Clinical Characteristics of Ovarian Cancer Classified by \textit{BRCA1}, \textit{BRCA2}, and \textit{RAD51C} Status

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We evaluated homologous recombination deficient (HRD) phenotypes in epithelial ovarian cancer (EOC) considering \textit{BRCA1}, \textit{BRCA2}, and \textit{RAD51C} in a large well-annotated patient set. We evaluated EOC patients for germline deleterious mutations (n = 899), somatic mutations (n = 279) and epigenetic alterations (n = 482) in these genes using NGS and genome-wide methylation arrays. Deleterious germline mutations were identified in 32 (3.6%) patients for \textit{BRCA1}, in 28 (3.1%) for \textit{BRCA2} and in 26 (2.9%) for \textit{RAD51C}. Ten somatically sequenced patients had deleterious alterations, six (2.1%) in \textit{BRCA1} and four (1.4%) in \textit{BRCA2}. Fifty two patients (10.8%) had methylated \textit{BRCA1} or \textit{RAD51C}. HRD patients with germline or somatic alterations in any gene were more likely to be high grade serous, have an earlier diagnosis age and have ovarian and/or breast cancer family history. The HRD phenotype was most common in high grade serous EOC. Identification of EOC patients with an HRD phenotype may help tailor specific therapies.

Ovarian cancer represents the fifth most common cause of cancer mortality in women due in part to the advanced stage at which patients typically present, with an estimated 14,030 deaths and 22,240 cases in 2013 in the United States\(^1\). The most common ovarian cancer is invasive epithelial ovarian cancer, with five common histological subtypes: high grade serous (70%), endometrioid (10%), clear cell (10%), low grade serous (5%), and mucinous (3%). A family history of breast or ovarian cancer in first degree relatives increases the risk for ovarian cancer\(^2\); family-based studies have revealed high- and moderate-penetrance genes including \textit{BRCA1}\(^3\), \textit{BRCA2}\(^4\), DNA mismatch repair genes\(^5\), \textit{RAD51C}\(^6,7\), \textit{RAD51D}\(^8\) and \textit{BRIP1}\(^6\), and case-control studies have identified eleven common variants associating with modestly increased risks\(^9–15\). Estimates of the contribution of germline \textit{BRCA1} and \textit{BRCA2} mutations to EOC vary widely from 5% to 20%\(^16\); somatic mutations in \textit{BRCA1} and \textit{BRCA2} occur less frequently (in 2%–8% patients)\(^17,18\) and, as has \textit{BRCA1} methylation\(^19–22\) been reported in ovarian cancer patients with no family history\(^23\). Ovarian cancer patients who are \textit{BRCA1} and \textit{BRCA2} germline mutation carriers have been reported to have an improved outcome compared to non-carriers and the mechanism underlying this benefit has been hypothesized as a high response rate to platinum agents, particularly among patients with high grade serous histology\(^24–27\). This is because patients with germline \textit{BRCA1} or \textit{BRCA2} mutations have an impaired ability to repair double stranded DNA breaks through homologous recombination\(^28\). More recently, favorable responses to poly (adenosine diphosphate-ribose) polymerase (PARP) inhibitors have been noted in ovarian cancer patients carrying deleterious germline \textit{BRCA1} and \textit{BRCA2} mutations\(^29\). However, some ovarian cancer patients without germline \textit{BRCA1} and \textit{BRCA2} mutations also respond to PARP inhibitors\(^30\), suggesting that broader dysfunction of homologous recombination than \textit{BRCA1} and \textit{BRCA2}, a homologous recombination deficient (HRD) phenotype may
be important. HRD may be the result of deleterious germline mutation in other homologous recombination genes such as RAD51B, RAD51C, or RAD51D or somatic mutation or silencing of the same homologous recombination genes\textsuperscript{31,32}.

We evaluated the prevalence of HRD in a large EOC case series and correlated this with clinical outcomes. HRD was determined by sequencing BRCA1, BRCA2 and RAD51C in germline and tumor DNA and assessing DNA methylation of cytosines at CpG sites surrounding these genes.

Results
Table 1 shows the characteristics of patients and Figure 1 shows the number of samples with each data type, after QC exclusions, included in this analysis.

Frequency of methylation, germline and somatic mutations. Of the 899 germline-tested patients, deleterious mutations were seen in 83 (9%): 32 (3.5%) in BRCA1, 28 in BRCA2 (3%), and 26 in RAD51C (2.9%; Table 2). Three cases carried mutations in more than one gene: two patients carried deleterious mutations in BRCA1 and RAD51C, and one carried deleterious mutations in BRCA2 and RAD51C. For the 619 HGS cases, deleterious mutations were seen in 69 (11%), 27 (4%) in BRCA1, 24 (4%) in BRCA2 and 19 (3%) in RAD51C. One HGS patient carried a mutation in both BRCA2 and RAD51C. Of the six Ashkenazi Jewish patients, one carried a germline BRCA1 mutation (G61G) and none carried either somatic mutations or methylation. Table S1 and S2 provides a complete mutation listing, after exclusions (see Methods section). Another 187 women carried germline missense mutations, none of which had evidence for pathogenicity in the key clinical databases, 65 of these alterations were in BRCA1, 102 in BRCA2 and 32 in RAD51C. Twelve patients had a missense mutation in multiple genes. These variants were considered variants of unknown significance (VUS). In the patients with deleterious mutations, 13 patients also had a missense VUS that was identified. The remaining 629 patients had no deleterious or missense VUS mutations in BRCA1, BRCA2 or RAD51C.

Two hundred and seventy nine patients with available tumor tissue were screened for somatic mutations. Ten tumors (4%) were found to have deleterious somatic mutations, six in BRCA1, and four in BRCA2 (Table 2). Table S2 provides a complete listing for the somatic mutations. Missense VUS were also detected with one in BRCA1 and one in BRCA2. Two hundred and sixty seven patients had no deleterious or missense VUS mutations detected. No deleterious somatic variations were identified in RAD51C.

Forty five of 482 tumors (9%) exhibited a methylated BRCA1 phenotype, and seven tumors (1%) showed RAD51C methylation (Table 2). BRCA1 methylation was not detected in any individual carrying a germline BRCA1 deleterious mutation; similarly, RAD51C methylation was exclusive of germline RAD51C mutation carrier status. However, there were two patients with a mutation and methylation of another gene; one patient had a somatic mutation in BRCA2 and BRCA1 methylation and another carried a germline RAD51C mutation and had methylated BRCA1.

Clinical and pathological analyses. In combination, 143 patients exhibited a HRD phenotype (82 due to germline mutation only, nine due to somatic mutation only, 50 due to tumor methylation only, one due to germline mutation and methylation, and one additional due to somatic mutation and methylation, Table 2). A total of 79 HRD patients showed alterations in BRCA1, 30 in BRCA2, 29 in RAD51C and five patients showed alterations in more than one gene.

We examined the clinical features of HRD compared to patients with no abnormality detected (NAD) for those with both germline and somatic data (Tables 3, S3). HRD patients had younger age at diagnosis (P = 0.0001) and were more likely to be high grade serous (118/264, P = 0.0004) and have a family history of breast or ovarian
are in fact high grade serous EOC33. Other covariates (debulking, common pathology challenges is whether high grade endometrioid 2/23 patients with low grade endometrioid EOC. One of the most with high grade (10/27) endometrioid EOC were HRD, compared to

We also examined clinical features within the HRD patients and found only family history of breast or ovarian cancer was more common in mutated than methylated patients (P = 0.0001, data not shown).

Survival analyses were carried out on those patients with HRD and those with NAD for which all data (germline, somatic mutation and methylation) were available (n = 356). Forty percent (143/356) of all the patients were HRD, while 44.7% (118/264) of HGS patients were HRD. Overall survival for all patients was associated with the HRD phenotype (adjusted HR 0.73, 95% CI 0.54–0.98, P = 0.04, Table 4), but not within each of the defective classes, genes that were mutated at the germline or somatic level or were somatically methylated (respectively, adjusted HR 0.81, 95% CI 0.57–1.15; adjusted HR 0.56, 95% CI 0.20–1.54, adjusted HR 0.65, 95% CI 0.42–1.02, P = 0.18, Table 4), or when outcomes were examined by germline mutation carrier status (P = 0.09, Table 4). The hazard ratios data are similar to those previously reported in a large germline study26.

Results were similar whether VUS were included in the NAD category or excluded (data not shown). Analysis of time to recurrence showed no association with any of the HRD phenotypes (Table S3). Survival analyses were also conducted restricted to high grade serous (n = 618 for germline carrier analysis, 264 for HRD analysis) and showed an overall survival differences between HRD and NAD (adjusted HR 0.70, 95% CI 0.51–0.97, P = 0.03, Table 4, Figure 2), but not between germline mutation carriers and-non carriers (adjusted HR 1.07, 95% CI 0.65–1.76; adjusted HR 0.76, 95% CI 0.45–1.30, adjusted HR 0.66, 95% CI 0.37–1.18, P = 0.40, Table 4, Figure 2). Survival at 5 years was similar (adjusted HR 0.68 95% CI 0.48, 0.97, P = 0.04, data not shown). Analysis of time to recurrence was null (Table S4, Figure S3). Finally, we examined HRD phenotypes by debulking status in germline mutation carriers, as Alsop et al27 reported that improved survival was not evident in patients that were sub-optimally debulked. However, we observed no difference in germline mutation carriers stratified by debulking status (data not shown).

**Discussion**

Using a large homogeneous collection of ovarian cancer patients with clinical follow up unselected for family history, we evaluated

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### Table 2 | Summary of germline mutations, somatic mutations, and methylation of BRCA1, BRCA2 and RAD51C

| Gene, n | N Tested | BRCA1 | BRCA2 | RAD51C | Combined HRD |
|---|---|---|---|---|---|
| Germline deleterious mutation | 899 | 32 | 28 | 26 | 83 |
| Somatic deleterious mutation | 279 | 6 | 4 | 0 | 10 |
| Methylated | 482 | 45 | - | 7 | 52 |
| No deleterious mutation, not methylated | NA | 237 | 261 | 263 | NA |
| No deleterious germline mutation, unknown somatic mutation and/or tumor methylation status | NA | 592 | 606 | 604 | NA |
| Not methylated, germline and somatic mutation status unknown | NA | 151 | 164 | 163 | NA |
| Total | 1063 | 1063 | 1063 | 143 |

Deleterious mutations were frame shift insertion/deletion, splice site, rare missense supported by multiple methods to be damaging. NA, not applicable; one case carried germline deleterious mutations for both BRCA2 and RAD51C, and two patients carried germline deleterious mutations for BRCA1 and RAD51C, thus the combined number of germline deleterious mutation carriers is less than the sum of mutation carriers for each gene; one case carried a RAD51C germline deleterious mutation and methylated for BRCA1 and another case carried a BRCA2 somatic deleterious mutation and methylated for BRCA1, thus the total number of patients with a HRD phenotype is less than the sum of germline mutation carriers and patients with a somatically mutated or methylated gene.

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### Table 3 | Comparison of the clinical characteristics of HRD to NAD patients

| HRD (n = 143) | NAD (n = 213) | P value |
|---|---|---|
| Age at Diagnosis, years | 58.4 (10.6) | 63.1 (11.5) | 0.0001 |
| Histology | | | 0.0004 |
| High grade serous | 118 (45%) | 146 (55%) | |
| High grade endometrioid | 10 (37%) | 17 (63%) | |
| Low grade endometrioid | 2 (9%) | 21 (91%) | |
| Clear Cell | 5 (28%) | 13 (72%) | |
| Low grade Serous | 2 (15%) | 11 (85%) | |
| Mucinous | 0 (0%) | 5 (100%) | |
| Other/Unknown | 6 (100%) | 0 (0%) | |
| Grade | | | <0.0001 |
| High | 135 (44%) | 174 (66%) | |
| Low | 5 (11%) | 39 (89%) | |
| Unknown | 3 (100%) | 0 (0%) | |
| First Degree Family History of Breast or Ovarian Cancer | | | 0.0020 |
| No | 104 (36%) | 183 (64%) | |
| Yes | 39 (57%) | 30 (43%) | |
| First Degree Family History of Ovarian Cancer | | | 0.0344 |
| No | 132 (39%) | 207 (61%) | |
| Yes | 11 (65%) | 6 (35%) | |

Only patients with known HRD status (germline mutation, somatic mutation and somatic methylation) available were included; VUS carriers are included in NAD patient group; stage, debulking, presence of ascites, peritoneal cytology, smoking history, enrollment year, alcohol use were not significantly different between HRD and NAD patients.
Table 4 | Analysis of overall survival by HRD phenotypes

| By HRD | All patients | Covariate-adjusted | High grade serous | Covariate-adjusted |
|--------|--------------|---------------------|-------------------|---------------------|
|        | N N (%) deaths | HR (95% CI) | P value | N N (%) deaths | HR (95% CI) | P value | N N (%) deaths | HR (95% CI) | P value |
| NAD | 213 | 131 (62%) | ref | 0.27 | 146 | 100 (68%) | ref | 0.05 |
| HRD | 143 | 84 (59%) | 0.86 | 0.65,1.13 | 0.73 | 0.54,0.98 | 0.04 | 0.74 | 0.55,1.00 |

By Type of Alteration

| NAD | 213 | 131 (62%) | ref | 0.75 | 146 | 100 (68%) | ref | 0.05 |
| HRD | 143 | 84 (59%) | 0.86 | 0.65,1.13 | 0.73 | 0.54,0.98 | 0.04 | 0.74 | 0.55,1.00 |

By Germline Mutation

| NAD | 213 | 131 (62%) | ref | 0.75 | 146 | 100 (68%) | ref | 0.05 |
| None | 82 | 48 (59%) | 0.89 | 0.64,1.24 | 0.81 | 0.57,1.15 | 0.73 | 0.54,0.98 | 0.04 | 0.74 | 0.55,1.00 |
| Germline mutation | 9 | 4 (44%) | 0.67 | 0.25,1.82 | 0.56 | 0.20,1.54 | 0.73 | 0.54,0.98 | 0.04 | 0.74 | 0.55,1.00 |
| Somatic mutation | 50 | 32 (64%) | 0.88 | 0.60,1.30 | 0.65 | 0.42,1.02 | 0.73 | 0.54,0.98 | 0.04 | 0.74 | 0.55,1.00 |

Women with VUS in any of the three genes were included in the NAD and non-carrier groups; results did not differ when these individuals were excluded from the analyses. Adjusted results were adjusted for age at diagnosis, debulking status, stage, grade (low/high), and hormone receptor positivity. 

Strengths of this report include detailed methylation phenotyping of a subset of patients and sequencing of tumor DNA. Limitations include the lack of patients in each group and the small sample size of patients analyzed. Other future work includes sequencing of additional DNA repair genes in order to more comprehensively identify those patients with defective recombination pathway function.
Methods

Patient population and biospecimens. Women with EOC (n = 1063) provided written informed consent between 1992 and 2011 using IRB approved processes at the Mayo Clinic in Rochester, MN. Use of patient derived samples in this study was also approved and the work described was carried out in accordance with these approved guidelines. Eligible participants were women aged 20 years or older with diagnosis of pathologically confirmed invasive primary epithelial ovarian, fallopian tube or primary peritoneal cancer. Patients were followed for vital status using electronic medical records and linkage to vital statistics sources; the median time of follow up was 4.5 years, with a range of 0.1–10 years. Most patients were treated with a platinum agent (carboplatin or cisplatin) and taxane, the standard of care at time of diagnosis. Peripheral blood (n = 899) was used a source of germline DNA. Pre-chemotherapy tumor tissue (up to n = 482) was snap frozen immediately following surgery and stored at −80°C; tumor DNA was prepared from cryostat sections using Qiagen PureGene chemistry (Qiagen Inc, Valencia, CA, USA) and quantitated using Trinean (Trinean, Gentbrugge, Belgium). Tumor RNA was isolated from fresh frozen samples using the Qiagen RNEasy protocol (Qiagen Inc, Valencia, CA, USA) and quantitated using a Nanodrop Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Somatic methylation analyses preceded the mutation screening, thus samples with insufficient DNA for both analyses were not sequenced.

DNA sequencing and bioinformatics. Germline DNA (n = 899, 1.25 µl DNA at a concentration 75 ng/µl) and tumor DNA (n = 279) were sequenced at the University of Cambridge, Cambridge, UK at BRCA1, BRCA2 and RAD51C. The procedure entailed design of PCR primers, testing and optimization using Fluidigm Access Array (Fluidigm Corp., San Francisco, CA, USA). Briefly, the method used a 4-primer amplicon-tagging scheme. Tagged, target-specific primer pairs were combined with sample-specific primer pairs that contained barcoding sequences and the adaptor sequences used by the Illumina sequencing system. Using sample-specific barcodes, all PCR products generated in the 48.48 Access Array Integrated Fluidic Circuit were unique and pooled together to run in a single sequencing experiment. Sequencing reactions were carried out on an Illumina GAIIx next-generation sequencer (Illumina, Inc., San Diego, CA, USA).

GENOME_GPS 1.0 was used for analysis of DNA sequence data including read alignment, variant detection, and annotation (Figure S1). Reads were aligned to genome build hg19 using Novoalign and realignment, recalibration used the Genome Analysis Toolkit (GATK) v.1.6.7.44. The percent of reads mapped on target was used to exclude seven germline samples with <80% of amplicons covered at 20× and twenty one tumor samples with <80% of the amplicons covered at 20×. To avoid false-negative deleterious carrier status assignments for the somatic mutation data, non-carrier patients were excluded if either germline or tumor sequencing data exhibited <80% on-target sequence coverage at 20× or greater [mean coverage, 1st and 3rd quartiles: RAD51C 138.76 (121.68, 150.94); BRCA1 164.81 (142.59, 179.47); BRCA2 157.65 (133.68, 174.13); all three genes, 158.20 (136.06, 173.04)]. Germline variants were called through GATK Unified Genotyper. Germline filtering used genotype quality greater than 20 and read depth greater than 20. Somatic single nucleotide variants were called using SomaticSniper, whereas insertions and deletions were called by GATK Somatic Indel Detector. Somatic variants were considered with a depth of at least 20× coverage for the germline and tumor DNA at the given site as well as a quality score ≥ 20 and excluded if close to indel calls, aligned to multiple positions and/or occurred in a known repeat region. Indel calls required ≥ 10 supporting tumor sequencing reads, and no reads supporting the indel in the germline.

Each variant was annotated using the Targeted RE-sequencing Annotation Tool (TREAT)44. Missense coding variants were functionally annotated by snpEFF (http://snpeff.sourceforge.net/) and PolyPhen-2 to predict biological effects. Somatic variants were considered deleterious if they were stop-gains (nonsense) or occurred at splice sites junctions, along with frameshifts and insertion/deletions as deleterious. In

Figure 2 | Overall survival for high grade serous EOC by HRD and type of alteration. (A). Red: HRD; Black: NAD. (B). Above is a depiction of samples with type of alteration in each gene; below are the Kaplan Meier curves. Red: germline deleterious mutation; Green: somatic deleterious mutation; Blue: somatic methylation; Black: no germline mutation, somatic mutation or methylation.
Illumina Infinium HumanMethylation450 BeadChip as previously described. We estimated pairwise Spearman expression. Expression data were generated using 750 ng of high quality total RNA and suggested that BeadChip data were satisfactory; Pearson correlation of methylation deleteriousness could not be determined with confidence, or which were known IARC (www.iarc.fr), HGMD (www.hgmd.cf.ac.uk), and Alamut (http://www.association analysis. HRD patients were defined as those with deleterious germline mutations, deleterious somatic mutations or methylation of BRCA1, BRCA2, or RAD51C. Clinical characteristics of HRD and patients with NAD were compared using Fisher exact tests. Survival analysis with HRD status was conducted using multivariable Cox proportional hazards modeling and, for adjusted analyses, included age, stage, debulking, menopausal status, grade, and ascites, estimating hazard ratios (HRs) and 95% confidence intervals (CIs). Time at risk was defined from the date of diagnosis to date of death allowing for left truncation with right censoring at last date of follow up to 10 years. In addition to overall survival, we considered time-to-recurrence or death from any cause. We analyzed survival using the HRD phenotype defined by germline status and also by combined germline, somatic, and methylation status (using the subset with complete data). Analyses were for all patients and among patients with high grade serous EOC and were conducted both with VUS considered to be non-carriers or excluded. Each analysis used all available data (thus sample sizes vary) and was implemented in SAS, with corresponding Kaplan-Meier curves generated in R.

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**Author contributions**
E.L.G. and P.P.D.P. designed the study. J.M.C. coordinated the analyses and prepared the manuscript. All authors read and approved the final version of the manuscript. M.C.L., N.B.L., C.W., B.L.F., E.M.D. and J.D. performed statistical and informatics analyses. G.C., H.H. and G.E.K. provided the gene expression data. H.S., P.H. and P.P.D.P. performed the next generation sequencing. J.M.C. performed the methylation analysis. Sample provision and management were coordinated by B.W., N.M.L., M.W. and M.B. Technical support and advice were provided by M. C., J.C., M.B. and J.-B.F.

**Additional information**
Supplementary information accompanies this paper at http://www.nature.com/scientificreports

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