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Identification of a locus near *ULK1* associated with progression-free survival in ovarian cancer

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Abstract

Background—Many loci have been found to be associated with risk of epithelial ovarian cancer (EOC). However, although there is considerable variation in progression-free survival (PFS), no loci have been found to be associated with outcome at genome-wide levels of significance.

Methods—We carried out a genome-wide association study (GWAS) of PFS in 2352 women with EOC who had undergone cytoreductive surgery and standard carboplatin/paclitaxel chemotherapy.

Results—We found seven single nucleotide polymorphisms (SNPs) at 12q24.33 associated with PFS ($P < 5 \times 10^{-8}$), the top SNP being rs10794418 (HR = 1.24, 95% CI 1.15–1.34; $P = 1.47 \times 10^{-8}$). High expression of a nearby gene, *ULK1*, is associated with shorter PFS in EOC, and with poor prognosis in other cancers. SNP rs10794418 is also associated with expression of *ULK1* in ovarian tumors, with the allele associated with shorter PFS being associated with higher expression, and chromatin interactions were detected between the *ULK1* promoter and associated SNPs in serous and endometrioid EOC cell lines. *ULK1* knockout ovarian cancer cell lines showed significantly increased sensitivity to carboplatin *in vitro*.

Conclusions—The locus at 12q24.33 represents one of the first genome-wide significant loci for survival for any cancer. *ULK1* is a plausible candidate for the target of this association.

Impact—This finding provides insight into genetic markers associated with EOC outcome and potential treatment options.

Keywords

progression-free survival; overall survival; epithelial ovarian cancer; chemotherapy; SNPs

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AUTHOR CONTRIBUTIONS

MCJQ and SJ performed the statistical analyses. DG performed the HiChIP analysis. KMC performed 3C experiments and luciferase assays, along with AC. JB and TO'M performed in bioinformatic analyses. JPT and MR provided technical data support. AdeF performed expression analyses through kmPLOTTER. A-MP provided expression results from the AOCs. MJCG and GCT drafted the manuscript. JSO, PG, BG, SLE, YL and PDPP assisted with the design of the study. All other authors provided phenotypic data and DNA samples. All authors read and approved the final manuscript.

Conflicts of interest:

There are no conflicts of interest to disclose.

INTRODUCTION

Ovarian cancer is the leading cause of death from gynaecological cancers (1). Although EOC is among the most chemotherapy-sensitive of solid tumors and generally shows a high initial response to platinum/taxane treatment, the disease will recur in 60–80% of women with advanced disease within five years (2), (3). Considerable effort has been focused on identifying predictors of outcome at the somatic level, but to date no germline polymorphisms associated with outcome have been identified at genome-wide significance (4), (5), (6), (7). It is well recognised that the different histologic subtypes of EOC differ with respect to response to treatment and outcome, and much of the mortality associated with this disease is due to diagnosis only occurring at an advanced stage of the disease (8). The most common subtype is high-grade serous, which is usually very sensitive to platinum-based chemotherapy initially, with response rates to first-line treatment being as high as 85%. However, the five year survival rates can be less than 30% due to progressive development of resistance to chemotherapy, and the majority of women diagnosed with advanced serous EOC will eventually relapse and die from their disease (9), (10).

For the past two decades, the global standard of care for advanced ovarian cancer, consisting of cytoreductive surgery combined with carboplatin and paclitaxel chemotherapy, has remained essentially unchanged (11). However, new molecularly targeted therapies for recurrent EOC, such as the angiogenesis inhibitor, Bevacizumab, and PARP inhibitors that can increase PFS in specific sub-populations of patients, are currently entering clinical practice in the first line setting. The identification of germline variants associated with response to treatment and outcome may help guide the selection of treatment options and ultimately lead to a more personalised approach to treatment. We and others have used the candidate gene approach to identify predictors of disease progression, but promising candidates have not validated in large-scale association studies (12), (13). GWAS have successfully identified susceptibility loci for many cancers, including EOC. We previously reported a multi-phase GWAS that identified SNPs in an enhancer of *PSIP1* associated with PFS in serous EOC patients ($P = 7 \times 10^{-5}$, HR = 1.90 for rs7874043) (4). However, GWAS have not identified any loci associated with outcome from EOC below genome-wide significance thresholds ($P < 5 \times 10^{-8}$).

Here we present the results of a GWAS of PFS in 2,352 EOC patients who had undergone cytoreductive surgery and first line chemotherapy with at least four cycles of standard dose combined paclitaxel and carboplatin. Meta-analysis of results from 19 sites within the Ovarian Cancer Association Consortium (OCAC) and from the Cancer Genome Atlas (TCGA) study of predominantly high-grade serous EOC, identified one locus at 12q24.33 associated with PFS ($P = 1.47 \times 10^{-8}$), which may target *ULK1*.

MATERIALS AND METHODS

Study Populations

Data were derived from three sources: patients from OCAC genotyped on the OncoArray (14) ($n=5508$ with data on overall survival (OS), $n=4,956$ with data available on PFS); additional OCAC patients genotyped in the COGS study (15) ($n=317$ for OS, 303 for PFS)

and the publicly available TCGA data for high-grade serous EOC patients genotyped on the Affymetrix SNP 6.0 array (16) (n=335 for OS, 337 for PFS) (Supplementary Tables 1-3). The minimum criteria for inclusion in these studies were cytoreductive surgery as part of primary treatment, and European ancestry determined using the program LAMP to assign intercontinental ancestry (17). In total, data were available for overall survival for all 6,160 patients and for PFS in 5,596 patients (Supplementary Table 3). Of all 6,160 patients, 2,620 had chemotherapy consisting of at least four cycles of three-weekly paclitaxel and carboplatin at known or assumed doses of 135–175 mg/m² and AUC 5–7 respectively (7) which was considered ‘standard chemotherapy’; the remainder had no chemotherapy or chemotherapy that differed from ‘standard chemotherapy’ (henceforth ‘irrespective of chemotherapy’).

Our primary outcome of interest was PFS in all histologies in the ‘standard chemotherapy’ group (n=2,352). Additionally, we performed exploratory analyses for PFS in the ‘irrespective of chemotherapy’ group (n=5,596), for the serous subtype (n=4,392), and for overall survival (OS) (n=6,160). PFS was defined as the interval between the date of histological diagnosis and the first confirmed sign of disease progression based on CA125 criteria (Gynecologic Cancer Intergroup (18)), imaging, clinical assessment or death, as previously described (12). OS was the interval between the date of histological diagnosis and death from any cause. Patients who had an interval of >12 months between the date of histological diagnosis and DNA collection were excluded from the analysis to avoid survival bias. For any analysis, a minimum number of 10 women with ovarian cancer per study site was required for inclusion. Cases from the BAV, NCO, NOR and HJO sites were not included in the PFS analysis because baseline PFS estimates for these sites were significant outliers ($P < 10^{-5}$ compared to the largest site, AUS) (number used in each analysis given per site in Supplementary Table 1). The total numbers of cases available for each of these analyses are given in Supplementary Table 3. All studies received approval from their respective human research ethics committees, and all OCAC participants provided written informed consent. Data from TCGA (<http://cancergenome.nih.gov/>) were downloaded through the TCGA data portal and assessed for ancestral outliers to determine those of European descent (as described in (4)).

Genotyping and imputation

The OncoArray is a custom-designed Illumina array consisting of ~533,000 variants, 260,660 of which formed a GWAS backbone (14). Over 500,000 samples were genotyped on the OncoArray platform, including studies participating in the OCAC. Details of SNP quality control, imputation, and intercontinental ancestry and principal component (PC) analysis have been reported elsewhere (19). Further details of COGS and TCGA samples are provided in previous reports (4), (7). Imputation using 483,972 SNPs that passed quality control on the OncoArray was carried out to the 1000 Genomes phase 3 reference dataset, NCBI build b37 (October 2014 release) using IMPUTE2 (20). Data on over 12.1 million SNPs with a minimum imputation score of 0.3 were available for analysis from the OncoArray, on 11.6 million from COGS and on 10.6 million from TCGA (Supplementary Table 4). Only European samples for each study group, as determined by respective PC analysis, were used for analysis.

Statistical Analysis

Primary analysis was of PFS among women who had standard chemotherapy (n=2,352); additional exploratory analyses were of PFS irrespective of chemotherapy (n=5,596), and of OS (n=2,620 patients with standard chemotherapy, n=6,160 patients irrespective of chemotherapy) (Supplementary Table 3). We also performed exploratory analyses limited to the serous subtype for all of these four analyses. Cox proportional hazards (COXPH) models were used to estimate the SNP associations with PFS and OS, adjusting for study sites, grade (low versus high), FIGO stage (4 levels), residual disease (nil versus any), histology (five subtypes - serous, mucinous, endometrioid, clear cell, mixed epithelial - in the all histology analysis), and age at diagnosis (OS analysis only). COXPH results from the three data sources (OncoArray, COGS and TCGA) were meta-analyzed with the METAL program (21), using the standard error model weighting, with genomic control correction. Supplementary Table 4 shows the total number of SNPs analyzed and the lambda values for each analysis.

We primarily focused on SNPs with MAF > 2%, although SNPs with a MAF between 0.5 and 2% that reached genome-wide significance in the COXPH analysis were re-analyzed using the COXPHF model (Cox regression with Firth's Penalized Likelihood) (22), (23) see also <https://cran.r-project.org/web/packages/coxphf/coxphf.pdf> and also with up to 100 million permutations of the COXPH model where appropriate.

Expression quantitative trait loci analyses

Associations between germline genetic variants and ovarian tumor gene expression were conducted using samples from 310 Mayo Clinic patients with available genotype (Illumina Infinium OncoArray) and gene expression (Agilent whole human genome 4x44K expression microarray) data. For all SNPs and gene expression probes within a 1 MB region of the top association signal (rs10794418), a linear model was fit to predict gene expression adjusted for age. Expression values for most probes were normally distributed. Sensitivity analyses were also performed with a Van der Waerden rank transformation of expression values, restricted to the high-grade serous subtype (N=231) and including copy number adjustment (N=286 overall and N=218 high-grade serous). Similar analyses were performed for all SNPs and gene expression probes within a 1 MB region of *ULK1*. Multiple testing correction was implemented using false discovery rate (FDR) methods.

Cell Lines

We used two serous EOC cell lines (OVCAR8 and CaOV3) and three endometrioid EOC cell lines (A2780, IGROV1 and TOV112D). OVCAR8, A2780 and IGROV1 were grown in RPMI media supplemented with 10% fetal bovine serum. CaOV3, was grown in DMEM media supplemented with 10% serum and 1% non-essential amino acids. TOV112D was grown in MCDDB105 and Medium 199 (50:50) supplemented with 15% fetal bovine serum. TOV112Dluc, containing a luciferase tag, was grown with the addition of puromycin (2 mg/mL). All cell lines were routinely tested for *Mycoplasma* and profiled with short tandem repeats to confirm their identity.

HiChIP Library Generation

TOV112D cells were grown to ~80% confluence in 10-cm plates and fixed using 1% formaldehyde for 10 min. Formaldehyde was neutralised by washing twice with ice-cold 125 mM glycine/PBS. Cells were detached from tissue culture dishes using a cell scraper, centrifuged and washed with PBS before storage at -80°C . Samples for HiChIP were generated as per O'Mara et al (24) with modifications. Briefly, a HiC+ Kit (Arima Genomics) was used to isolate nuclei, digest and label chromatin with biotin, and perform proximity ligation *in situ* to create global chromatin contact libraries. Nuclei were then lysed and chromatin fragmented using a Covaris S220 Sonicator. Fragmented chromatin was incubated overnight with a monoclonal antibody raised against H3K27Ac (Abcam, EP16602) and the following day H3K27Ac-associated chromatin was captured using Protein A beads, which was purified with DNA concentrator columns (Zymo Research). Chromatin labelled with biotin was captured with streptavidin-coated beads and TDE1 enzyme (Illumina) used for tagmentation. HiChIP sequencing libraries were PCR-generated from tagmented samples using the Nextera DNA preparation kit (Illumina). Ampure XP beads were used to select for 300–700 bp fragments. Two independent sequencing libraries generated for Illumina NextSeq550 (QIMRB sequencing facility, Brisbane, Australia) for $2\times 150\text{bp}$ sequencing.

HiChIP Bioinformatic Analyses

HiChIP sequencing libraries were analyzed as per O'Mara et al (24) with modifications. HiC-Pro version 2.9.0 was used to align fastq files, generated from sequencing reads, to the human reference genome (hg19) (25). Duplicate reads were removed using default settings, and unique reads assigned to Arima restriction fragments and filtered for valid interactions. Valid reads were processed using the hichipper pipeline version 0.7.0 (26) and the MACS2 background model was used to identify H3K27Ac chromatin peaks. Hichipper selected for chromatin interactions between 5 kb and 2 Mb. We then filtered chromatin loops, supported by at least four unique paired-end reads, with a Mango (27) q -value < 0.0001 and replication in two independent experiments for further investigation. We also defined promoter-associated interactions as those loops within a HiChIP anchor located 3 kb from a gene transcription start site (GRCh37; accessed May 2019).

Chromatin Conformation Capture

Cross-linked DNA from OVCAR8 and CaOV3, A2780, IGROV1 and TOV112Dluc was digested with HindIII to generate chromatin conformation capture (3C) libraries as previously described (28). 3C interactions were quantified by ddPCR previously described (29) on three independent 3C libraries using primers designed against the HindIII restriction fragments across the 12q24.33 locus (Supplementary Table 5).

Plasmid Generation

A 1794-bp fragment containing the *ULK1* promoter using PCR primers (Supplementary Table 5) modified with KpnI and HindIII was cloned into the pGL3 basic luciferase reporter. A 1049 bp fragment of the putative regulatory element (PRE) containing SNPs rs12301971,

rs112786120 and rs11246872 was generated using PCR primers listed in Supplementary Table 5. This PRE was then cloned into the *ULK1* promoter construct.

Reporter Assays

We transiently transfected TOV112D cells with luciferase reporter constructs with the *ULK1* promoter and SNP-containing putative regulatory elements. Renilla was co-transfected as an internal control. After 24 hours, cell lysates were prepared using the Dual-Glo Luciferase kit (Promega) and luciferase luminescent activity measured with a Synergy H4 plate reader (Biotek). Luciferase activity was normalized to Renilla to correct for differences in transfection efficiencies or lysate preparation. The activity of each enhancer construct was compared to the construct with only the *ULK1* promoter. Allele-specific activity was calculated after log transforming the raw readings and performing two-way ANOVA, followed by Dunnett's multiple comparisons test using a custom Shiny web application (jbeesley.shinyapps.io/qimrb_luciferaseassay/).

ULK1 knockout in ovarian cancer cell lines

Ovarian cancer cell lines, TOV112D and OVCAR8, were transduced with a lentiviral vector expressing the Cas9 nuclease under blasticidin selection (Lenti-Cas9-2A-Blast). Guide RNAs (gRNAs) were designed using Broad sgRNA design tool (<http://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design>).

A non-target control sequence (NTC) and three gRNAs targeting *ULK1* (*ULK1* C1, *ULK1* C2 and *ULK1* C3, Supplemental Table 6) were cloned into a lentiviral vector (lentiGuide-Puro) (61). Cas9-expressing cell lines were transduced with either a lentiviral vector expressing NTC or a single *ULK1* gRNA under puromycin selection. After antibiotic selections, real time PCR (qRT-PCR) and Western blotting were performed to validate the depletion of *ULK1* mRNA and protein level in these cells. Total RNA was isolated from eight cell lines using the RNeasy Mini Kit (Qiagen). cDNA was synthesized using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) and amplified using PowerUp™ SYBR™ Green Master Mix (Thermo Scientific). Three primer pairs were used for *ULK1*, along with five housekeeping genes (Supplementary Table 7). The mRNA levels for each sample were measured in technical triplicates for each primer set. Experiments were performed using an ABI ViiA(TM) 7 System (Applied Biosystems), and data processing was performed using ABI QuantStudio™ Software V1.1 (Applied Biosystems). The average of Cts from all the primer pairs for *ULK1*, compared to the geometric mean of housekeeping genes were used to calculate C_T . The relative quantitation of each mRNA normalizing to that in the NTC line was performed using the comparative C_T method (2^{-C_T}). For Western blot analysis, *ULK1* (D8H5) Rabbit mAb was used (8054, Cell Signaling).

Paclitaxel and carboplatin sensitivity in *ULK1* knockout ovarian cancer cell lines

Paclitaxel (Focus Bioscience) was dissolved in DMSO to make a 50 mM stock solution and carboplatin (Focus Bioscience) was dissolved in water to make a 50 mM stock solution. All drugs were diluted in fresh medium immediately before each experiment. All additional dilutions were completed with the respective cell culture media for each cell line in order

to achieve serial dilution for the respective concentrations for treatment of the cells. Cell growth inhibition by paclitaxel or carboplatin was determined by proliferation assays in 96 well plates using InCuCyte. 3,500 of NTC, *ULK1 C1*, *ULK1 C2* or *ULK1 C3* cells per well were plated in 100 μ l for each dose in duplicates. Four images of each well were acquired to establish the base line confluence, and serial drug dilutions were administered in 100 μ l volumes to achieve the final concentrations in 200 μ l culture media. Doses for serial dilution of the drugs were in Supplementary Table 8. Images were taken every 4 hours in a 112 hour period to document the confluence in each well. Baseline-corrected confluence fold changes (growth curve) were calculated using GraphPad. The area under baseline-corrected growth curve (AUC) of NTC and *ULK1* knockout lines was calculated to measure their proliferation. The AUC of each dose was calculated and dose responses were generated using GraphPad function log (inhibitor) vs. response (three parameters). The IC_{50} was defined as the drug concentration required for a 50% reduction in baseline-corrected AUC. IC_{50} values were the outputs in the best-fit values for paclitaxel and carboplatin in the eight ovarian cancer cell lines with different gene editing (TOV112D NTC, TOV112D *ULK1 C1*, TOV112D *ULK1 C2*, TOV112D *ULK1 C3*, OVCAR8 NTC, OVCAR8 *ULK1 C1*, OVCAR8 *ULK1 C2* and OVCAR8 *ULK1 C3*). Five independent experiments were performed for each drug and each cell line. The IC_{50} ratios of the three *ULK1* knockout lines were compared to the IC_{50} of the corresponding NTC lines and the fold changes were tested using the one-way ANOVA function in GraphPad.

RESULTS

In our primary analysis of PFS for patients with all ovarian cancer histologies who had standard chemotherapy we identified 209 SNPs with $P < 10^{-5}$, of which 43 SNPs had $P < 10^{-6}$ (Figure 1; Supplementary Table 9). Seven correlated SNPs, all at 12q24, exceeded the nominal genome-wide threshold level of significance ($P < 5 \times 10^{-8}$) (Table 1). These seven SNPs were all imputed, with $r^2 > 0.99$. The top SNP was rs10794418 (HR 1.24, 95% CI 1.15–1.34; $P = 1.47 \times 10^{-8}$), at chr12:132041198 (MAF = 0.4), with the rarer T allele associated with shorter PFS. The results for this SNP were consistent in the OncoArray, COGS and TCGA cohorts (HR 1.22–1.61; heterogeneity P value = 0.45) and in each of the OCAC sites genotyped on the OncoArray (HR 1.05–1.87), except for two of the smallest sites, UHN and WMH (HR 0.97 and 0.61, respectively) (Supplementary Figure 1). Analysis of the OncoArray data stratified by histotype showed that the effect was significant in serous cases ($n=1,532$; HR 1.17, 95% CI 1.07–1.27), but significantly stronger ($P = 0.002$) in the endometrioid cases ($n=187$; HR 2.48, 95% CI 1.57–3.92) (Figure 2). Five years after diagnosis, 20.6% TT patients were still progression-free, compared to 25.4% for TC and 32.4% for CC patients (Supplementary Figure 2).

We conducted several exploratory analyses for PFS in: 1) 5,596 patients of all histologies irrespective of chemotherapy regimen, 2) 1,751 patients with serous EOC who had standard chemotherapy, 3) 4,037 patients with serous EOC irrespective of chemotherapy regimen (Supplementary Figures 3-5). None of these exploratory analyses of PFS identified any SNPs that reached genome-wide significance; all SNPs with $P < 10^{-5}$ for each analysis are shown in Supplementary Tables 10-12. We also looked for associations with OS in 1) 2,620 patients with cancers of all histologies who had standard chemotherapy, 2) 6,160 patients

with cancers of all histologies irrespective of chemotherapy regimen, 3) 1,901 patients who had serous EOC with standard chemotherapy, 4) 4,391 patients with serous EOC irrespective of chemotherapy regimen. None of these analyses of OS identified any SNPs that reached genome-wide significance.

We examined the results for rs10794418, the top SNP for PFS in women who had undergone standard chemotherapy, in all of these exploratory analyses (Table 1). The effect estimates for PFS were attenuated in analysis of patients of all histologies in the “irrespective of chemotherapy” group (HR 1.06; 95% CI 1.01–1.12, $P = 0.01$), and in the subsets of patients with serous histology treated with standard chemotherapy (HR 1.20, 95% CI 1.10–1.30, $P = 2 \times 10^{-5}$) and also in women with serous histology in the “irrespective of chemotherapy” group (HR 1.04, 95% CI 0.98–1.10, $P = 0.17$). Among patients who had standard chemotherapy, rs10794418 was significantly associated with OS, in women with any histotype (HR 1.13, 95% CI 1.04–1.22, $P < 0.01$) and in the subset of women with serous cancers (HR 1.11, 95% CI 1.01–1.22, $P = 0.02$).

In primary and some secondary analyses, we also identified several rare SNPs (with MAF 0.005–0.02) that reached genome-wide significance when using the COXPH model (Supplementary Table 13). However, the asymptotic approximation made in COXPH can provide unreliable results with small minor allele counts so we re-analysed these using the more computationally intensive COXPHF model which implements a Firth corrected Cox model (23). Given the OncoArray data set comprised 90% of our total sample size we used this to perform our COXPHF analysis. As a further check, we also used label swap permutation to derive empirical p-values using the COXPH model (up to 100 million permutation replicates, with replicate number varied depending on the p-value achieved). The robust p-values were then used instead of the asymptotic p-values. The statistical significance for all SNPs with MAF $< 2\%$ which previously showed associations indicative of genome-wide significance was decreased by at least an order of magnitude and so we did not take any of them forward for further meta-analysis (Supplementary Table 13).

The lead SNP, rs10794418, lies in an intergenic region between the genes *LOC338797* (alias *RP13-507P192*) and *SFSWAP* at 12q24.33 (Supplementary Figure 6). Further analysis of this region, indicates that the 43 putative causal variants (defined as those with a P-value within two orders of magnitude of that for rs10794418), lie within 1Mb of 14 protein-coding (*ADGRD1*, *DDX51*, *EP400*, *EP400NL*, *FBRSL1*, *GALNT9*, *LACAT8*, *MMP17*, *MUC8*, *NOC4L*, *PUS1*, *RAN*, *RIMPBP2*, *SFSWAP*, *STX2* and *ULK1*) and several non-coding genes, most of which are expressed in the normal ovary and in serous high grade ovarian tumor tissue (Supplementary Figure 6).

We used KMplotter (30) to evaluate associations between PFS following diagnosis of EOC and expression of the genes in the 1Mb region around rs10794418, for which data were available (*ADGRD1* (alias *GPR133*), *DDX51*, *EP400*, *FBRSL1*, *GALNT9*, *LINC01257* (alias *RP11-638F5.1*), *MMP17*, *MUC8*, *NOC4L*, *PUS1*, *RAN*, *STX2* and *ULK1*). First, we analysed patients with cancers of any histology who had been treated with any chemotherapy because the doses used for those treated with taxol and platin therapy are not known. Significant associations that met our Bonferroni-corrected significance threshold

of $P = 0.0036$ were observed for five genes (*ADGRD1*, $P = 0.0008$; *DDX51*, $P = 0.0001$; *FBRSL1*, $P = 1.7 \times 10^{-5}$; *LINC01257*, $P = 0.0016$; and *ULK1*, $P = 2.0 \times 10^{-5}$) (Figure 3 and Supplementary Figure 7). For each of these genes, high levels of expression were associated with shorter PFS. Second, we analysed serous cancers only, treated with any chemotherapy, and found a significant association for *STX2* ($P = 0.0001$), with *ULK1* remaining significantly associated with outcome ($P = 4.4 \times 10^{-5}$). Further analyses restricted to a smaller dataset of patients known to have received taxol and platin therapy (Figure 3), did not show any significant associations using this threshold, though the association with *ULK1* expression remained nominally significant ($P = 0.019$).

We carried out expression quantitative trait loci (eQTL) analysis to determine whether the SNPs associated with PFS were also associated with expression of any genes in the region in ovarian tumors. Among the 62 SNPs in the region with evidence of association with PFS ($P < 10^{-5}$; Supplementary Table 9), there were 21 eQTL signals with uncorrected $P < 0.01$ (Supplementary Table 14); all of these are associated with probes for *RAN* or *ULK1*. For rs10794418, the minor T allele was associated with increased expression of *RAN* (A_32_P506600, $\beta = 0.03$, uncorrected $P = 0.007$) and *ULK1* (A_23_P72550, $\beta = 0.03$, uncorrected $P = 0.009$); results were similar but attenuated when restricted to high grade serous tumors, and when adjusted for copy number. These probes have a moderate level of correlation ($R = 0.37$). The SNP with the strongest correlation with *ULK1* expression is rs6598157 ($\beta = 0.032$, $P = 0.003$), which is also associated with PFS ($P = 1.73 \times 10^{-7}$).

We used the H3K27Ac HiChIP data, which identifies genome-wide looping between promoters and enhancers, from the endometrioid EOC cell line, TOV112D, to identify target gene promoters that loop to any of the 43 PFS-associated variants (Supplementary Figure 8). This identified *ULK1* and *MMP17* as potential target genes. Given that the kmPLOTTER and eQTL analyses both implicated *ULK1* as a target gene, we validated the interaction between the PFS associated SNPs and the *ULK1* promoter using targeted 3C experiments in A2780, IGROV1, TOV112Dluc, OVCAR8 and CaOV3. This identified an interaction between the *ULK1* promoter and a PRE in all five cell lines (Figure 4).

The interacting restriction fragment identified by 3C is 16kb and contains 21 PFS-associated variants. Three SNPs (rs12301971, rs112786120 and rs11246872) fall within a region of H3K27ac enrichment identified by HiChIP in TOV112D cells (Supplementary Figure 8). We examined the regulatory capability of this PRE in TOV112D cells using a luciferase construct containing the two PFS-associated haplotypes ('risk' associated with shorter PFS, and 'protective' with longer PFS) to determine whether either enhancer haplotype was more active at transactivation. Reporter assays showed significant enhancer activity of the PRE on the *ULK1* promoter. However, there was no significant difference between the risk and protective haplotypes under the conditions tested (Figure 4).

Knockdown of *ULK1* in OVCAR8 and TV112D cells resulted in small changes in proliferation rates (both increased and decreased) in OVCAR8 and TOV112D (Supplementary Figure 9). However, when the cells were treated with carboplatin, the IC_{50} of *ULK1* OVCAR8 and TV112D knockout lines was reduced significantly and substantially, in comparison to the IC_{50} of the NTC lines (Figure 5). A less pronounced effect was

seen when the cells were treated with paclitaxel, with a decrease in IC₅₀ seen in both cell lines only following knockdown with ULK C1, and a less consistent effect seen following knockdown with ULK C2.

DISCUSSION

We performed a GWAS of survival time in 2,352 women diagnosed with EOC who had undergone cytoreductive surgery and been treated with a standard carboplatin and paclitaxel regimen as first line chemotherapy and identified a region at 12q24.33 associated with PFS. This study is the largest GWAS to date of PFS in EOC, using detailed clinical information to adjust for the main predictors of outcome – residual disease, stage, grade and histology - and to restrict the primary analysis to 2,352 patients who had standard chemotherapy. The minor T allele of the top SNP, rs10794418, was associated with shorter PFS in all except two of the smallest contributing sites from OCAC. This study, which expands on our previous studies with smaller sample sizes (7), (4), is the first to identify a locus associated with outcome in EOC that meets the genome-wide significance level. Wheeler *et al.*, (2013) performed a GWAS of carboplatin- and cisplatin-induced toxicity on lymphoblastoid cell lines (31), but did not identify an association with SNPs at 12q24.33, either because of lack of power, or issues of tissue-specificity. Sato *et al.* performed a small GWAS of survival in lung cancer patients treated with carboplatin and paclitaxel, but none of the three SNPs they reported as associated with outcome reached $P < 10^{-5}$ in our analyses (32).

The association signal for SNPs at 12q24.33 was strongest in the full data set (all histologies) although a similar HR was seen in the serous-only subset (1.24 versus 1.20). It was evident from a subtype specific analysis of the OncoArray samples, that the endometrioid subtype (n=187) had a higher hazard ratio (2.48 (95% CI: 1.57 –3.92), compared to other subtypes such as serous (n=1,532, HR of 1.17 (95% CI: 1.07–1.27) ($P = 0.002$ for difference in HR between serous and endometrioid subtypes). This may in part be due to the better PFS which has been reported for the endometrioid subtype (33) which has different molecular pathogenesis to the other subtypes. As we hypothesized, the effect was most marked in patients who had received standard chemotherapy, being attenuated in those who had no, or non-standard, chemotherapy which often included an additional drug. This may suggest that the target gene(s) of the association plays a role in response to paclitaxel or carboplatin. The association of the top SNP with OS was weaker than for PFS, but still significant for patients who received standard chemotherapy.

Bioinformatic analysis indicated that the candidate SNPs reside in an intergenic region of chromosome 12, suggesting they may have a regulatory function. Intersection of histone modification ChIP-seq data for whole ovary generated by the Roadmap Epigenomics Project revealed that the variants 12:132059205:C:T and 12:132058489:T:C lie within enhancer-associated H3K27ac and H3K4me1 marks. Examination of TCGA expression data showed that a number of genes in this region are expressed in serous ovarian tumors – for example *RIMBP2*, *STX2*, *RAN*, *SFSWAP*, *MMP17*, *ULK1*, *PUS1*, *EP400*, *SNORA49*, *EP400NL*, *DDX51*, *NOC4L* and *GALNT9*. Similarly, analysis of 114 serous ovarian tumor samples in the Australian Ovarian Cancer Study showed that 13 genes in this region were expressed at > 5 counts per million, in more than 10% of the samples

assayed (*RIMBP2*, *STX2*, *RAN*, *GPR133*, *SFSWAP*, *ULK1*, *PUS1*, *EP400*, *EP400NL*, *DDX51*, *NOC4L*, *GALNT9*, and *FBRSL1*) (34). Taken together, these data suggest that PFS-associated variants that lie within PREs, which are active in normal ovarian tissue, may influence expression of nearby genes expressed in ovarian tumors. Expression analysis using kmPLOTTER (www.kmplot.com/ovar/) showed that among patients who had been treated with any chemotherapy, *ADGRD1*, *DDX51*, *FBRSL10*, *LINC01257*, *STX2* and *ULK1* were associated with PFS in analyses of all histologies, or serous alone. This was most striking for *ULK1* with high expression associated with shorter PFS in those with any histology (HR 1.32, 95% CI 1.16–1.50), and in those with only serous EOC (HR 1.36, 95% CI 1.17–1.59). eQTL analysis found that the minor T allele of rs10794418 was associated with increased expression of *RAN* and *ULK1*. Because kmPLOTTER analysis indicated that *ULK1* is the most likely target of the association with PFS, we extended the eQTL analysis for *ULK1* to all the SNPs in the region and found that the top eQTL is also associated with PFS with a P-value within an order of magnitude of that for rs10794418. This would be consistent with the likelihood that the same SNP is driving both expression of *ULK1* and the association with PFS.

ULK1 (unc-51 like autophagy activating kinase 1) is a serine/threonine kinase which plays an integral role in human autophagy signalling (35–36). Interestingly, Nishikawa *et al.*, (2015) found that weak expression of ULK1 as measured by immunohistochemistry, was associated with better PFS in multivariate analysis in patients diagnosed with metastatic renal cell carcinoma and treated with the mTOR inhibitors everolimus and temsirolimus (37). mTOR inhibitors have also been investigated as a treatment in ovarian cancer with low response rates (38), although no activated mTOR signalling molecules have been shown to be convincing biomarkers for prognosis (39). In primary ovarian adenocarcinoma increased *ULK1* expression is associated with recurrence (40). High expression of ULK1 has also been found to be associated with poor prognosis in nasopharyngeal carcinoma (41), oesophageal squamous cell carcinoma (42), clear cell renal carcinoma (43), non-small cell lung cancer (44) and hepatocellular carcinoma (45). Conflicting reports have been published in relation to breast cancer with low ULK1 expression correlated with poor outcome (46) and low expression identified in TCGA breast cancer tissue samples and especially in triple negative breast cancer (47), but high levels of ULK1 correlated with poor prognosis in some breast cancer patients (48). A study in the ovarian cancer cell line, CaOV3, found that DUSP1-knockdown promoted autophagy via an ULK1 mediated mechanism (49) and there is a strong functional link between autophagy and both paclitaxel (50) and carboplatin (51) response. Additionally, there is evidence that autophagy may be integral to chemoresponse with an induction of autophagy limiting the efficiency of chemoresponse in oesophageal cancer cells (52), whilst both protumorigenic and antitumorigenic autophagy were observed treating breast cancer cells with various chemotherapeutics (53).

Our HiChIP and 3C experiments indicate that *ULK1* is a target gene at this locus. We have identified an interacting enhancer containing three PFS-associated variants. While there was no difference between the two haplotypes on the activity of the *ULK1* promoter in our reporter assays, such differential activity may be context dependent. It is also possible that other variants at the 12q24.33 locus may regulate *ULK1*, but our bioinformatics analyses indicate we have tested the most likely functional SNPs in our reporter assays.

Hypothesizing that ULK1 may act as a marker for drug response, we evaluated the effect of knocking it out on cell proliferation, and on carboplatin or paclitaxel sensitivity. Knockdown of ULK1 rendered the cells much more sensitive to carboplatin. An inhibitor of ULK1 has been shown to sensitize non-small cell lung cancer cells to cisplatin by modulating autophagy and apoptosis pathways (44). ULK1 inhibition by a small molecule inhibitor enhanced cytotoxicity of daunorubicin against acute myeloid leukaemia cells (54), and inhibition of ULK1 decreased autophagy and cell viability in high-grade serous ovarian cancer spheroids (55). The point at which autophagy is inhibited may be critical as an early stage ULK1/2 inhibitor potentiated chemosensitivity in mesothelioma, whilst a late stage inhibitor did not (56). Further experiments inhibiting ULK1 in ovarian cancer cells are required to determine whether tumor growth, chemoresponse, or progression free survival, would be affected *in vivo*.

In conclusion, we have identified a region at 12q24.33 associated with PFS, in all EOC subtypes in a cohort of clinically well-defined ovarian cancer patients. Our eQTL and functional analyses identified *ULK1* as the target gene at this locus, and high expression of ULK1 is associated with shorter PFS in ovarian cancer. Intriguingly, ULK1's central role in the autophagy process highlights its potential as a biomarker and drug target. Although the effect of the associated alleles is small, this does not negate the potential for therapeutic intervention, as has been noted for other GWAS target genes (57). Further functional evaluation of ULK1 in ovarian cancer, and other potential targets in this region, could help progress the development of novel treatment strategies for EOC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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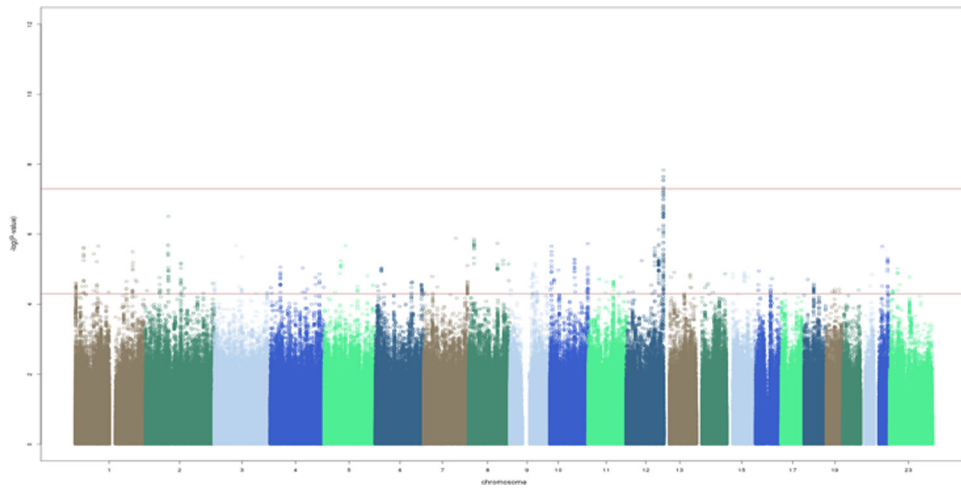


Figure 1. Manhattan Plot of results of the meta-analysis of association of PFS with patients (all histologies) who had standard chemotherapy. The top red line indicates the genome-wide significance threshold; the lower line indicates a suggestive significance level.

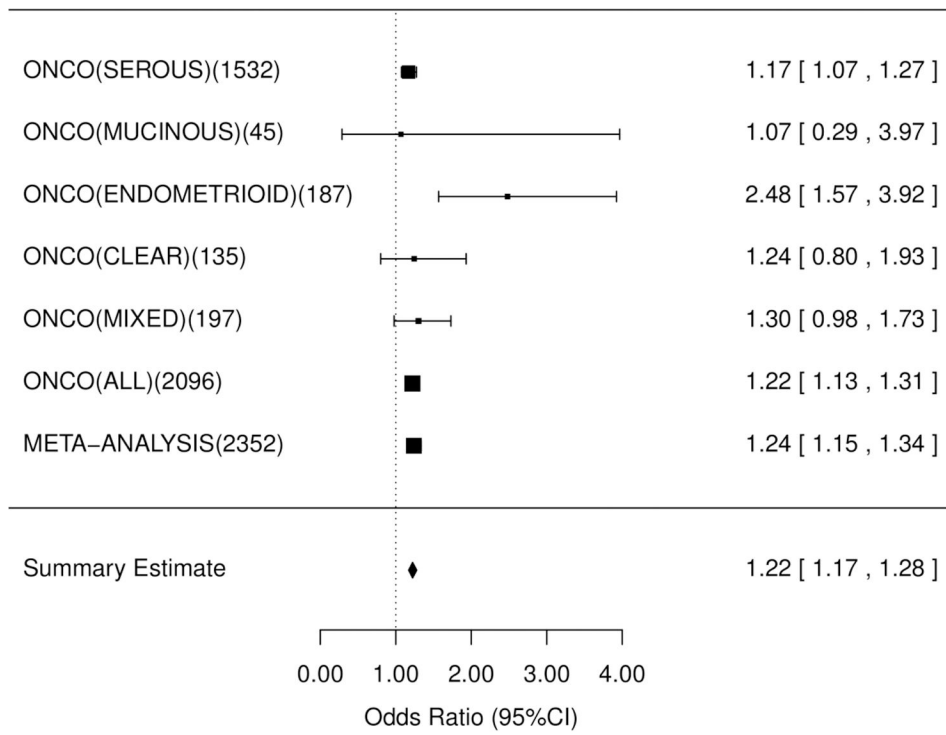


Figure 2. Association of rs10794418 with PFS in patients who had standard chemotherapy, stratified by histotypes in the OncoArray results. Hazard Ratios, along with upper and lower 95% confidence intervals are plotted. Sample sizes are indicated in brackets.

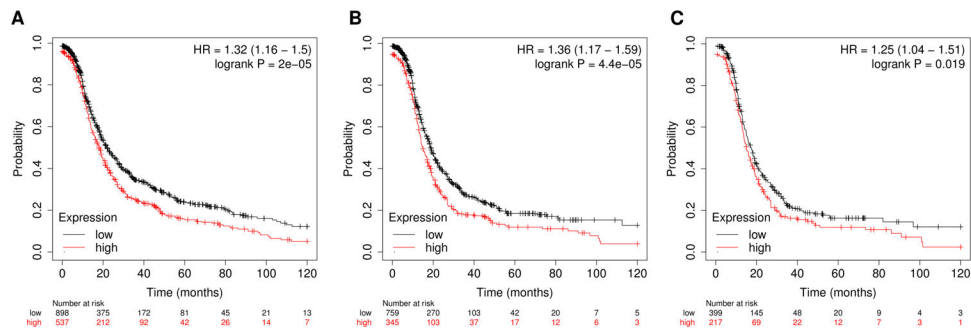


Figure 3.

Association of expression of *ULK1* and PFS using Kaplan-Meier plotter (<http://kmplot.com/>), an online tool to assess the expression levels of microarray-quantified genes using data from publically available datasets²². (A) Women diagnosed with ovarian cancer of any histotype (n = 1,435) treated with any chemotherapy. (B) Women diagnosed with serous ovarian cancer (n = 1,104) treated with any chemotherapy. (C) Women diagnosed with serous ovarian cancer (n = 616) treated with taxol and platin. Cases were divided into high and low expression using auto best cut-off and censored at 10 years.

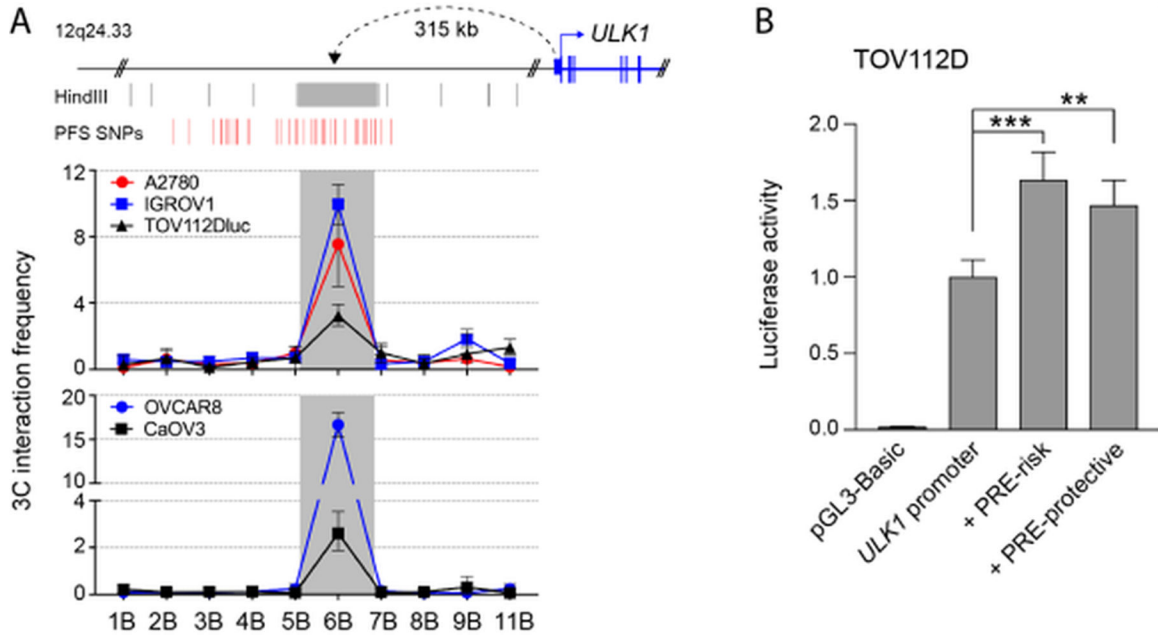


Figure 4.

PFS-associated variants lie in an enhancer of the *ULK1* gene (A) Chromosome conformation capture (3C) interactions between the *ULK1* promoter and regions containing PFS-associated variants in endometrioid (A2780, IGROV1 and TOV112Dluc) and serous (OVCAR8 and CaOV3) cells. 3C interactions were quantified by ddPCR on three independent 3C libraries using primers designed against the HindIII restriction fragments across the 12q24.33 locus. (B) Luciferase reporter assay showing the protective and risk associated haplotypes containing rs12301971 (T allele associated with risk), rs112786120 (A allele associated with risk) and rs11246872 (A allele associated with risk), cloned upstream of the *ULK1* promoter-driven luciferase reporter constructs. Cells were transiently transfected and assayed for luciferase activity 24 hours later. Error bars denote 95% confidence intervals from three independent experiments performed in triplicate. P-values were determined by two-way ANOVA followed by Dunnett’s multiple comparisons test (** p < 0.01, *** p < 0.001) on log transformed data; for ease of interpretation back transformed data have been graphed.

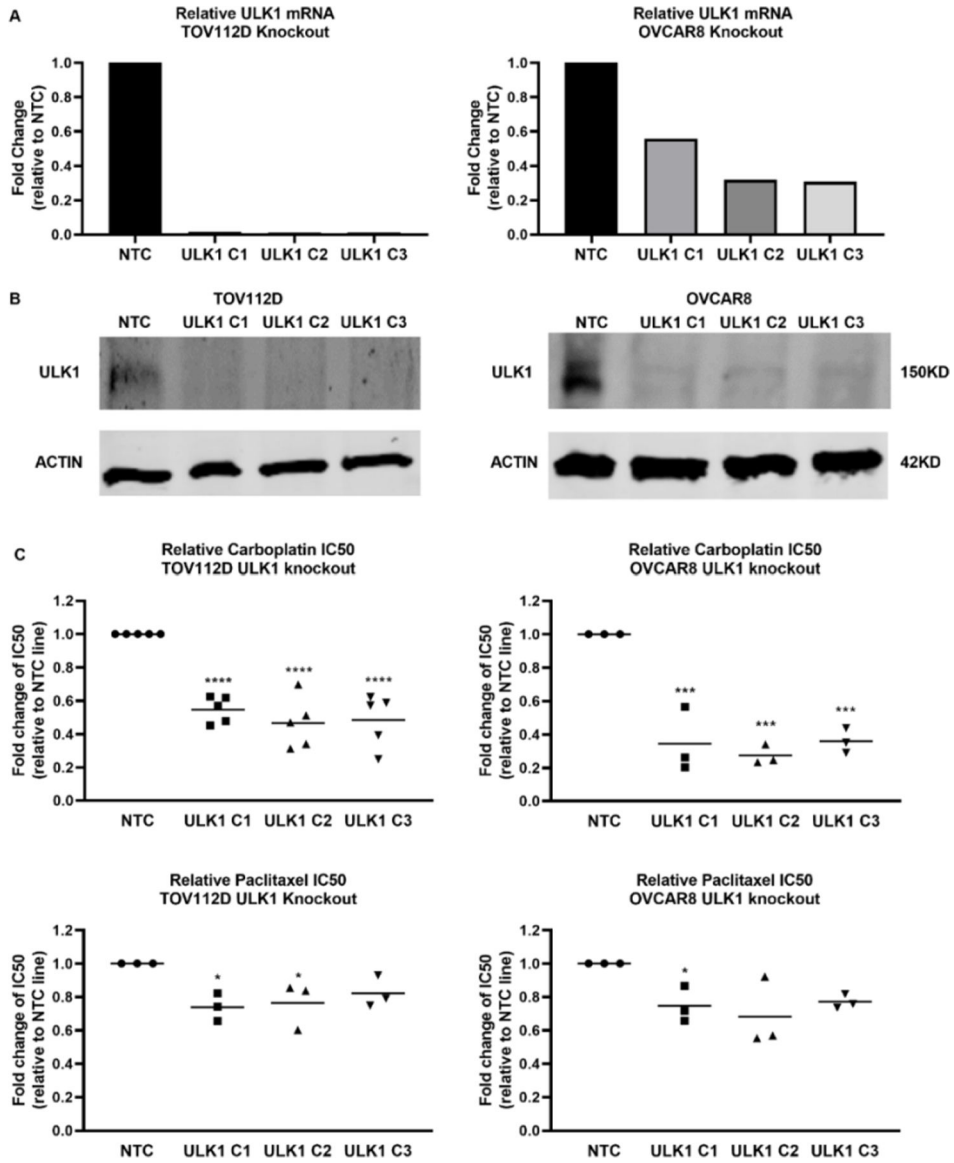


Figure 5. Knockdown of ULK1 showing increased sensitivity to carboplatin (A) Relative ULK1 mRNA level in TOV112D (left panel) and OVCAR (right panel) NTC and ULK1 knockout lines. (B) ULK1 protein level in TOV112D (left panel) and OVCAR (right panel) NTC and ULK1 knockout lines. (C) Relative IC₅₀ of Carboplatin (top panel) or Paclitaxel (bottom panel) in TOV112D (left panel) or OVCAR (right panel) NTC and ULK1 knockout lines.

Table 1: Association between rs10794418 and progression-free survival time in women diagnosed with EOC

Endpoint	Treatment	All Cases				Serous Cases Only			
		N	HR (95% CI)	P-value	Direction [‡]	N	HR (95% CI)	P-value	Direction [‡]
Progression-free survival	Standard chemotherapy	2352	1.24 (1.15–1.34)	1.47 × 10⁻⁸	+++	1751	1.20 (1.10–1.30)	0.00002	+++
	Irrespective of chemotherapy	5596	1.06 (1.01–1.12)	0.01	+++	4037	1.04 (0.98–1.10)	0.17	+++
Overall survival	Standard chemotherapy	2620	1.13 (1.04–1.22)	0.003	+++	1901	1.11 (1.01–1.21)	0.02	+++
	Irrespective of chemotherapy	6160	1.04 (0.99–1.10)	0.11	+++	4391	1.03 (0.97–1.10)	0.29	+++

[‡]COXPH model HR (Hazard Ratio) estimates adjusted for residual disease, FIGO ovarian cancer stage, tumor histology (only for All Cases - serous, mucinous, endometrioid, clear cell, mixed epithelial), grade (low vs high), OCAC site, age at diagnosis (only for OS) and 9 principal components

HR, hazard ratio estimating ordinal association per copy of minor T allele (frequency = 0.401)

CI, confidence interval

[‡]+, direction of effect for the T allele; order of studies for direction is OncoArray, COGS and TCGA