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14. ABSTRACT

The purpose of this project was to identify ovarian cancer susceptibility genes involved in stromal- epithelial cross talk. We have now genotyped 2138 samples for 1536 tagging, ns and miRNA binding site SNPs in 174 genes. Following Quality Control exclusions, the final dataset comprised 1839 samples (675 cases and 1164 controls) with genotype information for 1292 SNPs in 174 genes. We also genotyped 22 SNPs in 2985 cases and 2932 controls from six sites within the Ovarian Cancer Association Consortium, and the best 3 SNPs in PODXL (rs1013368), ITGA6 (rs13027811) and MMP3 (rs522616) in 7,139 cases and 7,851 controls from 12 sites in OCAC. None of these SNPs showed significant associations with ovarian cancer risk in pooled analysis, adjusted for study and age, under any model.

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INTRODUCTION

We proposed that subtle variation in the expression or function of genes expressed as a consequence of interactions between ovarian cancer cells and the host micro-environment could contribute to susceptibility to ovarian cancer. This idea was novel because this class of genes has not previously been tested for a role in ovarian cancer susceptibility. Our approach, and our choice of candidate genes, was based on extensive preliminary data we have accumulated from co-culture of fibroblast and epithelial ovarian cells. Our original aim was to identify all non-synonymous coding and putative promoter SNPs in 60 candidate genes highlighted by our analysis of cross talk between fibroblast and epithelial elements of ovarian tumors, as well as a set of tagging SNPs in 20 of these co-culture regulated genes which are altered in expression in serous tumours, compared with normal ovarian surface epithelial cells. However, at the start of this project we acquired an Illumina Bead Station and so we genotyped 1536 SNPs in the first stage, allowing us to genotype potentially functional as well as tagging SNPs in 174 genes of interest in 773 cases with invasive, serous ovarian adenocarcinoma, and 1365 controls. This task was followed by independent 'validation' of the most significant associations in up to 7,139 cases and 7,851 controls. None of these SNPs showed significant associations with ovarian cancer risk in pooled analysis, adjusted for study and age, under any model.

BODY

The statement of work was altered in December 2006 because we changed genotyping platforms in order to genotype many more SNPs, but with an altered the time frame. The tasks below are from the new SOW.

Task 1. *In silico* identification of SNPs in candidate genes (months 1-9)

1. *identification of 174 candidate genes involved in cross talk*

The original application proposed genotyping of candidate genes based on a series of *in vitro* experiments involving co-culture of ovarian epithelial and theca fibroblast cells. The genes were further prioritized based on elevated expression in two published ovarian cancer expression profiling studies, as well as an in house expression profile and we then generated a list of 255 candidate genes of interest.

2. *identification of 1536 tagging SNPs, nsSNPs and SNPs in putative microRNA binding sites in these 174 genes*

With Drs Ellen Goode and David Rider at the Mayo Clinic, and Illumina Inc., we then generated a list of SNPs within 5 kb of these 255 genes (58,114 SNPs in total). We then used the binning algorithm of LDSelect to identify 4567 tagSNPs among these, with (r2) $\geq$ 0.8 and minor allele frequencies (MAFs) > 0.05, using data from a variety of sources. Then we prioritized the list to 166 genes based on known function and the number of bins in each gene (excluding genes with a large number of bins), in an attempt to reduce the list to ~1500 SNPs.

We then requested from Illumina Inc the design scores for all SNPs within 5kb of these 166 genes and picked the best tagSNP in each bin (or two tagSNPs if there are

>10 tagging SNPs in a bin and none had an optimal design score).We also used www.patrocles.org to identify SNPs (with MAFs ≥ 0.05) in microRNA binding sites within these genes, and added nsSNPs (with MAFs ≥ 0.05) from the public databases to the potential SNP list. This identified 170 miRNA binding sites and nsSNPs with Illumina design scores > 0.6 in these 166 genes. In total this gave 1410 tagSNPs, miRNA binding site SNPs and nsSNPs, and so the list was supplemented by tag and supplemental SNPs in another 12 candidate genes, bringing the number of genes represented in the final list to 174, in which there were 1509 SNPs meeting the above criteria (some of the original 174 candidate genes had no appropriate SNPs in them). In order to reach the final total of 1536 SNPs for the Illumina OPA, the MAF of the supplemental SNPs was dropped to 0.01. The final list of 1536 SNPs included 106 supplemental SNPs and 1430 tagSNPs. The Illumina OPA for these 1536 SNPs was ordered in December 2006, and received early in February 2007.

Task 2. Genotyping of 900 cases and 1200 controls for 1536 SNPs using the Illumina Goldengate Assay (months 10-15)

While the design of the Illumina OPA was underway we completed the extraction and Quality Control of 1350 case and 1100 controls DNAs from the Australian Ovarian Cancer Study (AOCS), and the making of plates for Goldengate genotyping using cases and controls from both the AOCS and the Australian Cancer Study.

We have now genotyped 2138 samples for 1536 SNPs in 174 genes. There were 773 invasive serous cases from the Australian Ovarian Cancer Study (527), Australian Cancer Study (121) and Mayo Clinic (125), with 1365 controls from the same sources (893, 411 and 61 respectively). Insufficient DNA was available from the AOCS to achieve our original aim of genotyping 900 invasive serous cases, but additional power was obtained by using a larger number of controls.

Plates were prepared containing randomly mixed cases and controls, with two duplicated samples and one blank per plate. The Golden Gate assay was performed according to the manufacturer's instructions. Following completion of the assay for all 23 plates, analysis was carried out using Illumina BeadStudio software version 3.1.0.0. The following quality control measures were implemented:

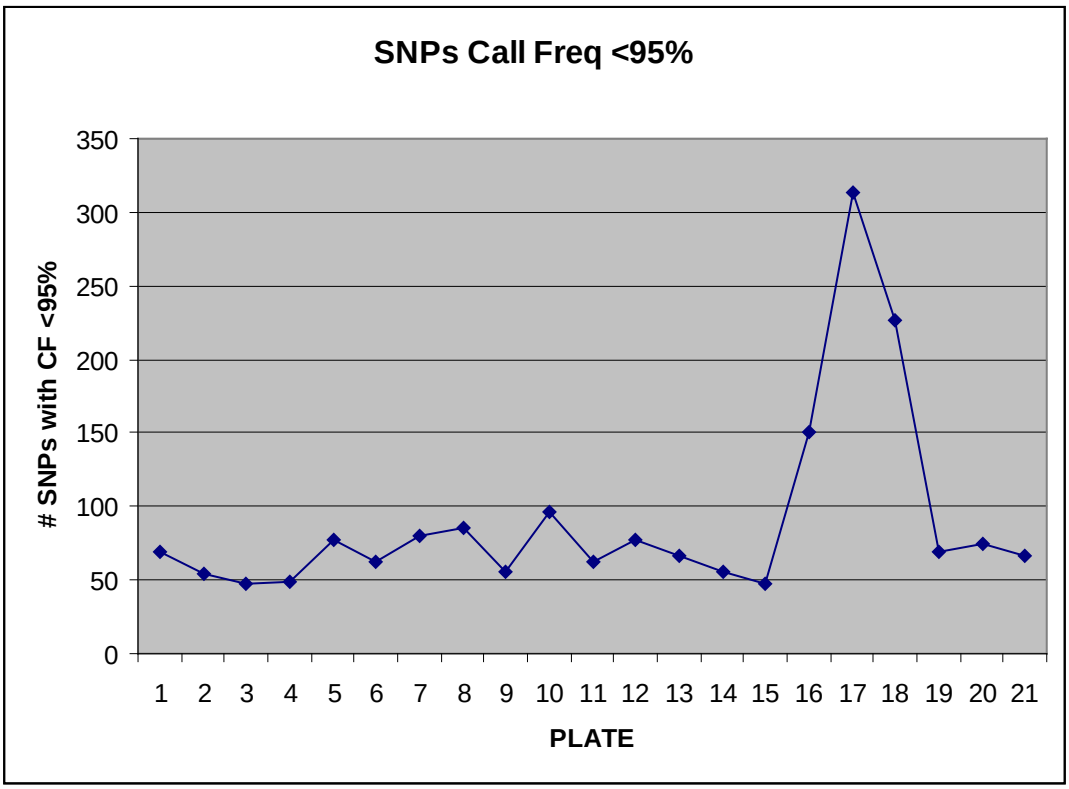
The original raw dataset contained genotype information for 2208 samples and 1536 SNPs. Following automatic clustering, SNPs were ranked using their "GenTrain" score, number between 0 and 1 indicating how well the samples clustered for this locus. SNPs with a low score were checked manually and re-clustered if possible. Subsequently all SNPs were checked for clustering quality.

Next, SNPs were filtered based on call rate with a call rate > 95% deemed as acceptable. Additional filter steps included removal of SNPs with a minor allele frequency of zero. Hardy Weinberg equilibrium was also tested for each SNP, and only those that passed a low threshold, with a p value > 0.0001, were included. SNPs with two or more discrepancies between duplicate pairs were excluded.

For sample quality control, a call rate threshold of 95% was used so that samples that failed for ≥95% SNPs were excluded which reduced the number of samples from 2208 to 2145. Analysis of signal intensities across all plates revealed three plates

(#16-18) with low intensity, just prior to the annual service of the laser. A separate analysis looking at call rates and concordance for each plate showed that these same plates failed quality control thresholds (Figure 1) and so they were omitted from further analysis.

Figure: Call frequencies for SNPs in each plate genotyped



The final dataset therefore comprised 1839 samples (675 cases and 1164 controls) with genotype information for 1292 SNPs in 174 genes. An analysis using the PLINK software package was then performed.

Task 3. **Genotyping of the AOCS/ACS test set for additional SNPs by Mass Array and statistical analysis of test set (months 16-21)**

1. *genotyping 900 cases and 1200 controls by Mass Array for 70 SNPs that were not amenable to Illumina genotyping in 13 key genes using 30-plexes*

- AOCS and ACS case (including non-serous invasive cases and LMP cases) and control DNAs were randomly plated in 8 x 384 well plates for iPLEX genotyping. We originally selected 174 genes for Golden Gate analysis. Many of these genes contain SNPs of interest that were either not amenable to the Golden Gate assay, or were genotyped on the OPA but failed quality control criteria. The genes of most *a priori* biological interest to us are *CXCL9*, *CTGF*, *LCN2*, *DCN*, and *VIL2*. In addition, we genotyped the AOCS samples for additional SNPs from our 'top hits' from the OPA analysis by iPLEX.

2. *statistical analysis of test set*

Analysis of the OPA data generated a list of SNPs for the Ovarian Cancer Association Consortium (OCAC) to genotype for further 'validation'. OCAC was founded in 2005 and is comprised of 21 groups from Australia, Europe and America (Gayther et al., 2007; Pearce et al., 2008).

All statistical analyses were conducted using the PLINK v0.99 Whole Genome Association Analysis toolset (http://pngu.mgh.harvard.edu/purcell/plink/) (Purcell et al., 2007). Of the 1536 SNPs genotyped using the using the Illumina Goldengate Assay, genotype data available for analysis consisted of a 1292 SNPs in a total of 1839 individuals following exclusions according to pre-determined quality control standards. Further quality control at the analytical level imposed by PLINK resulted in the exclusion of one SNP which failed the PLINK threshold of >10% of individuals with no genotype data, and three SNPs with a minor allele frequency (MAF) of <1%. Of the 1839 individuals with genotype data, three individuals were excluded by PLINK from all analyses because <10% of markers were successfully genotyped for these individuals. The final PLINK analysis data set consisted of a total of 1836 individuals for which genotype data on 1286 SNP were available. Summary statistics were obtained for each SNP on the frequency of missing genotype data among cases and controls as well as a comparison of missingness between cases and controls using the Fisher's exact test. A total of 37 (2.9%) SNPs had significantly different frequencies of missing genotype data between cases and controls ($p<0.05$). Deviations from expected Hardy Weinberg (HW) proportions were analyzed using the Fisher's exact test and minor allele frequencies (MAFs) were also estimated for all SNPs. A basic allelic association test for ovarian cancer and each SNP was conducted comparing allele frequencies in cases and controls. The odds ratio (ORs) and 95% confidence intervals (CI) generated by this analysis represents the risk of ovarian cancer associated with the minor allele (m) for each SNP, and the unadjusted p-values were derived from 2 x 2 tables of ovarian cancer (cases vs. controls) by allele (m vs. M) using the chi-square test on 1 degree of freedom (df). Additional tests for allelic association for each SNP were implemented in PLINK included the Cochran-Armitage Trend test (1df), the general genotypic association test (2df) of ovarian cancer (cases vs. control) by genotype (mm vs. Mm vs. MM), the dominant gene association test (1df) of ovarian cancer (cases vs. controls) by dominant genotype (mm/Mm vs. MM), and the recessive gene association test (1df) of ovarian cancer (cases vs. controls) by recessive genotype (mm vs. Mm/MM).

The following exclusion criteria were applied: SNPs with at least one failed duplicate, SNPs with a significantly different proportion of missing genotype data between cases and controls (P_{Miss} <0.05), SNPs not conforming to HW proportions (P_{HWE} <0.05) for either cases, controls or both, and SNPs with no significant trend in allelic dose response (P_{Trend} >0.05). From this list, we further estimated which SNPs are likely to be the best predictors of ovarian cancer (PPV) according to the p-values derived from the most robust test for allelic association i.e P_{Trend}, the power of the study to detect this association, and the prior probability of 0.0001. We selected SNPs for validation in Task 4 from this list.

Based on their Positive Predictive Values, the Ovarian Cancer Association Consortium decided to genotype three of these SNPs in *PODXL*, *ITGA6* and *MMP3*.

Task 4. **Genotyping of the replication set and statistical analysis of replication set (months 22-32)**

1. *genotyping 1200 cases and 3600 controls by Mass Array for 45-60 SNPs in 30-plexes, significantly associated with ovarian cancer risk in the test set (P< 0.001)*

We combined the iPLEX genotyping for Tasks 3 and 3 and genotyping was successfully completed on 2985 ovarian cancer cases and 2932 controls from six sites within the Ovarian Cancer Association Consortium (Gayther et al., 2007; Pearce et al., 2008). (AOCS/ACS, SEARCH (PI: Paul Pharoah), MALOVA (Estrid Hogdall), UKOPS (Simon Gayther), University of Southern California (Leigh Pearce) and Mayo Clinic (Ellen Goode)) by Mass Array for 22 SNPs in *CSF1, DDR2, FN1, ITGA6, ITGAV, LCN2, MMP26, MMP3, MMP7, PANX1, PLOD2, PODXL, PTEN, PTTG1,TERT* and *TIMP3.*

In addition, the three SNPs in *PODXL* (rs1013368), *ITGA6* (rs13027811) and *MMP3* (rs522616) were genotyped in 7,139 cases and 7,851 controls from 12 sites in OCAC.

2. *statistical analysis*

None of these 25 SNPs showed significant associations with ovarian cancer risk (P > 0.03) in pooled analysis, adjusted for study and age, under any model. The per allele adjusted OR for rs1013368 was 0.99 (95% CI 0.90-1.09), for rs13027811 was 0.97 (95% CI 0.85-1.09) and for rs522616 was 1.06 (95% CI 0.98-1.16).

In addition, we combined our data on 18 SNPs in *FGF2* (some of which showed a nominal association with ovarian cancer risk) with that of three other sites in OCAC from the United States who had genotyped 17 of these, and 7 additional ones. Analysis was restricted to non-Hispanic White women with serous ovarian carcinoma (1269 cases and 2829 controls). There were two nominally statistically significant associations between heterozygosity for two *FGF2* SNPs (rs308379 and rs308447; p<0.05) and serous ovarian cancer risk in the combined dataset, but rare homozygous estimates did not achieve statistical significance, nor were they consistent with the log additive model of inheritance. Overall genetic variation in *FGF2* does not appear to play a role in susceptibility to ovarian cancer.

Task 5. **DHPLC to identify putative functional SNPs in genes associated with serous invasive ovarian cancer in both the test and replication set (months 25-35)**

1. *design of DHPLC primers*
2. *DHPLC of coding and conserved regulatory regions of ~5 genes in 94 moderate familial risk ovarian cancer cases*

This task was not longer appropriate given the failure to validate our results in OCAC.

Task 6. **Functional evaluation of putative rSNPs (months 28-36)**

This task therefore was not undertaken.

Task 7. **Manuscript preparation (months 32-)**

The main manuscript is currently in preparation. In addition, we have submitted one on the analysis of SNPs in *FGF2.*

KEY RESEARCH ACCOMPLISHMENTS

We have genotyped 2138 samples (773 invasive, serous cases plus 1365 controls) for 1536 tagging, non-synonymous and miRNA binding site SNPs in 174 genes. Following Quality Control exclusions, the final dataset comprised 1839 samples (675 cases and 1164 controls) with genotype information for 1292 SNPs in 174 genes. The three SNPs in *PODXL* (rs1013368), *ITGA6* (rs13027811) and *MMP3* (rs522616) were genotyped in 7,139 cases and 7,851 controls from 12 sites in OCAC in an attempt to validate our positive results from the OPA. None of these SNPs (nor 22 others types in five of the OCAC sites) showed significant associations with ovarian cancer risk ($P > 0.03$) in pooled analysis, adjusted for study and age, under any model. Combined analysis of SNPs in *FGF2* with that from three other OCAC sites did not find any significant associations with ovarian cancer risk.

REPORTABLE OUTCOMES

Abstract presented at the AACR meeting on 'Approaches to complex pathways in molecular epidemiology' in Albuquerque in May 2007.

Talk presented at the annual kConFab/AOCS meeting in Queensland, Australia in August 2008.

Johnatty, S.E., Beesley, J., Chen, X., Spurdle, A.B., deFazio, A., Webb, P.M., Australian Ovarian Cancer Study Group, Australian Cancer Study (Ovarian Cancer), Goode, E.L., Rider, D.N., Vierkant, R.A., Anderson, S., Wu, A.H., Pike, M., Van Den Berg, D., Moysich, K., Ness, R., Doherty, J., Rossing, M-A., Pearce, C.L., Chenevix-Trench, G. Polymorphisms in the *FGF2* gene and risk of serous ovarian cancer: results from the Ovarian Cancer Association Consortium. *International Journal of Cancer* (submitted Feb 24th 2009) - attached

CONCLUSION

Although the results of this study have been disappointingly negative, they highlight the importance of consortium-based approaches to investigate putative ovarian cancer genetic associations given the need for large sample sizes. Without the ability to validate our 'positive' findings (most of which were of borderline significance) in OCAC, we would have published false-positive results, adding to the already flawed literature in this area. These data will complement those from Genome Wide Association Studies which do tag all genes with the same depth as we have for these 174 candidate genes.

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APPENDICES

Abstract from 2008 kConFab/AOCS conference:

Identification of epithelial ovarian cancer susceptibility genes involved in stromal-epithelial cross talk

Sharon E. Johnatty[1], Jonathan Beesley[1], Izhak Haviv[2], Anna deFazio[3], Natalie Gava[3], David N. Rider[4], Ellen L. Goode[4], Australian Cancer Study (Ovarian Cancer) [1], Australian Ovarian Cancer Study Group[1,2, 3], Penny Webb[1], Georgia Chenevix-Trench[1] and the Ovarian Cancer Association Consortium

1) Queensland Institute of Medical Research, Brisbane, Australia; 2) Peter MacCallum Cancer Center, Melbourne, Australia; 3) Westmead Institute for Cancer Research, University of Sydney at the Westmead Millennium Institute, Westmead Hospital Sydney, Australia; 4) Mayo Clinic, Rochester, Minnesota, USA

Several lines of evidence suggest a role for stromal cells in providing the oncogenic signal for the malignant transformation of epithelial cells. We therefore hypothesized that subtle variation in the expression or function of genes expressed as a consequence of interactions between ovarian cancer cells and the host micro-environment, and in genes differentially expressed in normal ovarian surface epithelial (OSE) cells compared with inclusion cysts (IC) and invasive serous ovarian tumours, could contribute to susceptibility to invasive serous ovarian cancer. Based on extensive preliminary data from co-culture of fibroblast and epithelial ovarian cells, we identified 1,536 tagSNPs, miRNA binding site SNPs and nonsynonymous SNPs with minor allele frequencies (MAFs) $\geq$ 0.05, from 174 candidate genes expressed in serous ovarian tumours as a result of cross talk between ovarian epithelial and stromal cells, of which a subset were also known to be differentially expressed between OSE cells and ICs/tumours. These were genotyped with the Illumina GoldenGate Assay™, and after quality control checks our final dataset consisted of 1282 SNPs genotyped in 675 invasive serous cases and 1164 controls from the Australian Ovarian Cancer Study, Australian Cancer Study and the Mayo Clinic. The risk of ovarian cancer associated with the minor allele for each SNP was estimated from a basic allelic association test for each SNP, as well as the Cochran-Armitage trend test.

The best predictors of ovarian cancer risk were SNPs in *PODXL, ITGA6* and *MMP3.* tgSNPs that had failed QC in these three genes were then genotyped by iPLEX, before SNPs were selected for validation. Three SNPs in *PODXL, ITGA6* and *MMP3* which had Positive Predictive Values of 33%, 4.5% and 4.1% respectively are currently being analysed in the whole of the Ovarian Cancer Association Consortium, as well as a further 19 SNPs in *PTTG1, CSF1, PLOD2, FGF2, TIMP3, DDR2, FN1, MMP7, PANX1, PTEN, ITGAV* and *LCN2* in a subset of OCAC studies (SEARCH, MALOVA, UKOPS and USC). Results of these validation studies will be presented.

List of personnel receiving pay from the research effort over the course of the project:

Jonathan Beesley
Xiaoqing Chen
Sharon Johnatty
Cameron Johnstone
Sibylle Kugler

Submitted to *International Journal of Cancer* 24th Feb 2009:

Polymorphisms in the *FGF2* gene and risk of serous ovarian cancer: results from the Ovarian Cancer Association Consortium

Sharon E. Johnatty[1], Jonathan Beesley[1], Xiaoqing Chen[1], Amanda B. Spurdle[1], Anna deFazio[3], Penelope M Webb[1], Australian Ovarian Cancer Study Group[1,3], Australian Cancer Study (Ovarian Cancer)[1], Ellen L. Goode[4], David N. Rider[4], Robert A. Vierkant[4], Stephanie Anderson[4], Anna H. Wu[5], Malcolm Pike[5], David Van Den Berg [6], Kirsten Moysich[7], Roberta Ness[8], Jennifer Doherty[9], Mary-Anne Rossing[9], Celeste Leigh Pearce[5], Georgia Chenevix-Trench[1]

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Key words: ovarian cancer, serous, basic fibroblast growth factor, polymorphisms, *FGF2*

Abbreviations: *FGF2*, fibroblast growth factor 2; OCAC, Ovarian Cancer Association Consortium; tgSNP, tagging single nucleotide polymorphism; MAF, minor allele frequency; OR, odds ratio; CI, confidence interval

Short Title: *FGF2* polymorphisms and ovarian cancer risk

Journal Category: Epidemiology

Grant support: National Health and Medical Research Council of Australia (199600, ACS study; GCT and PW), U.S. Army Medical Research and Materiel Command under DAMD17-01-1-0729, the Cancer Council Tasmania and Cancer Foundation of Western Australia (AOCS study); and NIH R01 CA122443, R01 CA112523 and R01 CA87538 (DOVE study; JAD and MAR); the Mayo Foundation (MAYO study; EG, DR, RV, SA). Some of this work was funded by the California Cancer Research Program grants 00-01389V-20170 and 2110200, U.S. Public Health Service grants CA14089, CA17054, CA61132, CA63464, N01-PC-67010 and R03-

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Statement on novelty and impact of this study

This is the first study to assess the association between tagging SNPs in the *FGF2* gene and serous ovarian cancer in a large study of US and Australian women. Our study showed no significant association between any of the 25 *FGF2* SNPs analyzed and risk of serous tumors among non-Hispanic White women, and highlights the value of large international consortia to thoroughly evaluate candidate susceptibility genes.

Abstract

Fibroblast growth factor (*FGF*)-2 (basic) is a potent angiogenic molecule involved in tumor progression, and is one of several growth factors with a central role in ovarian carcinogenesis. We hypothesised that common single nucleotide polymorphisms (SNPs) in the *FGF2* gene may alter angiogenic potential and thereby susceptibility to ovarian cancer. We analysed 25 *FGF2* tgSNPs using five independent study populations from the United States and Australia. Analysis was restricted to non-Hispanic White women with serous ovarian carcinoma (1269 cases and 2829 controls). There were no statistically significant associations between any *FGF2* SNPs and ovarian cancer risk. There were two nominally statistically significant associations between heterozygosity for two *FGF2* SNPs (rs308379 and rs308447; $p<0.05$) and serous ovarian cancer risk in the combined dataset, but rare homozygous estimates did not achieve statistical significance, nor were they consistent with the log additive model of inheritance. Overall genetic variation in *FGF2* does not appear to play a role in susceptibility to ovarian cancer. These results highlight the importance of consortium-based approaches to investigate putative ovarian cancer genetic associations given the need for large sample sizes.

Introduction

Ovarian cancer is the seventh leading cause of cancer mortality among women globally, accounting for 4.2% of cancer deaths[1]. Lethality of ovarian cancer is due in part to the absence of symptoms in the majority of cases who typically present with metastatic disease that has spread outside the pelvis[2]. The lack of practical screening methods and detectable symptoms in the early stages of tumor progression underscore the importance of a better understanding of the molecular aspects of disease to effective prevention and treatment[3]. Although the aetiology of ovarian cancer has not been fully elucidated, it is generally agreed that family history of ovarian or breast cancer is the most important risk factor for epithelial ovarian cancer[4]. Hereditary ovarian cancers occurring in breast/ovarian cancer families have been linked to mutations in the *BRCA1* and *BRCA2* genes, while cases occurring in association with Lynch syndrome have been linked to mutations in *MSH2* and *MLH1* [5, 6]. Given that only 3-5% of cases present as high-risk familial cases[3], it is plausible that several low-penetrance genes with relatively common alleles may account for a portion of the increased risk.

Fibroblast growth factor (*FGF*)-2 (basic) has been localized to 4q26-q27 and is a member of a large family of structurally related proteins that affect the growth and differentiation, migration and survival of a wide variety of cell types. It is highly conserved among eukaryotes with sequence homology of >90% across a wide range of species[7]. FGF2 is a potent angiogenic molecule and has been shown to induce migration and proliferation of endothelial cells which differentiate into new vascular structures[8]. Inactivation of FGF2 *in vivo* has been shown to suppress tumor growth through the inhibition of FGF2-induced angiogenesis[9]. Elevated levels of urinary FGF2 were shown to correlate significantly with metastatic disease in a wide range of cancers including ovarian tumors[10]. Expression studies in ovarian cancer cell lines have also demonstrated significant increases in mRNA expression of the FGF2 receptor, as well as dose-dependent increased cell numbers in response to exogenous stimulation by FGF2[11]. In addition, gene expression profiling of advanced ovarian tumors indicates that FGF2 signalling plays a central role throughout the carcinogenesis process[12].

We hypothesised that common single nucleotide polymorphisms (SNPs) in the *FGF2* gene may alter the angiogenic potential of FGF2 and thereby susceptibility to ovarian cancer. While there is much evidence that *FGF2* is functionally relevant to tumor development and metastasis, to the best of our knowledge no study to date has assessed common variations in this gene for a possible association with ovarian cancer susceptibility. The current study evaluates twenty-five *FGF2* SNPs for an association with ovarian cancer risk, and represents a collaborative effort using data from five case-control studies from the United States and Australia, all participants in the Ovarian Cancer Association Consortium (OCAC)[13]. OCAC is an international collaboration established to provide a forum for researchers to evaluate genetic associations with ovarian cancer with increased power.

Material and methods

Study Population

Details of study design, and case and control ascertainment for each study included in this analysis are summarized in Table 1. A total of five ovarian cancer case-control

studies contributed data to this analysis, four of which used population-based ascertainment methods and one (MAYO) that was clinic-based (Table 1). Individuals with missing data on tumor behaviour, histology or race, and controls with prior oophorectomies, were excluded from the analysis. The final combined dataset comprised 1457 serous invasive cases and 3137 controls, the majority of whom were reported to be non-Hispanic Whites. All studies have been previously described elsewhere[14-18]. Approval from respective human research ethics committees was obtained, and all participants provided written informed consent.

Single Nucleotide Polymorphism Selection and Genotyping

Genotype data for this analysis was obtained from two 1536-SNP Illimina Golden Gate Assays™ conducted at two OCAC centers: AOCS-ACS and MAYO samples were genotyped at the Queensland Institute of Medical Research (QIMR), Queensland, Australia; DOVE, HOPE and USC samples were genotyped at the University of Southern California (USC) Epigenome Center, California, USA. Genotyping was conducted according to customized GoldenGate genotyping procedures (Illumina Inc.).

At QIMR we examined genotypes within 5 kb of *FGF2* (June 2006) from the projects of the HapMap Consortium[19], Perlegen[20], NIEHS SNPs, and SeattleSNPs {http://pga.mbt.washington.edu/} and found HapMap to be the most informative for European-American samples using the binning algorithm of ldSelect[21] to identify tagging SNPs (tgSNPs) for SNPs with $r^2 \geq 0.8$ and minor allele frequencies (MAFs) > 0.05. Fifty-eight SNPs were sorted into 20 bins, yielding 20 tgSNPs, 2 of which failed assay conversion. At USC we selected tgSNPs for *FGF2* (including putative regulatory regions 20kb up and 10kb downstream of the gene) using the program SNAGGER[22]. We attempted to tag all SNPs in HapMap (Release #21 July 2006) in the CEU population with a MAF of 0.05 or greater with an $r^2 \geq 0.8$.

A total of 25 *FGF2* SNPs were selected across both collaborations, 17 of which were genotyped for all studies, one was genotyped for the AOCS-ACS and MAYO studies only and seven were genotyped for DOVE, HOPE and UCS studies only (Table 2). The performance of our selected tgSNPs in capturing known common variation across the *FGF2* gene was evaluated using Tagger[23] implemented in Haploview[24]. We estimate that 97% of the known common variants (MAF $\geq$ 0.05) across the *FGF2* locus (including 20kb 5' and 10 kb 3' of the gene) have been captured by these SNPs.

Samples with call rates below 95% (or 90%), and SNPs with call rates below 98% (or 95%), were excluded at QIMR (and USC). At QIMR, SNPs with GenTrain scores < 0.5 were manually checked and adjusted according to Illumina guidelines; all SNPs were manually checked at USC. Greater than 97% and 93% at of SNPs passed this initial quality assurance at QIMR and USC respectively. Two samples per 96 well plate were blindly duplicated (n=20). One inter- and one intra-plate duplicate samples were included on each plate to assist with genotype calling and ensuring against plate flips. In addition, 128 blinded duplicate samples were included at USC. Genotyping quality was also assessed using tests for Hardy-Weinberg equilibrium (HWE). SNPs with significant deviations from HWE in controls ($0.001<P<0.05$) were assessed and the genotype data was excluded if the clustering was found to be suboptimal. SNPs with HWE $P<0.001$ were excluded from the analysis. Overall, >84% and 91% of

SNPs passed all quality assurance criteria at QIMR and USC, respectively.

Statistical Analysis

Case-control analyses were restricted to White non-Hispanic women with serous invasive ovarian tumours. White women participating in Australian studies were assumed to be non-Hispanic. Genotype frequencies in non-Hispanic White controls for each *FGF2* SNP were assessed for departure from HWE using the χ^2 goodness-of-fit test. Each of the five contributing case-control studies was assessed for differences in age at interview among controls and age at diagnosis among cases using Student's *t*-test for comparison of means. The MAF for each SNP was estimated from the control population for each study.

The combined odds ratios (ORs) and their 95% confidence intervals (95% CIs) were obtained from unconditional logistic regression models. Assuming a log additive model of inheritance, the per-allele risks associated with serous invasive tumors among non-Hispanic Whites for each of the 25 *FGF2* SNP were estimated by fitting the number of rare alleles carried as a continuous covariate. All estimates were adjusted for study site, and age at diagnosis for cases or age at interview for controls. All tests for association were two-tailed and statistical significance was assessed at $p<0.05$ using STATA v. 9.0 (StataCorp, USA).

Results

Details of study design, and case and control ascertainment for each contributing study are summarized in Table 1. Genotype data across the different studies met the minimum quality assurance measure for inclusion in the analysis, with the exception of rs17473132 SNP which was out of HWE in the USC study (p=0.0002), resulting in the exclusion of 374 genotypes for this SNP from the final dataset. Cases were significantly more likely to be older than controls (p <0.0001), and ranged in age at diagnosis from 23.6 to 86 years (mean age 60.1 ± 10.3) while controls ranged in age at interview from 19.2 to 91 years (mean age 56.9 ± 11.2; see Table 1).

Estimates for the 25 *FGF2* SNPs and risk of invasive serous tumors were calculated among non-Hispanic White women, based on genotype data from a combined total of 1269 serous invasive cases and 2829 controls genotyped at both sites (Table 2). None of the 25 SNPs analysed were significantly associated with risk of ovarian cancer although, without correcting for multiple testing, two SNPs showed borderline evidence of an association. The per-allele estimate for the rs308447 SNP showed a borderline significant inverse association with serous tumors [$OR_{per\text{-}allele}$ = 0.87 (0.76 - 1.00), p = 0.04]. However, although the heterozygous estimate supports an association [OR_{Het} = 0.72 (0.59 – 0.87) p = 0.001], the odds ratio for rare homozygotes was neither statistically significant (p>0.4) nor consistent with the log additive model of inheritance. Similarly the rs308379 SNP was inversely associated with serous tumors among heterozygotes [OR_{Het} = 0.85 (0.74 – 0.98) p = 0.03] but no equivalent association was observed among rare homozygotes (p= 0.59), nor were the estimates consistent with the log additive model of inheritance (Table 2). These observations are likely to be due to chance alone, and we therefore conclude that there is no association between any of these 25 SNPs in *FGF2* and risk of invasive serous ovarian cancer in non-Hispanic White women.

Discussion

FGF2 is a potent angiogenic molecule that has been shown to promote tumour cell mitosis and has been implicated in the differentiation of stromal and epithelial cells from a dormant to an invasive phenotype[25]. We have evaluated the effects of 25 SNPs in the *FGF2* gene on the risk of invasive serous ovarian cancer in non-Hispanic White women enrolled in five case-control studies from the United States and Australia, and found no convincing evidence of an association of any *FGF2* SNPs with serous ovarian tumors in our combined dataset. We acknowledge that the potential for variation in estimates is inherent in analyses involving samples from different countries, given the likelihood of differences in case-control selection criteria and population differences attributable to environmental factors or genetic background. However, all contributing studies included in our analysis selected controls from the same source population as cases, participants were predominantly non-Hispanic White (Table 1), and indeed there was no evidence of heterogeneity between the studies (non-Hispanic Whites only) for any of the SNPs included in this analysis ($P_{\text{Heterogeneity}} \geq 0.14$).

The human *FGF2* gene encompasses 71.53 kb of genomic sequences on chromosome 4. Using Hapmap SNP genotype frequency data for *FGF2* SNPs, we estimated that the 25 SNPs presented in this report capture 97% of the known common variation (MAF $\geq$ 0.05) across the *FGF2* locus at $r^2 \geq 0.8$ for pairwise correlations. To the best of our knowledge, this is the first study to evaluate *FGF2* SNPs in a large multi-center study. Based on the method of Purcell et al [26] we estimated that we had $\geq$80% power to detect ORs of 1.20 at an alpha of 0.05 for the 19 SNPs with MAFs $\geq$0.1 (Table 2). However, we acknowledge that we had considerably less power to detect these effect sizes with the six SNPs with MAFs < 0.1.

Our study highlights the importance of consortium-based approaches to investigating putative genetic association in case-control analyses, particularly for low-risk genes that require large sample sizes to detect small SNP effects. We note that three SNPs, in addition to the rs308447, achieved the minimal level of significance of $p \leq 0.05$ in study-specific per-allele estimates (data not shown), but not in the combined analysis. If we had reported the results of these individual case-control studies, it may have led other groups to attempt replication but our combined analysis provides a more accurate assessment of these associations and reduces publication bias.

FGF2 has been the focus of a plethora of studies into human tumor biology and has important implications for cancer therapies and clinical outcomes. FGF2 is one of several fibroblast growth factor molecules that interact with various vascular endothelial growth factors and cell surface receptors that are known to play a role in tumor growth and angiogenesis[27, 28]. The correlation between angiogenesis and the extent of metastatic disease has been widely demonstrated in a large and diverse range of human cancers[29-31] including advanced stage ovarian carcinoma[32, 33]. Abnormally high concentrations of FGF2 have been found in the serum of patients with active metastatic cancers and have been shown to correlate significantly with extent of disease, clinical status and risk of future mortality[10]. These findings would support the assessment of *FGF2* polymorphisms with regard to ovarian cancer survival and prognosis in future studies. To date several functional angiogenic gene SNPs have been studied in solid cancers with varying results derived from sample sizes that are

too small to detect the modest effects anticipated from these low penetrance genes[34]. Large-scale epidemiologic studies of other genes involved in angiogenesis are therefore warranted to further enhance our understanding of tumor progression. This could lead to novel approaches to risk stratification or the use of anti-angiogenic treatment strategies, if angiogenic potential, and hence prognosis, can be predicted according to individual genotype.

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Table 1: Description of study populations according to contributing OCAC study

Study (Location)[Ref]	Case ascertainment	Cases				Control ascertainment	Controls	
		Total Number[1]	Age[2] (Mean ± Std. Dev.)	[4]Primary Site (ovary/tubal/ primary peritoneal)	[5]FIGO Stage (I/II/III/IV)		Total Number[1]	Age[2] (Mean ± Std. Dev.)
AOCS-ACS (Australia)[14]	Surgical treatment centres throughout Australia, and cancer registries of Queensland, South and Western Australia, New South Wales and Victoria;	549 (476)	60.4 (±10.1)	381/22/73	33/28/333/56	Population based: Commonwealth Electoral roll	1,028 (946)	57.4 (±11.6)
MAYO (USA)[15]	Cases attending the Mayo Clinic from six surrounding states	124 (124)	*63.0 (±11.9)	124/0/0	8/3/85/27	Clinic based: Women seeking general exams at Mayo Clinic	60 (60)	*62.5 (±12.5)
DOVE (USA)[16]	Cancer Surveillance System, SEER[3]	298 (274)	59.0 (±8.6)	298/0/0	n/a	Population based: Random digit dialling	726 (652)	55.9 (±9.5)
HOPE (USA)[17]	Registries, physician offices, pathology databases	168 (161)	60.1 (±11.3)	168/0/0	n/a	Population based: Random digit dialling	702 (671)	57.6 (±10.3)
USC (USA)[18]	Los Angeles Cancer Surveillance Program	318 (234)	59.3 (±10.9)	318/0/0	n/a	Population based: Neighbourhood recruits	621 (500)	55.7 (±12.9)
Totals	---	**1457 (1269)**	**60.1 (±10.3)**			---	**3137 (2829)**	**56.9 (±11.2)**

1: All serous invasive cases with genotype data available for analysis, with the number of non-Hispanic White in parentheses

2: Age of Non-Hispanic White serous cases (age at diagnosis) and controls (age at interview); mean and standard deviation based on total number; * no significant difference ($p \leq 0.05$) between mean age of cases and controls
3: SEER, Surveillance Epidemiology and End Results
4. Primary site of tumor among non-Hispanic White case (numbers may not sum to N because of missing data)
5. Stage of tumor among non-Hispanic White case (numbers may not sum to N because of missing data)
n/a – not available

Table 2: Risk estimates for the 25 *FGF2* SNPs among non-Hispanic White women with serous carcinoma

SNPid			Heterozygotes				Rare Homozygotes				Per-Allele			
	MAF[1]	Controls/Cases[2]	OR[3]	95%	CI	P-value	OR[3]	95%	CI	P-value	OR[3]	95%	CI	P-value
rs10003827[4]	0.14	1,823 667	0.82	(0.66	- 1.02)	0.08	1.00	(0.52	- 1.92)	0.99	0.87	(0.72	- 1.05)	0.14
rs10452197	0.14	2,816 1,265	0.99	(0.84	- 1.16)	0.87	1.37	(0.88	- 2.13)	0.16	1.04	(0.91	- 1.19)	0.55
rs11737764[4]	0.09	1,821 668	1.15	(0.91	- 1.45)	0.24	0.82	(0.30	- 2.26)	0.71	1.10	(0.89	- 1.36)	0.37
rs11938826	0.16	2,818 1,256	0.95	(0.81	- 1.11)	0.49	0.82	(0.53	- 1.29)	0.39	0.94	(0.82	- 1.07)	0.32
rs12506776	0.17	2,823 1,266	0.91	(0.78	- 1.06)	0.25	0.93	(0.63	- 1.38)	0.71	0.93	(0.82	- 1.06)	0.27
rs1476214	0.38	2,821 1,267	0.95	(0.82	- 1.09)	0.45	0.90	(0.73	- 1.10)	0.29	0.95	(0.86	- 1.04)	0.26
rs1476217[4]	0.37	1,822 669	0.84	(0.69	- 1.02)	0.08	0.88	(0.67	- 1.15)	0.35	0.91	(0.80	- 1.04)	0.16
rs 167428	0.27	2,826 1,267	1.03	(0.90	- 1.19)	0.64	0.94	(0.72	- 1.22)	0.63	1.00	(0.90	- 1.11)	0.96
rs17407577	0.06	2,828 1,269	0.89	(0.72	- 1.10)	0.29	2.63	(0.80	- 8.67)	0.11	0.95	(0.78	- 1.16)	0.61
rs17473132	0.07	2,536 1,159	1.20	(0.98	- 1.46)	0.08	0.18	(0.02	- 1.40)	0.10	1.11	(0.92	- 1.35)	0.26
rs1960669	0.14	2,825 1,267	0.93	(0.79	- 1.09)	0.39	1.16	(0.72	- 1.88)	0.53	0.97	(0.85	- 1.12)	0.69
rs308379	0.39	2,825 1,268	**0.85**	**(0.74**	**- 0.98)**	**0.03**	0.94	(0.77	- 1.16)	0.59	0.94	(0.85	- 1.04)	0.22
rs308382	0.16	2,822 1,269	0.92	(0.78	- 1.07)	0.26	1.18	(0.78	- 1.77)	0.43	0.97	(0.85	- 1.10)	0.63
rs308420	0.09	2,819 1,265	0.97	(0.81	- 1.17)	0.76	1.43	(0.71	- 2.89)	0.32	1.01	(0.86	- 1.20)	0.89
rs308428	0.14	2,826 1,268	0.99	(0.84	- 1.15)	0.86	0.95	(0.58	- 1.54)	0.82	0.98	(0.86	- 1.13)	0.80
rs308435[4]	0.15	1,822 669	0.87	(0.71	- 1.07)	0.20	1.14	(0.68	- 1.93)	0.62	0.94	(0.79	- 1.11)	0.46
rs308439	0.04	2,805 1,256	0.97	(0.76	- 1.24)	0.83	na	na	na	na	0.92	(0.73	- 1.17)	0.51
rs308441	0.20	2,821 1,266	1.03	(0.89	- 1.19)	0.69	0.93	(0.65	- 1.33)	0.70	1.00	(0.89	- 1.13)	0.94
rs308443	0.03	2,823 1,266	0.96	(0.71	- 1.28)	0.77	na	na	na	na	0.91	(0.68	- 1.21)	0.51
rs308447[4]	0.38	1,819 667	**0.72**	**(0.59**	**- 0.87)**	**0.001**	0.88	(0.67	- 1.16)	0.37	**0.87**	**(0.76**	**- 1.00)**	**0.04**
rs3789138[5]	0.42	996 598	1.13	(0.89	- 1.42)	0.32	0.89	(0.65	- 1.21)	0.44	0.97	(0.84	- 1.13)	0.70
rs3804158	0.45	2,819 1,255	1.01	(0.86	- 1.18)	0.92	0.92	(0.76	- 1.11)	0.37	0.96	(0.88	- 1.06)	0.42
rs6819187[4]	0.44	1,821 667	0.89	(0.73	- 1.09)	0.27	0.92	(0.71	- 1.19)	0.51	0.95	(0.84	- 1.08)	0.42
rs7694627[4]	0.17	1,823 669	0.97	(0.79	- 1.18)	0.76	1.01	(0.59	- 1.74)	0.96	0.98	(0.83	- 1.16)	0.83
rs7700205	0.17	2,825 1,268	1.03	(0.89	- 1.19)	0.72	1.05	(0.71	- 1.54)	0.81	1.03	(0.91	- 1.16)	0.69

"na" represents SNPs with insufficient homozygote numbers for calculation of risk estimates; bold indicates $p < 0.05$

1: Minor allele frequency estimated from control population
2: Sample sizes reflect differences in genotype data available for analysis and exclusions based on HWE threshold
3: Odds Ratios (ORs) are adjusted for study and age (at interview for controls; at diagnosis for cases). Reference genotypes for case-control comparisons are common homozygotes
4: Indicates SNPs genotyped for DOVE, HOPE and USC studies only
5: Indicates SNPs genotyped for AOCS-ACS study only