

Methylation status of nine tumor suppressor genes in multiple myeloma

Esteban Braggio · Angelo Maiolino · Maria E. Gouveia · Roberto Magalhães · João T. Souto Filho · Márcia Garnica · Marcio Nucci · Ilana Zalberg Renault

Received: 25 August 2009 / Revised: 20 November 2009 / Accepted: 25 November 2009 / Published online: 26 December 2009
© The Japanese Society of Hematology 2009

Abstract Aberrant methylation in promoter-associated CpG islands has been recognized as a major mechanism for tumor suppressor gene silencing in several malignancies. We determined the methylation status of nine tumor suppressor genes in 68 newly diagnosed MM patients by methylation-specific PCR. The frequency of promoter hypermethylation for individual genes was: *CDH1*, 50%; *p16^{INK4a}*, 42.8%; *p15^{INK4b}*, 16.2%; *SHP1*, 14.7%; *ER* and *BNIP3*, 13.2%; *RARβ*, 11.8%; *DAPK* 5.9%; and *MGMT* 0%. Overall, 79% of patients presented at least one hypermethylated gene. By univariate analysis, hypermethylation of *DAPK* ($P < 0.001$) and *RARβ* ($P = 0.01$) genes were identified as adverse prognostic features. Median OS of patients with hypermethylation in *DAPK* (4 months) and *RARβ* (34 months) was significantly lower than in patients without hypermethylation (median survival not reached), with values of $P < 0.001$ and $P = 0.01$, respectively. Our

data suggest that *DAPK* and *RARβ* hypermethylation are adverse prognostic factors in MM. The relevance of these findings as poor prognosis indicators requires confirmation in a larger sample with longer follow-ups.

Keywords Multiple myeloma · Hypermethylation · MSP · *DAPK*

1 Introduction

Multiple myeloma (MM) is an incurable plasma cell tumor that affects the bone marrow compartment, and is characterized by the production of monoclonal immunoglobulin (M protein) [1]. It accounts for more than 10% of all hematological malignancies and approximately 2% of cancer deaths [2]. In the recent years, the knowledge of the molecular and cytogenetics abnormalities involved in the origin and progression of the MM has increased considerably. Recently, two subsets of patients have been distinguished based on the cytogenetic findings: (a) the hyperdiploid variant

Electronic supplementary material The online version of this article (doi:10.1007/s12185-009-0459-2) contains supplementary material, which is available to authorized users.

E. Braggio · M. E. Gouveia · I. Z. Renault
Laboratory of Molecular Biology,
Bone Marrow Transplantation Center,
Instituto Nacional de Câncer, Rio de Janeiro, Brazil
e-mail: ebraggio@hotmail.com

M. E. Gouveia
e-mail: megouveia@yahoo.com.br

A. Maiolino · R. Magalhães · J. T. Souto Filho · M. Garnica · M. Nucci
Hematology Service, Department of Internal Medicine,
Universidade Federal do Rio de Janeiro,
Rio de Janeiro, Brazil
e-mail: maiolino@hucff.ufrj.br

R. Magalhães
e-mail: robmag@superig.com.br

J. T. Souto Filho
e-mail: drjoatadeu@yahoo.com.br

M. Garnica
e-mail: marciagarnica@hucff.ufrj.br

M. Nucci
e-mail: mnucci@hucff.ufrj.br

I. Z. Renault (✉)
Laboratory of Molecular Biology,
CEMO, INCa, Praça da Cruz Vermelha 23, 6th Floor,
Rio de Janeiro, RJ 20230-130, Brazil
e-mail: zalberg@inca.gov.br; ilanazalberg@yahoo.com.br

(>46 to <76 chromosomes); and (b) the non-hyperdiploid variant, including the hypodiploid (up to 44–45 chromosomes), pseudodiploid (44/45 to 46/47 chromosomes with gains/losses compared with the normal karyotype), and the near tetraploid or hypotetraploid (75 or more chromosomes) [3, 4]. These subgroups are preferentially associated with additional cytogenetics abnormalities and distinct clinical outcome [3–7]. Additional data from gene expression profiling lead to the proposition that up-regulation of at least one gene of cyclin *D* family might represent a common, early oncogenic event [8], a reason why a molecular classification, based on cyclin *D* expression pattern and presence of *IgH* translocations, has been proposed [9].

While the knowledge of the genetic and cytogenetic abnormalities associated with the origin and progression of MM is becoming more apparent, the role of epigenetic modifications is still poorly understood. Aberrant methylation at promoter-associated CpG islands has been recognized as a major mechanism for silencing tumor suppressor genes in several malignancies, representing an epigenetic equivalent to mutations and deletions in carcinogenesis [10, 11]. To date, the role of aberrant promoter methylation in MM has been reported in several studies, mainly focusing on the methylation status of *p16^{INK4a}*, *p15^{INK4b}*, *CDH1* (E-cadherin), *SOCS1*, and *DAPK* [12–20]. However, these studies have shown a highly variable prevalence of promoter hypermethylation within a same gene [12, 15, 17–19], as well as conflicting results between aberrant methylation and clinical characteristics and outcome [13, 14, 17, 19, 20].

These findings prompted our comprehensive analysis to determine the methylation status of nine tumor-suppressor gene promoters by methylation-specific PCR (MSP) in a group of 68 recently diagnosed MM patients. These genes were selected in view of: (a) their potential involvement in the pathogenesis of hematological diseases, and (b) their relevant involvement in several cellular pathways, such as DNA repair (*MGMT*), cell cycle regulation (*p15^{INK4b}* and *p16^{INK4a}*), cell–cell adherence (*CDH1*), apoptosis regulation (*DAPK* and *BNIP3*), hormonal response (*RAR β* and *ER*), and Jak/STAT3 signaling pathway (*SHP1*). In order to obtain further insights on the prognostic implications resulting from epigenetic silencing, we analyzed the relationship between methylation status, clinical characteristics, and overall survival (OS).

2 Patients and methods

2.1 Patients

Bone marrow (BM) aspirates from 68, previously untreated, MM patients and of ten healthy donors were collected at the Hospital Universitário Clementino Fraga Filho, Rio de Janeiro, Brazil. Samples were collected after informed

consent was obtained in accordance with the Declaration of Helsinki. Diagnosis and staging classification of MM followed standard criteria [21, 22]. Treatment consisted of 3–4 cycles of VAD (vincristina, doxorubicin, and dexamethasone), followed by mobilization of peripheral blood hematopoietic stem cells with cyclophosphamide (4 g/m²) and G-CSF (10 μ g/kg/day). After collecting a minimum of 4×10^8 CD34+ cells/Kg, patients received melphalan (200 mg/m²) and autologous hematopoietic stem cell infusion. After day +100 of transplant, patients were consolidated with thalidomide (200 mg/day) \pm dexamethasone (40 mg/day for 4 days once a month), during a total of 12 months. The clinical and laboratorial characteristics of these 68 patients at diagnosis were extracted from medical charts. The characteristics analyzed were age, gender, MM subtype, Durie-Salmon and ISS staging systems, percentage of plasma cells in bone marrow, hemoglobin (Hb) level, white blood cell and platelets counts, serum calcium, serum creatinine, serum lactate dehydrogenase, albumin and serum β 2 microglobulin levels (Table 1).

Table 1 Clinical characteristics of myeloma patients at diagnosis

Number of patients	68
Median age, years (range)	55 (28–74)
Male/female	38/30
MM subtype (%)	
IgG	38 (56)
IgA	13 (19)
Light chain	11 (16)
Non-secretory	6 (9)
Durie-Salmon classification (%)	
Ia	1 (1.5)
Ib	0 (0)
IIa	13 (19)
IIb	3 (4.4)
IIIa	33 (48.5)
IIIb	18 (26.5)
ISS classification (%)	
I	18 (26.5)
II	26 (38)
III	17 (25)
Unknown	7 (10.3)
Hemoglobin, g/L (range)	9.25 (3.6–15.1)
PTL/mm ³ (range)	195,000 (15,000–904,000)
Serum calcium, mg/dL (range)	9.4 (1.24–15.4)
Creatinine, mg/dL (range)	1.1 (0.7–31)
LDH, U/L (range)	246 (75–745)
Serum albumin, g/dL (range)	3.35 (1.6–6.1)
β 2 microglobulin, mg/L (range)	3.73 (1.1–63)
Median follow-up, months (range)	15.5 (1–184)

ISS international staging system; PTL platelets; LDH lactate dehydrogenase

2.2 Genomic DNA extraction

Mononuclear cells were separated by Ficoll-Hypaque[®] (Sigma, St. Louis, MO, USA) density-gradient centrifugation. Cell lyses and DNA extraction were performed with DNAzol[®] according to the recommendations of the manufacturer (Invitrogen, Carlsbad, CA, USA).

2.3 Sodium bisulfite treatment and methylation-specific polymerase chain reaction (MSP)

Conversion of unmethylated cytosine to uracil residues by bisulfite treatment [23] was carried out by denaturing 1–3 µg of DNA with 3 M NaOH and sodium bisulfite for 16 h at 50°C, purification with the Wizard DNA Purification System (Promega, Madison, WI, USA), desulfuration with 0.3 M NaOH, ethanol precipitation, and resuspension in water. Samples were stored at –20°C.

Cell lines used as controls for methylated alleles (Supplementary Table S1) were kindly provided by Dr. Marco Ladetto, University of Torino, Italy and Dr. Martino Introna, Mario Negri Institute, Milan, Italy. Cells were grown in RPMI 1640 (Life Technologies, Rockville, MD, USA) supplemented with 10% fetal bovine serum, and incubated in 5% CO₂ at 37°C.

MSP assays were performed into two separate reactions with different specific primer pairs for discriminating between unmethylated (U-MSP) and methylated (M-MSP) regions. Bisulfite-treated DNA of ten bone marrow healthy donors and cell lines were used as controls for U-MSP and M-MSP, respectively, in analyses of nine suppressor tumor genes (*p15^{INK4b}*, *p16^{INK4a}*, *DAPK*, *CDH1*, *SHP1*, *ER*, *MGMT*, *BNIP3*, and *RARβ*). Primer sequences and annealing temperatures are listed in Table 2.

MSP reactions were carried out in a final volume of 25 µl, containing 10 ng of bisulfite-treated DNA, 0.5× PCR Buffer, 2 mM MgCl₂, 0.01% (wt/vol) gelatine, 0.25 mM dNTPs, 10 pmol of each primer and 0.5 U *Taq* Platinum DNA polymerase (Invitrogen). Amplifications, carried out in a Flexigene PCR Thermocycler (Techne, Burlington, New York, USA), consisted of an initial denaturing step at 94°C (2 min) followed by 40 cycles at 94°C (30 s), annealing at a specific temperature (see Table 2) for 30 s, 72°C (30 s), and a final extension step at 72°C (10 min). PCR products were run in vertical electrophoresis plates at 10 V/cm in 7.5% non-denaturing polyacrylamide gels and visualized by silver staining. Results were based on two separate MSP reactions.

2.4 DNA sequencing

Unmethylated and methylated alleles of all genes were subsequently sequenced to confirm the efficiency of

bisulfite treatment and the specificity of MSP reactions. PCR products were purified with the GFX TM PCR DNA and Gel Band Purification Kit H (GE Healthcare, Schenectady, NY, USA), bi-directionally sequenced following labeling with the DYEnamyc TM ET Terminator Cycle Sequencing Premix Kit H (GE Healthcare) in an ABI PRISM 377 automatic sequencer (Applied Biosystems, Foster City, CA, USA) and analyzed with the Sequence Navigator software (Applied Biosystems).

2.5 MSP sensitivity

The sensitivity of MSP assays was tested by serially diluting (10⁻¹–10⁻⁴) methylated DNA from control cell lines with unmethylated DNA, treatment with sodium bisulfite, and subsequently amplifying them with specific primers.

2.6 Identification of chromosomal abnormalities by cIg-FISH

The presence of chromosome 13 deletion, t(4;14)(p16;q32) and t(11;14)(q23;q32) was analyzed by cIg-FISH, which combines FISH with cytoplasmic immuno-fluorescence designed to identify PCs [4, 6]. In all cases, specific commercial probes were used (Abbott Laboratories, Santa Clara, CA, USA).

2.7 Statistical analysis

The correlation between methylation status and clinical and laboratorial characteristics was assessed by Fisher's exact test or Chi-square test (categorical variables) and Student's *t* test (continuous variables). Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated for binomial variables. Overall survival (OS) was estimated using the Kaplan–Meier method. Differences between survival curves were estimated by the log-rank test. Results were considered significant with *P* < 0.05. Data were analyzed by using SPSS software, version 10.0.

3 Results

3.1 MSP sensitivity and specificity

We analyzed CpG islands spanning along the promoter region of nine tumor suppressor genes. In all cases, the promoter hypermethylation of these genes was previously found to be associated with transcriptional silencing [14, 23–31]. The MSP specificity was confirmed by DNA sequencing, with U-MSP amplifications showing that all cytosines had been converted to uracils by bisulfite

Table 2 Primer sequences, chromosomal location of the target genes and annealing conditions

Primer	Sequence (5'-3')		Cytoband	Annealing <i>t</i> (°C)	Reference
P16-MSP1	TTA TTA GAG GGT GGG GTG GAT TGT	U-MSP	9p21	60	[23]
P16-MSP2	CAA CCC CAA ACC ACA ACC ATA A				
P16-MSP3	TTA TTA GAG GGT GGG GCG GAT CGC	M-MSP		60	
P16-MSP4	GAC CCC GAA CCG CGA CCG TAA				
P15-MSP1	TGT GAT GTG TTT GTA TTT TGT GGT T	U-MSP	9p21	60	[23]
P15-MSP2	CCA TAC AAT AAC CAA ACA ACC AA				
P15-MSP3	GCG TTC GTA TTT TGC GGT T	M-MSP		60	
P15-MSP4	CGT ACA ATA ACC GAA CGA CCG A				
CDH1-MSP1	GGT GGG TGG GTT GTT AGT TTT GT	U-MSP	16q22	60	[24]
CDH1-MSP2	AAC TCA CAA ATC TTT ACA ATT CCA ACA				
CDH1-MSP3	GTG GGC GGG TCG TTA GTT TC	M-MSP		60	
CDH1-MSP4	CTC ACA AAT ACT TTA CAA TTC CGA CG				
ER-MSP1	ATG AGT TGG AGT TTT TGA ATT GTT T	U-MSP	6q25	60	[25]
ER-MSP2	ATA AAC CTA CAC ATT AAC AAC AAC CA				
ER-MSP3	CGA GTT GGA GTT TTT GAA TCG TTC	M-MSP		60	
ER-MSP4	CTA CGC GTT AAC GAC GAC CG				
DAPK-MSP1	GGA GGA TAG TTG GAT TGA GTT AAT GTT	U-MSP	9q34	60	[26]
DAPK-MSP2	CAA ATC CCT CCC AAA CAC CAA				
DAPK-MSP3	GGA TAG TCG GAT CGA GTT ACC GTC	M-MSP		60	
DAPK-MSP4	CCC TCC CAA ACG CCG A				
SHP1-MSP1	GTG AAT GTT ATT ATA GTA TAG TGT TTG G	U-MSP	12p13	60	[27]
SHP1-MSP2	TTC ACA CAT ACA AAC CCA AAC AAT				
SHP1-MSP3	GAA CGT TAT TAT AGT ATA GCG TTC	M-MSP		60	
SHP1-MSP4	TCA CGC ATA CGA ACC CAA ACG				
RAR β -MSP1	TTG GGA TGT TGA GAA TGT GAG TGA TTT	U-MSP	3p24	60	[17]
RAR β -MSP2	CTT ACT CAA CCA ATC CAA CCA AAA CAA				
RAR β -MSP3	TGT CGA GAA CGC GAG CGA TTC	M-MSP		60	
RAR β -MSP4	CGA CCA ATC CAA CCG AAA CGA				
BNIP3-MSP1	TAG GAT TTG TTT TGT GTA TG	U-MSP	8p21	58	This study
BNIP3-MSP2	ACC ACA TCA CCC ATT AAC CAC A				
BNIP3-MSP3	TAG GAT TCGT TTC GCG TAC G	M-MSP		64	
BNIP3-MSP4	ACC GCG TCG CCC ATT AAC CGC G				
MGMT-MSP1	TTT GTG TTT TGA TGT TTG TAG GTT TTT GT	U-MSP	10q26	58	[28]
MGMT-MSP2	AAC TCC ACA CTC TTC CAA AAA CAA AAC A				
MGMT-MSP3	TTT CGA CGT TCG TAG GTT TTC GC	M-MSP		54	
MGMT-MSP4	GCA CTC TTC CGA AAA CGA AAC G				

treatment, while in the M-MSP amplifications, the methylated cytosine residues in CpG dinucleotides had remained unaffected and all the unmethylated cytosines had been changed to uracils (Fig. 1). The sensitivity of M-MSP varied between 10^{-2} for *BNIP3* and *MGMT* to 10^{-3} for *p15^{INK4b}*, *p16^{INK4a}*, *CDH1*, *ER*, *DAPK*, *RAR β* , and *SHP1* (Fig. 2).

To confirm the absence of non-specific reactions, we tested ten peripheral blood samples obtained from bone marrow health donors. In all the ten-health donor's samples, the nine genes were successfully amplified with

specific U-MSP primers but none of them was amplified by using M-MSP specific primers.

3.2 Methylation status in MM patients

We examined the DNA methylation status of nine genes in 68, previously untreated, MM patients (Fig. 3). The frequency of hypermethylation at promoters was: *CDH1*, 50%; *p16^{INK4a}*, 42.6%; *p15^{INK4b}*, 16.2%; *SHP1*, 14.7%; *ER* and *BNIP3*, 13.2%; *RAR β* , 11.8%; and *DAPK* 5.9% (Fig. 4a). No patient showed hypermethylation at the

Fig. 1 Sequencing of bisulfite-converted methylated (a) and unmethylated DNA samples (b) for *p16^{INK4a}* promoter region. The converted cytosines in the CpG dinucleotides are showed inside boxes

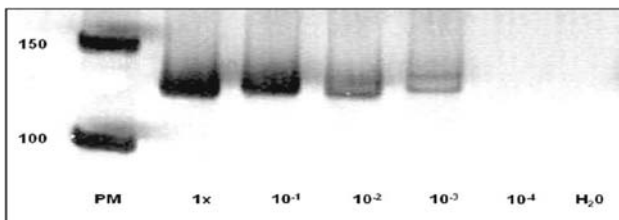
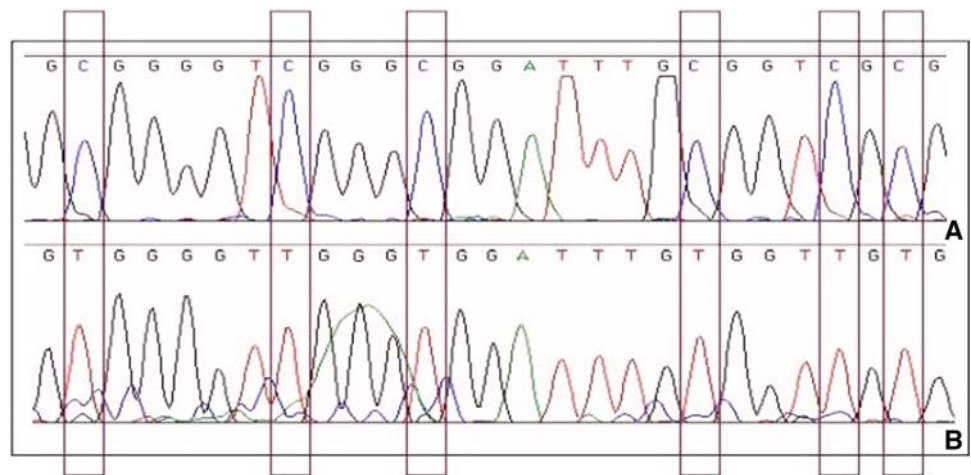


Fig. 2 The sensitivity of MSP assays was tested by serially diluting (10^{-1} – 10^{-4}) methylated DNA from control cell lines with unmethylated DNA, treatment with sodium bisulfite, and subsequently amplifying them with specific primers

MGMT promoter. At least one hypermethylated gene was identified in 79.4% of patients (54 out of 68). One hypermethylated gene was observed in 27.9% of patients (19/68), two genes in 25% (17/68), three genes in 17.6% (12/68), four genes in 7.3% (5/68), and five hypermethylated genes in 1.5% (1/68; Fig. 4b). The methylation profile of the nine tumor suppressor genes analyzed is shown in Fig. 5.

3.3 Association between aberrant methylation, clinical characteristics, and prognosis

We looked for potential correlations between methylation status and clinical parameters such as age, gender, MM subtype, Durie-Salmon and ISS staging systems, Hb level, white blood cell and platelets counts, serum calcium, serum creatinine, serum lactate dehydrogenase, albumin and serum $\beta 2$ microglobulin levels.

CDH1 and *p16^{INK4a}* were initially analyzed, as they were the two most commonly methylated genes. No differences in prognostic factors were observed between methylated and unmethylated groups. Durie-Salmon and ISS staging systems were also analyzed, and no correlation was found (Supplementary Table S2).

Patients with *DAPK* hypermethylation were more likely to have a serum creatinine level >2.0 mg/dL ($P = 0.006$), serum calcium >9.5 mg/dL ($P = 0.05$), and Durie-Salmon stage III ($P = 0.04$). The correlation between *DAPK* methylation status and clinical characteristics is shown in Table 3. Aberrant *ER* methylation was associated with lower albumin levels ($P = 0.02$), lower Hb levels ($P = 0.03$), high (>5.5 mg/L) serum $\beta 2$ -microglobulin levels ($P = 0.03$), and advanced ISS stages ($P = 0.04$). The methylation status of the other genes did not correlate with any clinical parameter.

We also search for correlations between number of methylated genes per patient and prognostic factors (Hb, platelets, serum creatinine, and $\beta 2$ -microglobulin levels), but there were no differences. Durie-Salmon and ISS staging systems were also analyzed, and no correlation was found with number of methylated genes (Supplementary Tables S3, S4). Of note, the patient with methylation in five genes was staged as 3 in ISS and 3b in DS system.

Likewise, we did not find any correlation between the methylation status of any gene and the presence of t(4;14)(p16;q32), chromosome 13 deletion, or t(11;14)(q23;q32) by cIg-FISH, which are associated with poor, intermediate, and good prognosis, respectively.

Univariate analysis identified hypermethylation of *DAPK* ($P < 0.001$) and *RAR β* ($P = 0.01$), platelet counts $<100,000/\text{mm}^3$ ($P < 0.001$) and serum calcium >9.5 mg/dL ($P = 0.03$) as adverse prognostic features (Table 4), while a trend to high serum creatinine levels was not statistically significant ($P = 0.064$). The median OS estimates of patients with *DAPK* or *RAR β* hypermethylated (4 and 34 months, respectively) was considerably lower than in patients without aberrant methylation (median survival not reached, $P < 0.001$ and $P = 0.01$, respectively; Fig. 6). Patients with and without *CDH1* and *p16^{INK4a}* had median

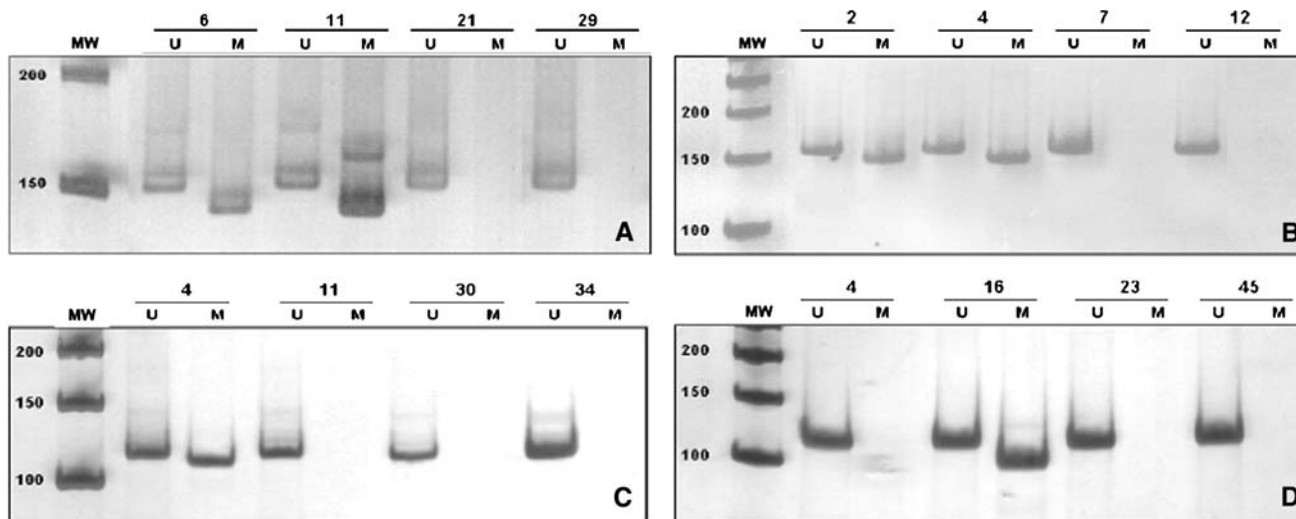


Fig. 3 MSP employing specific primers for unmethylated (U) and methylated (M) DNA. In the gels is shown the methylation status in four patients for genes *p15^{INK4b}* (a), *ER* (b), *RARβ* (c), and *SHP1* (d)

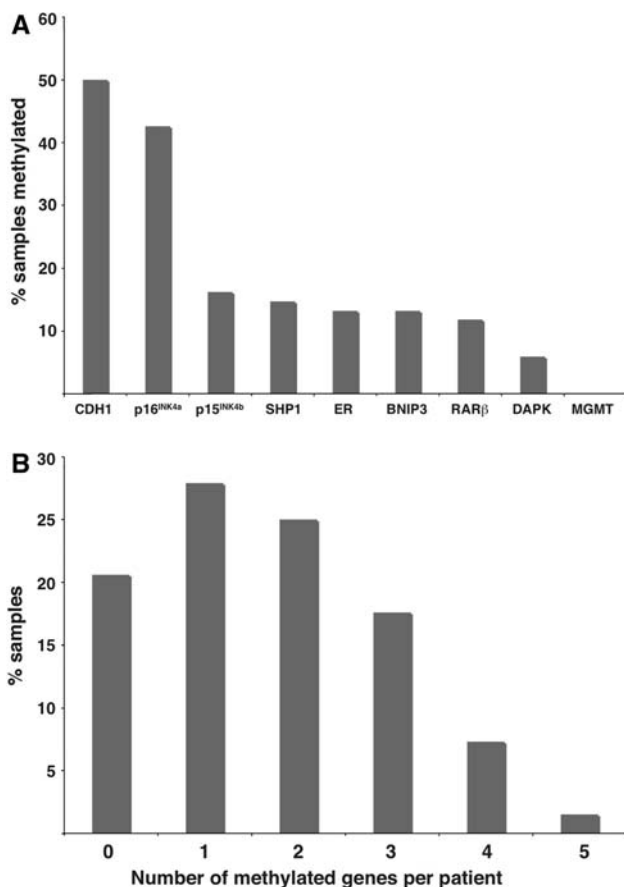


Fig. 4 a Histogram showing the prevalence of the hypermethylated alleles of the different tumor suppressor genes included in this study. b Distribution of genes hypermethylated, showing the prevalence of patients with 0, 1, and more genes affected

survival of 60 months versus not reached median survival (p log rank = 0.79) and 34 versus 60 months (p log rank = 0.92), respectively.

4 Discussion

In recent years, aberrant methylation of 5' promoter regions has been clearly shown to operate as a transcription regulator, and its role in several human cancers has been reported [10, 11, 32–34]. The aim of this study was to determine the methylation profile of nine tumor suppressor genes from several cellular pathways in MM patients and its association with clinical parameters and survival.

A critical point in MM genomic studies is the separation of the CD138+ cell fraction, which includes the tumor clone. However, one benefit of MSP technique is the sensitivity. Here, we calculated a sensitivity of 10^{-3} for the majority of genes, thus allowing the identification of methylated alleles present in at least 1% fraction. For that reason, the tumor clone isolation through sorting is not mandatory for MSP, and the technique can be performed from the mononuclear fraction.

Hypermethylation was commonly detected in >40% of cases at *CDH1* and *p16^{INK4a}* promoters, and in less extent (<20%) at *p15^{INK4b}*, *SHP1*, *ER*, *BNIP3*, *RARβ*, and *DAPK* promoters, while it was absent at the *MGMT* promoter. At all, we found at least one hypermethylated gene in 79.4% of MM patients. These data suggest that aberrant methylation is a commonly observed process, affecting a variety of tumor suppressor genes controlling different pathways in MM patients.

In this study, *DAPK* hypermethylation showed a statistically significant association with other unfavorable clinical variables, such as higher serum creatinine levels, serum calcium, and Durie-Salmon stage III ($P = 0.04$). Furthermore, our data show lower median OS estimates in patients with *DAPK* hypermethylation than those without

Fig. 5 Methylation profile of nine tumor suppressor genes in 68 MM patients. *Gray boxes* indicate methylated promoters, *white boxes* indicate unmethylated promoters

Patient	p15 ^{INK4b}	p16 ^{INK4a}	CDH1	ER	DAPK	RAR β	SHP1	BNIP3	MGMT
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									
14									
15									
16									
17									
18									
19									
20									
21									
22									
23									
24									
25									
26									
27									
28									
29									
30									
31									
32									
33									
34									
35									
36									
37									
38									
39									
40									
41									
42									
43									
44									
45									
46									
47									
48									
49									
50									
51									
52									
53									
54									
55									
56									
57									
58									
59									
60									
61									
62									
63									
64									
65									
66									
67									
68									

the aberrant methylation status ($P = 0.01$). Ng et al. [12] have found an association between *DAPK* hypermethylation and a threefold reduction in the median survival of MM patients when compared with the group without aberrant methylation, but this association was not statistically significant ($P = 0.38$). The *DAPK* protein is a serine/threonine kinase implicated in diverse apoptosis pathways, including those involved in neuronal cell death and tumor suppression [35]. Loss of *DAPK* expression by promoter

hypermethylation has been commonly identified in a number of B-cell malignancies, especially in follicular lymphoma, MALT lymphoma, diffuse large B-cell lymphoma, mediastinal large B-cell lymphoma, and Burkitt's lymphoma [18]. The inactivation of apoptotic pathways product of *DAPK* silencing may collaborate with the survival advantage conferred to lymphoma cells by BCL-2 deregulation in FL and NF- κ B activation in MALT lymphoma [36].

Table 3 Clinical and laboratorial characteristics of myeloma patients according to the *DAPK* methylation status

	Methylated (<i>N</i> = 4)	Unmethylated (<i>N</i> = 64)	<i>P</i> value
Age, median (range)	61 (53–68)	54.5 (28–74)	0.22
Male (%)	2 (50)	36 (56)	1.0
MM type (%)			0.19
IgG	3 (75)	35 (55)	
IgA	0	13 (20)	
Light chain	1 (25)	10 (16)	
Non-secretor	0	6 (9)	
Durie-Salmon stage (%)			0.038
1	0	1 (2)	
2	0	16 (26)	
3	4 (100)	44 (72)	
ISS			0.43
1	0	18 (28)	
2	2 (50)	24 (37)	
3	2 (50)	7 (11)	
Hemoglobin level, median	7.5	9.3	0.11
PTL counts (cells/mm ³), median	159,000	197,000	0.24
Serum calcium (mg/dL), median	12	9	0.02
Creatinine (mg/dL), median	3.6	1	0.18
LDH (U/L), median	233	246	0.67
Serum albumin (g/dL), median	3.2	3.3	0.73
β 2 microglobulin (mg/dL)	4.9	3.75	0.49
Death (%)	3 (75)	13 (20)	0.038

ISS international staging system; *PTL* platelets; *LDH* lactate dehydrogenase

Table 4 Univariate analysis of prognostic factors for overall survival using Cox's regression

	<i>N</i>	Univariate		<i>P</i> value
		50% survival (months)	Mean survival (months)	
Platelets				
<10 ⁵ /mm ³	10	17	17	0.0003
>10 ⁵ /mm ³	57	Not reached	53	
Serum creatinine				
>2 mg/dL	20	Not reached	35	0.0644
<2 mg/dL	47	60	50	
Serum calcium				
>9.5 mg/dL	33	Not reached	43	0.0283
<9.5 mg/dL	35	60	107	
<i>DAPK</i>				
Methylated	4	4	9	0.0017
Unmethylated	64	Not reached	110	
<i>RAR</i> β				
Methylated	8	34	22	0.0102
Unmethylated	60	Not reached	117	

RAR β hypermethylation was also associated with a poor outcome. This gene can mediate response to retinoid therapies, and it was suggested that lack of response might be associated to promoter hypermethylation [37, 38]. Aberrant *RAR* β methylation was detected with a range of

very low [17] to intermediate frequency in MM patients [30], without any identifiable association between methylation status and clinical variables. In this study, we identified *RAR* β hypermethylation as an adverse prognostic feature by univariate analysis with the median OS in

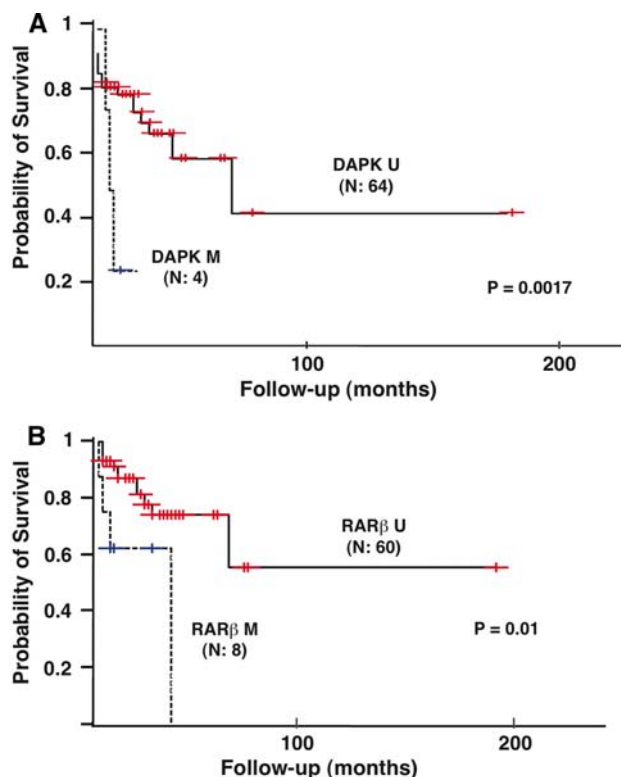


Fig. 6 Overall survival of 68 MM patients according to the methylation status of *DAPK* (a) and *RARβ* (b) genes. Solid line unmethylated cases; dotted lines methylated cases

patients with *RARβ* hypermethylation of 34 months compared with median survival not reached in patients without abnormal methylation profile ($P = 0.01$).

Methylation at any other gene promoter herein investigated was not associated with differences in OS. The methylation status of *p16^{INK4a}* has been previously studied, but its biological and clinical implications in multiple myeloma are still unclear. Initial reports have shown an association between *p16^{INK4a}* hypermethylation and poor outcome [13, 14, 17]. Our results did not show a negative prognostic impact of *p16^{INK4a}* hypermethylation, according with the two larger MM cohorts previously studied, in which overall survival differences between patients with or without *p16^{INK4a}* methylated could not be demonstrated [19, 20]. It is therefore likely that *p16^{INK4a}* hypermethylation, though not associated with a poor outcome, might be a marker of disease progression.

In summary, this study showed that aberrant gene promoter methylation is a common phenomenon, affecting several metabolic pathways in MM patients. Our data suggested that *DAPK* hypermethylation might be an adverse prognostic factor in MM. To assess the relevance of this indicator of poor prognosis in addition to classical prognostic factors, studies with larger cohorts and longer follow-ups are required. The apparent lack of clinical

impact resulting from hypermethylation of several genes herein studied suggested that their inactivation could be indicators of disease progression.

Finally, hypermethylation-associated gene silencing is a potentially reversible phenomenon, and demethylation agents have been shown to exert clinical activity in patients with myelodysplastic syndromes and acute myeloid leukemia [39, 40]. Thus, the simultaneous aberrant methylation of multiple gene promoters in MM not only provides new insights into the biology of MM but also suggests that these epigenetically affected regions may become potential targets of demethylating therapeutic strategies.

Acknowledgments We are grateful to Dr. Héctor N Seuánez (INCa - UFRJ, Brazil) for critical review of this manuscript. We thank Dr. Ladetto, Alberto Rocci (University of Torino, Italy) and Dr. Inrona (Mario Negri Institute, Milan, Italy) for providing the cell lines. We thank the Instituto Nacional de Câncer (INCa), Fundação Ary Frauzino (FAF), and SwissBridge Foundation for their financial support. The work was supported in part by the Conselho Nacional de Pesquisas - CNPq - Brazil and Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP - Brazil.

Conflict of interest statement The authors have no interests to declare.

References

- Kyle RA, Rajkumar SV. Multiple myeloma. *N Engl J Med*. 2004;351:1860–73.
- Ries LAG, Eisner MP, Kosary CL. SEER cancer statistics review 1975–2001. National Cancer Institute; 2004.
- Smadja NV, Fruchart C, Isnard F, Louvet C, Dutel JL, Cheron N, et al. Chromosomal analysis in multiple myeloma: cytogenetic evidence of two different diseases. *Leukemia*. 1998;12:960–9.
- Debes-Marun C, Dewald G, Bryant S, Picken E, Santana-Dávila R, González-Paz N, et al. Chromosome abnormalities clustering and its implications for pathogenesis and prognosis in myeloma. *Leukemia*. 2003;17:427–36.
- Fonseca R, Barlogie B, Bataille R, Bastard C, Bergsagel PL, Chesi M, et al. Genetics and cytogenetics of multiple myeloma: a workshop report. *Cancer Res*. 2004;64:1546–58.
- Fonseca R, Debes-Marun CS, Picken EB, Dewald GW, Bryant SC, Winkler JM, et al. The recurrent IgH translocations are highly associated with nonhyperdiploid variant multiple myeloma. *Blood*. 2003;102:2562–7.
- Smadja NV, Leroux D, Soulier J, Dumont S, Arnould C, Taviaux S, et al. Further cytogenetic characterization of multiple myeloma confirms that 14q32 translocations are a very rare event in hyperdiploid cases. *Gene Chromosome Cancer*. 2003;38:234–9.
- Bergsagel P, Kuehl M. Critical roles for immunoglobulin translocations and cyclin D dysregulation in multiple myeloma. *Immunol Rev*. 2003;194:96–104.
- Bergsagel PL, Kuehl M, Zhan F, Sawyer J, Barlogie B, Shaughnessy J. Cyclin D dysregulation: an early and unifying pathogenic event in multiple myeloma. *Blood*. 2005;106:296–303.
- Laird P, Jaenisch R. DNA methylation and cancer. *Hum Mol Genet*. 1994;3:1487–95.

11. Jones P, Baylin S. The fundamental role of epigenetic events in cancer. *Nat Rev Genet.* 2002;3:415–28.
12. Ng M, To W, Lo KW, Chan S, Tsang KS, Cheng SH, et al. Frequent death-associated protein kinase promoter hypermethylation in multiple myeloma. *Clin Cancer Res.* 2001;7:1724–9.
13. Mateos M, García-Sanz R, López-Pérez R, Moro M, Ocio E, Hernández J, et al. Methylation is an inactivating mechanism of the p16 gene in multiple myeloma associated with high plasma cell proliferation and short survival. *Br J Haematol.* 2002;118:1034–40.
14. Guillemin G, Depil S, Wolowicz D, Quesnel B. Different prognostic values of p15INK4b and p16INK4a in multiple myeloma. *Haematologica.* 2003;88:476–8.
15. Chim CS, Kwong YL, Fung TK, Liang R. Methylation profiling in multiple myeloma. *Leuk Res.* 2004;28:379–85.
16. Chim C, Fung T, Cheung W, Liang R, Kwong Y. SOCS1 and SHP1 hypermethylation in multiple myeloma: implications for epigenetic activation of the Jak/STAT pathway. *Blood.* 2004;103:4630–5.
17. Galm O, Wilop S, Reichelt J, Jost E, Gehbauer G, Herman J, et al. DNA methylation changes in multiple myeloma. *Leukemia.* 2004;18:1687–92.
18. Rossi D, Capello D, Glohini A, Franceschetti S, Paulli M, Bhatia K, et al. Aberrant promoter methylation of multiple genes throughout the clinico-pathologic spectrum of B-cell neoplasia. *Haematologica.* 2004;89:154–64.
19. Seidl S, Ackermann J, Kaufmann H, Keck A, Nösslinger T, Zielinski C, et al. DNA-methylation analysis identifies the E-cadherin gene as a potential marker of disease progression in patients with monoclonal gammopathies. *Cancer.* 2004;100:2598–606.
20. Gonzalez-Paz N, Chng W, McClure R, Blood E, Oken M, Van Ness B, et al. Tumor suppressor p16 methylation in multiple myeloma: biological and clinical implications. *Blood.* 2007;109:1228–32.
21. Durie B, Salmon S. A clinical staging system for multiple myeloma. Correlation of measured myeloma cell mass with presenting clinical features, response to treatment, and survival. *Cancer.* 1975;36:842–54.
22. The International Myeloma Working Group. Criteria for the classification of monoclonal gammopathies, multiple myeloma and related disorders: a report of the International Myeloma Working Group. *Br J Haematol.* 2003;121:749–57.
23. Herman J, Graff J, Myohanen S, Nelkin B, Baylin S. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA.* 1996;93:9821–6.
24. Graff J, Herman J, Myohanen S, Baylin S, Vertino P. Mapping patterns of CpG island methylation in normal and neoplastic cells implicates both upstream and downstream regions in de novo methylation. *J Biol Chem.* 1997;272:22322–9.
25. Lapidus R, Nass S, Butash K, Parl F, Weitzman S, Graff J, et al. Mapping of ER gene CpG island methylation-specific polymerase chain reaction. *Cancer Res.* 1998;58:2515–9.
26. Katzenellenbogen R, Baylin S, Herman J. Hypermethylation of the DAPKinase CpG islands is a common alteration in B cell malignancies. *Blood.* 1999;93:4347–53.
27. Oka T, Ouchida M, Koyama M. Gene silencing of the tyrosine phosphatase SHP1 gene by aberrant methylation in leukemias/lymphomas. *Cancer Res.* 2002;62:6390–4.
28. Esteller M, Garcia-Foncillas J, Andion E, Goodman S, Hidalgo O, Vanaclocha V, et al. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. *N Engl J Med.* 2000;343:1350–4.
29. Esteller M, Gaidano G, Goodman S, Zagonel V, Capello D, Botto B, et al. Hypermethylation of the DNA repair gene O(6)-methylguanine DNA methyltransferase and survival of patients with diffuse large B-cell lymphoma. *J Natl Cancer Inst.* 2002;94:26–32.
30. Takahashi T, Shivapurkar N, Reddy J, Shigematsu H, Miyajima K, Suzuki M, et al. DNA methylation profiles of lymphoid and hematopoietic malignancies. *Clin Cancer Res.* 2004;10:2928–35.
31. Murai M, Toyota M, Satoh A, Suzuki H, Akino K, Mita H, et al. Aberrant DNA methylation associated with silencing BNIP3 gene expression in hematopoietic tumours. *Br J Cancer.* 2005;92:1165–72.
32. Jones P, Laird P. Cancer epigenetics comes of age. *Nat Genet.* 1999;21:163–7.
33. Baylin S, Herman J. DNA hypermethylation in tumorigenesis: epigenetics joins genetics. *Trends Genet.* 2000;16:168–74.
34. Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer. *Cancer Res.* 2001;61:3225–9.
35. Deiss L, Feinstein E, Berissi H, Cohen O, Kimchi A. Identification of a novel serine/threonine kinase and a novel 15-kD protein as potential mediators of the gamma interferon-induced cell death. *Genes Dev.* 1995;9:15–30.
36. Sanchez-Beato M, Sanchez-Aguilera A, Piris MA. Cell cycle deregulation in B-cell lymphomas. *Blood.* 2003;101:1220–35.
37. Cote S, Momparler R. Activation of the retinoic acid receptor beta gene by 5-aza-2'-deoxycytidine in human DLD-1 colon carcinoma cells. *Anticancer Drugs.* 1997;8:56–61.
38. Virmani A, Rathi A, Zochbauer-Muller S, Sacchi N, Fukuyama Y, Bryant D, et al. Promoter methylation and silencing of the retinoic acid receptor-beta gene in lung carcinomas. *J Natl Cancer Inst.* 2000;92:1303–7.
39. Silverman LR, Holland JF, Weinberg RS, Alter BP, Davis RB, Ellison RR, et al. Effects of treatment with 5-azacytidine on the in vivo and in vitro hematopoiesis in patients with myelodysplastic syndromes. *Leukemia.* 1993;7(Suppl 1):21–9.
40. Silverman L, Demakos E, Peterson B, Kornblith AB, Holland JC, Odchimar-Reissig R, et al. Randomized controlled trial of azacytidine in patients with the myelodysplastic syndrome: a study of the cancer and leukemia group B. *J Clin Oncol.* 2002;20:2429–40.