Non-equivalent gene expression and copy number alterations in high-grade serous ovarian cancers with BRCA1 and BRCA2 mutations

Running title: BRCA mutation non-equivalence in high-grade serous cancer

Joshy George1,2 *, Kathryn Alsop1,2 *, Dariush Etemadmoghadam1,3,4, Heather Hondow1, Thomas Mikeska1,4, Alexander Dobrovic1,3,4, Anna deFazio5 for the Australian Ovarian Cancer Study Group1,5,6, Gordon K. Smyth7,8, Douglas A. Levine9, Gillian Mitchell1,3, and David Bowtell1,2,3,4

* co-first authors.

1 Peter MacCallum Cancer Centre, East Melbourne, Victoria, Australia;

2 Department of Biochemistry and Molecular Biology, University of Melbourne, Melbourne, Victoria, Australia

3 Sir Peter MacCallum Cancer Centre, Department of Oncology, University of Melbourne, Melbourne Australia;

4 Department of Pathology, University of Melbourne, Melbourne, Victoria, Australia;

5 Westmead Institute for Cancer Research, University of Sydney at Westmead Millennium Institute, and Departments of Gynaecological Oncology, Westmead Hospital, NSW, Australia;

6 Queensland Institute of Medical Research, Brisbane, QLD, Australia;

7 Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria Australia

8 Department of Mathematics and Statistics, University of Melbourne, Melbourne, Victoria, Australia
Gynaecology Service/Department of Surgery, Memorial Sloan-Kettering Cancer Centre, New York.

Correspondence to: Professor David Bowtell, Australian Ovarian Cancer Study, Peter MacCallum Cancer Centre, Locked Bag No. 1 A'Beckett St., Victoria, 8006, Australia. Ph: +613 9656 1356, E-mail: d.bowtell@petermac.org.

Financial Support: This study was supported by the Ovarian Cancer Research Program of the US Department of Defense (W81XWH-08-1-0684 and W81XWH-08-1-0685); Cancer Australia and the National Breast Cancer Foundation (ID509303, CG-08-07, ID509366); the Peter MacCallum Cancer Centre Foundation and the Cancer Council Victoria. The Australian Ovarian Cancer Study (AOCS) was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-01-1-0729, The Cancer Council Victoria, Queensland Cancer Fund, The Cancer Council New South Wales, The Cancer Council South Australia, The Cancer Foundation of Western Australia, The Cancer Council Tasmania and the National Health and Medical Research Council of Australia (NHMRC; ID400413, ID400281).

Conflict of interest statement: The authors have no conflicts of interest to declare.

Word count: 4025 words
**Translational Summary:**

Disruption of the BRCA pathway is a feature of approximately half of all high grade serous ovarian carcinomas (HGSC) but it is unclear whether aberrations in the pathway are functionally equivalent. This study for the first time systematically investigates differences in gene expression, in combination with DNA copy number, in HGSC arising in association with *BRCA1* or *BRCA2* mutations. We show that *BRCA1* mutant tumors are associated with a specific molecular subtype of HGSC and have a distinct gene expression signature, which is heavily influenced by specific amplification events at 8q24 and on the X chromosome. By contrast, *BRCA2* mutant tumors more closely resemble ‘wild-type’ HGSC. High frequency of amplification involving 8q24 and loci on the X chromosome in *BRCA1* HGSC resembles basal-like breast cancer (BLBC). Our work has important implications for the design of clinical trials in mutation carriers and in understanding the molecular features of HGSC and BLBC.

**Word Count:** 149 words
Abstract

**Purpose:** High-grade serous carcinoma (HGSC) accounts for the majority of epithelial ovarian cancer deaths. Genomic and functional data suggest that approximately half of unselected HGSC have disruption of the BRCA pathway and defects in homologous recombination repair (HRR). Pathway disruption is regarded as imparting a *BRCAness* phenotype. We explored the molecular changes associated with HGSC arising in association with specific *BRCA1/BRCA2* somatic/germline mutations, and in those with *BRCA1* DNA promoter methylation.

**Experimental Design:** We describe gene expression and copy number analysis of two large cohorts of HGSC in which both germline and somatic inactivation of HRR has been measured.

**Results:** *BRCA1* disruptions were associated with the C2 (immunoreactive) molecular subtype of HGSC, characterised by intense intra-tumoral T-cell infiltration. We derived and validated a predictor of *BRCA1* mutation or methylation status, but could not distinguish *BRCA2* from wild type tumors. DNA copy number analysis showed that cases with *BRCA1* mutation were significantly associated with amplification both at 8q24 (frequencies: *BRCA1* tumors 50%, *BRCA2* tumors 32% and wild type (WT) tumors 9%) and regions of the X-chromosome specifically dysregulated in basal-like breast cancer (BLBC; *BRCA1* 62%, *BRCA2* 34% and WT 35%). Tumors associated with *BRCA1/BRCA2* mutations shared a negative association with amplification at 19p13 (*BRCA1* 0%, *BRCA2* 3% and WT 20%) and 19q12 (*BRCA1* 6%, *BRCA2* 3% and WT 29%).

**Conclusion:** The molecular differences between tumors associated with *BRCA1* compared with *BRCA2* mutations accords with emerging clinical and pathological data, and support a growing appreciation of the relationship between HGSC and BLBC.

**Word count:** 250 words

**Key words:** serous ovarian cancer, *BRCA1, BRCA2*
Introduction:

*BRCA1* and *BRCA2* encode proteins that are critical for the integrity of the cellular genome, particularly through their roles in homologous recombination repair (HRR) of DNA double strand breaks (1). Germline mutations in either gene confer greatly increased risk of breast or epithelial ovarian cancer. Epithelial ovarian cancer (EOC) is a histologically diverse disease with serous, mucinous, endometrioid and clear cell cancers typically regarded as the most common histotypes. Among these, high-grade serous carcinoma (HGSC) is the most important histological subtype, accounting for about two thirds of EOC deaths. Recent studies have highlighted molecular differences between EOC histotypes (2, 3), defined distinct cellular origins (4, 5) and have revised the histological (6) and molecular (2, 7) classification of EOC. Secretory cells of the distal fallopian tube appear to be the progenitors of a substantial proportion of HGSC that are diagnosed as being of ovarian, fallopian tube or peritoneal origin.

Germline mutations in *BRCA1* or *BRCA2* had been thought to occur at a frequency of 5-10% in women diagnosed with EOC, irrespective of histological subtype. However, among EOC patients, *BRCA1/2* germline mutation may be essentially a feature of HGSC (8, 9) and we recently reported a higher combined *BRCA1/2* germline mutation frequency in HGSC (17%) When germline mutations were associated with other histotypes, this was probably due to pathological misclassification of tumors at initial diagnosis (8). Consistent with the importance of *BRCA1/2* to the genesis of HGSC, somatic point mutations are seen in these genes in ~5-7% of these tumors (7) and promoter methylation of *BRCA1* is found in a further 11% of HGSC (7). Inactivation of both *BRCA1* or *BRCA2* is rarely seen together (7), suggesting that disruption of either gene is functionally equivalent or lethal in combination. Collectively, changes in *BRCA1/2*, together with germline or somatic mutation, methylation or amplification of other members of the HRR pathway
including *EMSY*, *FANC*-family genes, *RAD51C*, and *PTEN* occur in ~50% of HGSC, a figure that accords with functional assays of defective HRR in HGSC (10).

Little is known of the molecular differences that underlie clinical and pathological variation between *BRCA1*- and *BRCA2*-associated HGSC. A supervised analysis of microarray-based gene expression data identified distinct gene expression profiles of *BRCA1*- and *BRCA2*-mutated tumors and suggested that sporadic HGSC resembled one or the other germline mutated samples (11). This data was subsequently used to generate a classifier of *BRCA*ness, which could predict response to platinum-based therapies or poly(ADP-ribose) (PARP) inhibitors (12). More recently, germline mutation in *BRCA1* or *BRCA2* was found to be anti-correlated with amplification of the *CCNE1* gene, which encodes the cell cycle regulator Cyclin E1 (7).

Here we explore the molecular biology of HGSC arising in association with BRCA aberrations, finding further evidence of fundamental differences between *BRCA1* mutated/methylated and *BRCA2*-mutated tumors or their wild type counterparts.

**Materials and Methods:**

*Patient samples and associated genomic information.* Previously published gene expression data was obtained from three independent ovarian cancer cohorts: The Cancer Genome Atlas (TCGA) dataset of 316 HGSC (7), a cohort of 132 HGSC from the Australian Ovarian Cancer Study (AOCS) (2), and 61 ovarian tumors of mixed histologies from the Memorial Sloan Kettering Cancer Center, which we refer to as the Jazaeri dataset (11, 12). Further information about the cohorts and their respective genomic datasets are provided in Supplementary Methods.
**Bioinformatic analyses.** Methodology for evaluation of the gene expression and DNA copy number data, including the generation of a gene expression-based classifier, are provided in the Supplementary Methods. In addition, to facilitate reproducibility of the research, a Sweave formatted file, capable of reproducing all the figures and tables, can be provided on request.

**qPCR validation of copy number associations.** Gene copy number for MYC, PTK2 and PYCRL were assessed by real-time quantitative-PCR using the 7900HT Fast Real-Time PCR system (Applied Biosystems) as described previously (13). Primers were designed to amplify exonic regions, avoiding known SNPs and amplification of homologous sequences, using Primer3 (14). Further information, including primer sequences, can be found in the Supplementary Methods.

**Results:**

**High frequency disruption of the BRCA1/2 pathway in HGSC tumors.**

To explore the molecular features of tumors arising in BRCA mutation carriers and non-carriers, we first screened for germline (8) and somatic BRCA1/2 mutation information in a subset of 132 women recruited to the Australian Ovarian Cancer Study (AOCS) for which we had previously obtained Affymetrix U133+2.0 gene expression data on tumor samples (2). Germline mutations were identified 15.9% of cases (14 BRCA1, 7 BRCA2; Table 1), a similar frequency to that reported previously for HGSC (7, 9) and slightly below that seen for the overall AOCS cohort (8). A further 6.1% (8/132) of patients had a germline BRCA1/2 sequence variant of unknown, but likely low, pathogenic significance and were considered wild type (Supplementary Table 1). Consistent with previous reports (7, 15, 16), pathogenic somatic mutations in BRCA1/2 were found in 6.1% of our samples (4 BRCA1, 4 BRCA2, Table 1). Methylation of the BRCA1 promoter (17) and several other members of the HRR pathway have been described previously, including PALB2 (18) and FANCF (19). Extensive BRCA1 promoter methylation was observed in 15.9% of AOCS samples.
Supplementary Table 4), however, no significant methylation of either the PALB2 (20) or FANCF promoter regions was observed, a finding that was consistent with independent TCGA data (7). Overall, 37.9% of the AOCS samples showed evidence of disruption of the BRCA1/2 pathway by either BRCA1/2 germline or somatic mutation or BRCA1 methylation, with the different type of disruption being almost completely mutually exclusive (Figure 1A).

Carcinomas associated with BRCA1 mutant tumors cluster with the C2 molecular subtype of HGSC.

We previously described four molecular subtypes (C1, C2, C4, C5) of HGSC (2) that were subsequently validated in the TCGA analysis (7). One-hundred and eleven of the AOCS tumors profiled for BRCA pathway disruption, and 210 of those from the TCGA analysis were subsequently classified as being HGSC, and were included in one of these four subtypes. BRCA1 disrupted tumors (methylated, or somatically/germline mutated) were observed to be markedly enriched in the C2 (immunoreactive) molecular subtype for both the TCGA (Figure 1B, Supplementary Table 6) and AOCS datasets (Figure 1C, Supplementary Table 7). A focused statistical test designed to detect this enrichment was strongly significant for both the TCGA (p=0.0002, Figure 1B) and AOCS cohorts (p=0.017, Figure 1C). By contrast, BRCA2 mutant tumors were not significantly associated with any of the molecular subtypes in either the AOCS or TCGA datasets. The C2 subtype is characterised by an intense infiltration of T cells in the epithelial fraction of the tumor and generally favourable patient overall survival (2) (21).

Gene expression distinguishes BRCA-mutated and wild type tumors.

A microarray-based gene expression profile has been previously described that distinguished BRCA1 and BRCA2 mutant tumors and classified sporadic cancers as either BRCA1-like or BRCA2-like (11). We were unable to replicate these findings within the same dataset (Jazaeri) or with the
TCGA dataset (described in Supplementary Methods and Supplementary Figures 1, 2 and 3). We were also unable to distinguish \textit{BRCA1/2} mutated tumors from those arising in non-carriers in either the AOCS or TCGA datasets when we used a more recently reported \textit{BRCA}-like gene expression signature, also derived from the Jazaeri dataset (12) (Supplementary Figure 5). We therefore sought to develop a novel classifier that could identify ovarian tumors carrying any mutation in \textit{BRCA1} or \textit{BRCA2}. We made use of the TCGA expression dataset for gene discovery (200 expression profiled cases with known \textit{BRCA} status; 27 \textit{BRCA1}, 28 \textit{BRCA2} and 145 wild type) and then validated findings in the AOCS cohort.

It is unclear whether \textit{BRCA1} methylation impacts on HGSC biology to the same extent as somatic or germline mutations, as patients with \textit{BRCA1} methylation have been reported to have similar clinical outcomes to those with wild-type HGSC (7). Therefore, to maximize the opportunity to discover a signature associated with either \textit{BRCA1} or \textit{BRCA2} mutation, we initially excluded \textit{BRCA1} methylated samples and focussed on \textit{BRCA} germline or somatically mutated tumors for our analyses. Sixty five genes were identified that were differentially expressed between \textit{BRCA}-mutated and wild-type samples, after correcting for multiple testing and allowing for a false discovery rate of <5\% (Supplementary Methods and Supplementary Table 8). The differences in expression of individual genes were modest, in most cases involving less than a 2-fold change in expression between mutated and wild type cancers (Supplementary Table 8). There was no overlap between the 65 genes identified here and those associated with the previously described \textit{BRCA-like} signature (12).

A classifier was created using the differentially expressed gene-list derived from TCGA data (Supplementary Methods) and applied to the AOCS cohort. The distribution of scores in tumors with either germline or somatic \textit{BRCA1/2} mutations, and \textit{BRCA1} promoter methylated samples was
highly significantly different to wild-type AOCS tumors (Figure 2A, p<0.0001; student t-test). A receiver operating characteristic (ROC) curve was computed for the TCGA, AOCS and Jazaeri datasets, demonstrating that our classifier outperformed the previously-described \textit{BRCA-like} signature in all three instances (Supplementary Table 10).

\textit{A distinct pattern of gene expression distinguishes BRCA1 from BRCA2 and wild type tumors.}

Separation of the tumors by mutation type showed that median classifier values of \textit{BRCA1} mutated or methylated samples were significantly different to wild type tumors, however, there was less discrimination between wild type and \textit{BRCA2} mutated samples (Figure 2B). As these findings suggested that \textit{BRCA1} mutated samples contributed most of the discriminatory power of the classifier we repeated the gene selection process, but this time seeking markers specifically associated with either \textit{BRCA1} or \textit{BRCA2} mutation when compared to wild-type tumors. Within the TCGA data we identified 34 genes that were differentially expressed between \textit{BRCA1} mutated and wild-type tumors, 24 of which were common to the initial \textit{BRCA1/2} signature (Supplementary Table 9). No differentially expressed genes were associated with \textit{BRCA2} mutated tumors, at a false discovery rate of <5\% and after correcting for multiple testing. Collectively these findings suggest that \textit{BRCA1} mutant and methylated tumors have a common distinct pattern of gene expression, whereas \textit{BRCA2} mutant tumors more closely resemble those arising in a wild type background.

Independent validation of the 34 gene-classifier using AOCS samples demonstrated clear separation of \textit{BRCA1} mutant and methylated tumors from wild type samples, with \textit{BRCA2} mutant tumors having intermediate values (Figure 2C, 2D). Using the 34-gene list and a k-nearest neighbour classification method to predict the \textit{BRCA1} status, we achieved a positive predictive value of 0.77 and a negative predictive value of 0.92, with an overall accuracy of 89\% (Supplementary Table 12).
Women with tumors deemed to be *BRCA1-like* according to the classifier were shown to have a longer progression-free (PFS) and overall survival (OS) compared with wild type, in both the TCGA and AOCS cohorts in univariate analyses (log rank test: TCGA PFS p = 0.027, OS p = 0.027: AOCS PFS p = 0.010, OS p = 0.008) ([Supplementary Figure 7](#)), supporting distinct underlying biology or chemo-responsiveness of *BRCA-like* tumors.

*Chromosomal alterations at 8q24, 19q12 and X are associated with BRCA1 disruption.*

The inability to identify significantly differentially expressed genes between *BRCA1* and *BRCA2* tumors, and between *BRCA2* and wild type tumors prompted us to consider a gene set analysis (GSA) (22). GSA identified genes associated with chromosomal regions 8q24 and Xq28 as being differentially expressed between *BRCA1* mutated and other samples ([Supplementary Table 14](#)). By contrast, there was no obvious enrichment of genes associated with specific chromosomal loci among *BRCA2* mutated samples ([Supplementary Table 15](#)). HGSC are characterised by genomic instability, including amplifications and deletions (23) and we therefore considered whether copy number differences in the *BRCA1*-associated tumors contributed to their specific gene expression signature. We made use of the TCGA cohort, for which there were 204 HGSC samples available with annotated *BRCA*-pathway events and copy-number data (34 *BRCA1* mutated, 30 *BRCA2* mutated, 140 wild-type). The proportion of samples with genomic copy number changes in *BRCA1/2* carriers and wild type patients were compared and p-values estimated after correcting for the false discovery rate. We observed a general increase in amplifications in *BRCA1* mutant tumors and deletions in tumors from *BRCA2* carriers ([Supplementary Figure 8](#)). Importantly, several chromosomal regions were significantly differentially amplified, including 8q24, 19q12-13 and regions on the X chromosome, in tumors arising in *BRCA1* mutant samples versus those in non-carriers ([Figure 3A](#)). The 8q24 amplicon is gained in 63.4% of TCGA HGSC samples and...
amplified in 23.7% of cancers (7, 23), however, amplification was much more common in BRCA1-mutant compared with wild type cancers (p<0.0001). Amplification of 8q24 is the most common copy number variant in HGSC (7) and the MYC proto-oncogene is a putative driver of the amplification.

We wanted to exclude the possibility that the increase in the frequency of 8q24 amplification simply reflected a general increase in DNA copy number in BRCA1-associated tumors. We therefore examined amplification on chromosome 3, involving another commonly gained region at 3q26. The 3q26 amplicon is gained in 64.9% of TCGA samples, and amplified in 17.2%. Unlike the 8q24 and X-chromosome loci, there was no significant difference in the level of amplification at 3q26 in BRCA1 mutated HGSC when compared to BRCA2 mutated or wild type HGSC (Figure 3A). As a further control, the BRCA1-mutated tumors were stratified by their MYC status, and the extent of overall genomic alterations compared. There was no significant relationship between the degree of 8q24 gain/amplification and the extent of overall genomic alteration in individual samples (Supplementary Figure 14). The control data confirm that aberrations at 8q24 and the X-chromosome are specifically enriched in tumors associated with BRCA1 mutations. We have previously shown that MYCN amplification is specifically associated with the C5 molecular subtype of HGSC (21) but unlike MYC, we found no evidence of an association between MYCN copy number and BRCA1/2 status (data not shown).

Amplification of the 8q24 locus has broad and complex implications, and in addition to involving MYC it frequently involves genetic risk loci associated with colorectal and breast cancer (24). For example, the non-coding RNA, Pvt-1, which is adjacent to MYC also appears to contribute to the oncogenic effects of amplification at 8q24 (25). We plotted the strength of association of copy
number variation at 8q24 with BRCA1 mutation status, finding that the strongest association lay telomeric to MYC, adjacent to the PTK2 gene (Figure 3B).

To independently validate the association of the 8q24 amplification with BRCA1 mutation status we performed quantitative PCR analysis of DNA derived from AOCS tumors, using three genes (MYC, PCRYL, PTK2) that collectively spanned the region of 8q24 that was most closely associated with BRCA1 mutation. Germline/somatic BRCA mutation, and BRCA1 promoter methylation were each associated with amplification of 8q24 in the AOCS dataset, with the strongest association seen with MYC amplification in both BRCA1 methylated and mutated samples (Figure 4A). Interestingly, we also observed a differential association between amplification of 8q24, and germline versus somatically BRCA2 mutated samples, although the number of samples available for this subset analysis was limited (Figure 4A). Finally we considered whether MYC status influenced clinical outcome in carriers, who generally have a more favourable overall survival compared to non-carriers (8). In a pooled analysis of data from TCGA and AOCS there was no evidence that MYC amplification affected survival in BRCA1-mutated tumors (Figure 4B and C; n=52).

**Interaction between BRCA1/2 mutation and regions in chromosome 19 and the X chromosome.**

Amplifications at 19q12 and on the X chromosome were also significantly differentially altered in BRCA-mutated tumors. As reported previously (7), amplification of CCNE1 at 19q12 was mutually exclusive to BRCA-pathway disruption (Figure 3A) and this extended to 19q13, which is partially co-amplified with 19q12 (26). In addition, amplification of several regions on the X chromosome (Xq21, Xq25, Xq26, Xq27 and Xq28) were also associated with BRCA1 mutation, and genes associated with these regions were enriched among the list of 34 genes we had found to be BRCA1-specific (Supplementary Table 9).
Amplification at Xq28 appears to be even more specific to BRCA1 mutation than the association with 8q24 (Figure 3A). Uniparental X-chromosomal isodisomy, gain of Xq28, and over expression of a subset of X chromosome genes has been reported previously in basal-like breast cancers, which are associated with BRCA1 germline mutations (27). Similar to the findings in basal-like cancers, we found no evidence of a global change in expression of X chromosome genes in BRCA1 or BRCA2 mutant HGSC (Supplementary Figure 15). We did observe however, that a subset of X-chromosome genes that were previously identified as over expressed in basal-like breast cancers (27) were also significantly over expressed in BRCA1-mutation associated HGSC (Supplementary Table 19).

Discussion:

Consistent with previous reports, we observed somatic or germline mutations in the BRCA1 and BRCA2 genes associated with a large proportion of HGSC tumors, and these were almost completely mutually exclusive. Mutual exclusivity may reflect a functional equivalence of the mutations, in which there is no selective advantage to a tumor cell by possessing more than one defect in the BRCA pathway. Sensitivity to platinum-based therapy in the primary (28) and relapse setting (8), as well as significant responses to poly(ADP-ribose) polymerase (PARP) inhibitors (29) are all consistent with the notion of a shared BRCAness phenotype of tumors arising in BRCA1/2 carriers (30). However, recent evidence points to important clinical and pathological differences in the behaviour of tumors arising in women with BRCA1 compared to BRCA2 mutations.

Although both genes encode proteins that participate in the HRR pathway, BRCA1 has both an earlier and wider role in DNA damage response (31-33) and additional cellular functions, including cell-cycle regulation (32). BRCA1 loss may therefore have more extensive molecular and clinical consequences when compared to a BRCA2 mutation. Indeed, germline BRCA1 mutation confers a
higher risk of developing ovarian cancer than germline BRCA mutation (34) and on average, ovarian tumors arise a decade earlier in BRCA1 carriers compared with those in women with BRCA2 mutations or with wild-type BRCA1/2 genes (8). Women with either BRCA1 and BRCA2 germline mutations generally have a better response to therapy and a longer overall survival compared to non-carrier ovarian cancer patients, and some have reported that those with a BRCA2 mutation survive longer than BRCA1 carriers despite usually being older at diagnosis (35).

Differences are also observed between BRCA1 and BRCA2-associated breast cancer. Distinct pathological features, including high rates of mitosis and pushing margins, are seen in BRCA1-associated breast cancers (36), whereas tumors arising in BRCA2 carriers more closely resemble those of non-carriers. In addition, germline mutations in BRCA1 are strongly associated with basal-like, estrogen-receptor negative breast cancers, whereas both ER-positive and ER-negative tumors are seen in BRCA2 carriers. Recently, necrosis, high mitotic counts, prominent intraepithelial lymphocytes and nuclear atypia have been specifically associated with BRCA1 rather than BRCA2 mutation in high grade serous cancers (37) (38). Our finding of a strong association of BRCA1 inactivation with the C2 molecular subtype of HGSC is consistent with these reports, as this subtype is characterised by intense intraepithelial T-cell infiltration (2). Not all C2 tumors in our set had detectable inactivation of BRCA1, and it is possible that other mechanisms of BRCA1/HRR deficiency are operative in these tumors. It is unclear whether BRCA1-associated tumors tend to be more strongly immunogenic and/or are less capable of suppressing a cytotoxic immune response, however, these findings suggest that knowledge of BRCA mutation status should be considered in the design of future immunotherapy trials in HGSC.

BRCA1- and BRCA2-associated gene expression signatures have been reported previously (11, 12), yet we were unable to validate these signatures in independent datasets. Furthermore, the previous
observation that \textit{BRCA1} and \textit{BRCA2} tumors have distinct patterns of expression, and that wild type tumors resembled one or the other profile, was not supported by our study. By using a more homogenous tumor cohort, well-annotated for \textit{BRCA} status, we found that \textit{BRCA1}-mutated tumors are the outlier in gene expression, with \textit{BRCA2} and wild type tumors being more closely related. Interestingly, both the signature and the association with C2 molecular subtype were also observed in \textit{BRCA1} methylated cancers, even though patients with methylated \textit{BRCA1} alleles apparently do not share the same survival advantage of HGSC patients with germline \textit{BRCA1} mutations (7). The similar molecular phenotype of germline and methylated \textit{BRCA1} HGSC is consistent with recent results in breast cancer (39). A number of genes associated with the \textit{BRCA1} gene expression signature in HGSC are associated with DNA damage and/or \textit{BRCA1}, including \textit{BMI1} and \textit{CDKN1C} (40, 41), and \textit{HSF1}, possibly reflecting genomic stress and altered multiprotein complex stoichiometry (42). The tumor suppressor \textit{CDKN1C} has previously been identified in a gene expression signature found in the fallopian tubes of \textit{BRCA1} mutation carriers with pre-neoplastic lesions (41). In conjunction with \textit{BRCA}-loss being an initiating event in the development of HGSC, the down regulation of \textit{CDKN1C} may also be important for tumorigenesis in mutation carriers.

A gene-expression signature that can help identify mutation carriers could be clinically useful in several ways. Patients with germline \textit{BRCA1/2} mutations have high response rates to PARP inhibitors but responses are also seen in non-carriers; therefore identifying biomarkers of HGSC patients who are likely to respond to therapy, or have a \textit{BRCA}ness phenotype, is a high priority. A signature that can interrogate the overall activity of the \textit{BRCA} pathway, rather than the need to perform a series of gene-specific tests, would be desirable. At present this signature is not sufficiently reliable to be used as a surrogate for genetic testing of probands, however, the classifier may complement other tools for assessing the likely pathogenicity of \textit{BRCA} sequence variants of unclassified significance uncovered during diagnostic \textit{BRCA} testing. Reversion of germline \textit{BRCA1}
and BRCA2 alleles and partial restoration of HRR following platinum treatment is associated with resistance to platinum-based therapies and PARP inhibitors (12, 43, 44). It is important to know whether reversion of BRCA1 alleles is associated with a gene expression signature more typical of wild type tumors.

Gene set enrichment analysis demonstrated that the BRCA1-specific gene signature was substantially driven by chromosomal aberrations at 8q24 and on the X chromosome, and these regions were also identified by a supervised analysis of copy number data. The strongest association between 8q24 amplification and BRCA1 mutation localised to PTK2 within the TCGA dataset, however, this shifted to MYC in validation studies with the AOCS dataset. Functional studies are needed to further refine the contribution of one or more genes in the 8q24 locus to an interaction with BRCA1 mutation.

We have previously noted a relationship between HGSC and basal-like breast cancer (BLBC) with both tumor types sharing a propensity for widespread chromosomal copy number change, almost ubiquitous TP53 mutation, frequent disruption of the BRCA pathway, MYC gain, and CCNE1 amplification together with RB loss (45). Our findings suggest that MYC amplification is particularly a feature of BRCA1 mutated or BRCA1-methylated HGSC and is less common in BRCA2 germline mutant or non-mutant HGSC. Genomic amplification of MYC has previously been linked to BRCA1-mutated and BRCA1-methylated breast tumors (46) and BLBC (45). The significance of a special relationship between BRCA1 protein loss and MYC amplification is unclear, however, we note that these proteins have been shown to physically interact, with BRCA1 repressing MYC-mediated transcription (47). In the absence of BRCA1 function, cells may enjoy a selective advantage from MYC amplification. Although we did not observe a difference in survival...
of BRCA1 carriers with or without amplification of the MYC locus, this analysis made use of a limited number of tumor samples and is worthy of further consideration.

We also identified specific chromosomal aberrations and over expression of a subset of X chromosome genes in HGSC that were previously identified in BLBC (27), providing a further parallel between these tumor types and implying a common molecular relationship between specific X chromosome loci and BRCA1 (48-50).

Although epithelial ovarian cancer is still largely treated as a single entity, molecular and pathological studies of the last decade have underscored the diverse nature of the disease (2, 4-6, 37). Here we provide additional evidence of this molecular diversification, segregating even those tumors that share a common pathway deficiency. Understanding the molecular changes associated with tumors arising in distinct genetic backgrounds will help provide an integrated picture of their circuitry and thereby offer novel approaches to therapeutic intervention.
Acknowledgements: This study was approved by the Human Research Ethics Committees at the Peter MacCallum Cancer Centre. AOCS was approved by the Human Research Ethics Committees at the Peter MacCallum Cancer Centre, Queensland Institute of Medical Research, University of Melbourne, New South Wales Cancer Council, The Cancer Council South Australia, The Cancer Foundation of Western Australia and all participating hospitals.

We gratefully acknowledge the cooperation of the participating institutions in Australia and worldwide, and also acknowledge the contribution of the study nurses, research assistants and all clinical and scientific collaborators to both the AOCS and TCGA studies. The complete AOCS Study Group can be found at www.aocstudy.org. Information regarding the TCGA can be found at http://cancergenome.nih.gov/. We would like to thank all of the women who participated in these research programs.
References


Tables:

Table 1: Pathogenic mutations identified (germline or somatic) in the 132 AOCS serous ovarian cases included in this analysis. Germline pathogenic mutations identified using Sanger sequencing of peripheral blood DNA (8). Somatic mutations identified using a high-resolution melt analysis (Supplementary Methods).

<table>
<thead>
<tr>
<th>AOCS Study ID</th>
<th>Gene</th>
<th>Exon</th>
<th>Nomenclature</th>
<th>Location</th>
<th>Molecular Subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>6399</td>
<td>BRCA1</td>
<td>2</td>
<td>c.68_69delAG</td>
<td>Germline 3</td>
<td></td>
</tr>
<tr>
<td>6708</td>
<td>BRCA1</td>
<td>2</td>
<td>c.68_69delAG</td>
<td>Germline 2</td>
<td></td>
</tr>
<tr>
<td>1880</td>
<td>BRCA1</td>
<td>3</td>
<td>c.131G&gt;T</td>
<td>Germline 4</td>
<td></td>
</tr>
<tr>
<td>8515</td>
<td>BRCA1</td>
<td>11</td>
<td>c.1961delA</td>
<td>Germline 2</td>
<td></td>
</tr>
<tr>
<td>2595</td>
<td>BRCA1</td>
<td>11</td>
<td>c.1961delA</td>
<td>Germline 1</td>
<td></td>
</tr>
<tr>
<td>1563</td>
<td>BRCA1</td>
<td>11</td>
<td>c.2504_2505delInsAAGTATCCATTGGACA</td>
<td>Germline 4</td>
<td></td>
</tr>
<tr>
<td>4112</td>
<td>BRCA1</td>
<td>11</td>
<td>c.2716A&gt;T</td>
<td>Germline 1</td>
<td></td>
</tr>
<tr>
<td>2846</td>
<td>BRCA1</td>
<td>11</td>
<td>c.2836_2837delInsC</td>
<td>Germline 2</td>
<td></td>
</tr>
<tr>
<td>4065</td>
<td>BRCA1</td>
<td>11</td>
<td>c.3627_3682delInsA</td>
<td>Germline 1</td>
<td></td>
</tr>
<tr>
<td>5874</td>
<td>BRCA1</td>
<td>11</td>
<td>c.3756_3759delGTCT</td>
<td>Germline 5</td>
<td></td>
</tr>
<tr>
<td>3158</td>
<td>BRCA1</td>
<td>11</td>
<td>c.3756_3759delGTCT</td>
<td>Germline 4</td>
<td></td>
</tr>
<tr>
<td>4947</td>
<td>BRCA1</td>
<td>11</td>
<td>c.4065-4068delTCAAG</td>
<td>Germline 2</td>
<td></td>
</tr>
<tr>
<td>4194</td>
<td>BRCA1</td>
<td>11</td>
<td>c.4066_4069delCAAG</td>
<td>Germline 4</td>
<td></td>
</tr>
<tr>
<td>2039</td>
<td>BRCA1</td>
<td>14</td>
<td>c.4484G&gt;A</td>
<td>Germline 1</td>
<td></td>
</tr>
<tr>
<td>4956</td>
<td>BRCA1</td>
<td>11</td>
<td>c.3645delG</td>
<td>Somatic 2</td>
<td></td>
</tr>
<tr>
<td>435</td>
<td>BRCA1</td>
<td>11</td>
<td>c.3762delGAACA</td>
<td>Somatic 1</td>
<td></td>
</tr>
<tr>
<td>9046</td>
<td>BRCA1</td>
<td>11</td>
<td>c.3827T&gt;A</td>
<td>Somatic n/a</td>
<td></td>
</tr>
<tr>
<td>2378</td>
<td>BRCA1</td>
<td>22</td>
<td>c.5380G&gt;T</td>
<td>Somatic 4</td>
<td></td>
</tr>
<tr>
<td>958</td>
<td>BRCA2</td>
<td>11</td>
<td>c.2456delInsA</td>
<td>Germline 1</td>
<td></td>
</tr>
<tr>
<td>3202</td>
<td>BRCA2</td>
<td>11</td>
<td>c.3136G&gt;T</td>
<td>Germline n/a</td>
<td></td>
</tr>
<tr>
<td>5129</td>
<td>BRCA2</td>
<td>11</td>
<td>c.4163delCTinsA</td>
<td>Germline 5</td>
<td></td>
</tr>
<tr>
<td>1251</td>
<td>BRCA2</td>
<td>11</td>
<td>c.5350_5351delAA</td>
<td>Germline 4</td>
<td></td>
</tr>
<tr>
<td>6867</td>
<td>BRCA2</td>
<td>11</td>
<td>c.5576_5579delTTAA</td>
<td>Germline 4</td>
<td></td>
</tr>
<tr>
<td>3142</td>
<td>BRCA2</td>
<td>11</td>
<td>c.5946deldelT</td>
<td>Germline 1</td>
<td></td>
</tr>
<tr>
<td>9540</td>
<td>BRCA2</td>
<td>16</td>
<td>c.7757G&gt;A</td>
<td>Germline 5</td>
<td></td>
</tr>
<tr>
<td>1819</td>
<td>BRCA2</td>
<td>6</td>
<td>c.476-1G&gt;A</td>
<td>Somatic 1</td>
<td></td>
</tr>
<tr>
<td>8794</td>
<td>BRCA2</td>
<td>11</td>
<td>c.4357A&gt;T</td>
<td>Somatic 1</td>
<td></td>
</tr>
<tr>
<td>1603</td>
<td>BRCA2</td>
<td>11</td>
<td>c.4540G&gt;T</td>
<td>Somatic 1</td>
<td></td>
</tr>
<tr>
<td>1511</td>
<td>BRCA2</td>
<td>11</td>
<td>c.4945A&gt;T</td>
<td>Somatic 1</td>
<td></td>
</tr>
</tbody>
</table>
Figure Legends:

**Figure 1:** A Disruption of the *BRCA*-pathway in high grade serous cancers in 50/132 (37.9%) in the AOCS dataset. Pathogenicity of sequence variants was assessed by use of the Alamut program (Interactive Biosoftware) as described in (8). With the exception of two cases with both *BRCA2* germline mutation and *BRCA1* methylation, the various mutations were mutually exclusive. Molecular subtype of serous and endometrioid ovarian cancers (2): C1 (red), C2 (green), C3 (yellow), C4 (aqua), C5 (orange), C6 (pink), N/A (white). **B** Association plot depicting the relationship between *BRCA1* or *BRCA2* dysfunction with the four molecular subtypes of HGSC (C1, C2, C4, C5). *BRCA1* tumors (mutated and methylated) were highly significantly associated with the C2 (immunoreactive) molecular subtype in the TCGA dataset (p=0.0002; two-sided likelihood ratio test. N=210 HGSC; 37 *BRCA1*=27 mutated, 10 methylated, 28 *BRCA2*, 145 wild type). Observed counts greater or less than expected are depicted as boxes above or below the baseline. The area of each box is proportional to the difference between the observed and expected count and the height represents the standardized residual as shown of the side-bar (see [Supplementary Methods](#) and [Supplementary Table 6](#)). **C** *BRCA1* tumors (mutated and methylated) were also associated with the C2 molecular subtype in the AOCS dataset (p=0.017, two-sided likelihood ratio test, N=111 HGSC (ie, excluding 21 cases that did not cluster into any of the subtypes); 33 *BRCA1*=16 mutated, 17 methylated, 10 *BRCA2*, 68 wild type, [Supplementary Table 7](#)).

**Figure 2:** Gene expression signatures associated with *BRCA1/2* mutation status **A.** Validation of a 65 gene classifier developed using TCGA data and tested using AOCS samples. Classifier scores for wild types samples were significantly different to those with *BRCA4*-pathway inactivation (*BRCA*=germline/somatic *BRCA* mutation; *BRCA1* promoter methylation; non-*BRCA*= wild-type).
B. Scores obtained with the 65-gene *BRCA*-classifier and samples segregated by mutation type. C. Distribution of scores obtained with a *BRCA1*-classifier, based on 34 genes identified as differentially expressed between *BRCA1*-mutated and wild-type tumors in the TCGA dataset, and applied to AOCS tumors. D. Scores obtained with the 34-gene *BRCA1*-classifier and applied to AOCS samples, segregated by mutation type. p values reported in each case obtained with Student’s t-test.

**Figure 3:** A. Amplifications of genomic loci chr8q24, chr19q12 and chrXq28 are significantly associated with *BRCA* mutation status. Significantly differentially amplified genomic regions were identified by comparing the proportion of samples amplified in *BRCA1* mutated, *BRCA2* mutated and wild type samples using Fisher’s exact test. The p-values were adjusted to correct for false discovery rate. The 3q26 locus is gained in 64.9% of TCGA samples, and amplified in 17.2%. The 8q24 amplicon is gained in 63.4% of TCGA samples, and amplified in 23.75%. Amplifications in chromosomal locus 8q24 and Xq28 are significantly higher in *BRCA1* mutated tumors, whereas amplification of chr19q12 is more frequently observed in wild type tumors. The proportion of samples with amplification at 3q26 is shown as a control. This region is frequently gained in high-grade serous cancer but there was no difference in the amplification status of this locus with *BRCA* mutation status. B. Statistical significance of difference in the proportion of samples amplified at the 8q24 locus. Negative log2 transformed false discovery rate is plotted for all the three pair-wise comparisons. Selected genes mapping to this region are shown at the actual genomic location along the x-axis. A full list of genes found in the amplicon can be found in **Supplementary Table 16**.

**Figure 4:** A. Validation of the 8q24 amplification by q-PCR in the AOCS dataset. All three genes validated (*MYC*, *PTK2* and *PYCRL*) are found in the identified 8q24 amplicon. *MYC* copy number is higher in *BRCA1*-mutated, *BRCA1*-methylated and *BRCA2* somatically-mutated tumors when
compared to wild-type (two-tailed t-test; pvalues *** = <0.0001 – 0.001; ** = 0.001-0.01; * = 0.01-0.05). Expression levels of PTK2 and PYCRL are not significantly higher in BRCA1 somatically mutated or BRCA2 germline mutated tumors when compared to wild-type. B. Progression-free survival time (time from diagnosis to first relapse or last follow-up) and C. overall survival (time from diagnosis to death or last follow-up) in the BRCA1-mutated tumors, stratified by MYC amplification status. Patients whose tumors had both a BRCA1 mutation and MYC amplification did not have a shorter disease free interval (p=0.764, log-rank test) or overall survival (p=0.493, log-rank test). Analysis is from AOCS and TCGA datasets combined.
1A

HGSC Molecular Subtype (Germline)
HGSC Molecular Subtype (Somatic)
HGSC Molecular Subtype (HGSOM cohort)
HGSC Molecular Subtype (AOCS cohort)
HGSC Molecular Subtype (TCGA cohort)
HGSC Molecular Subtype (FANCF cohort)
HGSC Molecular Subtype (PALE2 cohort)
HGSC Molecular Subtype (Germline Unclassified Variant)

1B

HGSC Molecular Subtype (TCGA cohort)

p = 0.0002

BRC1
BRC2
WT

C1 C2 C4 C5

1C

HGSC Molecular Subtype (AOCS cohort)

p = 0.017

BRC1
BRC2
WT

C1 C2 C4 C5
A

B
A

MYC

PTK2

PYCRL

B

C

Log rank test p-value = 0.764

Log rank test p-value = 0.493
Non-equivalent gene expression and copy number alterations in high-grade serous ovarian cancers with BRCA1 and BRCA2 mutations

Joshy George, Kathryn Alsop, Dariush Etemadmoghadam, et al.

Clin Cancer Res  Published OnlineFirst April 30, 2013.

Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-13-0066

Supplementary Material  Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2013/04/30/1078-0432.CCR-13-0066.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.