Identification of a Locus Near *ULK1* Associated With Progression-Free Survival in Ovarian Cancer

Michael C.J. Quinn[1], Karen McCue[1], Wei Shi[1], Sharon E. Johnatty[1], Jonathan Beesley[1], Andrew Civitarese[1], Tracy A. O'Mara[1], Dylan M. Glubb[1], Jonathan P. Tyrer[2], Sebastian M. Armasu[3], Jue-Sheng Ong[1], Puya Gharahkhani[1], Yi Lu[1], Bo Gao[4,5], Ann-Marie Patch[1], Peter A. Fasching[6,7], Matthias W. Beckmann[7], Diether Lambrechts[8,9], Ignace Vergote[10], Digna R. Velez Edwards[11], Alicia Beeghly-Fadiel[12], Javier Benitez[13], Maria J. Garcia[13,14], Marc T. Goodman[15,16], Thilo Dörk[17], Matthias Dürst[18], Francesmary Modugno[19,20,21], Kirsten Moysich[22], Andreas du Bois[23,24], Jacobus Pfisterer[25], Klaus Bauman[26]; for the AGO Study Group, Beth Y. Karlan[27], Jenny Lester[27], Julie M. Cunningham[28], Melissa C. Larson[3], Bryan M. McCauley[3], Susanne K. Kjaer[29,30], Allan Jensen[31], Claus K. Hogdall[29], Estrid Hogdall[30,31], Joellen M. Schildkraut[33], Marjorie J. Riggan[34], Andrew Berchuck[34], Daniel W. Cramer[35], Kathryn L. Terry[35,36], Line Bjorge[37,38], Penelope M. Webb[39]; for the OPAL Study Group, Michael Friedlander[40], Tanja Pejovic[41,42], Melissa Moffitt[41,42], Rosalind Glasspool[43], Taymaa May[44], Gabrielle E.V. Ene[44], David G. Huntsman[45,46,47,48], Michelle Woo[49], Michael E. Carney[50], Samantha Hinsley[51], Florian Heitz[23,24,52], Sian Fereday[53], Catherine J. Kennedy[5,54], Stacey L. Edwards[1], Stacey J. Winham[3], Anna deFazio[5,54,55]; for Australian Ovarian Cancer Study Group, Paul D.P. Pharoah[2,56], Ellen L. Goode[3], Stuart MacGregor[1], and Georgia Chenevix-Trench[1]

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 2,352 women with EOC who had undergone cytoreductive surgery and standard carboplatin/paclitaxel chemotherapy.

Results: We found seven 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.

See related commentary by Peres and Monteiro, p. 1604

[1]Department of Genetics and Computational Biology, QIMR Berghofer Medical Research Institute, Brisbane, Queensland, Australia. [2]Department of Oncology, Centre for Cancer Genetic Epidemiology, University of Cambridge, Strangeways Research Laboratory, Cambridge, United Kingdom. [3]Department of Quantitative Health Sciences, Mayo Clinic, Rochester, Minnesota. [4]Crown Princess Mary Cancer Care Centre, Westmead Hospital, Sydney, New South Wales, Australia. [5]Centre for Cancer Research, The Westmead Institute for Medical Research, Sydney, New South Wales, Australia. [6]Division of Hematology and Oncology, Department of Medicine, University of California at Los Angeles, David Geffen School of Medicine, Los Angeles, California. [7]Department of Gynecology and Obstetrics, University Hospital Erlangen, Friedrich-Alexander-University Erlangen-Nuremberg, Comprehensive Cancer Center Erlangen-EMN, Erlangen, Germany. [8]VIB Center for Cancer Biology, VIB, Leuven, Belgium. [9]Laboratory for Translational Genetics, Department of Human Genetics, University of Leuven, Leuven, Belgium. [10]Division of Gynecologic Oncology, Department of Obstetrics and Gynaecology and Leuven Cancer Institute, University Hospitals Leuven, Leuven, Belgium. [11]Department of Obstetrics and Gynecology, Vanderbilt Epidemiology Center, Vanderbilt Genetics Institute, Vanderbilt University School of Medicine, Nashville, Tennessee. [12]Division of Epidemiology, Department of Medicine, Vanderbilt Epidemiology Center and Vanderbilt-Ingram Cancer Center, Vanderbilt University School of Medicine, Nashville, Tennessee. [13]Human Genetics Group, Spanish National Cancer Centre (CNIO), and Biomedical Network on Rare Diseases (CIBERER), Madrid, Spain. [14]Computational Oncology Group, Spanish National Cancer Centre (CNIO), Madrid, Spain. [15]Cancer Prevention and Control, Samuel Oschin Comprehensive Cancer Institute, Cedars-Sinai Medical Center, Los Angeles, California. [16]Department of Biomedical Sciences, Community and Population Health Research Institute, Cedars-Sinai Medical Center, Los Angeles, California. [17]Gynaecology Research Unit, Hannover Medical School, Hannover, Germany. [18]Department of Gynecology, Jena University Hospital – Friedrich Schiller University Jena, Jena, Germany. [19]Division of Gynecologic Oncology, Department of Obstetrics, Gynecology and Reproductive Sciences, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania. [20]Department of Epidemiology, University of Pittsburgh Graduate School of Public Health, Pittsburgh, Pennsylvania. [21]Womens Cancer Research Center, Magee-Womens Research Institute and Hillman Cancer Center, Pittsburgh, Pennsylvania. [22]Division of Cancer Prevention and Population Sciences, Cancer Pathology & Prevention, Roswell Park Cancer Institute, Buffalo, New York. [23]Department of Gynecology and Gynecologic Oncology, Kliniken Essen-Mitte, Essen, Germany. [24]Department of Gynecology and Gynecologic Oncology, Dr. Horst Schmidt Kliniken Wiesbaden, Wiesbaden, Germany. [25]Gynecologic Oncology Center, Kiel, Germany. [26]Department for Gynecology, Philipps University of Marburg, Marburg, Germany. [27]Department of Obstetrics and Gynecology, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, California. [28]Department of Laboratory Medicine and

Introduction

Ovarian cancer is the leading cause of death from gynecological 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% to 80% of women with advanced disease within 5 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 (4–7). It is well recognized 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 5-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 subpopulations 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 personalized 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 multiphase GWAS that identified SNPs in an enhancer of *PSIP1* associated with PFS in patients with serous EOC ($P = 7 \times 10^{-5}$; HR = 1.90 for rs7874043; ref. 4). However, GWAS have not identified any loci associated with outcome from EOC below genome-wide significance thresholds ($P \leq 5\times10^{-8}$).

Here we present the results of a GWAS of PFS in 2,352 patients with EOC 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 ($P = 1.47\times10^{-8}$), which may target *ULK1*.

Materials and Methods

Study populations

Data were derived from three sources: patients from OCAC genotyped on the OncoArray [$n = 5{,}508$ with data on overall survival (OS), $n = 4{,}956$ with data available on PFS; ref. 14]; additional OCAC patients genotyped in the COGS study ($n = 317$ for OS, 303 for PFS; ref. 15) and the publicly available TCGA data for patients with high-grade serous EOC genotyped on the Affymetrix SNP 6.0 array ($n = 335$ for OS, 337 for PFS; Supplementary Tables S1–S3; ref. 16). 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 S3). Of all 6,160 patients, 2,620 had chemotherapy consisting of at least four cycles of 3-weekly paclitaxel and carboplatin at known or assumed doses of 135 to 175 mg/m^2 and AUC 5 to 7 respectively (7), which was considered "standard chemotherapy"; the remainder had no

Pathology, Mayo Clinic, Rochester, Minnesota. [29]Department of Gynecology, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark. [30]Department of Virus, Lifestyle and Genes, Danish Cancer Society Research Center, Copenhagen, Denmark. [31]Department of Lifestyle, Reproduction and Cancer, Danish Cancer Society Research Center, Copenhagen, Denmark. [32]Department of Pathology, Herlev Hospital, University of Copenhagen, Copenhagen, Denmark. [33]Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, Georgia. [34]Department of Obstetrics and Gynecology, Duke University Medical Center, Durham, North Carolina. [35]Obstetrics and Gynecology Epidemiology Center, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts. [36]Department of Epidemiology, Harvard School of Public Health, Boston, Massachusetts. [37]Department of Gynecology and Obstetrics, Haukeland University Hospital, Bergen, Norway. [38]Centre for Cancer Biomarkers CCBIO, Department of Clinical Science, University of Bergen, Bergen, Norway. [39]Department of Population Health, QIMR Berghofer Medical Research Institute, Brisbane, Queensland, Australia. [40]Prince of Wales Clinical School, University of New South Wales, Sydney, Australia. [41]Department of Obstetrics and Gynecology, Oregon Health & Science University, Portland, Oregon. [42]Knight Cancer Institute, Oregon Health & Science University, Portland, Oregon. [43]Beatson West of Scotland Cancer Centre and University of Glasgow, Glasgow, United Kingdom. [44]Division of Gynecologic Oncology, Princess Margaret Hospital, University Health Network, Toronto, Ontario, Canada. [45]British Columbia's Ovarian Cancer Research (OVCARE) Program, Vancouver General Hospital, BC Cancer Agency and University of British Columbia, British Columbia, Canada. [46]Department of Pathology and Laboratory Medicine, The University of British Columbia, Vancouver, British Columbia, Canada. [47]Department of Obstetrics and Gynaecology, The University of British Columbia, Vancouver, British Columbia, Canada. [48]Department of Molecular Oncology, The University of British Columbia, Vancouver, British Columbia, Canada. [49]British Columbia's Ovarian Cancer Research (OVCARE) Program, Department of Molecular Oncology, British Columbia Cancer Research Centre, Vancouver, British Columbia, Canada. [50]Department of Obstetrics and Gynecology, John A. Burns School of Medicine, University of Hawaii, Honolulu, Hawaii. [51]Cancer Research UK Glasgow Clinical Trials Unit, University of Glasgow, Glasgow, United Kingdom. [52]Department for Gynecology with the Center for Oncologic Surgery Charité Campus Virchow-Klinikum, Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, Berlin Institute of Health, Berlin, Germany. [53]Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia. [54]Department of Gynaecological Oncology, Westmead Hospital, Sydney, New South Wales, Australia. [55]The University of Sydney, Sydney, New South Wales, Australia. [56]Strangeways Research Laboratory, Department of Public Health and Primary Care, Centre for Cancer Genetic Epidemiology, University of Cambridge, Worts Causeway, Cambridge, United Kingdom.

Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (http://cebp.aacrjournals.org/).

M.C.J. Quinn, K. McCue, S. MacGregor, and G. Chenevix-Trench contributed equally to this article.

Corresponding Author: Georgia Chenevix-Trench, Department of Genetics and Computational Biology, QIMR Berghofer Medical Research Institute, Brisbane Q4029, Australia. E-mail: georgia.trench@qimrberghofer.edu.au

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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 ($n = 2,352$). In addition, we performed exploratory analyses for PFS in the "irrespective of chemotherapy" group ($n = 5,596$), for the serous subtype ($n = 4,392$), and for OS ($n = 6,160$). PFS was defined as the interval between the date of histologic diagnosis and the first confirmed sign of disease progression based on CA125 criteria (Gynecologic Cancer Intergroup; ref. 18), imaging, clinical assessment, or death, as described previously (12). OS was the interval between the date of histologic diagnosis and death from any cause. Patients who had an interval of >12 months between the date of histologic 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 with the largest site, AUS; number used in each analysis given per site in Supplementary Table S1). The total numbers of cases available for each of these analyses are given in Supplementary Table S3. 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 ref. 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 1,000 genomes phase III 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 S4). 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 S3). 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 vs. high), FIGO stage (four levels), residual disease (nil vs. 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 SE model weighting, with genomic control correction. Supplementary Table S4 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; refs. 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 4×44K 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 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% FBS. CaOV3, was grown in DMEM media supplemented with 10% serum and 1% nonessential amino acids. TOV112D was grown in MCDB105 and Medium 199 (50:50) supplemented with 15% FBS. 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 minutes. Formaldehyde was neutralized by washing twice with ice-cold 125 mmol/L 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 and colleagues (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 mAb 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 labeled 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 to 700 bp fragments. Two independent sequencing libraries generated for Illumina NextSeq550 (QIMRB sequencing facility, Brisbane, Australia) for 2×150bp sequencing.

HiChIP bioinformatic analyses

HiChIP sequencing libraries were analyzed as per O'Mara and colleagues (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; ref. 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 *Hin*dIII to generate chromatin conformation capture (3C) libraries as described previously (28). 3C interactions were quantified by ddPCR previously described (29) on three independent 3C libraries using primers designed against the *Hin*dIII restriction fragments across the 12q24.33 locus (Supplementary Table S5).

Plasmid generation

A 1794-bp fragment containing the *ULK1* promoter using PCR primers (Supplementary Table S5) modified with *Kpn*I and *Hin*dIII 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 S5. 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 PREs. Renilla was cotransfected 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 with 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 (gRNA) were designed using Broad sgRNA design tool (http://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design).

A nontarget control sequence (NTC) and three gRNAs targeting *ULK1* (*ULK1* C1, *ULK1* C2, and *ULK1* C3; Supplementary Table S6) were cloned into a lentiviral vector (lentiGuide-Puro; ref. 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 Fisher Scientific) and amplified using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). Three primer pairs were used for *ULK1*, along with five housekeeping genes (Supplementary Table S7). 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 with the geometric mean of housekeeping genes were used to calculate $\Delta C H$. The relative quantitation of each mRNA normalizing to that in the NTC line was performed using the comparative Ct method ($\Delta\Delta C H$). For Western blot analysis, ULK1 (D8H5) Rabbit mAb was used (8054, Cell Signaling Technology).

Paclitaxel and carboplatin sensitivity in ULK1 knockout ovarian cancer cell lines

Paclitaxel (Focus Bioscience) was dissolved in DMSO to make a 50 mmol/L stock solution and carboplatin (Focus Bioscience) was dissolved in water to make a 50 mmol/L 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 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 μL for each dose in duplicates. Four images of each well were acquired to establish the base line confluence, and serial drug dilutions were administrated in 100 μL volumes to achieve the final concentrations in 200 μL culture media. Doses for serial dilution of the drugs were in Supplementary Table S8. 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) versus 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 with 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 $P < 10^{-6}$ (**Fig. 1**; Supplementary Table S9). Seven correlated SNPs, all at 12q24, exceeded the nominal genome-wide threshold level of significance ($P < 5\times10^{-8}$; **Table 1**).

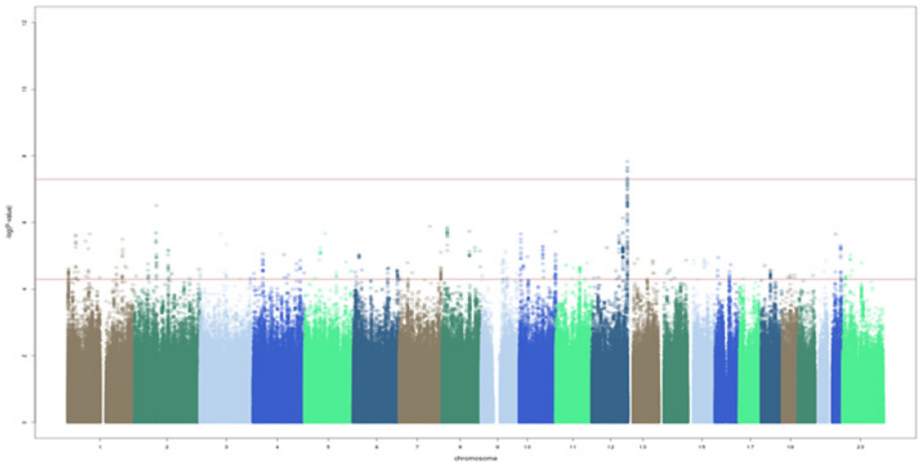

Figure 1.
Manhattan plot of results of the meta-analysis of association of PFS with patients (all histologies) who had standard chemotherapy. The top red line indicates the genome-wide significance threshold; the lower line indicates a suggestive significance level.

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; $P = 1.47\times10^{-8}$), 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 Fig. S1). 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; **Fig. 2**). Five years after diagnosis, 20.6% TT patients were still progression-free, compared with 25.4% for TC and 32.4% for CC patients (Supplementary Fig. S2).

We conducted several exploratory analyses for PFS in: (i) 5,596 patients of all histologies irrespective of chemotherapy regimen; (ii) 1,751 patients with serous EOC who had standard chemotherapy; (iii) 4,037 patients with serous EOC irrespective of chemotherapy regimen (Supplementary Figs. S3–S5). 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 S10 to S12. We also looked for associations with OS in (i) 2,620 patients with cancers of all histologies who had standard chemotherapy; (ii) 6,160 patients with cancers of all histologies irrespective of chemotherapy regimen; (iii) 1,901 patients who had serous EOC with standard chemotherapy; and (iv) 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; $P = 2\times10^{-5}$) 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 S13). However, the asymptotic approximation made in COXPH can provide unreliable results with small minor allele counts so we re-analyzed 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

Table 1. Association between rs10794418 and PFS time in women diagnosed with EOC.

		All cases				Serous cases only			
Endpoint	**Treatment**	***N***	**HR (95% CI)**	***P*-value**	**Direction[a]**	***N***	**HR (95% CI)**	***P*-value**	**Direction[a]**
PFS	Standard chemotherapy	2,352	1.24 (1.15–1.34)	1.47×10^{-8}	+++	1,751	1.20 (1.10–1.30)	0.00002	+++
	Irrespective of chemotherapy	5,596	1.06 (1.01–1.12)	0.01	+++	4,037	1.04 (0.98–1.10)	0.17	+++
OS	Standard chemotherapy	2,620	1.13 (1.04–1.22)	0.003	+++	1,901	1.11 (1.01–1.21)	0.02	+++
	Irrespective of chemotherapy	6,160	1.04 (0.99–1.10)	0.11	+++	4,391	1.03 (0.97–1.10)	0.29	+++

Note: 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 nine PCs.
Abbreviation: HR, hazard ratio estimating ordinal association per copy of minor T allele (frequency = 0.401).
[a]+, direction of effect for the T allele; order of studies for direction is OncoArray, COGS, and TCGA.

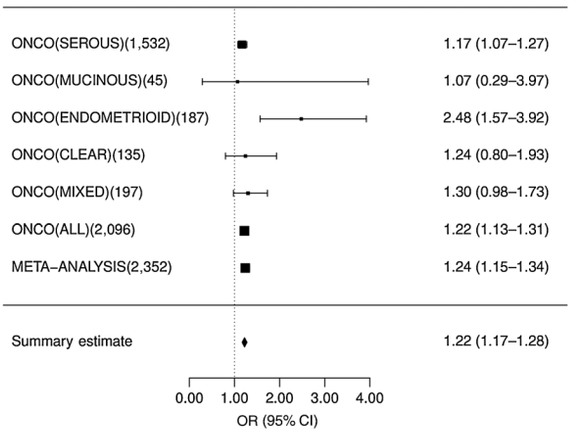

Figure 2.
Association of rs10794418 with PFS in patients who had standard chemotherapy, stratified by histotypes in the OncoArray results. HRs, along with upper and lower 95% CIs, are plotted. Sample sizes are indicated in brackets.

decreased by at least an order of magnitude and so we did not take any of them forward for further meta-analysis (Supplementary Table S13).

The lead SNP, rs10794418, lies in an intergenic region between the genes *LOC338797* (alias *RP13–507P192*) and *SFSWAP* at 12q24.33 (Supplementary Fig. S6). 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 noncoding genes, most of which are expressed in the normal ovary and in serous high-grade ovarian tumor tissue (Supplementary Fig. S6).

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 analyzed 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 $P \le 0.0036$ were observed for five genes (*ADGRD1,* $P = 0.0008$; *DDX51,* $P = 0.0001$; *FBRSL1,* $P = 1.7\times10^{-5}$; *LINC01257,* $P = 0.0016$; and *ULK1,* $P = 2.0\times10^{-5}$; **Fig. 3**; Supplementary Fig. S7). For each of these genes, high levels of expression were associated with shorter PFS. Second, we analyzed serous cancers only, treated with any chemotherapy, and found a significant association for *STX2* ($P = 0.0001$), with *ULK1* remaining significantly associated with outcome ($P = 4.4\times10^{-5}$). Further analyses restricted to a smaller dataset of patients known to have received taxol and platin therapy (**Fig. 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 S9), there were 21 eQTL signals with uncorrected $P < 0.01$ (Supplementary Table S14); 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 Fig. S8). 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,

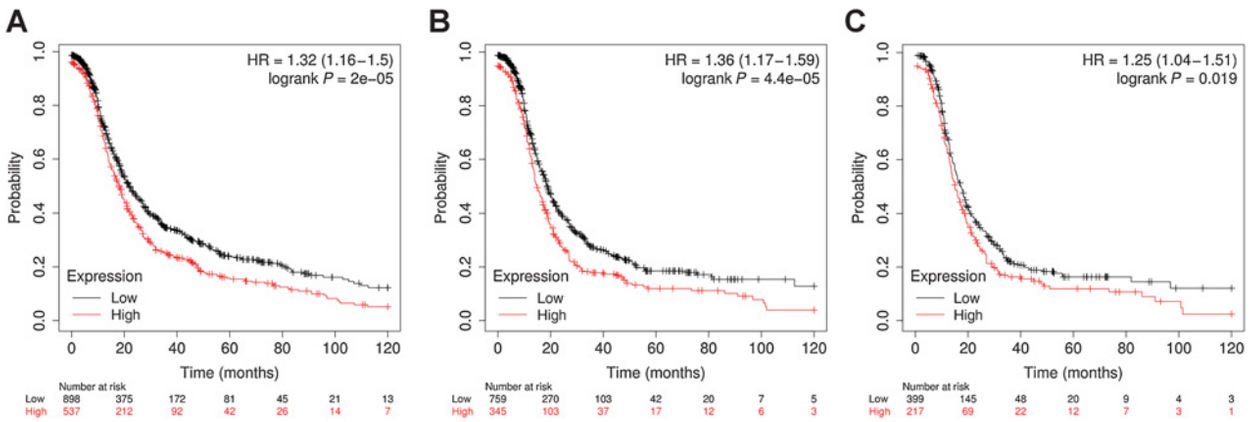

Figure 3.
Association of expression of *ULK1* and PFS using Kaplan–Meier plotter (http://kmplot.com/), an online tool to assess the expression levels of microarray-quantified genes using data from publicly available datasets (22). **A,** Women diagnosed with ovarian cancer of any histotype ($n = 1,435$) 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.

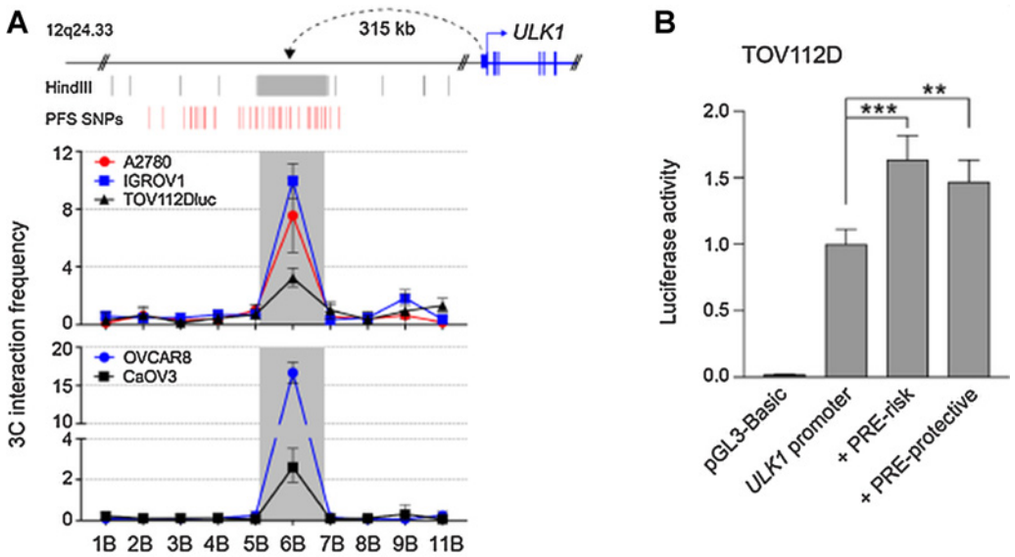

Figure 4.

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 *Hin*dIII 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% CIs 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.

IGROV1, TOV112Dluc, OVCAR8, and CaOV3. This identified an interaction between the *ULK1* promoter and a PRE in all five cell lines (**Fig. 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 Fig. S8). 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 (**Fig. 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 Fig. S9). 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 (**Fig. 5**). A less pronounced effect was seen when the cells were treated with paclitaxel, with a decrease in IC_{50} 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 (4, 7) is the first to identify a locus associated with outcome in EOC that meets the genome-wide significance level. Wheeler and colleagues (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 and colleagues 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 vs. 1.20). It was evident from a subtype-specific analysis of the OncoArray samples, which the endometrioid subtype ($n = 187$) had a higher hazard ratio (2.48; 95% CI, 1.57–3.92), compared with other subtypes such as serous ($n = 1{,}532$; HR = 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

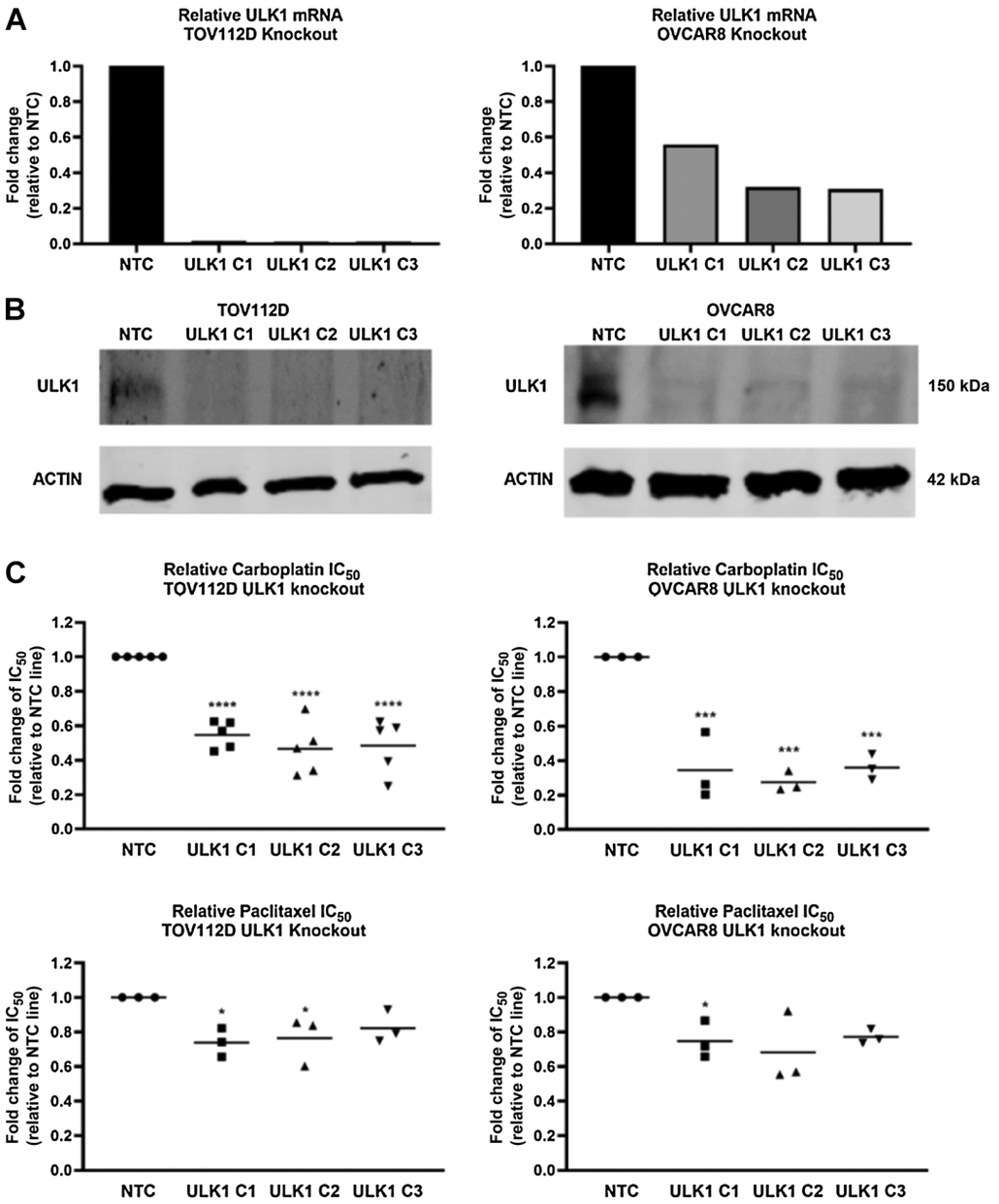

Figure 5.

Knockdown of ULK1 showing increased sensitivity to carboplatin. **A,** Relative *ULK1* mRNA level in TOV112D (left) and OVCAR (right) NTC and ULK1 knockout lines. **B,** ULK1 protein level in TOV112D (left) and OVCAR (right) NTC and ULK1 knockout lines. **C,** Relative IC_{50} of carboplatin (top) or paclitaxel (bottom) in TOV112D (left) or OVCAR (right) NTC and ULK1 knockout lines.

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, NOC4 L*, 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*; ref. 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 signaling (35, 36). Interestingly, Nishikawa and colleagues, (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), esophageal 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 patients with breast cancer (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. In addition, there is evidence that autophagy may be integral to chemoresponse with an induction of autophagy limiting the efficiency of chemoresponse in esophageal cancer cells (52), whereas 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 leukemia 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, whereas a late-stage inhibitor did not (56). Further experiments inhibiting ULK1 in ovarian cancer cells are required to determine whether tumor growth, chemoresponse, or PFS, 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 patients with ovarian cancer. 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.

Authors' Disclosures

No disclosures were reported.

Authors' Contributions

M.C.J. Quinn: Formal analysis. **K. McCue:** Methodology. **W. Shi:** Formal analysis, investigation, writing–original draft. **S.E. Johnatty:** Data curation. **J. Beesley:** Supervision. **A. Civitarese:** Methodology. **T.A. O'Mara:** Visualization, writing–review and editing. **D.M. Glubb:** Methodology, writing–original draft. **J.P. Tyrer:** Data curation. **S.M. Armasu:** Formal analysis. **J.-S. Ong:** Data curation. **P. Gharahkhani:** Data curation. **Y. Lu:** Resources. **B. Gao:** Data curation. **A.-M. Patch:** Formal analysis. **P.A. Fasching:** Resources. **M.W. Beckmann:** Resources. **D. Lambrechts:** Resources. **I. Vergote:** Resources. **D.R.V. Edwards:** Resources. **A. Beeghly-Fadiel:** Resources. **J. Benitez:** Resources. **M.J. Garcia:** Resources. **M.T. Goodman:** Resources. **T. Dörk:** Resources. **M. Dürst:** Resources. **F. Modugno:** Resources. **K. Moysich:** Resources. **A. du Bois:** Resources. **J. Pfisterer:** Resources. **K. Bauman:** Resources. **B.Y. Karlan:** Resources. **J. Lester:** Resources. **J.M. Cunningham:** Resources. **M.C. Larson:** Resources. **B.M. McCauley:** Resources. **S.K. Kjaer:** Resources. **A. Jensen:** Resources. **C.K. Hogdall:** Resources. **E. Hogdall:** Resources. **J.M. Schildkraut:** Resources. **M.J. Riggan:** Resources. **A. Berchuck:** Resources. **D.W. Cramer:** Resources. **K.L. Terry:** Resources. **L. Bjorge:** Resources. **P.M. Webb:** Resources. **M. Friedlander:** Resources. **T. Pejovic:** Resources. **M. Moffitt:** Resources. **R. Glasspool:** Resources. **T. May:** Resources. **G.E.V. Ene:** Resources. **D.G. Huntsman:** Resources. **M. Woo:** Resources. **M.E. Carney:** Resources. **S. Hinsley:** Resources. **F. Heitz:** Resources. **S. Fereday:** Resources. **C.J. Kennedy:** Resources. **S.L. Edwards:** Supervision. **S.J. Winham:** Formal analysis. **A. deFazio:** Resources, supervision. **P.D.P. Pharoah:** Resources. **E.L. Goode:** Investigation. **S. MacGregor:** Conceptualization, data curation, formal analysis, writing–original draft. **G. Chenevix-Trench:** Conceptualization, supervision, funding acquisition, writing–original draft, writing–review and editing.

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