Molecular Cancer Therapeutics

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

Craig P. Carden[1,2,3], Adam Stewart[1,2], Parames Thavasu[1,2], Emma Kipps[1,2,3], Lorna Pope[1,2], Mateus Crespo[1,2], Susana Miranda[1,2], Gerhardt Attard[1,2], Michelle D. Garrett[1], Paul A. Clarke[1], Paul Workman[1], Johann S. de Bono[1,2,3], Martin Gore[3], Stan B Kaye[1,2,3], and Udai Banerji[1,2,3]

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

Authors' Affiliations: [1]Cancer Research UK Cancer Therapeutics Unit, Division of Cancer Therapeutics, [2]Drug Development Unit, Division of Cancer Therapeutics and Division of Clinical Studies, The Institute of Cancer Research; and [3]The Royal Marsden NHS Foundation Trust, Sutton, United Kingdom

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Corresponding Author: Udai Banerji, Drug Development Unit, The Royal Marsden Hospital, Downs Road, Sutton SM2 5PT, United Kingdom. Phone: 44-020-8661-3984; Fax: 44-020-8642-7979; E-mail: udai.banerji@icr.ac.uk

doi: 10.1158/1535-7163.MCT-11-0996

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 analysis using a commercially available triplex ELISA, which measured phosphorylation of AKT, p70S6K, and GSK3β. There is evidence that AKT, p70S6K, and GSK3β are regulated by activation of PI3K, although the interactions are complex [for e.g., there is evidence that GSK3β is regulated by AKT (31), although the exact nature of this is unclear (32)]. We considered measuring the phosphorylation of mTOR, S6, and 4EBP1, but the quantity of each individual sample collected was insufficient to conduct further assays so we made a pragmatic decision to use triplex ELISA plates to simultaneously measure activation of AKT, p70S6K, and GSK3β. Thus, we aimed to study the feasibility of measuring the signaling output of the PI3K pathway by quantifying p-AKT, p-p70S6K, and p-GSK3β in ovarian cancer cells isolated from ascites cells by ELISA. We also aimed to correlate the activation of the pathway with response to subsequent treatment. In addition, we investigated genetic factors that are known to influence the PI3K pathway such as *PIK3CA* and *AKT* mutations/amplifications and *PTEN* loss.

Materials and Methods

Patients

Patients undergoing palliative ascitic taps for symptom relief were asked to consent to the use of their ascitic fluid for research purposes. This protocol was reviewed and approved by the Institutional Review Board and Research Ethics Committee (Committee for Clinical Research, Royal Marsden Hospital, Sutton, UK; Reference No: CCR2932).

Details of patient treatment were recorded in the electronic patient record at our institution. Patient response to the subsequent line of chemotherapy after the ascitic drainage was assessed radiologically by Response 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 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 Data and Supplementary Fig. 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 for the sample to be considered to have an amplification or deletion.

Differences in responses to subsequent chemotherapy and between groups of patients, who had *PIK3CA* or *AKT* 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% CO_2 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.

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

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

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-GSK3β 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).

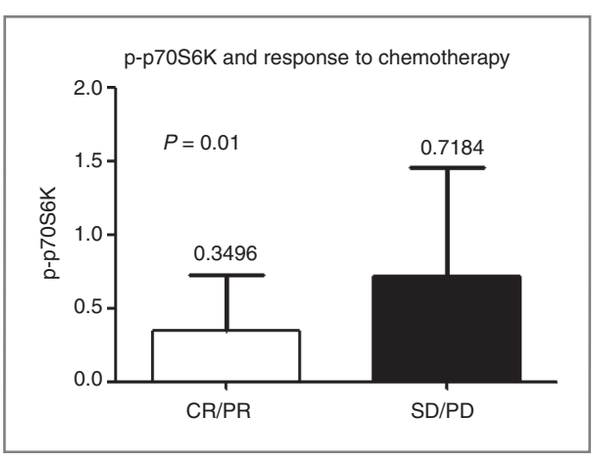

Figure 1. Association of clinical response and signaling output of the PI3K pathway. The differences in the phosphorylation of p70S6K, AKT, and GSK3β in patients, who had a best response of complete response (CR) or partial response (PR) versus patients who had stable disease (SD) or progressive disease (PD), were measured by ELISA in immunomagnetically separated cancer cells from ascites of patients undergoing palliative paracentesis.

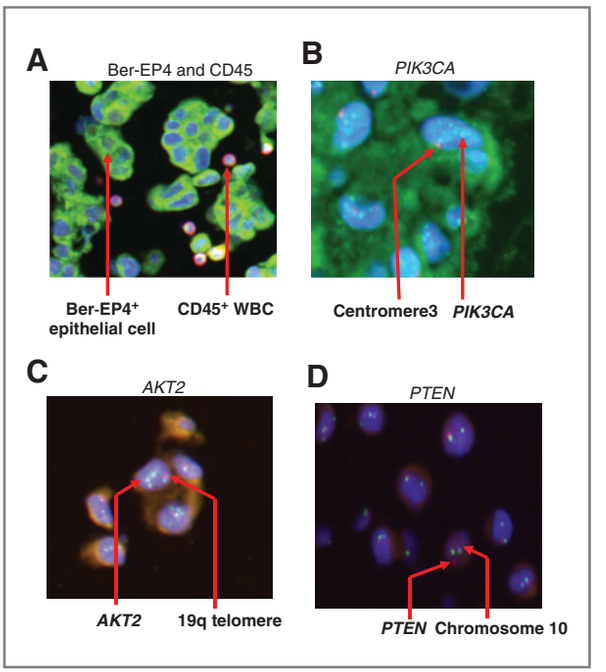

Figure 2. Representative samples of *PIK3CA* and *AKT* amplification and *PTEN* deletion. FISH analysis of *PIK3CA, AKT2* and *PTEN*. Each slide underwent immunofluorescence to positively identify cancer cells (Ber-EP4) and negatively identify noncancer cells, CD45 (CD45; white blood cells), CD68 (macrophages), and podoplanin (mesothelial cells). A, an example of a sample of ascites with cells expressing Ber-EP4 and CD45 are presented at a ×20 magnification. B, in excess of 2 copies of the *PIK3CA* genes (green) in relation to markers for centromere 3 in a cell (red). C, an excess of 2 copies of the *AKT2* gene (green) in relation to 19q telomere in a cell (red). D, less than 1 *PTEN* gene (red) copy in relation to markers for centromere 10 (green). All FISH images are at ×63 magnification.

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 *PIK3CA, AKT2* and loss of *PTEN*

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

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

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

Marker	*PIK3CA* amplified ($n = 6$)	*PIK3CA* not amplified ($n = 57$)	Statistical significance
p-AKT, mean (SD)	0.1833 (0.18)	0.3318(0.5272)	$P = 0.7786$
p-p70S6K, mean (SD)	0.4317 (0.2581)	0.5240 (0.6345)	$P = 0.8698$
p-GSK3β, mean (SD)	1.360 (1.023)	1.055 (0.6914)	$P = 0.7342$
Marker	***AKT2* amplified $n = 6$**	***AKT2* not amplified $n = 57$**	**Statistical significance**
p-AKT, mean (SD)	0.1650 (0.0809)	0.3337 (0.5288)	$P = 0.8513$
p-p70S6K, mean (SD)	0.3883 (0.3662)	0.5286 (0.6289)	$P = 0.5740$
p-GSK3β, mean (SD)	0.9400 (0.2175)	1.099 (0.7575)	$P = 0.7430$
Marker	***PTEN* loss $n = 17$**	**No *PTEN* loss $n = 46$**	**Statistical significance**
p-AKT, mean (SD)	0.1700 (0.2428)	0.3287 (0.4685)	$P = 0.0786$
p-p70S6K, mean (SD)	0.3112 (0.2813)	0.5907 (0.6781)	$P = 0.0631$
p-GSK3β, mean (SD)	1.004 (0.6364)	1.114 (0.7583)	$P = 0.5459$
Marker	**Mutations in *PIK3CA*, *KRAS* or *CMET* or amplification of *PIK3CA*, *AKT2* or loss of *PTEN* ($n = 30$)**	**No mutations in *PIK3CA*, *KRAS* or *CMET* or amplification of *PIK3CA*, *AKT2* or loss of *PTEN* ($n = 33$)**	**Statistical significance**
p-AKT, mean (SD)	0.2133 (0.2252)	0.3518 (0.5409)	$P = 0.7462$
p-p70S6K, mean (SD)	0.4150 (0.3107)	0.6064 (0.7817)	$P = 0.6062$
p-GSK3β, mean (SD)	0.9957 (0.6765)	1.165 (0.7662)	$P = 0.1730$

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.

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-GSK3β in 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 Q61H) 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-GSK3β 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 (Supplementary Table S2). 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

phosphorylation of Ser^{473} and Thr^{308} 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 chemonaive, 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 *AKT*2 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 and design 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, he has received inventors' 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 Chroma Therapeutics. He has been on advisory boards of Piramed Pharma, Chroma Therapeutics, Novartis, Wilex, and Nextech Ventures. As an employee of the ICR, which operates a rewards-to-inventors scheme, intellectual property has been licensed to Piramed Pharma and Genentech (both acquired by Roche) and Astex Pharmeceuticals 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. Gore 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

Development of methodology: C.P. Carden, A. Stewart, P. Thavasu, M. Crespo, S. Miranda, G. Attard, 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/A8274, C309/A11566). 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

1. Parkin DM. Global cancer statistics in the year 2000. Lancet Oncol 2001;2:533–43.
2. Cooke SL, Brenton JD. Evolution of platinum resistance in high-grade serous ovarian cancer. Lancet Oncol 2011;12:1169–74.
3. Yap TA, Carden CP, Kaye SB. Beyond chemotherapy: targeted therapies in ovarian cancer. Nat Rev Cancer 2009;9:167–81.
4. Altomare DA, Wang HQ, Skele KL, De Rienzo A, Klein-Szanto AJ, Godwin AK, et al. AKT and mTOR phosphorylation is frequently detected in ovarian cancer and can be targeted to disrupt ovarian tumor cell growth. Oncogene 2004;23:5853–7.
5. Yuan ZQ, Sun M, Feldman RI, Wang G, Ma X, Jiang C, et al. Frequent activation of AKT2 and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/Akt pathway in human ovarian cancer. Oncogene 2000;19:2324–30.
6. Faratian D, Um I, Wilson DS, Mullen P, Langdon SP, Harrison DJ. Phosphoprotein pathway profiling of ovarian carcinoma for the identification of potential new targets for therapy. Eur J Cancer 2011;47:1420–31.
7. Bunkholt Elstrand M, Dong HP, Odegaard E, Holth A, Elloul S, Reich R, et al. Mammalian target of rapamycin is a biomarker of poor survival in metastatic serous ovarian carcinoma. Hum Pathol 2010;41:794–804.

8. Woenckhaus J, Steger K, Sturm K, Munstedt K, Franke FE, Fenic I. Prognostic value of PIK3CA and phosphorylated AKT expression in ovarian cancer. Virchows Arch 2007;450:387–95.
9. Castellvi J, Garcia A, Rojo F, Ruiz-Marcellan C, Gil A, Baselga J, et al. Phosphorylated 4E binding protein 1: a hallmark of cell signaling that correlates with survival in ovarian cancer. Cancer 2006;107:1801–11.
10. Levine DA, Bogomolniy F, Yee CJ, Lash A, Barakat RR, Borgen PI, et al. Frequent mutation of the PIK3CA gene in ovarian and breast cancers. Clin Cancer Res 2005;11:2875–8.
11. Huang J, Zhang L, Greshock J, Colligon TA, Wang Y, Ward R, et al. Frequent genetic abnormalities of the PI3K/AKT pathway in primary ovarian cancer predict patient outcome. Genes Chromosomes Cancer 2011;50:606–18.
12. Abubaker J, Bavi P, Al-Haqawi W, Jehan Z, Munkarah A, Uddin S, et al. PIK3CA alterations in Middle Eastern ovarian cancers. Mol Cancer 2009;8:51.
13. Kolasa IK, Rembiszewska A, Felisiak A, Ziolkowska-Seta I, Murawska M, Moes J, et al. PIK3CA amplification associates with resistance to chemotherapy in ovarian cancer patients. Cancer Biol Ther 2009;8: 21–6.
14. Nakayama K, Nakayama N, Kurman RJ, Cope L, Pohl G, Samuels Y, et al. Sequence mutations and amplification of PIK3CA and AKT2 genes in purified ovarian serous neoplasms. Cancer Biol Ther 2006;5:779–85.
15. Campbell IG, Russell SE, Choong DY, Montgomery KG, Ciavarella ML, Hooi CS, et al. Mutation of the PIK3CA gene in ovarian and breast cancer. Cancer Res 2004;64:7678–81.
16. Willner J, Wurz K, Allison KH, Galic V, Garcia RL, Goff BA, et al. Alternate molecular genetic pathways in ovarian carcinomas of common histological types. Hum Pathol 2007;38:607–13.
17. Carpten JD, Faber AL, Horn C, Donoho GP, Briggs SL, Robbins CM, et al. A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. Nature 2007;448:439–44.
18. Bleeker FE, Felicioni L, Buttitta F, Lamba S, Cardone L, Rodolfo M, et al. AKT1(E17K) in human solid tumours. Oncogene 2008;27: 5648–50.
19. Nakayama K, Nakayama N, Jinawath N, Salani R, Kurman RJ, Shih Ie M, et al. Amplicon profiles in ovarian serous carcinomas. Int J Cancer 2007;120:2613–7.
20. Obata K, Morland SJ, Watson RH, Hitchcock A, Chenevix-Trench G, Thomas EJ, et al. Frequent PTEN/MMAC mutations in endometrioid but not serous or mucinous epithelial ovarian tumors. Cancer Res 1998;58:2095–7.
21. Lee YK, Park NH. Prognostic value and clinicopathological significance of p53 and PTEN in epithelial ovarian cancers. Gynecol Oncol 2009;112:475–80.
22. Baker AF, Dragovich T, Ihle NT, Williams R, Fenoglio-Preiser C, Powis G. Stability of phosphoprotein as a biological marker of tumor signaling. Clin Cancer Res 2005;11:4338–40.
23. Taylor CR, Levenson RM. Quantification of immunohistochemistry–issues concerning methods, utility and semiquantitative assessment II. Histopathology 2006;49:411–24.
24. Passebosc-Faure K, Li G, Lambert C, Cottier M, Gentil-Perret A, Fournel P, et al. Evaluation of a panel of molecular markers for the diagnosis of malignant serous effusions. Clin Cancer Res 2005;11: 6862–7.
25. Kielhorn E, Schofield K, Rimm DL. Use of magnetic enrichment for detection of carcinoma cells in fluid specimens. Cancer 2002;94: 205–11.
26. Nagrath S, Sequist LV, Maheswaran S, Bell DW, Irimia D, Ulkus L, et al. Isolation of rare circulating tumour cells in cancer patients by microchip technology. Nature 2007;450:1235–9.
27. Pecot CV, Bischoff FZ, Mayer JA, Wong KL, Pham T, Bottsford-Miller J, et al. A novel platform for detection of CK+ and CK- CTCs. Cancer Discov 2011;1:580–6.
28. No JH, Jeon YT, Park IA, Kim YB, Kim JW, Park NH, et al. Activation of mTOR signaling pathway associated with adverse prognostic factors of epithelial ovarian cancer. Gynecol Oncol 2011;121:8–12.
29. Meng Q, Xia C, Fang J, Rojanasakul Y, Jiang BH. Role of PI3K and AKT specific isoforms in ovarian cancer cell migration, invasion and proliferation through the p70S6K1 pathway. Cell Signal 2006;18: 2262–71.
30. Ip CK, Cheung AN, Ngan HY, Wong AS. p70 S6 kinase in the control of actin cytoskeleton dynamics and directed migration of ovarian cancer cells. Oncogene 2011;30:2420–32.
31. Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 1995;378:785–9.
32. Lu Y, Muller M, Smith D, Dutta B, Komurov K, Iadevaia S, et al. Kinome siRNA-phosphoproteomic screen identifies networks regulating AKT signaling. Oncogene 2011;30:4567–77.
33. Attard G, Clark J, Ambroisine L, Fisher G, Kovacs G, Flohr P, et al. Duplication of the fusion of TMPRSS2 to ERG sequences identifies fatal human prostate cancer. Oncogene 2008;27:253–63.
34. Sircar K, Yoshimoto M, Monzon FA, Koumakpayi IH, Katz RL, Khanna A, et al. PTEN genomic deletion is associated with p-Akt and AR signalling in poorer outcome, hormone refractory prostate cancer. J Pathol 2009;218:505–13.
35. Reid AH, Attard G, Ambroisine L, Fisher G, Kovacs G, Brewer D, et al. Molecular characterisation of ERG, ETV1 and PTEN gene loci identifies patients at low and high risk of death from prostate cancer. Br J Cancer 2010;102:678–84.
36. Fumagalli D, Gavin PG, Taniyama Y, Kim SI, Choi HJ, Paik S, et al. A rapid, sensitive, reproducible and cost-effective method for mutation profiling of colon cancer and metastatic lymph nodes. BMC Cancer 2010;10:101.
37. Peng DJ, Wang J, Zhou JY, Wu GS. Role of the Akt/mTOR survival pathway in cisplatin resistance in ovarian cancer cells. Biochem Biophys Res Commun 2010;394:600–5.
38. Liu LZ, Zhou XD, Qian G, Shi X, Fang J, Jiang BH. AKT1 amplification regulates cisplatin resistance in human lung cancer cells through the mammalian target of rapamycin/p70S6K1 pathway. Cancer Res 2007;67:6325–32.
39. Xu R, Nakano K, Iwasaki H, Kumagai M, Wakabayashi R, Yamasaki A, et al. Dual blockade of phosphatidylinositol 3′-kinase and mitogen-activated protein kinase pathways overcomes paclitaxel-resistance in colorectal cancer. Cancer Lett 2011;306:151–60.
40. Bian CX, Shi Z, Meng Q, Jiang Y, Liu LZ, Jiang BH. P70S6K 1 regulation of angiogenesis through VEGF and HIF-1alpha expression. Biochem Biophys Res Commun 2010;398:395–9.
41. Raynaud FI, Eccles SA, Patel S, Alix S, Box G, Chuckowree I, et al. Biological properties of potent inhibitors of class I phosphatidylinositide 3-kinases: from PI-103 through PI-540, PI-620 to the oral agent GDC-0941. Mol Cancer Ther 2009;8:1725–38.
42. Mabuchi S, Altomare DA, Connolly DC, Klein-Szanto A, Litwin S, Hoelzle MK, et al. RAD001 (Everolimus) delays tumor onset and progression in a transgenic mouse model of ovarian cancer. Cancer Res 2007;67:2408–13.
43. Wang Y, Helland A, Holm R, Kristensen GB, Borresen-Dale AL. PIK3CA mutations in advanced ovarian carcinomas. Hum Mutat 2005;25:322.
44. Kurose K, Zhou XP, Araki T, Cannistra SA, Maher ER, Eng C. Frequent loss of PTEN expression is linked to elevated phosphorylated Akt levels, but not associated with p27 and cyclin D1 expression, in primary epithelial ovarian carcinomas. Am J Pathol 2001;158:2097–106.
45. Wing LY, Chen HM, Chuang PC, Wu MH, Tsai SJ. The mammalian target of rapamycin-p70 ribosomal S6 kinase but not phosphatidylinositol 3-kinase-Akt signaling is responsible for fibroblast growth factor-9-induced cell proliferation. J Biol Chem 2005;280: 19937–47.
46. Tabancay AP Jr, Gau CL, Machado IM, Uhlmann EJ, Gutmann DH, Guo L, et al. Identification of dominant negative mutants of Rheb GTPase and their use to implicate the involvement of human Rheb in the activation of p70S6K. J Biol Chem 2003;278:39921–30.
47. Theurillat JP, Metzler SC, Henzi N, Djouder N, Helbling M, Zimmermann AK, et al. URI is an oncogene amplified in ovarian cancer cells and is required for their survival. Cancer Cell 2011;19:317–32.

48. O'Brien C, Wallin JJ, Sampath D, GuhaThakurta D, Savage H, Punnoose EA, et al. Predictive biomarkers of sensitivity to the phosphatidylinositol 3′ kinase inhibitor GDC-0941 in breast cancer preclinical models. Clin Cancer Res 2010;16:3670–83.

49. Janku F, Tsimberidou AM, Garrido-Laguna I, Wang X, Luthra R, Hong DS, et al. PIK3CA mutations in patients with advanced cancers treated with PI3K/AKT/mTOR axis inhibitors. Mol Cancer Ther 2011;10:558–65.

50. Clarke PA, Workman P. Phosphatidylinositide-3-kinase inhibitors: addressing questions of isoform selectivity and pharmacodynamic/predictive biomarkers in early clinical trials. J Clin Oncol 2012;30:331–3.