Protein western array analysis in human pituitary tumours: insights and limitations

Antônio Ribeiro-Oliveira Jr1,2, Giulia Franchi1,3, Blerina Kola1, Paolo Dalino1,4, Sérgio Veloso Brant Pinheiro2, Nabila Salahuddin1, Madalina Musat1, Miklós I Góth5, Sándor Czirják6, Zoltán Hanzély6, Deivid Augusto da Silva2, Eduardo Paulino Jr2, Ashley B Grossman1 and Mártia Korbonits1

1Department of Endocrinology, Barts and the London School of Medicine and Dentistry, London EC1M 6BQ, UK
2Department of Internal Medicine, Pediatrics and Pathology of Federal University of Minas Gerais, 30330-120 Belo Horizonte/Minas Gerais, Brazil
3Endocrinology Unit, ‘Vita-Salute’ San Raffaele University Hospital, via Olgettina 48, 20132 Milan, Italy
4Endocrinology Unit, Department of Endocrinology, Niguarda Ca’ Granda Hospital, Piazza Ospedale Maggiore, 3, 20162 Milan, Italy
5Endocrinology Unit, Internal Medicine, National Medical Center, 1135 Budapest, Hungary
6Endocrinology Unit, National Institute of Neurosurgery, 1143 Budapest, Hungary

(Correspondence should be addressed to A Ribeiro-Oliveira Jr who is now at Laboratório de Pesquisas em Endocrinologia, Federal University of Minas Gerais-Brasil, Avenida Alfredo Balena, 190, sala 151, Belo Horizonte, Brazil; Email: brolivei@uol.com.br)

Abstract

The molecular analysis of pituitary tumours has received a great deal of attention, although the majority of studies have concentrated on the genome and the transcriptome. We aimed to study the proteome of human pituitary adenomas. A protein array using 1005 monoclonal antibodies was used to study GH-, corticotrophin- and prolactin-secreting as well as non-functioning pituitary adenomas (NFPAs). Individual protein expression levels in the tumours were compared with the expression profile of normal pituitary tissue. Out of 316 proteins that were detected in the pituitary tissue samples, 116 proteins had not previously been described in human pituitary tissue. Four prominent differentially expressed proteins with potential importance to tumorigenesis were chosen for validation by immunohistochemistry and western blotting. In the protein array analysis heat shock protein 110 (HSP110), a chaperone associated with protein folding, and B2 bradykinin receptor, a potential regulator of prolactin secretion, were significantly overexpressed in all adenoma subtypes, while C-terminal Src kinase (CSK), an inhibitor of proto-oncogenic enzymes, and annexin II, a calcium-dependent binding protein, were significantly underexpressed in all adenoma subtypes. The immunohistochemical analysis confirmed the overexpression of HSP110 and B2 bradykinin receptor and underexpression of CSK and annexin II in pituitary adenoma cells when compared with their corresponding normal pituitary cells. Western blotting only partially confirmed the proteomics data: HSP110 was significantly overexpressed in prolactinomas and NFPAs, the B2 bradykinin receptor was significantly overexpressed in prolactinomas, annexin II was significantly underexpressed in somatotrophinomas, while CSK did not show significant underexpression in any tumour. Protein expression analysis of pituitary samples disclosed both novel proteins and putative protein candidates for pituitary tumorigenesis, though validation using conventional techniques are necessary to confirm the protein array data.

Endocrine-Related Cancer (2008) 15 1099–1114

Introduction

Pituitary tumours are very common neoplasms, with a reported prevalence of 17% in the general population, and they constitute 10% of intracranial neoplasms (Asa & Ezzat 2002, Ezzat et al. 2004). Although these tumours cause considerable morbidity and mortality due to their hormonal and local space-occupying effects, they only very rarely transform into true metastatic cancers (Kaltsas et al. 2005). Pituitary adenomas therefore offer an important model to study how cell proliferation is...
regulated, and how dysregulation may be compatible with adenoma transformation.

Studies over recent years have established that sporadic pituitary tumours rarely show somatic mutational changes in genes implicated in genetic pituitary tumour syndromes: menin (in multiple endocrine neoplasia-1 (MEN 1) syndrome (Chandrasekharappa et al. 1997)), CDKNIB (causing extremely rarely the MEN1 syndrome (Pellegrata et al. 2006, Georgitis et al. 2007)), protein kinase A regulatory subunit-1A (PRKAR1A), in a proportion of Carney complex cases (Kischner et al. 2000) and AIP (aryl hydocarbon receptor-interacting protein, causing a proportion of familial isolated pituitary adenoma cases (Vierimaa et al. 2006, Daly et al. 2007, Leontiou et al. 2008)). In terms of mRNA expression, MEN1, CDKNIB and PRKAR1A mRNA expression is not changed in sporadic pituitary adenomas (Dahia et al. 1998, Satta et al. 1999, Kaltas et al. 2002) while AIP mRNA is up-regulated rather than down-regulated in sporadic pituitary adenomas (Leontiou et al. 2008). In terms of protein expression, MEN1 and PRKAR1A show no change while CDKNIB protein is down-regulated in pituitary adenomas, especially in corticotropicinomas (Lidhar et al. 1999, Lloyd et al. 2002) and, again contrary to expectation, AIP protein is up-regulated in sporadic pituitary adenomas (Leontiou et al. 2008). Transgenic animal models suggest that changes in the regulation of the cell cycle are likely to be important in pituitary oncogenesis (Fero et al. 1996), although it is probable that many of these changes are secondary to more proximal abnormalities in cell signalling pathways. Therefore, it has been of benefit to explore the phenotypic and genotypic changes seen in pituitary adenomas as compared with normal human pituitary tissue in order to identify the dysregulated pathway(s).

Several dysregulated genes and proteins are expected to occur in different pituitary tumours, and we and others have recently published analyses of pituitary tumour mRNA using microarray technology (Zhan et al. 2003, Moreno et al. 2005, Morris et al. 2005). However, it is well known that a number of cell cycle proteins in particular are regulated at the protein level, and it is probable that mRNA analysis would not detect such changes. We have chosen to investigate the level of protein expression in pituitary tumours with a protein array using pituitary adenomas, and compared with normal pituitary protein expression patterns, in order to identify novel expressed proteins in human pituitary tissue and to search for putative proteins involved in pituitary tumorigenesis. Furthermore, we used both immunohistochemistry and immunoblotting to quantify protein expression individually on a subset of these proteins in order to attempt validation of the findings. Our results confirm that protein array may be a useful approach to identify novel candidate proteins, but the results obtained must be confirmed by more established techniques. This in turn suggests that the results of proteomic analysis must always be considered in the light of the other data.

Subjects and methods

Tissue specimens

Samples from pituitary adenomas were removed at transsphenoidal surgery and frozen immediately. Autopsy normal pituitary samples were collected within 24 h of death from patients with no evidence of any endocrine abnormality. For the BD PowerBlot Western array (BD Biosciences Pharmingen, La Jolla, CA, USA), a total of 20 human pituitary adenomas were selected, representative of each of the main adenoma subtypes (growth hormone-(GH), prolactin-(PRL) and corticotrophin (ACTH)-secreting tumours and non-functioning pituitary adenomas (NFPA)), whilst 5 normal pituitaries were used as controls. For the immunohistochemistry, three GH-, three PRL-, three ACTH-secreting tumours, three NFPA and four normal pituitary samples were studied. For the western blotting, 10 GH-, 7 PRL-, 7 ACTH-secreting tumours, 10 NFPA and 12 normal pituitary samples were studied. The tumour type was determined on the basis of clinical and biochemical findings before surgery, and by morphological and immunohistochemical analysis of the removed tissue samples, as described previously (Lidhar et al. 1999). Informed consent was obtained from all patients, and the protocol was approved by the institutional Research Ethics Committee.

Protein extraction and quantification

For the BD PowerBlot Western array, five samples for each tumour subtype and five samples of normal pituitary were combined in order to obtain a single sample of 150 mg tissue for each diagnosis. Samples were lysed and the protein concentration measured as previously described (Musat et al. 2005).

BD powerblot western array

The BD PowerBlot was performed by BD Biosciences Pharmingen utilising a high-resolution two-dimensional electrophoresis as previously described (Phelan & Nock 2003). Protein concentrations were quantified...
and samples were run on six high-resolution 4–15% gradient SDS–polyacrylamide gels in triplicate. Each of the four subtypes of pituitary adenomas and the normal pituitary tissue was studied using a single array. After electrophoresis, the gels were transferred to PVDF membranes. Each membrane was divided into 41 lanes by applying a chamber-forming grid and probed with 1005 high-quality monoclonal antibodies per run. After 1 h of incubation at room temperature, the chambers were rinsed and incubated with single fluorescent secondary antibody under the same conditions (Alexa 680-labelled goat anti-mouse IgGs; Molecular Probes, Eugene, OR, USA). Image data were captured using the Odyssey Infrared Imaging System (LI-COR). Molecular masses were determined using molecular weight standards (BD Biosciences).

Samples were run in triplicate and analysed using a 3×3 matrix comparison method. For example, run 1 of the control was compared with runs 1, 2 and 3 of the experimental groups. Run 2 of the control was compared with runs 1, 2 and 3 of the experimental groups. Run 3 of the control was compared with runs 1, 2 and 3 of the experimental groups – altogether 9 comparisons. Comparative analysis (i.e. fold change in expression level in treated versus control) followed, and protein expression level changes were determined. Changes were divided into 10 categories based on reproducibility and signal intensity, with ‘level 10’ (the highest) representing changes greater than twofold in all 9 comparisons that were from good quality signals and passed a visual inspection. Only the proteins which were expressed in pituitary samples and reached at least ‘level 1’ of confidence are reported (316 altogether from the 1005 tested antibodies).

We then filtered the data in order to identify the most overexpressed or underexpressed proteins, corresponding to those with good replicates (coefficient of variation <10%) and a confidence level of 10 for each pituitary adenoma subtype. We then selected four proteins with prominent changes seen in all pituitary adenoma subtypes, and with potential relevance to human tumorigenesis, as putative protein candidates for further study and confirmation using immunohistochemistry and western blotting.

Immunohistochemistry

In order to study the distribution of the four selected proteins (heat shock protein (HSP110), B2 bradykinin receptor, CSK (C-terminal Src kinase) and annexin II), and also to compare their presence in neoplastic cells with their respective non-neoplastic cells, we utilised confocal immunofluorescent analysis of the GH-, PRL- and ACTH-secreting adenomas and normal pituitary tissue, or the streptavidin–biotin method (DakoCytomation Inc., Carpinteria, CA, USA) for the NFPAs. The clinical-pathological diagnosis of all pituitary adenomas had been confirmed using immunohistochemical determination of the expressed pituitary hormones: for the adenoma samples, all slides utilised showed more than 90% of the specific tumour, as confirmed by haematoxylin and eosin. Normal pituitary architecture was evaluated by reticulin staining. For confocal immunofluorescent analysis of GH-, PRL- and ACTH-secreting adenomas, after deparaffinisation and hydrating procedures, the sections were incubated in blocking solution (1% BSA and 0.1% Tween 20) at room temperature for 1 h and then incubated overnight at 4 °C with one of the primary antibodies (purified mouse anti-HSP110 antibody (1:200), anti-mouse B2 bradykinin receptor antibody (1:200), goat polyclonal to CSK antibody (1:200) and goat polyclonal to annexin II antibody (1:400)). In order to co-localise the selected proteins in different non-neoplastic cells, rabbit anti-GH (1:200), rabbit anti-prolactin (1:200) or rabbit anti-ACTH (1:200), supplied by Dako (Oxford, UK), were also added to the incubation buffer used in normal pituitary samples. After an overnight incubation, slides were rinsed five times in PBS and then incubated for 1 h in the dark at room temperature with anti-mouse IgG-conjugated with FITC (1:400, Jackson ImmunoResearch, West Grove, PA, USA) or anti-goat conjugated with Cy5 (1:400, Jackson), according to the primary antibody previously used. An anti-rabbit conjugated with Cy3 (1:400, Jackson) was also added to the incubation buffer used on normal pituitary samples. Following washes with PBS, sections were mounted in 90% glycerol/10% TRIS 1M and images were captured through confocal microscope (Zeiss LSM 510), 40× objective, and 400× original magnification. All confocal settings were determined at the beginning of imaging session and remained unchanged. For quantitative analysis, images were captured at eight bits and analysed in grey scale. Three to four images were captured from each sample and three measurements were obtained for each image. ImageJ (NIH, Bethesda, USA) software was used to quantify fluorescence intensity and area. Background fluorescence was then subtracted from the region of interest. The intensity of fluorescence corresponded to the unit ‘grey level’, varying from zero (black) to 255 (white), as an average of the area (sum of grey value of all pixels divided by the number of pixels/area).

For the non-functional pituitary adenomas, while it is accepted that the majority of them are of gonadotrophic origin, many do not express gonadotrophins uniformly or may express only one subunit (follicle-stimulating...
hormone, FSH, luteinizing hormone and/or human chorionic gonadotrophin, HCG) rendering the double-labelling technique difficult. The NFPAs were therefore selected on the basis of their immunohistochemical expression of FSH using monoclonal anti-FSH (Novocastra, Newcastle-upon-Tyne, UK). Conventional immunohistochemistry using consecutive cuts was performed, as described previously (Lidhar et al. 1999), and FSH staining positive areas were compared between NFPAs and normals. Sections were assessed by a single observer blinded to the diagnosis. In each section, at least 200 cells were analysed. The percentage of positive cells was then expressed as a ratio of positive cells to the total number of cells counted. Photographs were taken with a Canon PowerShot A530 colour video camera and processed in Corel photo-Paint, version 12.0.0.458, 2003, Corel Corporation.

Western blotting

Human pituitary adenoma tissue and normal pituitary tissue were studied for the assessment of the four selected proteins: HSP110, B2 bradykinin receptor, CSK and annexin II. In each case, 10 µg protein samples were subjected to 10% SDS-PAGE separation, with protein transfer to a PVDF membrane. The membrane was blocked with 5% non-fat milk for 90 min and incubated overnight at 4°C with the primary antibody. The membrane was washed six times with PBS containing 0.01–0.05% Tween and subsequently incubated for 90 min at room temperature. A chemiluminescent peroxidase substrate, ECL (Amersham-Pharmacia), was applied according to the manufacturer’s instructions, and the membranes were exposed briefly to X-ray film. The PVDF membrane was then stripped with 0.985 g Tris–Hydrochloride, 2 g SDS, 781 µl 2-mercaptoethanol (pH 4), and reprobed. The antibodies and the respective dilutions that were used are the following: purified mouse anti-HSP110 antibody at 1:1000 (BD Biosciences Pharmingen), mouse anti-B2 Bradykinin Receptor antibody at 1:1000 (Fitzgerald, Concord, MA, USA), polyclonal goat anti-CSK (1:500) and anti-annexin II (1:200) antibody (Abcam, Cambridge, UK) and for equal loading rabbit polyclonal anti-GAPDH antibody at 1:10 000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). An anti-goat antibody at 1:5000, an anti-rabbit antibody at 1:2000, and an anti-mouse antibody at 1:2000 Dako were the secondary antibodies used. Densitometric readings of the resulting bands were evaluated using the ImageJ program, and the ratio to GAPDH was calculated and compared with controls.

Statistical analysis

The data were tested for statistical significance with the non-parametric Mann–Whitney test or multivariate analysis using the Kruskal–Wallis test followed by Conover–Inman analysis (StatsDirect, Ian Buchan, Cambridge, UK). Significance was taken as P < 0.05. All values given are means ± S.E.M.

Results

The 1005 antibodies utilised in the BD PowerBlot Western array detected a total of 316 proteins from the human pituitary tissues. These proteins were categorised into different functional groups, according to their areas of activity, and they are summarised in Fig. 1 and listed with their functional categories in Supplementary Table 1, which can be viewed online at http://erc.endocrinology-journals.org/supplemental/. Out of these 316 proteins, we found through a literature survey and interrogation of a human pituitary database (Zhan & Desiderio 2003, Zhao et al. 2005a) that 116 proteins had not previously been described as being expressed in human pituitary tissue, and 81 of these have also not been studied in the pituitary of any other species.

Expression profile of adenomas

The expression profiles for GH-, PRL-, ACTH-secreting and non-functioning adenomas and NFPAs, and the expression profile comparisons to normal autopsy pituitary tissues, using the BD PowerBlot Western array, were analysed. The main criteria used to select potential candidate proteins were ‘level 10’ of confidence for all pituitary adenoma subtypes. We also considered in this selection process the consistency within the replicates in the array, our previous microarray data (Morris et al. 2005), and the possible relevance of the proteins for human tumorigenesis. The most significant and consistently over- and underexpressed ‘level 10’ proteins occurring in all adenoma types studied are shown in Table 1. ‘Level 10’ proteins that were specific to only one or two adenoma subtypes are shown in Table 2. Interestingly, there were some ‘level 10’ proteins that were present only in certain tumours and not present in normal pituitary tissue, while others were present in normal pituitary tissue but not present in certain tumours (Table 3).

In all pituitary adenomas subtypes, the number of ‘level 10’ overexpressed proteins was higher than the number of ‘level 10’ underexpressed ones (Table 4). However, the fold-change of underexpression was higher for the underexpressed proteins when compared with the fold change for the overexpressed ones (Table 4).
Identification of potential candidate proteins

We selected four proteins which showed significant changes in all four adenoma subtypes, two being overexpressed (HSP110 and B2 bradykinin receptor) and two underexpressed (CSK and annexin II) for further study. HSP 110 and C-terminal Src kinase had not specifically been described before as part of the human pituitary proteome database (Zhan & Desiderio 2003, Zhao et al. 2005a).

HSP110 showed an average of 17-fold overexpression in all adenoma subtypes as compared with normal pituitary tissues (33-, 9-, 10- and 14-fold overexpression for GH-, PRL-, ACTH-secreting adenomas and NFPA respectively) in the protein array analysis. In our previous pituitary microarray study HSP110 gene expression showed underexpression although this was not significant (Morris et al. 2005).

The B2 bradykinin receptor showed an average of 11-fold overexpression in all adenomas subtypes as compared with normal pituitary tissues (16-, 20-, 5- and 4-fold overexpression for GH-, PRL-, ACTH-secreting adenomas and NFPA respectively). No data from our previous microarray were available for the B2 bradykinin receptor. Other studies have suggested a role for this receptor in anterior pituitary regulation (Sharif et al. 1988, Kuan et al. 1990), and the importance of its up-regulation in other human cancers has been emphasised (Ikeda et al. 2004, Dlamini & Bhoola 2005, Zhao et al. 2005b).

The CSK protein showed an average of 117-fold underexpression in all adenoma subtypes as compared with normal pituitary tissues (25-, 168-, 134- and 140-fold underexpression for GH-, PRL-, ACTH-secreting adenomas and NFPA respectively). Our previous microarray data also showed reduced mRNA expression in pituitary adenomas (Morris et al. 2005).

Finally, annexin II showed an average of 27-fold underexpression in all adenoma subtypes as compared with normal pituitary tissues (25-, 20-, 32- and 31-fold underexpression for GH-, PRL-, ACTH-secreting adenomas and NFPA respectively). A significant level of underexpression had also been shown in our previous microarray data, where an average fourfold underexpression was found (Morris et al. 2005).
Immunohistochemistry for HSP110, B2 bradykinin receptor, CSK and annexin II

In order to compare the expression levels of an individual protein between specific cell types in the normal pituitary and the specific cell type-derived tumours, we performed confocal immunofluorescent analysis for GH-, PRL- and ACTH-secreting adenomas. For the NFPA, conventional immunohistochemistry with consecutive cuts was performed and FSH-positive tumour areas were compared with FSH-positive areas from the normal pituitary. To this end, 12 pituitary tumours and 4 normal pituitaries were selected.

The confocal immunofluorescent analysis showed that HSP110 was expressed in the normal pituitary, with mild coexpression in normal GH, PRL and ACTH cells. It also showed the overexpression of HSP110 in GH-, PRL- and ACTH-secreting tumour cells when compared with respective normal cells from normal pituitary \((P < 0.01 \text{ for all comparisons})\). HSP110 was also overexpressed in FSH-staining tumour when compared with FSH cells from normal pituitary \((P < 0.01)\). These data confirm the overexpression of this protein in tumorous cells when compared with the corresponding normal pituitary cells (Fig. 2).

### Table 1 Most consistently changing proteins in all adenoma subtypes

<table>
<thead>
<tr>
<th>Protein</th>
<th>Swiss-Prot ID</th>
<th>GH</th>
<th>PRL</th>
<th>ACTH</th>
<th>NFPA</th>
<th>Direction of change</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARF3 (ADP-ribosylation factor 3)</td>
<td>P16587</td>
<td>14</td>
<td>26</td>
<td>N/A</td>
<td>28</td>
<td>+</td>
</tr>
<tr>
<td>Annexin I</td>
<td>P46193</td>
<td>12</td>
<td>13</td>
<td>31</td>
<td>24</td>
<td>−</td>
</tr>
<tr>
<td>Annexin II</td>
<td>P07355</td>
<td>25</td>
<td>20</td>
<td>32</td>
<td>31</td>
<td>−</td>
</tr>
<tr>
<td>B2 bradykinin receptor</td>
<td>P30411</td>
<td>16</td>
<td>20</td>
<td>05</td>
<td>04</td>
<td>+</td>
</tr>
<tr>
<td>CSK tyrosine-protein kinase CSK (C-Src Kinase)</td>
<td>P32577</td>
<td>25</td>
<td>168</td>
<td>134</td>
<td>140</td>
<td>−</td>
</tr>
<tr>
<td>Heat shock-110 kDa</td>
<td>Q60446</td>
<td>33</td>
<td>09</td>
<td>10</td>
<td>14</td>
<td>+</td>
</tr>
<tr>
<td>MEF 2D (myocyte-specific enhancer factor 2D)</td>
<td>Q63943</td>
<td>20</td>
<td>15</td>
<td>06</td>
<td>21</td>
<td>+</td>
</tr>
<tr>
<td>P54nrb (54 kDa nuclear RNA- and DNA-binding protein)</td>
<td>Q15233</td>
<td>39</td>
<td>50</td>
<td>29</td>
<td>68</td>
<td>+</td>
</tr>
<tr>
<td>Rab3 (Rab-protein 3, member RAS oncogene)</td>
<td></td>
<td>04</td>
<td>N/A</td>
<td>14</td>
<td>11</td>
<td>+</td>
</tr>
<tr>
<td>RBP (retinol-binding protein I, cellular)</td>
<td>P09455</td>
<td>06</td>
<td>177</td>
<td>N/A</td>
<td>26</td>
<td>−</td>
</tr>
<tr>
<td>SCAMP1 (secretory carrier membrane protein 1)</td>
<td>O15126</td>
<td>12</td>
<td>10</td>
<td>N/A</td>
<td>06</td>
<td>+</td>
</tr>
</tbody>
</table>

Average fold change for each adenoma subtype of the most consistent over- (+) or underexpressed (−) ‘level 10’ proteins from the array in alphabetical order. N/A, not available.

### Immunohistochemistry for HSP110, B2 bradykinin receptor, CSK and annexin II

In order to compare the expression levels of an individual protein between specific cell types in the normal pituitary and the specific cell type-derived tumours, we performed confocal immunofluorescent analysis for GH-, PRL- and ACTH-secreting adenomas. For the NFPA, conventional immunohistochemistry with consecutive cuts was performed and FSH-positive tumour areas were compared with FSH-positive areas from the normal pituitary. To this end, 12 pituitary tumours and 4 normal pituitaries were selected.

The confocal immunofluorescent analysis showed that HSP110 was expressed in the normal pituitary, with mild coexpression in normal GH, PRL and ACTH cells. It also showed the overexpression of HSP110 in GH-, PRL- and ACTH-secreting tumour cells when compared with respective normal cells from normal pituitary \((P < 0.01 \text{ for all comparisons})\). HSP110 was also overexpressed in FSH-staining tumour when compared with FSH cells from normal pituitary \((P < 0.01)\). These data confirm the overexpression of this protein in tumorous cells when compared with the corresponding normal pituitary cells (Fig. 2).

### Table 2 Most consistently changing proteins for each adenoma subtype

<table>
<thead>
<tr>
<th>Protein</th>
<th>Swiss-Prot ID</th>
<th>Adenoma subtype</th>
<th>Average fold change</th>
<th>Direction of change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine receptor b</td>
<td>P25109</td>
<td>ACTH</td>
<td>14</td>
<td>+</td>
</tr>
<tr>
<td>ASS (argininosuccinate synthase)</td>
<td>P00966</td>
<td>PRL</td>
<td>21</td>
<td>+</td>
</tr>
<tr>
<td>Apoptosis regulator BAX</td>
<td>Q07812</td>
<td>NFPA</td>
<td>15</td>
<td>+</td>
</tr>
<tr>
<td>Dematin</td>
<td>Q08495</td>
<td>ACTH and GH</td>
<td>10 and 10</td>
<td>+</td>
</tr>
<tr>
<td>EBP 50 (Ezrin–radixin–moesin-binding phosphoprotein 50)</td>
<td>O14745</td>
<td>PRL and NFPA</td>
<td>19 and 15</td>
<td>+</td>
</tr>
<tr>
<td>ECA39 (branched-chain-amino-acid aminotransferase)</td>
<td>P54687</td>
<td>NFPA</td>
<td>14</td>
<td>+</td>
</tr>
<tr>
<td>Epithelial-cadherin precursor</td>
<td>P12830</td>
<td>PRL</td>
<td>39</td>
<td>+</td>
</tr>
<tr>
<td>HILP/XIAP (inhibitor of apoptosis protein 3/X-linked IAP)</td>
<td>P98170</td>
<td>NFPA and ACTH</td>
<td>18 and 11</td>
<td>+</td>
</tr>
<tr>
<td>Lamin A/C</td>
<td>Q06981</td>
<td>ACTH</td>
<td>14</td>
<td>−</td>
</tr>
<tr>
<td>L-Caldesmon</td>
<td>Q05682</td>
<td>ACTH</td>
<td>18</td>
<td>+</td>
</tr>
<tr>
<td>MEPHX (microsomal epoxide hydrolase)</td>
<td>P07099</td>
<td>GH, PRL and NFPA</td>
<td>14, 17 and 10</td>
<td>−</td>
</tr>
</tbody>
</table>

The 11 most consistent ‘level 10’ proteins for pituitary adenoma subtypes in alphabetical order, including average fold change and direction of change (+/−).
The confocal immunofluorescent analysis showed that B2 bradykinin receptor was expressed in the normal pituitary, with mild coexpression in normal GH, PRL and ACTH cells. It also showed overexpression of this protein in GH-, (P < 0.05), PRL-, (P < 0.01) and ACTH-secreting tumour cells (P < 0.01) when compared with their respective pituitary cells (P < 0.01). B2 bradykinin receptor was also overexpressed in FSH-staining tumour when compared with FSH cells from normal pituitary (P < 0.01). These data confirm the overexpression of B2 bradykinin receptor in tumorous cells when compared with the corresponding normal pituitary cells (Fig. 3).

For the CSK protein, the confocal immunofluorescent analysis showed expression of this protein in normal pituitary, with coexpression in GH, PRL and ACTH cells. It also showed underexpression in GH-, PRL- and ACTH-secreting tumour cells when compared with their respective normal pituitary cells (P < 0.01). CSK was also underexpressed in FSH-staining tumour when compared with FSH cells from normal pituitary (P < 0.01). These data confirm the underexpression of CSK in all adenoma tumour cells compared with the corresponding normal pituitary cells (Fig. 3).

### Table 3
Proteins that were not detected in normal pituitary or in one adenoma type

<table>
<thead>
<tr>
<th>Protein</th>
<th>Swiss Prot ID</th>
<th>Adenoma subtype</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>14-3-3e-23KD 14-3-3 protein epsilon (mitochondrial import stimulation factor L subunit)</td>
<td>P42655</td>
<td>Underexpression in GH secreting adenomas and NFPA</td>
<td>Cannot be detected in GH secreting tumours and NFPA</td>
</tr>
<tr>
<td>CPG16/Calbin kinase VI (calcium/calmodulin-dependent protein kinase type I-like)</td>
<td>O08875</td>
<td>Overexpression in ACTH-secreting adenomas</td>
<td>Cannot be detected in normal pituitary</td>
</tr>
<tr>
<td>GRIM-19 (cell death-regulatory protein 19)</td>
<td>P13693</td>
<td>Underexpression in ACTH-secreting adenomas</td>
<td>Cannot be detected in ACTH-secreting tumours</td>
</tr>
<tr>
<td>HRF (histamine-releasing factor)</td>
<td>P35268</td>
<td>Overexpression in PRL-secreting adenomas</td>
<td>Cannot be detected in normal pituitary</td>
</tr>
<tr>
<td>L22-18-19 KD (60s ribosomal protein L22)</td>
<td>Q13163</td>
<td>Overexpression in PRL-secreting adenomas</td>
<td>Cannot be detected in normal pituitary</td>
</tr>
<tr>
<td>MEK5-53-57KD (dual specificity mitogen-activated protein kinase kinase 5; MAP kinase kinase 5)</td>
<td>O54916</td>
<td>Overexpression in GH-secreting adenomas</td>
<td>Cannot be detected in normal pituitary</td>
</tr>
<tr>
<td>PP2A catalytic α-34KD (protein phosphatase 2, catalytic subunit, alpha)</td>
<td>Q04912</td>
<td>Overexpression in GH-secreting adenomas</td>
<td>Cannot be detected in normal pituitary</td>
</tr>
<tr>
<td>Reptilin (RalBP1-associated Eps domain-containing protein 1; RalBP1-interacting protein 1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RONa (macrophage-stimulating protein receptor precursor; MSP receptor; p185-Ron; CD136 antigen; CDw 136)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Consistent level 10 proteins for specific adenoma subtypes that showed gross over or underexpression compared with normal pituitary. NFPA, non-functional pituitary adenoma.

### Table 4
The number of the most consistently changing proteins in each adenoma subtype

<table>
<thead>
<tr>
<th>‘Level 10’ proteins</th>
<th>GH</th>
<th>PRL</th>
<th>ACTH</th>
<th>NFPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of ‘level 10’ overexpressed proteins</td>
<td>29</td>
<td>29</td>
<td>36</td>
<td>36</td>
</tr>
<tr>
<td>Number of ‘level 10’ underexpressed proteins</td>
<td>12</td>
<td>11</td>
<td>22</td>
<td>20</td>
</tr>
<tr>
<td>Means±S.E.M. of fold changes in ‘level 10’ overexpressed proteins</td>
<td>8.9±0.8*</td>
<td>10.9±1.1†</td>
<td>6.7±0.5*</td>
<td>9.0±1.1†</td>
</tr>
<tr>
<td>Means±S.E.M. of fold changes in ‘level 10’ underexpressed proteins</td>
<td>10.6±1.0</td>
<td>25.7±7.0</td>
<td>13.8±2.0</td>
<td>16.4±2.4</td>
</tr>
</tbody>
</table>

Number of ‘level 10’ proteins and profile of their over and underexpressions in each adenoma subtype compared with controls. *P < 0.05 for comparisons between fold changes in ‘level 10’ over and underexpressed proteins. †P < 0.01 for comparisons between fold changes in ‘level 10’ over and underexpressed proteins.
when compared with the corresponding normal pituitary cells (Fig. 4).

The confocal immunofluorescent analysis showed expression of annexin II in normal pituitary, with coexpression in GH, PRL and ACTH cells. It also showed underexpression of this protein in GH-, PRL- and ACTH-secreting tumour cells when compared with their respective normal pituitary cells \( (P < 0.01 \text{ for all comparisons}) \). Annexin II was also underexpressed in FSH-staining tumour when compared with FSH cells from normal pituitary \( (P < 0.01) \). These data confirm the underexpression of annexin II in all adenoma tumour cells when compared with the correspondent normal pituitary cells (Fig. 5).

**Western blotting for HSP110, B2 bradykinin receptor, CSK and annexin II**

In order to validate the expression patterns of HSP110, B2 bradykinin receptor, CSK and annexin II as shown by protein array, conventional western blotting was also performed on the proteins from 34 pituitary tumours and 12 normal pituitaries. Certain samples were the same as the ones included in the protein array, to these we added additional tumours and normal pituitary samples for this validation process.

HSP110 immunoblotting confirmed overexpression in NFPAs \( (P < 0.01 \text{ compared with normal controls}) \) and prolactin-secreting adenomas \( (P < 0.05) \), but no
significant overexpression was detected in GH- or ACTH-secreting adenomas (Fig. 6A). These data therefore confirmed in part, the protein overexpression shown in the protein array to all adenomas subtypes. Immunoblotting for the B2 bradykinin receptor confirmed overexpression in prolactin-secreting adenomas ($P < 0.05$), correlating well with the degree of overexpression shown for this adenoma subtype in the protein array; ACTH-secreting adenomas also showed a trend towards overexpression ($P = 0.1$), but neither the NFPAs nor the GH-secreting adenomas showed significant changes (Fig. 6B). These data thus confirmed the overexpression of B2 bradykinin receptor shown in the protein array for the prolactin-secreting adenomas alone.

For the underexpressed CSK protein, the results on the conventional western blotting were not confirmed; none of the adenoma subtypes showed significantly reduced expression by immunoblotting (Fig. 6C). Annexin II, another prominent underexpressed protein in the protein array, was underexpressed in GH-secreting adenomas ($P < 0.01$) and there was also a trend for underexpression in the NFPAs ($P = 0.09$). However, this was not seen in either ACTH- or PRL-secreting adenomas (Fig. 6D).

Discussion

We have utilised a proteomics technique to identify a series of proteins that are differentially expressed in pituitary adenomas when compared with the normal
pituitary. Due to the magnitude of the amount of protein required for protein array, and the relatively small amount of material available from each tumour, we had to amalgamate tissue from five separate tumours for each tumour subtype sample. Following the analysis of protein array data, we explored the expression of four grossly over- and underexpressed proteins seen in all tumour subtypes by immunohistochemistry and conventional western blotting. The proteomic array data were fully reproduced by the immunohistochemistry but only partially reproduced by western blotting.

High-resolution two-dimensional gel electrophoresis (2-DE) has been the standard tool for expression proteomics since its introduction more than 30 years ago. The protein array that we describe here covered 1005 proteins with monoclonal antibodies, out of which 316 were expressed in pituitary tissue. Excellent previous studies have set up a database of proteins expressed in pituitary tumours (Zhan & Desiderio 2003, Zhao et al. 2005a) and now our data add a large number of novel proteins into this database. These proteins are shown in Supplementary Table 1 according to their functional categories and direction of changes observed in adenomas. Despite various improvements of the 2-DE technique, one of the main inherent limitations of this method is the fact that many proteins that are expressed at low levels may escape detection. On the other hand, the findings shown in Table 4 of many-fold changes in underexpression suggest a considerable sensitivity for this array.

Figure 4 Double immunofluorescent staining using CSK (red staining) and polyclonal GH, PRL and ACTH (green staining) antibodies in normal pituitary, and CSK (red staining) GH-, PRL- and ACTH-secreting adenomas. Colocalisation is shown by orange colour (scale bar 50 μm). In figure 4D, conventional immunohistochemistry staining using CSK (brown) in consecutive cuts of normal pituitary tissue and non-functional pituitary adenomas (FSH positive); scale bar 25 μm. All data are shown as means ± s.e.m.; AU, arbitrary units; *P < 0.05, **P < 0.01.
The molecular analysis of pituitary tumours has been extensively studied, and a number of factors involved in pituitary tumorigenesis have been described. The genome of a given cell or organism is relatively static, subject to epigenetic modulation, whereas the transcriptome and proteome are more dynamic, and change with time and conditions reflecting the different state of the disease. Indeed, the proteome is much more complex than the transcriptome due to post-translational modifications, translocation, protein–protein interactions and regulation, but otherwise may provide more direct information when compared with the transcriptome in relation to oncogenesis. An important aim of proteomics has been to understand the cellular function at the protein level by the dynamic proteome, clarifying the functions of proteins and disclosing new biomarkers for disease states.

The four proteins chosen from the protein array for validation by immunoblotting showed consistent prominent over- or underexpression in all adenoma subtypes in the original array. Furthermore, two of these proteins (HSP110 and CSK) had not previously been described as expressed in normal human pituitary tissue. The data obtained from the immunohistochemical analysis confirmed these changes for all four proteins. Immunoblotting showed only partial parallelism with the array data; it confirmed the results shown in the protein array of overexpression of HSP110 in NFPAs and prolactin-secreting adenomas.

**Figure 5** Double immunofluorescent staining using annexin II (red staining) and polyclonal GH, PRL and ACTH (green staining) antibodies in normal pituitary, and annexin II (red staining) in GH-, PRL- and ACTH-secreting adenomas. Colocalisation is shown by orange colour (scale bar 50 μm). In figure 5D, conventional immunohistochemistry staining using annexin II (brown) in consecutive cuts of normal pituitary tissue and non-functional pituitary adenomas (FSH positive); scale bar 25 μm. All data are shown as means ± S.E.M.; AU, arbitrary units; *P < 0.05, **P < 0.01.
and also overexpression of the B2 bradykinin receptor in prolactin-secreting adenomas. Furthermore, it confirmed the underexpression of annexin II in GH-secreting adenomas, although it completely failed to mirror the results for CSK. These data implicate a role for HSP110, the B2 bradykinin receptor, CSK and annexin II in the process of pituitary tumorigenesis in these tumour subtypes, although whether these changes are causal or consequential remains unclear.

HSP110 is expressed in most tissues, and like other stress proteins, HSP110 is a chaperone protein associated with protein folding. It can be induced by heat shock and its induction is associated with thermotolerance. HSP’s overexpression has been recently described as important in a series of human cancers (Feng et al. 2005, Melle et al. 2006). Our previous microarray data showed a trend for HSP110 in the opposite direction (Morris et al. 2005), suggesting that some form of post-transcriptional modification accounts for these results. The novel data regarding the overexpression of molecular chaperone HSP110 in NFPAs and prolactin-secreting tumours demonstrates the added value of proteomic analysis subsequent to mRNA analysis. Recent proteomic studies on breast cancer cells have shown that HSPs, including HSP110, showed protein expression patterns that might be altered by 17β-oestradiol treatment (Lee et al. 2006). Furthermore, APG-2 protein, a member of the HSP110 family, has shown developmental changes in its expression at the protein level (Okui et al. 2000). These data demonstrate the utility of an analysis at the protein level. As recent publications have characterised HSP110 and other HSPs as potential anti-tumour agents (Wang et al. 2001, Casey et al. 2003), it is possible that HSP110 overexpression in these adenomas may be a protective mechanism to reduce adenomatous proliferation, and may relate to the benign nature of the majority of such tumours. Other heat shock-induced proteins have also been demonstrated to be differentially induced in tumorous tissue (Feng et al. 2005, Melle et al. 2006).

The B2 bradykinin receptor is a member of the kallikrein system, a subgroup of the serine protease family of enzymes, which also includes the enzyme known as prostate-specific antigen, an important clinical marker in human prostate cancers (Diamandis & Yousef 2002). The B2 bradykinin receptor was overexpressed in prolactin-secreting tumours; previous work has shown that bradykinin increases prolactin, but not GH release, and that this stimulation could be antagonised by a B2-type bradykinin receptor antagonist and inhibited by dopamine (Kuan et al. 1990). Other studies have suggested a role for this receptor in anterior pituitary regulation (Sharif et al. 1988). The B2 bradykinin receptor belongs to the kallikrein-kinin system, and several of its components have been reported to be tumour markers for many malignancies (Diamandis & Yousef 2002), especially those of endocrine-related organs (Yousef & Diamandis 2003). The importance of its up-regulation in other human cancers has also been described (Ikeda et al. 2004, Dlamini & Bhoola 2005, Zhao et al. 2005).

Furthermore, it has been suggested that some kallikreins may be part of a novel enzymatic cascade pathway that is activated in some cancers, with a particular potential role for kallikreins in central nervous system (Yousef & Diamandis 2003, Yousef et al. 2003). While its function in pituitary oncogenesis remains obscure, it may offer a novel therapeutic target (Stewart et al. 1997).
CSK is a non-receptor protein tyrosine kinase, its major function being to specifically phosphorylate a conserved C-terminal tyrosine of Src family kinases. As a consequence of down-regulation of the Src kinase protein expression and presumably its activity, proto-oncogenic enzymes controlling cell growth and proliferation would diminish. It is well known that Src-family protein tyrosine kinases are tightly regulated in normal cells and aberration in their regulation can lead to constitutive activation, which is associated with certain types of cancer (Kim et al. 2004, Chong et al. 2005, Martin 2006).

As a calcium-dependent phospholipids-binding protein, annexin II has been suggested to play a role in exocytosis from anterior pituitary secretory cells (Turgeon et al. 1991, Senda et al. 1994). It has previously been reported as being down-regulated in some human cancers (Xin et al. 2003, Gillette et al. 2004), although overexpression of annexin II on the cell surface has been suggested to contribute to the development of metastases by enhancing tumour cell detachment and tissue invasion (Choi et al. 2000, Ma et al. 2000). Annexin II was described in a previous human pituitary proteomics analysis (Zhan & Desiderio 2003); in this case our finding of underexpression in GH-secreting adenomas is in accordance with our previous microarray showing down-regulation of annexin II mRNA in GH-secreting adenomas (Morris et al. 2005). The importance of the annexin family as potential regulatory factor in pituitary tumours has been described, and expression of annexins I and V have been demonstrated in pituitary tumours (Traverso et al. 1999, Mulla et al. 2004). Specifically, annexin II overexpression has previously been shown in a GH-secreting carcinoma (Mulla et al. 2004). It is therefore possible that carcinomatous transformation per se leads to a specific annexin II increase.

While factors involved in pituitary tumorigenesis have been subject to intensive study (Asa & Ezzat 2002, Heaney & Melmed 2004, Kaltas et al. 2005, Farrell 2006) there have been relatively few studies of the specific pituitary protein changes using proteomics (Zhan & Desiderio 2003, Zhan et al. 2003, Asa & Ezzat 2005, Moreno et al. 2005, Zhao et al. 2005a). This study was performed using a screening commercial protein array to track many different proteins simultaneously in order to obtain greater insight into cellular function. However, there are problems associated with the limited sampling that is often employed, and also some uncertainty regarding the use of autopsy controls for comparison with fresh adenomatous tissue. In our study all tissues were normalised for protein content, and such tissue is the best currently available for comparison to tumours. Reassuringly, a considerable proportion of proteins showed higher expression in the normal autopsy tissue than in the surgical adenoma samples.

Despite the recent advances in human proteomics (Garbis et al. 2005, Senzer et al. 2005), we believe confirmation with additional technique(s) can provide further useful data. Our proteomics data were corresponding with the immunohistochemistry but the immunoblotting results were more variable. Especially, the fold changes over- or underexpressions shown by the protein array were only weakly correlated with the quantitative changes observed in western blotting.

Indeed, several shortcomings have been described in proteomic studies, and while there are few reports on proteomics for pituitary adenomas, the findings may be even more difficult to interpret with these small tumours when compared with other human cancers. Furthermore, this study was performed utilising a commercial screening array that was not designed specifically to focus on the pituitary. As a consequence, novel proteins were shown to be present in pituitary while others were not assessed. However, our findings of the expression of the pituitary transcription factor Pit 1 in controls, up-regulation of this protein in GH- and PRL-secreting adenomas, and the absence of this protein in ACTH-secreting adenomas and non-functional pituitary adenomas, corroborates the hypothesis that this is indeed a reliable protein array. Although whole tissue samples lead to frank admixture of tumour cells with various other cells (stromal cells, macrophages, red blood cells, etc.) it may be argued that these cells may also be important for the physiology of the tumour, and that they should therefore be included. On the other hand, it would have been ideal that this study had matched for age and gender tumour bearing patients and control pituitaries, although the small availability of pituitary material for research generally precludes this selection. It is also known that the comparisons between adenomatous tissues consisting of a monoclonal, homogeneous tumour cell population and normal pituitaries representing various proportions of endocrine cell types may also carry some bias. This study, however, overcame this important obstacle, showing by immunofluorescent and conventional immunohistochemistry the expected over- or underexpression of the four selected proteins in human pituitary tumour cells when compared with their normal pituitary cells. Interestingly, these results were much less obvious in the immunoblotting study, and in some cases were absent. The concordance of the proteomic results with the immunohistochemistry suggests that conventional immunoblotting in this situation is of much less sensitivity.
In summary, our data show the limitations of proteomic profiling, but do reveal novel proteins in human pituitary tissue and some possible candidates for pituitary oncogenesis. Our data also encourage future studies targeting these novel protein candidates as possible biomarkers to this diagnosis, monitoring of disease progression, and developing of novel drug therapeutic approaches to some specific pituitary adenomas. However, we caution using proteomic array data without validating its findings using alternative more conventional techniques.

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

Funding
This work was supported by the Cancer Research Committee of Saint Bartholomew’s Hospital and by the OTKA K68660 grant. A R O Jr was supported by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico).

Acknowledgements
We are grateful for the support of Cancer Research Committee of Saint Bartholomew’s Hospital and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico).

References
Casey DG, Lysaght J, James T, Bateman A, Melcher AA & Todryk SM 2003 Heat shock protein derived from a non-autologous tumour can be used as an anti-tumour vaccine. Immunology 110 105–111.
Chong YP, Mulhern TD & Cheng HC 2005 C-terminal Src kinase (CSK) and CSK-homologous kinase (CHK)-endogenous negative regulators of Src-family protein kinases. Growth Factors 23 233–244.
Farrell WE 2006 Pituitary tumours: findings from whole genome analyses. Endocrine-Related Cancer 13 707–716.
Chong YP, Mulhern TD & Cheng HC 2005 C-terminal Src kinase (CSK) and CSK-homologous kinase (CHK)-endogenous negative regulators of Src-family protein kinases. Growth Factors 23 233–244.
Farrell WE 2006 Pituitary tumours: findings from whole genome analyses. Endocrine-Related Cancer 13 707–716.


