

Differential gene expression profiles of invasive and non-invasive non-functioning pituitary adenomas based on microarray analysis

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Abstract

Non-functioning pituitary adenomas (NFPAs) may be locally invasive. Markers of invasiveness are needed to guide patient management and particularly the use of adjuvant radiotherapy. To examine whether invasive NFPAs display a specific gene expression profile relative to non-invasive tumors, we selected 40 NFPAs (38 of the gonadotroph type) and classified them as invasive ($n=22$) or non-invasive ($n=18$) on the basis of magnetic resonance imaging and surgical findings. We then performed pangenomic analysis with the 44k Agilent human whole genome expression oligonucleotide microarray in order to identify genes with differential expression between invasive and non-invasive NFPAs. Candidate genes were then tested in qRT-PCR. Prediction class analysis showed that the expression of 346 genes differed between invasive and non-invasive NFPAs ($P<0.001$), of which 233 genes were up-regulated and 113 genes were down-regulated in invasive tumors. On the basis of Ingenuity networks and the degree of up- or down-regulation in invasive versus non-invasive tumors, 35 genes were selected for expression quantification by qRT-PCR. Overexpression of only four genes was confirmed, namely *IGFBP5* ($P=0.02$), *MYO5A* ($P=0.04$), *FLT3* ($P=0.01$), and *NFE2L1* ($P=0.02$). At the protein level, only myosin 5A (MYO5A) immunostaining was stronger in invasive than in non-invasive NFPAs. Molecular signature allows to differentiate 'grossly' invasive from non-invasive NFPAs. The product of one of these genes, *MYO5A*, may be a useful marker of tumor invasiveness.

Endocrine-Related Cancer (2010) 17 361–371

Introduction

Pituitary adenomas represent at least 10% of intracranial neoplasms (Melmed 2003, Ezzat *et al.* 2004). Non-functioning pituitary adenomas (NFPAs) are distinguished from functioning forms on the basis of clinical and biochemical features. Most NFPAs are gonadotroph adenomas expressing steroidogenic factor 1 (Asa *et al.* 1996, Al-Shraim & Asa 2006), required for differentiation of gonadotroph cells, and producing follicle-stimulating hormone (FSH) β and/or luteinizing hormone (LH) β and/or α -subunit. Although classified as slow-growing benign brain tumors, ~40% of NFPAs show characteristics of local invasion: they erode the sella turcica and spread into neighboring tissues, invading the sphenoid bone inferiorly and the cavernous sinus laterally. Total surgical removal may be impossible, owing to the risk of damaging vital vascular or nervous structures. Recurrences may develop from tumor remnants, necessitating additional surgery or radiation, with a risk of further complications. The potential for proliferation and for invasion may be totally independent: some huge tumors remain enclosed, while some microadenomas are locally invasive. Generally, preoperative imaging and/or peroperative findings show whether or not a pituitary adenoma is invasive. However, equivocal cases require long-term surveillance, and adjuvant treatment may be delayed. There is thus a need for markers of invasiveness based on surgical specimens.

Numerous genetic alterations may play a role in pituitary tumorigenesis (Ezzat *et al.* 1995, 2002, Zhang *et al.* 1999, 2002, 2003, Pagotto *et al.* 2000, Evans *et al.* 2001, 2003, Korbonits *et al.* 2002, Simpson *et al.* 2002, Heaney *et al.* 2003, Bahar *et al.* 2004, Emery *et al.* 2006, Leontiou *et al.* 2008), but few data are available on the molecular factors underlying the invasiveness of sporadic pituitary adenomas, and particularly NFPAs. The role of *PTTG* (Zhang *et al.* 1999, Salehi *et al.* 2008) and the N-terminally truncated isoform of fibroblast growth factor receptor 4 (FGFR4) (ptd-FGFR4; Qian *et al.* 2004) has been suggested. Overexpression of polysialylated neural cell adhesion molecule has been related to pituitary adenoma invasion (Trouillas *et al.* 2003), and overexpression of matrix metalloproteases (MMPs), and particularly MMP-9, has been detected in various types of invasive adenoma (Turner *et al.* 2000, Gong *et al.* 2008, Hussaini *et al.* 2007). Bone morphogenic protein, a factor involved in gonadotroph tumor development, might also play a role along with retinoic acid-inducible neural-specific protein 3 (Shorts-Cary *et al.* 2007).

Here, we investigated whether invasive NFPAs display a specific gene expression profile relative to

non-invasive tumors. We used microarrays to analyze 40 NFPAs (mainly gonadotroph tumors), of which 22 NFPAs were invasive and 18 NFPAs were non-invasive. The aim was to find a specific expression profile for invasive tumors, in order to guide therapeutic decision making and particularly the use of adjuvant radiotherapy.

Materials and methods

NFPA tumors and tissue characterization

Forty pituitary NFPA specimens were obtained after transsphenoidal surgery. The patients were managed in our center at Bicêtre Hospital, and relevant clinical and hormonal findings, preoperative imaging studies, immunohistochemical findings, and post-operative outcome were available. Tissue portions not used for histology were frozen at -80°C in isopentane and stored in liquid nitrogen directly after surgical resection, pending RNA extraction. Gonadotroph tumors were further characterized by immunostaining for FSH β and LH β and/or α -subunit. Tumor size and invasiveness were defined on the basis of preoperative magnetic resonance imaging (MRI) and perioperative findings, using the modified Hardy criteria (Hardy 1979), as follows: grade I, enclosed microadenoma (tumor < 10 mm); grade II, enclosed macroadenoma (tumor > 10 mm); grade III, localized perforation of the sellar floor; and grade IV, diffuse destruction of the sellar floor. The site of extrasellar extension was designated by a letter as follows: suprasellar extension (O, A, B, or C) with O: none, A: occupies the suprasellar cistern, B: recesses of the third ventricle obliterated, C: grossly displaced third ventricle; parasellar extension (D and E) with D: intracranial (intradural) and E: into or beneath the cavernous sinus (extradural). Grade III and IV tumors were considered invasive. Tumor invasion was based on evidence of bone destruction and/or tumor extension within the sphenoid and/or cavernous sinuses and/or brain, as confirmed at surgery. The longest anterior–posterior, vertical, and transverse diameters were measured, and tumor volume was calculated as $0.5 \times \text{width} \times \text{length} \times \text{height}$ (Lundin & Pedersen 1992). After surgery, all the patients were periodically reassessed clinically and radiologically to check for signs and symptoms of disease recurrence. Tumor recurrence was diagnosed if radiological signs of regrowth of a tumor remnant or a new tumor were found (in patients with no surgical remnants). Remnant tumors were considered stable if there was no evidence of growth on two MRI within 1 year interval and no signs of disease reactivation.

On this basis, there were 22 invasive and 18 non-invasive NFPAs, and all but one of the NFPAs were macroadenomas. The clinical and morphological characteristics of these tumors are summarized in Table 1. This series and an independent series of 19 NFPAs (all of gonadotroph type), 10 invasive and 9 non-invasive followed at our department in Bicêtre Hospital, were used to validate the set of genes differentially expressed from microarray analysis. The protocol was approved by the Review Board of our institution, and the patients gave their informed consent.

Total RNA extraction

Total RNA was isolated from frozen tissue by using Trireagent (Sigma–Aldrich), and was purified on RNeasy columns (Qiagen) according to the manufacturer's protocols. RNA quality, based on the 28S:18S ribosomal RNA ratio, was assessed with an Agilent bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). All specimens included in this study had a ratio higher than 1.5. RNA samples were frozen in nuclease-free water until use (Promega Corporation).

Table 1 Clinical and pathological characteristics of 40 non-functioning pituitary adenomas

Pituitary adenomas (PA) number	Age at surgery (years)/sex	Hormonal secretion	Immunohistochemistry	Tumor volume (cm ³)	Tumor grading (Hardy)	Follow-up time from surgery (years)/outcome
PA01	61/M	NF	FSH β	10.0	IV, C, D, E	4.5/recurrence
PA02	58/M	NF	FSH β , LH β , and α SU	2.2	II, B, E	4.5/recurrence
PA03	74/M	NF	LH β and α SU	77.0	IV, C, D, E	3.5/stably remnant
PA04	75/M	NF	LH β and FSH β	7.8	III, B	4/stably remnant
PA05	51/M	NF	LH β and FSH β	33.3	IV	4/stably remnant
PA06	54/M	NF	LH β , FSH β , and α SU	21.4	IV, C	4/stably remnant
PA07	71/M	NF	FSH β	4.0	IV	4/stably remnant
PA08	90/M	NF	FSH β and α SU	4.0	IV, B, E	2.5/stably remnant
PA09	77/M	NF	LH β and FSH β	4.0	IV, B, E	3.5/stably remnant
PA10	83/M	NF	LH β , FSH β , and α SU	10.5	IV, C, E	3.5/stably remnant
PA11	70/M	NF	LH β , FSH β , and α SU	2.9	III, B, E	3.5/stably remnant
PA12	73/F	NF	LH β	13.5	IV, B, E	3.5/stably remnant
PA13	52/M	NF	FSH β	7.8	IV, B, E	3/stably remnant
PA14	59/F	FSH	FSH β	7.8	IV, B, E	5.5/recurrence
PA15	59/F	NF	FSH β	7.8	III, B, E	3/remnant
PA16	76/M	NF	FSH β	7.8	IV, B, E	3/remnant
PA17	48/M	NF	FSH β	7.8	III, C, E	3/remnant
PA18	63/M	NF	LH β and FSH β	13.5	IV, B, E	3/remnant
PA19	74/F	NF	LH β , FSH β , and α SU	6.9	IV, B, E	3/remnant
PA20	47/M	FSH	LH β , FSH β , and α SU	2.1	IV, B, E	2.5/remnant
PA25	70/M	NF	LH β and FSH β	7.4	III, A	9.5/recurrence inv
PA30	60/M	NF	α SU	7.8	III, B	4/recurrence inv
PA21	67/M	NF	α SU	2.9	II, O	6.5/no remnant
PA22	82/M	NF	LH β and α SU	9.4	II, B	6.5/no remnant
PA23	68/F	NF	Negative	6.5	II, B	6.5/no remnant
PA24	60/F	NF	FSH β	3.5	II, C	6.5/no remnant
PA26	43/M	FSH	FSH β	9.2	II, O	10.5/stably remnant
PA27	37/F	NF	LH β , FSH β , and α SU	1.6	II, A	3.5/stably remnant
PA28	35/F	FSH, LH	LH β and FSH β	1.7	II	4/no remnant
PA29	56/M	NF	LH β	4.0	II, B	4/no remnant
PA31	42/F	NF	LH β and FSH β	0.5	I	4/no remnant
PA32	82/M	NF	LH β and FSH β	4.0	II, B	4/no remnant
PA33	64/F	NF	FSH β	4.0	II, B	4/no remnant
PA34	63/F	NF	Negative	7.8	II, C	4/no remnant
PA35	55/M	NF	FSH β	2.5	II, B	4/no remnant
PA36	56/F	NF	LH β , FSH β , and α SU	7.8	II, B	4/no remnant
PA37	51/M	NF	LH β and FSH β	10.2	II, B	4/no remnant
PA38	51/M	NF	LH β , FSH β , and α SU	4.0	II, B	4/no remnant
PA39	55/M	NF	FSH β	4.3	II, A	3.5/stably remnant
PA40	30/F	NF	LH β and FSH β	8.0	II, B	3/no remnant

PA, pituitary adenoma; NF, non-functioning; α SU, α -subunit.

Microarray hybridization

Microarray analysis was performed with Agilent competitive hybridization of each sample versus a common reference. Because the reference sample should preferentially have an expression profile that resembles that of the test samples, we used reference as a pool of equal amounts of total RNA from all samples (Knapen et al. 2009). Five microgram aliquots of total RNA from each sample and from the reference pool were used to generate labeled antisense cRNAs with T7 RNA polymerase. We used Agilent custom-designed 60-mer oligonucleotide microarrays and dual-color analysis in which probes from all samples and from the reference RNA are labeled with cyanine 5-CTP and cyanine 3-CTP respectively. These microarrays had 44 000 features representing the whole genome (44K Agilent Human Genome, Agilent Technologies). Reverse transcription, linear amplification, cRNA labeling, and purification were performed with the Agilent Linear Amplification kit. Hybridization was allowed to proceed for 17 h at 60 °C, with 1 µg of cyanine 5-labeled cRNA from each tumor mixed with the same amount of cyanine 3-labeled cRNA from the reference pool. The arrays were then washed with 0.6× and 0.01× SSC buffers containing Triton, and were dried with a nitrogen gun before scanning with an Agilent DNA microarray scanner. The fluorescence images thus obtained were quantified with Feature Extraction software (Agilent Technologies). All the microarray data have been submitted to Array Express at the European Bioinformatics Institute (<http://www.ebi.ac.uk/arrayexpress/>). Array Express is a public repository for microarray data, created to store well-annotated data in accordance with microarray gene expression data recommendations (<http://www.mged.org>).

Reverse transcription and quantitative real-time PCR analysis

Reverse transcription was performed using 1 µg total RNA from each sample in a reaction volume of 20 µl containing 50 U Moloney murine leukemia virus reverse transcriptase, 20 U ribonuclease inhibitor (Applied Biosystems, Foster City, CA, USA), 1 mmol/l dNTPs (Amersham Pharmacia Biotech), 5 mmol/l MgCl₂, 10 mmol/l Tris-HCl (pH 8.3), 10 mmol/l KCl, and 50 pmol/l random hexamers (Perkin Helmer Corp PE Applied Biosystems). A subset of 35 genes was tested by quantitative real-time PCR (qRT-PCR). Oligonucleotide primers and Taqman probes specific for each gene were designed to be intron spanning using PrimerExpress software

(Applied Biosystems; Supplementary Table 1, see section on supplementary data given at the end of this article). Sequences were obtained from the GenBank database, and the oligonucleotides were purchased from MWG Biotech (Courtaboeuf, France). Primers and probes for other specific genes and housekeeping genes were obtained from Assays-On-Demand (Applied Biosystems; Supplementary Table 1). Quantitative RT-PCR used TaqMan Universal PCR Master Mix and was run on an ABI PRISM 7700 sequence detection system (Applied Biosystems). Each reaction was performed with cDNA equivalent to 20 ng total RNA per tube in a final volume of 20 µl, and the expression of each target gene was determined simultaneously for all samples in 96-well plates. Each target gene was normalized to the human acidic ribosomal phosphoprotein P0 gene *RPLP0*. Gene expression values were calculated with the $2^{-\Delta\Delta C_t}$ method, where the ΔC_t value of the sample was determined by subtracting the average C_t value of the target gene from the average C_t value of the *RPLP0* gene. The reference pool, corresponding to the microarray reference RNA, was used as calibrator (1× sample).

Immunohistochemical analysis

Formalin-fixed paraffin-embedded pituitary tumor samples (five invasive and six non-invasive) selected from among those used for microarrays were studied by immunohistochemistry, along with two necropsy samples of normal anterior pituitary tissue. Five-micron-thick tissue sections were deparaffinized, rehydrated, and microwaved in pH6 citrate buffer at 100 °C for 15 min. After a wash in PBS and 15 min of preincubation with a commercial serum-free protein block (Dako, Carpinteria, CA, USA), the sections were incubated overnight at 4 °C in a humid chamber with the following primary antibodies: rabbit anti-human insulin-like growth factor binding protein 5 (IGFBP5) (GroPep Ltd, Adelaide, SA, Australia; dilution: 0.04 µg/ml), which does not cross-react with other IGFBP isoforms; rabbit anti-myosin 5A (MYO5A; GeneTex, Inc., Irvine, CA, USA; dilution: 1/300); monoclonal anti-pituitary tumor transforming gene (PTTG) (Novocastra Laboratories; dilution 1/50); monoclonal anti-Ki67 (Dako; dilution 1/100); and monoclonal antihuman p53 (Dako; dilution: 1/50). Pre-immune rabbit and mouse immunoglobulin at the appropriate dilutions were applied as negative controls to serial sections of each sample. Bound antibodies were revealed with a commercial peroxidase-labeled streptavidin immunohistochemical kit (LSAB 2, Dako), according to the manufacturer's instructions.

Aminoethylcarbazole was used as chromogen. Finally, some sections were counterstained with Meyers hematoxylin and mounted in aqueous medium (Glycergel, Dako). Protein expression was scored blindly by two observers using a conventional optical microscope (Provis, Olympus, Tokyo, Japan). A score was calculated as the percentage of immunostained tumor cytoplasm (for IGFBP5 and MYO5A) or nuclei (Ki67 and p53) in the most representative field ($\times 400$). Immunostaining intensity was scored as negative (-); weak (+/-); moderate (+); or strong (++) . Several sections of each tumor were examined, and the highest score was used for statistical analysis.

Bioinformatics and statistical analysis

All data were filtered to eliminate low-intensity values using a threshold (under 100 arbitrary units for both colors) based on the linearity test. Genes showing minimal variation across the set of arrays were excluded from further analysis. To select only genes with differential expression, we applied a 1.5-fold change cutoff, before any other statistical analysis, as previously used in our group (Raslova *et al.* 2007). We focused on gene showing a difference in expression of at least 1.5-fold from the median in at least 20% of the arrays. Genes significantly different between the classes, with P values of at least 0.001, were used for class prediction to discriminate between invasive and non-invasive tumors. Bioinformatic analysis of microarray data was performed with BrB-ArrayTools version 3.7. This model was developed to identify gene expression profiles predicting the class of future samples. The models are based on the Compound Covariate Predictor (Radmacher *et al.* 2002), Diagonal Linear Discriminant Analysis (Dudoit *et al.* 2002), Nearest Neighbor Classification (Dudoit *et al.* 2002), and Support Vector Machines with linear kernel (Ramaswamy *et al.* 2001). The models incorporated genes that were differentially expressed among genes at the 0.001 significance level, as assessed with the random variance t -test (Wright & Simon 2003). We estimated the prediction error of each model by leave-one-out cross-validation (LOOCV) as described by Radmacher *et al.* (Simon *et al.* 2003). For each LOOCV training set, the entire model-building process was repeated, including the gene selection process. We also examined whether the cross-validated error rate estimate for a given model was significantly less than would be expected from random prediction. The class labels were randomly permuted, and the entire LOOCV process was repeated. The significance

level is the proportion of random permutations that gave a cross-validated error rate no greater than the cross-validated error rate obtained with the real data. One thousand random permutations were used.

The class prediction dataset was analyzed by using Ingenuity Pathway Analysis software (www.ingenuity.com). A dataset containing gene identifiers and their corresponding expression values, such as fold changes and P values, was uploaded as a tab-delimited text file. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. A 1.5-fold change cutoff and a P value of < 0.001 were used to identify genes with differential expression. These genes, called 'focus genes', were then used as the starting point to generate biological networks.

Unpaired t -tests with Welch's correction (Prism 4, Graphpad Software, Inc., San Diego, CA, USA) were applied to qRT-PCR data for invasive and non-invasive NFPAs. P values below 0.05 were considered to denote significant differences.

Results

Clinical and morphological characteristics

The patients consisted of 27 men and 13 women, aged from 30 to 90 years. Mean age was 66 and 55 years in the invasive and non-invasive subgroups respectively ($P=0.02$; Table 1). All the patients had biochemically NFPAs, except for four patients who had above normal serum levels of FSH ($n=3$) or both FSH and LH ($n=1$). All but two tumors showed positive immunostaining for FSH β , LH β , and/or α -subunit. On imaging, the volume of invasive and non-invasive tumors was not significantly different ($P=0.07$). Maximal tumor volume before surgery ranged from 2.1 to 77.0 cm³ in the invasive subgroup (mean, 12.1 cm³) and from 0.5 to 10.2 cm³ (mean 5.2 cm³) in the non-invasive subgroup. Post-operative follow-up ranged from 2.5 to 10.5 years (mean 4 years) and was not significantly different between the groups ($P=0.07$). During follow-up, 17 patients with invasive tumors had stable remnants, while 5 patients had recurrences (invasive in two cases) after a mean of 3.8 years. In the non-invasive subgroup, 15 patients had no remnant and 3 patients had stable remnants; there were no recurrences in this subgroup during a mean follow-up of 4.8 years.

Differentially expressed genes

Using BrB software, a fold change- and intensity-based filtering approach (> 1.5 -fold change and $P < 0.001$) selected 3771 features from among the 44 000 present on the 60-mer oligonucleotide microarray.

Prediction class analysis showed that 346 genes discriminated between invasive and non-invasive NFPAs ($P < 0.001$); 233 genes were up-regulated and 113 were down-regulated in invasive tumors (Supplementary Table 2, see section on supplementary data given at the end of this article). As expected, the expression pattern of invasive adenomas was highly homogeneous, whereas non-invasive tumors fell into two subgroups (Fig. 1): one with a pattern very similar to that of invasive adenomas and one with a more heterogeneous pattern. Some of the differentially regulated genes have known functions in cancer, cell cycling, and cell death (up-regulated: *BAX*, *BCL2*, *BBC3*, *CDC2L2*, *CDK3*, *HNRPU*, *FLT3*, *IGFBP5*, *MYO5A*, *LTBP4*, *ADAMTS7*, *MMP17*, *RASA4*, *RAB18*, *SKI*, and *MAG*; down-regulated: *DST*, *DICER1*, *CITED1*, *PRDX2*, *TFG*, *RAB1A*, *RAC1*, and *PIK3CB*), cellular assembly, morphology, and motility (up-regulated: *FLNA*, *OSBP*, *MYO5A*, *TPM3*, and *TPM4*; down-regulated: *MAP1LC3B*, *SNAP25*, *HMGB1*, and *DST*), transcription factor and gene expression regulation (up-regulated: *NFE2L1*,

HNRPU, *HSP90AA1*, and *PARP10*; down-regulated: *CXADR*, *SNX3*, *SRP9*, and *SON*), and metabolism or other functions (up-regulated: *OGDH*, *OSBP*, *UCP3*, *TBXAS1*, and *GRIN1*; down-regulated: *SSTR1* and *UBE2L1*). To further analyze the biological significance of these genes, we used Ingenuity pathway analysis. This approach identified seven networks involving a majority of the genes listed above. Two networks involved *IGFBP5*.

Quantitative gene expression analysis

On the basis of Ingenuity networks and the degree of up- or down-regulation in invasive versus non-invasive tumors, expression of 35 genes was quantified by qRT-PCR, and overexpression of only four genes was confirmed, namely *IGFBP5* ($P = 0.02$), *MYO5A* ($P = 0.04$) (Fig. 2), *FLT3* ($P = 0.01$), and *NFE2L1* ($P = 0.02$). However, only two of these genes, *IGFBP5* ($P = 0.03$) and *MYO5A* ($P = 0.03$), were confirmed to be overexpressed in an independent series of 19 gonadotroph tumors (data not shown).

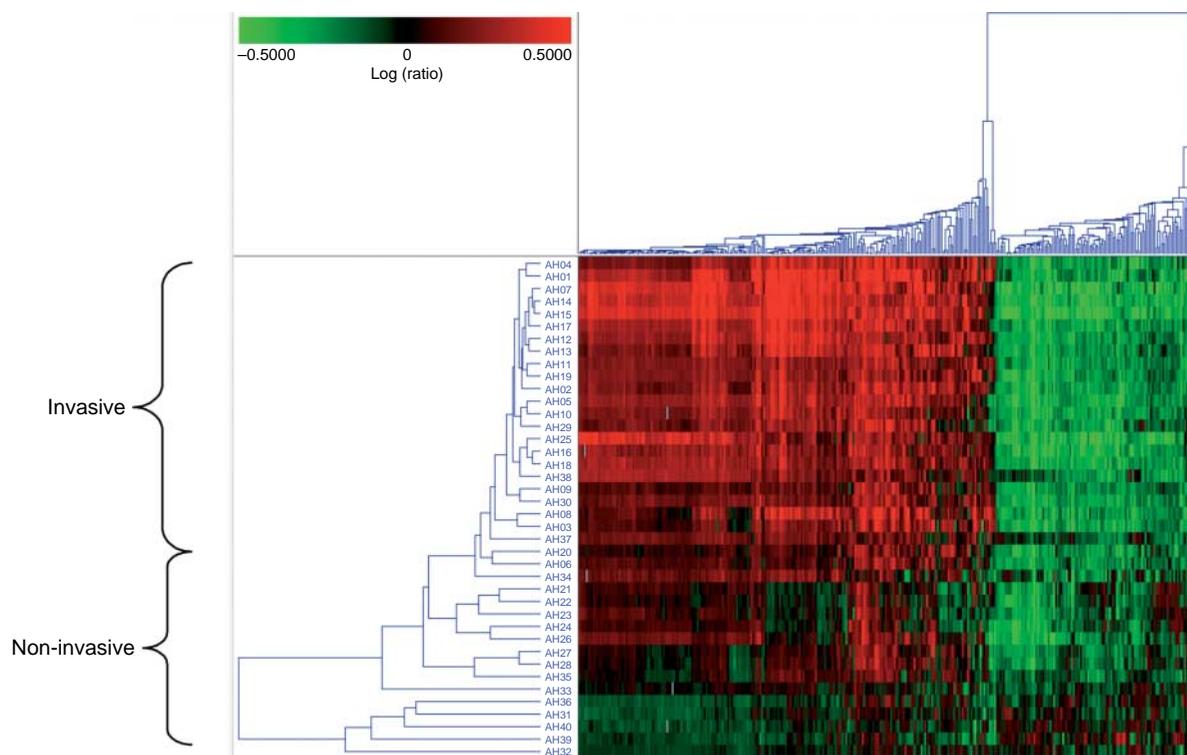


Figure 1 Cluster two-dimensional expression profile of the 346 genes differentially expressed between 22 invasive and 18 non-invasive non-functioning pituitary adenomas in microarray analysis. Red, up-regulated genes in invasive versus non-invasive tumors; green: down-regulated genes in invasive versus non-invasive tumors. The expression profile in the invasive subgroup was very homogeneous, contrary to the non-invasive subgroup.

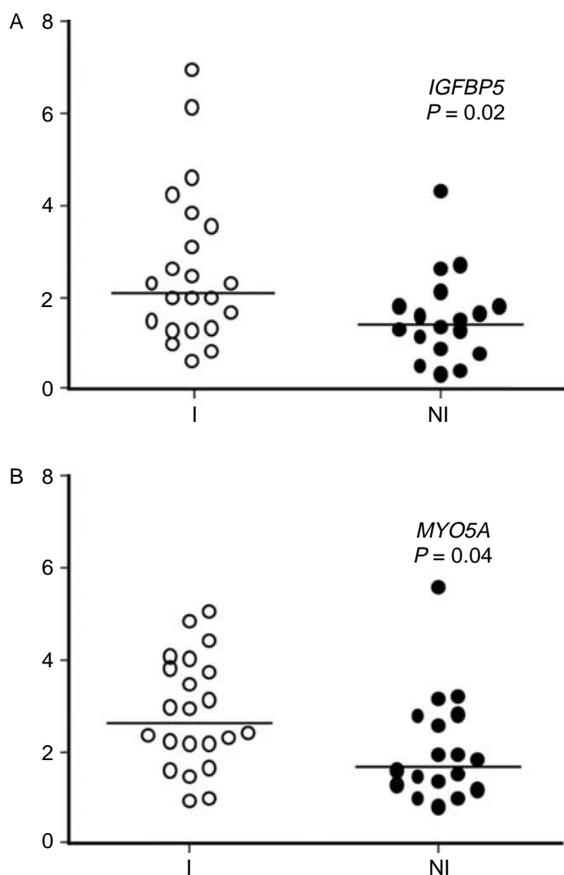


Figure 2 mRNA expression levels (arbitrary units) of *IGFBP5* and *MYO5A* normalized to *RPLPO* in invasive, I (○), and non-invasive, NI (●), non-functioning pituitary adenomas. The horizontal line corresponds to the median value in each group.

Protein expression

Two gene products (*MYO5A* and *IGFBP5*) were selected for immunohistochemical studies. Positive immunostaining for *MYO5A* and *IGFBP5* was not observed in normal pituitary tissue. In pituitary adenomas, *MYO5A* exhibited cytoplasmic immunostaining. Both the intensity of *MYO5A* immunostaining and the number of labeled cells were higher in invasive tumors than in non-invasive tumors: 70–100% of cells scored (+) or (++) in invasive tumors, while only 5–25% of cells scored (–) or (+/–) in non-invasive tumors (Fig. 3). *IGFBP5* immunostaining (Fig. 3) was localized in the cell cytoplasm in both invasive and non-invasive tumors, with no clear difference in the number of stained cells or in staining intensity between the groups. Nuclear Ki67 immunostaining was present in only a minority of cells and, as expected, was not significantly different between the two groups (Supplementary Table 3, see section on supplementary data given at the end of this article). We found a

good correlation between *MYO5A* and PTTG immunostaining but not between *MYO5A* and Ki67 immunostaining (Supplementary Figure 1, see section on supplementary data given at the end of this article).

Discussion

In the present study, using microarray analysis of 40 NFPAs, we were able to identify 346 genes which discriminated between invasive and non-invasive tumors, and to demonstrate that a set of two genes (*MYO5A*, *IGFBP5*) is overexpressed by qRT-PCR in invasive tumors. At the protein level, only the overexpression of *MYO5A* was confirmed.

Microarray technology has previously been used to evaluate gene expression during pituitary tumorigenesis, but most studies compared pituitary adenomas to normal pituitary tissues (Evans *et al.* 2001, Moreno *et al.* 2005, Morris *et al.* 2005, Farrell 2006, Gurlek *et al.* 2007, Shorts-Cary *et al.* 2007, Wierinckx *et al.* 2007) in order to identify genes associated with tumor pathogenesis. To gain insight into the mechanisms involved in pituitary adenoma invasiveness, we sought to identify genes that were differentially expressed in invasive and non-invasive forms. We found that a 346-gene signature discriminated between invasive and non-invasive tumors, some of these genes being involved in known biological functions such as cell cycling, cell death, cell morphology and motility, and gene expression regulation (transcription factors). After bioinformatic analysis of the molecular signature, among the selected 35 genes, only 4 of these genes (*MYO5A*, *IGFBP5*, *FLT3*, and *NFE2L1*) were overexpressed. After control in an independent series of NFPAs, *MYO5A* and *IGFBP5* were found to be differentially expressed ($P < 0.05$) between the two tumor subgroups. Immunocytochemical analysis of protein expression showed that only *MYO5A* expression differed between the tumor subgroups, with stronger immunostaining in invasive tumors.

MYO5A is a member of the unconventional myosin family and shows broad tissue expression (Rodriguez & Cheney 2002) principally in brain cells. Multiple functions have been assigned to this actin-dependent molecular motor, including the transport of melanosomes, smooth endoplasmic reticulum, recycling endosomes, neurotransmitter vesicles, mRNA (Reck-Peterson *et al.* 2000, Langford 2002, Salerno *et al.* 2008), and neuroendocrine vesicles (Rudolf *et al.* 2003). A role for *MYO5A* in tumor cell migration, invasion, and metastasis has recently been described (Du *et al.* 2007, Lan *et al.* 2010). Thus, our finding that *MYO5A* gene and protein expression is

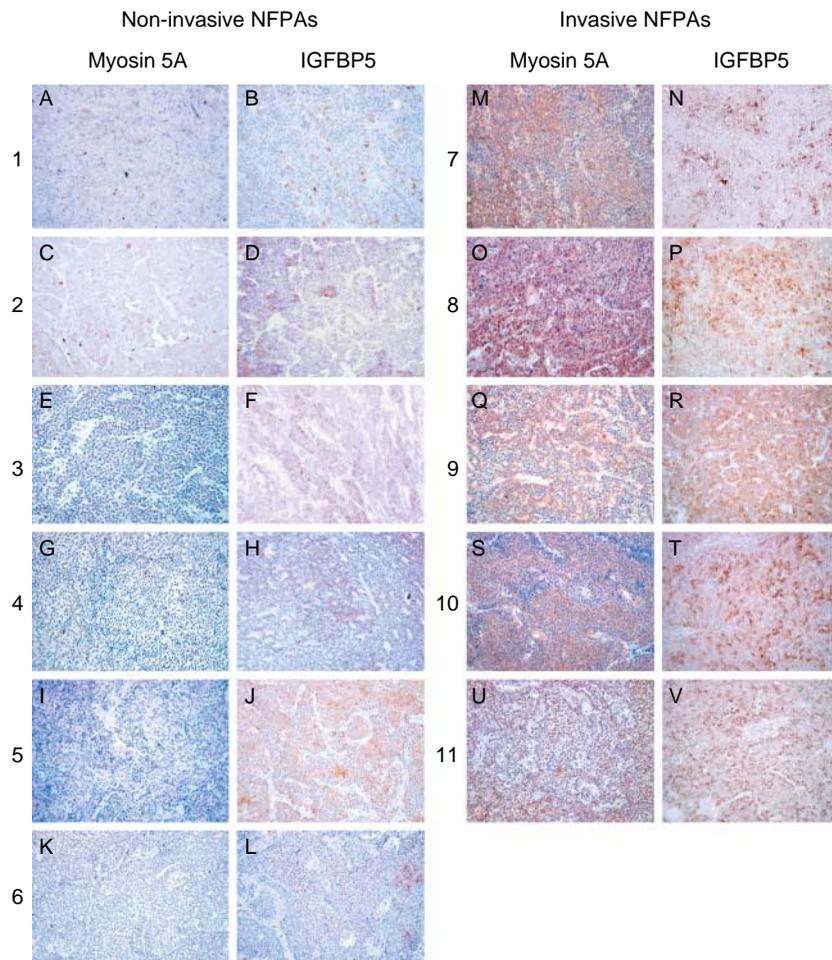


Figure 3 Expression of myosin 5A and IGFBP5 in six non-invasive NFPAs (numbered 1–6) and five invasive NFPAs (numbered 7–11) studied by immunohistochemistry. Myosin 5A is weakly to moderately expressed in non-invasive tumors; however the immunolabeling is limited to a reduced number of cells: in tumors 1 (A), 2 (C), and 5 (I), the expression of this marker is found in 15, 25, and 10% of cells respectively, and in <5% of labeled cells (E, I, and K) in the other tumors. IGFBP5 is moderately expressed in most non-invasive adenomas. The number of labeled cells is 10, 40, 30, 55, 70, and 30% in tumors 1 (B), 2 (D), 3 (F), 4 (H), 5 (J), and 6 (L) respectively. Myosin 5A expression was moderate to strong in all invasive tumors. The immunolabeling was present in 80, 75, 70, 100, and 70% of the cells of adenomas 7 (M), 8 (O), 9 (Q), 10 (S), and 11 (U) respectively. IGFBP5 expression was moderate to strong in all invasive adenomas. The immunolabeling was present in 50, 40, 100, 50 and 50% of the cells of adenomas 7 (N), 8 (P), 9 (R), 10 (T), and 11 (V) respectively.

higher in invasive than non-invasive NFPAs may have important implications, if confirmed in a larger series of NFPAs and also in other adenomas.

IGFBP5 was also overexpressed in our invasive tumors. The product of this gene has been shown to play a role in malignancy, and particularly in breast, ovary, and prostatic cancer (Hwa *et al.* 1997, Schneider *et al.* 2002, Johnson *et al.* 2006, Wang *et al.* 2006a,b, 2008, Nishino *et al.* 2008). However, IGFBP5 protein expression did not differ between invasive and non-invasive NFPAs. This could have several explanations including an extracellular secretion of IGFBP5, a post-translational modification or a degradation of the protein.

Overall, the difference in gene expression between invasive and non-invasive tumors is not particularly striking. This is not unexpected, however, when comparing two grades of tumoral tissue, contrary to comparisons of tumoral and normal tissue. Interestingly, microarray analysis showed that invasive tumors had a very homogeneous gene expression profile (Fig. 1), contrary to non-invasive tumors. Some of the latter displayed an expression profile resembling the invasive expression profile, suggesting that they might be ‘pre-invasive’ or carry an increased risk of recurrence. A subsequent tumor outcome will be particularly informative.

We cannot exclude that the 11-year difference in mean age of the patients with invasive tumors and non-invasive tumors has any impact on the data from microarray analysis as well as the validation by qRT-PCR and immunohistochemistry: the influence of age in expression of some genes or proteins in pituitary tumors has not been previously studied.

Our definition of invasive pituitary tumors was based on preoperating imaging studies, gross intraoperative observation, and post-operative histopathology. Some investigators claim that the definition of invasion requires histological evidence of dural invasion, but this is not routinely obtained in most centers and it has no long-term significance (Meij *et al.* 2002). The 2004 WHO classification (Al-Shraim & Asa 2006) introduced the term ‘atypical adenoma’ for tumors with metastatic potential, based on prognostic markers such as the number of mitoses and Ki67 and p53 immunostaining, but the relationship of these markers with invasiveness is less clear (Asa 2008, Salehi *et al.* 2009). In our series, Ki67 immunostaining was similar in invasive and non-invasive NFPAs, but the predictiveness of the Ki67 labeling index for aggressive behavior is greater in functioning pituitary adenomas (including prolactinomas) than in NFPAs (Gurlek *et al.* 2007). Moreover, we have previously shown that the Ki67 labeling index is more reliable for predicting the recurrence of pituitary tumors (Filippella *et al.* 2006).

PTTG and *MMP9* were also up-regulated in the invasive tumors, as measured by qRT-PCR (data not shown), in keeping with previous findings (Turner *et al.* 2000, Trouillas *et al.* 2003, Filippella *et al.* 2006, Gong *et al.* 2008, Hussaini *et al.* 2007, Wierinckx *et al.* 2007). At the protein level, PTTG staining was stronger in invasive tissues and displayed a good correlation with that of MYO5A. By contrast with a previous report (Hussaini *et al.* 2007), *MMP9* immunostaining was not different between the two groups.

In conclusion, microarray analysis of a large series of 40 NFPAs identified a molecular signature designating ‘grossly’ invasive tumors by comparison with non-invasive tumors. The product of one of these genes, MYO5A protein, may be a useful marker of invasiveness. The role of these genes in the invasion process of some pituitary adenomas – not only NFPAs but also other types – remains to be determined, along with their potential as predictive biomarkers of recurrence.

Data availability

The microarray data gathered in this study have been submitted to the Array Express data repository at

the European Bioinformatics Institute (<http://www.ebi.ac.uk/arrayexpress/>) with the accession number E-TABM-899.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1677/ERC-10-0018>.

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 a grant from the French Ministry of Health (Programme Hospitalier de Recherche Clinique Ile de France No. AOR03 052) and a grant of doctoral fellowship from Pfizer for F.A.

Acknowledgements

The authors thank J Bertherat and X Bertagna for providing information about some of their patients included in this study.

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