Enhanced protein kinase B/Akt signalling in pituitary tumours

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Abstract

Pituitary tumours have previously been shown to harbour several abnormalities that cause deregulation of the cell cycle, particularly down-regulation of expression of the cyclin-dependent kinase inhibitor p27. However, it has been unclear whether these are the primary initiating events, or are secondary to other more proximate alterations in signalling pathways. In other cellular systems the Akt signalling pathway has been associated with downstream modulation of cell-cycle control. The aim of the present study was to test the hypothesis that Akt signalling is enhanced in pituitary tumours, and to see if changes in Akt expression are related to previous findings on low expression levels of the nuclear cell-cycle inhibitor p27 in pituitary tumours. We examined normal and adenomatous human pituitary tissue for mRNA and protein expression of Akt1, Akt2 and p27, and the activation of Akt, as well the phosphatase involved in the inactivation of Akt, phosphatase and tensin homologue deleted on chromosome 10 (PTEN). In pituitary adenomas Akt1 and Akt2 mRNA were found to be over-expressed compared with normal pituitary, while PTEN transcripts showed similar levels between the two tissue types. Immunohistochemical expression of phospho-Akt was found to be higher in the tumours than normal pituitaries, while the protein expression of nuclear p27 and PTEN was lower in the adenomas. However, the expression of p27 and Akt were not directly correlated. PTEN sequencing revealed no mutation in the coding region of the gene in pituitary adenomas, and thus we did not locate a cause for the increased phosphorylation of Akt. In summary, we have shown over-expression and activation of the Akt pathway in pituitary tumours, and we speculate that cell-cycle changes observed in such tumours are secondary to these more proximate alterations. Since Akt is a major downstream signalling molecule of growth factor-liganded tyrosine kinase receptors, our data are most compatible with an abnormality at this level as the primary driver of pituitary tumorigenesis.

Introduction

Pituitary tumours account for 10–15% of all intracranial neoplasms. Despite extensive research, the pathogenesis of the majority of pituitary adenomas remains to be clarified. Deregulation of various cell-cycle pathways have been reported to be a frequent event in these tumours (Hibberts et al. 1999, Simpson et al. 1999, Lidhar et al. 1999, Zhang et al. 1999, Farrell & Clayton 2000, Simpson et al. 2000, 2001, Ruebel et al. 2001, Asa & Ezzat 2002, Korbonits et al. 2002, Musat et al. 2002). Progression through the cell cycle is a finely regulated process controlled by cyclically operating biochemical machinery constructed from a set of interacting proteins that induce and coordinate proper progression through the cycle; these include cyclins and cyclin-dependent kinases (CDKs), which promote cell-cycle progression, and their inhibitors that oppose cell proliferation.

Our previous studies and those of others on the cell-cycle inhibitor p27 showed that nuclear p27 protein...
expression in human pituitary adenomas was lower than in normal pituitary tissue, and this was especially true for corticotroph adenomas and pituitary carcinomas (Bamberger et al. 1999, Lidhar et al. 1999, Lloyd 2001), despite p27 mRNA expression being similar in tumour samples in comparison with normal pituitaries. Furthermore, the p27 gene has not been found to be mutated in human pituitary tumours (Tanaka et al. 1997, Dahia et al. 1998). This suggested that p27 might be subject to excessive proteolytic degradation in such tumours. The fall in nuclear p27 expression may play an important role in leading to over-expression of cyclin E via CDK2, and hence the increased proliferation of adenomas compared with normal pituitary and subsequent derangements in mitotic regulation. We then showed that there was a negative association between the nuclear expression of p27 and Skp2 (the F-box protein addressing it for ubiquitination), suggesting that increased Skp2 might play at least a part in this process (Musat et al. 2002). However, Skp2 expression was not altered overall in tumours compared with normal pituitary. Furthermore, we studied several other agents involved in the degradatory pathways of p27 via phosphorylation at Thr187 (Körbonits et al. 2002), including Jab1, which enables p27 to be exported from the nucleus (Claret et al. 1996), and the Jab1 inhibitor macrophage inhibitory factor (MIF) (Pyle et al. 2003). However, we were unable to account for the abnormal cellular localization of p27 in pituitary tumours in terms of known regulators of its degradatory pathways.

Mitogenic signalling by receptor tyrosine kinases that increase phosphatidylinositol 3-kinase (PI3K) activity lead to activation of Akt (protein kinase B, PKB), which in turn triggers a cascade of responses — cell growth, proliferation, survival and increased motility — which drive tumour progression in breast, ovarian, prostate, pancreatic and thyroid cancers. PI3K is negatively regulated by phosphatases such as phosphatase and tensin homologue deleted on chromosome 10 (PTEN), which has been demonstrated to be an important tumour-suppressor gene that blocks the PI3K pathway (Datta et al. 1999). The serine/threonine protein kinase PKB/Akt has emerged as a crucial regulator of growth, proliferation, differentiation and apoptosis, and is a major downstream effector of PI3K. Three isoforms of Akt (Akt1, Akt2, Akt3) have been cloned that are in general broadly expressed, although different tissue-expression patterns have been described (Vivanco & Sawyers 2002). The most prominent isoforms involved in cell proliferation are Akt1 and Akt2. Amplification of Akt2 has been reported in breast, ovarian (Bellacosa et al. 1995) and pancreatic cancers (Cheng et al. 1996), and over-expression and over-activation of Akt1 and Akt2 have been described recently in thyroid cancer (Ringel et al. 2001). The role of Akt in supporting tumorigenesis is due to phosphorylation and relocalization of key regulatory molecules involved in apoptosis, cell growth and proliferation. With respect to cell-cycle progression, Akt has been shown to be involved in preventing cyclin D1 degradation, and more recently has been shown to negatively influence the expression and localization of cell-cycle inhibitors such as p21 and p27 (Vivanco & Sawyers 2002). It was for this latter reason that we speculated that changes in Akt expression or activation may occur in pituitary adenomas. In the present study we therefore aimed to determine whether enhanced Akt signalling or PTEN loss-of-function mutations are a feature of pituitary adenomas.

Materials and methods

Samples for real time reverse transcriptase (RT)-PCR

Human pituitary tumours (n=65; Table 1) removed at trans-sphenoidal surgery were classified histologically, using haematoxylin and eosin, reticulin and immunohistochemical hormone staining as somatotroph adenomas (n=20), corticotroph adenomas (n=8), prolactinomas (n=7) and non-functioning pituitary adenomas (NFPAs; n=30). Pituitaries removed at autopsy from patients who had died from

<table>
<thead>
<tr>
<th>Clinical diagnosis</th>
<th>Samples studied for Akt1 and Akt2 (size of tumour)</th>
<th>Samples studied for PTEN (size of tumour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Somatotroph adenomas</td>
<td>n=13 (11 macro; 2 micro)</td>
<td>n=16 (14 macro; 2 micro)</td>
</tr>
<tr>
<td>Corticotroph adenomas</td>
<td>n=7 (2 macro; 5 micro)</td>
<td>n=6 (3 macro; 3 micro)</td>
</tr>
<tr>
<td>Prolactinomas</td>
<td>n=7 (4 macro; 3 micro)</td>
<td>n=7 (4 macro; 3 micro)</td>
</tr>
<tr>
<td>NFPAs</td>
<td>n=17 (10 macro; 7 NA)</td>
<td>n=25 (20 macro; 1 micro; 4 NA)</td>
</tr>
<tr>
<td>Normal pituitary tissue</td>
<td>n=9</td>
<td>n=12</td>
</tr>
</tbody>
</table>

NA, data not available.

Table 1 Clinical details of patients studied with real time RT-PCR
normal pituitary

NA, data not available.

NFPAs

Prolactinomas

adenomas

Corticotroph immunostaining (all pituitary hormones expressed)

gery were used that proved normal pituitary on resection specimens removed at trans-sphenoidal surgery and prepared for pathological examination by formalin fixation and paraffin embedding. These were classified histologically as somatotroph adenomas (n = 10), corticotroph tumours (n = 10), prolactinomas (n = 10) or NFPAs (n = 10). For normal pituitary controls (n = 10) part of the resection specimens removed at trans-sphenoidal surgery were used that proved normal pituitary on immunostaining (all pituitary hormones expressed) and on reticulin staining (normal reticulin pattern; Lidhar et al. 1999).

RNA preparation

Total RNA was extracted from tissue using the Promega SV isolation kit and protocol (Promega, Southampton, UK), which includes a DNase step. RNA was quantified by spectrophotometry (Cecil CE Computing Double Beam UV Spectrophotometer; Cecil Instruments, Cambridge, UK) and by using the RiboGreen RNA Quantitation Reagent Kit (Molecular Probes Europe BV, Leiden, The Netherlands) in a 1420 Multilabel Counter Wallac Victor2. The RNA was diluted to 50 ng/μl for use in the RT-PCR assay and stored at –80°C.

Real time RT-PCR

Real-time RT-PCR primers and intron-spanning probes for Akt1, Akt2, PTEN and 18 S rRNA were designed using Primer Express software (PE-Applied Biosystems, Warrington, Cheshire, UK) based on the published sequence data of the genes (GenBank accession numbers: Akt1, NM_005163; Akt2, NM_001626; PTEN, NM_000314) and synthsized by MWG Biotech (Ebersberg, Germany). The TaqMan probes were labelled with a reporter dye (6-carboxy-fluorescein, FAM) at the 5’ end and a quencher dye (6-carboxy-tetramethylrhodamine, TAMRA) at the 3’ end.

Two-step real-time RT-PCR was performed for relative quantification of Akt1, Akt2 and PTEN transcripts using a TaqMan Gold assay (PE-Applied Biosystems). Total RNA (1 μg) for each sample was reverse transcribed in a 50 μl reaction. Another five dilutions of mRNA from a different sample (i.e. 1, 0.2, 0.04, 0.008 and 0.0016 μg) were used in an RT reaction to produce DNA for the real-time quantitative PCR (RT-PCR) standard curve.

RQ-PCR was performed separately for each Akt isoform, PTEN and 18 S rRNA together with a five-point standard curve for each target gene. cDNA equivalent of 20 ng total RNA (1 μl room-temperature RT reaction mixture) was used in a 10 μl PCR containing TaqMan PCR Universal MasterMix (PE-Applied Biosystems) primers and probes. The PCR reactions for each gene were performed in triplicates on a 384-well plate by the ABI 7900 Sequence Detection System. Fluorescence detection was performed during each PCR cycle (real-time run type) and transformed into a relative quantity of gene expression (referred to as the logarithmic input amount) using a five-point-dilution standard curve run on each plate. Normalized results for Akt1, Akt2 and PTEN

<table>
<thead>
<tr>
<th>Clinical diagnosis</th>
<th>No. of samples (size of tumour)</th>
<th>Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Somatotroph adenomas</td>
<td>n = 10 (8 macro; 2 micro)</td>
<td>M = 8, F = 2</td>
</tr>
<tr>
<td>Corticotroph adenomas</td>
<td>n = 10 (3 macro; 7 micro)</td>
<td>M = 2, F = 8</td>
</tr>
<tr>
<td>Prolactinomas</td>
<td>n = 10 (7 macro; 3 micro)</td>
<td>M = 4, F = 6</td>
</tr>
<tr>
<td>NFPAs</td>
<td>n = 10 (10 macro)</td>
<td>M = 4, F = 6</td>
</tr>
<tr>
<td>Normal pituitary tissue</td>
<td>n = 10</td>
<td>M = 2, F = 8</td>
</tr>
</tbody>
</table>

non-endocrine disease were used as normal controls (n = 12). Informed consent was obtained from each subject or subject’s guardian, and the local Ethics Committee approved all studies.

Samples for sequencing

33 pituitary tumours (Table 2) were used for PTEN sequencing. Benign adenomas were categorized as growth hormone-secreting tumours (n = 4), adrenocorticotropic hormone (corticotropin)-secreting tumours (n = 4), prolactinomas (n = 2) and NFPAs (n = 23).

Samples for immunohistochemistry

Pituitary tissue (n = 50; Table 3) was collected at trans-sphenoidal surgery and prepared for pathological examination by formalin fixation and paraffin embedding. These were classified histologically as somatotroph adenomas (n = 10), corticotroph tumours (n = 10), prolactinomas (n = 10) or NFPAs (n = 23).

Table 2. Clinical details of patients studied for PTEN sequencing

<table>
<thead>
<tr>
<th>Clinical diagnosis</th>
<th>No. of samples (size of tumour)</th>
<th>Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Somatotroph adenomas</td>
<td>n = 4 (4 macro)</td>
<td>M = 2, F = 2</td>
</tr>
<tr>
<td>Corticotroph adenomas</td>
<td>n = 4 (1 macro; 3 micro)</td>
<td>M = 1, F = 3</td>
</tr>
<tr>
<td>Prolactinomas</td>
<td>n = 2 (2 macro)</td>
<td>M = 1, F = 1</td>
</tr>
<tr>
<td>NFPAs</td>
<td>n = 23 (22 macro; 1 NA)</td>
<td>M = 13, F = 10</td>
</tr>
</tbody>
</table>

NA, data not available.

Table 3. Clinical details of patients studied with immunohistochemistry

<table>
<thead>
<tr>
<th>Clinical diagnosis</th>
<th>No. of samples (size of tumour)</th>
<th>Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Somatotroph adenomas</td>
<td>n = 10 (8 macro; 2 micro)</td>
<td>M = 8, F = 2</td>
</tr>
<tr>
<td>Corticotroph adenomas</td>
<td>n = 10 (3 macro; 7 micro)</td>
<td>M = 2, F = 8</td>
</tr>
<tr>
<td>Prolactinomas</td>
<td>n = 10 (7 macro; 3 micro)</td>
<td>M = 4, F = 6</td>
</tr>
<tr>
<td>NFPAs</td>
<td>n = 10 (10 macro)</td>
<td>M = 4, F = 6</td>
</tr>
<tr>
<td>Normal pituitary tissue</td>
<td>n = 10</td>
<td>M = 2, F = 8</td>
</tr>
</tbody>
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were calculated as the ratio between mean logarithmic input amount of the target gene and 18 S rRNA (see

Several samples were run on electrophoresis on agarose gels to confirm the product: all showed a unique band of the expected size for each amplicon.

PTEN sequencing

Direct sequencing of the entire coding region of PTEN was performed on cDNA from 33 pituitary tumours (Table 2). PCR products were prepared using three overlapping primer sets. PCR using 5 μl cDNA was performed in a 25 μl reaction volumes containing 4 μM primers, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.625 U Taq (Promega). The PCR product size was checked by ethidium bromide-stained 2% agarose gel electrophoresis before purification using the ExoSAP-IT kit protocol (USB Corp, Cleveland, OH, USA). Each PCR product was sequenced in both the forward and reverse directions using a 10 μl sequencing reaction consisting of 4 μl purified PCR product, 4 μl Big Dye terminator ready reaction mix (PE-Applied Biosystems) and 10 μM primer. The primers were the same as those used for the PCR amplification. The cycle sequencing was performed on the GeneAmp 9700 with 25 cycles at 96 °C for 10 s, 50 °C for 15 s and 60 °C for 4 min. The extension products obtained were purified using Sephadex column precipitation plates (Amersham Life Science, Little Chalfont, Bucks, UK) to remove the excess of dye terminators, which can interfere with base calling. Electrophoresis of the samples was performed on the ABI Prism 3700 DNA analyzer (PE-Applied Biosystems). The obtained sequences were analyzed using BIOEDIT software (http://jwbrown.mbio.ncsu.edu/RnaseP/info/programs/BIOEDIT/bioedit.html) and compared with the published PTEN sequence.

Immunohistochemistry

Each section underwent heat-mediated antigen-retrieval treatment before immunohistochemical analysis with the standard avidin–biotin complex immunoperoxidase system (Vectastain Elite; Vector Labs, Peterborough, UK). The rabbit polyclonal anti-(phospho-Ser 473 Akt) antibody (Cell Signalling Technology, Beverly, MA, USA) was used at a dilution of 1:50. Consecutive sections were also stained for p27 (anti-p27 antibody from clone SX53G8, used at a 1:200 dilution, was a gift from Dr X Lu; Lidhar et al. 1999) and PTEN (anti-PTEN antibody, dilution 1:100, from clone 28H6; Novacastra Laboratories, Newcastle, UK). Western blotting on human pituitary tissue using rabbit polyclonal anti-(phospho-Ser 473 Akt) antibody (Cell Signalling Technology), mouse monoclonal anti-PTEN antibody and mouse monoclonal anti-p27 (BD Biosciences, Oxford, UK) revealed bands of the appropriate size in each case.

Human tonsil tissue, removed with consent given for experimentation, was used as a positive control as this contains lymphoid tissue with variable proliferative activity. Negative control studies used a specific blocking peptide for the anti-(phospho-Ser 473 Akt) antibody, while the specificity of the p27 antibody had been well demonstrated previously by pre-absorption of the antibody by the appropriate blocking peptide that abolished p27 staining (Lidhar et al. 1999). Novocastra Laboratories confirmed the PTEN antibody specificity by reabsorbing the antibody with the recombinant protein prior to immunohistochemical application. For assessment of cytoplasmic phospho-Akt in the pituitary, 500 cells/slide were scored as strong (score 3), moderate (score 2), weak (score 1) or negative (score 0) cells per section. Strongly and moderately stained cells were subsequently combined to produce the percentage of cells that stained positive for analysis (Lidhar
et al. 1999, Korbonits et al. 2002). Nuclear immunostaining was assessed for p27 and PTEN using the same scoring, while cytoplasmic staining was noted as focal or diffuse, and as negative, weak, moderate or strong. Sections were chosen on each slide from a randomized grid array, and were counted blind to the diagnosis.

Photographs of slides were taken using a Leica DMR microscope and a Leica DC 200 digital camera (Leica GmBH, Solms, Germany) at a ×630 magnification and were printed on a HP inkjet printer on HP Premium Photo paper.

**Protein extraction**

Whole lysates were prepared from 50–250 mg frozen fresh human pituitary tissue (from five normal autopsy pituitaries and five pituitary adenomas) by homogenization in 500 µl ice-cold cell lysis buffer (Cell Signalling Technology) with 1 mM PMSF added, followed by a brief sonication and centrifugation at 12,000 g for 20 min at 4 °C. The supernatant was saved and stored at −80 °C. Protein yield was quantified using the Bradford protein assay (BioRad, Hemel Hempstead, Herts, UK).

Subcellular protein fractionation was performed using Subcellular Proteome Extraction Kit (S-PEK; Calbiochem/Merck Biosciences, Nottingham, UK) and protocol. Briefly, 50–75 mg frozen fresh human pituitary tissue (from four normal autopsy pituitaries and four pituitary adenomas) was fragmented and sequentially lysed in four extraction buffers supplemented with protease- and phosphatase-inhibitor cocktails to release proteins according to their subcellular localization. The purity of nucleic and cytosolic fractions was assessed by immunoblotting with c-Jun antibody (Calbiochem) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology, Santa Cruz, CA, USA) respectively. Protein yield was quantified using interfering protein assay (Calbiochem) and stored at −80 °C.

**Western blotting and immunoprecipitation**

50µg total protein lysate were denatured in SDS sample buffer, separated by electrophoresis on a SDS–PAGE gel (8%) and transferred to a PVDF membrane (Immobilon-P; pore size 0.45µm; Millipore, Watford, Herts, UK). The membrane was dried and subsequently incubated with the primary antibody (anti-(phospho-Ser 473 Akt), anti-Akt1/2 (Santa Cruz Biotechnology) and anti-β-actin (Abcam, Cambridge, UK)) in 5% BSA, respectively in 5% non-fat dry milk in Tris-buffered saline/Tween 20 overnight at 4 °C. After three washes in Tris-buffered saline/Tween 20, membranes were incubated with the secondary antibody conjugated with horseradish peroxidase. Immunodetection was performed using the ECL Plus detection system (Amersham Life Science). Autoradiography of the immunoblots was performed on Hyperfilm (Amersham Life Science). Akt extracts from Jurkat cells (Cell Signalling Technology) were used as positive controls for Akt and phospho-Akt. Cross detection between phospho-Akt and Akt was avoided by stripping the membrane and by using different species-raised primary antibodies. The optical density of the appropriately sized bands was measured using Kodak 1D Image Analysis Software (Scientific Imaging Systems, Eastman Kodak Company, Rochester, NY, USA). The relative expression of phospho-Akt was calculated as a ratio to total Akt optical density. Total Akt expression was reported relative to that of β-actin. Assessment of p27 in the cytosolic and nucleic proteome fractions was performed using 25 µg of each protein fraction run on 12% Tris/HCl gel (BioRad), by the above immunoblot technique, using a monoclonal p27 antibody (BD Biosciences).

Immunoprecipitation studies were performed for the detection of phospho-Thr 157 p27 in the cytosolic proteome fraction, using Seize X immunoprecipitation kit (Pierce Biotechnology, Rockford, IL, USA) and protocol. A 1 mg cytosolic proteome fraction was incubated with monoclonal p27 immobilized antibody (BD Biosciences). Immunoprecipitates were eluted, denatured and run on a 12% Tris/HCl gel, then transferred on a PVDF membrane. p27 immunoprecipitates were subject to immunoblot analysis using anti-(phospho-Thr 157 p27) (a gift from Dr G Viglietto, Institute of Endocrinology and Experimental Oncology, Naples, Italy) as described in Viglietto et al. (2002).

**Statistical analysis**

Statistical analysis was performed with StatsDirect software (Buchan I; Addison Wesley Longman, Cambridge, UK). After the Shapiro–Wilk test showed the data to be non-normally distributed, the data were analysed with the non-parametric, two-tailed Mann–Whitney U test and Kruskal–Wallis test, as appropriate. Significance was taken at P < 0.05. The results are shown as means ± S.E.M.

**Results**

Akt is over-expressed and over-activated in human pituitary tumours

Using RQ-PCR we determined the expression of Akt1 and Akt2 mRNA isoforms from human pituitary tissue samples: 44 human pituitary tumours and nine
normal pituitaries (from autopsy material) were assessed (Table 1). Both Akt isoforms were significantly over-expressed in pituitary tumours compared with the normal pituitaries (Fig. 1A; \( P < 0.05 \)). Within the individual groups of tumours, the NFPAs showed the highest level of both Akt1 and Akt2 expression compared with other tumour types (Fig. 1B; \( P < 0.05 \)).

We next assessed the protein expression of Akt1 and Akt2 by means of immunohistochemistry and Western blotting. 40 human pituitary tumours and 10 normal pituitaries (part of resected tissue at surgery) were assessed for expression of Ser 473-phosphorylated Akt1 and Akt2 by means of immunohistochemistry. Elevated levels of phosphorylated Akt were found in pituitary tumours (mean percentage of positive cells, 60.34 ± 5.25%) compared with normal pituitaries (21.2 ± 5.7%; \( P < 0.01 \)), and this was consistent over the individual tumour types (Fig. 2A). Western blotting and immunoprecipitation studies were applied to assess the ratio of total Akt versus phospho-Akt in five normal pituitaries (autopsy material) and five pituitary tumours (Fig. 2B). The immunoblots for phospho-Akt and total Akt were analyzed by band optical densitometry and normalized to \( \beta \)-actin. The results revealed an increase in both the total Akt/\( \beta \)-actin and phospho-Akt/total Akt band-intensity ratios (Fig. 2B) in the tumours. While over-phosphorylation of Akt in pituitary tumours (mean phospho-Akt/total Akt 2.70 ± 1.68 in tumours versus 0.24 ± 0.12 in normal pituitaries; \( P < 0.05 \)) was highly significant, the difference in total Akt expression did not reach statistical significance (mean Akt/\( \beta \)-actin 0.55 ± 0.12 in tumours versus 0.4 ± 0.07 in normal pituitaries; \( P > 0.05 \)).

**p27 may be displaced from the nucleus in pituitary tumours**

In order to test the hypothesis that Akt sequesters p27 in the cytoplasm, away from its nuclear targets, we assessed nuclear p27 protein expression in the pituitary samples which had been assessed for cytoplasmic phospho-Akt expression by means of
The pattern of p27 immunostaining was opposite to that of phospho-Akt. As reported previously by ourselves and others (Bamberger et al. 1999, Lidhar et al. 1999), normal pituitary samples were found to express more nuclear p27 (mean percentage of positive cells 72.44 ± 4.93%) than the pituitary tumours (mean percentage of positive cells 47.3 ± 8.81%), such that there was an inverse relationship to that seen for cytoplasmic phospho-Akt. Expressing the ratio of these two agents enhanced the difference between normal pituitary and pituitary adenomas: phospho-Akt/p27 ratio 3.26 ± 1.07 in tumours versus 0.29 ± 0.07 in normal samples; P < 0.01 (Fig. 3B). However, there was no statistically significant correlation between individual tumours when comparing nuclear p27 with cytoplasmic phospho-Akt. As reported previously, it was difficult to quantitate p27 cytoplasmic staining in pituitary tissue.

Immunoblot studies for p27 content in cytosolic and nucleic proteome fractions were used to support the immunohistochemistry findings. While normal pituitary samples were found to express more nuclear p27 than the pituitary tumours, the tumours showed a trend towards higher levels of p27 in the cytosolic compartment (Fig. 3C). However, immunoprecipitation studies failed to consistently reveal the presence of phospho-p27 on Thr 157 in the cytosolic fractions when using 1 mg protein (results not shown).

**PTEN is not mutated or under-expressed in human pituitary tumours**

In order to explore upstream regulators of Akt activation, we further investigated the phosphatase PTEN with the hypothesis that this is negatively regulated to permit over-activation of Akt. Direct sequencing revealed no mutations of **PTEN** in the coding region of the gene in 33 pituitary tumours. RQ-PCR studies were performed on 54 pituitary tumours (25 NFPAs, 16 somatotroph adenomas, six corticotroph adenomas...
and seven prolactinomas) and 12 normal pituitaries (Table 1). PTEN mRNA was similarly transcribed in the tumours compared with normal pituitary tissue (Fig. 4A). Further studies on PTEN protein expression were performed on consecutive sections of the same pituitary samples analysed for phospho-Akt. Very little PTEN cytoplasmic staining was noted (see Fig. 3A), and this could not be quantitated. By contrast, there was clear nuclear expression of PTEN, and overall the pituitary tumour samples expressed less nuclear PTEN protein (mean percentage of positive cells 41.54 ± 4.88%) compared with the normal pituitary (85.12 ± 3.21%; P < 0.01; Fig. 4B). The level of nuclear PTEN was significantly correlated with that of p27 (Spearman's rank correlation coefficient +0.311; P < 0.05)

Discussion

Growth factor-mediated activation of the PI3K/Akt transduction cascade has been implicated in a large spectrum of human malignancies. In response to mitogens Akt is activated in close proximity of the cell membrane by phosphorylation at residues Thr-308 and Ser 473 by phosphoinositide-dependent kinases PDK1 and PDK2, respectively. The process is dependent on conversion of phosphatidylinositol 4,5-bisphosphate into phosphatidylinositol 3,4,5-trisphosphate by activated PI3K, which serves to anchor Akt in the cell membrane. Activity of the PI3K/Akt pathway is in turn negatively regulated by phosphatases such as PTEN, SHIP1 and SHIP2 which remove phosphates from phosphatidylinositol 3,4,5-trisphosphate. Inactivating mutations/deletions of these phosphatases can lead to tumour progression. As a primary mediator of PI3K signalling, Akt has a number of downstream substrates that may contribute to malignant transformation: these include apoptotic factor Bad, procaspase-9, inhibitor of nuclear factor kappa B kinase (IKK), cAMP-response-element-binding protein (CREB), the forkhead family of transcription factors, glycogen synthase kinase 3 (GSK-3), mammalian target of rapamycin (mTOR), p21 and p27 (Vivanco & Sawyers 2002).

A novel link between activation of the Akt pathway and cell-cycle progression has been established recently with the finding that cytoplasmic phosphorylation of p27 on residue Thr 157 by Akt leads to cytoplasmic sequestration or mislocalization of p27 (Liang et al. 2002, Shin et al. 2002, Viglietto et al. 2002), impairing its function as a CDK inhibitor. The Akt phosphorylation site in p27 is within its nuclear localization sequence (amino acids 151–166), explaining how this process may affect cellular localization of p27 while not changing its total levels.

The present study indicates that Akt signalling may be enhanced in human pituitary tumours, and supports a possible role for Akt with respect to p27 deregulation.

We first established over-expression of Akt1 and Akt2 mRNA in pituitary tumours compared with the normal pituitary by quantitative PCR, and showed increased levels of the active Ser 473-phosphorylated form of Akt by means of both immunohistochemistry and Western blotting with a phospho-Akt antibody. The pattern of expression for cytoplasmic phospho-Akt was opposite to that of nuclear p27 in the samples studied, although individual samples showed a lack of precise correlation. We were not able to reveal the presence of phospho-p27 (Thr157) in cytosolic extracts of pituitary tumours, using the only antibody currently available (in small quantities). However, a trend towards a higher content of total p27 in the cytoplasmic versus nucleic compartment of these tumours compared with normal pituitaries was noted.

We further explored the possibility that at least part of the increased phosphorylation of Akt would be secondary to a decrease in cytoplasmic PTEN expression, or to a defunctioning mutation in its expressed RNA sequence. The level of PTEN mRNA expression was similar in pituitary tumours compared with normal pituitary, and no mutation of the PTEN gene was found in a series of 33 pituitary tumours. However, we were unable to clearly demonstrate the presence of PTEN protein in pituitary cell cytoplasm; instead, PTEN...
protein expression was found mainly in the nuclei in both normal and tumour samples, and at this site it was less expressed in the tumours compared with the normals, and directly correlated with the level of p27 expression. Despite the lack of a traditional nuclear localization sequence in the PTEN protein, an increasing number of reports have found nuclear localization of PTEN in breast, thyroid, endocrine pancreatic tumours, cutaneous melanoma, oesophageal squamous cell carcinoma and colorectal carcinoma (Gimm et al. 2000, Perren et al. 2000, Tachibana et al. 2002, Whiteman et al. 2002, Zhou et al. 2002, Ginn-Pease & Eng 2003). In these studies a shift from nuclear to cytoplasmic PTEN correlated with increasing neoplasia for all tissues. Furthermore, it has been suggested that PTEN may play a role within the nucleus to induce cell-cycle arrest in G1, and thus that function of PTEN as a cell-cycle regulator may extend beyond the suppression exerted on the PI3K/Akt pathway in the cytosol (Ginn-Pease & Eng 2003). It would appear that most PTEN is nuclear in both normal and tumorous pituitary, and presumably in this location it is not acting as a phosphoinositide phosphatase. It is therefore difficult to establish whether the increased ratio of phospho-Akt to Akt is related to any change in cytoplasmic PTEN expression, but it is clear that mutations of PTEN, as seen in several other tumours, are not a common feature of pituitary tumours. We did not explore other mechanisms of PTEN inactivation as methylation or haploinsufficiency, since PTEN mRNA levels were similar in normal and tumorous pituitary tissue. It is unclear, therefore, as to the mechanism whereby Akt is over-transcribed and over-phosphorylated in pituitary adenomas as compared with normal pituitary tissue. However, Akt is one of the major signalling moieties in the downstream cascade from growth factor receptors of the tyrosine receptor kinase class, particularly of the epidermal growth factor (EGF), insulin-like growth factor (IGF) and fibroblast growth factor (FGF) receptor families (Nicholson & Anderson 2002). Our data would be most compatible with enhanced activity of these receptors, by constitutive and/or unliganded activation, as the proximate cause of the changes in Akt phosphorylation and subsequent abnormalities in the cell cycle; such defects in cell-cycle control would be anticipated to lead in turn to chromosomal instability (Hernando et al. 2004).

In summary, we have demonstrated that Akt is over-expressed and over-phosphorylated in human pituitary tumours, and that this is in an opposite direction to that of nuclear p27. Whether this is causally related to inappropriate p27 cytoplasmic localization is not clear at present, but such an explanation would be compatible with these results. We therefore suggest that enhanced levels of phosphorylated Akt may be responsible for accelerated and dysregulated progression through the cell cycle in these tumours. The increased activation of Akt appears not to be secondary to mutations of PTEN, but rather suggests a change at more proximate levels parts of the cell-signalling pathway. Furthermore, this may suggest the use of molecular therapies targeted at this pathway in the treatment of pituitary tumours resistant to conventional treatment (Wendel et al. 2004).

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