Activation of RAF/MEK/ERK and PI3K/AKT/mTOR pathways in pituitary adenomas and their effects on downstream effectors

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Abstract

Raf/MEK/ERK and phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) cascades are key signalling pathways interacting with each other to regulate cell growth and tumourigenesis. We have previously shown B-Raf and Akt overexpression and/or overactivation in pituitary adenomas. The aim of this study is to assess the expression of their downstream components (MEK1/2, ERK1/2, mTOR, TSC2, p70S6K) and effectors (c-MYC and CYCLIN D1). We studied tissue from 16 non-functioning pituitary adenomas (NFPAs), six GH-omas, six prolactinomas and six ACTH-omas, all collected at transsphenoidal surgery; 16 normal autopsy pituitaries were used as controls. The expression of phospho and total protein was assessed with western immunoblotting, and the mRNA expression with quantitative RT-PCR. The expression of pSer217/221 MEK1/2 and pThr183 ERK1/2 (but not total MEK1/2 or ERK1/2) was significantly higher in all tumour subtypes in comparison to normal pituitaries. There was no difference in the expression of phosphorylated/total mTOR, TSC2 or p70S6K between pituitary adenomas and controls. Neither c-MYC phosphorylation at Ser 62 nor total c-MYC was changed in the tumours. However, c-MYC phosphorylation at Thr58/Ser62 (a response target for Akt) was decreased in all tumour types. CYCLIN D1 expression was higher only in NFPAs. The mRNA expression of *MEK1*, *MEK2*, *ERK1*, *ERK2*, *c-MYC* and *CCND1* was similar in all groups. Our data indicate that in pituitary adenomas both the Raf/MEK/ERK and PI3K/Akt/mTOR pathways are upregulated in their initial cascade, implicating a pro-proliferative signal derangement upstream to their point of convergence. However, we speculate that other processes, such as senescence, attenuate the changes downstream in these benign tumours.

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Introduction

Pituitary tumours are common neoplasms, with a reported overall prevalence of 16.7% in the general population, 14.4% in autopsy studies and 22.5% in radiological studies (Ezzat *et al.* 2004). These tumours

are mostly benign, but cause significant morbidity through mass effects and/or the inappropriate secretion of pituitary hormones (Ezzat *et al.* 2004). Despite intensive study, the molecular pathogenesis of the majority of sporadic pituitary adenomas remains

unclear (Asa & Ezzat 2002, Melmed 2008). There are several genetic syndromes associated with pituitary tumours, but abnormalities in the genes mutated in these syndromes are rarely abnormal in sporadic tumours (Beckers & Daly 2007, Boikos & Stratakis 2007, Karhu & Aaltonen 2007, Horvath & Stratakis 2008, Leontiou *et al.* 2008). There are also a number of tumour suppressor genes which are inactivated in sporadic tumours, often by promoter methylation, but no single causal agent or mutation has been identified (Dworakowska & Grossman 2009b).

Two major signalling pathways responsible for regulating cell growth and proliferation, downstream of growth factor receptors, are the phosphatidylinositol 3-kinase (PI3K)/Akt and Raf/mitogen-activated protein kinase (MAPK) pathways (McCubrey *et al.* 2007). These have been shown to be overactive in many human tumours (Adjei & Hidalgo 2005, Roberts & Der 2007). It has been reported that Akt is overexpressed and overactivated in non-functioning pituitary adenomas (NFPAs; Musat *et al.* 2005), and there is recent evidence that B-Raf, an upstream regulator of the MAPK cascade, is also overexpressed predominantly in NFPAs (Ewing *et al.* 2007). Such activation may account for the changes in cell-cycle components which are a frequent characteristic of pituitary tumours, especially p27 (Lloyd *et al.* 1997, Lidhar *et al.* 1999). In addition, we have shown that somatostatin analogues, which cause tumour regression in certain types of pituitary adenoma, downregulate the MAPK pathway in pituitary tumours (Hubina *et al.* 2006). However, it has been unclear as to whether the changed expression of the upstream components of these two converging pathways is associated with downstream modulation of their subsequent effectors.

Raf/MEK/ERK is a hierarchical cascade, originating at the cell membrane with receptors for mitogens or growth factors, which recruit, via adapter proteins and exchange factors, the small guanosine triphosphatase Ras (Adjei & Hidalgo 2005). Ras then activates Raf (MAPK kinase kinase, MAPKKK), which in turn activates MAPK kinase (MAPKK; MEK1/2): MEK1/2 proteins are dual-specificity kinases containing two consensus kinase motifs, one motif being involved in phosphorylation of serine/threonine residues while the other is involved in phosphorylation of tyrosine residues. Two MEK homologues, MEK1 and MEK2, are activated through phosphorylation of two serine residues at positions 217 and 221 by Raf-like molecules, and then MEK1/2 sequentially phosphorylate ERK1 and ERK2 at two sites, Tyr185 followed by Thr183 (Haystead *et al.* 1992). ERK1/2 in turn phosphorylates and activates ribosomal S6 kinase and

transcription factors such as c-MYC, Elk1, c-Fos or CYCLIN D1 (Terada *et al.* 1999a,b), resulting in the modulation of genes associated with proliferation (Guan 1994) and leading to cell transformation (Joneson & Bar-Sagi 1997). ERK1 and 2 activate CYCLIN D1 promoter activity and CYCLIN D1 expression, whereas p38MAPK works in an opposing manner, inhibiting CYCLIN D1 transcription and expression (Lavoie *et al.* 1996).

In terms of the PI3K/Akt/mammalian target of rapamycin (mTOR) pathway, PI3K is activated as a result of the ligand-dependent activation of tyrosine kinase receptors, G-protein-coupled receptors, or integrins. Receptor-independent activation of PI3K can also occur in cells expressing constitutively-active Ras protein (Kauffmann-Zeh *et al.* 1997, Rodriguez-Viciano *et al.* 1997). The best-characterised phosphorylation target of PI3K is Akt (protein kinase B), resulting in the phosphorylation of a host of other proteins that affect cell growth, cell cycle entry, and cell survival. Akt phosphorylation at Thr308 and Ser473 activates a serine–threonine kinase mTOR at Ser2448, which further activates 40S ribosomal protein S6 kinase (p70S6K) at Thr229 and Thr389 (Volarevic & Thomas 2001) and inactivates 4E-binding protein (4E-BP1). The tuberous sclerosis complex (TSC) – which mediates between PI3K/Akt and mTOR – is germline mutated in tuberous sclerosis, and there is evidence that neuroendocrine and pituitary tumours are a feature of this disease (Dworakowska & Grossman 2009a). Mitogenic stimuli activating Akt phosphorylate TSC2 at Ser939 and Thr1462. This phosphorylation destabilises TSC2, inhibits the formation of the TSC1/2 complex, and leads to an increase in mTOR activity (Inoki *et al.* 2002). Changes in 4E-BP1 lead to the initiation of translation of mRNA for c-MYC or CYCLIN D1 through its association with eIF-4E (Rosenwald *et al.* 1995, Sonenberg & Gingras 1998), while Akt also activates CYCLIN D1 indirectly via glycogen synthase kinase-3 (GSK-3 β ; Diehl *et al.* 1998, Onofri *et al.* 2006). There is also further evidence that these two pathways interact, e.g. ERK1/2 indirectly activates mTOR by phosphorylation of TSC2 at Ser669. Thus, both pathways are involved in the regulation of c-MYC and CYCLIN D1 transcription. ERK1/2 also directly activates phosphorylation of c-MYC protein at Ser62 leading to its accumulation, whereas Akt decreases phosphorylation (via GSK-3 β) at Thr58 which diminishes c-MYC degradation (Sears *et al.* 2000).

The aim of this study was to investigate in more detail the status of the intervening components of

Raf/MEK/ERK and PI3K/Akt/mTOR pathways as well as their two principal targets – c-MYC and CYCLIN D1. In different types of human pituitary adenomas and normal pituitary we assessed the protein expression of phosphorylated/total MEK1/2, ERK1/2, p38MAPK, TSC2, mTOR, p70S6K, c-MYC and CYCLIN D1. In addition, we performed a quantitative analysis of mRNA expression of all of the components which had show alterations at the protein level (*MEK1*, *MEK2*, *ERK1*, *ERK2*, *c-MYC* and *CCND1*).

Materials and methods

Tissues

We studied tissue from 50 human pituitaries (Table 1) including 16 NFPAs, six somatotrophinomas (GH-omas), six prolactinomas (PRL-omas) and six ACTH-oma, removed at transsphenoidal surgery and immediately ‘flash-frozen’ and stored at -80°C . A total of 16 normal pituitary samples were used as a control group. These were collected at autopsy (which was performed within 24 h of death from non-endocrine disease), ‘flash-frozen’ and stored at -80°C . The tumour study group consisted of 18 men (53%) and 16 women (47%), with a mean age of 58.3 years (age range 19–77 years). The controls consisted of 13 men (81%) and 3 women (19%), with a mean age of 64.7 years (age range 42–77 years). All patients gave written informed consent for the protocol, which was approved by the local Institutional Review Board (IRB). The autopsy collection was also approved by the relevant local IRB.

Protein extraction and western blot analysis

Whole lysates were prepared from 50 to 250 μg frozen fresh human pituitary tissue by homogenisation in 500 μl ice-cold cell lysis buffer with added 1 mM phenylmethylsulphonyl fluoride, 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 BCA protein

assay kit (Pierce–Perbio, Cramlington, UK). Fifty microgram of total protein lysate were denatured in SDS sample buffer, separated by electrophoresis on a SDS-PAGE gel (10%) and transferred to a nitrocellulose membrane (Immobilon-P; pore size 0.45 mm; Millipore, Watford, Herts, UK). The membrane was subsequently incubated with the primary antibody in 0.1% or 5% BSA, or in 5% non-fat dry milk in Tris-buffered saline/Tween 20 overnight at 4°C (Table 2). After six washes in Tris-buffered saline/Tween 20, the membranes were incubated with the secondary antibody for 90 min. Immunodetection was performed using the Odyssey infrared imaging system (LI-COR Biosciences, Cambridge, UK). Each membrane was incubated and double-stained with two primary antibodies (phospho-protein and β -actin or total protein and β -actin). Cross-detection between analysed protein and β -actin was avoided by using different species-raised against primary antibodies. To assess expression of each protein we have constructed a set consisting of three membranes (15 wells), including all sample types (four normal pituitaries, two PRL-omas, two ACTH-omas, two GH-omas and four NFPAs). One well was reserved for the size marker. The optical density of the appropriately sized bands was measured using the Odyssey molecular imaging software (LI-COR Biosciences).

The expression of phospho-mTOR, phospho-TSC2, phospho-p70S6K, phospho-ERK1/2, phospho-MEK1/2, phospho-c-MYC and phospho-p38MAPK was normalised to β -actin from the corresponding membrane and was calculated as a ratio of phospho-peptide/ β -actin. The expression of total-mTOR, total-TSC2, total-p70S6K, total-ERK1/2, total-MEK1/2, total-c-MYC, total-p38MAPK and CYCLIN D1 was normalised to β -actin from the corresponding membrane and was calculated as a ratio of total/ β -actin. The relative expression of each phospho-protein was calculated as well as the proportion of phospho/total protein. All results normalised to β -actin were compared between subgroups of pituitary tumours, and are shown as % of control.

Table 1 Clinical details of patients included in the study

Clinical diagnosis	Samples studied (tumour size)	Patient gender	Patient age (mean + range)
NFPA	$n=16$ (15 macro, 1 micro)	4 F, 12 M	56 years (28–75)
GH-oma	$n=6$ (1 macro, 5 micro)	4 F, 2 M	48 years (31–64)
PRL-oma	$n=6$ (2 macro, 4 micro)	3 F, 3 M	33 years (19–46)
ACTH-oma	$n=6$ (2 macro, 4 micro)	5 F, 1 M	48.3 years (24–77)
All adenomas	$n=34$	16 F, 18 M	58.3 years (19–77)
Normal pituitary	$n=16$	3 F, 13 M	64.7 years (42–77)

Table 2 Antibodies used for western blotting in the current study

Antibody name	Origin	Concentration	Source	Primary/ secondary	Monoclonal/ polyclonal
Anti-phospho-MEK1/2 (Ser217/221)	Rabbit	1:1000	Cell Signalling Technology, Hitchin, UK	Primary	Monoclonal
Anti-MEK1/2 (total)	Rabbit	1:1000	Cell Signalling Technology	Primary	Monoclonal
Anti-phospho-ERK1/2 (Thr183)	Rabbit	1:4000	Promega Corporation	Primary	Polyclonal
Anti-ERK1/2 (total)	Rabbit	1:5000	Promega	Primary	Polyclonal
Anti-phospho-c-myc (Ser62)	Mouse	1:200	Spring Bioscience, Fremont, CA, USA	Primary	Monoclonal
Anti-phospho-c-myc (Thr58/Ser62)	Rabbit	1:1000	Cell Signalling Technology	Primary	Polyclonal
Anti-c-myc (total)	Rabbit	1:1000	Cell Signalling Technology	Primary	Polyclonal
Anti-cyclin D1	Rabbit	1:1000	Cell Signalling Technology	Primary	Monoclonal
Anti-phospho-p38MAPK (Thr180/Tyr182)	Rabbit	1:1000	Cell Signalling Technology	Primary	Monoclonal
Anti-p38MAPK (total)	Rabbit	1:1000	Cell Signalling Technology	Primary	Monoclonal
Anti-phospho-mTOR (Ser2448)	Rabbit	1:1000	Cell Signalling Technology	Primary	Polyclonal
Anti-mTOR (total)	Rabbit	1:2000	Cell Signalling Technology	Primary	Polyclonal
Anti-phospho-tuberin (Thr1462)	Rabbit	1:1000	Cell Signalling Technology	Primary	Polyclonal
Anti-phospho-tuberin (Ser939)	Rabbit	1:1000	Cell Signalling Technology	Primary	Polyclonal
Anti-tuberin (total)	Rabbit	1:1000	Cell Signalling Technology	Primary	Polyclonal
Anti-phospho-S6K (Thr389)	Rabbit	1:1000	Cell Signalling Technology	Primary	Polyclonal
Anti-S6K (total)	Rabbit	1:1000	Cell Signalling Technology	Primary	Polyclonal
Anti-β-actin	Mouse	1:5000	Abcam, Cambridge, UK	Primary	Monoclonal
Anti-rabbit IRDye_ 800	Goat	1:2000	LI-COR Biosciences, Cambridge, UK	Secondary	–
Anti-mouse IRDye_ 680	Goat	1:10 000	LI-COR Biosciences	Secondary	–

mRNA extraction and ‘real-time’ PCR

Total RNA from tissue was extracted using an RNeasy Mini kit (Qiagen) and the protocol was followed according to the manufacturers’ instructions. A DNase treatment step was performed to limit contamination of the total RNA with genomic DNA. One microgram of total RNA was reverse-transcribed (RT) in a 25 µl reaction using 5 µl 5× first strand buffer (Invitrogen), 100 mM dithiothreitol (Invitrogen), 20 mM of deoxynucleotide triphosphate (Promega), 20 µg/µl random hexamer primers (Promega), MMW-RT (Maloney murine leukemia virus transcriptase; Invitrogen) and 40 U/µl RNase inhibitor (Promega). Thermal cycling conditions were as follows: 26 °C for 10 min, 37 °C for 60 min and 92 °C for 10 min. cDNA was amplified by real-time (RT) PCR using TaqMan Universal Master Mix (Applied Biosystems, Warrington, UK), 20× assay primers and their respective assay ID for human *MEK1* Hs00983256_g1, *MEK2* Hs00360961_m1, *MAPK1* (encoding for ERK2) Hs00177066_m1, *MAPK3* (encoding for ERK1) Hs00385075_m1, *CCND1* Hs00277039_m1, *c-MYC* Hs0015348_m1 and the β-actin Vic-MGB probe 4326315E. cDNA was amplified in a 7900HT Taqman machine (Applied Biosystems). All data were expressed in relation to β-actin and then calculated relative to the percentage of control.

Statistical analysis

Statistical analysis was performed with StatsDirect software (Buchan I; Addison Wesley Longman, Cambridge, UK). The data were noted to be non-normally distributed. Statistical analysis was performed with the use of the Kruskal–Wallis test followed by the Conover–Inman comparison, the Mann–Whitney test and the Spearman’s correlation test. Statistical significance was defined at $P < 0.05$ and all data are shown as means ± S.E.M.

Results

Western blotting

The expression of pSer217/221 MEK1/2 (Fig. 1A), but not total MEK1/2 (Fig. 1B), was significantly higher in PRL-omas ($277 \pm 27\%$, $P < 0.001$), ACTH-omas ($287 \pm 14\%$, $P < 0.001$), GH-omas ($175 \pm 39\%$, $P = 0.009$) and NFPA’s ($281 \pm 15\%$, $P < 0.001$) in comparison to normal pituitaries. Furthermore, the ratio between pSer217/221 MEK1/2 and total MEK1/2 was significantly higher in all groups of pituitary tumours in comparison to normal pituitaries ($271 \pm 13\%$ for PRL-omas, $P = 0.001$; $227 \pm 30\%$ for ACTH-omas, $P < 0.001$; $187 \pm 15\%$ for GH-omas; $P = 0.01$ and $371 \pm 45\%$ for NFPA’s; $P < 0.001$; Fig. 1C).

Figure 1 MEK1/2 expression in normal pituitaries and pituitary adenomas. The phospho-MEK1/2 at Ser217/221 normalized to β -actin and total-MEK1/2 was elevated in all types of pituitary adenomas ($P < 0.001$ and $P < 0.001$ respectively). (A and C) There was no difference in total-MEK1/2 expression between control and adenomas groups ($P = 0.48$). (B) The results for all groups in order are shown as % of control for the average of pMEK1/2 and tMEK1/2, normalized to β -actin, and pMEK1/2 normalized to tMEK1/2. Error bars represent S.E.M. ** $P < 0.05$ versus control for individual comparisons.

The expression of pThr183 ERK1/2 (Fig. 2A), but not total ERK1/2 (Fig. 2B), was significantly higher in PRL-omas ($174 \pm 16\%$, $P = 0.01$), ACTH-omas ($231 \pm 46\%$, $P < 0.001$), GH-omas ($184 \pm 38\%$, $P = 0.01$) and NFPA ($317 \pm 38\%$, $P < 0.001$) in comparison to normal pituitaries. Furthermore, the ratio between pThr183 ERK1/2 and total ERK1/2 was also significantly higher in all groups of pituitary tumours in comparison to normal pituitaries ($274 \pm 13\%$ for PRL-omas, $P < 0.001$; $290 \pm 30\%$ for ACTH-omas, $P < 0.001$; $198 \pm 15\%$ for GH-omas; $P = 0.01$ and $214 \pm 12\%$ for NFPA; $P < 0.001$; Fig. 2C).

There was no difference in either total mTOR ($P = 0.46$) or its phospho-derivative Ser2448 ($P = 0.25$); in total TSC2 ($P = 0.78$) or its derivatives

Ser939-TSC2 ($P = 0.88$) or Thr1462-TSC2 ($P = 0.84$); or in total p70S6K ($P = 0.72$) or its phospho-derivative Thr389 ($P = 0.47$) between any type of pituitary adenoma and normal pituitary.

There was no difference in the expression of Ser62 c-MYC between pituitary adenomas and normal pituitary ($P = 0.75$). However, the expression of c-MYC doubly-phosphorylated at Ser62/Thr58 (Fig. 3A), but not total c-MYC (Fig. 3B), was significantly decreased in all types of adenoma in comparison to normal pituitary ($45 \pm 4\%$ for PRL-omas, $P = 0.01$; $40 \pm 6\%$ for ACTH-omas, $P = 0.01$; $42 \pm 6\%$ for GH-omas, $P = 0.04$ and $54 \pm 9\%$ for NFPA, $P = 0.06$). Furthermore, the ratio between c-MYC doubly-phosphorylated at Ser62/Thr58 and total c-MYC was also significantly lower in all secretory groups of pituitary tumours in comparison to normal pituitaries, and tending towards significance in the

Figure 2 ERK1/2 expression in normal pituitaries and pituitary adenomas. The phospho-ERK1/2 at Thr183 normalized to β -actin and total-ERK1/2 was elevated in all types of pituitary adenomas ($P < 0.001$ and $P < 0.001$ respectively). (A and C) There was no difference in total-ERK1/2 expression between control and adenomas groups ($P = 0.42$). (B) The results for all groups in order are shown as % of control for the average of pERK1/2 and tERK, normalized to β -actin, and pERK1/2 normalized to tERK1/2. Error bars represent S.E.M. ** $P < 0.05$ versus control for individual comparisons.

Figure 3 c-MYC expression in normal pituitaries and in pituitary adenomas. The phospho-c-MYC at Ser62/Thr58 normalized to β-actin and total-c-MYC was decreased in all types of pituitary adenomas ($P=0.03$ and $P=0.02$ respectively). (A and C) There was no difference in total c-MYC expression between control and adenomas groups ($P=0.12$). (B) The results for all groups in order are shown as % of control for the average of doubly-phosphorylated c-MYC and total c-MYC, normalized to β-actin, and doubly-phosphorylated c-MYC normalized to total c-MYC. Error bars represent S.E.M. ** $P<0.05$ versus control for individual comparisons.

NFPAs ($37 \pm 9\%$ for PRL-omas, $P=0.01$; $33 \pm 12\%$ for ACTH-omas, $P=0.01$; $35 \pm 11\%$ for GH-omas; $P=0.01$ and $56 \pm 26\%$ for NFPAs, $P=0.09$; Fig. 3C).

A significant increase in CYCLIN D1 expression was observed in NFPAs compared to normal controls ($136\% \pm 10$, $P=0.01$), but not in any of the other adenoma groups (Fig. 4). There was no difference in the amount of pThr180/Tyr182 p38MAPK between the normal or abnormal tissue ($P=0.17$; data not shown).

Representative western blots are shown for pMEK1/2, tMEK1/2 and β-actin (Fig. 5A), pERK1/2, tERK1/2 and β-actin (Fig. 5B), doubly-phosphorylated c-MYC, total c-MYC and β-actin (Fig. 5C), and CYCLIN D1 and β-actin expression (Fig. 5D), in normal pituitaries, PRL-omas, ACTH-omas, GH-omas and NFPAs in Fig. 5.

Quantitative RT-PCR

Quantification of mRNA showed similar expression for *MEK1*, *MEK2*, *ERK1*, *ERK2*, *c-MYC* and *CCND1* in all tissue groups ($P=0.78$ for *MEK1*; $P=0.46$ for *MEK2*; $P=0.73$ for *ERK1*; $P=0.23$ for *ERK2*; $P=0.59$ for *c-MYC* and $P=0.62$ for *CCND1*, respectively). Representative results for mRNA of analysed genes normalized to β-actin in controls and NFPAs are shown in Fig. 6. There was no correlation between the amount of protein product assessed by western blotting and mRNA assessed by RT-PCR for any of the molecular species ($P=0.10$ for *ERK1*; $P=0.41$ for *ERK2*; $P=0.54$ for *c-MYC* and $P=0.84$ for *CCND1* respectively).

Discussion

The Raf/MEK/ERK and PI3K/PTEN/Akt pathways diverge from common growth factor receptors on the cell surface, modulating phosphorylation of many downstream targets involved in cell survival, proliferation, and in some cases in malignant transformation. In this study of human pituitary adenomas, we demonstrate that our previously-reported overexpression of B-Raf (MAPKKK) was associated with increased phosphorylation, and hence activation, of its immediate downstream targets MEK1/2 (MAPKK) and ERK1/2 (MAPK) in all adenoma subtypes. However, we were only able to show a small increase in CYCLIN D1 expression, in NFPAs alone, and we did not find the increase in pSer62 c-MYC expression that would be expected with activation of the MAPK pathway. In terms of the PI3K/Akt pathway, we did not see any increased phosphorylation of TSC2 by Akt nor the anticipated changes secondary to Akt in mTOR or p70S6K. Two N-terminal phosphorylation sites in c-MYC, Thr58 and Ser62, are known to be regulated

Figure 4 CYCLIN D1 expression in normal pituitaries and in pituitary adenomas. CYCLIN D1 normalized to β-actin was elevated in non-functioning pituitary adenomas ($P=0.01$), but not in other types of adenomas. The results for all groups in order are shown as % of control for the average of CYCLIN D1 normalized to β-actin. Error bars represent S.E.M. ** $P<0.05$ versus control for individual comparisons.

Figure 5 Representative western blots for: (A) pMEK1/2, tMEK1/2, and β-actin expression in normal pituitaries ($n=4$), and PRL-omas, ACTH-omas, GH-omas, and NFPAs ($n=2$ for each, $n=3$ for NFPAs). (B) pERK1/2, tERK1/2, and β-actin expression in normal pituitaries ($n=4$), PRL-omas, ACTH-omas, GH-omas, and NFPAs ($n=2$ for each, $n=3$ for NFPAs). (C) Doubly-phosphorylated c-MYC, total c-MYC, and β-actin expression in normal pituitaries ($n=4$), PRL-omas, ACTH-omas, GH-omas, and NFPAs ($n=2$ for each, $n=3$ for NFPAs). (D) CYCLIN D1 and β-actin expression in normal pituitaries ($n=4$), PRL-omas, ACTH-omas, GH-omas, and NFPAs ($n=2$ for each, $n=4$ for NFPAs).

by mitogen stimulation. Phosphorylation of Ser62 mediated by ERK1/2 is required for Ras-induced stabilisation of c-MYC, but conversely, phosphorylation of Thr58, mediated by GSK-3β (DePaoli-Roach 1984) but dependent on the prior phosphorylation of Ser62, is associated with degradation of c-MYC (Cross *et al.* 1995, Sears *et al.* 2000). In this study we found decreased c-MYC phosphorylation at Thr62/Ser58 concordant with previously described Akt over-activation in pituitary adenomas, an effect thought to occur via Akt-mediated phosphorylation of GSK-3β. We were unable to check the status of TSC2 phosphorylated at the site activated by ERK1/2, Ser669, since there is no commercially available antibody to detect this site.

In a very recent animal study performed in a mouse model, which spontaneously develops TSH-secreting pituitary adenomas (Lu *et al.* 2008), Akt and its downstream effectors mTOR and p70S6K were activated and contributed to increased cell proliferation

and pituitary growth. In addition, activation of Akt led to decreased apoptosis by inhibiting pro-apoptotic activity of Bcl-2-associated death promoter, further contributing to the aberrant cell proliferation

Figure 6 Representative mRNA expression for MEK1, MEK2, ERK1, ERK2, c-MYC, and CCND1 in normal pituitaries and non-functioning pituitary adenomas. mRNA expression of MEK1, MEK2, ERK1, ERK2, c-MYC, and CCND1 was similar between controls and NFPA, as revealed by RT-PCR studies. The average ratio of mRNA for each gene normalized to β-actin was shown as % of control. Error bars represent S.E.M.

(Lu *et al.* 2008). In our study we did not show any alteration in mTOR and p70S6K status in different types of pituitary adenomas, although the very rare human TSH-omas were not included in this analysis.

It is recognised that ligand-dependent or mutational activation of receptor tyrosine kinase, or mutational activation of pathway intermediates such as RAS and BRAF, lead to activation of ERK signalling with demonstrably high intracellular levels of pERK (Cobb & Goldsmith 1995, Lewis *et al.* 1998). However, it has been suggested that expression of pERK might sometimes be a poor measure of activation of ERK pathway signalling and not necessarily reflect substrate flux through this pathway, although this is a common assumption (Pratilas *et al.* 2009). Nevertheless, the present data, together with previous studies, do suggest that the two major signalling pathways are likely to be over-active in pituitary tumours, and that therefore the fundamental change in pituitary adenoma cell status occurs either at, or upstream to, the point of convergence of these two pathways; this would be at the level of growth factor receptor(s), or their related control mechanisms. Indeed, a recent study has demonstrated that the membrane anchor E-cadherin is detached from the membrane of adenoma cells compared to normal pituitary (Elston *et al.* 2009), suggesting that membrane changes and/or matrix-related events are a major feature of such tumours. Our results are compatible with the concept that they may be driving the process rather than occurring in response to cytoplasmic or nuclear signals. Furthermore, this may be therapeutically important as growth factor receptors signal through tyrosine receptor kinases (TRKs), and such TRKs may be blocked by small-molecule TRK inhibitors (TKIs). It is possible that the TKIs may be of value in the treatment of recurrent or resistant pituitary tumours in the future.

Our findings on CYCLIN D1, which is regulated by both ERK1/2 and Akt pathways, are particularly interesting. CYCLIN D1 is a proto-oncogene (11q13) which acts in the pRb/p16/CDK4 pathway on G₁-S progression of the cell cycle. *CCND1* gene amplification (Hibberts *et al.* 1999) and polymorphisms (Gazioglu *et al.* 2007), and enhanced nuclear (Jordan *et al.* 2000, Gazioglu *et al.* 2007) and/or cytoplasmic (Hibberts *et al.* 1999, Jordan *et al.* 2000, Gazioglu *et al.* 2007) accumulation, have been described in sporadic pituitary adenomas, especially in NFPAs (Hibberts *et al.* 1999, Jordan *et al.* 2000). Over-expression of CYCLIN D1 in NFPAs in comparison to other tumour types, as observed in our study, is essentially in agreement with the study by Hibberts *et al.* (1999) where CYCLIN D1 was found to be specifically

over-expressed in NFPAs in comparison to somatotrophinomas. However, the fact that in some cases of pituitary adenomas CYCLIN D1 over-expression occurred in the absence of *CCND1* allelic imbalance suggested that additional mechanisms responsible for deregulated CYCLIN D1 expression are involved in human pituitary tumourigenesis (Hibberts *et al.* 1999). The mitogen-dependent induction of CYCLIN D1 expression is one of the earliest cell cycle-related events to occur during the G₀/G₁ to S-phase transition (Lavoie *et al.* 1996). Activation of the Raf/MEK/ERK cascade has been shown to be sufficient to induce CYCLIN D1 promoter activity and CYCLIN D1 expression, even in the absence of growth factors. In contrast, the p38MAPK cascade showed an opposite effect on the regulation of CYCLIN D1 expression (Lavoie *et al.* 1996). Based on western blot analysis, we confirmed previous reports of CYCLIN D1 over-expression in NFPAs, but the increase was slight, was not seen in other tumour subtypes, and was not associated with any change in *CCND1* mRNA. We speculated that the lack of change in transcription might be secondary to changes in p38MAPK, which decreases CYCLIN D1 transcription, but we did not see any changes in either p38MAPK activation or in the total amount of this protein.

Our results therefore demonstrate that while there is indeed evidence for over-activation of both signalling pathways, their effects are to a major extent attenuated downstream. This suggests that, in these benign tumours, overdrive of the signalling pathways is blunted by protective anti-tumour mechanisms (Aoki *et al.* 2007, Chesnokova *et al.* 2008). One such mechanism is the recently – described process of senescence, particularly oncogene-induced senescence, whereby early neoplastic cells are eliminated from the proliferative pool. High pituitary p21 levels have been shown to promote senescence and restrain pituitary tumour growth, particularly in somatotroph tumours, and may underlie the failure of invariably benign pituitary tumours to progress to true malignancy (Chesnokova *et al.* 2008). In addition, the pleiotropic cytokine interleukin-6 (IL-6) has been demonstrated to mediate senescence in a cell-autonomous, i.e. non-paracrine, mode, and this is compatible with previous data that pituitary adenomas specifically immunostain for IL-6 (Tsagarakis *et al.* 1992, Jones *et al.* 1994).

In conclusion, in this study we describe over-activation of the downstream kinases to B-Raf in pituitary adenomas, as well as a slight increase in CYCLIN D1 in non-functioning tumours, but no change in c-MYC phosphorylation and hence stabilisation by ERK1/2. In contrast, the PI3K/Akt pathway

shows no increased activity in its major downstream TSC/mTOR components, but there was decreased phosphorylation and thus increased stabilisation of c-MYC by this pathway. We speculate that the causal agent(s) in sporadic pituitary tumourigenesis are upstream to the convergence of these two Ras-dependent pathways, and that induced senescence, possibly involving p21 and/or IL-6, is responsible for the benign nature of the great majority of such tumours.

Declaration of interest

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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