Research Article

Molecular Profiling and Clinical Outcome of High-Grade Serous Ovarian Cancer Presenting with Low- versus High-Volume Ascites

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Epithelial ovarian cancer consists of multiple histotypes differing in etiology and clinical course. The most prevalent histotype is high-grade serous ovarian cancer (HGSOC), which often presents at an advanced stage frequently accompanied with high-volume ascites. While some studies suggest that ascites is associated with poor clinical outcome, most reports have not differentiated between histological subtypes or tumor grade. We compared genome-wide gene expression profiles from a discovery cohort of ten patients diagnosed with stages III-IV HGSOC with high-volume ascites and nine patients with low-volume ascites. An upregulation of immune response genes was detected in tumors from patients presenting with low-volume ascites relative to those with high-volume ascites. Immunohistochemical studies performed on tissue microarrays confirmed higher expression of proteins encoded by immune response genes and increased tumor infiltrating cells in tumors associated with low-volume ascites. Comparison of 149 advanced-stage HGSOC cases with differential ascites volume at time of primary surgery indicated low-volume ascites correlated with better surgical outcome and longer overall survival. These findings suggest that advanced stage HGSOC presenting with low-volume ascites reflects a unique subgroup of HGSOC, which is associated with upregulation of immune related genes, more abundant tumor infiltrating cells and better clinical outcomes.

1. Introduction

Epithelial ovarian cancer (EOC) is the leading cause of gynecologic cancer-related death in developed countries, with nearly a quarter million women diagnosed worldwide annually [1]. Of the various EOC histotypes, which have distinct precursor lesions, genomic profiles, and clinical course [2, 3], high-grade serous ovarian cancer (HGSOC) accounts for the majority of cases and a disproportionate number of
deaths. Adding to the complexity, recent large-scale gene expression studies identified at least four molecular subtypes within HGSOC [4, 5], with some evidence associating these subtypes with differences in overall patient survival [4, 6].

Ovarian cancer is typically diagnosed at an advanced stage, with high-volume ascites a common presenting feature [7]. While some studies have indicated better prognosis in cases presenting with no ascites, most reports to date have grouped all histological subtypes and tumor grades together. Such an approach makes it difficult to assess if differences in ascites volume are an independent predictor of survival and better surgical outcome. The association of ascites with poor outcome in EOC could reflect the fact that HGSOC is an aggressive cancer that tends to present with high-volume ascites and carries poor prognosis compared to other histological subtypes and low-grade disease [8–11]. For this reason, we focused on a homogeneous group of patients diagnosed exclusively with HGSOC, to assess the significance of differences in ascites volume at the time of diagnosis. Although ascites often resolves early in therapy, reaccumulation occurs frequently and becomes a significant quality of life issue, particularly with chemoresistance and disease progression. Shortness of breath, abdominal bloating, pain, nausea, and early satiety caused by ascites contribute to cachexia with eventual compromise of the patient’s mobility and often with respiratory distress and bowel obstruction [12]. While the pathogenesis of malignant ascites is incompletely understood, increased vascular permeability and tumor neovascularization due to high concentrations of vascular endothelial growth factor (VEGF) and decreased rates of lymphatic drainage are considered critical [13–17]. Despite its clinical importance, ascites volume has not been captured as a parameter in molecular profiling studies.

In the present study, we focused on the impact of differences in ascites volume on patients diagnosed with advanced stage HGSOC. We compared gene expression profiles in a discovery cohort of these tumors. Our analysis revealed a unique subset of immune-related genes upregulated in the low-volume ascites group. Immunohistochemistry performed on a larger cohort of primary tumors validated these results and showed increased number of tumor infiltrating immune cells in the low-volume ascites group. This group also had better surgical outcome, defined as optimal (<1.0 cm residual tumor) versus suboptimal cytoreduction, and overall survival that was consistent with a stronger tumor immune response seen in those patients.

2. Material and Methods

2.1. Whole Genome Transcriptome Profiling. A discovery cohort of snap-frozen, stage IIIC primary HGSOC specimens from 12 patients presenting with low- (≤200 cc) or high-volume (>1000 cc) ascites was obtained from the University Health Network Biobank. A gynecology pathologist (BC) reviewed each specimen to confirm the diagnosis and ensure presence of more than 70% epithelial tumor cells. The University Health Network Research Ethics Board approved this study and all patients consented to the use of their tissue and clinical data for research.

RNA was extracted from tumor tissue using an RNeasy Mini Kit (Qiagen). Quality and quantity of RNA as well as cDNA were confirmed prior to hybridization to Illumina HumanHT-12 v4r2 BeadChip microarrays. Only samples passing quality control metrics in the Illumina BeadStudio and R (version 2.14.1; Lumi Bioconductor package) software programs were included in the final analysis (9 of the 12 low-volume and 10 of the 12 high-volume ascites). Array data were converted to logs, quantile, and median-normalized and analyzed for differential expression between groups using GeneSpring (v12.1, Agilent). Unsupervised hierarchical clustering using average linkage rules and a Pearson centered distance metric was performed to assess the overall degree of gene expression similarity among samples [18]. All probes were filtered prior to analysis to remove those showing little or no signal in either sample group. Only probes reacting with at least 80% of samples in either group, with expression in the 20–100th percentile of measured signal values, were retained. A moderated student’s t-test [19] without multiple testing corrections was used to identify probes whose mean expression was different between low- and high-volume ascites samples. A Westfall and Young Family Wise Error Rate (FWER) multiple testing correction was also applied to the moderated t-test. All probes found significant were ranked by fold change. Gene ontology (GO) analysis using a hypergeometric test with a false discovery rate (FDR) cutoff of q < 0.2 was used to find significantly altered categories. For gene set enrichment analysis (GSEA), version 3.1 of mSigDB was used with a cutoff FDR of q < 0.1 [20] using all unfiltered probes on the array. Gene expression array data have been deposited in the gene expression omnibus repository, accession number GSE51831.

2.2. Validation by Immunohistochemistry. A tissue microarray was constructed using a semiautomated TMArrayer (Pathology Devices, Inc.) from archived formalin-fixed paraffin-embedded tumors from a total of 54 patients with stages III–IV HGSOC presenting with low- or high-volume ascites, including the 24 patients initially considered for the discovery cohort plus an additional 30 patients identified in the ovarian cancer database at Princess Margaret Cancer Center, Toronto, ON. Only patients with sufficient archived primary and metastatic tissue were included. Two cores of each tissue were selected. Three cores were excluded prior to analysis due to poor quality; hence a total of 51 cores were included in the final analysis. Immunohistochemical studies were performed for detection of proteins encoded by genes highly expressed in the low-volume ascites group. Sections (4 μm thick) of the tissue microarray were deparaffinized and antigen retrieval or unmasking procedures were applied, if necessary, by heating the sections in 10 mM citrate buffer at pH 6.0 or Tris-EDTA buffer at pH 9.0 using a microwaveable pressure cooker. Endogenous peroxidase was blocked with 3% hydrogen peroxide. After blocking with appropriate serum, sections were incubated with primary antibody using previously optimized conditions. Primary antibodies included anti–CD3 (1:500, cat. A0452, DAKO), anti–CD8 (1: 200, cat. NCLCD8-4B11, Vector Labs), anti–CD20 (1: 300, cat. M0755, DAKO), anti–CD74 (1: 500, cat. Ab9514, Abcam),
anti-CD48 (1:7500, cat. 962-M01, Novus Biological), anti-
TAP2 (1:1000, cat. HPA001312, ATLAS), and anti-HLA-DR
(1:7500, cat. NB120-17844, Novus Biological). A Super Sensi-
tive Polymer-HRP Kit (BioGenex QD440-XAK) was used to
amplify primary antibody staining. Immunostaining was
visualized using freshly prepared dianaminobenzidine solution
(DAKO). Sections were lightly counterstained with Mayer's
hematoxylin.

Tissue cores stained for HLA-DR, CD74, CD48, and
TAP2 were scored as zero (no staining of tumor), one (intermediate staining), or two (strong staining in ≥50% of
tumor) by a pathologist blinded to patient clinical infor-
mation. The tissue microarray was also stained for markers of
infiltrating T (CD3, CD8) and B cells (CD20). For each
patient, one area of primary tumor and one area of metastasis
were selected after scanning all cores for tumor epithelium
mostly enriched with tumor infiltrating leukocytes (TILs),
in accordance with a previously described protocol [21].
The number of TILs was counted in one representative
high power field up to a maximum of 50 TILs per high
power field. Staining intensity scores for tumor antigens
were described using frequencies and proportions and compared
between patients with low- and high-volume cancers using the
Wilcoxon rank-sum test and between primary tumor and
metastases using the Wilcoxon signed-rank test, with each
sample acting as its own control. Immune cell scores
were compared between the two patient groups using Wilcoxon
rank-sum test. Generalized estimating equations were used
to determine ordinal and logistic regression parameters while
adjusting for repeated measurements on patients to compare
those with low- and high-volume ascites on pooled tumor
staining scores and on pooled numbers of immune cells in the
epithelium. Statistical significance was set to \( P < 0.05 \).
All analyses were implemented using SAS software, version
9.3 TS level 1 M1.

2.3. Patient Outcome Assessment. A search of the Princess
Margaret Cancer Center Ovarian Cancer Database identified
240 stages III-IV HGSOC cases that underwent up-front
cytoreductive surgery between January 2003 and August 2011.
Clinical data extracted from real-time synoptic operative
reports and/or electronic medical records for these patients
included age at diagnosis, FIGO stage, ascites volume at
time of surgery, surgical outcome, and date of death. Where
applicable, date of death or survival duration as of April 2012
was validated using the Ontario Cancer Registry. Only those
patients with a clear indication of ascites volume ≥1000 cc
(high-volume) or ≤200 cc (low-volume) were included in
further analysis (\( n = 149 \)). Continuous variables were com-
pared between patients with high- and low-volume ascites
using the Wilcoxon rank-sum test. Categorical variables were
compared using Fisher's exact test. Overall survival was
measured from the date of surgery to the date of death from
any cause. Patients alive at last followup were censored.
The log-rank test was used to compare outcomes for patients
with high- and low-volume ascites, and a Cox proportional
hazards regression model was used to compare the two
groups while adjusting for stage. Kaplan-Meier plots were
generated to estimate one-, three-, and five-year survival
probabilities. Statistical significance was set to \( P < 0.05 \).

3. Results

3.1. Gene Expression Profiling Reveals a Distinct Signature
for Tumors Associated with Low- versus High-Volume Ascites.
Nine samples with low-volume ascites and ten samples with
high-volume ascites met the requirements for RNA and
cDNA quality and constituted the discovery cohort. Median
age for patients with low- and high-volume ascites was 68
(range 44–84) and 58.5 years (range 46–85), respectively (\( P =
0.57 \)). All patients were diagnosed with stage IIIC disease.
Unsupervised two-way hierarchical cluster analysis of both
the entire set of the filtered array probes (35433 probes)
and a subset of probes (371 probes) that showed the most
overall variability (overall standard deviation >1.0) did not
segment the samples according to ascites volume (data not
shown). An uncorrected moderated \( t \)-test found 198 probes
statistically different between ascites volume groups using
\( P < 0.05 \) and a minimum fold change of 1.5. Of these, 103
probes representing 98 unique genes were upregulated in the
low-volume ascites tumors and 95 probes representing 84
unique genes were upregulated in the high-volume ascites
tumors (see Supplemental Table S1 in Supplementary Mate-
rial available online at http://dx.doi.org/10.1155/2014/367103).
A clustering of samples based upon these 198 probes is shown
in Figure 1. Fifteen probes using a Westfall and Young FWER
corrected moderated \( t \)-test were found to be significant using an
\( P < 0.1 \) threshold and a minimum fold change of 1.5. These
overlapped entirely with the 198 probes found with the
uncorrected moderated \( t \)-test (Supplemental Table S1).

Using GO analysis, an enrichment of GO terms such as
antigen processing and presentation, MHC protein complex
(particularly MHC II), and cytokine activity was found in the
low-volume ascites group (Table I). Not surprisingly, the
9 (out of 15) probes found upregulated in the low-
volume ascites using the Westfall and Young corrected results
were also enriched for immune related categories (data not
shown). This enrichment of immune-related categories in the
low-volume ascites group was further confirmed with GSEA testing (Supplemental Data Table S2), which uses \( a \)
priori defined gene sets to find statistically significant (FDR-
corrected) changes between two defined groups of samples.
In high-volume ascites cases, GO analysis revealed genes
associated with extracellular matrix (Supplemental Table S3).

3.2. Increased Immune Response in Tumors from HGSOC
Patients with Low-Volume Ascites. CD74, HLA-DR, and
TAP2 were expressed at significantly higher levels in the
epithelium of cancer cells derived from tumors of patients
with low- versus high-volume ascites (Figure 2; \( P = 0.046,
P = 0.006, \) and \( P = 0.002 \), resp.), consistent with our
microarray data. CD48 staining did not show the differential
expression suggested by the RNA data. Staining intensity
for the four antibodies was similar between the primary
tumors and the corresponding metastatic lesions. While
tumor infiltrating T lymphocytes were more abundant than
B cells in the tumor epithelium, infiltrating T and B cells
High-volume ascites

Low-volume ascites

Figure 1: Gene expression profiling of a discovery cohort reveals an immune gene signature for HGSOC tumors presenting with low-volume ascites. Hierarchical clustering of patient samples based on 198 probes differentially expressed by ≥1.5-fold as determined by a moderated t-test ($P < 0.05$) in tumors associated with high- and low-volume ascites. Each line of the cluster tree shown at the top represents one patient sample. The ascites volume group is indicated by the bar at the bottom and by the line color. Magenta bars on the side indicate probes corresponding to the 20 genes that overlap with genes within the TCGA immunoreactive group and are upregulated in the low-volume ascites group.

were both more common in the tumor epithelium of the low-volume ascites group based on staining for CD20, CD8, and CD3 ($P = 0.02, P = 0.001,$ and $P = 0.01$) (Table 2). The number of TILs did not differ between primary tumors and corresponding metastatic lesions.

3.3. Low-Volume Ascites Is Associated with Better Surgical Outcome and Survival. Overall 149 patients were included in the clinical data and outcome study: 65 with low-volume ascites and 84 with high-volume ascites. Mean age at diagnosis was similar and over 70% of patients were stage IIIc at the time of diagnosis in the two groups (Figure 3). While a higher percentage of patients with low- as compared to high-volume ascites had undergone aggressive surgery (defined as at least one of the following: diaphragmatic stripping, peritoneal resection, bowel resection, or splenectomy) to achieve optimal surgical outcome, this difference was not statistically significant (73.8% versus 64.6%; $P = 0.28$). However, the clinical course of disease differed between the two groups; the outcome of primary surgery was better for the low-volume ascites group with 63.1% of patients having no macroscopic evidence of disease at the end of surgery, compared with only 29.8% in the high-volume ascites group. Overall, only 13.9% of the low-volume ascites cases, as compared to 49.2% of the high-volume ascites cases, were suboptimally debulked ($P < 0.0001$). This difference in debulking success remained after adjusting for stage. Moreover, 75.4% of patients in the low-volume ascites group were alive at last followup (median = 27.3 months) versus 51.2% in the high-volume ascites group (median = 27.5 months). Since the 50th percentile survival had not yet been reached in the low-volume ascites group, we compared the 25th percentile for overall survival, which showed a value of 33.7 months versus 25 months for the low-versus high-volume ascites groups ($P = 0.009$) (Figure 3). After adjusting for stage, the high-volume ascites group was still associated with higher risk of death (hazard ratio = 2.1, 95% confidence interval: 1.18, 3.78, $P = 0.01$) (Figure 3).
### Table 1: Gene ontology (GO) categories enriched in the low-volume ascites group.

<table>
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<th>GO term</th>
<th>GO accession</th>
<th>P value</th>
<th>Corrected P value</th>
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4. Discussion

Our results demonstrate molecular differences between HGSOC associated with low-volume ascites compared to those with high-volume ascites. The most significant finding is an upregulation of immune response genes in the low-volume ascites group. The corresponding proteins are involved in immune response: HLA-DR, HLA-DM, and CD74, which play a crucial role in HLA class II antigen processing and presentation; HLA-A and HLA-F, MHC class I molecules, and their intracellular transport proteins TAP1 and TAP2, involved in stimulation of cytotoxic responses; chemokines such as CXCL9, CXCL10, and CXCL16 and cytokines such as CCL5, which participate in T-cell activation and chemotaxis. Consistent with the microarray transcript data, immunohistochemical staining confirmed increased expression of HLA-DR, CD74, and TAP2 proteins in the tumor epithelium of the low-volume ascites group. In support of these differences in immune response gene expression, our results demonstrate that low-volume ascites tumors are characterized by more abundant infiltrating immune cells. In addition, our data show that patients presenting with HGSOC and low-volume ascites are more likely to have successful cytoreductive surgery and to experience longer survival than those presenting with high-volume ascites, despite similar stage and grade at presentation.
It has long been established that EOC can stimulate host immune response and that the presence of infiltrating T cells, particularly CD8⁺ cytotoxic T lymphocytes (CTL), is associated with a better outcome [22–26]. In accordance with these studies, our results indicate better clinical outcome for the low-volume ascites group, whose tumors are characterized by more abundant tumor infiltrating cells. High expression of HLA-DM and HLA-DR by the tumor epithelium of

<table>
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<th>Low-volume ascites</th>
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<td><strong>FIGO stage</strong></td>
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<td>IIA</td>
<td>4 (4.8%)</td>
<td>6 (9.2%)</td>
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<td>IIIB</td>
<td>6 (7.1%)</td>
<td>4 (6.2%)</td>
<td>0.13</td>
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<tr>
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<td>60 (71.4%)</td>
<td>50 (76.9%)</td>
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<tr>
<td>IV</td>
<td>14 (16.7%)</td>
<td>5 (7.7%)</td>
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<td>62 (73.8%)</td>
<td>42 (64.6%)</td>
<td></td>
</tr>
<tr>
<td><strong>Outcome of primary surgery</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No macroscopic disease</td>
<td>25 (29.8%)</td>
<td>41 (63.1%)</td>
<td></td>
</tr>
<tr>
<td>Optimal debulking (≤1 cm)</td>
<td>23 (27.4%)</td>
<td>15 (23.1%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Suboptimal debulking (≥1 cm)</td>
<td>36 (42.9%)</td>
<td>9 (13.9%)</td>
<td></td>
</tr>
<tr>
<td><strong>Months of followup from surgery</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>27.5 (0.3–108.4)</td>
<td>27.3 (0.5–96.8)</td>
<td>n/a</td>
</tr>
<tr>
<td><strong>Status at last followup</strong></td>
<td>Alive</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>43 (51.2%)</td>
<td>49 (75.4%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dead</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>41 (48.8%)</td>
<td>16 (24.6%)</td>
<td></td>
</tr>
<tr>
<td><strong>Months from surgery to death</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25th percentile (95% conf. interval)</td>
<td>25.0 (17.1, 32.8)</td>
<td>33.7 (18.3, 68.1)</td>
<td>0.009</td>
</tr>
<tr>
<td>Median (95% conf. interval)</td>
<td>38.2 (34.0, 48.8)</td>
<td>Median not reached</td>
<td></td>
</tr>
</tbody>
</table>

*P* values shown in the table were determined by Fisher’s exact test for categorical variables, the Wilcoxon rank-sum test continuous and ordered variables, and the long-rank test for days to death. n/a: statistical test not applied. The predicted probability of overall survival in patients with low- and high-volume ascites diagnosed with stages III-IV HGSOC is shown by the Kaplan-Meier plot. Data included in this plot were analyzed by a log-rank test.
HGSOC correlates with tumor infiltrating T cells and better overall survival [27]. HLA class I antigens bind tumor-associated peptides during their intracellular assembly, which are then presented to CTL. In general, EOC lesions display downregulated expression of HLA class I or the processing intermediates, TAP1 and TAP2. In view of the crucial role of the HLA class I complex in presenting tumor-associated peptides to CTL, it is not surprising that downregulation of HLA class I and its processing machinery is associated with advanced stage and disease progression [28] and has a negative impact on patient survival [29]. Thus, our finding that both HLA classes I and II genes are upregulated in tumors from patients with low-versus high-volume ascites could help to explain their better clinical outcome. This interpretation is supported by a recent cross-platform study of six gene array datasets (including the TCGA and the Tothill datasets) by Yoshihara et al. [6] that found decreased overall survival in HGSOC associated with reduced expression of immunoreactive genes in the tumor.

Large-scale, genome-wide gene expression profiling studies of HGSOC by Tothill et al. [4] and TCGA [5] indicate the existence of distinct molecular subgroups, with both studies identifying a subgroup enriched in immune response genes. While these studies incorporated a wide range of presenting clinical parameters, ascites volume was not included. A preliminary comparison of our upregulated genes in tumors associated with low-volume ascites with the genes upregulated in the TCGA immune group indicates an overlap (shown in Figure 1) that is not expected by chance alone \( \left( P < 0.01, \text{hypergeometric test} \right) \). The number of samples available for our discovery cohort was limited in size due to the variable capturing and inconsistent quantification of ascites volume in tumor banks and clinical databases, which resulted in our inability to find an external dataset to validate our findings. Nonetheless, our results are highly suggestive that low-volume ascites may be an associating clinical parameter of the “immune” molecular subgroup of HGSOC.

The surgical outcome at the end of primary cytoreductive surgery is one of the most important independent predictors of survival for advanced stage EOC. While resection of all macroscopic residual tumor is the optimal goal, debulking of tumor lesions to <1.0 cm in their greatest dimension is considered beneficial [30]. Other independent prognostic factors identified by a large retrospective study of 1895 patients diagnosed with stage III EOC include age, histological type, and performance status [31]. In our study, age was similar between the two groups and histological type was restricted to HGSOC; performance status was not addressed. Our results show that patients with advanced stage HGSOC presenting with low-volume ascites have improved survival compared to patients with high-volume ascites. It remains unclear as to whether this is the result of improved surgical outcome, an enhanced immunoresponsiveness, or an interaction between these two factors.

## 5. Conclusion

This study shows that HGSOC presenting with low-volume ascites has molecular features distinct from HGSOC presenting with high-volume ascites and is characterized by an enhanced immunoreactive phenotype, better surgical outcome, and prolonged overall survival. While further prospective studies are required, our findings suggest that these patients are likely to achieve favorable outcome at the end of primary cytoreductive surgery. Our results also indicate that adjuvant immunotherapy may be a reasonable future approach for the treatment of ascites. We believe that ascites volume is an important clinical parameter that should be accurately captured to enable future research on differences in ascites volume in advanced ovarian cancer.

### Abbreviations

- CTL: Cytotoxic T lymphocytes
- EOC: Epithelial ovarian cancer
- FDR: False discovery rate
- FWER: Family wise error rate
- GO: Gene ontology
- GSEA: Gene set enrichment analysis
- HGSOC: High-grade serous ovarian cancer
- IQR: Interquartile range
- TCGA: The cancer genome atlas
- TILS: Tumor infiltrating lymphocytes
- VEGF: Vascular endothelial growth factor

### Conflict of Interests

The authors declare that there is no conflict of interests.

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References


