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BRCA1 and *BRCA2* mutations correlate with *TP53* abnormalities and presence of immune cell infiltrates in ovarian high-grade serous carcinoma

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We characterized *BRCA1* and *BRCA2* status (mutation/methylation) in a consecutive series of cases of ovarian carcinoma in order to identify differences in clinicopathological features, molecular characteristics, and outcome between the pelvic high-grade serous cancers with (i) germline or somatic mutations in *BRCA1* or *BRCA*2, (ii) methylation of *BRCA1*, and (iii) normal *BRCA1* or *BRCA2*. In all, 131 women were identified prospectively, who were undergoing surgical staging and agreed to germline testing for *BRCA1* and *BRCA*2 mutations. Histopathology, germline and somatic *BRCA1* or *BRCA*2 mutations, *BRCA1* methylation, and *BRCA1* and *BRCA2* mRNA expression levels distinguished four subgroups. In all, 103 cases were high-grade serous carcinoma and of these 31 (30%) had germline or somatic *BRCA1 or BRCA2* mutations (20% *BRCA1* and 10% *BRCA2*) (group 1), 21 (20%) had methylation of *BRCA1* (group 2), and in 51 (50%) there was no BRCA loss (group 3). Group 4 consisted of 28 cases of non-high-grade serous, none of which had BRCA loss. *BRCA1* and *BRCA2* mRNA expression levels correlated with designated group ($P = 0.0008$). Among high-grade serous carcinomas, there were no differences between groups 1–3 with respect to stage, ascites, CA125 level, platinum sensitivity, cytoreduction rate, neoadjuvant chemotherapy, or survival. Tumors with *BRCA1* or *BRCA2* mutations had increased immune infiltrates (CD20 and TIA-1) compared with high-grade serous without mutations ($P = 0.034$, 0.027). *TP53* expression differed between groups ($P < 0.0001$), with abnormal *TP53* expression in 49/50 tumors from groups 1 and 2. Wild-type *TP53* expression was associated with worse outcome in high-grade serous ($P < 0.001$). BRCA loss (mutation/methylation) is a common event in the pelvic high-grade serous (50%). *TP53* abnormalities and increased immune cell infiltrates are significantly more common in high-grade serous with germline and somatic mutations in *BRCA1* or *BRCA2*, compared with tumors lacking BRCA abnormalities.
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Ovarian cancer is the leading cause of death due to gynecological malignancies and the fifth most common cause of cancer death in North America. Advances in surgical technique and chemotherapy have done little to change overall survival statistics with the majority of women diagnosed with

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advanced stage ovarian cancer experiencing a recurrence within two years of diagnosis, and ultimately succumbing to their disease. The median progression-free survival and overall survival from landmark phase III trials range between 12 and 24 months and 29–65 months, respectively.[1,2] Factors that are believed to impact survival include patient age, performance status, preoperative CA125, volume of ascites, stage, grade, extent of cytoreductive surgery, chemotherapeutic agents, route of delivery, duration of treatment, histological subtype, host immune response, and specific genetic alterations, including BRCA mutations.[2–15] There remains, however, limited ability to accurately prognosticate in patients with advanced stage high-grade serous carcinoma based on features of the primary tumor at the time of diagnosis.

Seventy percent of ovarian carcinomas are high-grade serous histological subtype, accounting for the majority (90%) of ovarian carcinoma deaths. A large majority of high-grade serous ovarian cancers have *TP53* mutations, which appear to occur as an early event in disease progression.[16,17] Tumors from women with high-grade serous cancers frequently show a host immune response, and the presence of cytotoxic T lymphocytes, and T-regulatory cells is consistently associated with favorable prognosis.[18–24] Germline mutations in *BRCA1* and *BRCA2* are present in $\sim$18% of ovarian cancer patients with high-grade serous carcinoma.[25–27] When combined with BRCA deficiencies that result from somatic mutations or epigenetic silencing, it appears that up to half of all high-grade serous ovarian cancers (hereditary and sporadic) have BRCA dysfunction.[15,26–30]

BRCA1 and *BRCA2* genes encode functionally related proteins that play a critical role in repair of DNA double-strand breaks.[31–33] Loss of BRCA function results in development of chromosomal instability. The chromosomal instability phenotype associated with ‘BRCAness’ (loss of BRCA function or BRCA-null) correlates with sensitivity to DNA cross-linking agents in preclinical models.[34–36] Standard first line chemotherapy for high-grade serous cancer includes a platinum compound, for example, cisplatin or carboplatin, the mechanism of action which is, in part, the cross-linking of DNA. There are data to suggest that tumors with a BRCA mutations profile have improved responsiveness to platinum as compared with those with functioning *BRCA1* and *BRCA2* genes,[37–41] and patients with mutations in *BRCA1* or *BRCA2* have slightly better (hazard ratio (HR) $\sim$0.7) survival, compared with patients whose tumors lack BRCA mutations.[27,42–46] Methylation of the *BRCA1* promoter, resulting in epigenetic silencing, is not associated with improved outcomes [15]. Our objective was to determine whether the BRCA status in high-grade serous cancer correlates with other known clinical, pathological, immune, or genetic prognostic or predictive factors.

Materials and methods

Patient Selection and Clinicopathological Parameters

This was a prospective study; patients were recruited from the Vancouver General Hospital and British Columbia Cancer Agency in Vancouver, British Columbia, Canada, which is the primary referral center for patients with ovarian carcinoma for the province of BC and the Yukon Territory. Ethical approval was obtained from the University of British Columbia Ethics Board. All women undergoing debulking surgery (primary or delayed) for non-mucinous carcinoma of ovarian/peritoneal/fallopian tube origin were approached for informed consent for the banking of tumor tissue and were referred to our hereditary cancer genetic counselors to discuss germline BRCA testing. Patients with borderline tumors (tumors of low-malignant potential) were excluded. All germline testing results were provided to the participants through a post-test counseling session, and the family members of all germline mutation carriers were subsequently offered genetic counseling and testing, through the Hereditary Cancer Program. Forty-nine of the cases in this series were the subject of a previous report on characterization of *BRCA1* and *BRCA2* abnormalities in ovarian carcinoma (recruitment beginning 2004).[26] This previous study did not include analysis of clinical features, including patient outcomes. Recruitment continued until the spring of 2009 with the goal of minimum 2 years follow-up in all individuals. Pathology review was performed in the entire cohort.

Clinical and outcome data was collected on the cohort including age at diagnosis, CA125 level preoperatively, stage, grade, histological subtype, cytoreduction (to no residual, <1 cm or >1 cm), ascites, primary debulking *vs* neoadjuvant chemotherapy, response to therapy (using the 2010 Gynecologic Cancer InterGroup Fourth Ovarian Consensus Conference criteria) based on time since last treatment with either cisplatin or carboplatin, time to recurrence, time to death, and last date of follow-up. Clinical data collection was done without knowledge of the molecular test results. Similarly, histological, immunohistochemical, and molecular testing were done independently, without knowledge of clinical data, including outcome.

Tissue Banking, DNA, and RNA Extraction

Cancer tissue was stored at -80° and corresponding tissue was also placed in paraffin blocks. Hematoxylin and eosin (H&E) sections corresponding to the selected frozen tissue samples were reviewed to ensure that samples consisted of at least 70% tumor cells. Cores were taken from paraffin blocks. DNA and RNA were extracted using the RecoverAll Total Nucleic Acid Isolation kit for FFPE (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions.

Multiplex Ligation-Dependent Probe Amplification Screening and Sequencing for Germline Mutations

For the identification of germline *BRCA1* and *BRCA2* single and multiple exon deletions or duplications, multiplex ligation-dependent probe amplification analysis kits (P002-C1, P090-A2, MRC Holland, Amsterdam, NL) were used according to the manufacturer's protocol. *BRCA1* reference sequence: NM_007294.4 *BRCA2* reference sequence: NM_000059.3. Single exon deletions were independently confirmed. The sequence of *BRCA1* and *BRCA2* was determined from peripheral blood derived gDNA via bidirectional dideoxy sequencing. Analysis of the amplification and sequencing products was performed using an ABI3730 Analyzer (Applied Biosystems). *BRCA1* and *BRCA2* variants that were not considered pathogenic and/or were not recognized by the Breast Cancer Information Core (BIC) (http://research.nhgri.nih.gov/bic/) were not reported.[47]

BRCA1 and *BRCA2* Somatic Mutation Testing

Genomic DNA was extracted from flash frozen tumors using the Ambion DNA extraction kit as per manufacturer's protocol (Ambion). 500 ng was used for Illumina library construction as previously described.[48] The library then underwent a selected gene capture step consisting of 15 genes that included *BRCA1* and *BRCA2* using cDNA as the capture probe. The select gene capture data were first aligned to the whole genome using the BWA (LD09)[49] aligner, and the alignment results were converted to bam files (LHW + 09). Bam files were then converted to fastq files using picard (http://picard.sourceforge.net/index.shtml), and the fastq files were realigned to the 15 targeted gene coordinates using BWA (LD09). Point mutations were called by SNVMix (GSM + 10),[50] and indels were called by samtools (LHW + 09).[51] The called point mutations were filtered through dbsnp and the mutations which were not in dbsnp were run through mutationassessors (http://mutationassessor.org/). Sanger sequencing confirmed the mutations revealed by the analysis. A subset of tumors (from original cohort, cases from 2004 to 2005) had been tested for somatic mutations using denaturing high-performance liquid chromatography and the precise mutation identified through Sanger's sequencing as previously described.[26] We searched the BIC, PubMed, and our own databases in order to determine, which mutations are predicted to be of clinical significance (functional impact on protein product). Unclassified variants were not reported.

BRCA1 Promoter Methylation

Purified DNA (500 ng) was bisulfite converted using the EpiTect bisulfite kit (Qiagen) according to the manufacturer's instructions. PCR was done for both methylated and unmethylated DNA using previously published primers[52] except that the forward primer for each was labeled with a FAM fluorescent label. The PCR products were run on a $\times$3130 L genetic analyzer (Applied Biosystems) and analyzed with associated software.

BRCA1 and *BRCA2* mRNA Expression Levels for *BRCA1* and *BRCA2*

Extracted RNA (1 μg) was treated with DNaseI (Invitrogen, Carlsbad, CA) before creating cDNA using the SuperscriptIII First Strand Synthesis System (Invitrogen) with random hexamers. Applied Biosystems (ABI) Taqman primer/probe kits (Hs00173233_m1 (*BRCA1*), Hs00609060_m1 (*BRCA2*), Hs01920652_s1 (PTEN), and Hs00907966_m1(PIK3CA)) were used to quantify mRNA expression levels using an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems).[37] Relative gene expression was quantified according to the comparative Ct method using human 18s ribosomal RNA expression as the endogenous reference (Applied Biosystems) and commercial RNA controls (Stratagene, La Jolla, CA). Relative quantification was determined by the ABI software as follows: $2^{(\Delta\text{Ct sample}-\Delta\text{Ct calibrator})}$. Ratios (tumor relative gene expression: average of all tumors) of $\leq$1.0 were scored as decreased mRNA expression, to allow for analysis of mRNA expression levels as a categorical variable.

Immunohistochemistry

H&E stained sections of the primary tumor were reviewed and representative areas of tumor were selected and marked. Corresponding areas on the paraffin blocks were marked and two tissue cores from the representative areas in the donor blocks were removed using TMArrayer by Pathology Devices with a 0.6-mm diameter needle and inserted into a single recipient paraffin block. Sections were cut from the tissue microarray block using a standard microtome (4 μm) and baked at 60 °C for 1 h before staining. The following proteins were tested for with immunohistochemistry and the details on supplier, catalog, clone, concentration, antigen retrieval, and scoring methods are provided in a (Supplementary Appendix 1): ANXA4, *BRCA1*, Cyclin E, MDM2, NDRG1, p16, p21, p27, *TP53*, pAKT, PRKDC, SERBP1, PTEN, CD8, CD3, CD4, CD20, FOXP3, and TIA1. Scoring was binarized as zero for absent or minimal (<1% of cells staining) expression and one for expression, with the following exceptions: cyclin E, p16, p27, and pAKT were scored as zero, one, or two for no staining, weak staining, and strong staining, respectively. For *TP53*, scoring was as follows: zero for complete loss of expression, one for focal expression (1–50% of

cells), or two for overexpression (defined as >50% of tumor cells showing strongly positive nuclear staining), as described previously.[16] The immune cell markers CD8, CD3, CD4, CD20, FoxP3, and TIA1 were scored as zero for no intraepithelial immune cells present in either 0.6 mm tissue microarray core, or one for one or more positively stained intraepithelial lymphocytes (Supplementary Appendix 1).

Statistical Analysis

Clinicopathological parameters and immunohistochemical staining data were considered to be categorical data. Patient age and mRNA data were considered continuous unless otherwise specified. Descriptive statistics were computed from simple frequency distributions. Contingency analysis was used to compare the associations of categorical variables and *P*-values were derived using Pearson's Chi Square statistic. Associations between categorical and continuous variables were measured using the Tukey–Kramer analysis for multiple comparisons. Progression-free survival and overall survival were assessed using Kaplan–Meier curves and differences were quantified with the Log-Rank Statistic. For the purposes of this study, uncorrected *P*-values were reported and levels of <0.05 were considered statistically significant. All analyses were computed with JMP v9.0.1, SAS Institute, Cary, NC, USA.

Results

Cases were grouped as follows: Group1: Patients with high-grade serous carcinomas with identified germline or somatic mutations in either *BRCA1* or *BRCA2* ($n=31$, or 30% of high-grade serous). In all, 19/21 *BRCA1* mutations and 7/10 *BRCA2* mutations were germline. Within group 1 there were 21 patients with *BRCA1* mutations (20%) and 10 with *BRCA2* mutations (10%). Group 2: Patients with high-grade serous carcinoma whose tumors demonstrated methylation of *BRCA1* ($n=21$, or 20% of high-grade serous). Group 3: Patients with high-grade serous carcinoma who were not included in group 1 or 2 above ($n=51$, or 50%), that is, no *BRCA1*/*BRCA2* mutation and no methylation of *BRCA1*. Group 4: Non-high-grade serous carcinoma ($n=28$). Neither *BRCA1*/*BRCA2* mutations nor *BRCA1* methylation were identified in the non-high-grade serous cohort.

The demographic and clinicopathological characteristics of the cohort are provided in Table 1. Within high-grade serous carcinomas (groups 1–3) there are no statistically significant differences between groups for any of the parameters tested (stage, ascites, cytoreduction, neoadjuvant therapy, and CA125 levels) with the exception of age, which was significantly lower in the *BRCA1 and BRCA2*

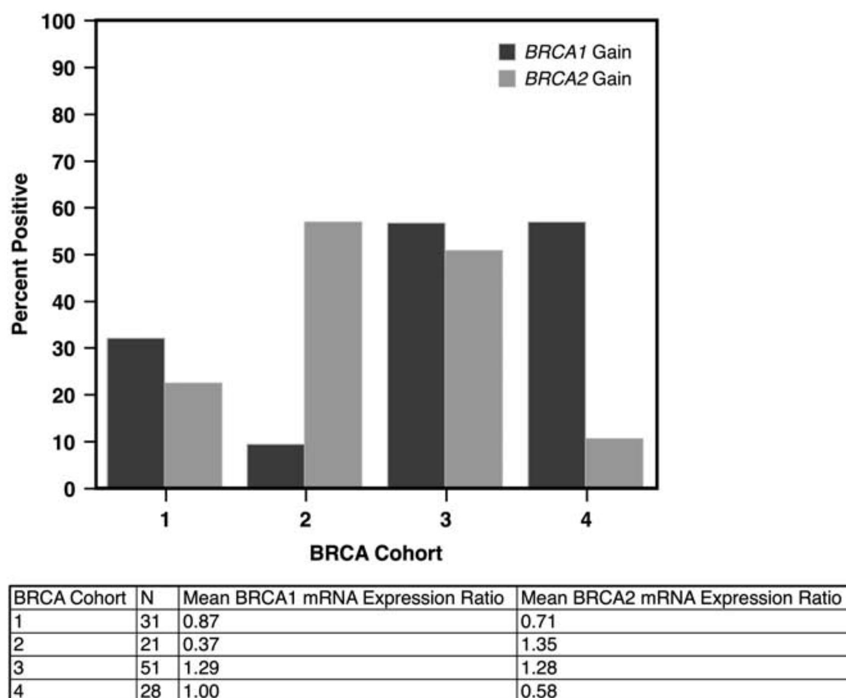
Table 1 Clinicopathological characteristics and clinical outcomes of the cohort ($n=131$ ovarian carcinomas, including 103 high-grade serous cancers)

	Group 1 (consolidated)	*Group 1 (stratified)*		*Group 2*	*Group 3*	*Group 4*	*All HGS*
		BRCA 1	*BRCA 2*				
N	31	21	10	21	51	28	103
Mean age (95% CI)	52.3 (49.3–55.3)	51.4 (47.4–55.4)	54.2 (49.2–59.2)	56.9 (51.6–62.2)	63.8 (60.8–66.8)	53.1 (48.6–57.7)	58.9 (56.7–61.1)
Stage I (%)	3 (9.7)	2 (9.5)	1 (10.0)	1 (4.8)	3 (5.9)	9 (32.1)	7 (6.8)
Stage II (%)	2 (6.4)	1 (4.8)	1 (10.0)	2 (9.5)	6 (11.8)	9 (32.1)	10 (9.7)
Stage III (%)	20 (64.5)	14 (66.7)	6 (60.0)	18 (85.7)	34 (66.7)	10 (35.8)	72 (69.9)
Stage IV (%)	6 (19.4)	4 (19.0)	2 (20.0)	0 (0)	8 (15.7)	0 (0.0)	14 (13.6)
Cytoreduction status (none) (%)	8 (25.8)	7 (33.3)	1 (10.0)	4 (19.1)	11 (21.6)	16 (57.1)	23 (22.3)
Cytoreduction status (<1 cm) (%)	13 (41.9)	9 (42.9)	4 (40.0)	7 (33.3)	17 (33.3)	5 (17.9)	37 (35.9)
Cytoreduction status (>1 cm) (%)	10 (32.3)	5 (23.8)	5 (50.0)	10 (47.6)	23 (45.1)	7 (25.0)	43 (41.8)
Platinum sensitive (%)	25 (80.6)	15 (71.4)	10 (100)	16 (76.2)	38 (74.5)	22 (78.6)	79 (76.7)
Ascites (none) (%)	6 (19.3)	3 (14.3)	3 (30.0)	5 (23.8)	15 (29.4)	14 (50.0)	26 (25.2)
Ascites (<0.5 l) (%)	7 (22.6)	5 (23.8)	2 (20.0)	5 (23.8)	15 (29.4)	9 (32.1)	27 (26.2)
Ascites (>0.5 l) (%)	18 (58.1)	13 (61.9)	5 (50.0)	11 (52.4)	21 (41.2)	5 (17.9)	50 (48.5)
Mean CA125 (95% CI)	1897 (1008–2786)	2191 (1128–3253)	1279 (592–3152)	2011 (584–3438)	1332 (480–2185)	1084 (93–2075)	1643 (1083–2204)
Neoadjuvant chemotherapy (%)	7 (22.6)	6 (28.6)	1 (10.0)	1 (4.8)	7 (13.7)	1 (3.6)	15 (14.6)
Mean follow-up (mo) (95% CI)	43.5 (35.1–52.0)	36.3 (27.8–44.7)	58.7 (40.8–76.7)	47.1 (37.3–56.8)	39.5 (33.7–45.4)	41.9 (32.2–51.7)	42.3 (38.0–46.5)
Number with recurrence (%)	24 (77.4)	17 (80.9)	7 (70.0)	19 (90.5)	39 (76.5)	12 (42.9)	82 (79.6)
Number without recurrence (%)	7 (22.6)	4 (19.1)	3 (30.0)	2 (9.5)	12 (23.5)	16 (57.1)	21 (20.4)

germline or somatic mutation group (group 1) and in the *BRCA1* methylated group (group 2), compared with group 3 ($P<0.0001$ and 0.028, respectively). The overall cytoreduction rate to <1 cm or no residual was 58% with only 14.5% of cases receiving neoadjuvant chemotherapy in this series. Patients were given intravenous carboplatin AUC 6 and paclitaxel 175 mg/m^2 post debulking surgery or as neoadjuvant chemotherapy with only three patients receiving intraperitoneal chemotherapy protocol with day 1 intravenous paclitaxel 175 mg/m^2 and intraperitoneal carboplatin AUC 6, day 8 intraperitoneal paclitaxel 60 mg/m^2. The median progression-free survival and overall survival for high-grade serous carcinomas was 18 and 73 months, respectively, with an average follow-up time of 3.5 years (range 2.3–7 years). Within the advanced stage (stage III/IV) HGS carcinomas ($n=86$) median progression-free survival and overall survival was 16 and 72 months.

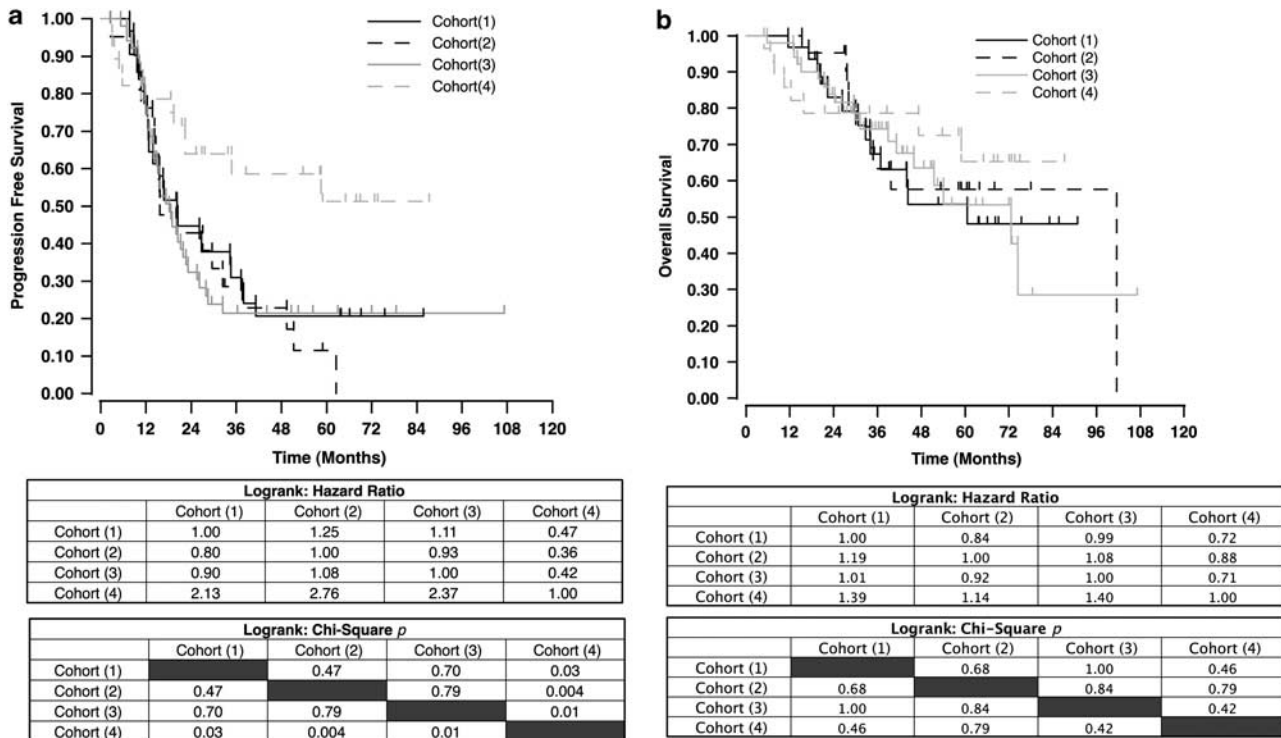
mRNA expression level ratios for *BRCA1* and *BRCA2* correlated with group assignment with the lowest mRNA expression level ratios in groups 1 and 2 as compared with group 3 ($P=0.0008$) (Figure 1). Within group 1 analysis of variance was performed and demonstrated significantly lower *BRCA1* mRNA expression ratios in the *BRCA1* mutation group as compared with those with mutations in *BRCA2* ($P=0.008$). For non-high-grade serous carcinomas the mean *BRCA1* mRNA expression ratio was 1.0 justifying consideration of a cutoff in our categorization of low/loss of mRNA to be ⩽1.0 *vs* high/gain mRNA ratio of >1. *BRCA2* expression levels were also significantly different between groups ($P=0.0011$) with low ratios in group 1 and group 4, and particularly in those individuals ($n=10$) with known germline or somatic *BRCA2* mutations. The cohort of *BRCA1* methylated cases (group 2) had the highest proportion of cases with elevated *BRCA2* mRNA expression (Figure 1). In group 1, all patients with *BRCA2* germline or somatic mutations had a low ratio of *BRCA2* mRNA (mean ratio 0.499). However, of the 21 patients who had *BRCA1* germline or somatic mutations there were three germline *BRCA1* mutation carriers who had high-*BRCA1* mRNA levels (mean ratio 2.13). Two of the women with 'discordant' *BRCA1* mutation status and mRNA expression levels were carriers of the well characterized exon 2185 del AG *BRCA1* mutation, and the third had an inherited exon 11 Q563X.

Kaplan–Meier survival curves were generated for groups 1–4 (progression-free survival and overall survival). In group 4, median progression-free survival was never reached but is estimated at 5 years or 60 months. Within high-grade serous carcinomas, median progression-free survival in groups 1–3 was 20, 16, and 18 months, respectively. A statistically significant difference in progression-free survival could only be demonstrated between Group 4 *vs* Groups 1,2 and 3 ($P=0.03$) with no differences in outcome discerned between the three



BRCA Cohort	N	Mean BRCA1 mRNA Expression Ratio	Mean BRCA2 mRNA Expression Ratio
1	31	0.87	0.71
2	21	0.37	1.35
3	51	1.29	1.28
4	28	1.00	0.58

Figure 1 mRNA expression level ratios for *BRCA1* and *BRCA2* assessed as a categorical variable (⩽1.0 was used to categorize as 'loss' or low-mRNA expression ratio and >1 as 'gain' or high-mRNA expression ratio) differ between groups 1–4 ($P=0.0008$ for both *BRCA1* and *BRCA2*). The mean mRNA expression level ratio within each group is shown.



(a)

Logrank: Hazard Ratio				
	Cohort (1)	Cohort (2)	Cohort (3)	Cohort (4)
Cohort (1)	1.00	1.25	1.11	0.47
Cohort (2)	0.80	1.00	0.93	0.36
Cohort (3)	0.90	1.08	1.00	0.42
Cohort (4)	2.13	2.76	2.37	1.00

Logrank: Chi-Square *p*				
	Cohort (1)	Cohort (2)	Cohort (3)	Cohort (4)
Cohort (1)		0.47	0.70	0.03
Cohort (2)	0.47		0.79	0.004
Cohort (3)	0.70	0.79		0.01
Cohort (4)	0.03	0.004	0.01	

(b)

Logrank: Hazard Ratio				
	Cohort (1)	Cohort (2)	Cohort (3)	Cohort (4)
Cohort (1)	1.00	0.84	0.99	0.72
Cohort (2)	1.19	1.00	1.08	0.88
Cohort (3)	1.01	0.92	1.00	0.71
Cohort (4)	1.39	1.14	1.40	1.00

Logrank: Chi-Square *p*				
	Cohort (1)	Cohort (2)	Cohort (3)	Cohort (4)
Cohort (1)		0.68	1.00	0.46
Cohort (2)	0.68		0.84	0.79
Cohort (3)	1.00	0.84		0.42
Cohort (4)	0.46	0.79	0.42	

Figure 2 Kaplan–Meier survival analysis where (**a**) progression-free survival and (**b**) overall survival are assessed for groups 1–4.

high-grade serous carcinomas subgroups ($P>0.47$) (Figure 2a). In the assessment of overall survival between groups, the majority of the cohort was censored, with 82 of 131 patients alive at the time of final analysis. However, of those who did succumb to their disease, no statistically significant differences in overall survival were as observed between the groups ($P>0.42$) (Figure 2b).

Immunohistochemistry demonstrated decreased expression of MDM2, ANXA4, and p21 and increased expression of p16 in high-grade serous carcinomas (groups 1–3) as compared with non-high-grade serous (group 4) ($P=<0.0001$ for all). Loss of PTEN was noted in 25% of non-high-grade serous cases in contrast to 5% of high-grade serous cases ($P=0.008$). Expression of the above proteins did not differ significantly between the three groups of high-grade serous carcinomas. Loss of PTEN was associated with worse outcome ($P=0.012$) (Table 2). No significant differences were seen between groups 1–3 with respect to MDM2, ANXA4, p21, p16, PTEN, NDRG1, SERBP1, cyclin E, p27, or pAKT immunoexpression (Table 2). There was a significant difference in CD20 ($P=0.034$) and TIA1 ($P=0.027$) cell infiltrates between groups 1–3, and a trend towards difference in CD8 ($P=0.057$), with tumors in group 1 more likely to show these immune cell infiltrates. No association was found between the presence of any of the immune cell infiltrates and outcomes in high-grade serous carcinomas in this series (Table 2).

TP53 expression was strongly associated with group ($P<0.0001$). *TP53* was completely absent (immunohistochemistry score 0) in 33.3% of high-grade serous, and overexpressed (immunohistochemistry score 2) in 58.3% of high-grade serous carcinoma, with wild-type *TP53* expression pattern (immunohistochemistry score 1) in only 8.3% of high-grade serous. All of the *BRCA1* methylated cases and all but one of the *BRCA1* and *BRCA2* mutation carriers showed either absence or overexpression of *TP53* (Figure 3a). Within high-grade serous carcinomas *TP53* expression was significantly different when comparing those tumors with BRCA abnormalities (groups 1 and 2) and those without (group 3) ($P=0.012$). Survival analysis for all high-grade serous carcinomas that was evaluable for *TP53* immunohistochemistry ($n=96$) revealed the worst outcomes in those women whose tumors had wild-type *TP53* (immunohistochemistry score 1), and more favorable outcomes where loss or gain of *TP53* expression was noted. HRs for wild-type *TP53* (immunohistochemistry score 1) are tabulated, ranging from 2.8 to 4.2 (Figure 3b and c).

BRCA1 immunohistochemistry was assessed with Ab-1 monoclonal antibody at four different dilutions (1:10, 1:25, 1:50, and 1:75) and there was no association demonstrated between immunohistochemistry staining and group assigned (Figure 4) ($P=0.27$). Even within group 1, immunohistochemistry with *BRCA1* antibody could not distinguish between those with *BRCA1 vs BRCA2* mutations

Table 2 Immunohistochemical staining results for groups 1–3

Biomarker	*Group 1*[a]	*Group 2*[a]	*Group 3*[a]	*Difference between HGS subgroups (groups 1–3)* (P-*value*)	*Associated with PFS in HGS (groups 1–3)* (P-*value*)
CD3	26/29	17/21	36/45	0.506	0.594
CD8	26/28	16/21	34/46	0.057	0.383
CD20	14/29	7/21	14/44	**0.034**	0.411
TIA-1	25/29	18/21	28/45	**0.027**	0.156
FOXP3	20/29	16/21	24/42	0.281	0.696
MDM2	0/29	0/21	3/44	0.097	0.851
ANAXA4	0/29	2/20	1/43	0.140	0.857
p16	18/29	15/21	29/46	0.742	0.680
p21	8/29	3/21	11/46	0.508	0.631
PTEN loss	1/29	2/21	2/46	0.631	**0.012**[b]

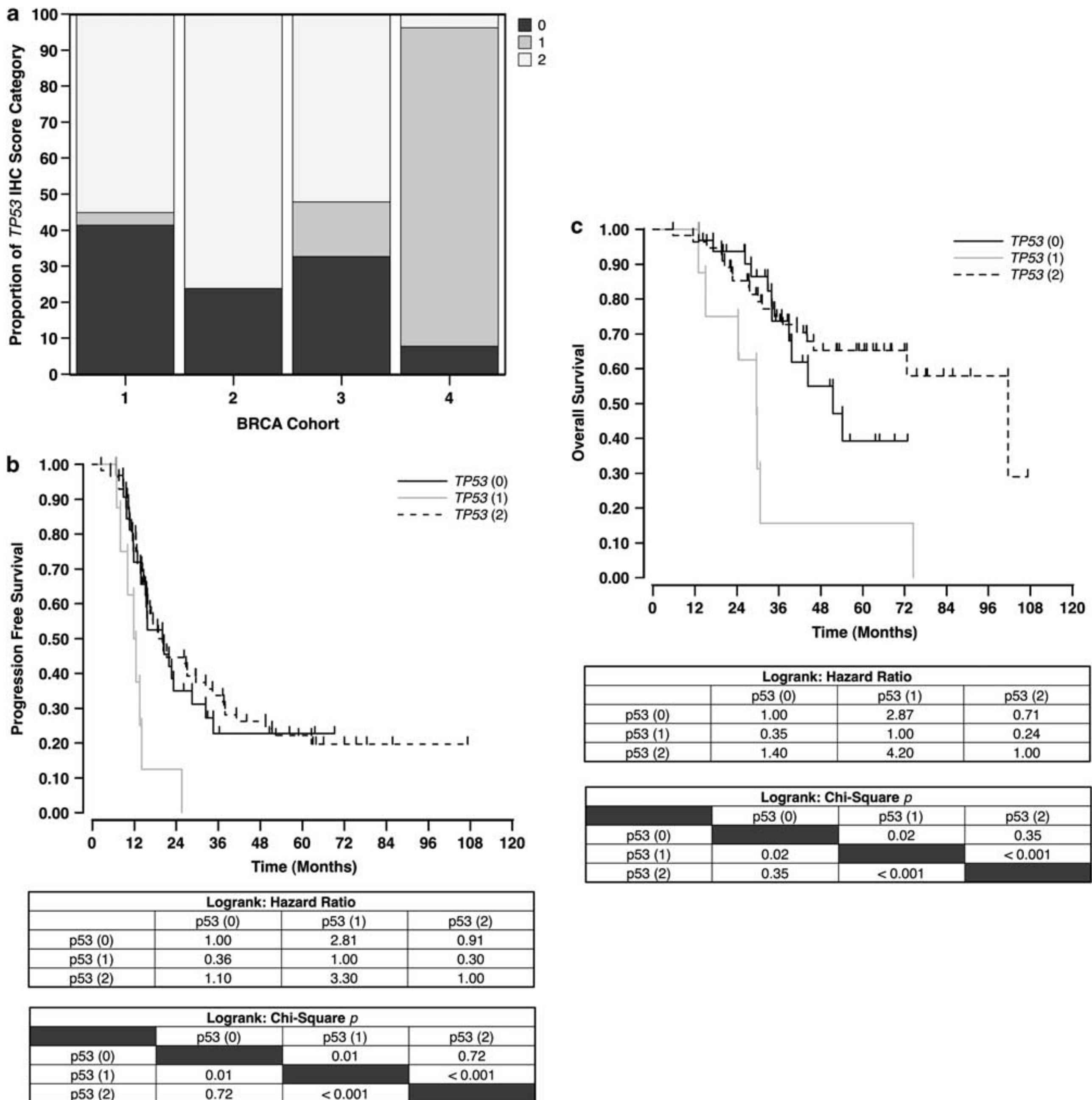
[a]The number of immunohistochemistry (IHC) positive cases are shown as the numerator over the number of cases tested by IHC. The exception is PTEN where the numerator reflects the number of cases with loss of PTEN expression. Significant associations are shown in bold.
[b]Based on few events, that is, majority of high-grade serous (HGS) carcinomas had undetectable expression levels.

($P = 0.98$), nor did *BRCA1* immunohistochemistry correlate with *BRCA1* mRNA expression levels ($P = 0.089$). Unstained slides from a subset of cases were sent to a separate institution for independent validation of *BRCA1* immunohistochemistry. Of the 11 cases where *BRCA1* germline mutations had been identified, 3 tested positive by *BRCA1* immunohistochemistry. Of the 33 cases with no *BRCA1* mutations, 17 tested negative by immunohistochemistry. Sensitivity, specificity, positive predictive value, and negative predictive values for *BRCA1* immunohistochemistry as a test for *BRCA1* mutation were 27.3%, 51.5%, 15.8%, and 75%, respectively, in this independent assessment of *BRCA1* immunohistochemistry.

Discussion

The importance of histological subtype in ovarian carcinomas has been increasingly appreciated.[3,53] High-grade serous carcinomas are distinct from non-high-grade serous carcinomas, differing with respect to clinical presentation, distribution of disease, response to therapy, survival, and site of origin.[3,53–56] High-grade serous carcinomas are characterized by ubiquitous *TP53* abnormalities, BRCA abnormalities in ~50% of cases, and chromosomal instability. This study confirmed that BRCA abnormalities are exclusively a feature of high-grade serous carcinomas, with germline or somatic mutations present in 30% of high-grade serous carcinomas (24% of the entire ovarian carcinoma cohort), *BRCA1* methylation identified in 20% of high-grade serous carcinomas, and neither *BRCA1* nor *BRCA2*2 mutations nor methylation of *BRCA1* seen in the 28 non-high-grade serous carcinomas. Although this does not prove that BRCA abnormalities never occur in non-high-grade serous ovarian carcinoma subtypes, if they do occur they are rare. Previous studies correlating BRCA mutation status with subtype have suffered from not having contemporary pathology review; histological subtyping based on current criteria is highly reproducible[3] and reflects underlying molecular abnormalities. The results reported herein indicate that testing for *BRCA1* and *BRCA2* mutations in patients with non-high-grade serous ovarian carcinomas is not routinely indicated.

Attention is now focusing on the molecular abnormalities within high-grade serous subtype that might explain the observed differences in patient outcomes. Patient and treatment factors (such as age, timing and aggressiveness of surgery, type and delivery route of chemotherapy, and host immune response) and tumor genetic alterations may all impact clinical course and survival. A recent report on patients from 27 international studies demonstrated improved survival of *BRCA1* and *BRCA2* mutation carriers relative to non-carriers.[57] Unlike previous series (and the current case series) where the number of *BRCA1* and *BRCA2*-null patients from which conclusions were drawn was relatively small, Bolton's multi-center study had outcome data on over 1400 *BRCA1* and *BRCA2* mutation carriers and ~2400 non-carriers, allowing them to take into account clinical factors known to influence outcome. The recently published data from The Cancer Genome Atlas has confirmed the association of BRCA mutations with a favorable prognosis, and also showed no prognostic effect with *BRCA1* promoter methylation (compared with tumors lacking BRCA mutations or methylation).[15] Our failure to see significant differences in outcome in patients with high-grade serous carcinoma, based on BRCA status, can be attributed to a lack of sufficient cases to detect a relatively modest difference in prognosis in the current series. The fact that no differences were observed in this series of 103 high-grade serous cases, when patients were stratified based on BRCA status, highlights the inability of BRCA mutation testing to serve as a tool that can accurately predict outcome in individual patients with high-grade serous carcinoma. The HR of ~0.7 associated with BRCA mutation in the Bolton's series is less



(b)

Logrank: Hazard Ratio			
	p53 (0)	p53 (1)	p53 (2)
p53 (0)	1.00	2.81	0.91
p53 (1)	0.36	1.00	0.30
p53 (2)	1.10	3.30	1.00

Logrank: Chi-Square *p*			
	p53 (0)	p53 (1)	p53 (2)
p53 (0)		0.01	0.72
p53 (1)	0.01		< 0.001
p53 (2)	0.72	< 0.001	

(c)

Logrank: Hazard Ratio			
	p53 (0)	p53 (1)	p53 (2)
p53 (0)	1.00	2.87	0.71
p53 (1)	0.35	1.00	0.24
p53 (2)	1.40	4.20	1.00

Logrank: Chi-Square *p*			
	p53 (0)	p53 (1)	p53 (2)
p53 (0)		0.02	0.35
p53 (1)	0.02		< 0.001
p53 (2)	0.35	< 0.001	

Figure 3 (**a**) Contingency analysis of *TP53* immunostaining results showing significant differences between groups 1–4 ($P<0.0001$). Kaplan–Meier survival analysis for high-grade serous carcinomas (groups 1–3) based on *TP53* expression category (0, 1, and 2) for progression-free (**b**) and overall (**c**) survival reveals a survival disadvantage in tumors with wild-type *TP53* (immunohistochemistry score 1) as compared with the other expression categories (progression-free survival: $P=0.0035$, overall survival: $P=0.0005$).

impressive than HRs associated with cytoreductive status, stage, and *TP53* immunohistochemistry (wild-type pattern expression *vs* overexpression or complete absence of expression). Response to conventional chemotherapy and molecular targeted therapy is not limited to BRCA mutant phenotypes, as platinum sensitivity is high in all high-grade serous carcinomas. A recent series assessing efficacy of poly (ADP-ribose) polymerase (PARP) inhibitors in heavily pretreated women with ovarian and breast carcinoma revealed that the success of treatment with PARP inhibitors in ovarian carcinoma was not limited to BRCA mutation carriers nor platinum sensitive cases.[58] There are presumably other molecular parameters that are equally if not more important than BRCA status in influencing response to treatment and outcome.

Within high-grade serous cancers there are immunohistochemistry features (ie, *TP53* and immune cell infiltrates) that were significantly associated with genetic changes in *BRCA1* and *BRCA2* (group 1). This supports there being molecular differences

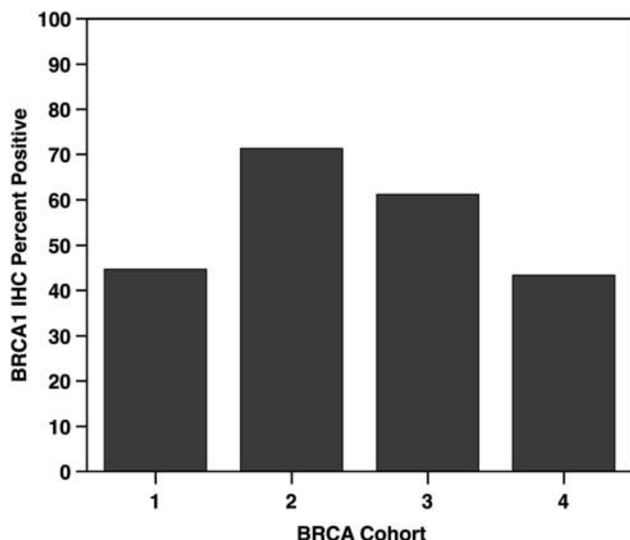


Figure 4 Frequency distribution of *BRCA1* immunohistochemistry scoring in groups 1–4 revealing no detectable differences between groups ($P = 0.2731$).

between group 1 (*BRCA1* and *BRCA2* mutations) and groups 2 and 3 that are most probably a result of the BRCA mutation. Expression of most markers was identical, however, and the striking clinical, pathological, and molecular similarities between the groups suggest that group 3 tumors have as yet undetermined abnormalities that are functionally equivalent to *BRCA1* and *BRCA2* mutations. Increased presence of immune cell markers CD20 and TIA-1 was observed in patients with *BRCA1* or *BRCA2* mutations as distinct from groups 2 and 3. These markers were not associated with improved outcomes in our relatively small cohort of high-grade serous carcinomas but other series with greater case numbers have consistently demonstrated immune cell infiltrates to be a favorable prognostic factor.[18,19,22,24,59] *TP53* abnormalities (loss of expression, which correlates with nonsense mutations or deletions, or overexpression, which correlates with missense mutations) were essentially ubiquitous in high-grade serous cancers with *BRCA1* or *BRCA2* abnormalities and also were associated with favorable outcome. Thus BRCA mutations, immune cell infiltrates, and *TP53* abnormalities co-vary in high-grade serous carcinomas. In this series only *TP53* was of prognostic significance. A sufficiently large series of cases to allow multivariate analysis is needed to determine which of these three features are of prognostic significance independent of the others.

This series and the Cancer Genome Atlas data[15] showed no survival advantage in the *BRCA1* methylated cohort (group 2). Discordant data from previous series could be at least partly attributed to challenges in methodology surrounding methylation testing.

BRCA1 immunohistochemistry did not correlate with subgroup of ovarian carcinoma nor were any differences in outcome according to *BRCA1* immunohistochemistry appreciated. The use of *BRCA1* immunohistochemistry as a surrogate for *BRCA1* genetic status or functional status, using currently available reagents, is doubtful based on these results.

BRCA mRNA expression level ratios clearly correlate with group, particularly in patients with identified *BRCA1* and *BRCA2* germline or somatic mutations, but the correlation is imperfect and assessment of mRNA levels cannot act as a surrogate for BRCA mutational analysis. It is possible that these results may be confounded by contaminating populations of non-tumour cells, for example, lymphocytes,[60,61] as these BRCA expressing cells, which are associated with a favorable prognosis, may lead to increased expression levels even when tumor cells lack expression. It was interesting to observe increased *BRCA1* mRNA expression levels in three *BRCA1* germline mutation carriers, two of which have 185 del AG mutations. These may be secondary to contamination by normal cells as described above, or reveal a true difference between germline and tumor DNA suggesting the tumor has undergone a secondary mutation with restoration of the open reading frame and restoration of a functional *BRCA1* gene.[62–64]

We have demonstrated that BRCA abnormalities are only associated with the high-grade serous subtype of ovarian carcinoma. *TP53* abnormalities (as detected by immunohistochemistry) and immune cell infiltrates were associated with BRCA mutations; there is at present no data to indicate whether BRCA mutation, *TP53* abnormalities, and host immune cell infiltrates, all of which are favorable prognostic factors in univariate analysis in large case series, are of independent prognostic significance. High-grade serous carcinomas without BRCA mutations are very similar, clinically, to high-grade serous carcinomas with BRCA mutations with respect to stage at presentation, ability to optimally surgically cytoreduce, and outcome. This suggests there are other as yet unidentified molecular changes in high-grade serous carcinomas that will be able to predict response to treatment and outcome better than BRCA mutation status.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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