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Promoter CpG methylation of multiple genes in pituitary adenomas: Frequent involvement of *Caspase-8*

M. JOSEFA BELLO[1], JOSE M. DE CAMPOS[2], ALBERTO ISLA[3], CACILDA CASARTELLI[4] and JUAN A. REY[1]

[1]Unidad de Investigación, Laboratorio de Oncogenética Molecular y Epigenética del Cáncer, Hospital Universitario La Paz, Paseo Castellana 261, 28046 Madrid; [2]Departmento de Neurocirugía, Hospital del Rio Hortega, 47010 Valladolid; [3]Departmento de Neurocirugía, Hospital Universitario La Paz, 28046 Madrid, Spain; [4]Laboratorio de Oncogenetica, Departamento de Genetica, Facultade de Medicina de Ribeirão Preto, Universidade de São Paulo (FMR-USP), Ribeirão Preto-São Paulo, Brazil

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Abstract. The epigenetic changes in pituitary adenomas were identified by evaluating the methylation status of nine genes (*RB1*, *p14^{ARF}*, *p16^{INK4a}*, *p73*, *TIMP-3*, *MGMT*, *DAPK*, *THBS1* and *caspase-8*) in a series of 35 tumours using methylation-specific PCR analysis plus sequencing. The series included non-functional adenomas (n=23), prolactinomas (n=6), prolactinoma plus thyroid-stimulating hormone adenoma (n=1), growth hormone adenomas (n=4), and adrenocorticotropic adenoma (n=1). All of the tumours had methylation of at least one of these genes and 40% of samples (14 of 35) displayed concurrent methylation of at least three genes. The frequencies of aberrant methylation were: 20% for *RB1*, 17% for *p14^{ARF}*, 34% for *p16^{INK4a}*, 29% for *p73*, 11% for *TIMP-3*, 23% for *MGMT*, 6% for *DAPK*, 43% for *THBS1* and 54% for *caspase-8*. No aberrant methylation was observed in two non-malignant pituitary samples from healthy controls. Although some differences in the frequency of gene methylation between functional and non-functional adenomas were detected, these differences did not reach statistical significance. Our results suggest that promoter methylation is a frequent event in pituitary adenoma tumourigenesis, a process in which inactivation of apoptosis-related genes (*DAPK, caspase-8*) might play a key role.

Introduction

Pituitary tumours are mostly benign adenomas that grow slowly and frequently secrete hormone gene products, which can lead to disturbed endocrine functions. Aggressively growing adenomas, showing signs of local invasion, are, however, relatively common (1,2). These neoplasms represent approximately 10% of brain tumours, and are the most common adult intracranial neoplasm.

There is some information on the genetic abnormalities leading to pituitary tumour formation. Cytogenetic and CGH studies have identified consistent gains of chromosomes X, 19, 12, 9, 7 and 3, and losses of chromosomes 10, 11, 13, 14, 19 and 22, thus suggesting that tumour-related genes located in these genomic regions may play a role in pituitary tumorigenesis (3-7). Point mutations in the GS protein gene (leading to constitutive activation of the cAMP pathway) (8), or *MEN1* gene (9), as well as high pituitary tumour transforming (PTT) gene expression (10), have been associated with pituitary tumorigenesis and invasiveness. Early studies on the cell-cycle regulators, *RB1*/*p16^{INK4a}*, demonstrated non-random abnormalities of these genes parallel to the loss of their corresponding protein products. Simpson *et al* (11) demonstrated the absence of pRB1 in 27% of somatotrophinomas and 4% of non-functional tumours, but this finding was not associated with mutations at the *RB1* locus. Similarly, early studies of *p16^{INK4a}* in pituitary tumours showed loss of the protein without evident mutations (detected by SSCP) and only a small proportion of adenomas (~12%) displayed homozygous deletions of this gene (12,13). These data thus demonstrated that the loss of function by these cell-cycle regulator genes is a non-random event in pituitary tumorigenesis, and suggested that gene-silencing mechanisms other than mutation or deletion might be responsible for their inactivation.

Methylation of gene regulatory elements is an epigenetic change that is an alternative mechanism to genetic alteration for gene inactivation. CpG islands are 0.5-2.0 Kb regions that are rich in cytosine-guanine dinucleotides and are present in the 5' region of approximately half of all human genes. Methylation of the CpG island located within a promoter element is generally associated with delayed replication and inhibition of transcription initiation (14), thus contributing to the pathogenesis and progression of malignant neoplasms. Examples of this process have been reported in a wide range of tumour types and cancer-related genes (15) and some

Correspondence to: Dr Juan A. Rey, Unidad de Investigacion, Lab. Oncogenetica Molecular y Epigenetica del Cancer, Hospital Universitario La Paz, Paseo Castellana 261, 28046 Madrid, Spain
E-mail: jarey.hulp@salud.madrid.org

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information is available on pituitary tumours (reviewed in ref. 16). A study by Simpson *et al* (17) detected aberrant *RB1* promoter methylation in six of ten cases that failed to express pRB1, and three additional tumours presented deletions within the protein-binding pocket domain. In contrast, most adenomas and all normal pituitary tissues that expressed pRB1 displayed unmethylated *RB1* promoter (17). In addition to the well-documented gene deletion, aberrant promoter methylation has also been described as an alternative mechanism to silencing of the $p16^{INK4a}$ gene (18-21). Some differences with respect to the frequency of $p16^{INK4a}$ hypermethylation in distinct pituitary tumour subtypes were found; the common data of these reports indicate that non-functional tumours are sites of epigenetic change, and that methylation of this gene is infrequent in somatotrophinomas (18-21).

Additional reports recently demonstrated that the loss of *DAPK* (death associated protein kinase) expression, frequently parallel to aberrant hypermethylation or homozygous deletion, was associated with invasive pituitary tumours (22), whereas high methylation frequencies of the *RASSF1A* promoter were identified in the most aggressive adenomas (23).

All of these findings thus suggest that inappropriate methylation is a frequent event in pituitary tumorigenesis and may affect multiple genes in this neoplasm. In the present study, we determined the frequency of methylation of nine genes in a series of 35 pituitary adenomas, and controls consisting of two normal pituitary tissue samples, using polymerase chain reaction (PCR)-based techniques involving sodium bisulphite modification of DNA (MSP) plus sequencing.

Materials and methods

Tissue specimens and DNA extraction. Thirty-five sporadic human pituitary adenomas with matched blood samples were obtained from patients who had undergone hypophysectomy. The age range at diagnosis was from 20 to74 years (mean age 45.68 years). Eighteen patients were male and seventeen were female. Clinical and immunohistochemical studies allowed tumour classification as follows: non-functional (NF) adenomas, 23 cases; functional (F) including prolactinomas, six cases; prolactinoma plus thyroid-stimulating hormone adenoma, one case; growth hormone adenomas, four cases; and adrenocorticotropic adenoma, one case. In addition to tumours, two histologically normal (postmortem) pituitary samples were also studied. Genomic DNA was extracted from frozen samples as described previously (24).

MSP and direct sequencing. We analysed the status of nine genes frequently showing promoter methylation in other neoplasms. The genes studied were *RB1*, $p14^{ARF}$, $p16^{INK4a}$, *p73*, *TIMP-3* (the tissue inhibition of metalloproteinase 3 gene) *MGMT* (O^6-methylguanine DNA-methyl transferase), *DAPK*, *THBS1* (thrombospondin-1) and *caspase-8* (cyteine-aspartic acid protease 8). These genes were chosen for study on the basis of their critical cancer-related function, since they are frequently hypermethylated and silenced in other neoplasms, as well as being located in genomic regions known to be involved in chromosome deletions in pituitary adenomas. Bisulphite modification of genomic DNA was performed as reported (25). Briefly, 2 μg of genomic DNA was denatured with 2 M of NaOH and modified with sodium bisulphite treatment. After purification with the DNA clean-up kit (Promega, Madison, WI), the DNA was treated with NaOH, precipitated with ethanol, and resuspended in water. PCR was conducted with primers specific for either the methylated or the unmethylated alleles in standard conditions with variable annealing temperatures (55-66˚C), using the Biotools DNA polymerase kit (Madrid, Spain). We used the primer sets described previously (26). Water was substituted for DNA as a negative control, and as a positive control for the methylated alleles we treated DNA (from healthy volunteers' lymphocytes) with Sss1 methyl-transferase (New England Biolabs, Beverly, MA, USA) and then subjected it to bisulphite treatment. PCR products were loaded onto 6% polyacrylamide gels or 2-3% agarose gels, stained with ethidium bromide and visualised under UV. Samples giving signals approximately equivalent to the positive control were designated as methylated. To verify the identity of PCR products, they were purified and sequenced using the ABI PRISM Big-Dye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA) on AB model 3100 or 377 DNA sequencers. A given tumour, identified by MSP, was confirmed as methylated whenever at least 51% of the studied CpG sites were methylated.

Results

The results of the MSP analysis for the nine loci are shown in Table I. Nearly all of the tumours showing methylation by MSP, showed complete methylation at almost every individual CpG site analysed in the corresponding gene promoter. Partially methylated promoters (with <50% methylated GpG sites) constituted <10% of the methylated genes identified by MSP, and were considered as non-methylated for the study. All 35 samples showed methylation in at least one of these loci, and 14 adenomas (40%) displayed concurrent methylation of three or more loci. Methylation of only one gene was observed in 11 adenomas corresponding to eight NF and three functional (F) tumours. Overall methylation values of ≥15% were obtained for seven genes: *RB1* (20%), $p14^{ARF}$ (17%), $p16^{INK4a}$ (34%), *p73* (29%), *MGMT* (23%), *THBS1* (43%) and *caspase 8* (54%). The remaining two genes (*TIMP-3* and *DAPK*) presented methylation frequencies of <15%. Both samples of normal pituitary tissue displayed the unmethylated alleles of all nine genes analysed; representative examples of the MSP analysis are shown in Fig. 1.

We used the methylation index (MI) defined as the number of methylated loci/the number of tested loci, to determine the frequency of methylation of multiple genes in a given sample or tumour group. The MI ranged from 0.11 to 0.55, with an overall mean of 0.260. As shown in Table II, the overall MI value was slightly higher for NF adenomas (0.267) than F tumours (0.247), but these differences did not reach statistical significance. Similar MI values were also detected between the distinct F adenoma groups (except the ACTH group that included a single case): PRL, 0.267; GH adenomas, 0.24. The frequency of aberrant gene methylation in NF versus F tumours was similar for *MGMT*, *DAPK*, *THBS1* and *caspase 8*. In contrast, *RB1*, $p16^{INK4a}$ and TIMP-3 were more frequently methylated in the NF group of adenomas; $p14^{ARF}$ and *p73* displayed a higher frequency of methylation in the F tumours.

SPANDIDOS PUBLICATIONS ummary of clinicopathological data and methylation of all nine genes in pituitary adenomas.

Sample	Sex/Age	Imm-Chem	*RB1*	*p14^{ARF}*	*p16I^{NK4a}*	*TP73*	*TIMP3*	*MGMT*	*DAPK*	*THBS1*	*Caspase 8*	MI
PA-1	F/67	NF	+	-	-	+	-	-	-	+	+	0.44
PA-2	M/45	NF	-	-	-	+	-	-	-	+	-	0.22
PA-3	F/30	NF	-	-	-	-	-	-	-	+	-	0.11
PA-4	M/54	NF	-	-	-	-	-	-	-	+	-	0.11
PA-6	M/74	NF	+	-	-	-	-	-	-	-	-	0.11
PA-7	M/72	NF	-	-	-	-	-	-	-	-	+	0.11
PA-8	M/55	NF	-	-	-	-	-	-	-	-	+	0.11
PA-9	M/46	NF	-	-	-	-	-	+	-	-	+	0.22
PA-11	F/45	NF	-	+	+	-	-	+	-	+	+	0.55
PA-12	M/39	NF	+	+	-	+	-	-	-	-	+	0.44
PA-17	M/74	NF	-	-	+	-	+	+	-	+	+	0.55
PA-18	M/56	NF	+	-	-	+	-	+	-	-	-	0.33
PA-19	M/ 57	NF	-	+	+	-	-	+	-	+	+	0.55
PA-21	M/45	NF	-	-	-	+	+	-	-	-	+	0.33
PA-22	F/56	NF	-	-	+	-	-	-	-	+	+	0.33
PA-23	F/62	NF	-	-	-	-	-	-	-	-	+	0.11
PA-25	F/32	NF	-	-	+	-	-	-	-	-	-	0.11
PA-26	M/22	NF	-	-	+	-	-	-	-	-	-	0.11
PA-29	F/46	NF	-	-	+	+	+	-	-	-	-	0.33
PA-30	F/45	NF	+	-	+	-	-	-	-	-	+	0.33
PA-32	F/48	NF	+	-	-	-	-	-	-	+	-	0.22
PA-33	F/44	NF	-	-	+	-	-	-	-	-	+	0.22
PA-35	F/46	NF	-	-	+	-	-	-	+	-	-	0.22
Total non-functional adenomas			6/23	3/23	10/23	6/23	3/23	5/23	1/23	9/23	13/23	0.24
		%	26	13	43	26	13	22	4	39	57	
PA-5	M/66	PRL	-	-	-	-	-	-	-	+	-	0.11
PA-13	F/20	PRL	-	+	-	-	-	-	-	+	-	0.22
PA-14	F/21	PRL	-	+	-	+	-	-	-	+	+	0.44
PA-15	M/37	PRL	-	-	+	+	-	+	-	-	+	0.44
PA-24	F/21	PRL	+	-	-	+	-	-	-	-	-	0.22
PA-34	F/24	PRL	-	-	+	-	-	-	-	-	+	0.22
PA-20	F/25	PRL + TSH	-	-	-	-	+	-	-	-	+	0.22
		Total PRL	1/7	2/7	2/7	3/7	1/7	1/7	0/7	3/7	4/7	0.26
		%	14	29	29	43	14	14	0	43	57	
PA-10	M/44	GH	-	-	-	+	-	-	-	+	+	0.33
PA-27	F/44	GH	-	-	-	-	-	-	+	-	-	0.11
PA-28	M/45	GH	-	-	-	-	-	+	-	-	+	0.22
PA-31	M/46	GH	-	+	-	-	-	+	-	+	-	0.33
		Total GH	0/4	1/4	0/4	1/4	0/4	2/4	1/4	2/4	2/4	0.24
		%	0	25	0	25	0	50	25	50	50	
PA-16	M/46	ACTH	-	-	-	-	-	-	-	+	-	0.11
Total functional adenomas			1/12	3/12	2/12	4/12	1/12	3/12	1/12	5/12	6/12	0.26
		%	8	25	17	33	8	25	8	42	50	
Total series			7/35	6/35	12/35	10/35	4/35	8/35	2/35	15/35	19/35	0.26
		%	20	17	34	29	11	23	6	43	54	

M, male; F, female; Age, years; NF, non-functional adenomas; PRL, prolactinoma; TSH, thyroid-stimulating hormone adenoma; GH, growth hormone adenoma; ACTH, adrenocorticotropic adenoma.

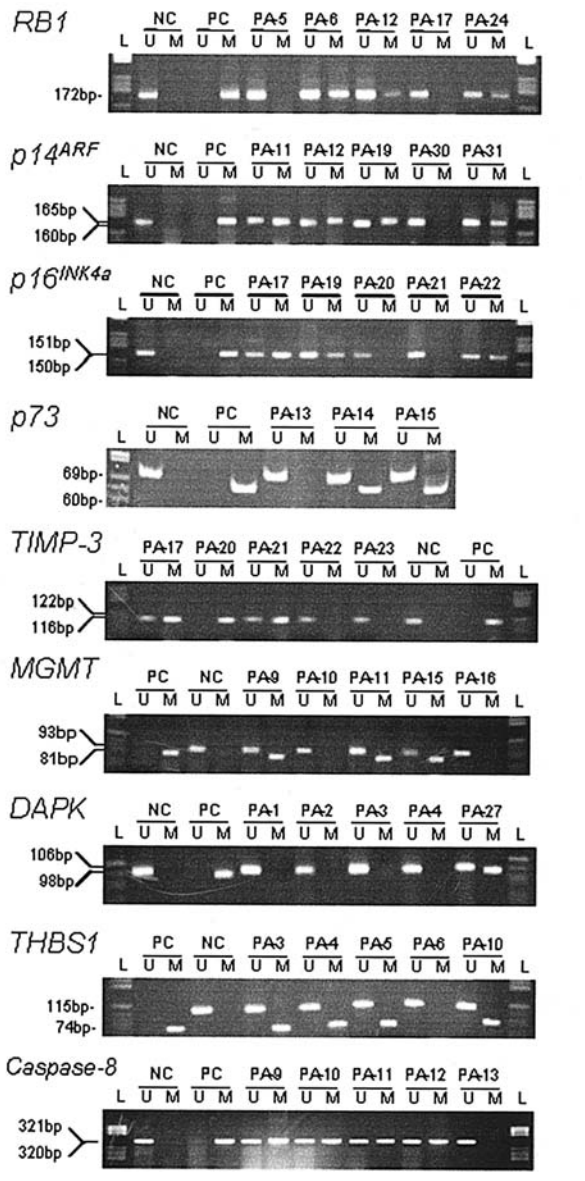

Figure 1. Methylation analysis of pituitary adenomas by MSP. The gene studied is given at the left. Lane U, amplified product with primers recognising unmethylated sequence; Lane M, amplified product with primers recognising methylated sequence. PC, positive control for methylation. NC, negative control for methylation. The PCR product sizes of all genes are shown to the left. L, ladder.

However, these differences did not reach statistical significance (χ^2 and Fisher's exact tests).

There was no significant association between overall MI and sex/age distribution, although the tumours arising in male patients, or in patients under 46 years of age presented higher MI values.

Discussion

This study presents the first analysis of the methylation profile of nine tumour-related genes in pituitary adenomas. The results demonstrate that this epigenetic change is a relatively frequent event in this neoplasm since at least one gene was methylated in all of the analysed samples. Although hypermethylation varied according to the gene, there were significant MI values per tumour, ranging from 0.11 to 0.55 (mean 0.26). We also identified non-random frequencies of aberrant promoter methylation in most analysed genes, but only seven of them (*RB1*, *p14^{ARF}*, *p16^{INK4a}*, *p73*, MGMT, *THBS1* and *caspase 8*) were methylated in at least 15% of the analysed samples.

Table II. Summary of the overall MI findings related to immunohistochemical and clinicopathological data.

Characteristics	No. of cases examined	MI value
Total tumours	35	0.260±0.141
Tumours arising in female patients	17	0.258±0.128
Tumours arising in male patients	18	0.267±0.156
Tumours arising in patients <46 years	18	0.268±0.131
Tumours arising in patients >46 years	17	0.252±0.154
Non-functional tumours	23	0.267±0.154
Functional tumours	12	0.247±0.116
Prolactinomas	7	0.267±0.124
GH adenomas	4	0.24±0.09
ACTH adenomas	1	0.11

In 21 adenomas for which enough DNA was available, we performed mutational analysis of *p16^{INK4a}* (exons 1, 2 and 3) and *RB1* (exons 20-24 and the essential promoter region) (unpublished data). No mutations or deletions were observed in *RB1*. A polymorphic variant at the 3'UTR of *p16^{INK4a}* was identified in three tumours (PA-5, PA-7 and PA-21) and a homozygous deletion of this gene (as determined by multiplex PCR) could be identified in two cases (PA-16 and PA-18). Accordingly, these data confirm that mutation or homozygous deletions of *RB1* pathway genes would be a rare event in pituitary adenomas (12,13,17,20,21,27). In agreement with previous studies suggesting that *RB1* pathway gene expression may be altered by epigenetic changes in most pituitary adenomas (16-21,27), we identified aberrant promoter methylation of either *RB1* or *p16^{INK4a}* in 51% of the cases in this series, primarily the non-functional adenomas and prolactinomas. However, no GH adenoma in our series presented these epigenetic changes. Previous studies, primarily focusing on *p16^{INK4a}* aberrant methylation, showed controversial findings in these GH adenomas since the reported frequency of hypermethylated alleles varied from 8-9.5% (18,20) to 42.9% or 81.8% (21,29).

In addition to *p16^{INK4a}*, the same locus at 9p21 encodes *p14^{ARF}*, as a result of alternative spliced RNAs. The p14 protein binds to mdm2 and stabilises both TP53 and mdm2 (30) thus forming a gene pathway that participates in regulation of gene transcription, induction of G1/S arrest and apoptosis promotion. Mutations of *TP53* may be present in a subset of pituitary adenomas and a role for these genetic alterations in the acquisition of malignant phenotypes has been proposed (reviewed in ref. 31). The epigenetic inactivation of *p14^{ARF}* due to aberrant promoter methylation that we detected in

amples in our series may represent an alternative m for inactivating *TP53/mdm2/p14^{ARF}* gene function in pituitary adenoma development.

Our results also showed that *p73*, *TIMP-3*, *MGMT* and *THBS1* were methylated at significant rates in both functional and non-functional adenomas. Expression silencing by aberrant promoter methylation of these genes has been well-documented in a variety of neoplasms (15). These genes play key roles (angiogenesis or invasion inhibition, DNA repair) in tumour development; accordingly, their epigenetic alteration may represent a mechanism that could disrupt key cellular functions and contribute to pituitary adenoma development. These biological implications, however, need to be studied further in larger tumour series.

Apoptosis-related genes, such as *DAPK* and *PTAG* (pituitary tumour associated gene), have recently been identified as participating in the formation of these tumour types (22,32). The loss of *DAPK* expression in these studies was significantly associated with invasive pituitary tumours (59% of cases versus 7% in non-invasive cases). Aberrant promoter methylation and, less frequently, homozygous deletion have been identified as the main mechanisms responsible for *DAPK* silencing (22). Two of the adenomas that we studied (6%) present aberrant promoter methylation of this gene; this low frequency may be due to the fact that all of the cases in our series were non-invasive. However, it should not be forgotten that Simpson *et al* (22) suggested that these 'non-invasive' adenomas with *DAPK* silencing may be predisposed toward an invasive or metastatic phenotype. We detected high rates of aberrant methylation (54%) affecting another apoptosis-related gene: *caspase-8*. Binding of TNF-related apoptosis-inducing ligands (TRAIL) to death receptor 4 and 5 allows caspase-8 to bind to the receptor, leading to the proteolytic activation that initiates a cascade of caspases causing apoptosis (33). Silencing of *caspase-8* due to aberrant CpG island methylation has previously been documented in medulloblastomas and neuroblastomas (26,34-36). Those tumours that do not express *caspase-8* may avoid apoptosis and acquire a more aggressive and therapy-resistant tumour phenotype (35). Demethylation with 5-aza-2' deoxycytidine has been demonstrated to restore *caspase-8* mRNA expression and, thus, TRAIL sensitivity in medulloblastoma-derived cell lines, suggesting that *caspase-8* could be a target for chemotherapy with inhibitors of DNA methylation (35). Therefore, in addition to the previously well-established epigenetic inactivation of cell-cycle control genes, apoptosis-related genes are frequently silenced through aberrant promoter methylation in pituitary tumours and may represent targets for new therapeutic options on the basis of their epigenetic status.

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