR*. SNPs found within each gene as well as SNPs within 25 kb upstream and downstream of each gene were assessed. Only SNPs with an imputation $r^2 \geq 0.5$ and a minor allele frequency (MAF) ≥ 0.05 were considered in the analyses. Hence, our final analyses included 2185 SNPs.

Statistical analysis

The program LAMP was used to assign intercontinental ancestry based on genotype frequencies in European, Asian, and African populations [21]. Participants with 90% or more European ancestry were classified as European. To adjust for ancestry within the European population, a principal components analysis (PCA) was performed within our subjects using a set of 37,000 unlinked markers as well as an in-house program written in C++ that used the Intel MKL library for eigenvectors (http://ccge.medschl.cam.ac.uk/software/pccalc).

Coverage referred to the total proportion of genetic variation being captured for each gene at an $r^2 \geq 0.8$. It was determined by calculating the pairwise r^2 between the SNPs we had genotypes for (with an imputation $r^2 \geq 0.5$ and a MAF ≥ 0.05) and all of the 1000 Genomes SNPs in each gene that had a MAF ≥ 0.05.

Unconditional logistic regression was used to assess the association between each SNP in the gonadotropin signaling pathway genes and ovarian cancer risk. All analyses took into consideration study set and adjusted for population substructure by including the first five PCA eigenvalues in the model. This was done for all invasive ovarian cancer as well as for the four ovarian cancer histological subtypes (high-grade serous, mucinous, endometrioid, clear cell). Per-allele log odds ratios (ORs) and 95% confidence intervals (CIs) were estimated. All reported p-values were two-sided.

Because we were interested in identifying whether or not individual genes (versus the individual SNPs) were associated with ovarian cancer risk, a burden test was performed using the admixture likelihood (AML) method [22] to determine whether overall genetic variation in each gonadotropin signaling gene was associated with disease risk after accounting for the correlation between the SNPs in each gene.

The AML method postulates that within a set of SNPs, a given proportion α is associated with the outcome, and the effect size of each associated SNP will fall on a non-central χ^2 distribution with non-centrality parameter η. The η parameter is a measure closely related to that SNP's contribution to the genetic variance of the outcome variable. The AML method estimates values for α and η using a pseudo-maximum likelihood method. Due to a lack of independence among our SNPs, it is not possible to assess the significance of the estimated parameters by a simple likelihood ratio test so the AML method was used to assess significance by simulation instead.

We used the AMLcalc program (http://ccge.medschl.cam.ac.uk/software/aml) to perform the burden test using 1000 simulations with the maximum proportion of associated SNPs set to 0.2 on the genotyped and imputed data and adjusting for the first five ancestry principal components. p-Values for the AML trend test are provided.

Results

The number of SNPs analyzed for each gene and the number of SNPs statistically significantly associated with invasive ovarian cancer risk (at a nominal $p \leq 0.05$ significance level) are presented in Table 1. Neither *GNRH* nor *GNRHR* harbored a SNP associated with overall invasive ovarian cancer at the $p \leq 0.05$ level (Table 1). *LHCGR* had the most strongly associated single SNP, rs72618637 ($p = 0.001$; Table 1), but *FSHB* showed some evidence of a gene-level association ($p = 0.036$; Table 1). A borderline statistically significant gene-level association between *INHA* and invasive ovarian cancer was also observed ($p = 0.060$). However, when all SNPs across the 11 genes were considered together ("global"), no evidence of an association between genetic variation in the gonadotropin signaling pathway and invasive ovarian cancer risk was observed ($p = 0.33$).

Results of the most significant association for each gene by histological subtype are presented in Tables 2A and 2B. *FSHB* had the most strongly associated single SNP, rs7951733 ($p = 4.62 \times 10^{-5}$; Table 2B and Supplementary Fig. S1) across all histological subtypes, including overall invasive, and also showed suggestive evidence of a gene-level association with the invasive endometrioid subtype ($p = 0.063$). In addition, while genetic variation across the 11 genes did not show evidence of a global association with any of the four histological subtypes, there was evidence of gene-level associations between *LHCGR* and *GNRH* and the invasive high-grade serous subtype ($p = 0.046$ and $p = 0.041$, respectively) as well as between *INHBB* and the invasive mucinous subtype ($p = 0.045$). However, there was no evidence of any gene-level association with the invasive clear cell subtype.

Discussion

The gonadotropin hypothesis has been one of the leading hypotheses concerning ovarian cancer development. After evaluating this pathway, we found individual SNP associations at the $p \leq 0.05$ level, which is intriguing, but none was statistically significant after considerations for multiple comparisons. Rs7951733, which was an imputed SNP in our dataset ($r^2 = 0.95$) with a MAF = 0.08, was particularly interesting given its strong association with the endometrioid ovarian cancer subtype ($p = 4.62 \times 10^{-5}$, Supplementary Fig. S1). It is located approximately 5 kb downstream of the *FSHB* gene, which encodes the beta polypeptide of FSH, a gonadotropin involved in reproduction. However, the relevance of this SNP remains uncertain.

In this case where many nominally significant associations were observed, an alternative method for evaluating whether variation in this pathway is associated with risk is needed. A standard approach would be to attempt replication. However, to our knowledge, our analysis includes the majority of ovarian cancer cases and controls available worldwide for genetic studies. Hence, we conducted burden testing using the AML approach to evaluate evidence of gene-level associations. This method did provide some evidence of gene-level associations. A total of 55 burden tests were carried out (i.e., 11 genes × 5 types of ovarian cancer (overall invasive plus the four subtypes of ovarian cancer)), in which four significant associations (*FSHB*, *LHCGR*, *GNRH*, and *INHBB*) and one borderline significant association (*INHA*) were observed, compared to 2.8 expected by chance at the $p \leq 0.05$ level. This lends support to a possible role for genetic variation in this pathway with ovarian cancer risk.

Given that most ovarian cancer patients present at a postmenopausal stage when circulating FSH and LH levels are high and remain high due to the lack of negative feedback mechanisms by ovarian steroids, an association between chronically elevated gonadotropin levels and ovarian carcinogenesis has been suggested [23]. This is further supported by elevated gonadotropin levels in cyst and peritoneal fluid from ovarian cancer patients, although their relevance when the carcinogenesis has already occurred is uncertain [24]. Epidemiological evidence indirectly supporting the role of gonadotropins in ovarian carcinogenesis includes the well-established protective effects of pregnancies and OC use, which suppress gonadotropin secretion by the pituitary gland [25]. The gonadotropin hypothesis has also prompted substantial work on the potential risk-enhancing effects of infertility treatments, which include high-doses of gonadotropins to induce ovulation [26]. Hence, while gonadotropins alone may not reconcile all of the data pertaining to ovarian cancer etiology and progression, the evidence suggests that they are at least partly involved; it has been hypothesized that high gonadotropin levels could stimulate ovarian epithelial cells and initiate or promote tumorigenesis.

Table 1
Gene-level AML test and most significantly associated SNP (MAF[a] ≥ 0.05) in each gonadotropin signaling gene for all invasive ovarian cancers.

Gene	Coverage (at imputation $r^2 \geq 0.8$)	Number of SNPs	Number of significant SNPs (at ≤0.05)	AML burden test[b]	Most significant SNP	MAF[a]	Imputation r^2	OR[c]	p-Value
ACVR1	0.55	101	1	0.73	rs35160507	0.07	0.77	1.07	0.030
ACVR2	0.98	163	1	0.52	rs17742573	0.07	0.56	0.93	0.046
CGA	0.97	140	15	0.29	rs7745823	0.24	0.83	0.96	0.035
FSHB	0.99	111	18	0.036	rs12805742	0.22	0.94	1.04	0.018
FSHR	0.90	746	15	0.57	chr2:49204261:D	0.07	0.70	0.91	0.007
GNRH	1.00	108	0	0.65	N/A	–	–	–	–
GNRHR	0.94	126	0	0.59	N/A	–	–	–	–
INHA	0.86	81	15	0.060	rs12720063	0.21	1.00	0.95	0.007
INHBA	1.00	77	1	0.80	rs17776182	0.15	1.00	0.95	0.010
INHBB	0.82	109	22	0.098	rs11900747	0.06	0.55	1.13	0.004
LHCGR	0.97	423	52	0.091	rs72618637	0.19	0.68	1.07	0.001
Global	–	2185	140	0.33	–	–	–	–	–

Note: "N/A" reflects genes with no significant SNPs at p ≤ 0.05. "Global" refers to when all genes are considered.
[a] MAF = minor allele frequency.
[b] AML = admixture maximum likelihood, taking into account the first five ancestry principal components; p-values for trend reported.
[c] OR = per allele odds ratio, taking into account study set and the first five ancestry principal components.

It was previously suggested that variants in *FSHR* (rs6165 and rs6166) may affect susceptibility of women to ovarian cancer [15–17]. However, the results from three studies that investigated this were conflicting. Our analysis of 746 SNPs in this gene included these two variants, but neither was found to be associated with risk of ovarian cancer (p = 0.46 and p = 0.45, respectively).

Our results suggest that there may be other genes involved in the gonadotropin signaling pathway that might affect risk of developing ovarian cancer and specific subtypes of ovarian cancer as well. In addition, the molecular function of such genes makes their involvement in ovarian carcinogenesis biologically plausible. *GNRH* is predominantly responsible for the release of the pituitary gonadotropins, FSH and LH, which makes it a likely gene to be involved in this pathway. *INHBB* or inhibin beta B is a subunit of inhibin, which is produced by the granulosa cells of the ovary and has been found to be a possible marker of ovarian cancer [27]. The potential association we report with the mucinous ovarian cancer subtype is interesting given case reports describing a combination of granulosa cell tumor and mucinous cystadenoma of the ovary [28,29]. It joins the alpha subunit of inhibin (*INHA*) to form a pituitary FSH secretion inhibitor. *LHCGR* or luteinizing hormone/choriogonadotropin receptor produces a protein that acts as a receptor for two ligands: LH, which plays a role in ovulation, and chorionic gonadotropin, which is necessary for pregnancy. In addition, evidence suggests an association between genetic variability in *LHCGR* and risk of ovarian hyperstimulation syndrome [30]. *FSHB* encodes the beta polypeptide of FSH, which has long been thought to play a role in ovarian carcinogenesis due to well-established protective factors that suppress its secretion [25].

Despite three genome-wide scans and multiple large-scale genotyping efforts, only 18 genome-wide significant ovarian cancer susceptibility alleles have been identified, compared to 76 in breast and 77 in prostate [unpublished results, 31]. This is largely attributable to limited sample numbers available in each study, which emphasizes the importance of collaborative efforts, such as OCAC. In addition, because of limited numbers, new statistical methods are needed to better understand the role of genetics in ovarian cancer etiology. This study highlights one such method. By linking biology with burden testing, we were able to find some evidence of an association between the gonadotropin signaling genes *GNRH*, *INHBB*, *FSHB*, and *LHCGR* and ovarian cancer risk.

None of the individual SNP associations we observed showed genome-wide significance ($p = 5 \times 10^{-8}$) and are thus circumstantial. However, we applied burden testing to evaluate gene-level effects as well, and although this does not allow us to identify the specific disease-associated variant, it does shed some light on potential genes to focus on in future studies. In addition, although much of the genetic data used were imputed, we restricted our analyses to only SNPs with an imputation $r^2 \geq 0.5$ and a MAF ≥ 0.05, which is a common method used for filtering imputed data [32].

While these findings cannot be directly applied to a clinical setting, they demonstrate both a biological and genetic basis to the role of gonadotropins in ovarian carcinogenesis, which could impact how

Table 2A
Gene-level AML test and most significantly associated SNP (MAF[a] ≥ 0.05) in each gonadotropin signaling gene for invasive high-grade serous and mucinous ovarian cancers.

Gene	Coverage (at $r^2 \geq 0.8$)	Serous high grade (n = 6258)							Mucinous (n = 991)						
		No. of signif. SNPs	AML burden test[b]	Most significant SNP	MAF[a]	Imput. r^2	OR[c]	p-Value	No. of signif. SNPs	AML burden test[b]	Most significant SNP	MAF[a]	Imput. r^2	OR[c]	p-Value
ACVR1	0.55	0	0.47	N/A	–	–	–	–	1	0.76	rs17804523	0.07	0.67	0.79	0.042
ACVR2	0.98	1	0.66	rs57870939	0.05	0.60	1.13	0.038	6	0.38	rs11681013	0.08	0.74	1.23	0.025
CGA	0.97	0	0.69	N/A	–	–	–	–	1	0.50	chr6:87820107:I	0.29	0.89	1.11	0.039
FSHB	0.99	1	0.35	rs7925340	0.21	0.99	1.05	0.049	37	0.11	rs12577729	0.22	0.95	1.14	0.018
FSHR	0.90	31	0.52	rs116044731	0.07	0.80	0.87	0.003	45	0.33	rs12997920	0.39	0.88	1.13	0.010
GNRH	1.00	12	0.041	chr8:25283745:D	0.42	0.62	1.07	0.010	0	0.40	N/A	–	–	–	–
GNRHR	0.94	0	0.34	N/A	–	–	–	–	0	0.78	N/A	–	–	–	–
INHA	0.86	5	0.24	rs77120825	0.06	0.51	1.16	0.013	0	0.43	N/A	–	–	–	–
INHBA	1.00	2	0.52	rs17719440	0.08	0.98	1.11	0.008	0	0.55	N/A	–	–	–	–
INHBB	0.82	12	0.15	rs4328642	0.05	0.58	0.86	0.010	26	0.045	rs6542591	0.39	0.67	1.17	0.003
LHCGR	0.97	90	0.046	rs13426172	0.12	1.00	0.91	0.005	21	0.67	rs55871926	0.10	0.80	1.26	0.004
Global	–	154	0.24	–	–	–	–	–	137	0.54	–	–	–	–	–

Note: "N/A" reflects genes with no significant SNPs at p ≤ 0.05. "Global" refers to when all genes are considered.
[a] MAF = minor allele frequency.
[b] AML = admixture maximum likelihood, taking into account the first five ancestry principal components; p-values for trend reported.
[c] OR = per allele odds ratio, taking into account study set and the first five ancestry principal components.

Table 2B
Gene-level AML test and most significantly associated SNP (MAF[a] ≥ 0.05) in each gonadotropin signaling gene for invasive endometrioid and clear cell ovarian cancers.

Gene	Coverage (at $r^2 \geq 0.8$)	Endometrioid (n = 2148)							Clear cell (n = 1013)						
		No. of signif. SNPs	AML burden test[b]	Most significant SNP	MAF[a]	Imput. r^2	OR[c]	p-Value	No. of signif. SNPs	AML burden test[b]	Most significant SNP	MAF[a]	Imput. r^2	OR[c]	p-Value
ACVR1	0.55	2	0.47	chr2:158719453:D	0.06	0.85	1.14	0.042	0	0.87	N/A	–	–	–	–
ACVR2	0.98	1	0.25	rs17742573	0.07	0.56	0.84	0.045	12	0.21	rs55816333	0.42	0.97	0.89	0.018
CGA	0.97	0	0.95	N/A	–	–	–	–	0	0.58	N/A	–	–	–	–
FSHB	0.99	27	0.063	rs7951733	0.08	0.95	0.76	4.62×10^{-5}	0	0.46	N/A	–	–	–	–
FSHR	0.90	19	0.34	rs191446440	0.05	0.62	1.30	0.002	45	0.20	rs55926033	0.12	0.87	1.21	0.007
GNRH	1.00	0	0.13	N/A	–	–	–	–	1	0.35	chr8:25290793:D	0.09	0.55	1.23	0.038
GNRHR	0.94	2	0.53	rs148964181	0.13	0.55	1.12	0.045	3	0.31	rs17637021	0.17	0.52	1.18	0.026
INHA	0.86	3	0.14	rs6436158	0.40	0.52	0.91	0.042	0	0.86	N/A	–	–	–	–
INHBA	1.00	0	0.58	N/A	–	–	–	–	0	0.94	N/A	–	–	–	–
INHBB	0.82	15	0.16	rs12475606	0.47	0.55	0.90	0.016	2	0.70	rs4528762	0.26	0.60	0.81	0.003
LHCGR	0.97	8	0.65	rs4293599	0.20	1.00	1.12	0.005	9	0.69	rs3884614	0.07	0.66	1.29	0.005
Global	–	77	0.33	–	–	–	–	–	72	0.53	–	–	–	–	–

Note: "N/A" reflects genes with no significant SNPs at $p \leq 0.05$. "Global" refers to when all genes are considered.
[a] MAF = minor allele frequency.
[b] AML = admixture maximum likelihood, taking into account the first five ancestry principal components; p-values for trend reported.
[c] OR = per allele odds ratio, taking into account study set and the first five ancestry principal components.

researchers and clinicians view ovarian cancer etiology. Based on our results, the gonadotropin hypothesis would be worth re-evaluating when larger samples with denser genotyping chips become available so that there is more power with less reliance on imputation, allowing for fine mapping to be carried out.

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Conflict of interest statement

Andrew Berchuck serves on the PARP inhibitor advisory board for Astra Zeneca and Usha Menon owns shares of Abcodia Ltd., a biomarker validation company.

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