

## ASSOCIATION STUDIES ARTICLE

# Exome genotyping arrays to identify rare and low frequency variants associated with epithelial ovarian cancer risk

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## Abstract

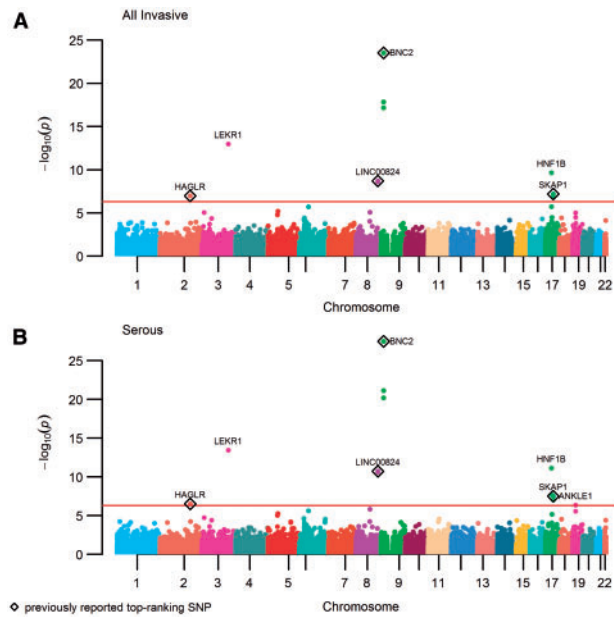
Rare and low frequency variants are not well covered in most germline genotyping arrays and are understudied in relation to epithelial ovarian cancer (EOC) risk. To address this gap, we used genotyping arrays targeting rarer protein-coding variation in 8,165 EOC cases and 11,619 controls from the international Ovarian Cancer Association Consortium (OCAC). Pooled association analyses were conducted at the variant and gene level for 98,543 variants directly genotyped through two exome genotyping projects. Only common variants that represent or are in strong linkage disequilibrium (LD) with previously-identified signals at established loci reached traditional thresholds for exome-wide significance ( $P < 5.0 \times 10^{-7}$ ). One of the most significant signals ( $P_{\text{all histologies}} = 1.01 \times 10^{-13}$ ;  $P_{\text{serous}} = 3.54 \times 10^{-14}$ ) occurred at 3q25.31 for rs62273959, a missense variant mapping to the LEKR1 gene that is in LD ( $r^2 = 0.90$ ) with a previously identified 'best hit' (rs7651446) mapping to an intron of TIPARP. Suggestive associations ( $5.0 \times 10^{-5} > P \geq 5.0 \times 10^{-7}$ ) were detected for rare and low-frequency variants at 16 novel loci. Four rare missense variants were identified (ACTBL2 rs73757391 (5q11.2), BTDR rs200337373 (3p25.1), KRT13 rs150321809 (17q21.2) and MC2R rs104894658 (18p11.21)), but only MC2R rs104894668 had a large effect size (OR = 9.66). Genes most strongly associated with EOC risk included ACTBL2 ( $P_{\text{AML}} = 3.23 \times 10^{-5}$ ;  $P_{\text{SKAT-o}} = 9.23 \times 10^{-4}$ ) and KRT13 ( $P_{\text{AML}} = 1.67 \times 10^{-4}$ ;  $P_{\text{SKAT-o}} = 1.07 \times 10^{-5}$ ), reaffirming variant-level analysis. In summary, this large study identified several rare and low-frequency variants and genes that may contribute to EOC susceptibility, albeit with possible small effects. Future studies that integrate epidemiology, sequencing, and functional assays are needed to further unravel the unexplained heritability and biology of this disease.

## Introduction

Epithelial ovarian cancer (EOC) has a strong heritable component, with an estimated three-fold increased risk among women with a first-degree relative having the disease (1). The excess familial risk that is not attributed to high penetrance mutations in genes such as BRCA1 and BRCA2 may be due to a combination of common and rare alleles that confer low- to moderate penetrance (2,3). Genome-wide association studies (GWAS) of EOC that have been conducted using most of the samples included in the current investigation have identified common variants at approximately 22 loci that collectively account for 4% of the estimated heritability (4–13). Few data exist regarding the contribution of rare (minor allele frequency (MAF) <0.5%) and low frequency (MAF 0.5–5%) protein-coding variants to EOC risk. This reflects the fact that protein-coding

variants have not been targeted by conventional GWAS (14) despite prediction that their effects could be substantial (15) and imputation is known to be challenging for rare variants (16).

Following GWAS arrays of the mid-2000s, exome-based arrays were developed in 2012. The Affymetrix Axiom® Exome Genotyping Array and the Illumina HumanExome Beadchip each contain >245,000 putative functional coding variants and other categories of variants selected from 16 exome sequencing initiatives that included approximately 12,000 individuals of diverse ethnic backgrounds and a range of diseases (17) (Supplementary Material, Table 1). Variants were included as 'fixed' content on the arrays if they occurred at least three times and were seen in two or more of the 16 studies (17). Here, we report the first large-scale genetic association study of uncommon



**Figure 1.** Manhattan plot of association for 98,299 variants from a pooled analysis of Affymetrix and Illumina exome genotyping arrays. Plots show the strength of association versus chromosomal position for (A) all invasive EOC risk and (B) serous invasive EOC risk. The red line represents exome-wide significance ( $5.0 \times 10^{-7}$ ). Exome-wide significant variants are annotated for the gene in which they are located. Known variants previously reported to have the strongest association signal are indicated by a black diamond.

exome-wide variants and EOC risk among nearly 20,000 women (Supplementary Material, Table 2).

## Results

Of the 98,299 polymorphic variants successfully genotyped as part of EOC case-control set 1 and set 2 (7,308 cases and 10,773 controls; Supplementary Material, Fig. 1), most (68%) were rare ( $MAF < 0.5\%$ ), many (20%) were common ( $n = 19,565$ ,  $MAF > 5\%$ ), and 12% ( $n = 12,175$ ) were low frequency ( $MAF$  between 0.5% and 5%). The majority of these variants were non-synonymous (87%) with 81% missense, 1% nonsense, 4% located in splice sites, and  $< 1\%$  resulting in a frame shift.

### Single variant associations

The quantile-quantile (Q-Q) plot of the distribution of test statistics for the comparison of genotype frequencies in cases and controls showed slight inflation in the median test statistics of the likelihood ratio tests ( $\lambda = 1.15$ ; Supplementary Material, Fig. 2). This slight inflation may be explained by the properties of the likelihood ratio test which make it sensitive to rare variants (18). No rare or low-frequency variants were statistically significantly associated with EOC risk ( $P < 5.0 \times 10^{-7}$ ); only common variants that represent or are in strong linkage disequilibrium (LD) with previously-identified signals at established loci (2q31.1, 3q25.31, 8q24.21, 9p22.2, 17q12, 17q21.3, and 19p13.1) reached traditional thresholds for exome-wide significance ( $P < 5.0 \times 10^{-7}$ ) (Fig. 1A and B, Supplementary Material, Table 3). Briefly, the most statistically significant association was observed at 9p22.2 for a previously identified intronic variant near the *BNC2* (*basonuclin2*) gene (4), rs38114113, with an odds ratio (OR), 95% confidence interval (CI), and P-value of 0.78 (0.75-0.82)

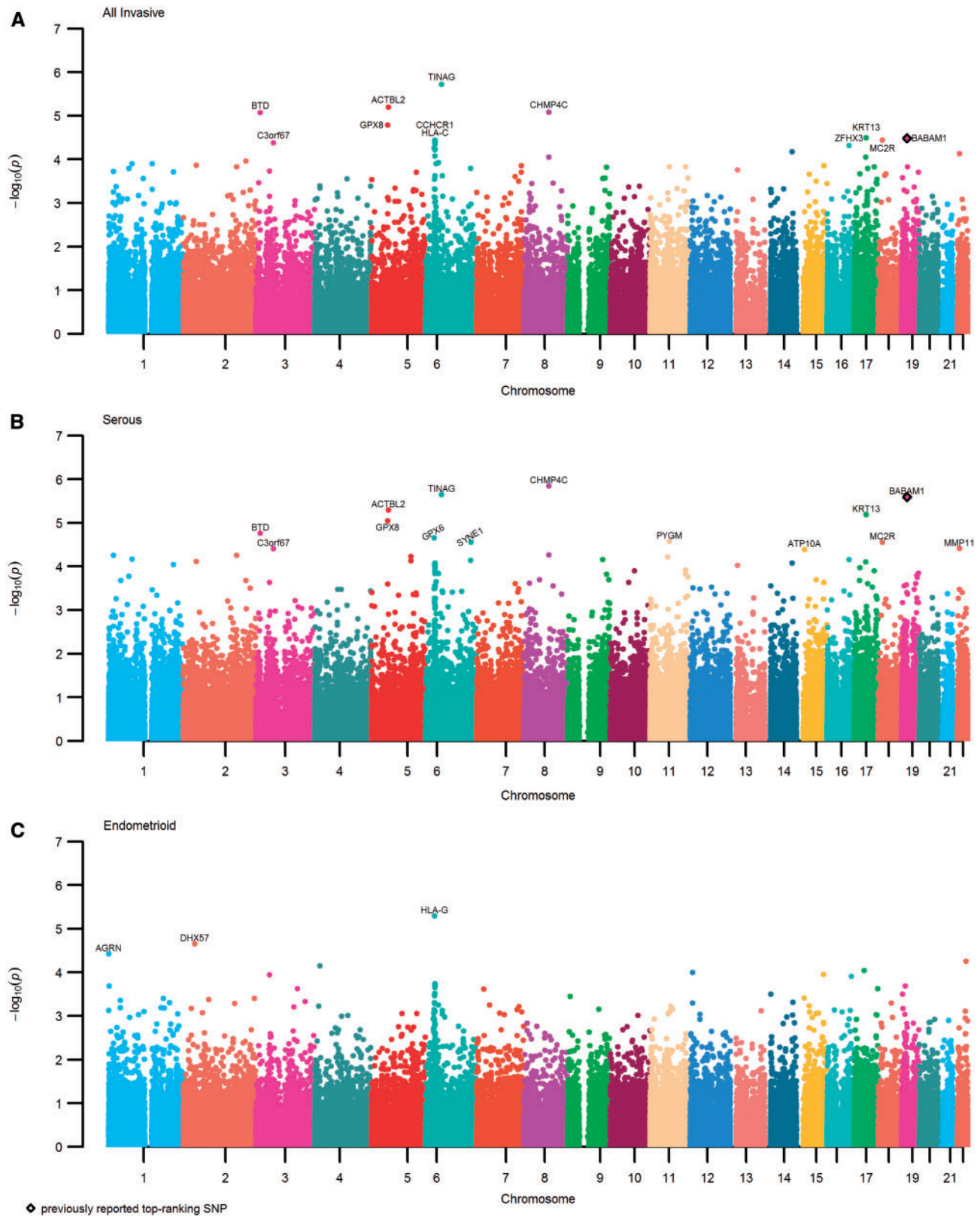
( $P = 2.96 \times 10^{-24}$ ) and 0.75 (0.72-0.79) ( $P = 3.32 \times 10^{-28}$ ) among all histologies and serous histology, respectively. rs38114113 is correlated ( $r^2 = 0.57$ -0.95) with two other detected SNPs ( $P = 10^{-18}$ ) near *BNC2*. The full genome-wide set of summary association statistics are given in Supplementary Material, Table 4.

The next most significant signal was at 3q25.31, with rs62273959  $P = 1.01 \times 10^{-13}$  and  $P = 3.54 \times 10^{-14}$  in all histologies (OR = 1.41) and serous only (OR = 1.45) analyses. rs62273959 is a missense variant mapping to the *LEKR1* (*leucine, glutamate and lysine rich 1*) gene which is in LD ( $r^2 = 0.90$ ) with a previously identified 'best hit', rs7651446(12) that is located in an intron of *TIPARP* (*TCDD-inducible poly(ADP-ribose) polymerase*). Imputation of the region (see Supplementary Material, Methods) identified rs78561123 (T > C) ( $P = 2.97 \times 10^{-15}$ ), a novel top-ranking variant that maps within 0.5kb of the 3'UTR for *LINC00886* (*long intergenic non-protein coding RNA 886*) and is in strong LD with rs7651446 ( $r^2 = 0.97$ ) and rs62273959 ( $r^2 = 0.93$ ) (Supplementary Material, Fig. 3A). The minor alleles of these three variants are located within the same haplotype associated with an increased risk among all histologies (OR (95% CI) = 1.41 (1.29-1.55),  $P = 3.14 \times 10^{-13}$ ). A fixed-effect meta-analysis of our study with an imputed dataset from the COGS genotyping initiative (7) also revealed stronger associations for variants located near *TIPARP* and *LINC00886* (Supplementary Material, Fig. 3B). The combined analysis of set 1 and set 2 also confirmed the existence of known common EOC susceptibility alleles or their proxies at 17q12, 8q24.21, 17q21.3, and 19p13.1 ( $P < 5.0 \times 10^{-7}$ ) (Supplementary Material, Table 3).

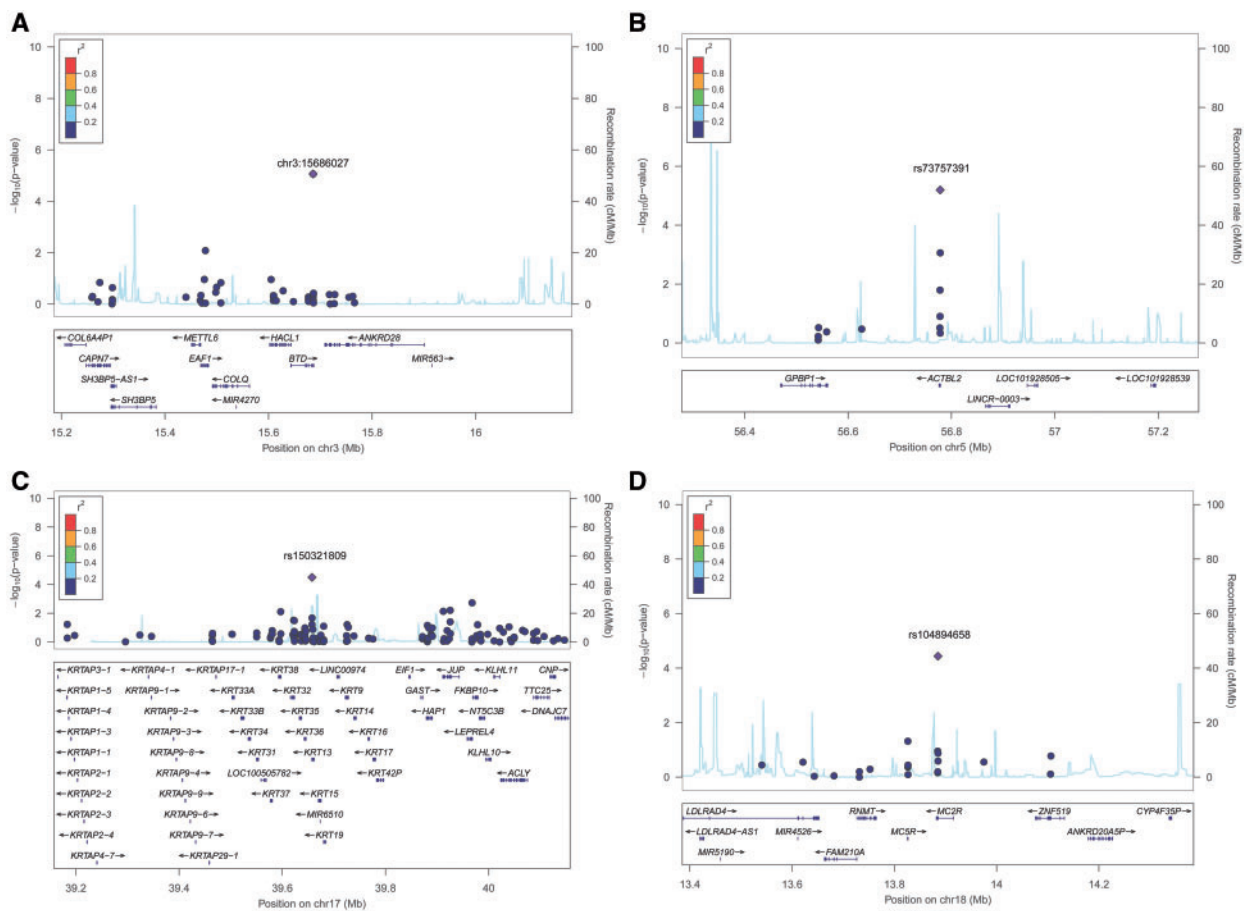
Associations for variants reaching a less stringent threshold ( $5.0 \times 10^{-5} > P \geq 5.0 \times 10^{-7}$ ) were detected among all histologies, serous histology, or endometrioid histology at 16 novel loci (1p36.33, 2p22.1, 3p25.1, 3p14.2, 5q11.2, 6p22.1, 6p21.33, 6q25.2, 6p12.1, 8q21.13, 11q13.1, 15q12, 16q22.3, 17q.21.2, 18p11.21, and 22q11.2) (Table 1; Fig. 2A and B). Of the novel variants that were identified, most were common and four were rare ( $MAF_{controls} < 0.003$ ). The four rare missense variants (map to *actin, beta-like 2*, *ACTBL2* (5q11.2), *biotinidase*, *BTD* (3p25.1), *keratin 13 type I*, *KRT13* (17q21.2), and *melanocortin 2 receptor*, *MC2R* (18p11.21). Visual inspection of cluster plots for all four rare variants underscored that the variant calling was good. Regional association plots for each of these rare variants reveal that they do not appear to be strongly correlated with other genotyped variants (Fig. 3). The identified rare variants mapping to *ACTBL2*, *BTD*, and *MC2R* are predicted to be damaging per Polyphen-2 (Table 1). Due to low heterozygous genotype counts, it was not possible to estimate ORs for variants at *ACTBL2* and *BTD*. For rs150321809 in *KRT13* and rs104894658 in *MC2R*, the magnitudes of association were relatively high, with ORs of 2.24 and 9.66, respectively, among all histologies. Analysis of 883 invasive endometrioid cancers identified three common variants at  $P < 5.0 \times 10^{-5}$  (Fig. 2C; Table 1). We recently described the contribution of deleterious coding variants in seven putative EOC susceptibility genes (*BRIP1*, *BARD1*, *PALB2*, *NBN*, *RAD51B*, *RAD51C*, and *RAD51D*) to EOC risk (19,20). The pooled exome dataset was used to examine associations for 68 variants that reside in these seven genes. None of these variants reached levels of statistical significance ( $P < 5.0 \times 10^{-5}$ ) in overall, serous, or endometrioid specific analyses. The most significant rare variant in overall and serous analyses was *BRIP1* rs4988345 ( $MAF = 0.0047$ ;  $P = 0.022$  and  $P = 0.024$ , respectively), whereas *PALB2* rs7605939 ( $MAF = 0.0002$ ) was the variant most significantly associated with endometrioid cancer risk ( $P = 0.007$ ). Similarly, we followed up on an exome sequencing study of 429 serous EOC

Table 1. Variants at novel loci associated with epithelial ovarian cancer susceptibility with  $5.0 \times 10^{-5} > P \geq 5.0 \times 10^{-7}$ 

Region <sup>a</sup>	rsID >(major> minor allele)	Position (hg19)	Nearest Gene(s)	Function (Polyphen score/ prediction)	Case MAF	Control MAF	All Invasive (7308 cases, 10773 controls)			Serous (5955 cases, 10773 controls)			Endometrioid (883 cases, 10773 controls)		
							OR (95% CI) <sup>b</sup>	P value	OR (95% CI) <sup>b</sup>	P value	OR (95% CI) <sup>b</sup>	P value	OR (95% CI) <sup>b</sup>	P value	
1p36.33	rs138031468 (G>T)	977028	AGRN	Missense: A375S	0.005	0.007	0.79 (0.60-1.03)	8.34E-02	0.91 (0.7-1.2)	5.20E-01	0.08 (0.01-0.56)	3.80E-05			
2p22.1	rs61757604 (C>T)	39095403	DHX57	Missense: C49S (0.318/B)	0.02	0.02	0.82 (0.68-0.99)	3.55E-02	0.92 (0.75-1.11)	3.69E-01	0.26 (0.12-0.56)	2.21E-05			
3p25.1	rs200337373 (G>A)	15686027	BTD	Missense: D222N (0.999/D)	0.001	0	NE <sup>c</sup>	8.61E-06	NE <sup>c</sup>	1.75E-05	NE <sup>c</sup>	4.74E-02			
3p14.2	rs4679621 (C>T)	59324733	C3orf67 (289kb) FHIT (410kb)	Intergenic	0.47	0.44	1.1 (1.05-1.14)	4.19E-05	1.1 (1.05-1.15)	3.95E-05	1.03 (0.93-1.14)	5.82E-01			
5q11.2	rs381852 (G>A)	54459961	GPX8 CDC20B	Missense: K182R (0/B), Intron	0.21	0.18	1.13 (1.07-1.2)	1.63E-05	1.14 (1.08-1.21)	9.07E-06	1.05 (0.92-1.2)	4.72E-01			
5q11.2	rs73757391 (C>T)	56778213	ACTBL2	Missense: E108K (1.0/D)	0.001	0	NE <sup>c</sup>	6.37E-06	NE <sup>c</sup>	5.15E-06	NE <sup>c</sup>	4.24E-02			
6p22.1	rs114979098 (G>T)	29785235	HLA-G (10kb)	Intergenic	0.42	0.41	1.11 (1.05-1.18)	4.82E-04	1.08 (1.02-1.15)	1.15E-02	1.37 (1.19-1.56)	5.15E-06			
6p21.33	rs149771958 (C>T)	31079994	C6orf15	Missense: C48R (0.895/PD)	0.07	0.08	0.84 (0.77-0.91)	4.24E-05	0.84 (0.77-0.92)	1.25E-04	0.86 (0.71-1.03)	1.01E-01			
	rs116682468 (C>T)	31112484	CCHCR1	Missense: R627Q (1.0/D)	0.07	0.07	0.83 (0.76-0.91)	3.90E-05	0.84 (0.77-0.92)	2.86E-04	0.82 (0.68-1)	4.55E-02			
	rs116151586 (T>C)	31118019	CCHCR1	Intron	0.32	0.32	0.88 (0.83-0.94)	4.60E-05	0.88 (0.83-0.94)	9.03E-05	0.84 (0.73-0.97)	1.39E-02			
	rs114470046 (C>A)	31125257	CCHCR1	Nonsense: E41Stop	0.07	0.07	0.83 (0.76-0.91)	3.61E-05	0.84 (0.77-0.92)	2.75E-04	0.82 (0.67-1)	4.31E-02			
	rs115538919 (C>T)	31129707	TCF19	Missense: P241L (0.906/PD)	0.07	0.07	0.83 (0.76-0.91)	3.76E-05	0.84 (0.77-0.93)	3.09E-04	0.82 (0.68-1)	4.49E-02			
6p21.33	rs113935384 (G>A)	31231989	HCG27 (60kb) HLA-C (4.5kb)	Intergenic	0.36	0.36	0.89 (0.84-0.94)	3.66E-05	0.89 (0.84-0.94)	1.09E-04	0.9 (0.79-1.02)	9.54E-02			
6p12.1	rs2297980 (A>G)	54173413	TINAG	Missense: Q22R (0/B)	0.09	0.11	0.84 (0.78-0.9)	1.90E-06	0.83 (0.77-0.9)	2.25E-06	0.91 (0.76-1.07)	2.47E-01			
6q25.2	rs199761238 (T>C)	152652052	SYNE1	Missense: N4590D (0.818/PD)	0	0.001	0.15 (0.03-0.63)	1.06E-03	NE <sup>c</sup>	2.78E-05	1.33 (0.29-6.08)	7.22E-01			
11q13.1	rs145514333 (C>T)	64527189	PYGM	Missense: R61H (1.0/D)	0.004	0.0001	3.18 (1.69-6.01)	1.49E-04	3.59 (1.91-6.77)	2.67E-05	0.84 (0.19-3.77)	8.18E-01			
15q12	rs147432497 (G>A)	25940059	ATP10A	Missense: R999C (1.0/D)	0	0.001	0.16 (0.04-0.67)	1.61E-03	NE <sup>c</sup>	4.13E-05	NE <sup>c</sup>	1.31E-01			
16q22.3	rs147445846 (G>C)	72992910	ZFX3	Missense: L379V (0.979/D)	0.001	0.002	0.37 (0.22-0.62)	4.86E-05	0.34 (0.19-0.61)	7.01E-05	0.42 (0.13-1.34)	9.42E-02			
17q21.2	rs150321809 (C>T)	39657599	KRT13	Missense: R429H (0.266/B)	0.003	0.001	2.24 (1.52-3.31)	3.20E-05	2.45 (1.65-3.65)	6.49E-06	1.03 (0.4-2.67)	9.48E-01			
18p11.21	rs104894658 (C>A)	13885297	MC2R	Missense: S74I (1.0/D)	0.002	0.0003	9.66 (2.73-34.24)	3.66E-05	10.15 (2.86-35.99)	2.76E-05	NE <sup>c</sup>	5.78E-02			
22q11.2	rs141200301 (C>T)	24123521	MMP11	Missense: R334C (1.0/D)	0.001	0	NE <sup>c</sup>	7.49E-05	NE <sup>c</sup>	3.91E-05	NA	NA			



**Figure 2.** Manhattan plot of association for sub-exome-wide ( $P \geq 5.0 \times 10^{-7}$ ) variants from a pooled analysis of Affymetrix and Illumina exome genotyping arrays. SNPs with  $P < 5.0 \times 10^{-7}$  were filtered out and the strength of genetic association versus chromosomal position was plotted for the remaining 98,287 SNPs for the risk of (A) all invasive EOCs, (B) serous invasive EOCs, and (C) endometrioid invasive EOCs. Known variants previously reported to have the strongest association signal are indicated by a black diamond. Sub-exome-wide significant SNPs ( $P < 5.0 \times 10^{-5}$ ) are annotated for the gene in which they are located.



**Figure 3.** Regional association plots for rare variants associated with EOC susceptibility. (A) *BTD* rs200337373 (3p25.1), (B) *ACTBL2* rs73757391 (5q11.2), (C) *KRT13* rs150321809, (17q21.2), and (D) *MC2R* rs104894658 (18p11.21). Linkage disequilibrium (LD,  $r^2$ ) between the strongest signal (noted by a purple diamond) and other variants is indicated by the color scheme.

cases and 557 controls by Kanchi et al. (21) in which rare truncation and missense variants were detected in known EOC susceptibility genes including *BRCA1*, *BRCA2*, *CHEK2*, and *PALB2* and in genes not previously associated with EOC susceptibility such as *NF1* and *CDKN2B*. Only four of the rare truncation or missense variants they (21) identified were represented on either of the genotyping arrays utilized in the current investigation. Applying a threshold of  $P < 0.05$  for these four variants (*BRCA1\_772*, *CLTC\_1498*, *ERCC2\_635*, and *ITK\_448*) only *BRCA1\_772* (p.Val772Ala; rs80357467, MAF=0.00033) was associated with overall EOC susceptibility in our pooled analysis (OR (95%CI)=4.64 (1.22–17.7)), with  $P = 0.014$  (serous OR=3.79,  $P = 0.043$ ). This variant is classified as non-pathogenic for the purposes of clinical management but may have a mild to moderate impact on risk (22). Thus, previously-detected rare variants were not strongly associated with EOC susceptibility in our larger dataset.

We also evaluated association results for the 80,178 set 1 variants ( $n = 5,431$  case and 5,639 controls) that were not in the pooled dataset (Supplementary Material, Fig. 1). Results for the most statistically significant ( $P < 5.0 \times 10^{-5}$ ) set 1 variants are displayed in Supplementary Material, Fig. 4A–C and are summarized in Supplementary Material, Table 5. Of six set 1 variants that were detected at the  $P < 5.0 \times 10^{-5}$  threshold of statistical significance, the most statistically significant association was again with a common variant at a known locus rs62273902

(MAF=0.06) in the 5' untranslated region of *LEKR1* showing an increased EOC risk among all histologies (OR=1.42,  $P = 1.91 \times 10^{-9}$ ) and serous histology (OR=1.46,  $P = 9.48 \times 10^{-10}$ ) that is strongly correlated ( $r^2 = 0.95$ ) with rs62273959, a variant identified in the pooled analysis (Supplementary Material, Table 3). Of the remaining five variants, one is rare and two have low frequencies. Rare variant rs115783655 ( $T > C$ ) (MAF=0.005) maps to an intron in *IZUMO4* (*IZUMO* family member 4) and was associated with endometrioid cancer risk ( $P = 3.32 \times 10^{-5}$ ) while low frequency missense variants chr19:38572993 (A > G, MAF=0.02) in *SIPA1L3* (*signal-induced proliferation-associated 1 like 3*) and rs148738146 (T > C, MAF=0.01) in *PLA2G12A* (*phospholipase A2, group XIII A*) were associated with decreased risks of EOC in all histologies and serous histology analyses (Supplementary Material, Table 5).

Eight of 9,600 indels assessed in set 1 only reached a threshold of  $P < 9.0 \times 10^{-4}$ , and only one of these (rs147613544 at 8p21.3) is rare (MAF=0.0009) and was associated with a decreased risk for EOC (OR=0.16,  $P = 5.0 \times 10^{-4}$ ). Set 1 also assessed 146 variants in the mitochondrial genome; only one rare (MAF=0.003) non-synonymous variant c.6480G > A (p.Val193Ile) located within cytochrome *c* oxidase subunit 1 (*COI*) was strongly associated with decreased EOC susceptibility among all histologies (OR=0.54,  $P = 0.0009$ ) and serous histology (OR=0.24,  $P = 0.0008$ ). G6480A has been associated with an increased risk for prostate cancer in African Americans (23).

## Gene-level associations

In a combined analysis of Affymetrix- and Illumina-based data, thirteen genes had  $P$ -values less than  $5 \times 10^{-4}$  for an association with EOC susceptibility overall based on the RAML test (24) (Fig. 4). Consistency was observed when comparing gene-level findings from RAML to those based on the SKAT-O (25) tests (Table 2). The genes that were most strongly associated with EOC risk using RAML included actin, beta-like 2, ACTBL2 ( $P_{AML} = 3.23 \times 10^{-5}$ ;  $P_{SKAT-O} = 9.23 \times 10^{-4}$ ) and keratin 13, KRT13 ( $P_{AML} = 1.67 \times 10^{-4}$ ;  $P_{SKAT-O} = 1.07 \times 10^{-5}$ ); these genes contained individual variants (rs73757391 and rs150321809) associated with EOC risk ( $5.0 \times 10^{-5} > P \geq 5.0 \times 10^{-7}$ ) highlighted in Table 1. Details regarding the set of rare variants that contributed to gene-level findings for ACTBL2 and KRT13 are summarized in Supplementary Material, Table 6. ACTBL2 and KRT were also statistically significant in the serous- only analysis after multiple correction testing using an FDR threshold of 15% (26). Of the genes featured in Table 2, MC2R also contained individual rare variants associated with EOC risk ( $5.0 \times 10^{-5} > P \geq 5.0 \times 10^{-7}$ ) (Table 1). When comparing primary high-grade serous EOC tumors and normal fallopian tube tissues in the TCGA dataset, two of the aforementioned genes were differentially expressed: KRT13 was overexpressed in tumor versus normal tissue ( $P = 0.034$ ) while MC2R was under-expressed ( $P = 0.004$ ), though neither finding was significant after adjustment for multiple comparisons.

Gene-level results for the 15,042 genes encompassed by set 1 only variants did not highlight ACTBL2, KRT13, or MC2R. Rather, leukocyte receptor tyrosine kinase (LTK) ( $P = 2.22 \times 10^{-5}$ ), ATPase NA+/K+ transporting alpha 3 polypeptide (ATP1A3) ( $P = 8.33 \times 10^{-5}$ ), and son of sevenless homolog 2 (SOS2) ( $P = 4.55 \times 10^{-5}$ ) were identified as the most strongly associated genes among all histologies, serous histology, and endometrioid histology, respectively (Supplementary Material, Table 7; Supplementary Material, Fig. 5). Collectively, all genotyped uncommon variants (MAF < 0.05%) explained 4.7% of the phenotypic variation in our subjects (27). Only 2% (11/4.7) of this variation can be attributed to variants with  $P < 5.0 \times 10^{-5}$  (Supplementary Material, Methods).

## Discussion

We report an EOC risk association analysis of 98,299 variants enriched for rare and low frequency protein-coding changes among nearly 20,000 women using commercially available genotyping arrays. Assuming a disease prevalence of 1.4%, our sample size was adequately powered (~89%) to detect associations with low frequency variants included on the exome arrays and moderate effect sizes ( $OR > 1.35$ ) should they exist, but we did not identify any novel uncommon variants at exome-wide levels of statistical significance ( $P < 5.0 \times 10^{-7}$ ). Instead, association with common variants (MAF > 5%) at known EOC loci (2q31.1, 3q25.31, 8q24.21, 9p22.2, 17q12, 17q21.3, and 19p13.1)(4–6,10) were identified; most of these variants were (or were in strong LD with) the previously reported top-ranking variant at the locus. Importantly, 16 novel loci with low-frequency or rare variants at  $P < 5.0 \times 10^{-5}$  were detected. Four rare variants were identified (ACTBL2 rs73757391 (5q11.2), BTDRS200337373 (3p25.1), KRT13 rs150321809 (17q21.2), and MC2R rs104894658 (18p11.21)), and gene-level analyses revealed statistically significant associations with variation in three of these genes. These results are consistent with the known landscape of common genetic variation in EOC risk and the utility of multi-marker testing for rare variation. They suggest that the effect sizes of rare coding variants with MAF that are in the range included on the

exome arrays may be less than 1.35, requiring larger sample sizes and the use of a family-based approach for their discovery. Indeed, recent simulation studies suggest that sample sizes of 60,000–100,000 will be needed to detect small effect sizes for rare variants (MAF < 0.5%) when using exome genotyping arrays (28).

Among the four rare variants that were identified, rs73757391, rs200337373, and rs104894658 are non-synonymous and predicted to be damaging. Moreover, according to the ClinVar database (22), BTDRS200337373 (G > A) and MC2R rs104894658 (C > A) are reported to be pathogenic for biotinidase deficiency and adrenocorticotropic hormone (ACTH) resistance observed in familial glucocorticoid deficiency (29), respectively. Importantly, gene-level analysis using different methods highlighted ACTBL2, KRT13, and MC2R as being strongly associated with EOC risk overall and serous disease. Whereas ACTBL2 is a cytoskeletal protein abundantly expressed in vascular smooth muscle cells (30) that has no reported link to cancer, BTDR (biotinidase) is a putative biomarker of breast cancer (31), papillary thyroid cancer aggressiveness (32), and lymph node involvement in patients with early stage cervical cancer (33). *In vivo* a deficiency of biotinidase affects the expression of central-carbon metabolism genes (34), a pathway important in the development and progression of EOC (35,36). KRT13 encodes a cytoskeletal protein downregulated in an estrogen receptor (ER) positive ovarian cancer cell line (37) and contributes to breast cancer growth and metastasis through its interaction with estradiol and the selective estrogen receptor modulators, tamoxifen and raloxifene (38). MCR2 belongs to a family of melanocortin receptors involved in the regulation of food intake, inflammation, skin pigmentation, sexual function, and steroidogenesis, in part by binding to adrenocorticotropic hormone (ACTH) (39,40). ACTH-producing ovarian tumors have been reported (41–43), but this has been in the context of Cushing's syndrome and non-epithelial ovarian cancers. Taken together, there is biological plausibility to explain some but not all of the current association results.

Independent replication of novel rare variant associations is important but challenging because of the lack of appropriate replication panels. The large COGS EOC meta-GWAS (7) with imputation to Phase I 1000 genome project data was completed after the onset of this study. As a form of replication, we attempted to interrogate this dataset (7) for the most strongly associated novel rare variants. Unfortunately, rare variants and their proxies were not represented in the imputed dataset, precluding the possibility of replication and the opportunity to evaluate associations between germline genotype and gene expression via expression quantitative trait locus analysis. Furthermore, our attempt to replicate associations with rare variants identified in studies of EOC that were much smaller than ours (19–21) did not yield statistically significant findings.

The limited evidence for novel rare or low frequency coding variants at exome-wide levels of significance is consistent with studies of other complex diseases (myocardial infarction (44), Alzheimer's disease (45), and insulin processing and secretion (46) that used these exome genotyping arrays. Published investigations of exome genotyping array data are limited for other cancers, precluding comparison of findings across cancer types. The limited evidence for rare or low-frequency coding variants may be expected when using rare variant chips for cohorts or diseases for which they were not originally designed. Integration of sequencing data in very large sample sizes may be an effective strategy for discovering additional rare EOC alleles in the future since the arrays do not provide complete



coverage of all functional variants at each locus and the accuracy of imputation for rare variants is suboptimal. Even with such limitations, the current study suggests that rare coding variants with large effects may exist, although they did not account for a significant fraction of EOC heritability within our data. In total, rare variants accounted for 4.7% of the phenotypic variation in our subjects (27) with only 2% (.11/4.7) of this variation from variants with  $P < 5.0 \times 10^{-5}$  (Supplementary Material, Methods). The remaining 98% of variance attributable to rare variants in this study could be due to small effect sizes that did not reach statistical significance. In the absence of opportunities to significantly increase sample sizes, future studies should rely on closer integration of epidemiology and laboratory assays of functional effects to further unravel the etiology of this disease.

## Materials and Methods

### Study population and genotyping

Study participants came from 27 independent studies in the international Ovarian Cancer Consortium (OCAC) (47) (Supplementary Material, Table 2). In brief, cases were women with pathologically-confirmed primary invasive EOC, fallopian tube cancer, or peritoneal cancer, and controls were women without EOC, with at least one ovary intact, and for most studies were frequency-matched to controls on age group and self-reported race. Specimens and data were collected according to protocols approved by local institutional review and ethics boards. Germline DNA samples from 19 studies (Set 1, 7,060 EOC cases and 6,712 controls) were genotyped on the Affymetrix Axiom Exome Genotyping Array at the Affymetrix Service Lab (Santa Clara, CA, USA), and those from eight studies (Set 2, 2,109 cases, 5,646 controls) were genotyped on the Illumina HumanExome Beadchip at the Strangeways Research Lab (University of Cambridge, UK).

### Genotyping quality control (QC)

Set 1 genotyping was performed in batches grouped according to sample type (genomic blood, genomic saliva, whole genome-amplified (WGA) blood, WGA saliva). Affymetrix Genotyping Console™ Software was used for automated allele calling for each batch, followed by initial sample and variant QC performed per protocol (<http://media.affymetrix.com/support/downloads/manuals/axiom>). Since significant batch effects were observed, intensity data from the genomic samples were combined into a single batch to enable the automated clustering algorithm to more accurately detect rare variants (48). WGA samples were not recalled as one batch because of known chemistry differences between the component batches (personal communication, Affymetrix, Inc). Four hundred thirty-seven samples were genotyped in duplicate and were identified with 99.8% concordance. As shown in Supplementary Material, Fig. 1, of 13,772 unique samples that were genotyped, 454 (3.3%) were excluded because they failed Affymetrix QC metrics (<97% call rate or dish QC < 0.82) and an additional 545 samples were excluded because of ambiguous gender, replicate discordance, sample relatedness, or failure to meet eligibility criteria for the primary analysis. Of 302,461 variants on the Affymetrix array, 123,934 variants (41%) were excluded for QC reasons which mostly included failed Affymetrix cluster QC, monomorphism, deviation from Hardy Weinberg Equilibrium (HWE)  $P < 10^{-7}$  in controls, or discordant B allele frequencies between the genomic and WGA samples. A total of 12,773 samples (6,288 case and 6,485 controls) and 178,527 variants genotyped on the Affymetrix platform passed QC steps. HapMap DNA samples for

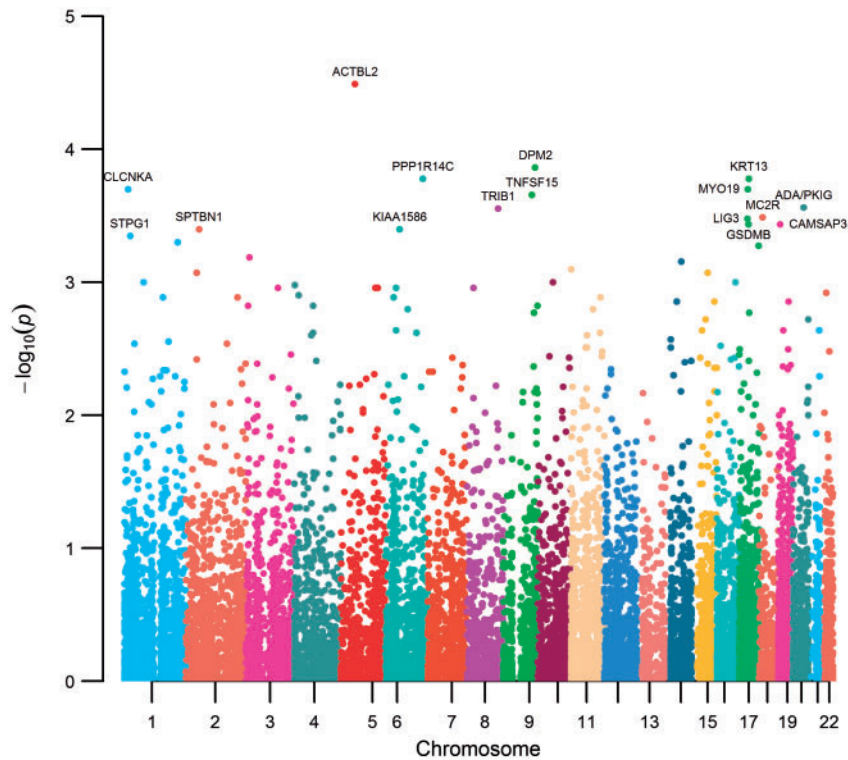
European (CEU,  $n = 60$ ), African (YRI,  $n = 53$ ) and Asian (JPT + CHB,  $n = 88$ ) populations were also genotyped, and the program LAMP (49) was used to estimate intercontinental ancestry based on the HapMap (release no. 23) genotype frequency data for the European, Asian, and African populations. Subjects with greater than 90% European ancestry were included in analyses (5,431 cases, 5,639 controls) (Supplementary Material, Fig. 1). Genotype data for set 1 are being released into dbGAP per NIH guidelines.

Set 2 genotyping was performed for 7,612 samples, and genotype calling was carried out according to Best Practice Guidelines (48) using the GenCall (50) module in Illumina's Genome Studio with a default GenCall threshold of 0.15. After initial sample QC, zCall (51) was calibrated to a z-value of 8 and run for all variants. One hundred and forty-three samples were genotyped in duplicate and identified with 92% concordance. Initial sample QC excluded 248 samples with low call rates (<70%). After zCall calibration and variant recall, we further excluded 221 (3%) samples for reasons including <99% call rate, high or low heterozygosity at a significance level of  $10^{-16}$ , ambiguous gender, relatedness, or genotypes discordant with prior genotypes from the international Collaborative Oncological Gene-Environment Study (iCOGS) genotyping array (7). Genotyping also included HapMap DNA samples (CEU,  $n = 95$ ; YRI,  $n = 82$ ; JPT + CHB,  $n = 93$ ), and ancestry was assessed using the IBS matrix for all samples combined with HapMap samples over the uncorrelated variants. Using this multi-dimensional scaling on a weighted identity by state matrix, non-European samples at a distance of greater than 10% were excluded ( $n = 97$ ) (Supplementary Material, Fig. 1). Of the 247,870 markers on the Illumina array, we excluded 94,231 variants (38%) for reasons including call rate <95%, poor cluster separation, duplicate probes, monomorphism, and deviation from HWE (Supplementary Material, Fig. 1). We tested for HWE using a Robertson and Hill test statistic stratified by study (52) and an exact test. Variants that failed both tests were excluded using exclusion thresholds of  $P$ -values <  $10^{-12}$  and  $10^{-6}$  for cases and controls, respectively. After all exclusions there were 7,046 European ancestry samples (1,878 cases and 5,168 controls) and 153,639 variants genotyped on the Illumina platform (Supplementary Material, Fig. 1). Thirty-five samples were genotyped in common as part of set 1 and set 2; the genotype concordance rate was 99.66%.

Thus, a combined total of 18,081 unique subjects of European ancestry (7,308 cases, 10,773 controls) were genotyped and passed sample QC. Of the 18,081 subjects, 5,138 (997 cases and 4,141 controls) were not previously genotyped as part of a previously described EOC GWAS or post-GWAS initiative (4,5,7). Of the variants passing QC for each set, 98,543 were present on both platforms and available for pooled analysis (Supplementary Material, Fig. 1), excluding non-autosomal variants ( $n = 1,983$ ) and those with discordant B allele frequencies between the two sets ( $n = 32$ ). On this combined dataset, we carried out principal components analysis (PCA) to examine the sub-European population structure using a linkage-disequilibrium-pruned set of 27,335 autosomal markers with  $MAF > 1\%$  and HWE  $P$  value  $> 10^{-7}$ . We inspected the first 10 principal components (PC) for evidence of population stratification in the pooled samples.

### Single variant analysis

Each variant was tested for a per allele association with EOC risk using a likelihood ratio test comparing the deviance ( $-2 \times \log$ -likelihood) of two generalized linear models with and without the



**Figure 4.** Gene-level association of rare variants (MAF < 1%) using the Rare Admixture Maximum Likelihood (RAML) association test. Results of association with all invasive EOC risk are shown for 15,118 genes, adjusting for study and first 5 PCs. Genes with  $P < 5.0 \times 10^{-4}$  are annotated.

**Table 2.** Genes most strongly associated with epithelial ovarian cancer risk, with  $P < 5.0 \times 10^{-4}$  by RAML

Gene Information		Rare Admixture Maximum Likelihood (RAML) test <sup>1</sup>						Sequence Kernel Association Test (SKAT) <sup>1</sup>						
All Invasive				Serous		Endometrioid		All Invasive		Serous		Endometrioid		
Gene	Region	N SNPs (total/rare)	P-value <sup>2</sup>	FDR	P-value <sup>2</sup>	FDR	P-value <sup>2</sup>	FDR	P-value <sup>2</sup>	FDR	P-value <sup>2</sup>	FDR	P-value <sup>2</sup>	FDR
ACTBL2	5q11.2	6/6	3.23E-05	0.38	<b>2.00E-05</b>	0.15	2.99E-01	1	9.23E-04	0.56	1.50E-03	0.46	2.60E-01	0.98
KRT13	17q21.2	7/7	1.67E-04	0.38	<b>2.94E-05</b>	0.15	2.65E-01	1	<b>2.04E-05</b>	0.15	<b>5.24E-06</b>	0.08	3.62E-01	0.98
CLCNKA	1p36.13	3/3	2.00E-04	0.38	8.33E-05	0.25	5.60E-01	1	2.98E-04	0.48	<b>6.08E-05</b>	0.14	5.61E-01	0.98
MYO19	17q12	14/11	2.00E-04	0.38	7.50E-04	0.37	1.84E-02	1	5.41E-03	0.64	9.30E-03	0.66	1.76E-02	0.74
TNFSF15	9q32	8/3	2.20E-04	0.38	5.56E-05	0.21	2.56E-01	1	2.79E-04	0.48	<b>4.39E-05</b>	0.14	2.62E-01	0.98
TRIB1	8q24.13	4/2	2.80E-04	0.38	3.67E-04	0.29	7.76E-01	1	4.90E-04	0.51	3.09E-04	0.26	7.25E-01	0.99
MC2R	18p11.21	6/6	3.25E-04	0.38	2.00E-04	0.29	9.62E-02	1	1.32E-01	0.91	2.01E-01	0.93	8.26E-02	0.98
LIG3	17q12	7/6	3.33E-04	0.38	1.70E-03	0.46	3.59E-02	1	4.91E-03	0.64	9.80E-03	0.66	4.62E-03	0.55
CAMSAP3	19p13.2	4/3	3.67E-04	0.38	2.40E-04	0.29	3.54E-01	1	1.10E-04	0.37	<b>6.76E-05</b>	0.14	4.20E-01	0.98
GSDMB	17q21.1	15/7	3.67E-04	0.38	4.33E-04	0.31	5.41E-01	1	2.78E-01	0.95	2.47E-01	0.93	2.49E-01	0.98
KIAA1586	6p12.1	3/2	4.00E-04	0.38	3.33E-04	0.29	1.80E-01	1	4.70E-04	0.51	2.00E-04	0.26	1.53E-01	0.98
SPTBN1	2p16.2	9/8	4.00E-04	0.38	2.10E-03	0.48	2.06E-01	1	<b>1.16E-05</b>	0.15	2.20E-04	0.26	3.88E-02	0.97
STPG1	1p36.11	9/3	4.50E-04	0.40	7.50E-04	0.37	2.21E-01	1	4.50E-01	0.95	1.71E-01	0.92	1.84E-01	0.98
GPATCH2	1q41	8/7	5.00E-04	0.42	3.67E-04	0.29	7.06E-01	1	3.26E-02	0.81	5.37E-02	0.84	1.00E+00	1.00
CCDC136	7q33	12/11	5.33E-04	0.42	1.43E-04	0.26	7.47E-01	1	1.07E-02	0.75	8.68E-03	0.66	7.53E-01	0.99
WDR59	16q23.1	10/8	1.00E-03	0.50	3.67E-04	0.29	2.58E-01	1	2.81E-03	0.61	2.67E-03	0.55	4.51E-01	0.98
NEXN	1p31.1	3/3	1.00E-03	0.50	3.67E-04	0.29	1.91E-01	1	5.46E-03	0.64	3.64E-03	0.61	4.53E-01	0.98
MMP11	22q11.23	5/4	1.20E-03	0.50	4.33E-04	0.31	2.98E-01	1	2.69E-01	0.95	4.25E-02	0.83	1.87E-01	0.98
BCL9L	11q23.3	2/2	1.30E-03	0.50	1.57E-04	0.26	4.52E-01	1	1.31E-03	0.57	1.58E-04	0.26	4.99E-01	0.98
PYGM	11q12- q13.2	11/11	3.10E-03	0.66	3.33E-04	0.29	3.52E-01	1	1.56E-01	0.92	5.24E-02	0.83	3.13E-01	0.98
ATMIN	16q23.2	7/6	5.06E-02	0.94	3.27E-01	1	3.67E-04	1	1.23E-01	0.91	5.30E-01	0.94	9.09E-04	0.30
SOS2	14q21	9/9	1.30E-01	0.99	3.71E-01	1	6.88E-05	1	8.85E-02	0.89	1.83E-01	0.92	6.34E-02	0.98
OR7G1	19p13.2	9/3	1.97E-01	1.00	3.85E-01	1	2.40E-04	1	9.47E-02	0.90	3.00E-01	0.94	4.23E-04	0.23
PRR5	22q13	5/4	3.71E-01	1.00	6.75E-01	1	3.67E-04	1	4.08E-01	0.95	8.36E-01	0.94	7.86E-02	0.98

<sup>1</sup>Both the RAML and SKAT methods were limited to rare variants (MAF < 1%). No weighting by minor allele frequency was used in either method.

<sup>2</sup>Analyses are adjusted for site and the first five principal components representing European ancestry.

variant. The models were adjusted for set (1 versus 2) and the first five PCs representing sub-European ancestry. When adjusted for study alone, there was an inflation of the test statistics ( $\lambda = 1.20$ ,  $\lambda_{1000} = 1.024$ ) which was reduced to  $\lambda = 1.15$ , ( $\lambda_{1000} = 1.018$ ) after adjustment for five principal components (53). Visual inspection of intensity cluster plots resulted in the elimination of 244 variants with poor differentiation between a heterozygote and homozygote calls (Supplementary Material, Fig. 1). Subgroup analysis was conducted for the two most common histologic subtypes: serous and endometrioid. Using a stringent Bonferroni correction for 98,299 tests, we considered variants with  $P < 5.0 \times 10^{-7}$  to be statistically significant. Because of the greater number of variants and samples in set 1, we also explored associations for 80,178 variants from set 1 that passed visual cluster inspection and were not included in the Illumina array; for these analyses, we adjusted for the first three PCs.

### Gene-level analysis

Given the emphasis of each array on exomic coverage (54), gene-level tests were also conducted, mapping variants within 50 kb to genes based on Genome Build 37 coordinates and gene annotation that was curated by Affymetrix from UCSC Genome Browser data tables. In total, 71,044 variants mapped to 15,118 genes of which 12,123 genes contained more than one variant and were evaluated for the pooled gene-level analysis. Two methods were used for gene-level analyses because of some similarity in assumptions and the ability to include covariates in the underlying regression model: a) the rare admixture maximum likelihood test (RAML) (55), which makes no assumptions about the proportion of variants that are associated with the phenotype of interest or the magnitude and direction of their effect and b) the Sequence Kernel Association Test -Optimal unified test (SKAT-O) (56), a score-based variance-component test that is powerful when the direction of association for variants can be increased or decreased. Both methods considered only rare variants (MAF < 1%) and were not weighted based on MAF. False discovery rate (FDR) is used to adjust for multiple comparisons and FDR of 15% is used to declare significance. Similarly, we also conducted gene-level analysis with the larger set of variants in set 1 which totaled 15,042 genes and 128,992 variants. For genes that were most strongly associated with EOC susceptibility, we mined publicly available gene expression data from the Cancer Genome Atlas Project (TCGA) (57) and compared gene expression between 568 high-grade serous ovarian tumors and 10 normal fallopian tube tissues according to previously described methods (9).

### Supplementary Material

Supplementary Material is available at HMG online.

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