Intracellular signalling via the AKT axis and downstream effectors is active and prognostically significant in cancer of unknown primary (CUP): a study of 100 CUP cases

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Received 14 December 2011; revised 14 February 2012; accepted 15 February 2012

Background: Hypothesising that cancer of unknown primary (CUP) may harbour unique characteristics, we present a translational study of the immunohistochemical expression and clinical correlation of key PTEN/AKT pathway molecules.

Patients and methods: We collected 100 paraffin-embedded CUP tissue blocks. We studied using tissue microarrays the expression of PTEN, phospho-AKT, Cyclin D1, p21, phospho-RPS6. From the percentage of staining tumour cells and the literature, we selected cut-offs to classify the expression of each biomolecule. We correlated IHC expression with clinical data.

Results: PTEN, pAKT, and pRPS6 showed frequent expression. At univariate analysis, high IHC expression of pAKT and pRPS6 displayed statistically significant association with worse survival. Prognosis was worse upon concurrent high IHC expression of pMAPK and pAKT [median overall survival = 8 months [95% confidence interval (CI) 5.3–10.7] versus 17 months [95% CI 13.1–20.9]]. In multivariate analysis, high p21 was associated with better survival (risk ratio [RR] = 0.34 [95% CI 0.16–0.73], P = 0.005). High expression of pAKT (RR = 2.39 [95% CI 1.23–4.66], P = 0.01) or pRPS6 (RR = 2.76 [95% CI 1.31–5.84], P = 0.008) was associated with worse survival.

Conclusions: p21 expression conferred favourable prognosis, while high pAKT or pRPS6 expression predicted worse prognosis. Concurrent MAPK and pAKT expression had a marked adverse impact on survival.

Key words: AKT, cancer of unknown primary (CUP), p21, PTEN, RPS6, translational study

Introduction

Cancer of unknown primary (CUP) is defined as histologically confirmed presence of malignancy in the absence of identifiable primary after a standardized diagnostic workup. It represents ~3% of all cancers diagnosed [1]. The recognition of ‘favourable’ prognosis subsets has been important for improving patient outcome by proposing primary site-specific therapeutic approaches derived from the management of known primary site tumours [2]. However, for the majority of CUPs that are classified in the ‘unfavourable’ subsets, no treatment can provide enough clinical benefit to be considered standard of care [3]. Several studies tried to identify a potential known primary site tumour using genomic technologies to better understand the biology of CUP [4], with mixed success [5]. The bizarre clinical course of these tumours and the resistance to therapy has predictably led to the notion that CUP may harbour unique biological characteristics and that treatment targeting molecular defects common in CUP may provide some benefit to the patients [6].

The PTEN/AKT pathway has been proven central in many cellular processes contributing to cancer initiation and progression, mostly those involved in cellular growth, proliferation, and inhibition of apoptosis [7]. Deregulation can occur through mutations, overexpression, or aberrant activation of various biomolecules and is detected in various
tumour types. This pathway exhibits extensive crosstalk with others controlling pivotal cellular processes. The central molecules PTEN and AKT, as well as direct (mTOR, PDK1) or indirect (cyclin D1, p21) downstream molecules, present possible targets for therapeutic inhibition.

Few data exist on the status of the PTEN/AKT pathway in CUP. To elucidate the protein expression and activation status of the components of the pathway, we explored by immunohistochemistry (IHC) key molecules of the cascade. We present a retrospective translational study of the immunohistochemical expression of phosphorylated (activated) epitopes of PTEN (phosphatase and tensin-like protein), AKT (RAC-alpha serine/threonine-protein kinase), RPS6 (40S ribosomal protein S6), p21 (cyclin-dependent kinase inhibitor 1A, CDKN1A), and CCND1 (cyclin D1) in 100 clinically annotated CUP tumours, and the correlation of IHC expression with clinical and pathological characteristics and outcome data.

**methods**

Among 150 patients with CUP managed at Hellenic Cooperative Oncology Group (HeCOG)-affiliated hospitals and in oncological departments from Valencia University Hospital, Spain, and Cluz, Romania, from 1997 to 2009, we retrospectively collected formalin-fixed paraffin-embedded tissue blocks from 100 patients (Figure 1). We received written permission to use their clinical data and tumour material from all patients. Detailed patients’ characteristics as well as data regarding the CUP pathology, clinical subgroups, management, and outcome are summarised in supplemental Table S1 (available at Annals of Oncology online).

An experienced pathologist (MB) reviewed haematoxylin- and eosin-stained slides from tissue blocks for adequacy of material and calculation of the percentage of tumour cells in each case. Two tissue cores of 1.5 mm from 100 clinically annotated CUP tumours were loaded in four tissue microarrays (TMAs) in duplicate microarray paraffin blocks and studied for IHC expression of signal transducers and regulators (full-length PTEN, phospho-AKT1, 2, 3 at Thr308), cell cycle controllers (Cyclin D1, p21), effector of protein synthesis (phospho-RPS6 at Ser235/236). The TMA was

![Figure 1. REMARK flow chart.](image-url)
constructed using the Beecher Instruments MTA-1 Tissue Arrayer (Beecher Instruments, Sun Prairie, WI). Each TMA block contained 30–87 tissue cores from the original tumour blocks, while cores from various neoplastic and non-neoplastic tissues were also included, serving as assay controls.

Serial 2.5-μm-thick sections from the TMA blocks were cut at the Laboratory of Molecular Oncology of the Hellenic Foundation of Cancer Research (School of Medicine, Aristotle University of Thessaloniki) mounted on adhesive microscope slides and subjected to IHC labelling using Bond Max™ (Leica Microsystems, Germany) and i6000 (Biogenex, San Ramon, CA) autostainers. The sections were stained with antibodies against PTEN, clone 6H2.L, Dako, Glostrup, DK, at dilution 1:200, for 30 minutes; phosphorylated Akt1/2/3 at Threonine 308 (SC, San Ramon, CA), at dilution 1:1000 overnight at 4°C; Cyclin D1, clone SP4 (Spring, Pleasanton, CA) at dilution 1:70 for 30 minutes; p21, clone SX118 (Dako) at dilution 1:60 for 30 minutes; and phospho-S6 ribosomal protein at Serine 235/236, clone D57.2.2E (CST, Danvers, MA) at dilution of 1:100 overnight at 4°C. Binding of antibodies was visualized using Bond Polymer Refine Detection (Leica Biosystems, Newcaste Upon Tyne, UK). DAB (3,3-diaminobenzidine) was used as a chromogen and haematoxylin as a counterstain. The quality of IHC staining was evaluated using the internal positive controls.

The percentage of staining tumour cells was calculated and the median value from the two microarray cores was recorded by two independent pathologists (AG, VS). After a thorough review of the literature, we concluded that no firmly established cut-off values existed for the biomolecules under study. We applied distributional analyses of recorded percentages of staining tumour cells in all samples and compared them with the cut-off values used in the medical literature in order to select cut-offs that separated each distribution to natural, though not equal, groups of cases. We used these cut-offs for classification of CUP cases to negative or positive for expression of each biomolecule, without performing exploratory analyses for prognostic significance. CUP cases considered positive for IHC expression of total PTEN protein, phospho-AKT1/2/3 at Thr308, phospho-RPS6 at Ser235/236, total p21, and total Cyclin D1 were those with median percentage of staining tumour cells of higher than 60% (PTEN), 85% (phospho-AKT), 60% (RPS6), 10% (p21), and 20% (Cyclin D1). Prognostic analyses were subsequently carried out only with the chosen cut-offs. At the final stage of the analysis, in order to avoid missing cut-offs with prognostic utility for patient outcome, we carried out receiver-operator curve (ROC) analysis of all biomolecule IHC staining percentages as test variables with death as the state variable. ROC analysis did not detect a missed cut-off with robust prognostic value.

Prognostic significance correlation among all markers under study was assessed using the Spearman correlation coefficient (ρ). The univariate Cox proportional hazard regression model was applied to study prognostic significance of variables for survival and the χ² exact test for the predictive significance of variables for response to therapy. Progression-free survival (PFS) was calculated from diagnosis to disease progression or death; overall survival (OS) was calculated from diagnosis to date of death or last follow-up with the Kaplan–Meier product-limit method. In multivariate analysis, a backward selection procedure with a removal criterion of $P > 0.10$ based on likelihood ratio test was carried out. All calculated $P$ values were two-sided and findings were considered significant when $P < 0.05$. Analyses were carried out with the use of the SPSS 17.0 statistical software package (SPSS Inc., Delaware, IL).

results

patient, tumour, and IHC demographics

Adenocarcinoma was the most frequent histological subtype, and >90% of tumours were of moderate or poor differentiation. Predominantly visceral involvement, midline nodal, and peritoneal or pleural carcinomatosis were the most frequent CUP subgroups under study. About three-quarters of the patients were of good performance status [Eastern Cooperative Oncology Group/World Health Organisation (ECOG/WHO) performance status 0 or 1] and most received platinum-based chemotherapy through institutional treatment protocols, half of whom responded (supplemental Table S1, available at Annals of Oncology online). Various CUP subtypes were represented in our patient sample, with 30% of patients belonging to the unfavourable predominantly visceral group.

At a median follow-up of 28 months, 61 patients had died. Median PFS was 7 months (95% CI 4.8–9.2) and median OS was 12 months (95% CI 8.7–15.3) for all patients. According to CUP subgroups, median OS was 11 months (95% CI 6.8–15.2) for predominantly nodal involvement, 17 months (95% CI 0.0–34.0) for peritoneal or pleural carcinomatosis, and 9 months (95% CI 4.1–13.9) for predominantly visceral involvement. No statistically significant difference was detected for OS among the CUP subgroups.

PTEN IHC results were available for 98 samples, AKT results for 97 samples, RPS6 results for 99 samples, p21 results for 94 samples, and cyclin D1 results for 97 samples. The localization of staining was in the membrane and cytoplasm for all studied biomolecules. PTEN, pAKT, and pRPS6 showed frequent expression (high median percentage of staining tumour cells) in the read-outs of the tissue arrays (Table 1 and Figure 2).

IHC correlations with clinicopathologic characteristics

p21 and Cyclin D1 showed statistically significant correlations with different CUP subgroups: high p21 expression was seen in 76% of predominantly nodal versus 63% of predominantly visceral versus 44% of the peritoneal or pleural carcinomatosis subgroup ($P = 0.025$). In addition, high Cyclin D1 staining was observed in 60% of predominantly nodal versus 42% of predominantly visceral versus 22% of the peritoneal or pleural carcinomatosis subgroup ($P = 0.04$). Cyclin D1 showed statistically significant correlation with histological type, as 70% of squamous carcinoma exhibited high expression versus only 39% of adenocarcinoma and 29% of unspecified carcinoma.

prognostic and predictive utility of biomarkers

We examined whether the IHC expression of the biomolecules under study correlated with response to chemotherapy. We

<table>
<thead>
<tr>
<th>Molecule</th>
<th>N</th>
<th>Staining tumour cells, median % (IQR)</th>
<th>Cut-off (%)</th>
<th>Positive cases (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTEN</td>
<td>98</td>
<td>60 (0–89)</td>
<td>60</td>
<td>49</td>
</tr>
<tr>
<td>pAKT</td>
<td>97</td>
<td>97 (71–100)</td>
<td>85</td>
<td>71</td>
</tr>
<tr>
<td>pRPS6</td>
<td>99</td>
<td>72 (27–90)</td>
<td>60</td>
<td>59</td>
</tr>
<tr>
<td>p21</td>
<td>94</td>
<td>17 (4–61)</td>
<td>10</td>
<td>57</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>97</td>
<td>12 (1–52)</td>
<td>20</td>
<td>43</td>
</tr>
</tbody>
</table>

IQR, interquartile range.
found no statistically significant associations, with the exception of increased p21 expression that displayed a trend towards improved response rate ($P = 0.09$).

At univariate analysis of prognostic impact on survival, high IHC expression of phospho-AKT and phospho-RPS6 displayed a statistically significant association with adverse outcome (Table 2). In fact, the adverse prognostic impact was enhanced in tumours with concurrent high IHC expression of both p44/42 MAPK Thr202/Tyr204 (data not shown) and pAKT Thr308, as the median OS of these patients was 8 months (95% CI 5.3–10.7) versus 17 months (95% CI 13.1–20.9) for those without concurrent IHC expression of both molecules (Figure 3). Clinicopathologic factors that were significantly correlated with lower OS were male gender (median OS 9 versus 17 months, $P = 0.0005$), ECOG/WHO performance status of two or worse (median OS 5 versus 14 months, $P = 0.001$), and non-platinum chemotherapy (median OS 7 versus 15 months, $P = 0.029$).

The prognostic impact of each biomolecule was additionally analysed in each CUP clinicopathologic subgroup as a hypothesis-generating exploratory analysis, despite the small sample size. Factors associated with worse prognosis were found in the visceral CUP subgroup: high Cyclin D1 expression (median OS 5 versus 13 months, $P = 0.027$), high RPS6 expression (median OS 8 versus 19 months, $P = 0.034$), and concurrent high expression of both AKT and MAPK (median OS 8 versus 15 months, $P = 0.011$).

Multivariate analysis was undertaken within a broader investigation exploring the impact of IHC expression of biomolecules from different pathways on CUP survival. Apart from the molecules presented here, Notch1-3, Jagged, phospho-p44/42 MAPK at Thr202/Tyr204, and cMet were also explored in the full analysis and will be presented separately.

For the pathway under study, high p21 was associated with reduced risk of death (risk ratio [RR] = 0.34 [95% CI 0.16–0.73], $P = 0.005$) (Figure 4), while high expression of phospho-AKT (RR = 2.39 [95% CI 1.23–4.66], $P = 0.01$), and high expression of phospho-RPS6 (RR = 2.76 [95% CI 1.31–5.84], $P = 0.008$) were associated with increased risk of death.

Table 2. Univariate analysis of prognostic utility of pAKT<sup>Thr308</sup> and pRPS6<sup>Ser235/236</sup> expression

<table>
<thead>
<tr>
<th>Protein</th>
<th>Median OS (months)</th>
<th>95% CI (months)</th>
<th>Breslow $P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAKT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>11</td>
<td>7.3–14.7</td>
<td>0.047</td>
</tr>
<tr>
<td>Low</td>
<td>15</td>
<td>8.7–21.1</td>
<td></td>
</tr>
<tr>
<td>pRPS6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>9</td>
<td>6.0–12.0</td>
<td>0.030</td>
</tr>
<tr>
<td>Low</td>
<td>17</td>
<td></td>
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</tr>
</tbody>
</table>

Our study showed that most of the PTEN/AKT pathway molecules we examined exhibited high IHC expression in our sample of 100 CUP patients. Although only a median of 17% of tumour cells stained for p21, its expression was associated with a favourable prognosis in multivariate analysis. In the same analysis, high AKT or RPS6 expression was associated with worse prognosis.

These results suggest that signalling through the PTEN/AKT pathway may be important for the clinical outcome of CUP patients. To enhance the validity of our results, we tested for the phosphorylated (activated) form of AKT and the activated
form of RPS6, suggesting that their activation rather than apparent accumulation, mediates the tumour effects of the pathway. The hypothesized effects are supported by the known mechanisms of action for these molecules. Specifically, AKT is a central molecule in the initiation and proliferation drive of tumours. It is activated by PI3K and, through crosstalk, with

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**Figure 3.** Overall survival of CUP patients by concurrent pAKT and pMAPK IHC expression (8 months [95% CI 5.3–10.7] versus 17 months [95% CI 13.1–20.9]). CI, confidence interval.

**Figure 4.** Overall survival of CUP patients by p21 IHC expression (RR = 0.34 [95% CI 0.16–0.73],  P = 0.005). CI, confidence interval.
Deregulation of the PTEN/AKT pathway has been observed in various tumour types [7]. Therapeutic targeting of this pathway, either upstream (e.g. with trastuzumab in breast cancer, erlotinib and gefitinib in non-small-cell lung cancer, cetuximab and panitumumab in colorectal cancer) or downstream (e.g. with mTOR inhibitors in clear-cell renal cancer) has been proven beneficial and is in widespread clinical use. As the pathway exhibits extensive crosstalk with several other intracellular pathways [8], novel agents that inhibit signalling at various single or multiple sites are possibly active [12] and are in development [13]. We expect this pathway to be relevant in CUP, as well, although no other studies have tested this assumption directly. Rather, our results provide further evidence that CUP tumours display to some extent certain common characteristics that may allow us to approach them as a coherent group, both diagnostically and therapeutically.

Our conclusions are somewhat limited by the fact that not all patients belong to a single CUP subtype, especially the unfavourable prognosis group, which has been recognized as the true CUP subtype. Although there is no proof that even this is a coherent group, it would be useful to include only patients whom we cannot otherwise discern in favourable subgroups. The moderate size of our study cohort precludes drawing reliable data from further subgroup analysis. About 80% of the patients received systemic treatment, with the majority receiving platinum-based chemotherapy. These results cannot discern whether the correlation of IHC biomolecule expression with survival is prognostic or predictive; although management was similar for most patients, it was not uniform for all. Moreover, very few patients received best supportive care only.

To the best of our knowledge, this is the only analysis published to date investigating the involvement of pivotal signalling pathways in the biology of CUP. Although our results provide insights into the molecular characteristics of CUP, they should only be seen as exploratory and hypothesis generating. If validated in prospective studies, they could inform the rational development of agents targeting the underlying mechanisms that provide CUP with its seminal features of rapid growth, widespread metastases, and resistance to chemotherapy.

**Disclosure**

The authors have declared no conflicts of interest.

8. Cully M, You H, Levine AJ, Mak TW. Beyond PTEN mutations: the PI3K pathway and Cyclin D1, which are also included in this study), and enhances cellular metabolism. On the other hand, PTEN is the most important suppressor of the PI3K/AKT pathway, although not the only regulating molecule of the cascade [8]. RPS6 encodes a component of the 40S ribosomal subunit and is a major substrate of protein kinases in the ribosome. It probably contributes to the control of cellular growth and proliferation, as well as apoptosis, through the selective translation of particular classes of messenger RNA [9, 10]. Cyclin D1 and p21 are mostly opposing cell cycle regulators, with Cyclin D1 promoting and p21 inhibiting the G1/S transition, while also interacting with other tumour suppressors, as Rb and p53 [11]. Our findings, therefore, that high activated AKT and activated RPS6 expression correlate with worse prognosis while high p21 expression correlates with superior outcome are consistent with the established knowledge of cellular molecular dynamics.

Obviously, although our study reveals aspects of the molecular dynamics of CUP, it is not sufficient to differentiate these tumours from cancer of known primary sites. Our conclusions are somewhat limited by the fact that not all patients belong to a single CUP subtype, especially the unfavourable prognosis group, which has been recognized as the true CUP subtype. Although there is no proof that even this is a coherent group, it would be useful to include only patients whom we cannot otherwise discern in favourable subgroups. The moderate size of our study cohort precludes drawing reliable data from further subgroup analysis. About 80% of the patients received systemic treatment, with the majority receiving platinum-based chemotherapy. These results cannot discern whether the correlation of IHC biomolecule expression with survival is prognostic or predictive; although management was similar for most patients, it was not uniform for all. Moreover, very few patients received best supportive care only.

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