Molecular Medicine in Practice

The Association of PI3 Kinase Signaling and Chemoresistance in Advanced Ovarian Cancer

Craig P. Carden1,2,3, Adam Stewart1,2, Parames Thavasu1,2, Emma Kipps1,2,3, Lorna Pope1,2, Mateus Crespo1,2, Susana Miranda1,2, Gerhardt Attard1,2, Michelle D. Garrett1, Paul A. Clarke1, Paul Workman1, Johann S. de Bono1,2,3, Martin Gore3, Stan B Kaye1,2,3, and Udai Banerji1,2,3

Abstract

Evidence that the phosphoinositide 3-kinase (PI3K) pathway is deregulated in ovarian cancer is largely based on the analysis of surgical specimens sampled at diagnosis and may not reflect the biology of advanced ovarian cancer. We aimed to investigate PI3K signaling in cancer cells isolated from patients with advanced ovarian cancer. Ascites samples were analyzed from 88 patients, of whom 61 received further treatment. Cancer cells were immunomagnetically separated from ascites, and the signaling output of the PI3K pathway was studied by quantifying p-AKT, p-p70S6K, and p-GSK3β by ELISA. Relevant oncogenes, such as PIK3CA and AKT, were sequenced by PCR-amplified mass spectroscopy detection methods. In addition, PIK3CA and AKT2 amplifications and PTEN deletions were analyzed by FISH. p-p70S6K levels were significantly higher in cells from 37 of 61 patients who did not respond to subsequent chemotherapy (0.7184 vs. 0.3496; P = 0.0100), and this difference was greater in patients who had not received previous chemotherapy. PIK3CA and AKT mutations were present in 5% and 0% of samples, respectively. Amplification of PIK3CA and AKT2 and deletion of PTEN was seen in 10%, 10%, and 27% of samples, respectively. Mutations of PIK3CA and amplification of PIK3CA/AKT2 or deletion of PTEN did not correlate with levels of p-AKT, p-p70S6K, and p-GSK3β. In patients with advanced ovarian cancer, there is an association between levels of p-p70S6K and response to subsequent chemotherapy. There is no clear evidence that this is driven specifically by PIK3CA or AKT mutations or by amplifications or deletion of PTEN. Mol Cancer Ther; 11(7); 1609–17. ©2012 AACR.

Introduction

Ovarian cancer is the most common cause of death from gynecologic malignancies in the Western world, with global estimates of approximately 192,000 new cases and 114,000 deaths a year (1). Although ovarian cancer is sensitive to platinum-based chemotherapy in 60% to 70% of cases, patients eventually become resistant to these agents (2). There is an urgent and unmet need to improve outcomes in patients with platinum-relapsed and -refractory ovarian cancer (3).

The phosphoinositide 3-kinase (PI3K) pathway is deregulated in multiple cancers, including ovarian cancer (3). The activation of the PI3K pathway in ovarian cancer, as evidenced by the levels of phosphorylated proteins such AKT or mTOR (4, 5), has been linked with adverse prognosis (6, 7), although not all studies have confirmed this (8, 9). Drivers of the PI3K pathway in ovarian cancer are thought to be multifactorial. Amplification of PIK3CA occurs in 10% to 36% of ovarian cancers and mutations are seen in 2% to 12% (10–16). Amplifications of AKT2 are seen in 12% to 18%, and AKT mutations are present in 0% to 2% (14, 17–19) of ovarian cancer specimens. Deletions of PTEN are found uncommonly in ovarian cancer, except for the endometrioid subgroup, in which up to 45% of cases exhibit loss of heterozygosity, whereas mutations are present in 6% to 11% of ovarian cancers (12, 16, 20, 21).

The majority of samples used in the studies cited above were obtained during diagnostic biopsy or surgery early in the course of the disease and may not reflect the biology of advanced disease, the setting in which patients are considered for clinical trials of PI3K pathway inhibitors. In addition, most work done on studying the relevance of the PI3K pathway in ovarian cancer is based upon formalin-fixed, paraffin-embedded (FFPE) tissue, which has technical limitations due to the inherent instability of the phospho-epitopes (22) and the semiquantitative nature of the technology (23). We hypothesized that cancer cells isolated from ascites in patients with advanced ovarian cancer...
cancer may more accurately represent the biology of patients with ovarian cancer receiving treatment for metastatic disease. We used immunomagnetically separated cells using epithelial cell adhesion molecule (EpCAM) as a marker for positive selection. EpCAM has been previously validated as a marker of cancer cells in serous effusions (24) and used to immunomagnetically separate cancer cells (25). However, although it is used extensively to enrich cancer cells, when isolating circulating tumor cells (26), it does not detect subsets of cancer cells with low or absent levels of EpCAM (27), which may be biologically relevant.

There are multiple possible measures of the signaling output of the PI3K pathway, including the phosphorylation of AKT (4, 5) and other proteins downstream of AKT such as mTOR (28), S6K (29, 30), S6 (9), GSK3β (31, 32), and 4EBP1 (9, 28). We embarked upon this evaluation Criteria in Solid Tumors (RECIST) and, in the absence of radiologically assessable disease, GCIG CA125 criteria were used. The ascitic taps were carried out when patients were symptomatic. When the procedure was conducted, the number of lines of previous chemotherapy and decisions about postprocedure chemotherapy (including interval and type of chemotherapy) were varied and could not be controlled for. Thus, survival was calculated from ascitic tap to death and not from diagnosis to death or start/end of subsequent chemotherapy to death.

**Isolation of ovarian cancer cells**

One liter of ascitic fluid was collected in bags containing 5,000 units of unfractionated heparin. One liter of the sample was transferred to four 250-mL centrifuge flasks and centrifuged at 1,000 × g for 10 minutes at 4°C (Eppendorf 5810-R). In one sample of 250 mL, all supernatant was removed and the unseparated cells were formalin-fixed and embedded in paraffin. In the remaining 3 samples, the supernatant was discarded and the soft pellet resuspended in 1-mL ascitic fluid for isolation of ovarian cancer cells. One milliliter aliquots of the cell suspension were hybridized with 25 μL of EpCAM-coated Dynal beads (Dynabeads Epithelial Enrich 161.02, Invitrogen), hybridized at 4°C for 30 minutes, magnetically separated according to the manufacturer’s instructions, flash-frozen, and then stored at −80°C. Validation of the process of immunomagnetic separation is presented in the Supplementary Data and Supplementary Fig. S1.

**ELISA**

Multiplex ELISA kits using electrochemiluminescence (ECL) technology were used to quantify phosphorylation of AKT (Ser 473), p70S6K (Thr 421, Ser 424), GSK3β (Ser 9), and respective total proteins (K1115D-1 and K11133D-1, Meso Scale Discovery). Plates were read on a SECTOR 6000 Imager (Meso Scale Discovery) as per the manufacturer’s instructions. The ELISA did not differentiate between p70S6K1 and p70S6K2. Twenty micrograms of protein was loaded in each well of the ELISA plate. The results of the ELISA were expressed in ECL counts and the values of phosphoprotein were normalized to respective total protein expression of each kinase, that is, AKT, p70S6K, and GSK3β. As this was effectively a ratio of ECL counts, no units were assigned to phosphoprotein levels.

Validation of the reproducibility of the immunomagnetic separation and ELISA is presented in the Supplementary Data and Supplementary Fig. S2.

The differences in p-AKT, p-p70S6K, and p-GSK3β between patients who responded to subsequent chemotherapy and those who did not were studied using nonparametric methods (Mann–Whitney test) as the data were non-Gaussian. The differences in p-AKT, p-p70S6K, and p-GSK3β between patients who had either PIK3CA or AKT2 amplifications or PTEN deletion were analyzed using Mann–Whitney tests (GraphPad Prism V5). The difference in survival (defined as the time between the sample was taken and death) between groups of patients stratified by median p-AKT, p-p70S6K, and p-GSK3β was calculated using the Kaplan–Meier method (GraphPad Prism V5).
p-p70S6K, and p-GSK3β were compared by log-rank tests (GraphPad Prism V5).

**Amplification of PIK3CA, AKT2 and deletion of PTEN**

**Immunofluorescence.** Paraffin-embedded blocks were cut to a thickness of 4 μm and slides were deparaffinized followed by xylene and ethanol washes. Antigen retrieval was carried out under pressure. Primary antibodies used were Ber-EP4 (M0804, Monoclonal mouse, Dako) at a dilution of 1:150, CD45 and CD68 (sc-25590 and sc-9139, rabbit polyclonal, Santa Cruz Biotechnology, Inc.) at dilutions of 1:200, and podoplanin (Ab10274, rabbit polyclonal, Abcam) at a dilution of 1:400. Secondary antibody Alexa Fluor 488 (A21200, anti-mouse, Invitrogen) was used at 1:1,000 and Alexa Fluor 555 (A21429, anti-rabbit, Invitrogen) at 1:1,000 dilution. Tissue images were captured with an Ariol SL-50 Automated Slide Scanner (Leica Microsystems) with Review V3.4 software (Genetix Ltd.).

**FISH.** FISH studies were carried out using previously described methods (33). Appropriate bacterial artificial chromosomes (BAC) were identified on the Ensembl Genome Browser and purchased (Children’s Hospital and Research Centre, Oakland, CA). RP11-115H8 (PIK3CA) and RP11-639F21 (AKT2) were directly labeled in spectrum green and RP11-846G17 (PTEN) in spectrum orange. Control probes for PIK3CA, AKT2 and PTEN were CEP3 and TelVysion 19q (labeled in spectrum orange) and CEP10 labeled in fluorescein isothiocyanate, respectively (6J3603, 5J0419, and 6J3710, Abbott Molecular). The genes of interest covered by the FISH probes are listed in the Supplementary Table S3. The probe used to detect PTEN has been used previously (34) and has considerable overlap with the BACs used by our group previously (35).

Slides previously processed for immunofluorescence were exposed to boiling ethanol and followed by boiling pretreatment solution (00-8401, SPot-Light Tissue Pretreatment Kit, Invitrogen). Samples were then treated with pepsin solution (00-3009, Digest-All 3, Invitrogen), ethanol-washed, and the appropriate FISH probe applied. Subsequently, the samples were denatured at 75°C and left to hybridize overnight and mounted.

Images were then recaptured on the Ariol SL-50 Automated Slide Scanner (Leica Microsystems) and stored for analysis. This allowed us to conduct side-by-side analysis of individual cells, scoring FISH results only from those cells positive for Ber-EP4 (and negative for CD45, CD68, and podoplanin). A minimum of 50 nuclei per sample were analyzed, discarding any in which the cell boundaries were not clearly delineated. When analyzing data, we defined amplification as any population in which the gene:control ratio was equal to or greater than 2.0 and loss as any population with a ratio of less than 1.0. Fifteen percent or more cells needed to have the abnormality to be considered to have an amplification or deletion.

Differences in responses to subsequent chemotherapy and between groups of patients, who had PIK3CA or AKT2 amplification or PTEN deletion, were analyzed by Fisher exact test (GraphPad Prism V5).

**Mutations in PIK3CA and AKT2**

DNA extraction was conducted on an ABI PRISM 6100 Nucleic Acid PrepStation (Applied Biosystems) according to the manufacturer’s protocols. DNA quantity (ng/μL) and quality (260/280) were recorded with a spectrophotometer (ND-1000, NanoDrop, Thermo Scientific). Using a previously validated PCR and extension-based method (36), 238 somatic mutations were profiled across 19 oncogenes with the OncoCarta Panel v1.0 and detected by massARRAY (Sequenom). A full list of mutations tested in the OncoCarta 1 panel is listed in Supplementary Table S1.

**Cell lines**

Cell lines used for validation experiments, SKOV3 and A2780, were purchased from the European Collection of Cell Cultures (Health Protection Agency). HCC827 was bought in from the American Type Culture Collection (LGC Standards). Cells were grown in Dulbecco’s Modified Eagle’s Medium containing 10% fetal calf serum and 5 mmol/L glutamine in a humidified atmosphere of 5% CO2 at 37°C and passaged for up to 6 months before renewal from frozen stocks.

**Results**

**Demographics and histologic subtypes**

Eighty-eight consecutive patients, who underwent ascitic drainage for symptom relief and who had consented to have samples analyzed, were studied. The median age of patients was 64 years (range, 37–90 years). The histologic subtypes of the majority of patients were 89% (78 of 88) high-grade serous, 6% (5 of 88) clear cell, 2% (2 of 88) mixed mullerian, 1% (1 of 88) borderline, 1% (1 of 88) endometrioid, and 1% (1 of 88) unknown. Of the total of 88 patients, 23 had not received prior chemotherapy and 65 were previously treated. Of the total of 88 patients, 61 patients received subsequent chemotherapy (22 previously untreated and 39 previously treated). The chemotherapy received after the paracentesis comprised predominantly platinum-, taxane-, and liposomal doxorubicin-based regimens (Table 1).

**Activation of the PI3K pathway and response to subsequent chemotherapy**

Twenty-four of the 61 (39%) who received subsequent chemotherapy were considered responders (complete or partial response), whereas 37 of 61 (61%) who had progressive or stable disease as their best response were classified as nonresponders.
There was a significantly higher level of p-p70S6K in cancer cells isolated from ascites in patients who did not respond to subsequent chemotherapy when compared with samples of patients who did respond (0.7184 vs. 0.3496; \( P = 0.0100 \); see Fig. 1). The p-AKT and p-GSK3\(\beta\) levels were not significantly different in samples from patients who did not respond to subsequent chemotherapy and those who did (0.3872 vs. 0.2283; \( P = 0.0968 \) and 1.058 vs. 1.120; \( P = 0.9647 \), respectively).

Interestingly of the 61 patients, 22 had not previously received chemotherapy, and of these patients, 15 (68%) had a complete or partial response as their best response. In this subgroup, both p-p70S6K and p-AKT were significantly higher in samples from patients who did not respond to chemotherapy than samples from patients who responded (1.216 vs. 0.34; \( P = 0.0288 \) and 0.5929 vs. 0.1680; \( P = 0.0407 \), respectively).

Amplification of \textit{PIK3CA}, \textit{AKT2} and loss of \textit{PTEN}

Of the 88 patients, 63 samples were available for FISH analysis for amplification of \textit{PIK3CA}, \textit{AKT2} and deletion of \textit{PTEN}.

Amplification of \textit{PIK3CA} and \textit{AKT2} was seen in 6 of 63 (10%) and 6 of 63 (10%) of samples, respectively, and these were mutually exclusive. Loss of \textit{PTEN} by FISH was seen in 17 of 63 (27%) samples (Fig. 2). For the samples reported to have amplification, \textit{PIK3CA} (\( n = 6 \)) and \textit{AKT2} (\( n = 6 \)), the median gene copy number:control ratios for \textit{PIK3CA} and \textit{AKT2} were 2 (range, 2–2.25) and 2 (range, 2–3.35), respectively. Sixteen patients were

**Table 1. Demographic profile of patients with ascites included in the study and subsequent chemotherapy received**

<table>
<thead>
<tr>
<th>Age (median)</th>
<th>62 y (range, 37%–90%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histology</td>
<td></td>
</tr>
<tr>
<td>High-grade serous</td>
<td>78/88 (89%)</td>
</tr>
<tr>
<td>Clear cell</td>
<td>5/88 (6%)</td>
</tr>
<tr>
<td>Mixed Mullerian</td>
<td>2/88 (2%)</td>
</tr>
<tr>
<td>Borderline</td>
<td>1/88 (1%)</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>1/88 (1%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>1/88 (1%)</td>
</tr>
<tr>
<td>Lines of previous chemotherapy</td>
<td></td>
</tr>
<tr>
<td>No previous chemotherapy</td>
<td>23/88 (26%)</td>
</tr>
<tr>
<td>Had previous chemotherapy</td>
<td>65/88 (74%)</td>
</tr>
<tr>
<td>Median lines of chemotherapy for whole cohort</td>
<td>2 (0–11)</td>
</tr>
<tr>
<td>Subsequent chemotherapy</td>
<td></td>
</tr>
<tr>
<td>Carboplatin combinations q3 weekly</td>
<td>19/61</td>
</tr>
<tr>
<td>Single-agent carboplatin q3 weekly</td>
<td>13/61</td>
</tr>
<tr>
<td>Weekly paclitaxel</td>
<td>10/61</td>
</tr>
<tr>
<td>Single-agent liposomal doxorubicin</td>
<td>10/61</td>
</tr>
<tr>
<td>Others</td>
<td>9/61</td>
</tr>
</tbody>
</table>

There was a significantly higher level of p-p70S6K in cancer cells isolated from ascites in patients who did not respond to subsequent chemotherapy when compared with samples of patients who did respond (0.7184 vs. 0.3496; \( P = 0.0100 \); see Fig. 1). The p-AKT and p-GSK3\(\beta\) levels were not significantly different in samples from patients who did not respond to subsequent chemother-

Figure 1. Association of clinical response and signaling output of the PI3K pathway. The differences in the phosphorylation of p70S6K, AKT, and GSK3\(\beta\) in patients, who had a best response of complete response (CR) or partial response (PR) versus patients who had stable disease (SD) or progressive disease (PD), were measured by ELISA in immunomagnetically separated cancer cells from ascites of patients undergoing palliative paracentesis.

Figure 2. Representative samples of \textit{PIK3CA} and \textit{AKT} amplification and \textit{PTEN} deletion. FISH analysis of \textit{PIK3CA}, \textit{AKT2} and \textit{PTEN}. Each slide underwent immunofluorescence to positively identify cancer cells (Ber-EP4) and negatively identify noncancer cells, CD45 (CD45; white blood cells), CD68 (macrophages), and podoplanin (mesothelial cells). A, an example of a sample of ascites with cells expressing Ber-EP4 and CD45 are presented at a \( \times 20 \) magnification. B, in excess of 2 copies of the \textit{PIK3CA} genes (green) in relation to markers for centromere 3 in a cell (red). C, an excess of 2 copies of the \textit{AKT2} gene (green) in relation to 19q telomere in a cell (red). D, less than 1 \textit{PTEN} gene (red) copy in relation to markers for centromere 10 (green). All FISH images are at \( \times 63 \) magnification.
reported to have PTEN loss with a median copy number:control ratio of 0.5 (range, 0–0.75). There were no differences in the levels of p-AKT, p-p70S6K, and p-GSK3 between samples with amplification of PIK3CA and AKT2 or loss of PTEN (Table 2).

**Mutations in a panel of oncogenes**

Of the 88 patients, 63 samples were available for sequencing of a panel of oncogenes. Of the panel of mutations tested, 3 PIK3CA (N345K, N345K, and H1047R), 3 CMET (T992I, T992I, and T992I), and 2 KRAS (Q61H and Q66H) mutations were detected. Of the PIK3CA mutations, 2 of the 3 were in clear cell cancers, and the rest of the mutations were found in high-grade serous cancers. There were no differences in the levels of p-AKT, p-p70S6K, and p-GSK3 between patients who had a mutation and those that did not (data not shown). The types and number of mutations are too heterogeneous to conduct meaningful statistical tests.

**Other correlative analyses**

The median survival of the entire cohort of 88 patients was 147 days and reflects the survival of patients with advanced ovarian cancer who undergo palliative ascitic drainage. There was no difference in patient survival with values above and below median of p-AKT, p-p70S6K, or p-GSK3β in patients who did and did not have amplification of PIK3CA and AKT2 or deletion of PTEN (Table 2). In addition, survival analysis using cutoff levels of p-AKT, p-p70S6K, or p-GSK3β derived from patients who did and did not respond to subsequent chemotherapy (n = 61) was applied to the entire cohort of 88 patients and did not show any statistically significant difference between the subgroups (Supplementary Table S2). These analyses were exploratory and were not statistically powered to show significant differences.

**Discussion**

The signaling output of the PI3K pathway has been studied previously in ovarian cancer as measured by

---

**Table 2.** Study of activation of the components of the PI3K pathway and association with PIK3CA and AKT2 amplification and PTEN loss

<table>
<thead>
<tr>
<th>Marker</th>
<th>PIK3CA amplified (n = 6)</th>
<th>PIK3CA not amplified (n = 57)</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-AKT, mean (SD)</td>
<td>0.1833 (0.18)</td>
<td>0.3318 (0.5272)</td>
<td>P = 0.7786</td>
</tr>
<tr>
<td>p-p70S6K, mean (SD)</td>
<td>0.4317 (0.2581)</td>
<td>0.5240 (0.6345)</td>
<td>P = 0.8698</td>
</tr>
<tr>
<td>p-GSK3β, mean (SD)</td>
<td>1.360 (1.023)</td>
<td>1.055 (0.6914)</td>
<td>P = 0.7342</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Marker</th>
<th>AKT2 amplified n = 6</th>
<th>AKT2 not amplified n = 57</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-AKT, mean (SD)</td>
<td>0.1650 (0.0809)</td>
<td>0.3337 (0.5288)</td>
<td>P = 0.8513</td>
</tr>
<tr>
<td>p-p70S6K, mean (SD)</td>
<td>0.3883 (0.3662)</td>
<td>0.5286 (0.6289)</td>
<td>P = 0.5740</td>
</tr>
<tr>
<td>p-GSK3β, mean (SD)</td>
<td>0.9400 (0.2175)</td>
<td>1.099 (0.7575)</td>
<td>P = 0.7430</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Marker</th>
<th>PTEN loss n = 17</th>
<th>No PTEN loss n = 46</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-AKT, mean (SD)</td>
<td>0.1700 (0.2428)</td>
<td>0.3287 (0.4685)</td>
<td>P = 0.0786</td>
</tr>
<tr>
<td>p-p70S6K, mean (SD)</td>
<td>0.3112 (0.2813)</td>
<td>0.5907 (0.6781)</td>
<td>P = 0.0631</td>
</tr>
<tr>
<td>p-GSK3β, mean (SD)</td>
<td>1.004 (0.6364)</td>
<td>1.114 (0.7583)</td>
<td>P = 0.5459</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Marker</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-AKT, mean (SD)</td>
<td>0.2133 (0.2252)</td>
</tr>
<tr>
<td>p-p70S6K, mean (SD)</td>
<td>0.4150 (0.3107)</td>
</tr>
<tr>
<td>p-GSK3β, mean (SD)</td>
<td>0.9957 (0.6765)</td>
</tr>
</tbody>
</table>

NOTE: Sixty-three patients were included in this analysis, where data for mutation of PIK3CA, AKT, KRAS, and CMET, in addition to amplification of PIK3CA and AKT and deletion of PTEN were available. The levels of p-AKT, p-p70S6K, and p-GSK3β were quantified and differences between groups that did and did not have amplification (PIK3CA and AKT2) and deletion (PTEN), known to activate the PI3K pathway, were studied and the differences between the signaling output are shown. As only 8 mutations (3 PIK3CA, 3 CMET, and 2 KRAS) were found, the differences in signaling output have not been presented because of low numbers of individual mutations. In addition, samples with mutations, amplification, or deletion of genes studied were pooled and compared with a cohort that did not have any of these features and the signaling output is presented in the lower section of the table.
phosphorylation of Ser10 and Thr308 sites of AKT (4–9), in most instances when samples had been obtained at primary surgery or at diagnostic biopsy. This approach has advantages, as patients can be followed up over considerable periods of time and most patients are chemotherapy-eligible, thus it is possible to interrogate predictive biomarkers of response to primary chemotherapy. However, these samples may not represent the biology of the tumors in advanced ovarian cancer. The present study provided a unique opportunity to gain an insight into the activation of the PI3K pathway in advanced ovarian cancer. Analyzing tumors from heavily pretreated patients for biologic–clinical correlations can be difficult because of the confounding effect of multiple lines of prior chemotherapy, and thus multivariate analysis using parameters, such as stage, grade, residual tumor mass, commonly used following debulking surgery and first-line chemotherapy for ovarian cancer is less meaningful in this setting. There remain technical challenges when using immunohistochemistry (IHC) as a readout to quantify phosphoproteins due to instability of the phospho-epitope (22) and lack of clarity as to the best methods to quantify signals (23). Interestingly, one previous study used pleural effusion and ascites samples while studying the PI3K pathway by counting number of cells positively staining for p-AKT and p-mTOR in EpCAM-positive and CD45-negative cells on flow cytometry. The authors also quantified p-AKT by Western blot analysis but used cell pellets that were not enriched for malignant cells (7).

The present study is unique, as it is to our knowledge the first time that signaling output of the PI3K signaling has been studied using quantitative electrochemiluminescent ELISA technology in immunomagnetically enriched ovarian cancer cells isolated from ascites specimens in patients with advanced ovarian cancer. Only patients with ascites were included in this study, which could have biased the study to a subset of patients with ovarian cancer with a poor prognosis. In addition, types of further chemotherapy received by subjects were varied and could have influenced the interpretation of data. Future studies limited to patients on specific chemotherapy regimens will add further clarity to the results of this study. Despite its limitations, in the present study of patients with advanced ovarian cancer, we have shown that patients who did not respond to subsequent chemotherapy had significantly higher p-p70S6K in ovarian cancer cells than those who did respond. While markers of the signaling output of the PI3K pathway such as p-AKT and p-mTOR have been linked to adverse prognosis in ovarian cancer (4, 5), to our knowledge, these are the first data relating PI3K pathway activation with response to chemotherapy in the setting of advanced ovarian cancer. One previous study has shown p-4EBP1 but not p-p70S6K to be linked to chemoresistance in clinical samples. That study, like many others, was carried on tissue collected at diagnosis and studied in paraffin-embedded tissue by immunohistochemical techniques (28). It was interesting to note that in this present study, levels of both p-p70S6K and p-AKT were significantly higher in cancer cells isolated from patients who did not respond to subsequent chemotherapy than those who did, in a cohort of previously untreated cases. The clinical data from this present study relate to experimental cancer models that have suggested that p-AKT and p-p70S6K play an important role in chemosensitivity/resistance to cisplatin and paclitaxel (37–39). It would be interesting to quantify p-p70S6K and p-AKT in flash-frozen fresh ovarian tissue sampled at diagnosis in larger patient cohorts in the future.

p70S6K has been shown to be crucial in ovarian cancer cell migration and metastasis in preclinical models (29, 30). Also, p70S6K and S6 have also been shown to be associated with hypoxia and angiogenesis in ovarian cancer models (40). The finding that higher levels of p-p70S6K in cancer cells derived from patients who did not respond to chemotherapy could be used to strengthen the argument that antiangiogenic agents, PI3K, AKT, and mTOR inhibitors may be useful for the management of patients with advanced chemoresistant ovarian cancer (3, 41, 42).

In our present study of cancer cells isolated from ascites of patients with advanced ovarian cancer, 3 of 63 (5%) and 0 of 63 of samples were detected to have PIK3CA and AKT mutations, respectively. Mutations of PIK3CA and AKT in samples predominantly taken at diagnosis have been previously described as less than 0% to 12% (11–13, 43). Our study found amplification of PIK3CA and AKT2 in 6 of 63 (10%) and 6 of 63 (10%), respectively, which is not obviously different from studies in which samples were predominantly taken at diagnosis; these reported an amplification of PIK3CA and AKT in the range of 13% to 35% (11–14, 19). The finding that the incidence of PIK3CA and AKT mutations and amplifications in advanced and often pretreated ovarian cancer is not different from a majority of studies conducted at diagnosis suggests that these mutations and amplifications are not late events occurring in advanced ovarian cancer either as part of the natural history of the disease or in response to chemotherapy.

The incidence of PTEN deletion in our study was assessed by FISH and was 27%. Previous studies have shown a loss of heterozygosity in approximately 40% (20, 44). PTEN loss by IHC has been reported in ovarian cancer and was in the range of 27% to 69% of samples (12, 21, 44). It was not possible to quantify PTEN protein by IHC in our study as paraffin-embedded tissue in this study had unseparated cells spun down from ascites, which did not have the tumoral architecture crucial to such analysis. The incidence of PTEN deletion in samples from patients in our study with advanced ovarian cancer was similar to the published incidence in samples taken at diagnosis, implying that this is not a major factor regulating secondary resistance to chemotherapy.

Of particular interest was the discovery that neither mutations and amplifications nor deletions of relevant genes in the PI3K pathway were associated with the signaling output of the pathway as evidenced by levels...
of p-AKT, p-p70S6K, and p-GSK3β. The modest size of the sample set led to a small number of mutations detected in this cohort and this is a possible reason why there was no association between mutations and signaling output in this study. However, it is possible that there are mutations and amplifications of receptor tyrosine kinases that are not frequently studied in ovarian cancer. As part of this study, in addition to the mutations we did detect in PIK3CA, KRAS, and CMET, we looked for and did not detect mutations of EGFR, NRAS, ERBB2, CKIT, FGFR1, and FGFR2. This is a comprehensive but not a complete list of mutations that could occur in advanced ovarian cancer. In addition to the analysis conducted in this study, the literature suggests that there are multiple factors independent of PI3K that influence p70S6K activation, such as the ligand FGF-9 (45), the signaling protein belonging to the RAS superfamily, Rheb (46), and amplification of the gene URI (47) to name a few. However, the volume of individual samples precluded us from analyzing a larger set of proteins or genes.

This present study has implications for the conduct of clinical trials of inhibitors of PI3K pathway in the setting of ovarian cancer. Some preclinical models have suggested that PIK3CA mutations predict sensitivity to PI3K pathway inhibitors (48), and there are hints that PIK3CA mutations may predict response to PI3K pathway inhibitors in early clinical trials (49). However, the status of individual predictive biomarkers sensitivity to PI3K pathway inhibitors is not always clear (50). This present study suggests that there are important factors that drive the PI3K pathway in advanced ovarian cancer in addition to the commonly studied markers such as mutations and amplifications of PIK3CA and AKT or loss of PTEN. The present study does strongly suggest an association between the signaling output of the PI3K pathway, as measured by p-p70S6K levels and chemoresistance in the setting of advanced ovarian cancer. Carefully conducted hypothesis-testing clinical trials of PI3K pathway inhibitors in advanced ovarian cancer, either as single agents or as combination therapy, are warranted.

**Disclosure of Potential Conflicts of Interest**

P.A. Clarke has been involved in a commercial collaboration with Yamanouchi (now Astellas Pharma) and with Piramed Pharma, and intellectual property arising from the program has been licensed to Genentech. Genentech and Piramed Pharma were acquired by Roche. As a result of the collaboration with Piramed, P.A. Clarke has received researchers’ bonus payments from The Institute of Cancer Research (ICR). P.A. Clarke is an employee of the ICR, which has a commercial interest in the development of PI3K inhibitors, including GDC-0941, and has received remuneration through a rewards-to-inventors scheme. P. Workman has received commercial research support from Yamanouchi (now Astellas), Piramed Pharma, Vernalis, and Astex Pharmaceuticals. He has declared ownership interests (including patents) as a scientific founder of Piramed Pharma (acquired by Roche) and Astex Pharmaceuticals and AstraZeneca. J.S. de Bono is employed as a professor in the ICR and has received honoraria from Speaker’s Bureau from Genentech. All authors with the exception of M. Core are employees of the ICR. The ICR has developed PI3K and AKT inhibitors in collaboration with Genentech, Astex Therapeutics, and AstraZeneca and receives payments for these.

**Authors’ Contributions**

**Conception and design:** C.P. Carden, P. Workman, J.S. de Bono, S.B. Kaye, U. Banerji


**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** C.P. Carden, A. Stewart, P. Thavasu, E. Kipps, S. Miranda, G. Attard, P. Workman, J.S. de Bono, M. Gore, S.B. Kaye, U. Banerji

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** C.P. Carden, A. Stewart, E. Kipps, L. Pope, P.A. Clarke, P. Workman, J.S. de Bono, S.B. Kaye, U. Banerji

**Writing, review, and/or revision of the manuscript:** C.P. Carden, L. Pope, G. Attard, M.D. Garrett, P.A. Clarke, P. Workman, J.S. de Bono, M. Gore, S.B. Kaye, U. Banerji

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** A. Stewart, P. Thavasu, P. Workman, J.S. de Bono, U. Banerji

**Study supervision:** P. Thavasu, M.D. Garrett, P. Workman, J.S. de Bono

**Grant Support**

M.D. Garrett, P.A. Clarke, P. Workman, J.S. de Bono, and U. Banerji were supported by Cancer Research UK grants (grant number C309/A0274, C309/A13566). M.D. Garrett, J.S. de Bono, S.B. Kaye, P. Workman, and U. Banerji were supported by an ECMC grant (C51/A7401,C12540/A15573). C.P. Carden, A. Stewart, P. Thavasu, E. Kipps, L. Pope, M. Crespo, S. Miranda, G. Attard, M.D. Garrett, P.A. Clarke, P. Workman, J.S. de Bono, M. Gore, S.B. Kaye, and U. Banerji acknowledge NHS funding to the NIHR Biomedical Research Centre.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 7, 2011; revised April 24, 2012; accepted April 26, 2012; published OnlineFirst May 3, 2012.

---

**References**


The Association of PI3 Kinase Signaling and Chemoresistance in Advanced Ovarian Cancer

Craig P. Carden, Adam Stewart, Parames Thavasu, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-11-0996

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2012/05/03/1535-7163.MCT-11-0996.DC1

Cited articles
This article cites 50 articles, 14 of which you can access for free at:
http://mct.aacrjournals.org/content/11/7/1609.full#ref-list-1

Citing articles
This article has been cited by 10 HighWire-hosted articles. Access the articles at:
http://mct.aacrjournals.org/content/11/7/1609.full#related-urls

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.