Epigenetic change in pituitary tumorigenesis

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Abstract
Throughout the genome CpG dinucleotides are found at one-fifth of their expected frequency and their rarity is further marked by the fact that 70% are methylated. In contrast, CpG islands (CGI), found associated with the promoters of many genes, have maintained their expected frequency of this dinucleotide, and remain unmethylated. Inappropriate methylation of CGIs is associated with histone deacetylation and gene silencing, while methylation of CpGs outside of CGIs is associated with significantly higher mutation rates. Methylation of CGIs is a frequent event in numerous tumour types including those that arise within the pituitary gland. Several studies now show highly frequent methylation of the p16 gene that is significantly associated with loss of cognate protein and that appears to be an early change in pituitary tumorigenesis. Collectively, studies show that somatotrophinomas are an infrequent target for p16 CGI methylation. However, in this pituitary tumour subtype, loss of pRb is associated with either CGI methylation or micro-deletion within the protein-pocket binding domain. As in other tumour types loss of p16 or RB1 appear to be mutually exclusive events in non-functional adenomas and somatotrophinomas respectively. Investigation of the Death Associated Protein Kinase gene shows that loss of its protein (DAPK), a pro-apoptotic molecule, in pituitary tumours is also associated with either methylation or deletion within its associated CGI. In the case of DAPK, however, these changes segregate with invasive pituitary tumours irrespective of tumour subtype. Methylation represents a positive signal that can be detected with exquisite sensitivity; in addition, this change targets multiple genes that show tumour type specificity. Taken together, the detection of DNA methylation changes, using either a panel of predefined marker-islands, or CGI arrays, provides the opportunity to generate ‘methylation profiles’. This new knowledge will increase our understanding of tumour biology and could ultimately aid medical management in these different tumour types, including those of pituitary origin.

DNA methylation
Through a process of post-replicative covalent modification, the DNA of mammalian cells contains a ‘fifth base’, namely 5-methylcytosine. The most frequent target for this modification is cytosine in the context of the dinucleotide CpG. Throughout the genome CpG dinucleotides are found at one-fifth of their predicted frequency, and the majority of those present (~70%) are methylated (reviewed in Baylin et al. 1998). In marked contrast to the genome-wide under-representation of CpGs, there are regions of the genome termed CpG islands (CGIs) that have maintained their expected frequency of this dinucleotide. CGIs are associated with the promoter regions of approximately 40–50% of all transcribed genes and, with the general exception of the inactive X-chromosome in females and the imprinted genes in both sexes, CGIs remain unmethylated (for review see Baylin et al. 1997, Zingg & Jones 1998).

Both pre-existing (normal) methylated CpG dinucleotides and methylation changes which take place as part of an aberrant biological process play important roles in tumorigenesis. A significantly higher DNA mutation rate, through spontaneous deamination of 5-methylcytosine, characterises ~30% of inherited diseases and cancer (Zingg & Jones 1997) and more than half of the described mutations in the p53 gene occur at CpG dinucleotides (Baylin et al. 1998). A second but distinct role of this epigenetic change relates to inappropriate methylation of CGIs in tumorigenesis. Consequently to this modification is an associated gene silencing which will, in the context of pituitary tumorigenesis, be the focus of this review.
CGI methylation and gene silencing

The role of CGI methylation as causal in, or secondary to, gene silencing has not been fully resolved (reviewed in Jones & Laird 1999, Baylin & Herman 2000), although more recently published views promote the latter perspective (Clark & Melkin 2002, Turker 2002). CGI methylation is, however, correlated with condensed nuclease-resistant heterochromatin during the establishment or reinforcement of transcriptional incompetence (Kass et al. 1997). The functional consequences, relating CGI methylation to transcriptionally repressive chromatin are outlined in Fig. 1. In this model an inactive, condensed, chromatin structure is established through histone deacetylation. Deacetylation is linked to methylation through the methyl-binding domain proteins, for example MeCP1 and MeCP2, which are components of or recruit histone deacetylases (HDACs). More recently, a direct link between methylation and deacetylation has been established whereby both the maintenance methyltransferase enzyme (DNMT1) and de novo methylases (DNMT3a and 3b) bind to HDACs and repress gene transcription through HDAC activity (reviewed in Burgers et al. 2002). These studies also showed that the actual methyltransferase activity of the Dnmts was dispensable for transcriptional silencing. In addition to reorganisation of chromatin structure, CpG methylation can result in a more direct mechanism of inhibition. In this case, direct inhibition of binding of sequence-specific transcription factors, whose consensus binding sites contain CpG dinucleotides, may repress gene expression (Tate & Bird 1993).

Figure 1 Schematic diagram showing how CGI methylation within a promoter region may contribute to gene silencing. (A) Transcriptionally active chromatin is shown schematically. Histone cores are shown as cylinders, with DNA, indicated by the black line, wrapped around the cylinders. The unmethylated CpG dinucleotides are shown as unfilled ovals along the DNA strand. The ‘relaxed’ nucleosomes are associated with acetylated histones shown as the filled diamond on the tail (thin, freeform line). (B) Methylation of CpG dinucleotides (filled ovals) has led to the recruitment of proteins with affinity for methylated DNA (see text for details and exceptions to this model) including those with HDAC activity. Deacetylated histone tails (shown as the unfilled diamonds) lead to a condensed chromatin configuration and transcriptional repression.

CGI methylation and cancer

Almost half of the known tumour-suppressor genes (TSGs) that cause familial cancers through germline mutation are associated with promoter-region CGI methylation in sporadic cancers, highlighting the importance of this change in tumour biology (Baylin & Herman 2000). Genes implicated in tumour evolution or progression, including those involved in DNA repair, drug resistance and detoxification, differentiation, apoptosis, angiogenesis, metastasis and invasion are associated with methylation-associated gene silencing in different tumour types (for review see Baylin & Herman 2000). Pituitary tumours, in common with other tumours, are subject to inappropriate methylation-associated gene silencing. It is now some 3 years since we published our review of methylation in pituitary tumorigenesis (Farrell & Clayton 1999) and in that time new findings have justified an update of this area of research.

Methylation-associated silencing of G1/S regulators

Transit from G1 to S phase of the cell cycle is mediated through the downstream effector molecule pRb and the principal regulatory molecules in this pathway are outlined in Fig. 2. In its phosphorylated form pRb releases specific transcription factors responsible for cell cycle progression. Conversely, hypophosphorylated pRb binds these factors thereby inhibiting progress through this checkpoint. The phosphorylation status of pRb is in turn regulated by upstream
mediators, namely, the cyclins, the cyclin-dependent kinases (CDKs), and the CDK inhibitors (CDKIs). Among the CDKIs, p16 (the protein product of the CDKN2A gene), plays a pivotal role in maintaining pRb in its hypophosphorylated form. Control of cell cycling, by these cell cycle regulators (p16 or pRb), is lost in virtually all tumour types (Sherr 1996). The mechanisms responsible for p16 or pRb functional loss include deletion, point mutation or promoter methylation, where the frequency of each mechanism varies with tumour type. The incidence of functional disruption of the p16 gene, especially when promoter methylation is included, indicates that this gene rivals p53 as the most frequently inactivated TSG in cancer (Baylin et al. 1998). However, for the RB1 gene, deletion and point mutation are the most common mechanisms of gene inactivation, and methylation-mediated silencing has until recently (see below) only been reported in a proportion of sporadic retinoblastomas (Greger et al. 1994, Stirzaker et al. 1997).

**Methylation-associated loss of p16 in pituitary tumours**

Early studies of p16 in pituitary tumours suggested universal loss of this protein as determined by Western blotting; however, this was not associated with mutation as determined by SSCP analysis. In the same study employing differential PCR, a proportion of adenomas (12%) were found to harbour a homozygous deletion (Woloschak et al. 1996). These authors suggested loss of p16 to be an early event in pituitary tumorigenesis, and that other mechanisms of gene silencing, in addition to deletion, were most probably responsible for the loss of p16 protein. However, our own studies using a panel of microsatellite markers failed to detect losses that encompassed the p16 gene (Woloschak et al. 1997, Simpson et al. 1998). Subsequent studies showed that the highly frequent loss of p16 protein in this tumour type was significantly associated with methylation of the p16 gene CGI (Woloschak et al. 1997, Simpson et al. 1998).
et al. 1999a). While the first study found methylation (~70% of adenomas; at all three methylation-sensitive sites tested) in various pituitary tumour subtypes (Woloschak et al. 1997), the second suggested it was frequent in non-functional tumours (70%) but infrequent in somatotrophinomas (9.5%), and represented an early event in pituitary tumorigenesis (Simpson et al. 1999a). Several subsequent publications reached similar conclusions of highly frequent methylation and/or infrequent mutation of the p16 gene in pituitary adenomas (Yoshimoto et al. 1997, Jaffrain-Rea et al. 1999, Ruebel et al. 2001, Seemann et al. 2001). In those studies that included protein expression data, significant correlations with loss of expression were found (Ruebel et al. 2001, Seemann et al. 2001). Ruebel et al. (2001) also described subtype-specific methylation of p16, with frequent methylation in non-functional (null-cell) adenomas (71%) and infrequent methylation in gonadotroph tumours (29%) and somatotrophinomas (0%). Seemann et al. (2001) also showed frequent methylation in the majority of pituitary tumour subtypes examined (~82%), with the exception of somatotrophinomas (8%) and corticotrophinomas (7%), and suggested loss of p16 to be a late event that correlated with tumour size.

Collectively, these data show non-functional tumours to be a frequent target for epigenetic change. There are some differences in the conclusions reached regarding the frequency of epigenetic change in other pituitary tumour subtypes, which may reflect the number of subtype-specific tumours investigated within the individual studies. Interestingly, three of those studies reached similar conclusions, of infrequent methylation of p16, in somatotrophinomas (Simpson et al. 1999a, Ruebel et al. 2001, Seemann et al. 2001).

Why methylation-associated silencing of p16 appears to be pituitary tumour subtype restricted is not clear. However, for this gene in other tumour types, both the overall frequency and mechanisms of inactivation (genetic vs epigenetic), appears to be tumour specific (Baylin & Herman 2000). In addition, in many other tumour types, disruptions at this cell cycle checkpoint show the target to be either p16 or RB, but not both (for review see Sherr 1996).

Methylation-associated loss of pRb in pituitary tumours

Early studies of RB in pituitary adenomas were in part prompted by the findings in the RB1 ‘knock-out’ mouse of pituitary tumours with almost complete penetrance; however, these studies did not reveal either mutation or changes in pRb expression (for review see Farrell & Clayton 2000). More recently, high-density allelotyping of the long arm of chromosome 13, together with pRb immunohistochemistry (IHC), showed that 27% of somatotrophinomas and 4% of non-functional tumours did not express pRb as determined by IHC, but that this was not associated with loss of an RB1 intragenic marker (Simpson et al. 1999b).

To gain mechanistic insight we examined the methylation status of the RB1 gene’s CGI, together with mutation and deletion analysis of the RB1 promoter, and the protein-binding pocket domain in a cohort of pituitary adenomas (Simpson et al. 2000). For the methylation analysis we used methylation-sensitive PCR (MS-PCR). Figure 3 shows examples of post-mortem normal pituitary glands and adenomas examined by this technique. The figure shows an unmethylated CGI in normal pituitary tissue and in a proportion of adenomas; however, it also shows evidence of methylation in some of the adenomas investigated. Of ten tumours that failed to express pRb, six were found to harbour methylation; a further three harboured deletions within the protein-binding pocket domain. Importantly, 18 of 20 adenomas and six of six normal pituitary glands that expressed pRb were unmethylated. In common with earlier studies, we did not find evidence for mutation within the coding region or in the RB1 gene promoter (Simpson et al. 2000). Methylation and deletion were mutually exclusive mechanisms associated with loss of pRb and segregated with the somatotrophinoma subtype. To date, there are only two other reports that document methylation-associated gene silencing of RB1 and both of these studies related to retinoblastomas (Greger et al. 1994, Stirzaker et al. 1997).

**RB1 and p16 as mutually exclusive targets**

Loss of p16 or RB1, irrespective of inactivation mechanism in other tumour types, are with high frequency mutually exclusive (reviewed in Sherr 1996), where loss of one would, in general, render the other functionally redundant. Indeed, the collective data (see above) for pituitary adenomas would support this view. In a recent study, we more rigorously addressed this question by examining a single cohort of tumours for aberrations in G1/S regulatory molecules (Simpson et al. 2001) This study showed that loss of p16 or pRb expression was indeed mutually exclusive in the majority of pituitary tumours examined. Furthermore, these results argue against the fact that methylation of these genes might simply reflect a generalised increase in methylase activity within particular adenomas. In the same study, we also examined oncogene-activating mechanisms that are beyond the scope of this review.

**Methylation-associated loss of the Death Associated Protein Kinase (DAP kinase) gene in pituitary tumours**

The DAP kinase gene was initially isolated as a positive mediator of interferon-γ-induced apoptosis (reviewed in
Figure 3 MS-PCR analysis of the RB1 promoter region in pituitary tumours and histologically normal post-mortem pituitary tissue. All DNA samples were sodium-bisulphite treated prior to MS-PCR analysis. Modified DNA samples were subjected to PCR analysis with oligonucleotides specific for unmethylated (U) or methylated (M) DNA. A PCR product is generated from in vitro methylated genomic DNA (C) with the oligonucleotide set specific for methylated but not unmethylated DNA. The absence of methylation in histologically normal post-mortem pituitary tissue is demonstrated by the presence of a PCR product generated by the oligonucleotides specific for unmethylated DNA. Representative PCR products corresponding to methylated (#20, 210, 230) and unmethylated (#2, 159) tumours are also shown. No methylation was found in any of the matched blood DNA samples examined (data not shown).

Feinstein et al. (1995). A role for DAP kinase in the activation of a p19ARF/p53 cell cycle checkpoint has been established (Raveh et al. 2001), thus providing a mechanism whereby loss might lead to inactivation of this critical apoptotic pathway in tumorigenesis. Indeed, several studies have suggested that loss of DAP kinase expression or methylation of its associated CGI may characterise highly invasive or metastatic tumours (Simpson et al. 2002 and references therein). Since none of these studies investigated associations between methylation and expression status we examined this gene and its protein product in a cohort of invasive and non-invasive pituitary tumours.

DAP kinase expression was investigated in 32 adenomas by RT-PCR analysis and Western blotting, and the methylation status was determined by MS-PCR. Loss of DAP kinase expression was significantly associated with invasive pituitary tumours (10 of 17; 59%) compared with their non-invasive counterparts (1 of 15; 7%). Examples of expression and methylation status in representative tumours are shown in Fig. 4. Of 11 tumours that failed to express DAP kinase five were found to be methylated and a further four were found to harbour homozygous deletions of the promoter region. Methylation and deletion were mutually exclusive events associated with loss of DAP kinase expression. We found no evidence of deletion or methylation in post-mortem normal pituitary tissue, and in those tumours that expressed DAP kinase only 2 of 21 (~10%) were methylated. Thus, this study showed for the first time, for any tumour type, an association between mutually exclusive alterations (methylation or deletion), within the DAP kinase promoter, and loss of DAP kinase expression. In addition, there is also a significant association between aberrations in this pathway and the invasive phenotype. While earlier studies, in other tumour types, show methylation or loss of expression to be associated with a metastatic phenotype it is well recognised that pituitary adenomas rarely progress to malignancy. However, two of the tumours included in this study were pituitary carcinomas and both failed to express DAP kinase in association with methylation of its CGI. Thus, the rarity of the transition to pituitary carcinoma, despite the frequent loss of DAP kinase in invasive adenomas, might simply reflect the possibility that other cooperating aberrations are necessary to complete this final transition.

Methylation profiles as biomarkers

With few exceptions, and irrespective of tumour type, conventional histological and biochemical assessment criteria are limited with respect to predicting or determining tumour behaviour or outcome. Equally, these types of analyses are challenged by those instances where, perhaps, tumour or pre-tumour cells represent the significant ‘minority’ among a ‘majority’ of normal cells. However, inappropriate methylation represents a positive signal, which can be detected by PCR-based methodologies with exquisite sensitivity against a background of essentially normal cells. Furthermore, emerging data show that different tumour types harbour methylation that targets multiple genes and a particular tumour type will frequently display a ‘methylation profile’ that is unique to that tumour (Esteller et al. 2001). Several studies have now reported methylation profiles in different tumour types as proof of principle for cancer risk assessment, early cancer diagnosis and prognosis and response to chemotherapy (reviewed in Baylin et al. 2001).
Methylation Sensitive PCR

Western Blotting

Figure 4 Representative examples of MS-PCR (top) analysis of the DAP kinase CGI together with Western blot analysis (bottom) in pituitary tumours and histologically normal post-mortem pituitary tissue. All DNA samples were treated as described in Fig. 3 and subject to MS-PCR. The absence of methylation in histologically normal post-mortem pituitary tissue is demonstrated by the presence of a PCR product generated by the oligonucleotides specific for unmethylated DNA. Representative PCR products corresponding to methylated (M) (#T13, T23 and T26) and unmethylated (U) (#T5 and T25) tumours are shown. No methylation was found in any of the matched blood DNA samples examined (data not shown). Patient numbers are shown below each lane. The Western blot analysis shows the same specimens as examined by MS-PCR in this case for DAP kinase protein expression. The panel shows expression of the 160 kDa DAP kinase protein in normal pituitary gland (NP22) and tumours T5 and T25, while the DAP kinase protein is not expressed in tumours T13, T23 or T26. In addition, the lower panel shows expression of the housekeeping gene vinculin in all normal pituitary tissues and tumours examined.

The emerging data on methylation-associated gene silencing in pituitary tumours would suggest that methylation profiling might provide a useful adjunct to more conventional assessment criteria. Although, to date, relatively few genes and their associated CGIs have been investigated it is already apparent that some appear to mark early change in particular pituitary tumour subtypes (p16), or show some subtype specificity (p16 and RB1) while still others appear to mark the boundary between non-invasive and invasive adenoma/carcinoma (DAPK). More recently, we examined the methylation status of p16 in adenomatous and non-adenomatous pituitary glands from patients with Cushing’s disease. In this analysis, we found frequent methylation irrespective of histological findings (DJ Simpson, RN Clayton & WE Farrell, unpublished observations. Thus, in the Cushing’s disease-associated non-adenomatous pituitary glands, detection of this molecular aberration represents either a change in pre-adenomatous cells within these specimens or perhaps, ‘scattered’ adenomatous cells that are not apparent by more conventional techniques. At the present time we are uncertain as to which of these two explanations is correct; however, the detection of this change does support the view that these cells harbour an intrinsic abnormality.

CGI methylation and future direction

While it is clear that inappropriate methylation may occur in multiple genes in different tumour types, and that these changes are frequently associated with changes in the expression level of the methyltransferase enzymes, there is still much to understand (Robertson & Jones 2000). In this
context, the gene target(s) for this aberration are frequently highly specific for a particular tumour type including those that arise within the pituitary gland. However, an explanation for the finding of target gene specificity is lacking at the present time. Equally, while there are emerging data suggesting that these changes are able to provide predictive indices, clearly for the pituitary and other tumour types these findings need to be rigorously assessed in prospective studies.

Many of the investigations of CGI methylation in both pituitary and other tumour types have employed a candidate gene approach; however, exciting data are now emerging on novel genes subject to CGI methylation. These studies have led to the generation of whole-genome CGI libraries, and the use of these libraries for the production of CGI arrays to screen tumour specimens and correlate findings with pathological parameters. Using arrays comprising ~1000 CGI tags, Yan et al. (2000) identified that close to 9% of these tags showed differential methylation in primary breast tumours compared with normal controls, and an association between CGI methylation pattern/profile and histological grade. Current investigations in our laboratory are directed toward the identification and characterisation of novel genes, silenced through CGI methylation in pituitary tumours, that may have useful application in array-based screening methodologies.

The past 5 years have seen exciting and novel findings that give new insight into pituitary tumorigenesis. Although we are still some way from a positive identification of the molecular defects responsible for tumour initiation and those that define progression, existing and emerging molecular techniques mean these goals are attainable.

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