REVIEW

Pituitary tumours: all silent on the epigenetics front

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Abstract

Investigation of the epigenome of sporadic pituitary tumours is providing a more detailed understanding of aberrations that characterise this tumour type. Early studies, in this and other tumour types adopted candidate-gene approaches to characterise CpG island methylation as a mechanism responsible for or associated with gene silencing. However, more recently, investigators have adopted approaches that do not require *a priori* knowledge of the gene and transcript, as example differential display techniques, and also genome-wide, array-based approaches, to 'uncover' or 'unmask' silenced genes. Furthermore, through use of chromatin immunoprecipitation as a selective enrichment technique; we are now beginning to identify modifications that target the underlying histones themselves and that have roles in gene-silencing events. Collectively, these studies provided convincing evidence that change to the tumour epigenome are not simply epiphenomena but have functional consequences in the context of pituitary tumour evolution. Our ability to perform these types of studies has been and is increasingly reliant upon technological advances in the genomics and epigenomics arena. In this context, other more recent advances and developing technologies, and, in particular, next generation or flow cell re-sequencing techniques offer exciting opportunities for our future studies of this tumour type.

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Sporadic pituitary adenomas

Sporadic pituitary adenomas account for 10–15% of diagnosed brain tumours and although some differences in their frequencies exist, each of the five hormone-secreting cell types within the gland can give rise to an adenoma (Kovacs & Hovarth 1987, Asa & Ezzat 2002, Melmed 2003). With the single exception of prolactinomas most adenomas are treated through surgical intervention with or without adjuvant radiotherapy (Pernicone *et al.* 1997). Although progression to pituitary carcinoma is exceedingly rare, a significant proportion of pituitary adenomas show invasive and or recurrent growth characteristics (reviewed in Melmed 2003, Heaney & Melmed 2004).

Pituitary tumour aetiology

A significant challenge to our deciphering of the aberrations that underlie the aetiology of this tumour type is that they do not follow the near classic progression paradigm that is apparent in multiple other tumour types, that is, initiation/transformation, hyperplasia, benign adenoma, invasive/aggressive adenoma and ultimately carcinoma. Therefore, although a significant body of literature exists that has uncovered

pathogenic changes in this tumour type (reviewed in, Asa & Ezzat 2002, 2005, Melmed 2003) it is not clear if these aberrations are responsible for the initiating, that is transforming event, or are those that promote progression. Indeed, with the notable exception of the *gsp* oncogene in somatotrophinomas (Landis *et al.* 1989), activating mutations in oncogenes and mutations that result in the loss or inactivation of tumour suppressor genes (TSGs) are an exceedingly infrequent finding. More recent studies have begun to uncover epigenetic changes in this tumour type. However, it is likely that these changes, either global or gene specific, act in concert with, as yet to be identified, genetic aberrations to drive the conversion of a normal cell to one with a propensity toward uncontrolled growth and tumour outgrowth. This review will focus on the major findings with respect to the epigenome in pituitary tumorigenesis and the techniques and emerging technologies that are allowing us to adopt unbiased whole-genome analyses.

Epigenetic gene silencing

The term epigenetic refers to a process that heritably influences the expression of a gene without genetic change to the underlying DNA sequence itself (Jaenish

& Bird 2003). DNA methylation is a key epigenetic modification and in mammals it is restricted to CpG dinucleotides. Although CpG dinucleotides are relatively infrequent (~ 1 per 100 bp) throughout the genome, where $\sim 80\%$ are methylated, they cluster at their predicted frequency (~ 1 per 10 bp) in repetitive sequences and in regions termed CpG islands, and in these cases, frequently encompass the transcription start site of genes (Gardiner-Garden & Frommer 1987). Under normal conditions, most promoter associated CpG islands remain unmethylated (Siegfried & Cedar 1997). However, exceptions include the inactive X-chromosome in females and the imprinted genes and some tissue specific genes in both sexes (Futscher *et al.* 2002). The silencing of TSGs, through or associated with CpG island methylation is recognized as a major mechanism of gene inactivation that frequently coexists with genetic lesions in most cancers studied to date (Esteller 2007). An association between CpG island methylation and modification to the underlying histones is also apparent and these changes either lead to or are a consequence of a preceding chromatin induced change. The modifications to the core histones, principally methylation and acetylation, are frequently referred to as the histone code and a detailed consideration of these has been reviewed elsewhere (Burgers *et al.* 2002). The cause and effect relationship between CpG island methylation, histone modification, chromatin remodeling and gene silencing events are presently not entirely clear. Thus, while early studies favoured a model where methylation of DNA (CpG islands) favoured recruitment of protein complexes that in turn led to histone modification and condensed chromatin structure, the reverse most likely also hold true (McGarvey *et al.* 2006). In these cases, CpG island methylation may result in the reinforcement of an already established histone-induced gene silencing event.

Methylation and gene silencing: candidate gene approaches

The first gene identified in sporadic pituitary tumours as subjected to epigenetic change was the TSG *CDKN2A*, frequently referred to as p16 (Woloschak *et al.* 1997). Our own studies were the first to describe a pituitary tumour subtype (somatotrophinomas) where this epigenetic aberration to the *CDKN2A* gene was an infrequent occurrence (Simpson *et al.* 1999). Subsequent to these reports, methylation and silencing of p16 in pituitary tumours, at high frequency and showing subtype specificity, has been confirmed in multiple other studies (reviewed in, Farrell 2005). Methylation of p16 also 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) and is consistent with its role as a *bona fide* cell cycle regulator and classification as a tumour suppressor. Subsequent studies that employ candidate gene approaches have described methylation mediated gene silencing in multiple other genes including, cell cycle regulators, as example, *RBI*, in other known and putative TSGs, including, RAS association domain family 1A (*RASSF1A*) and fibroblast growth factor receptor 2 (*FGFR2*) and also in genes with roles in apoptosis, invasion and metastasis, death associated protein kinase (*DAPK*), caspase 8 and Galectin 3. The role of these genes in pituitary tumorigenesis has been subjected to recent review (Farrell 2005).

Methylation and gene silencing: differential display approaches

Several studies have employed cDNA representational differential display to identify, at the transcript level, genes with roles in pituitary tumorigenesis. These studies have identified transcripts that show loss or significantly reduced expression in primary tumours relative to post-mortem normal pituitaries. Using this technique, the Klibanski group identified growth arrest and DNA damage-inducible gene (*GADD45 γ*) and maternally expressed gene 3 (*MEG3*) transcripts as differentially expressed in pituitary tumours (Zhang *et al.* 2002, 2003) and subsequent studies showed that loss of *GADD45 γ* and of the *MEG3* isoform, *MEG3a*, transcript was associated with methylation of their CpG islands (Bahar *et al.* 2004a,b, Zhao *et al.* 2005). Both of these genes were also shown to fulfil the criteria of *bona fide* tumour suppressors since enforced expression in cell lines including those in a pituitary lineage was responsible for growth inhibition and/or reduction in colony forming efficiency.

Methylation and gene silencing: genome-wide DNA approaches

Bahar *et al.* (2004a,b) exploited DNA methylation of CpG islands in tumours relative to normal pituitary to isolate and enrich sequences subjected to inappropriate methylation. The principle of the technique, methylation sensitive arbitrarily primed PCR, is outlined in Fig. 1 and was first described by Gonzalgo *et al.* (1997). The technique is reliant on restriction enzymes that recognise the methylation status of DNA to generate sequences refractory to digestion, that is methylated, and that can then be amplified using arbitrary primed PCR. These studies successfully isolated a novel pro-apoptotic gene (pituitary tumour apoptosis gene-*PTAG*)

Figure 1 Diagrammatic overview of methylation-sensitive arbitrarily primer PCR. Three hypothetical genes (A–C) are shown in normal pituitary (NP) and in a pituitary adenoma (PA). The CpG islands associated with the genes are shown as circles on a stick. Gene A is methylated in common between NP and PA. Gene B is methylated only in the adenoma. Gene C is not methylated in either specimen. The genomes are digested with a methylation-sensitive enzyme and post digestion, gene B (arrowed) in the PA is preserved (not digested). Both genomes are subjected to arbitrary primed PCR and differential display. For the NP, an amplicon representing gene A will resolve on the gel, whereas for the PA amplicons representing gene A and B will be resolved indicating B to be differentially methylated. Filled circles, methylation; unfilled circles, not methylated.

that showed significantly reduced expression in the majority of pituitary adenomas investigated. In these adenomas, loss of expression was associated with CpG island methylation in ~20% of cases, suggesting that mechanisms other than or in addition to methylation are responsible for loss.

Methylation and gene silencing: genome-wide reversal

Several recent studies in other tumour types have successfully identified novel silenced and methylated genes through epigenetic ‘unmasking’ techniques (Liang *et al.* 2002, Suzuki *et al.* 2002, Foltz *et al.* 2006). These studies have utilized pharmacological reversal of CpG island methylation in tumour cell lines in combination with whole-genome transcript microarrays to identify novel, previously silenced, genes in tumours. The reversal/erasure of methylation is reliant on several rounds of replication of cells in culture in the presence of a drug(s) that inhibits post-replicative re-methylation. In the absence of a suitable human pituitary cell line, we used the murine corticotroph adenoma cell line At-T20 to ‘unmask’ epigenetically silenced genes that were subsequently identified by

whole-genome transcript microarray analysis (Dudley *et al.* 2008). In these studies and in contrast to the pharmacological strategy thus far described, we used an RNA interference approach to knock down expression of the maintenance DNA methylase enzyme, DNMT1. Figure 2 shows that this approach led to significant and sustained depletion of DNMT1 in these cells and, by whole-genome array analysis, we identified 91 transcripts that were significantly differentially expressed relative to cells transfected with a non-target siRNA. The majority of these transcripts showed increased expression (Fig. 3).

To determine if this model system was useful in predicting genes that are silenced in primary human pituitary tumours, we selected several genes from our microarray analysis for further analysis. These studies showed that our model was indeed successful in predicting novel silenced genes, and for some of these genes silencing was associated with, or was a consequence of, CpG island methylation. Indeed, for one of the genes, neuronatin (*NNAT*), where we determined function, we showed that enforced expression in a pituitary cell line inhibited cell proliferation (Dudley *et al.* 2008).

In the context of enzymes thought to be responsible for maintenance or *de novo* methylation, Zhu *et al.* (2008a,b) recently described increased expression of the *de novo* methylase *DNMT3b* in primary human pituitary tumours and also apparent in AtT20 cells. In both the primary tumours and the cell line, no significant changes in DNA methylation (hypomethylation) was apparent to account for increased expression. Instead, by pharmacological unmasking techniques, they show that expression was modulated, principally by histone modifications. In addition, their study also showed, re- or increased expression of selected target genes post-pharmacological manipulations. In these

Figure 2 siRNA mediated knock-down of DNMT1 transcript and protein in AtT20 cells. Cells were subject to a consecutive transfection protocol over 8 days with siRNA specific for DNMT1 (siDNMT1) or a non-target control (siNT). Transcript levels of DNMT1 are expressed relative to sham transfected (transfection reagent alone). Inset figure shows a western blot of DNMT1 and a loading control GAPDH at the 8 day time point. At 8 days, there is a significant depletion of DNMT1 transcript and protein.

Figure 3 Volcano plot of transcripts in AtT20 cells differentially expressed post-siRNA treatments. Across the whole genome array, 91 transcripts showed significant ($P < 0.05$) differential expression following DNMT1 knock-down. The majority showed increased expression (right upper quadrant, shaded area). The three transcripts circled, showing the most significant decrease in expression are biological triplicate experiment each showing decrease in *DNMT1* and providing confidence in the approach.

cases, for these selected target genes, re-expression was also apparent following siRNA mediated knock-down of *DNMT3b*.

These combined studies provide insight with respect to the enzymes responsible for establishing or maintaining normal and perhaps mediating aberrant methylation and acetylation patterns in the pituitary and also in tumours emanating from this gland (Dudley *et al.* 2008, Zhu *et al.* 2008a,b). This new knowledge may also provide our next generation of therapeutic targets. Indeed, in other tumour types, drug interventions that target these enzymes are generating promising data (Tan *et al.* 2007 and references therein). However, a caveat to these approaches will be the role(s) of maintenance methylases, such as DNMT1, and those responsible for *de novo* methylation, principally DNMT3a and DNMT3b, in or across different species. Although beyond the scope and context of this review, significant differences are known to exist between murine and human cells with respect to the role(s) of the enzymes responsible for the maintenance and establishment of epigenetic change and those drugs that will mediate pharmacological reversal.

Methylation: the chromatin connection

Although methylation of gene promoter-associated CpG dinucleotides, individually, or in the context of CpG island can impact on gene expression, the principal mechanism leading to 'transcriptional incompetence', that is gene silencing, is through changes to the underlying histones themselves and manifest as

condensed chromatin. As already discussed (see above) changes to histones may be contingent upon prior CpG island methylation or conversely, modification of histone tails themselves may lead to CpG island methylation, and in this case, this change is responsible for reinforcing an already established silencing event.

Chemical modification of histones, which frequently targets lysine residues within their N and C terminal tails can significantly alter the degree of compaction, and hence the access of the transcription machinery to the DNA within. Histone protein modifications include methylation, acetylation, phosphorylation, sumoylation, ubiquitination and ADP-ribosylation. Therefore, the expression of the underlying genetic code is dependent upon the combinatorial modifications of the core histones and is frequently referred to as the 'histone code' (Turner 2000, Jenuwein & Allis 2001). Among these covalent modifications, the consequences of histone acetylation and methylation patterns on gene expression have received the most attention in multiple tumour types. However, with particular exception (see below), significantly fewer investigations, relative to changes in CpG island methylation patterns, have described this phenomenon in tumours emanating from within the pituitary gland.

Pituitary tumours: histone modifications

Where studied with respect to pituitary tumours, histone modification of candidate genes has been investigated employing chromatin immunoprecipitation assays (ChIP) and the principal findings are described in a subsequent section. In these studies, antibodies that recognize the specific histone modifications, as example, methylation or acetylation, are employed to immunoprecipitate cross-linked histone-DNA complexes, which are chromatin. It is therefore possible, post-reversal of the cross-linking and PCR amplification of the DNA, to derive a ratio of enrichment of precipitated DNA over input DNA. A caveat to this technique is the reliance on the specificity of the antibodies to the modification under investigation, it is therefore, important to include appropriate controls in these types of studies. An overview of the ChIP enrichment technique is shown in Fig. 4, together with the major methods for determining the presence or absence of gene-associated changes.

Decreased expression of *FGFR2* in a significant proportion of primary pituitary tumours is associated with DNA methylation of its associated CpG island (Abbass *et al.* 1997, Zhu *et al.* 2007b). A similar phenomenon is also apparent in the pituitary cell line, AtT20; however, ChIP assays also reveal evidence for histone methylation but not their deacetylation in the silencing event (Zhu *et al.* 2007b). Interestingly, in primary tumours showing reduced expression of

Figure 4 Overview of chromatin immunoprecipitation (ChIP) and post-enrichment analysis. Cross-linked and sonicated chromatin (DNA-histones and associated proteins) are immunoprecipitated with an antibody that in this case recognizes a particular histone modification (shown in red on the left side of the figure). Thus, fragmented chromatin on the left of the figure but not the right is specifically enriched. Following reversal of the cross linking, either a candidate gene (left part of figure) or genome-wide approach (right part of the figure) are adopted. In either case, PCR amplified and immunoprecipitated (IP) DNA is compared with total (input) DNA (T). For promoter or tiling arrays, two colour competitive hybridizations are employed and the relative enrichment may be quantified (see below the example array) and plotted (y-axis) relative to its genetic coordinates (x-axis).

FGFR2, the expression of the normally silent cancer-testis antigen, melanoma-associated antigen A3 (*MAGE-A3*) gene is apparent and is associated, in contrast to normal pituitary, with hypomethylation of this gene's CpG island (Zhu *et al.* 2008*a,b*). However, and of significant interest, a chromatin connection has also been established in that oestrogen also induces *MAGE-A3* expression and this case is associated with increased

histone acetylation and concomitant reduction in histone methylation (Zhu *et al.* 2008*a,b*). In other pituitary cell line studies, and in contrast to CpG island mediated silencing of *FGFR2*, the expression of a pituitary tumour derived isoform of *FGF4* is apparent following challenge with trichostatin A suggesting, in this case, that silencing is as a result of histone deacetylation (Ezzat *et al.* 2006 and references therein).

Histone modification: the Ikaros connection

Significant inroads, in a pituitary tumour context, to our understanding of histone modifications have been provided through studies of the transcription factor Ikaros (Ik) and the dominant negative (dn) isoform of this protein, Ik6, that is expressed in nearly half of all primary pituitary tumours (Ezzat *et al.* 2003). However, in primary tumours that do not express Ik, loss is associated with exon1 CpG island methylation, whereas in AtT20 cells, loss is associated with CpG island methylation and concomitant histone modification (Zhu *et al.* 2007a).

The consequences of Ik expression and of the dn isoform (Ik6) with respect to their influence on epigenetic events in the pituitary have been subjected to recent reviews (Ezzat *et al.* 2005a, Ezzat & Asa 2008). However, several novel findings from these studies are worthy of particular note with respect to their role in modification of histones in the pituitary and their derived cell lines. In GH4 cells, the differential effects of wild type Ik1 and of the dn isoforms (Ik6) have been explored. In these studies, the suppression of GH and stimulation of prolactin transcript expression, at least in part, is through Ik1 mediated influences on promoter acetylation (Ezzat *et al.* 2005b). The tumour specific dn isoforms, Ik6, also promotes AtT20 and GH4 cell growth that is associated with enhanced protection against apoptosis and up-regulation of the anti-apoptotic factor Bcl-XL. In these cells, Ik6 was responsible for selective acetylation of histone 3 sites within Bcl-XL gene; however, it did not influence methylation of the Bcl-XL promoter (Ezzat *et al.* 2006).

Future directions

In addition to the ChIP technique thus far described, specific enrichment, in this case of methylated DNA and termed methylated DNA immunoprecipitation (MeDIP), is being used to investigate differences between normal and tumour cells (Weber *et al.* 2005). Using this method of enrichment, a genome-wide approach is possible where the precipitated and enriched DNA is subsequently hybridized to whole-genome tiling arrays or to the more cost-effective options of promoter or specific CpG island arrays. Although, this technology has not thus far been applied to the analysis of pituitary tumours, it offers a future direction for study. Indeed, a similar technology, in this case termed-ChIP-chip is also directly applicable to study DNA-protein interactions. In these cases, antibody specificity dictates enrichment, thus it is possible to study, on genome-wide basis, the specificity of particular transcription factors for their DNA binding sites. Equally with antibodies directed toward particular histone

modification, it is possible to identify the DNA region that harbours the specific change. In these cases, as with the MeDIP hybridizations the chips, be they tiling or promoter arrays, facilitate whole-genome analysis without *a priori* knowledge of the DNA sequence itself.

Perhaps, likely to supersede these techniques and technological advances will be the exploitation of next generation or flow-cell, sequencing technologies. As with the other immunoprecipitation/enrichment techniques thus far described (MeDIP and ChIP-chip) this technology also usefully exploits DNA enrichment, however, in this case the enriched DNA is used for high-throughput parallel sequencing (ChIP-seq). A detailed consideration of this technology is beyond the scope of this review, however, the reader is directed to an excellent review of this technology and its advantages published elsewhere (Hoffman & Jones 2009).

Concluding remarks

The field of epigenomics research is providing rich and fertile ground for investigators interested in mechanisms responsible for pituitary tumour initiation and progression. As with many areas of investigation, new avenues of research and new research questions are now being addressed and the 'tools' that allow us to address these questions are either available or in rapid development. The haploid human genome comprises three billion base pairs, of these, 28 million are CpG dinucleotides. These represent formidable numbers; however, the technologies are either already available or are in development for us to characterize change at the single nucleotide level, and map those that influence or are consequences of change to the underlying histone code itself.

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.

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