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Citation

Published Version
doi:10.1371/journal.pgen.1001016

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Evaluation of Candidate Stromal Epithelial Cross-Talk Genes Identifies Association between Risk of Serous Ovarian Cancer and TERT, a Cancer Susceptibility “Hot-Spot”

Sharon E. Johnatty1•9, Jonathan Beesley1•, Xiaqing Chen1, Stuart Macgregor1, David L. Duffy1, Amanda B. Spurdle1, Anna deFazio2, Natalie Gava2, Penelope M. Webb1, Australian Ovarian Cancer Study Group1,2,3, Australian Cancer Study (Ovarian Cancer)1, Mary Anne Rossing4, Jennifer Anne Doherty5, Marc T. Goodman5, Galina Lurie5, Pamela J. Thompson5, Lynne R. Wilkens5, Roberta B. Ness6, Kirsten B. Moysich7, Jenny Chang-Claude8, Shan Wang-Gohrke9, Daniel W. Cramer10, Kathryn L. Terry10, Susan E. Hankinson11,12, Shelley S. Tworoger11,12, Montserrat Garcia-Closas13, Hannah Yang13, Jolanta Lissowska14, Stephen J. Chanock15, Paul D. Pharoah16, Honglin Song16, Alice S. Whittemore17, Celeste L. Pearce18, Daniel O. Stram18, Anna H. Wu18, Malcolm C. Pike18, Simon A. Gayther19, Susan J. Ramus19, Usha Menon19, Aleksandra Gentry-Maharaj19, Hoda Anton-Culver20, Argyrios Ziogas20, Estrid Hogdall21, Susanne K. Kjaer21,22, Claus Hogdall22, Andrew Berchuck23, Joellen M. Schildkraut23, Edwin S. Iversen23, Patricia G. Moorman23, Catherine M. Phelan24, Thomas A. Sellers24, Julie M. Cunningham25, Robert A. Vierkant25, David N. Rider25, Ellen L. Goode25, Izhak Haviv26,27, Georgia Chenevix-Trench1, Ovarian Cancer Association Consortium *

1 Queensland Institute of Medical Research, Brisbane, Australia, 2 Department of Gynaecological Oncology and Westmead Institute for Cancer Research, University of Sydney at the Westmead Millennium Institute, Westmead Hospital, Sydney, Australia, 3 Peter MacCallum Cancer Centre, East Melbourne, Australia, 4 Epidemiology Program, Division of Public Health Services, Fred Hutchinson Cancer Research Center, Seattle, Washington, United States of America, 5 Cancer Research Center of Hawaii, University of Hawaii, Hilo, Hawaii, United States of America, 6 University of Texas School of Public Health, Houston, Texas, United States of America, 7 Roswell Park Cancer Center, Buffalo, New York, United States of America, 8 Unit of Genetic Epidemiology, Division of Cancer Epidemiology, Deutsches Krebsforschungszentrum, Heidelberg, Germany, 9 Department of Obstetrics and Gynecology, University of Ulm, Ulm, Germany, 10 Obstetrics and Gynecology Epidemiology Program, Division of Cancer Epidemiology and Genetics National Cancer Institute, Krish, Maryland, United States of America, 11 Channing Laboratory, Department of Medicine, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts, United States of America, 12 Department of Epidemiology, Harvard School of Public Health, Boston, Massachusetts, United States of America, 13 National Cancer Institute, Division of Cancer Epidemiology and Genetics, Rockville, Maryland, United States of America, 14 Department of Cancer Epidemiology and Prevention, The M. Sklodowska-Curie Cancer Center and Institute of Oncology, Warsaw, Poland, 15 Laboratory of Translational Genomics Division of Cancer Epidemiology and Genetics National Cancer Institute, Bethesda, Maryland, United States of America, 16 Department of Oncology and Public Health and Primary Care, University of Cambridge, Cambridge, United Kingdom, 17 Department of Health Research and Policy, Stanford University School of Medicine, Stanford, California, United States of America, 18 Department of Preventive Medicine, Keck School of Medicine, University of Southern California Norris Comprehensive Cancer Center, Los Angeles, California, United States of America, 19 Department of Gynaecological Oncology, UCL EGA Institute for Women’s Health, University College London, London, United Kingdom, 20 Department of Epidemiology, School of Medicine, University of California Irvine, Irvine, California, United States of America, 21 Department of Virus, Hormones, and Cancer, Institute of Cancer Epidemiology, Danish Cancer Society, Copenhagen, Denmark, 22 Gynaecologic Clinic, The Juliane Marie Centre, Righospitalet, University of Copenhagen, Copenhagen, Denmark, 23 Division of Preventive Medicine, The Duke Comprehensive Cancer Center, Durham, North Carolina, United States of America, 24 Division of Cancer Prevention and Control, H. Lee Moffitt Cancer Center and Research Institute, Magnus Drive, Tampa, Florida, United States of America, 25 Department of Health Sciences Research, Mayo Clinic College of Medicine, Rochester, Minnesota, United States of America, 26 The Blood and DNA Profiling Facility, Baker IDI, Melbourne, Australia, 27 Department of Biochemistry, School of Medicine, University of Melbourne, Parkville, Australia

Abstract

We hypothesized that variants in genes expressed as a consequence of interactions between ovarian cancer cells and the host micro-environment could contribute to cancer susceptibility. We therefore used a two-stage approach to evaluate common single nucleotide polymorphisms (SNPs) in 173 genes involved in stromal epithelial interactions in the Ovarian Cancer Association Consortium (OCAC). In the discovery stage, cases with epithelial ovarian cancer (n = 675) and controls (n = 1,162) were genotyped at 1,536 SNPs using an Illumina GoldenGate assay. Based on Positive Predictive Value estimates, three SNPs—PODXL rs1013368, ITGA6 rs13027811, and MMP3 rs522616—were selected for replication using TaqMan genotyping in up to 3,059 serous invasive cases and 8,905 controls from 16 OCAC case-control studies. An additional 18 SNPs with Pper-allele < 0.05 in the discovery stage were selected for replication in a subset of five OCAC studies (n = 1,233 serous invasive cases; n = 3,364 controls). The discovery stage associations in PODXL, ITGA6, and MMP3 were attenuated in the larger replication set (adj. Pper-allele < 0.5). However genotypes at TERT rs7726159 were associated with ovarian cancer risk in the smaller, five-study replication study (Pper-allele = 0.03). Combined analysis of the discovery and replication sets for this TERT SNP showed an increased risk of serous ovarian cancer among non-Hispanic whites (adj. ORper-allele 1.14 (1.04–1.24) p = 0.003). Our study adds to the growing evidence that, like the 8q24 locus, the telomerase reverse transcriptase locus at 5p15.33, is a general cancer susceptibility locus.
Introduction

Ovarian cancer is the seventh leading cause of cancer mortality among women globally, accounting for 4.2% of cancer deaths [1], due in part to the lack of practical screening methods and detectable symptoms in the early stages of tumor progression [2]. 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 [3]. Hereditary ovarian cancer occurring in breast/ovarian cancer families has 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 [4,5]. Given that only 3% to 5% of ovarian cancer cases present from high-risk families and residual family history associations [2], it is likely that several low-penetrance genes with relatively common alleles that confer slightly increased risk may account for a portion of the risk of non-familial ovarian cancer. The Ovarian Cancer Association Consortium (OCAC) was established in 2005 to provide a forum for the identification and validation of common low-penetrance ovarian cancer susceptibility polymorphisms with increased power [6]. OCAC recently conducted a genome-wide association study (GWAS) and identified the first susceptibility locus associated with invasive ovarian cancer risk [7].

A number of hypotheses have been put forward to explain the pathogenesis of ovarian cancer [8,9], including that of incessant ovulation which causes repeated minor trauma to the surface of the ovary, leading to proliferation of ovarian epithelium and repair of the ovulatory wound [10]. However, it has also been hypothesized that fallopian tube epithelial cells migrating to the ovulatory wound could serve as precursors to ovarian cancer [11]. Research in the past two decades compellingly suggests that the neighbors of cancer cells, collectively termed stroma, are not uninvolved bystanders [12] and studies involving three-dimensional cell culture models underscore the involvement of the extracellular matrix surrounding cancer cells in the signalling pathways that promote cell survival [13]. Fibroblasts with a carcinoma-promoting phenotype [carcinoma-associated fibroblasts (CAFs)] residing in the breast cancer microenvironment lack the ability of normal fibroblasts to attenuate the growth of neighbouring transformed epithelial cells [14]. In addition, xenograft models have shown that CAFs accelerate cancer progression through their ability to secrete stromal cell-derived factor 1 [15]. Furthermore, expression profiling of ovarian tumor samples has identified a group of high-grade invasive cancers characterized by a reactive stromal gene expression signature and extensive desmoplasia, which confer an inherently poor prognosis [16]. If this CAF-dependent model of tumorigenesis is correct, it assigns a key role to the neighboring stroma in cancer initiation.

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 microenvironment could contribute to ovarian cancer susceptibility. We used a two-stage approach to comprehensively evaluate common variation in 173 genes selected for their putative role in stromal-epithelial interactions using a tagging-SNP approach and data from sixteen case-control studies participating in the Ovarian Cancer Association Consortium (OCAC).

Results

Candidate gene selection and justification are provided in Text S1 and Table S1. Characteristics of all case-control studies that contributed data to discovery and replication analyses are provided in Table S2. Comparison of the mean age at diagnosis for cases and age at interview for controls showed that cases were significantly older compared to controls (p<0.05). Figure S1 provides an overview of SNP and cases-controls numbers analysed in the discovery and replication stages of this study. Discovery samples consisted of serous invasive cases from the AUS (550 cases and 1,101 controls) and MAY (125 cases and 61 controls; all non-Hispanic Whites) studies. AUS participants were not selected for ethnicity, but comprised of predominantly non-Hispanic White women. Of the 1,837 women with genotype data, three were excluded by PLINK default thresholds because >10% of SNPs failed genotyping for these individuals. Of the 1,536 single nucleotide polymorphisms (SNPs) genotyped, 1,309 SNPs passed our initial quality control (QC) criteria, and of these, seven were excluded by PLINK default thresholds. The remaining 1,302 SNPs were subject to further pruning as follows: 37 SNPs with significantly different frequencies of missing genotype data between cases and controls (pMiss<0.05); 296 SNPs with duplicate
Author Summary

In this article, we report the findings from a large-scale analysis of common variation in genes that are expressed as a consequence of interactions between ovarian cancer cells and their host micro-environment that could influence serous ovarian cancer risk. We evaluated 1,302 common variants within or near 173 genes in two large case-control studies from the Ovarian Cancer Association Consortium (OCAC) and selected three variants for further evaluation in sixteen OCAC studies and an additional 18 for evaluation in five OCAC studies. We observed a significantly increased risk of serous ovarian cancer associated with a variant in the telomerase reverse transcriptase (TERT) gene. Although TERT variants have not been previously shown to contribute to ovarian cancer risk, several studies have recently reported associations between TERT variants and other forms of cancer, including gliomas, lung cancer, adenocarcinoma, basal cell carcinoma, prostate cancer, and multiple other cancers. TERT encodes a protein that is essential for the replication and maintenance of chromosomal integrity during cell division. In cancer cells, TERT has been linked to genomic instability and tumour cell proliferation. Further studies are necessary to confirm our findings and to investigate the mechanisms for the observed association.

discordance and/or failure to meet Hardy-Weinberg equilibrium (HWE) criteria (0.001 < PHWE < 0.05). Of the remaining 969 SNPs analysed in the discovery stage, 59 SNPs with PTrend < 0.05 were considered for the replication study (see Table S3).

Based on positive predictive value (PPV) estimates, the three SNPs selected for replication using TaqMan genotyping by the 16 OCAC studies were PODXL (podocylasin-like) rs1013368 (PPV 33.1%), ITGA6 (integrin, alpha 6) rs13027811 (PPV 4.5%) and MMP3 (matrix metalloproteinase 3) rs522616 (PPV 4.4%) (Table 1). These 16 OCAC studies included all histologic subtypes, and ethnicities. An additional 18 SNPs with PTrend < 0.05 which fitted into the iPLEX design were selected for replication by a subset of five of the 16 OCAC studies [AUS (additional samples not in the discovery set), MAL, SEA, UKO, and USC]. FG2 rs17473132 included among the 18 selected SNPs (PTrend = 0.008) has been previously reported elsewhere [17] and is therefore excluded from this report. Replication sample sizes varied by SNP depending on which participating OCAC study met QC criteria; MAY, NCO, NEC and NHS failed QC for PODXL rs1013368, and GER and STA failed QC for ITGA6 rs13027811. Table 2 provides the risk estimates adjusted for age and study site for SNPs included in the replication analysis. There was no evidence of between-study heterogeneity for any replication SNP with the exception of TERT rs7726159 (p = 0.005) (Table S4). Further examination of the site-specific Odds Ratios (ORs) showed that this was driven in part by the smaller USC study, the exclusion of which resulted in a p-value for between-study heterogeneity of 0.09. The associations observed in the discovery set for the three SNPs selected based on PPV values (PODXL rs1013368, ITGA6 rs13027811, and MMP3 rs522616), were completely attenuated in the larger replication analysis of 16 case control studies (adj. Pper-allele = 0.5) (Table 2).

However, adjusted log additive estimates for TERT (telomerase reverse transcriptase) rs7726159 retained a statistically significant p-value in the replication study of non-Hispanic White serous invasive cases and controls (Pper-allele = 0.03), and showed evidence of log additive effects across genotypes. We re-analysed this SNP combining discovery and replication data and observed some evidence of between-study heterogeneity (p = 0.027) which again improved with the exclusion of the smaller studies (USC and MAY; p = 0.16). Risk estimates for serous invasive ovarian cancer adjusted for age and study site remained statistically significant in the combined dataset [adj. ORper-allele 1.14 (1.04–1.24) p = 0.003; Table 3]. Likewise, in exploratory analyses of genotype data on all ethnicities stratified by histological subtype, a increased risk associated with this SNP was observed for serous invasive cases in models adjusted for age, sex and ethnicity [adj. ORper-allele 1.17 (1.08–1.27) p = 7.21 x 10−5]. TERT rs7726159 was also associated with serous borderline tumors, but not with any other invasive or borderline subtypes (Table 4, and Figure 1). For MMP7 rs17090236, the combined age- and site-adjusted estimate from the log additive model suggested an association with serous ovarian cancer but the point estimates were not in the same direction as those obtained in discovery analysis (0.84 vs.1.19; see Table S3 and Table 2). All other SNPs in the smaller replication study failed to replicate the significant associations observed in the discovery sample.

Discussion

Herein we report a large-scale analysis of 1,309 SNPs in 173 genes selected for their putative role in stromal epithelial cross talk, using a two-stage design for assessment of ovarian cancer risk. In the discovery stage we used data from two OCAC case-control studies (AUS and MAY) of predominantly non-Hispanic White women, and observed that SNPs in several genes were associated with risk of serous tumours in unadjusted log-additive models (Table S3). The most significant associations observed (PODXL rs1013368, ITGA6 rs13027811, and MMP3 rs522616; Table 1. Discovery analysis: risk estimates for serous ovarian cancer for three SNPs selected for replication by 16 OCAC studies.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>CHR</th>
<th>SNP</th>
<th>Minor Allele</th>
<th>Major Allele</th>
<th>*MAF</th>
<th>*PTrend</th>
<th>*OR (95% CI)</th>
<th>bPallel</th>
<th>bPTrend</th>
<th>dPower</th>
<th>dPPV</th>
</tr>
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<td>PODXL</td>
<td>7</td>
<td>rs1013368</td>
<td>G</td>
<td>A</td>
<td>0.34</td>
<td>1.00</td>
<td>1.32</td>
<td>(1.14–1.51)</td>
<td>0.0001126</td>
<td>0.0001037</td>
<td>0.51</td>
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<td>ITGA6</td>
<td>2</td>
<td>rs13027811</td>
<td>G</td>
<td>A</td>
<td>0.12</td>
<td>0.87</td>
<td>0.68</td>
<td>(0.54–0.85)</td>
<td>0.0008275</td>
<td>0.0008566</td>
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<tr>
<td>MMP3</td>
<td>11</td>
<td>rs522616</td>
<td>G</td>
<td>A</td>
<td>0.23</td>
<td>0.93</td>
<td>0.76</td>
<td>(0.64–0.90)</td>
<td>0.001178</td>
<td>0.001184</td>
<td>0.55</td>
</tr>
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</table>

*MAF and PTrend derived from controls.

*Odds ratios, 95% CI and p-values are derived from the allelic test for association using χ² test on 1 df.

*Cochran-Armitage trend test (1df).

*Power of the study to detect the association.

*Positive predictive value.

doi:10.1371/journal.pgen.1001016.t001
Table 2. Replication analysis: risk estimates for serous invasive ovarian cancer in non-Hispanic whites for SNPs selected for replication by indicated OCAC sites.

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>MAF</th>
<th>Controls</th>
<th>Cases</th>
<th>ORhet a (95% CI)</th>
<th>ORhet b (95% CI)</th>
<th>P</th>
<th>ORper-allele b (95% CI)</th>
<th>P</th>
<th>OCAC Studies c</th>
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<td>POXL</td>
<td>rs1013368</td>
<td>0.38</td>
<td>6,308</td>
<td>2,173</td>
<td>1.00 (0.89–1.10)</td>
<td>0.88 (0.86–1.18)</td>
<td>0.81</td>
<td>1.00 (0.87–1.01)</td>
<td>0.97</td>
<td>AUS, DOV, HAW, HOP, MAL, POL, SEA, STA, UCI, USC, UKO</td>
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<td>POXL</td>
<td>rs1327811</td>
<td>0.10</td>
<td>8,005</td>
<td>2,660</td>
<td>1.03 (0.92–1.16)</td>
<td>0.57</td>
<td>1.01 (0.86–1.27)</td>
<td>0.87</td>
<td>AUS, DOV, HAW, HOP, MAL, MAY, NCO, NEC, NHS, POL, SEA, UCI, USC</td>
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<tr>
<td>MMP3</td>
<td>rs522616</td>
<td>0.20</td>
<td>8,773</td>
<td>2,985</td>
<td>1.03 (0.94–1.12)</td>
<td>0.58</td>
<td>1.01 (0.88–1.04)</td>
<td>0.88</td>
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<tr>
<td>POXL</td>
<td>rs11768640</td>
<td>0.24</td>
<td>2,952</td>
<td>1,076</td>
<td>0.92 (0.79–1.06)</td>
<td>0.25</td>
<td>0.86 (0.62–1.18)</td>
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<td>ITGA6</td>
<td>rs1574028</td>
<td>0.09</td>
<td>2,958</td>
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<td>1.00 (0.83–1.21)</td>
<td>0.98</td>
<td>1.00 (0.85–1.15)</td>
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<td>0.98 (0.82–1.18)</td>
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<td>0.03</td>
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<td>0.87</td>
<td>AUS, MAL, SEA, UKO, USC</td>
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</table>

**Abbreviations:** MAF = minor allele frequency; OR = odds ratio; Het = heterozygous; ORhet = odds ratio for heterozygotes; ORper-allele = odds ratio per allele; OCAC = Ovarian Cancer Association Consortium.

**Notes:**
- MAF in controls.
- ORs, 95% CI and p-values are adjusted for age (at interview in controls, at diagnosis in cases) and study site.
- OCAC studies not listed for POXL rs1013368, ITGA6 rs13027811 and MMP3 rs522616 were excluded from analysis because of QC failures.
- doi:10.1371/journal.pgen.1001016.t002

**doi:** 10.1371/journal.pgen.1001016.t002

TERT Variant Associated with Serous Ovarian Cancer
Table 3. Combined discovery and replication analysis: site-specific and combined risk estimates for serous ovarian cancer for TERT rs7726159 among non-Hispanic whites.

<table>
<thead>
<tr>
<th>Study</th>
<th>Heterozygotes</th>
<th>Homozygotes</th>
<th>Per-allele</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>*OR (95% CI)</td>
<td>*OR (95% CI)</td>
<td>*OR (95% CI)</td>
</tr>
<tr>
<td></td>
<td>*P</td>
<td>*P</td>
<td>*P</td>
</tr>
<tr>
<td>SEA</td>
<td>1,213</td>
<td>383</td>
<td>1.35 (1.05–1.73)</td>
</tr>
<tr>
<td>AUS</td>
<td>1,202</td>
<td>636</td>
<td>1.30 (1.06–1.60)</td>
</tr>
<tr>
<td>MAL</td>
<td>764</td>
<td>264</td>
<td>1.50 (1.10–2.03)</td>
</tr>
<tr>
<td>UKO</td>
<td>564</td>
<td>235</td>
<td>0.95 (0.68–1.32)</td>
</tr>
<tr>
<td>USC</td>
<td>218</td>
<td>128</td>
<td>0.71 (0.45–1.13)</td>
</tr>
<tr>
<td>MAY</td>
<td>61</td>
<td>125</td>
<td>1.53 (0.80–2.94)</td>
</tr>
<tr>
<td>Combined (all studies)</td>
<td>4,022</td>
<td>1,771</td>
<td>1.23 (1.09–1.39)</td>
</tr>
</tbody>
</table>

a Estimates are adjusted for age (at interview in controls, at diagnosis in cases) and additionally for study site in combined (all studies) estimates.

Table 4. Combined discovery and replication analysis: risk estimates for TERT rs7726159 for all races according to tumor behaviour and histological subtypes.

<table>
<thead>
<tr>
<th>Tumor Behavior</th>
<th>Histological Subtype</th>
<th>*Controls</th>
<th>*Cases</th>
<th>bOR (95% CI)</th>
<th>bOR (95% CI)</th>
<th>bOR (95% CI)</th>
<th>bOR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>*P</td>
<td>*P</td>
<td>*P</td>
<td>*P</td>
</tr>
<tr>
<td>Invvasive</td>
<td>Serous</td>
<td>4138</td>
<td>2196</td>
<td>1.30 (1.16–1.45)</td>
<td>5.7 × 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>1.25 (1.05–1.49)</td>
<td>0.011</td>
</tr>
<tr>
<td></td>
<td>Mucinous</td>
<td>4138</td>
<td>271</td>
<td>1.15 (0.88–1.49)</td>
<td>0.31</td>
<td>1.01 (0.66–1.54)</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>Endometrioid</td>
<td>4138</td>
<td>454</td>
<td>0.81 (0.65–0.99)</td>
<td>0.045</td>
<td>1.09 (0.80–1.48)</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>Clear Cell</td>
<td>4138</td>
<td>261</td>
<td>1.03 (0.79–1.35)</td>
<td>0.83</td>
<td>1.12 (0.75–1.69)</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>All others</td>
<td>4138</td>
<td>355</td>
<td>1.08 (0.86–1.37)</td>
<td>0.50</td>
<td>1.49 (0.80–1.64)</td>
<td>0.45</td>
</tr>
<tr>
<td>Borderline/LMP</td>
<td>Serous</td>
<td>4138</td>
<td>251</td>
<td>1.63 (1.21–2.18)</td>
<td>0.001</td>
<td>2.04 (1.38–3.02)</td>
<td>0.0004</td>
</tr>
<tr>
<td></td>
<td>Mucinous</td>
<td>4138</td>
<td>249</td>
<td>1.13 (0.85–1.49)</td>
<td>0.40</td>
<td>0.85 (0.53–1.36)</td>
<td>0.51</td>
</tr>
</tbody>
</table>

#Cases and controls derived from AUS, MAL, MAY, SEA, UKO and USC studies.

*Estimates are adjusted for age (at interview in controls, at diagnosis in cases), race and study site.

doi:10.1371/journal.pgen.1001016.t004
with another functional or causal SNP within the gene. An alternative explanation for the observed association is population stratification, which occurs when allele frequencies differ within population subgroups, or when cases and controls are drawn from different subgroups. We suggest that this is a likely explanation because cases and controls were drawn from the same source populations within each study, and replication analyses were restricted to non-Hispanic White women or adjusted for ethnicity where applicable. However, it is possible that the association with serous ovarian cancer may vary across populations because of interaction with other genes or environmental factors, and additional studies would be required to confirm these findings.

Although TERT variants have not been previously reported to be associated with ovarian cancer, a recent meta-analysis of two GWAS identified another SNP in TERT, rs2736100, as significantly associated with gliomas (OR = 1.14; \( P = 1.50 \times 10^{-17} \)) [21]. GWAS have found that rs2736100 is also associated with lung cancer (OR = 1.14; \( P = 4 \times 10^{-5} \)) [22] and more specifically, with the adenocarcinoma subtype (OR = 1.23; \( P = 3.02 \times 10^{-7} \)) [23] (Figure 2A). Associations have also been reported between the TERT-CLPTM1L (cleft lip and palate transmembrane-1-like gene - cisplatin resistance-related protein 9-) locus and lung cancer (rs402710; OR = 1.17; \( P = 2 \times 10^{-6} \)) [22], basal cell carcinoma (rs401681; OR = 1.20; \( P = 4.8 \times 10^{-5} \)) [24], pancreatic cancer (rs401681; OR = 1.19; \( P = 3.66 \times 10^{-7} \)) [25], and multiple cancer types that are known to originate in the epithelium, including bladder, prostate and cervical cancer [26].

We genotyped rs2736100 in the discovery samples and found a borderline, but inverse, association with serous ovarian cancer (OR = 0.88 (0.77–0.99); \( P_{\text{trend}} = 0.06 \)). We also found a borderline association with rs1133719 and serous ovarian cancer risk (OR = 0.81 (0.67–0.98); \( P_{\text{trend}} = 0.025 \)) in discovery samples. Linkage disequilibrium (LD) estimation between the 11 TERT SNPs that we genotyped in stage 1 in 1,047 non-Hispanic White controls showed a moderate pairwise correlation between rs2736100 and rs7726159 (\( r^2 = 0.43 \); Figure 2B) but rs7726159, which we selected from NIEHS, is not in HapMap and so has not been genotyped in GWAS of ovarian or other cancers. Further analysis of this locus is necessary in order to definitively identify the causal SNP(s) at this locus.

To our knowledge, this is the first comprehensive evaluation of genes involved in stromal epithelial cross-talk and serous ovarian cancer. Candidate gene and SNP selection for discovery stage analysis was aimed at optimizing the likelihood of detecting a signal by including tagging and putatively functional SNPs with minor allele frequency (MAF) > 5%. Although a tagSNP approach has been shown to improve the power of the study for common variants [27], modest effects from SNPs with low MAFs may remain undetected. This was illustrated in a recent re-analysis of two SNPs in the DCN gene that failed to achieve the minimal \( P_{\text{trend}} \leq 0.05 \) in stage 1 analysis, but conferred a small but significantly decreased risk of serous ovarian cancer in a combined analysis of data from two additional studies [28]. We therefore suggest caution in interpreting null findings, and the need for large discovery and replication studies. Our discovery study was reasonably well powered, so the failure to find any associations with SNPs in genes involved in stromal epithelial cross-talk, except in DCN and TERT, suggests that genetic variation in this pathway is not a major determinant of serous ovarian cancer risk.

In summary, we have identified an association between TERT rs7726159 and serous ovarian cancer in a large sample of non-Hispanic White women participating in five OCAC case-control studies. We plan to further our investigation of this SNP and others in linkage disequilibrium with it, to determine whether TERT, CLPTM1L or another gene in the region is the functional target of this association. Our study adds to the growing evidence that, as well as the 8q24 locus [21,29,30–32], the TERT-CLPTM1L locus at 5p15.33, is a general cancer susceptibility locus. This is particularly interesting given the key roles of c-MYC (the nearest gene to the 8q24 locus) and TERT in tumorigenesis. TERT and MYC are both expressed in normal and transformed proliferating cells, and can induce immortalization when constitutively expressed [33]. The TERT promoter contains numerous MYC binding sites that mediate TERT transcriptional activation [34], suggesting that TERT is a target of MYC activity. Although TERT variants have not been previously reported to be associated with ovarian cancer, multiple genome-wide association studies have reported associations with this locus and risk of other cancers. Further analyses of this locus, including fine mapping, resequencing and functional assays, will be necessary to definitively identify the causal SNP(s).

#### Materials and Methods

**Study populations**

Approval from respective human research ethics committees was obtained, and all participants provided written informed consent. Approval from respective human research ethics committees was obtained, and all participants provided written informed consent.
consent. Sixteen OCAC case-control studies (summarized in Table S2) contributed data to this two-stage risk analysis. Samples in the discovery stage were derived from two case-control studies, AUS (550 cases and 1,101 controls) and MAY (125 cases and 61 controls). Cases in the discovery set were all diagnosed with serous carcinoma of the ovary, fallopian tube or peritoneum, and most of the participants were non-Hispanic white women. Cases and controls from an additional 14 OCAC studies, as well as an additional 284 AUS and 477 MAY samples, including cases with other histologies, were included in a stage 2 analysis designed to replicate the most promising SNPs from the discovery stage. Fifteen studies used population-based case and control ascertainment, and one (MAY) was clinic-based. All studies have been previously described [7,35,36]. The final combined dataset of all discovery and replication samples consisted of a total of 10,067 controls (9,953 were self-classified as non-Hispanic White) and 5,976 ovarian cancer cases of all histologies and morphologies, including 3,734 serous invasive cases (3,710 were self-classified as non-Hispanic Whites) (Table S2).

Candidate gene and SNP selection
Our approach and our choice of candidate genes was based on extensive preliminary data we have accumulated from gene expression profiles of co-cultured of theca fibroblast and epithelial ovarian cells (I. Haviv, personal communication), and expression profiles of murine ovarian epithelial cells identifying candidates that are regulated through the estrus cycle [37,38] (see Text S1). A compiled list of candidates was uploaded on the Ingenuity Pathway Analysis web interface and GeneSpring GX in order to obtain further candidates inferred from the literature. Prioritisation based on literature evidence for a plausible role in oncogenesis resulted in a list of 255 candidate genes of interest including CXCL9, CTGF, LCN2, DCN, and VIL2. CXCL9 is associated with ovarian cancer survival and acts by recruiting T-cells and inducing immune surveillance [39], and is expressed in epithelial cells co-cultured with fibroblasts. CTGF is likely to be the driver of the CAF phenotype. CTGF (TGFβ-stimulated) expression is associated with desmoplastic stroma [40] and elevated angiogenesis [41]. LCN2, DCN and VIL2 were regulated through the murine estrus.
cycle, and appear to be hormone responsive (either directly or indirectly) [37]. Furthermore, comparison with expression profiles of human ovarian carcinomas [42,43] showed that all three are differentially expressed in tumors compared with normal epithelial cells. Further details for candidate gene selection and justification are provided in Text S1 and Table S1.

We identified SNPs within 5 kb of these 255 genes (58,114 SNPs in total from dbSNP, Ensembl, the International HapMap Consortium [44], Perlegen Sciences [45], SeattleSNPs [pga.mbi.washington.edu/], NIEHS SNPs [http://ceg.gs.washington.edu/], and the Innate Immunity (IG) [http://www.ncbi.nlm.nih.gov/res/resources/pga/]). We used the binning algorithm of ldSelect [46] to identify 4,567 tagSNPs among these (r2 > 0.8) and minor allele frequencies (MAFs) > 0.05 based on the most informative available source (84% of genes used HapMap, 10% used SeattleSNPs, 3% used Perlegen Sciences, 2% used NIEHS SNPs, and 1% used Innate Immunity IG). 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 identify ~1,500 key SNPs. Based on Illumina design scores, we picked the best tagSNP in each bin (or two tagSNPs, if there were >10 tagSNPs in a bin but none of them had an optimal design score). We also used PATROCLES (www.patrocles.org) to identify supplemental SNPs with MAFs > 0.05 in microRNA binding sites or non-synonymous SNPs from public databases to the potential SNP list. This identified an additional 170 miRNA binding site SNPs and nsSNPs with Illumina design scores > 0.6. In total, this gave 1,410 tagSNPs, miRNA binding site SNPs and nsSNPs. In order to reach the final total of 1,536 SNPs for the Illumina GoldenGate assay, we added tagSNPs in another 12 candidate genes with MAF = 0.01. The final list of 1,536 SNPs included 106 supplemental SNPs and 1,430 tagSNPs in 173 genes (see Table S1).

Genotyping and quality control
The discovery samples were predominantly non-Hispanic White women with serous ovarian cancer and controls derived from two studies, the AUS and MAY studies, and were genotyped using the Illumina GoldenGate assay and Illumina BeadStudio software [47,48]. Plates were prepared containing randomly mixed cases and controls, with two duplicated samples and one blank per plate (n = 20). The Illumina GoldenGate assay was performed according to the manufacturer’s instructions. Following completion of the assay, all plates were analysed using Illumina BeadStudio software version 3.1.0.0. The original raw genotype dataset contained genotype information for 1,920 samples (including blanks and duplicates) and 1,536 SNPs. Following automatic clustering, SNPs were ranked using their GenTrain score (ranging from 0 to 1) and those with GenTrain scores < 0.5 were manually checked and adjusted according to Illumina guidelines. Samples with call rates below 95% and SNPs with call rates below 98% were excluded. A total of 1,292 SNPs passed this initial quality control (QC). Genotyping quality was also assessed using tests for Hardy-Weinberg equilibrium (HWE). Plots were examined for SNPs with significant deviations from HWE in controls (0.001 < P < 0.05) and the genotype data was excluded if the clustering was found to be suboptimal. SNPs with P_{HWE} < 0.001 were excluded from analysis. In addition, we genotyped 17 SNPs in CXCL9, CTGF, LGN2, DCN, and VIL2, that had not been amenable to the Illumina GoldenGate assay or failed QC criteria, at the Queensland Institute of Medical Research using MALDI-TOF mass spectrophotometric mass determination of allele-specific primer extension products with Sequenom’s MassARRAY platform and iPLEX Gold technology.

The final discovery dataset for analysis consisted of 675 cases and 1,162 controls with genotype data on 1,309 SNPs.

The three SNPs in POXL, ITGA6, and MMP3 selected for replication by all participating OCAC sites (with the exception of MMP3 at the MAM site) were genotyped with the TaqMan allele discrimination assay (TaqMan Applied Biosystems, Foster City, CA), using primers designed by Assays-by-Design (Applied Biosystems). MAY genotyping of MMP3 rs522616 was performed as part of a 1,536 Illumina GoldenGate Assay at the Mayo Clinic with cases and controls randomly mixed within each plate. Additional genotyping details are provided elsewhere [49].

Samples from five OCAC case-control studies (MAL, SEA, UKO, USC and additional samples from AUS) were genotyped for these and other replication SNPs, at the Queensland Institute of Medical Research using Sequenom iPLEX Gold technology. Primer design was carried out according Sequenom’s guidelines using MassARRAY Design software (version 1.0). Multiplex PCR amplification of fragments containing target SNPs was performed using Qiagen HotStart Taq Polymerase and a Perkin Elmer GeneAmp 2400 thermal cycler with 10 ng genomic DNA in 384 well plates. Shrimp Alkaline Phosphatase and allele-specific primer extension reactions were carried out according to manufacturer’s instructions for iPLEX GOLD chemistry. Assay data were analysed using Sequenom TYPER software (Version 3.4).

Only replication SNPs that met OCAC’s QC criteria (including > 95% call rate, and > 98% concordance between duplicates) were included in the analysis [50].

Statistical analysis
The primary test for association in stage 1 was univariate analyses of the relationship between SNP genotypes and risk of serous ovarian cancer using the PLINK v0.99 Whole Genome Association Analysis toolset [http://pngu.mgh.harvard.edu/purcell/plink/] [51]. Single-marker basic allelic association (x2 1df) tests (–assoc option) analyses were performed on each of the 1,390 post-QC SNPs in a total of 1,837 women. PLINK default thresholds were utilized, resulting in further exclusions: maximum missing genotypes per person ≤ 0.10 (–mind option), maximum failed genotypes per SNP ≤ 0.10 (–geno option), MAF ≥ 0.01 (–maf option). 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 (–test-missing option). Deviations from expected HWE proportions were analysed using the Fisher’s exact test and the MAFs were also estimated for all SNPs. The Cochran Armitage Trend test (x2 1df) assuming the log additive model (–model option) was performed to test the association between the minor allele of each SNP and serous ovarian tumors.

Selection of stage 1 SNPs for replication analyses in stage 2 was prioritized as follows: first, 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 HWE criteria (see Genotyping and quality control) for either cases, controls or both, and SNPs with no significant trend in allelic dose response (P_{trend} > 0.05) were excluded; secondly, we estimated from the remaining SNPs which were likely to be the best predictors of serous ovarian cancer risk by calculating the positive predictive value (PPV) using the P_{trend} values, the power of the study to detect this association, and the prior probability of 0.0001 [52]. Cases and controls from up to 14 additional studies participating in OCAC were included in replication analyses. We selected the three SNPs with the highest PPV for the larger replication analysis by all studies. Some
additional individuals from AUS and MAY (not in the discovery set) were included in the replication analysis. Replication samples were examined to determine the distribution of race/ethnicity across studies, and analyses were restricted to White non-Hispanic women with serous invasive ovarian tumors. Significant differences by study site between age at interview for controls and age and diagnosis for cases were assessed using the 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 (OR) and their 95% confidence intervals (95% CIs) were obtained from unconditional logistic regression models for each SNP genotype. Assuming a log additive model of inheritance, the per-allele ORs and their 95% CIs associated with serous invasive ovarian cancer in non-Hispanic Whites for each SNP selected for replication were estimated by fitting the number of rare alleles carried as a continuous covariate. Separate comparisons for women with one copy (heterozygotes) and women with two copies (rare homozygotes) of the minor allele vs. those with no copies (reference homozygotes) were conducted for all replication SNPs. Between-study heterogeneity was assessed using the likelihood ratio test to compare logistic regression models with and without a genotype-by-study interaction term. Risk estimates from all replication analyses were adjusted for age at diagnosis for cases or age at interview for controls and study site. Exploratory analyses combining all ethnicities were additionally adjusted for ethnicity. Forest plots generated in exploratory analyses according to histological subtype were obtained using the *rmeta* library [v2.15](http://www.r-project.org/) implemented in the R project for Statistical Computing. All tests for association were two-tailed, and unless otherwise specified, statistical significance was assessed at p<0.05 and tests for association in stage 2 were performed in STATA v. 9.0 (StataCorp, USA).

Supporting Information

**Figure S1** Study design for two-stage analysis of selected SNPs in genes involved in stromal-epithelial interactions in the Ovarian Cancer Association Consortium (OCAC).

Found at: doi:10.1371/journal.pgen.1001016.s001 (0.08 MB TIF)

**Table S1** Candidate genes, putative role/special justification for selection and reference list.

Found at: doi:10.1371/journal.pgen.1001016.s002 (0.05 MB DOC)

**Table S2** Characteristics of serous ovarian cancer cases and controls used in discovery and replication analyses according to contributing OCAC study.

Found at: doi:10.1371/journal.pgen.1001016.s003 (0.05 MB DOC)

**Table S3** SNPs successfully genotyped (Illumina & Sequenom) in the discovery stage with \( P_{\text{trend}} \leq 0.05 \) for serous ovarian cancer risk.

Found at: doi:10.1371/journal.pgen.1001016.s004 (0.12 MB DOC)

**Table S4** Study heterogeneity \( p \)-values for serous ovarian cancer risk estimates among non-Hispanic whites for SNPs reported in Table 2.

Found at: doi:10.1371/journal.pgen.1001016.s005 (0.04 MB DOC)

**Text S1** Candidate gene selection and justification.

Found at: doi:10.1371/journal.pgen.1001016.s006 (0.06 MB DOC)

Acknowledgments

We are grateful to the family and friends of Kathryn Sladek Smith for their generous support of OCAC through their donations to the Ovarian Cancer Research Fund. The PBCC thanks Dr. Louise Brinton and Mark Sherman from the Division of Cancer Epidemiology and Genetics of the National Cancer Institute, USA, Drs. Neomila Szczesniak-Dabrowoska and Beata Ppelonska of the Nofer Institute of Occupational Medicine (Lodz, Poland), Witold Zerwas of the Department of Cancer Epidemiology, Prevention, The M. Sklodowska-Curie Cancer Center and Institute of Oncology (Warsaw, Poland), and Pei Chao and Michael Stagner from Information Management Services (Silver Spring MD, USA), for their valuable contributions to the study. The GER study acknowledges Ursula Eilbre and Tanja Koehler for competent technical assistance for German Ovarian Cancer study. The AOCS and ACS Management Group (D. Bowtell, G. Chenevix-Trench, A. de Facio, D. Gerrig, A. Green, P. Webb) gratefully acknowledges the contribution of all the clinical and scientific collaborators (see [http://www.aocstudy.org/](http://www.aocstudy.org/)). The AOCS and ACS Management Group (A. Green, P. Parsons, N. Hayward, P. Webb, D. Whitehead) thank all of the project staff and collaborating institutions. We also thank all the participants in all the participating studies.

**The Ovarian Cancer Association Consortium**

Georgia Chenevix-Trench, Sharon E. Johnatty, Jonathan Bresley, Xinwei Chen, Penelope M. W. UN, Thomas Schildkraut (Ovarian Cancer Study Ovarian Cancer), The Australian Ovarian Cancer Study Group, The Queensland Institute of Medical Research, Queensland; Peter MacCallum Cancer Centre, Melbourne Victoria (AUSTRALIA); Anna H. Wu, Malcolm C. Pike, Celeste Leigh Pearce, Christopher K. Edlund, David J. Van Den Berg, University of Southern California, Keck School of Medicine, Los Angeles, CA; Montserrat Garcia-Closas, Hannah P. Yang, Shuwen Zhuanos, Nicola Kristensen, Danish Cancer Institute, National Institutes of Health, Rockville, MD; Hoda Anton-Culver , Argyrios Zogas, Wendy Brewster, School of Medicine, University of California, Irvine, CA; Ellen L. Goode, Brooke L. Friel, Robert A. Vierkant, Julie M. Cunningham, Mayo Clinic College of Medicine, Rochester, MN; Andrew Berchuck, Joellen M. Schildkraut, Edwin S. Vreesen, Jr, Patricia G. Moorman, Duke University Medical Center, Durham, NC; Maria T. Goodson, Michael E. Cerney, Pamela J. Thompson, Galina Lurie, Cancer Research Center of Hawaii, University of Hawaii, Honolulu, HI; Daniel W. Cramer, Margaret A. Gates, Immaculata DeVivo, Susan E. Hankinson, Shelley S. Tworoger, Kathryn L. Terry, Brigham and Women’s Hospital, Harvard School of Public Health, Boston, MA; Jennifer A. Doherty, Kara L. Cushing-Haagen, Chu Chen, Mary Anne Rossing, Fred Hutchinson Cancer Research Center, Seattle, WA; Linda S. Cook, Department of Internal Medicine, University of Colorado, CO; Kirsten Moysich, Richard C. DeOreo, Matthew T. Grasela, Roswell Park Cancer Institute, Buffalo, NY; Roberta B. Ness, University of Texas School of Public Health, Houston, TX; Alice S. Whitemore, Valerie McGuire, Weiva Sieh, Stanford University School of Medicine, Stanford, CA; Johnathan M. Lancaster, H. Lee Moffitt Cancer Center & Research Institute, Tampa, FL; Rachel T. Palmer-ium, University of North Carolina at Chapel Hill, NC; Harvey A. Risch, Yale University School of Public Health, New Haven, CT (UNITED STATES); Claus Hogdall, Estrid Hogdall, Susanne Krugger Kjaer, Danish Cancer Society/The Juliane Marie Centre, Copenhagen (DENMARK); Ralf Butzow, University of Helsinki, Haartman Institute, Helsinki (FINLAND); Simon A. Gayther , Aleksandra Gentry-Maharaj, Usha Menon, Susan J. Ramus, University College London, London, Paul D. Pharao, Barbara Perkins, Mitul Shah, Hongsin Song, University of Cambridge, Strangeways Research Laboratory, Cambridge (UNITED KINGDOM); Linda E. Kelenny, Alberta Health Services, Calgary (CANADA); Jacke Groslo, Jan Lubinski, Pomeranian Medical University, Szczecin; Jolanta Lisowska, Cancer Center and M Sklodowska-Curie Institute of Oncology, Warszawa (POLAND); Jenny Chang-Claude, Deutsches Krebsforschungszentrum, Heidelberg; Shan Wang-Gohrke, University of Ulm, Ulm (GERMANY).

Author Contributions

Conceived and designed the experiments: ABS Adf PMW MTG GL. JCC SEH MGC SJC PDP ASW CLP SAG SJR UM EH AB JMS TAS ELG GCT. Performed the experiments: JB XG NG IH. Analyzed the data: SEJ JB XC SM DLD NG CLP DNR. Contributed reagents/materials/analysis tools: SEJ SM DLD ABS Adf PMW MAR JAD MTG GL PJT LRW
References