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Aberrant gene promoter methylation in plasma cell dyscrasias

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ABSTRACT

The aberrant methylation of promoter CpG island is known to be a major inactivation mechanism of tumour-related genes. To determine the clinicopathological significance of gene promoter methylation in monoclonal gammopathies, we analysed the methylation status of 6 tumour suppressor genes and their association with loss of gene function. Methylation status of the genes p14, p15, p16, hMLH1, MGMT, and DAPK was determined by methylation-specific PCR in 52 cases: 30 MM, 13 MGUS, and 9 plasmacytomas, comparing them with their protein expression by immunohistochemistry, and association between methylation status, protein expression, and clinical characteristics was assessed. The methylation frequencies were 50% for p16, 17% for p15, 10% for hMLH1, 23% for MGMT and 30% for DAPK in MM samples, and 38%, 15%, 8%, and 15% for p16, p15, MGMT and DAPK respectively in MGUS samples. In plasmacytomas samples we found methylation of p16 in 55%, p15 in 22%, MGMT in 67% and DAPK in 44%. hMLH1 was unmethylated in all cases of MGUS and plasmacytomas. Immunohistochemistry showed that gene methylation was closely associated with a loss of protein expression. Our study demonstrates that methylation-mediated silencing is a frequent event in monoclonal gammopathies: 83% of MM, 46% of MGUS and 77% of plasmacytomas have at least one gene methylated, affecting different molecular pathways involved in cell cycle, DNA repair and apoptosis. This high prevalence of aberrant promoter hypermethylation suggests that monoclonal gammopathies carry a CpG island methylator phenotype, therefore the development of new DNA demethylation agents may be a potential therapeutic use in this disease.

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Introduction

Plasma cell dyscrasias are characterised by monoclonal proliferations of plasma cells composed of a number of clinicopathologic entities, including monoclonal gammopathy of undetermined significance (MGUS), solitary plasmacytoma, multiple myeloma (MM), and plasma cell leukaemia (PCL).

MM is a prototypical clonal B-cell malignancy with a terminally differentiated plasma cell phenotype. MM ranks as the second most frequently occurring haematological malignancy after non-Hodgkin lymphoma, and is characterised by multi-organ dysfunction as a result of bone marrow infiltration by malignant cells, and the systemic damage of monoclonal circulating protein (Kyle and Rajkumar, 2004). MGUS is a stable pre-malignant plasma cell tumour that can progress to malignant MM or a related malignant condition at a rate of 1% per year (Kyle and Rajkumar, 2006). Molecular genetic events associated with this transformation have not been identified.

Although molecular studies have largely focused on genetic alterations in MM, during the last few years there has been growing evidence that, in addition to genetic aberrations, epigenetic processes play a major role in carcinogenesis (Jones and Baylin, 2002). DNA methylation, catalysed by DNA methyltransferase, involves the addition of a methyl group to the carbon 5 position of the cytosine ring in the CpG nucleotide, leading to a conversion to methylcytosine. In many cancers, the CpG islands of selected genes are aberrantly methylated (hypermethylated), resulting in transcriptional repression of these genes. Tumour-specific methylation changes in different genes have been identified, and the methylation profile helps us to distinguish tumour types and, perhaps, their response to chemotherapeutic agents (Alizadeh et al., 2000; Choy et al., 2002; Murata et al., 2005; Takahashi et al., 2004: Yu et al., 2004). Hypermethylation affects different cellular pathways such as cell cycle regulation, DNA repair, and apoptosis, and has relevant consequences in human cancer (Das and Singal, 2004).

The *INK4A-ARF* locus, situated at 9p21, contains two tumour suppressor genes, $p14^{ARF}$ and $p16^{INK4A}$. $p14^{ARF}$ protein interacts physically with MDM2 and stabilises the p53 protein in the nucleus by blocking its cytoplasmic transport and MDM2-mediated degradation, indicating that it acts as an upstream regulation of p53 function, hence *p14* inactivation is an alternate mechanism of disrupting the p53 pathway (Quelle et al., 1995). *p14* is inactivated in various cancers through homozygous deletion, promoter methylation or mutations, however the latter is rare (Zheng et al., 2000). $p15^{INK4b}$ and $p16^{INK4a}$ proteins are cell cycle regulators involved in the inhibition of G1 phase progression. Both proteins associate with cyclin-dependent kinases 4 and 6 (CDK4, CDK6), and inhibit their kinase activities (Le and Yang, 2003). Homozygous deletion, point mutation, or

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Table 1
Clinical and laboratory characteristics of 52 patients with monoclonal gammopathies

Characteristics	No. of patients
Median patient age (range), years	68 (39–97)
Gender (*n*=52)	
Male	24
Female	28
Paraprotein isotype	
IgA	16
IgG	20
IgM	1
Light chain only	5
Non-secretory	1
Plasmacytomas (*n*=9)	
Extramedullary	5
Solitary bone	4
Stage	
MGUS	13
I	5
II	5
III	19
Plasma cell leukaemia (*n*)	1

methylation can inactivate the $p16^{INK4A}$ and $p15^{INK4B}$ genes. Hypermethylation of the *p16* promoter has been detected across many tumour types such as colorectal cancer, lung, and breast carcinomas and lymphomas, whereas hypermethylation of *p15* is only observed in haematological malignancies (Esteller et al., 2001).

DNA repair systems act to maintain genome integrity in the presence of replication errors, environmental insults, and the cumulative effects of ageing (Fink et al., 1998). hMLH1 and MGMT are two enzymes implicated in DNA repair, and we have recently shown that inactivation of the repair pathway plays an important role in genetic instability in plasma cell dyscrasias (Martin et al., 2006). *hMLH1* is an MMR gene responsible for correcting insertion/deletion loops and single base–base mismatched pairs that arise during normal DNA replication, especially in the repeated sequence motifs, such as microsatellites (Velangi et al., 2004; Jiricny and Nystrom-Lahti, 2000).

MGMT (O^6-methylguanine DNA methyl-transferase) encodes a DNA repair protein that removes mutagenics and cytotoxic adducts at O^6 of guanine and, therefore, plays an important role in maintaining normal cell physiology and genomic stability (Gerson, 2004). *MGMT* is closely related with cellular sensitivity to alkylating agents, and is inactivated by promoter hypermethylation in several human cancers. The presence of *MGMT* methylation is associated with better overall survival in diffuse large B-cell lymphoma (Esteller et al., 2002), and is involved in response to treatment in glioblastomas (Hau et al., 2007; Paz et al., 2004; Hiraga et al., 2006).

Death-associated protein kinase (DAPK) is a pro-apoptotic serine/threonine kinase involved in the extrinsic pathway of apoptosis, initiated by γ-interferon FAS ligand and tumour necrosis factor-α (Ng, 2002; Cohen et al., 1999). Downregulation of DAPK transcription by CpG methylation has been demonstrated in a variety of tumours, especially in B-cell malignancies (Katzenellenbogen et al., 1999), providing a selective growth advantage during tumour progression (Tang et al., 2000).

To explore the role of DNA methylation in plasma cell disorders we selected six tumour-related genes frequently silenced by aberrant methylation, and examined the methylation status for the following genes by methylation-specific PCR (MSPCR): *p16*, *p14*, *p15*, *DAPK*, *hMLH1* and *MGMT*. We also analysed protein expression of p15, p16, hMLH1 and MGMT using immunohistochemistry (IHC).

Materials and methods

Human tissue samples

A total of 54 cases was included in the study: 29 MM, 13 MGUS, 1 PCL, 2 polyclonal plasmacytosis and 9 plasmacytomas. Bone marrow aspirates (*n*=45) and bone marrow biopsies (*n*=29) from the posterior iliac crest were collected from 45 consenting patients during routine clinical assessment. Patients were classified according to the current WHO classification. Analyses of age, gender, haemoglobin levels, presence of lytic bone lesions, creatinine, serum calcium levels, LDH, β2 microglobulin, paraprotein subtype, percentage and morphology of plasma cells and stage of disease were performed for all patients.

Mononuclear cell suspensions (MCS) were prepared from bone marrow aspirates by Ficoll-Paque plus gradient centrifugation (Amersham Pharmacia AB, Uppsala, Sweeden), and plasma cell isolation from MCS was performed by immunomagnetic bead selection with CD138 using the Automacs system (Miltenyi, CA). Enriched fractions were assessed for purity by CD138-fluoresceina isotiocyanate (FITC) monoclonal antibody labelling (BB4, Cytognos). CD138, also known as syndecan-1, is expressed on plasma cells, but not on circulating B cells, T cells, or monocytes (Wijdenes et al., 1996). The purity of plasma cells obtained by this method was more than 90% as confirmed by flow cytometry (FAC sort: Becton Dickinson, San José) using the Paint a Gate program. Plasma cell leukaemia and plasmacytomas were not purified.

Frozen and paraffin embedded plasmacytomas were obtained from the Spanish National Tumour Bank Network, coordinated by the Spanish National Cancer Centre (CNIO), following the technical and ethical procedures of the network.

DNA was extracted from CD138 positive cells using Tri-Reagent (from Becton Dickinson, San José) according to the manufacturer's instructions.

Bisulphite modification and methylation-specific PCR

Bisulphite conversion was performed as previously described (Herman et al., 1996). DNA (1 µg) was denatured by NaOH. The denatured DNA was treated with sodium bisulphite to chemically modify the unmethylated cytosines to uracil. Modified DNA was purified using the Wizard DNA Clean-up system (Promega, Madison, WI). Samples

Table 2
Primers used for the analysis of methylation-specific PCR

Determination		Primer sequence 5′ to 3′	Product/Tª (°C)	
hMLH-1	Us	TTA ATA GGA AGA GTG GAT AGT G	107-bp	Tª 55 °C
	Ua	TCT ATA AAT TAC TAA ATC TCT TCA		
	Ms	TTA ATA GGA AGA GCG GAT AGC	106-bp	Tª 55 °C
	Ma	CTA TAA ATT ACT AAA TCT CTT CG		
MGMT	Us	TTT GTG TTT TGA TGT TTG TAG GTT TTT GT	93-bp	Tª 62 °C
	Ua	AAC TCC ACA CTC TTC CAA AAA CAA AAC A		
	Ms	TTT CGA CGT TCG TAG GTT TTC GC	81-bp	Tª 62 °C
	Ma	GCA CTC TTC CGA AAA CGA AAC G		
DAPK	Us	GGA GGA TAG TTG GAT TGA GTT AAT GTT	106-bp	Tª 60 °C
	Ua	CAA ATC CCT CCC AAA CAC CAA		
	Ms	GGA TAG TCG GAT CGA GTT AAC GTC	98-bp	Tª 60 °C
	Ma	CCC TCC CAA ACG A		
p16	Us	TTA TTA GAG GGT GGG GTG GAT TGT	151-bp	Tª 61 °C
	Ua	CAA CCC CAA ACC ACA ACC ATA A		
	Ms	TTA TTA GAG GGT GGG GCG GAT CGC	150-bp	Tª 67 °C
	Ma	GAC CCC GAA CCG CGA CCG TAA		
p15	Us	TGT GAT GTG TTT GTA TTT TGT GGT T	154-bp	Tª 62 °C
	Ua	CCA TAC AAT AAC CAA ACA ACC AA		
	Ms	GCG TTC GTA TTT TGC GGT T	148-bp	Tª 62 °C
	Ma	CGT ACA ATA ACC GAA CGA CCG A		
p14	Us	TTT TTG GTG TTA AAG GGT GGT GTA GT CAC AAA	132-bp	Tª 60 °C
	Ua	AAC CCT CAC TCA CAA		
	Ms	GTG TTA AAG GGC GGC GTA GC	122-bp	Tª 60 °C
	Ma	AAA ACC CTC ACT CGC GAC GA		

Us: unmethylated sense; Ua: unmethylated antisense; Ms: methylated sense; Ma: methylated antisense; Tª: annealing temperature.

Table 3
Results of methylation and immunohistochemical expression

	p16	p15	p14	hMLH1	MGMT	DAPK
MM and PCL						
MSPCR (*n*=30)	15 (50%)	5 (17%)	0	3 (10%)	7 (23%)	9 (30%)
IHC neg (*n*=16)	11 (69%)	ND	ND	8 (50%)	7 (44%)	ND
MGUS						
MSPCR (*n*=13)	5 (39%)	2 (15%)	0	0	1 (8%)	2 (15%)
IHC neg (*n*=11)	7 (64%)	ND	ND	0	4 (36%)	ND
Plasmacytomas						
MSPCR (*n*=9)	5 (55%)	2 (22%)	0	0	6 (67%)	4 (44%)
IHC neg (*n*=9)	6 (67%)	5 (55%)	ND	2 (22%)	6 (67%)	ND

MSPCR: methylation-specific PCR; ND: Not done; IHC neg: loss of immunohistochemical protein expression.

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