Methylation mechanisms in pituitary tumorigenesis

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Abstract

Methylation is essential for embryonic development, however aberrant methylation of CpG islands associated with the tumour suppressor genes (TSGs) and leading to gene silencing is found in numerous tumour types. The TSG p16/CDKN2A is involved in the genesis of many tumour types and frequent methylation of the CpG island of the p16/CDKN2A gene is associated with loss of protein expression in pituitary tumours. In addition, CpG sites are mutational hotspots and abnormal methylation patterns have been shown to lead to genetic instability, predisposing to, and preceding allelic loss. Although several studies of pituitary tumours have shown loss of genetic material at known and putative TSGs loci, studies of the retained alleles have revealed infrequent mutation. Equally, for several other TSGs no mechanisms have been described for their reduced expression. Methylation may represent a unifying theme, responsible in some cases for an absence or reduced expression and in other cases predisposing to allelic loss that may or may not encompass a TSG. In several tumour types treatment of tumours or their cognate cell lines with demethylating agents induces expression of previously methylated genes. Using the mouse corticotroph cell line AtT20 as a model system, transfection studies showed restoration of growth control through induction of ectopically expressed p16/CDKN2A. These effects were reversed by prior *in vitro* methylation of the constructs' CpG sites within the coding region of this gene. Methylation of an otherwise unmethylated CpG island renders a gene transcriptionally incompetent and clinically these genes represent attractive therapeutic targets since the gene is neither lost nor mutated, but may be reactivated. Future studies will no doubt describe more efficacious pharmacological interventions and identify the mechanisms responsible for the abnormal methylation patterns seen in tumours including those of pituitary origin.

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Mechanisms responsible for oncogene and tumour suppressor gene dysfunction

The detailed genetic changes involving oncogene and tumour suppressor genes (TSGs) in the initiation and progression of pituitary tumours have not been fully elucidated. However, in common with many other tumour types progression to invasive adenoma and the infrequent pituitary carcinoma is thought to be a consequence of a progressive accumulation of genetic abnormalities in these two types of genes which are responsible for controlling cell proliferation and differentiation (Thakker 1993, Melmed 1994). Collectively, numerous studies have put into place some of the changes in both oncogenes and TSGs in pituitary tumorigenesis and have recently been reviewed (Pei & Melmed 1996, Shimon & Melmed 1997, Farrell & Clayton 1998).

Oncogenes exert their action through a gain of function and the TSGs through a loss of function. The mechanisms responsible for oncogene activation are mutation, amplification and translocation. It is not the purpose of this review to detail the involvement of oncogenes in pituitary tumours and the reader is directed to a contribution by Melmed and colleagues in an accompanying article in this edition of *Endocrine-Related Cancer*. For the TSGs three principle mechanisms for loss or reduced expression have been described (Table 1). The first is loss of heterozygosity (LOH) that is accompanied by a concomitant mutation in the retained allele, and perhaps the classic example here is the retinoblastoma gene (*RBI*) in both familial and sporadic forms of this disease (Knudson 1971). The second mechanism responsible for loss of a TSG is homozygous deletion necessitating loss of both alleles. Table 1 shows that a

Table 1 Principle mechanisms responsible for loss of tumour suppressor genes in familial and sporadic tumours

Mechanism	Gene tumour
Loss of heterozygosity (concomitant mutation in the retained allele)	<i>RB1</i> Retinoblastomas
Homozygous deletion (loss of both alleles)	<i>p16/CDKN2A</i> Head and neck cancers
Methylation of CpG islands (reduced or absent expression)	<i>p16/CDKN2A</i> Multiple tumour types

particularly striking example is the TSG *p16/CDKN2A*, where in head and neck cancers nearly 70% of primary tumours have lost this TSG through this mechanism (Merlo *et al.* 1995, Reed *et al.* 1996). The third mechanism responsible for loss or reduced expression of a TSG is methylation of associated CpG islands. Many of the known TSGs have been shown to harbour CpG island methylation (for recent reviews see Baylin *et al.* 1991, Jones 1996, Baylin *et al.* 1998), that is associated with and frequently causal in gene silencing. For the TSG *p16/CDKN2A* gene on chromosome 9p, loss through methylation approaches 40% in primary colon tumours and increases to 92% in their equivalent cell lines (Herman *et al.* 1995 and reviewed in Baylin *et al.* 1998) and in non-functional pituitary tumours loss through methylation is found in 70% of this tumour subtype (and is discussed in detail in this review).

Role of DNA methylation in normal tissue and tumours

Approximately half of all transcribed genes, some 45 000, have CpG islands associated with them (Antequera & Bird 1993a,b). The default setting in normal tissue for the majority of these promoter-associated islands is that they are not methylated (Antequera & Bird 1993b). There are notable exceptions to this rule in normal cells that include the imprinted genes, Alu sequences and X-chromosome inactivation in females (reviewed in Jones 1996, 1999). Also, in normal, immortal and tumour cells methylation of CpG islands downstream of an active promoter does not block transcription (Jones 1999). Indeed, for several genes CpG island methylation downstream of the start site is frequently correlated with expression; this is in marked contrast to the inverse relationship seen at sites of transcriptional initiation (reviewed in Jones 1999). The majority of the remaining (non-island) CpG sites throughout the genome are methylated and constitute mutational hotspots (for an excellent review of this area the reader is directed to the cited review (Jones 1996)). Many of the known TSGs have been shown to harbour CpG island methylation (Table 2) in both primary tumours

and cell lines derived from these tumours. Table 2 shows not only the broad range of tumour types for which this phenomenon has been demonstrated but also genes involved in terminal differentiation (*MyoD*) and those involved in the suppression of the metastatic phenotype (*E-cadherin*). For the majority of these genes, in both primary tumours and tumour cell lines, methylation within their CpG islands has been shown to be significantly associated with and causal in gene silencing (reviewed in Baylin *et al.* 1998). Thus, for the TSG, the oncogenic mechanisms responsible for the progressive methylation of an associated CpG island may initially lead to reduced TSG expression. A selective growth advantage is thought to ensue and further methylation errors lead to eventual TSG silencing and monoclonal tumour outgrowth (Jones 1996). Although many of the known TSGs, in a variety of tumour types, have been shown to be silenced by this mechanism by far the most frequent TSGs affected by this mechanism of loss are the TSG *p16/CDKN2A* in solid tumours (Gonzalez-Zulueta *et al.* 1995, Herman *et al.* 1995, Merlo *et al.* 1995) and *p15/CDKN2B* in haematological malignancies (Herman *et al.* 1996a, 1997).

Detection of DNA methylation

The methylation of cytosine, usually in the context of the dinucleotide CpG is amenable to detection either by prior chemical modification and subsequent sequence analysis or the use of restriction enzymes that are sensitive to the methylation status of particular cytosines. Other novel strategies for the detection of methyl-cytosine are dependent on initial chemical modification and subsequent PCR amplification with primers specific for modified DNA. This technique, termed methylation-specific PCR, originally described by Herman *et al.* (1996b), is probably the method of choice if the available tumour sample is limited or heavily 'contaminated' with normal tissue. Frommer *et al.* (1992) pioneered direct sequence analysis following sodium bisulphite modification and subsequent conversion of non-methylated cytosines to uracil. This technique allows the identification of the number and location of specific methylated cytosine residues within a sequence.

Table 2 Genes associated with promotor region CpG island methylation in primary tumours and tumour cell lines

Tumour type	Gene
Retinoblastoma	<i>RB1</i>
Renal cell carcinoma	<i>VHL</i>
Common solid tumours and lymphomas	<i>p16, ER</i>
Bladder cancers	<i>Myo-D, E-Cadherin, p16</i>
Breast carcinomas	<i>BRCA1, Myo-D, E-Cadherin, HIC-1, MDG1</i>
Acute leukaemia and Burkitt lymphoma	<i>p15, ER</i>
Wilms tumour	<i>H19</i>
Prostate cancer	<i>GSTP1</i>
CML	<i>Bcr-abl</i>
Colon	<i>p16, E-Cadherin, ER, HIC-1</i>
Brain	<i>HIC-1</i>
Liver	<i>HIC-1</i>

The genes and tumour types are referenced throughout the text and are subject to detailed consideration in Jones (1996), Szyf (1996), Zingg & Jones (1997), Baylin *et al.* (1998).

CML; chronic myeloid leukemia.

Methylation-sensitive restriction digestion followed by Southern blotting or PCR amplification is frequently used for the detection of methylated regions. However, the technique relies on the methylated cytosine lying within the consensus sequence of the particular enzyme. An advantage of this technique is that it frequently allows analysis of several different regions by exploiting different methylation-sensitive restriction enzyme sites within an amplified sequence. We used methylation-sensitive restriction digestion of the *p16/CDKN2A* gene, followed by either PCR amplification or Southern blotting in a large number of pituitary tumours. Figure 1 shows the different methylation-sensitive restriction sites examined in exon 1 and exon 2 of the *p16/CDKN2A* gene. Since the CpG island of the *p16/CDKN2A* gene extends into the coding region this allowed us directly to assess the methylation status of this gene in this tumour type (see Fig.1).

The role of the tumour suppressor gene *p16/CDKN2A*

The downstream effector that signals a cell to transit from G₁ to S phase of the cell cycle is the phosphorylation status of the retinoblastoma protein (pRB). pRB in its hyperphosphorylated conformation releases specific transcription factors involved in cell cycle progression. In its hypophosphorylated state pRB binds these transcription factors and is responsible for inhibiting progress through this cell cycle checkpoint. Phosphorylation of pRB is, in turn, regulated by upstream effectors, in this case the cyclin-dependent kinases (CDKs). As their name

implies the CDKs are dependent on an interaction with the cyclins to phosphorylate pRB. Thus, a productive interaction between CDK4 and cyclin D1 is responsible for cell cycle progression. The role of p16 at this cell cycle checkpoint is to bind to or sequester CDK4 inhibiting this productive interaction. The p16 protein may therefore be viewed as a 'cell cycle brake'. For both p16 and indeed pRB, loss of these cell cycle regulators may be viewed as a removal of a cell cycle brake leading to uncontrolled cell proliferation (Sherr 1996).

Mechanisms responsible for the loss of *p16/CDKN2A* in pituitary tumours

Initial studies by our group focused on high-density microsatellite mapping of chromosome 9p, the location of the *p16/CDKN2A* gene. These studies were prompted by the frequent finding in numerous primary tumour types of loss of this TSG through homozygous deletion (Cairns *et al.* 1995). Using micro-dissected tumour material in comparison to patient-matched blood DNA in a large cohort of non-functional tumours, we failed to detect either LOH or homozygous deletion of the *p16/CDKN2A* gene in any of the tumours we studied (Farrell *et al.* 1997). However, we did find losses on chromosome 9p both telomeric and centromeric to this TSG. Losses were found at a frequency of 31% in non-functional pituitary tumours and at approximately equal frequency in both invasive and non-invasive tumours (summarised in Table 3). These results initially suggested the presence of another or other TSGs on chromosome 9p and that these changes occurred

Figure 1 Enzyme-mediated PCR analysis of CpG island methylation sites in the p16 gene. Schematic representation of PCR analysis of the *p16/CDKN2A* CpG island that extends into the gene-coding region. The figure shows normal pituitary and a pituitary tumour and the methylation sites examined in exons 1 and 2. Exons are shown as unfilled boxes and introns as interconnecting lines. Within the exons the methylation-sensitive sites are shown as either unmethylated (thin vertical bars) or as methylated (filled bars). After digestion DNA is subject to PCR amplification with primers for exon 1 and 2 (arrowed). A PCR amplicon is only generated from exon 1 in tumour DNA after digestion with any of the restriction enzymes, since in this example only these sites are methylated. X, no PCR amplicon.

early in pituitary tumorigenesis. In marked contrast, a similar analysis of somatotrophinomas with the same panel of microsatellite markers failed to detect losses in this tumour subtype (Simpson *et al.* 1999a). Using a complementary approach Woloschak *et al.* (1996) also showed retention of the *p16/CDKN2A* gene in the majority of pituitary tumours studied. These studies used differential PCR to show retention of the gene; however, in contrast to our data their analysis was suggestive of homozygous deletion in 8 out of 25 tumours. In the same study they failed to detect either expression of p16 protein as assessed by Western blotting or mutation in the coding region of the *p16/CDKN2A* gene by single strand conformation polymorphism (SSCP) analysis, suggesting other mechanisms of gene silencing.

Using methylation-sensitive restriction digestion followed by PCR amplification Woloschak and our own group determined the methylation status of the CpG island associated with the *p16/CDKN2A* gene in pituitary tumours. In addition, these studies were complemented with assessment of protein expression by either Western blot analysis or immunohistochemical expression (IHC). Woloschak *et al.* (1997) studied 20 tumours of various

subtypes although no details regarding their invasiveness were provided. They found 18 of 20 tumours were methylated in exon 1, and 3 of these tumours also showed methylation of an *HpaII* site in exon 2. Western blotting showed an absence or barely detectable p16 protein in all of the 20 tumours investigated. Since our studies had shown a difference with regard to chromosome 9p LOH in two pituitary tumour subtypes (Farrell *et al.* 1997, Simpson *et al.* 1999a) we investigated a large cohort of tumours comprising non-functional tumours and

Table 3 Chromosome 9p deletions in pituitary tumours. Summary of high-density chromosome 9p microsatellite mapping in non-functional tumours and somatotrophinomas

Non-functional adenomas	18/57 (31%)
Somatotrophinomas	0/21
Normal pituitary	0/15

Deletions flank but exclude the p16 gene. Losses in the non-functional tumours were found at approximately equal frequency in invasive and non-invasive tumours.

Figure 2 Methylation status of exons 1 and 2 of the *p16/CDKN2A* gene in a representative pituitary tumour found to be methylated at all three sites examined in exon 1, and normal pituitary in which none of the sites examined were found to be methylated. The left of the figure shows exon 1 and exon 2 boxed, vertical tick marks represent methylation sites. The filled circles surmounting the tick marks denote methylation at the sites examined. The methylation sites analysed are shown in Fig. 1 and are abbreviated as follows: *EagI* (*E*), *SacII* (*s*) and *SmaI* (*Sm*). Digested and undigested DNA (*U*) was subjected to multiplex PCR amplification for *p16/CDKN2A* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The *GAPDH* amplicon was designed not to contain any of the restriction sites. After digestion a PCR product was generated from exon 1 in the tumour irrespective of the enzyme used, showing that all three sites in the tumour were methylated. No PCR product was generated from exon 2 in any tumours examined. No PCR products were generated from normal pituitary after methylation-sensitive restriction enzyme digestion, indicating an absence of *p16/CDKN2A* methylation. Control PCR amplification of undigested DNA (*U*) and digested DNA for a *GAPDH* amplicon generated a product in all cases.

somatotrophinomas (Simpson *et al.* 1999a). In addition to this subdivision, tumours were divided on radiological criteria into invasive and non-invasive cohorts. Methylation-sensitive restriction digestion followed by PCR amplification showed that 70% of non-functional tumours were methylated compared with only 9.5% of somatotrophinomas (Table 4). When we subdivided the non-functional tumours into invasive and non-invasive we found an approximately equal frequency of methylation suggesting, as with the chromosome 9p LOH, that this represents an early change in pituitary tumorigenesis. In those tumours that were methylated we found that one, two or three sites within exon 1 were methylated. Figure 2 shows an example of this analysis in a tumour in which all three methylation sites within exon 1 of the *CDKN2A/p16* gene were found to be methylated. In addition, the figure shows a similar analysis of normal pituitary and an absence of methylation at any of the sites investigated. None of the tumours studied showed methylation with exon 2. In contrast, Woloschak *et al.* (1997) showed methylation within exon 2 using a similar technique; the likely explanation is the different methylation-sensitive enzymes used in these two studies. To further confirm methylation as assessed by the PCR-based technique, we used Southern blot analysis where sufficient tumour DNA

was available. After methylation-sensitive restriction digestion the Southern blotting data were completely concordant with the PCR-based technique for the detection of methylation within the CpG island of the *p16/CDKN2A* gene.

Next, we assessed IHC expression of p16 protein in all available tumours. Figure 3 shows representative examples of IHC expression of p16 protein in a normal pituitary, a tumour that was methylated and one that was not methylated at the sites investigated. Table 5 summarises IHC expression versus methylation status in

Table 4 Summary of *p16/CDKN2A* CpG island methylation in non-functional pituitary tumours and somatotrophinomas

Tumour	Methylated	Unmethylated
Non-functional adenomas	32/46 (70%)	14/46 (30%)
Somatotrophinomas	2/21 (9.5%)	19/21 (90.5%)

Methylation within exon 1 of the *p16/CDKN2A* gene is found at high frequency only in non-functional tumours. The frequency of methylation was approximately equal in invasive and non-invasive non-functional tumours.

Figure 3 Immunohistochemical analysis of p16 protein in normal pituitary (A) and pituitary adenomas in which the *p16/CDKN2A* was not methylated (B) and in a tumour shown to be methylated (C). Widespread nuclear and low level cytoplasmic reactivity for the *p16/CDKN2A* gene product is seen in normal pituitary (A). Nuclear reactivity for p16 is seen in a tumour shown not to be methylated at the restriction sites examined (B). Absence of nuclear p16 staining in a tumour shown to be methylated at all three sites examined within exon 1 of the *p16/CDKN2A* gene is shown in (C).

both non-functional tumours and somatotrophinomas. The table shows a highly significant correlation between methylation status and protein expression, where 78% of methylated non-functional tumours failed to express detectable p16 protein as assessed by IHC. In some cases we found loss of protein expression that was not associated with methylation (Table 5). However, complete sequence analysis of the coding region of the *p16/CDKN2A* gene did not reveal mutation in any of these tumours. SSCP analysis and sequencing of variants in a series of 31 sporadic pituitary tumours by Yoshimoto *et al.* (1997) also failed to detect mutation in either the *p16/CDKN2A* or juxtaposed *p15/CDKN2B* gene on chromosome 9p21; however, this study did not include analysis of protein expression.

Relationship of methylation to loss and/or reduced expression of other TSGs implicated in pituitary tumorigenesis

Several studies have shown loss of known or putative TSGs in pituitary tumorigenesis (reviewed in Pei &

Melmed 1996, Shimon & Melmed 1997, Farrell & Clayton 1998). Microsatellite and restriction fragment length polymorphism analysis have shown either singular or collective losses on the long arms of chromosomes 10, 11 and 13 that are significantly associated with invasive tumours in comparison to their non-invasive counterparts (Thakker *et al.* 1993, Boggild *et al.* 1994, Pei *et al.* 1995, Bates *et al.* 1997). However, studies of the retained allele at known TSGs associated with regions of loss have revealed infrequent mutation. The recent cloning of the multiple endocrine neoplasia type 1 (MEN1)-associated gene (Chandrasekharappa *et al.* 1997, European Consortium on MEN1 1997) allowed sequence analysis of this gene in sporadic pituitary tumours. A first report described presumed inactivating mutations in about 5% of sporadic pituitary (Zhuang *et al.* 1997). However, several subsequent studies have either failed to detect mutations (Prezant *et al.* 1998, Tanaka *et al.* 1998, Farrell *et al.* 1999) or detected mutation at extremely low frequency (Asa *et al.* 1998) in this gene. Three of these studies also examined transcript expression of the *menin* gene (Asa *et al.* 1998, Prezant *et al.* 1998, Farrell *et al.* 1999). While

Table 5 Summary of IHC expression of p16 protein in tumours with and without evidence of methylation of the *p16/CDKN2A* gene

	Methylated	Unmethylated
Non-functional adenomas		
p16 not expressed	25/32 (78%) ^a	2/14 (14%)
p16 expressed	7/32 (22%)	12/14 (86%)
Somatotrophinomas		
p16 not expressed	1/2 ^b	3/19 (16%)
p16 expressed	1/2	16/19 (84%)

Loss of p16 protein in non-functional tumours was significantly (^a $P=0.00007$) associated with methylation of its associated CpG island. In somatotrophinomas the majority of tumours were not methylated and expressed p16 protein. No mutations were found in the coding region of the *p16/CDKN2A* gene in those tumours that were unmethylated and failed to express p16 protein (^b $P=0.3$).

Asa *et al.* (1998) identified reduced expression to be a rare event, the other two studies failed to identify any tumours showing reduced expression (Prezant *et al.* 1998, Farrell *et al.* 1999). To date, no CpG island has been described that is associated with the *menin* gene. Thus, it appears unlikely, in the light of the expression of data from these three studies, that methylation plays a significant role in the regulation of the *menin* gene in pituitary tumorigenesis. Equally, studies of the *RB1* gene on chromosome 13q have failed to detect abnormalities in either the sequence or protein expression (reviewed in Farrell & Clayton 1998). This has led Melmed's group (Pei *et al.* 1995) and our own group (Bates *et al.* 1997) to suggest the presence of another or other TSGs on this chromosome in those tumours showing losses on chromosome 13q. However, very recent studies from our laboratory describing the fine mapping of chromosome 13q in non-functional tumours and somatotrophinomas show loss of pRB protein associated with somatotrophinomas (Simpson *et al.* 1999b). Current studies in our laboratory are directed towards identification of the mechanism of loss, since it is not associated with LOH of an intragenic marker to the *RB1* gene. Other studies, principally at the level of protein or transcript expression for the TSG products p53, nm23 and p27 have described changes associated with increasingly invasive behaviour. However, where studied no genetic abnormalities in these TSGs have been described (reviewed in Pei & Melmed 1996, Shimon & Melmed 1997, Farrell & Clayton 1998).

The apparent paradox of LOH at particular genetic loci without apparent mutation in known TSGs and reduced expression of some TSGs without apparent genetic abnormalities has not been resolved. Perhaps abnormal methylation patterns may represent a unifying mechanism. Abnormal methylation of normally unmethylated CpG islands may represent a mechanism in either the

reduction or silencing of gene expression. Although at present somewhat speculative, perhaps the silencing of *p16/CDKN2A* through abnormal methylation may prove to be the first reported example of a general mechanism for gene silencing or reduced expression in pituitary tumorigenesis. Equally, there is clear evidence in the literature, for example in fragile X syndrome (Oberle *et al.* 1991, Verkerk *et al.* 1991), and tumours of neural (Makos *et al.* 1993a), renal (Makos *et al.* 1993b) and colonic origin (Makos *et al.* 1992), where abnormal methylation may well precede and predispose towards genetic instability, leading to loss of genetic material. In this case, perhaps, losses already described in pituitary tumours may or may not be sites of unidentified TSGs loci. Indeed, if this were the case, methylation would initiate the loss; however, it would not necessarily encompass either a putative or known TSG. No doubt future studies will resolve this apparent paradox in both the pituitary and other tumour types.

Model pituitary systems

Numerous studies have documented the role of *p16/CDKN2A* as a TSG involved in G₁/S cell cycle arrest, as assessed by ectopic expression of p16 protein (Okamoto *et al.* 1994, Arap *et al.* 1995, Spillare *et al.* 1996). Equally, in both primary tumours and cell lines shown to harbour *p16/CDKN2A* methylation of its CpG island, pharmacological intervention with demethylating agents is associated with re-expression of p16 protein and growth arrest (Hsieh 1994, Gonzalgo *et al.* 1998). However, there are examples of normal tissue including colon (Gonzalgo *et al.* 1998), breast (van Zee *et al.* 1998) and brain (Costello *et al.* 1996) that do not express *p16/CDKN2A*, suggesting that alternative mechanisms are responsible for mediating cell cycle arrest. In addition, in experimental

models there are cell lines that are refractory to ectopically expressed p16/CDKN2A (Okamoto *et al.* 1994), suggesting the involvement of proteins other than p16 in cell cycle arrest. The *p16/CDKN2A* gene and protein product are expressed in normal pituitary (Woloschak *et al.* 1997, Simpson *et al.* 1999a). However, it is not clear for tumours that fail to express this protein if re-expression would induce either growth inhibition and/or cell cycle arrest. The question is further complicated by other unidentified mechanisms involved in pituitary tumorigenesis that may contribute an overriding influence on uncontrolled cell proliferation such as dominantly acting oncogenes. As there are no available human pituitary tumour cell lines and primary pituitary tumours are notoriously difficult to propagate *in vitro*, we used the mouse corticotroph cell line, AtT20, in an attempt to address some of these questions (SJ Frost *et al.* 1999) and this work will be reviewed briefly.

Genomic sequence analysis revealed that the *p16/CDKN2A* gene is homozygously deleted in the AtT20 cell line. Frost generated stable transfectants in which p16/CDKN2A was regulated by an inducible promoter. Induction of p16 protein resulted in a significant reduction in colony-forming efficiency (~85%) and a profound G₁ (~82%) arrest. Methylation of the *p16/CDKN2A* gene in human pituitary tumours is found within the coding region of its CpG island and most likely reflects, by association, methylation of more upstream regions of the gene. However, the model described allowed us to examine the effect of methylation within the coding region and was not thought to block transcription (Jones 1999). *In vitro* methylation of all available CpG sites in the coding region of the *p16/CDKN2A* gene was first carried out, prior to religation into the expression vector (SJ Frost, DJ Simpson, RN Clayton & WE Farrell, unpublished data). Transient transfection studies showed a restoration of colony-forming efficiency. Interestingly, using various methylase enzymes it was shown that methylation of as few as four of fifty-one available sites within this region was sufficient to restore colony-forming efficiency in AtT20 cells. These findings are somewhat at variance with the presumed role of CpG island methylation downstream of an initiation start site (review by Jones 1999). Ongoing studies are directed towards identifying if these findings represent a gene (*p16/CDKN2A*) or a pituitary-specific phenomenon.

Although aware of the danger of extrapolating from a mouse model to human tumours these results suggest that pharmacological restoration of p16 protein expression in human tumours may be sufficient to restore growth control.

Clinical perspective

Gene silencing through methylation represents a gene that is neither lost nor mutated but is present in a suppressed form. For growth regulatory genes and in particular the TSG this epigenetic mechanism of gene silencing represents a potentially attractive therapeutic target. In addition, very recent studies have shown the potential of this change in the diagnosis of tumours using peripheral blood samples. Since aberrant methylation can be detected in serum of patients with non-small cell lung cancer (Esteller *et al.* 1999) and in either serum or plasma of liver cancer patients (Wong *et al.* 1999) this type of analysis offers considerable potential in both the diagnosis and detection of recurrent and/or residual disease. Several studies have documented the effects of demethylating agents such as 5-aza-cytidine and 5-aza 2'-deoxycytidine (reviewed in Szyf 1996, Zingg & Jones 1997) and they have already been subject to clinical study (Mompalmer & De Vos 1990). However, the drugs are inherently cytostatic and there are questions relating to the carcinogenicity and mutagenicity of the azacytosine nucleosides (reviewed in Szyf 1996, Zingg & Jones 1996). Very recent studies in which significantly lower doses of 5-aza-2'-deoxycytidine were combined with an inhibitor of the enzyme histone deacetylase (trichostatin A) resulted in robust re-expression of methylated genes in a tumour cell line (Cameron *et al.* 1999). These studies directed towards pharmacological intervention and new insights into the mechanisms responsible for increased DNA methyltransferase enzyme activity offer considerable potential for the treatment of multiple tumour types including those arising in the pituitary.

Concluding remarks

Over the past several years, there has been an explosion of interest and in our knowledge regarding the role and mechanism of abnormal methylation patterns in tumour biology. Although many questions remain unanswered, perhaps the most pertinent relate to the mechanisms and signals that determine methylation patterns in normal cells and how they are deregulated in tumours. In this context, the role of the DNA methyltransferase enzymes responsible for both maintenance and *de novo* methylation will most likely be found to play pivotal roles in this process. In marked contrast to classical genetic changes associated with oncogenes and TSGs, methylation represents an essentially functional gene that is amenable to pharmacological intervention designed to induce re-expression. Identification and characterisation of treatment modalities with new generation drugs that act alone or in combination offer an exciting novel future direction. This review has focused as a principal theme on methylation of

the *CDKN2A/p16* gene in pituitary tumorigenesis. However, it is clear from other tumour types that many TSGs and indeed genes involved in both terminal differentiation and metastatic behaviour are silenced through this epigenetic change. No doubt the future will see new regulatory genes identified with abnormal methylation patterns in both the pituitary and in other tumour types, and new ways of reversing these changes.

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