Pituitary tumours: findings from whole genome analyses

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Abstract

Pituitary tumours are common intracranial neoplasms that cause significant morbidity through mass effects and/or the inappropriate secretion of pituitary hormones. Despite a considerable literature detailing potential pathogenic changes in these tumours, their aetiology remains largely unresolved. Recent studies have employed genome-wide profiling towards the identification of novel genes and pathways that are inappropriately expressed or regulated in this tumour type. The techniques employed vary in their complexity and interpretation; however, many of the findings from these types of studies have identified novel genes with potential and, in some cases, proven roles in pituitary tumorigenesis. These studies include comparative genomic hybridization, whole genome-wide allelotyping and methodologies for identification of novel genes associated with epigenetic silencing. In addition, differential display methodologies have been instrumental in the identification of transcripts inappropriately expressed including, pituitary tumour transforming gene, growth arrest and DNA damage-inducible protein (GADD)45γ and a maternal expressed gene 3 isoform, which in some cases have proven roles in pituitary tumorigenesis. Although few studies of whole genome transcript analysis, as determined by microarray or gene-chip technologies, are reported, these studies of human pituitary, in some cases combined with proteomics, are yielding useful data. In addition, these types of investigation have been applied to several animal models of pituitary tumorigenesis, and in these cases novel genes are highlighted as showing significant change. The identification of the initiating events responsible for the transformation of a normal pituitary cell into one with unrestrained proliferative capacity has so far eluded us. No doubt, these new technologies allowing an essentially unbiased genome-wide analysis, perhaps in combination with animal models that display a preceding hyperplasia, will allow us to identify genes critical to tumour evolution and progression.

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Pathogenesis of pituitary tumours

Pituitary adenomas

Pituitary adenomas are common intracranial neoplasms, comprising 10–15% of diagnosed brain tumours (Kovacs & Hovarth 1987). Tumours may arise from any of the five differentiated cell types within this gland, and reflecting their cellular origin frequently synthesize and secrete their respective hormone(s) (Asa & Ezzat 2002, Melmed 2003). In these cases, excess hormone secretion, for example, prolactin, growth hormone (GH) and adrenocorticotropic hormone (ACTH), are responsible for severe clinical syndromes that can be lethal. In addition, a significant percentage of tumours, typically macro-adenomas, are classified as clinically non-functioning and do not cause syndromes of hormone excess. Irrespective of hormonal status, tumours show a spectrum of growth characteristics and rapidly growing tumours give rise to symptoms of intracranial mass including headache, loss of normal anterior pituitary hormone production (through compressive effects) and visual field defects (Asa & Ezzat 2002, Melmed 2003). Pituitary tumours rarely metastasize; however, a significant proportion show invasive and or recurrent growth characteristics (Pernicone et al. 1997, Heaney & Melmed 2004).
Pituitary tumour clonality

The near-invariable finding of monoclonality in this tumour type supports the view that a somatic cell defect represents the primary initiating event (Alexander et al. 1990, Herman et al. 1990). However, clonal outgrowth is most likely facilitated by hypothalamic and pituitary-derived growth factors (Asa & Ezzat 2002, 2005). Although the majority of the pituitary tumours are sporadic, they are also found as components of several familial syndromes, including multiple endocrine neoplasia type 1, Carney complex and McCune–Albright syndrome (reviewed by Farrell 2005).

Pathogenic changes implicated in pituitary tumourigenesis

Considerable literature details the potential pathogenic changes in sporadic pituitary tumours that include hormones, growth factors, receptors, associated signal transduction pathways and cell-cycle regulators. A detailed consideration and analysis of these changes has been the subject of several recent reviews (Asa & Ezzat 2002, 2005, Melmed 2003, Heaney & Melmed 2004). However, despite our understanding of the aberrations that characterize this tumour type, their aetiology remains largely unresolved. Thus, it is difficult to determine if an identified change represents a primary (initiating) pathogenic event, responsible for cell transformation, or represents a change that promotes or facilitates tumour progression or, conversely, simply reflect tumour-associated epiphenomenon. In tumours, such as colon cancers, it is possible to delineate molecular aberrations associated with each stage of progression; however, pituitary tumours are not usually associated with preceding hyperplasia, non-invasive tumours do not necessarily progress to invasive adenoma and metastatic outgrowth is exceedingly rare (Asa & Ezzat 2002, Melmed 2003). In response to these challenges, recent studies have employed genome-wide approaches toward the identification of novel genes and/or pathways with potential, and in some cases proven roles in pituitary tumourigenesis. The primary focus of this review will be on the recent findings derived from whole genome analyses studies, and the principal techniques employed are detailed in Table 1.

Loss of heterozygosity (LOH) in sporadic pituitary tumours

The majority of studies employing LOH analyses to identify tumour suppressor gene loci in sporadic pituitary tumours utilize candidate gene approaches. For some of the loci identified, an increased frequency of LOH (at one or more loci) is apparent in invasive tumours and pituitary carcinomas relative to their non-invasive counterparts (Bates et al. 1997). The subsequent identification of the MEN1 gene, which maps to one of the regions identified (11q13), led to its characterization in sporadic pituitary tumours. Disappointingly, these studies largely discounted a significant role for this gene in sporadic pituitary tumours (Asa & Ezzat 2002, Melmed 2003). Studies of the RB1 gene that maps to 13q14, a region also identified as harbouring frequent LOH, also reached similar conclusions. However, the findings relating to the RB1 gene and its protein product may reflect the tumour subtypes investigated and/or mechanisms associated with inactivation and they will be reviewed in a subsequent section.

Whole genome cytogenetic alterations

Disruption in cell-cycle checkpoints leads to chromosomal instability and is a hallmark of tumourigenesis. Pituitary tumours are no exception and approximately half are reported as grossly aneuploid (Levy & Lightman 2003). A principal technique employed to define these changes, and that permits the simultaneous examination of all autosomes, is comparative genomic hybridization. This technique allows gross chromosomal changes (gains and losses) and ploidy to be determined in a single experiment. These studies have

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examined all the major pituitary tumour subtypes; however, the number of tumours examined is limited (Tanaka et al. 1997, Daniely et al. 1998, Harada et al. 1999, Hui et al. 1999, Metzger et al. 1999, Finelli et al. 2000, Fan et al. 2001, Rickert et al. 2001, Trautmann et al. 2001). Perhaps reflecting this limitation, these studies reached different conclusions with respect to gains and losses. For example, the combined data from these studies describe losses on chromosomes 1, 2, 3, 9, 10, 11, 13, 15, 16, 18 and 22. Although these studies reached similar conclusions for losses on the long arms of chromosome 1, 2, 11, 13 and 15, this was not the case for the losses of the other regions. In general, these studies show that chromosomal aberrations are more frequent in functional adenomas than their non-functional counterparts, and are more common in invasive and recurrent tumours than their benign counterparts. A technical limitation of this technique, with respect to identification of chromosomal losses, is that it is limited to analysis and identification of large regions of chromosomal deletion, whereas microdeletions may remain undetected.

Genome-wide microsatellite analysis

A genome-wide microsatellite analysis for LOH was made possible through essentially unbiased whole genome amplification (Simpson et al. 2003). Across the genome, the analysis identified discrete regions where LOH exceeded the frequency that accounts for (tumour-associated) random allelic loss. The study also identified regions and specific microsatellite markers that showed an increase in the frequency of LOH in invasive tumours relative to their non-invasive counterpart (Fig. 1). Microsatellite markers identified in this genome-wide analysis were subsequently employed to determine their value as predictive markers of tumour recurrence (Buch et al. 2004). This retrospective study identified particular loci in the initial tumour specimens that were indeed predictive of recurrent growth (Buch et al. 2004). It will be important to employ these markers in a prospective study to determine if this type of analysis is truly predictive of subsequent tumour behaviour. In addition, the whole-genome analysis identifies chromosomal hot-spots for the location of novel tumour suppressor gene (TSG).

Differential display techniques

Pituitary tumour transforming gene (PTTG)

The isolation and characterization of the PTTG1 gene transcript from a rat pituitary tumour cell line represented the first report in this tumour type employing mRNA differential display (Pei & Melmed 1997). PTTG is overexpressed in human pituitary tumours, and increased expression correlates with tumour invasiveness (Zhang et al. 1999). The in vitro transforming properties of PTTG, a human securin, were first demonstrated in NIH3T3 cells (reviewed in Melmed 2003) and its in vivo role in tumour development has been investigated in different animal models. These studies show, in experimentally induced rat pituitary adenomas, that tumour growth is blocked through expression of a dominant negative form of PTTG (Horwitz et al. 2003), and that PTTG deletion protects, or significantly delays, pituitary tumour development in Rb<sup>+/−</sup> mice (Chesnokova et al. 2005). Targeted expression of PTTG1 to the mouse pituitary, under the control of the α-GSU (glycoprotein
Bone morphogenetic protein 4 and noggin

A more recent study, employing mRNA differential display, identified decreased expression of the bone morphogenetic protein (BMP) inhibitor noggin in prolactinomas from D2-receptor-deficient mice (Paez-Pereda et al. 2003). In these tumours, confined to female mice, the decrease in noggin was associated with increased expression of BMP-4. Human prolactinomas, but not other pituitary tumour subtypes, showed increased expression of BMP-4 as determined by western blot analysis. Employing c-myc as a surrogate marker for cell proliferation, the study also showed that BMP-4 selectively stimulates, and noggin inhibits, cell proliferation in human prolactinomas, but not in other pituitary tumour subtypes. The same study also showed that enforced expression of noggin or a dominant negative form of Smad4 (a BMP-4 signal cotransducer) in GH3 cells reduced tumourigenicity in nude mice. A follow-on study by this group shows that most BMP-4 immunopositivity in normal pituitary is confined to somatotroph and corticotroph, but not lactotroph cell populations (Giacommini et al. 2006). In addition, this study shows that BMP-4 is expressed at low, but variable, levels in human corticotroph tumours associated with Cushing’s disease. In contradistinction to their earlier findings in cells of the somatotroph and corticotroph lineage (GH3 cells), enforced expression of BMP-4 in AtT20 in the corticotroph cell lineage inhibited proliferation in vitro and blocked tumour cell growth in vivo. These findings are thought to reflect the differences in cell lineage (corticotrophs vs somatotrophs), where BMP-4 either inhibits or stimulates cell growth respectively. In agreement with previously published data, retinoic acid inhibited AtT20 cell proliferation and in vivo tumour cell growth (Paez-Pereda et al. 2001). Interestingly, this subsequent study shows that retinoic acid induces BMP-4 transcription and translation. Although not addressed in these studies, it will be of interest to determine the consequence of noggin deficiency and targeted BMP-4 expression in appropriate mouse models.

GADD45γ

Zhang and colleagues (2002) used a similar genome-wide approach, in this case cDNA representational differential display, to isolate novel candidate genes subject to differential expression in human pituitary tumours. Their study differed from those described so far in that the primary source of RNA was normal human pituitary and clinically non-functioning pituitary tumours. They identified GADD45γ, a growth arrest and DNA damage inducible gene, as significantly underexpressed in the majority of pituitary tumours investigated. The growth suppressive properties of this gene, in a pituitary context, were investigated in vitro; transfection of GADD45γ significantly reduced tumour cell line proliferation as determined by colony-forming efficiency (CFE) assays. Similar conclusions, with respect to reduced expression of GADD45γ, in a significant proportion of pituitary tumours were reached in an independent study (Bahar et al. 2004b). This study also investigated the mechanisms responsible for or associated with loss of transcript expression and showed that loss of GADD45γ expression was significantly associated with methylation of this gene CpG island. Interestingly, the murine homologue of this gene is not expressed in the mouse pituitary cell line AtT20 and the Cpg island associated with murine GADD45γ is also heavily methylated. Treatment of these cells with the demethylating agent 5-Aza-2'-deoxycytidine resulted in robust re-expression of this gene (Bahar et al. 2004b). It is important to note that these findings do not show that methylation per se is responsible for gene silencing; however, they do show this change is causal in maintaining the silent epigenetic state. More recent studies from our laboratory have shown, in AtT20 cells, that methylation of the CpG island associated with the GADD45γ gene is present in late passage, but absent in earlier passage cells (Bahar A and Farrell WE, unpublished observation).

MEG3

MEG3, an imprinted, maternally expressed gene of unknown function, was initially identified as underexpressed in non-functioning pituitary tumours in the same study that first identified and investigated the role of GADD45γ in this tumour type (Zhang et al. 2002). In a subsequent study, the same group identified expression of an isoform of MEG3, the cDNA of which, they termed MEG3a, which is expressed in

hormone alpha subunit) promoter, was employed to more directly assess the role of PTTG in tumorigenesis (Abbud et al. 2005). These experiments showed that male transgenic animals have larger and irregularly shaped pituitaries. In some animals, PTTG expression was associated with focal pituitary hyperplasia and in some cases adenoma. The evidence for adenoma formation, that comprised luteinizing hormone (LH)- or GH- or thyroid-stimulating hormone (TSH)-cell adenoma, includes loss of the reticulin network, a ribbon like pattern of adenoma cells and extensive pituitary vacuolization.
normal human anterior lobe pituitary cells including gonadotrophs, but not expressed in most non-functioning tumours or somatotrophinomas (Zhang et al. 2003). The growth-suppressive properties of this isoform were investigated in different human tumour cell lines, including HeLa, MCF-7 and H4. Ectopic expression of MEG3a in these cell lines led to a significant decrease in CFE and the growth rate of HeLa cells as determined by cell proliferation experiments (Fig. 2). Additional investigations, as these authors point out, using an animal model will be critical to address whether the loss of MEG3 gene function is directly associated with tumour development.

To investigate the mechanisms associated with loss of MEG3 in non-functioning pituitary tumours, Zhao and colleagues (2005) undertook a genetic and epigenetic analysis of this gene. No genomic abnormality, as determined by LOH analysis or direct sequencing, was detected in tumours that failed to express the MEG3 transcript. Employing sodium bisulphite sequencing to determine the methylation status of the putative MEG3 promoter region, two CpG-rich regions in the 5' sequences of this single copy gene were identified as hypermethylated in non-expressing tumours. Sequencing of individual molecules by this technique revealed a methylation profile indicative of imprinting in normal pituitary. Furthermore, the functional importance of these regions was demonstrated in transient transfection reporter assays. Gene silencing was effectively reversed in cell lines treated with a demethylating agent, supporting a role for this epigenetic change in gene silencing.

**Methylation associated gene silencing in pituitary tumorigenesis**

The role of inappropriate methylation, histone deacetylation and histone modification in tumour-associated gene silencing has been subject to recent review (Jones & Baylin 2002). The first gene subject to detailed investigation for epigenetic change in sporadic pituitary tumours was the tumour suppressor gene CDKN2A (commonly referred to as p16). Numerous studies have shown methylation of this gene CpG island in pituitary tumours, and when studied show an association with gene silencing (Farrell 2005). Methylation appears to be an early change in pituitary tumorigenesis (Simpson et al. 2004) and enforced expression of this gene in pituitary cell lines inhibits cell proliferation (Frost et al. 1999) consistent with its role as a cell-cycle regulator. Subsequent studies, also employing candidate gene approaches, have described methylation-mediated gene silencing in other cell-cycle regulators, including the RB1 gene and also in genes with roles in apoptosis, invasion and metastasis (death-associated protein kinase and Galexatin 3). The role of these genes in pituitary tumorigenesis has been subject to recent review (Farrell 2005).
Identification of genome-wide differential methylation

Methylation-associated gene silencing of GADD45γ and MEG3 promoter sequences represent the first, albeit indirect, genome-wide approach employed in the identification of epigenetic change in pituitary tumorigenesis. Bahar and co-workers (2004a) adopted a more direct approach through analysis of DNA sequences that were inappropriately methylated in pituitary tumours relative to normal gland. The technique, methylation-sensitive arbitrarily primed PCR (Gonzalgo et al. 1997), identified DNA sequences, which were differentially methylated in pituitary tumours relative to the normal gland. One of the sequences identified corresponded to an open reading frame on chromosome 22 (C22orf3) of unknown function. The majority of pituitary tumours, irrespective of subtype, either did not express or expressed low levels of this transcript relative to normal gland. Sodium bisulphite sequencing of individual adenomas revealed dense, but heterogeneous methylation that was associated with loss of transcript expression; however, it was confined to approximately 20% of adenomas that failed to express this gene. These findings suggest that mechanisms other than, or in addition to, methylation as responsible for loss of transcript expression. The functional consequences, contingent on re-expression of this novel sequence, were examined in the pituitary tumour cell line AtT20. Induced expression had no effect on proliferation or cellular viability relative to wild-type cells. To investigate a possible role in apoptotic pathways, cells were challenged with bromocriptine, which was shown previously to elicit an apoptotic response in this cell line (Yin et al. 1994, 1999). In dose–response experiments, expression of the novel sequence significantly augmented an apoptotic response relative to wild-type cells. Further studies showed that apoptosis was mediated through a preceding caspase-activating pathway. The terminal stages of apoptosis, as determined by terminal deoxynucleotidyl transferase biotin–dUTP nick end labelling (TUNEL) labelling, also showed an increase in the number of apoptotic cells relative to cells harbouring an empty vector control. Thus, each of these end-points shows that this gene significantly augments a drug-induced apoptotic response. On this basis, we assigned the gene the acronym PTAG, pituitary tumour apoptosis gene. The ability of cells showing reduced expression of PTAG to evade or show a blunted apoptotic response may underlie oncogenic transformation in the pituitary and perhaps other tumour types.

In summary, methylation-mediated or associated gene silencing represents a fundamental aspect of tumorigenesis in multiple tumour types (Jones & Baylin 2002). Both candidate and novel gene approaches have provided new insight with regard to this change in pituitary tumours. Within the list of genes already identified, some show pituitary tumour subtype specificity, while others segregate with tumours that show invasive growth characteristics (reviewed by Farrell 2005). The specificity of this change with respect to tumour subtypes and growth characteristics together with functional consequences contingent on expression would suggest that this change does not simply reflect a tumour-associated epiphenomenon. As more data emerge, it will be possible to generate methylation profiles that provide a more detailed characterization of tumour subtypes and are perhaps predictive of growth characteristics. In contrast to gene loss, associated with deletion or mutation, methylation represents a potentially reversible change and thus offers exciting possibilities for pharmacological interventions strategies in tumour management.

Genome-wide microarray analysis of pituitary tumours

Microarray analysis has the advantage of permitting simultaneous analysis of thousands of genes at the level of transcript expression in a single experiment. Although few studies have applied this technology to the study of human pituitary tumours, the potential of this genome, or perhaps more accurately ‘transcriptome’ (Hu et al. 2005), wide parallel analysis toward identification of genes that underlie tumorigenesis, which are prognostic markers or therapeutic targets, cannot be overestimated. Technically, these experiments are relatively straightforward to perform; however, experimental design, inherent tumour cell heterogeneity and the complexity of data analysis represent significant challenges with respect to accurate interpretation (Fathallah-Shaykh 2005). In carefully designed studies that take into account of these multiple variables, these experiments generate reliable data pertaining to genome-wide transcript expression (Draghici et al. 2006).

Gene expression profiling and human pituitary tumours

To date, there are three published studies of gene expression profiling, as determined by microarray analysis of human pituitary tumours (Evans et al. 2001, Moreno et al. 2005, Morris et al. 2005). A further
three studies have applied this analysis to the study of rodent pituitary adenomas (Goidin et al. 2000, Wood et al. 2002, Mohammed et al. 2004b). In the three studies of human pituitary tumours, expression profiles were compared with that obtained from normal pituitary gland. These studies identified multiple transcripts as being significantly over- or under-expressed in tumours relative to normal pituitary and for selected transcripts were validated by quantitative RT-PCR. However, the conclusions reached should, at present, be interpreted with caution since the pituitary comprises an admix of different cell types, whereas the tumours comprise an expanded population of one major cell type. Thus, at this time, these data must be regarded as preliminary, requiring further validation where expression is compared between the tumour subtype and its specific normal cellular counterpart.

**Gene expression profiling and animal models**

Three studies of pituitary tumours in animal models have exploited whole genome microarray analysis (Goidin et al. 2000, Wood et al. 2002, Mohammed et al. 2004b). In comparison to gene expression profiling of human tumours, these studies, by their nature, are better controlled and have yielded useful data. Goidin and colleagues (2000) used microarrays, comprising 588 known gene cDNAs to identify gene expression profiles linked to the ageing-associated occurrence of spontaneous pituitary adenoma in a rat animal model. Expression profiles from 3-month-old rats and tumour-bearing 20–28-month-old rats identified expression of 79 genes. In older, tumour-bearing animals, 28 genes were expressed at higher levels, whereas in younger animals 15 genes were expressed at higher levels. Expression levels of selected genes were validated by semi-quantitative RT-PCR. The largest differences were found for expression of galanin and glutathione S-transferase in old and young rats respectively.

Wood et al. (2002) characterized early changes in gene expression that preceded thyroid hormone (TH)-induced involution of a TSH-secreting tumour grown in a hypothyroid host. Of the 1176 genes on the array, seven were upregulated and 40 downregulated subsequent to TH treatment. Observed changes in expression levels were validated by Northern blot analysis. Transcript levels for the cell-cycle regulators, cyclin-dependent kinase 2, cyclin A and p57 were decreased, whereas p15, a tumour suppressor, was induced by TH treatment. Several known, TH-responsive genes, including chromogranin B and C, inhibin, proconvertase 1, brain-derived neurotrophic factor, its receptor trkB and the receptor for thyrotrophin-releasing hormone showed decreased expression following treatment. Interestingly, p19ARF was dramatically induced by TH treatment and, although this protein can stabilize p53 by sequestering mdm2, no increase in p53 protein was apparent. Identification of these early changes in gene expression that precedes TH-induced growth arrest and tumour involution provides important information for further studies. As discussed by these authors, it will be important to determine the function of these TH-responsive genes.

Targeted overexpression of LH in transgenic mice results in ovary-dependent pituitary adenoma, i.e., preceded by hyperproliferation of cells expressing the transcription factor Pit-1 (Mohammed et al. 2004a). To identify genes involved in pituitary tumorigenesis in this mouse model, Mohammed et al. (2004b). Undertook global genes expression profiling using Affymetrix GeneChips interrogating more than 12 000 genes and expressed sequence tags (ESTs). A number of candidate genes were identified in tumour samples relative to wild-type littermates; 54 genes showing increased expression and 53 decreased. Of particular note, p8 (candidate of metastasis-1) was increased more than 12-fold in tumours relative to wild-type controls. Expression of p8, as determined by in situ hybridization, localized to tumour foci comprising lactotrophs suggesting a linkage with their oncogenic transformation. Further evidence, supporting a role of p8 in pituitary tumorigenesis, was obtained through antisense knockdown of p8 expression in GH3 (somatotroph lineage) and LβT2 (gonadotroph lineage) cells. Both cell lineages displayed attenuated tumour development or failed to grow when injected into athymic mice. These elegant studies suggest that p8 expression is required for maintaining a tumour phenotype. Importantly, these studies also suggest that p8 expression may represent only one of several distinct events in tumorigenesis. In this context, increased p8 expression was not found in hyperplastic tissue that precedes adenoma formation in this model. Increase expression of p8 is found in different human cancers and s.c. or i.p. injection of p8-expressing fibroblasts, but not their null counterparts, leads to tumour formation in nude mice (Iovanna 2002). Furthermore, recent studies have suggested that increase expression of p8 plays an important part in disease progression of papillary and medullary thyroid carcinomas (Ito et al. 2003, 2005). No doubt, future studies will seek to identify the molecular target of p8 in a pituitary context and the role of p8 homologues in human pituitary tumours.

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Concluding remarks

The application of essentially unbiased global genome profiling towards a clearer understanding of the molecular aberrations that underlie pituitary tumorigenesis has yielded novel data and new insights. The techniques thus far employed vary in their complexity and also in their subsequent interpretation. In this context, it is perhaps microarray experiments, together with the accurate interpretation of the derived data sets, that present the most significant challenge. In these cases, as more data emerge, and as methodologies and analyses are further refined, then these type of studies will allow us to draw more reliable conclusions. Identification of the primary molecular aberration(s) responsible for the evolution of human pituitary adenomas has, thus far, eluded investigators. Identification, at least in part, has been frustrated by the absence in the majority of cases of a hyperplasia preceding tumour development. In this context, our own studies identified methylation of the p16 gene CpG island in apparent normal pituitaries and corticotroph hyperplasia associated with Cushing’s disease (Simpson et al. 2004). Although these findings suggest that methylation of the p16 is an early change in pituitary tumourigenesis, they may simply reflect identification of tumour cells, albeit few in number, ‘scattered’ throughout the specimen. It is perhaps those animal models in which hyperplasia precedes adenoma formation that may prove informative with respect to the identification of bone fide initiating events. These models, combined with genome-wide scanning techniques, be they microarrays, proteomics, differential display or genome-wide methylation scans, may provide us with this new knowledge.

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