Aberrant *p15*, *p16*, *p53*, and *DAPK* Gene Methylation in Myelomagenesis: Clinical and Prognostic Implications

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Abstract

Aberrant DNA methylation is important in multiple myeloma pathogenesis. Methylation of *p15*, *p16*, *p53,* and *DAPK* genes was evaluated in bone marrow (94 patients; 8 controls) by methylation-specific polymerase chain reaction; 63% of patients with multiple myeloma and 39% of patients with monoclonal gammopathy of uncertain significance presented ≥1 hypermethylated gene (*P* < .05). This supports a relevant role for hypermethylation in the pathogenesis of the plasma cell neoplasms.

Background: Aberrant DNA methylation is considered a crucial mechanism in the pathogenesis of monoclonal gammopathies. We aimed to investigate the contribution of hypermethylation of 4 tumor suppressor genes to the multistep process of myelomagenesis. **Methods:** The methylation status of *p15*, *p16*, *p53*, and *DAPK* genes was evaluated in bone marrow samples from 94 patients at diagnosis: monoclonal gammopathy of uncertain significance (MGUS) (n = 48), smoldering multiple myeloma (SMM) (n = 8) and symptomatic multiple myeloma (MM) (n = 38), and from 8 healthy controls by methylation-specific polymerase chain reaction analysis. **Results:** Overall, 63% of patients with MM and 39% of patients with MGUS presented at least 1 hypermethylated gene (*P* < .05). No aberrant methylation was detected in normal bone marrow. The frequency of methylation for individual genes in patients with MGUS, SMM, and MM was *p15*, 15%, 50%, 21%; *p16*, 15%, 13%, 32%; *p53*, 2%, 12,5%, 5%, and *DAPK*, 19%, 25%, 39%, respectively (*P* < .05). No correlation was found between aberrant methylation and immunophenotypic markers, cytogenetic features, progression-free survival, and overall survival in patients with MM. **Conclusions:** The current study supports a relevant role for *p15*, *p16*, and *DAPK* hypermethylation in the genesis of the plasma cell neoplasm. *DAPK* hypermethylation also might be an important step in the progression from MGUS to MM.

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Introduction

Multiple myeloma (MM) is a plasma cell disorder characterized by monoclonal proteins in serum or urine, clonal plasma cells in bone marrow, and, if symptomatic, the presence of end-organ damage (CRAB criteria: hypercalcemia, renal insufficiency, anemia, bone lesions).[1] MM accounts for approximately 10% of all

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hematologic malignancies and is still incurable, mainly because of its high biological heterogeneity.[2-4]

According to several authors, MM is consistently preceded by a monoclonal gammopathy of uncertain significance (MGUS)—an asymptomatic (no CRAB criteria) premalignant entity that has been reported to have a transformation rate to MM of approximately 1% per year.[1] Smoldering multiple myeloma (SMM) is an intermediate asymptomatic malignant plasma cell disorder, with a risk of progression to symptomatic MM of 10% per year, for the first 5 years.[1,5-8] Indeed, both MGUS and SMM represent the ideal model for studying MM precursor disease and to define and develop early intervention strategies.

Aberrant CpG island methylation has been associated with various types of human malignancies, with increasing evidence suggesting that epigenetic events play a relevant role in carcinogenesis.[6,9-12]

The progression from MGUS to MM is the result of complex multistep genetic aberrations in addition to epigenetic modifications.[8,11,13] The aberrant CpG island methylation of gene promoter regions of a variety of regulatory genes, associated with gene function silencing (mainly of tumor suppressor genes), has been reported in human malignancies.[14]

It was previously demonstrated that hypermethylation of the cell cycle inhibitors *p15* and *p16,*[14-16] of the apoptosis regulator death-associated protein kinase (DAPK)[17,18] and the tumor suppressor gene *p53* occurs in plasma cell disorders.[9,12] However, its role in the transition from MGUS to MM has not been clarified.

The *p15* (INK4B) and *p16* (INK4A) genes are members of the INK4 family of cyclin-dependent kinase inhibitors (CDKIs). The *p15* gene is located 25 kb from the *p16* gene at 9p21, and their proteins share significant areas of homologous sequences.[19,20] The *p53* gene is a tumor suppressor gene that plays a critical role in cell cycle regulation, apoptosis, and cell senescence, involved in several physiologic and pathologic processes.[9]

DAP kinase, codified by the *DAP kinase (DAPK)* gene, localized in chromosome 9q34.1, is a calcium/calmodulin-dependent and cytoskeletal-associated serine/threonine kinase with death-inducing functions. *DAPK* is a positive mediator of apoptosis induced by interferon-γ, tumor necrosis factor-α, and FAS.[21-23] The loss of expression of *DAPK* is commonly associated with hypermethylation of the *DAPK* gene[10] and is frequently found in B-cell lymphoma and some carcinoma cell lines, which highlights its potential role as a tumor suppressor.[22] Furthermore, its expression was detected in some MM cell lines and in patient-derived primary cells.[21]

The characterization of the methylation profile of candidate genes that have been established to play a role in tumorigenesis may provide significant insight into the molecular pathogenesis of MGUS and MM, in particular, the role of the aberrant methylation of the 4 described genes. Moreover, although it has been shown that methylation patterns may be related to clinical behavior, response to therapy, and survival, data are scarce regarding MGUS, SMM, and MM.[24,25]

The aim of this study was to evaluate the role of aberrant methylation of promoter regions of tumor suppressor genes in the clonal evolution from MGUS to MM. Thus, we analyzed in MGUS, SMM, and symptomatic MM patients, the methylation status of 4 genes—*p15*, *p16*, *p53*, and *DAPK*—whose promoter hypermethylation was previously found to be associated with transcriptional silencing.[4,15] We also aimed to explore possible correlations between the clinical and laboratorial features of MGUS, SMM, and MM and the methylation pattern of the 4 evaluated genes at diagnosis. A third objective was to evaluate the impact of the methylation profile of *p15*, *p16*, *p53*, and *DAPK*, individually or combined, on progression-free and overall survivals.

Patients and Methods

Patient Characterization

A total of 94 patients with monoclonal gammopathies (38 patients with symptomatic MM, 8 patients with SMM, 48 patients with MGUS) and 8 healthy donors were studied. The diagnosis and staging of MM and MGUS followed International Myeloma Working Group criteria. All studies were performed in bone marrow aspirates of previously untreated patients, collected for diagnostic purposes. The study was approved by the Ethics Committee of Faculty of Medicine, University of Coimbra (FMUC) and samples were collected after informed consent was obtained in accordance with the Declaration of Helsinki. The main clinical and laboratory characteristics of the patient cohort are summarized in Table 1. The cytogenetic and immunophenotypic characteristics are shown in Table 2.

Overall survival was estimated only in patients with MM after a bortezomib-based regimen followed by high-dose therapy in transplant-eligible patients (aged $\leq$ 70 years) or after standard-dose therapy with thalidomide-based regimens or alkylating agents plus steroids in transplant-ineligible patients.

Methylation-Specific Polymerase Chain Reaction

One microgram of genomic DNA, obtained from unsorted bone marrow cells of patients with MM, SMM, and MGUS at diagnosis and healthy donors, was extracted using the QIAamp DNA kit (Qiagen, Chatsworth, Calif) and converted using sodium bisulfite according to the manufacturer's protocols (EpiTectBisulfite Kit, Qiagen). Methylation-specific polymerase chain reactions (PCRs) of *p15*, *p16*, *p53*, and *DAPK* were carried out as previously described,[15,16,26] and we used primer sequences for the methylated and unmethylated promoters (Supplemental Table 1 in the online version).[12,27,28] MSP was performed in a thermal cycler (Mycycler, Bio-Rad, Hercules, Calif) with the following conditions: 95°C for 5 minutes, 35 cycles of 95°C for 45 seconds, annealing temperature for 30 seconds, 72°C for 30 seconds, and a final extension of 10 minutes at 72°C (Supplemental Table 1 in the online version). The MSP mixture contained 50 ng of bisulfite-treated DNA, 0.2 mM 2′-deoxynucleoside 5′-triphosphates, 2 mM MgCl2, 1× PCR buffer, and 2 U of HotstarTaq DNA Polymerase (Qiagen). PCR products were resolved on 4% agarose gels, stained with ethidium bromide, and visualized under ultraviolet illumination.

Statistical Analysis

The association between methylation status and clinical and laboratorial characteristics was assessed by Fisher exact test or chi-square test (for categoric variables) and Student *t* test (for continuous variables). Survival was calculated from the date of diagnosis to the date of the patient's last follow-up or death. Overall survival was estimated according to the Kaplan−Meier method. All statistical

Table 1 Baseline Clinical and Laboratorial Features of Patients

	MGUS	SMM	MM
No. of patients	48	8	38
Gender, n (%)			
Male	28 (58%)	4 (50%)	17 (45%)
Female	20 (42%)	4 (50%)	21(55%)
Median age, y (range)	71 (39-86)	73 (58-89)	72 (41-87)
Paraprotein subtype, n (%)			
IgG	32 (67%)	6 (75%)	17 (45%)
IgA	12 (25%)	2 (25%)	16 (42%)
IgM	4 (8%)	0 (0%)	0 (0%)
Light chain only, n (%)			
K	0 (0%)	0 (0%)	4 (10%)
L	0 (0%)	0 (0%)	1 (3%)
ISS, n (%)			
1	NA	NA	8 (21%)
2	NA	NA	7 (18%)
3	NA	NA	23 (61%)
Bone marrow plasma cell, median (range) (%)	5 (1-9)	12 (10-16)	30 (3-95)
Bone disease, n (%)	0 (0%)	0 (0%)	27 (71%)
Hemoglobin, mean (range) (g/dL)	13.8 (11.8-15)	13.4 (11.6-15.2)	9.9 (8.1-16.1)
Serum calcium mean (range) (mg/dL)	9.6 (9.3-10)	9.6 (8.8-10.5)	9.5 (7.7-13.8)
Serum albumin mean (range) (g/dL)	4.4 (3.8-5.1)	4.5 (3.5-4.9)	3.5 (1.9-4.9)
Creatinine mean (range) (mg/dL)	0.71 (0.52-1.7)	0.8 (0.5-1.5)	0.99 (0.27-19.2)
LDH mean (range) (U/L)	182 (135-247)	172 (156-201)	168 (62-381)
β2-microglobulin mean (range) (μg/mL)	2.19 (1.31-5.74)	2.4 (1.3-6.2)	6.4 (1.6-11.7)

Abbreviations: Ig = immunoglobulin; ISS = international staging system; LDH = lactate dehydrogenase; MGUS = monoclonal gammopathy of uncertain significance; MM = multiple myeloma; NA = not applicable; SMM = smoldering multiple myeloma.

calculations were performed using Stata 12 statistical software (StataCorp LP, College Station, Tex). For all tests, $P < .05$ (2-sided) was regarded as statistically significant.

Results

Methylation Analysis in Patients With MM, SMM, and MGUS

The methylation frequencies of *p15*, *p16*, *p53*, and *DAPK* genes in bone marrow aspirates from 94 patients with monoclonal gammopathies (38 patients with MM, 8 patients with SMM, and 48 patients with MGUS) are summarized in Figure 1. The methylation status of the 4 studied tumor suppressor genes is shown in detail in Supplemental Figure 1 in the online version.

When we analyzed all studied patients with monoclonal gammopathies (n = 94), 52% presented at least 1 hypermethylated gene (63% of patients with MM and 39% of patients with MGUS, $P < .05$). At least 2 simultaneously aberrantly methylated genes were found in 29% of patients with MM and 10% of patients with patients ($P < .05$) (Figure 1).

Aberrant methylation of 1 and 2 genes was detected in 34% and 23% of MM samples and in 29% and 10% of MGUS samples, respectively. Five percent of patients with MM and none of the patients with MGUS exhibited methylation of 3 genes. None of the studied samples presented methylation of the 4 genes.

The prevalence of methylation of *DAPK* increased from MGUS to SMM to MM (16%, 25%, and 38%, respectively, $P < .05$). This observation was evident when we analyzed the 3 subgroups separately and when we analyzed MGUS plus SMM versus MM ($P = .035$). There were no statistical differences in the prevalence of methylation in MGUS, SMM, and MM for the 3 remaining genes (Figure 1).

None of the 8 normal control bone marrows presented aberrant methylation of the 4 evaluated genes (Supplemental Figure 1 in the online version). Examples of MSP are shown in Figure 2. The positive and negative controls showed the expected MSP results (Figure 2). When more than 1 gene was methylated, no correlation was identified between the methylation of a given gene and the methylation of any other given gene.

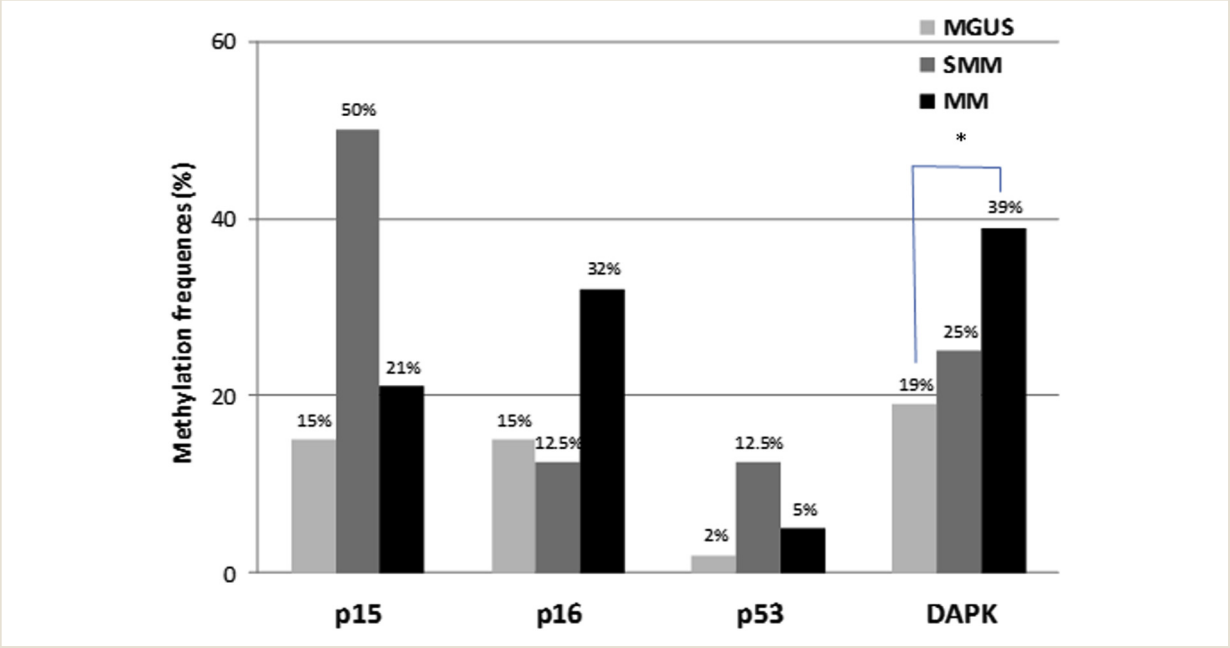
Association Between Aberrant Methylation of p15, p16, p53, DAPK, and Clinical Characteristics and Prognosis

We have also explored the potential correlations between the methylation pattern of the 4 studied genes and the baseline clinicopathologic features of patients with MM, including age, gender, type of paraprotein, International Staging System (ISS), hemoglobin, lactate dehydrogenase, β2-microglobulin, calcium and serum

Table 2 Baseline Cytogenetic and Immunophenotypic Features of Patients

Cytogenetic Abnormalities, n/N (%)			
t(14q32)	2/24 (8%)	1/8 (12.5%)	14/37 (38%)
Deleted 17p13.1 locus	0/24 (0%)	0/8 (0%)	1/37 (3%)
t (4;14)	0/24 (0%)	0/8 (0%)	5/37 (14%)
t (11;14)	1/24 (4%)	0/8 (0%)	4/37 (11%)
Immunophenotypic markers, n/N (%)			
CD27+	12/15 (80%)	4/6 (66%)	16/35 (46%)
CD28+	4/15 (21%)	1/6 (17%)	27/35 (77%)
CD56+	22/39 (56%)	6/7 (86%)	29/37 (78%)
CD117+	12/15 (80%)	4/6 (67%)	21/35 (60%)

Figure 1 Frequencies of Methylation of *p15*, *p16 p53*, and *DAPK* Genes in Patients With Symptomatic Multiple Myeloma (MM), Smoldering Multiple Myeloma (SMM), and Monoclonal Gammopathy of Uncertain Significance (MGUS). Results Are Expressed in Percentage (%). *$P < .05$

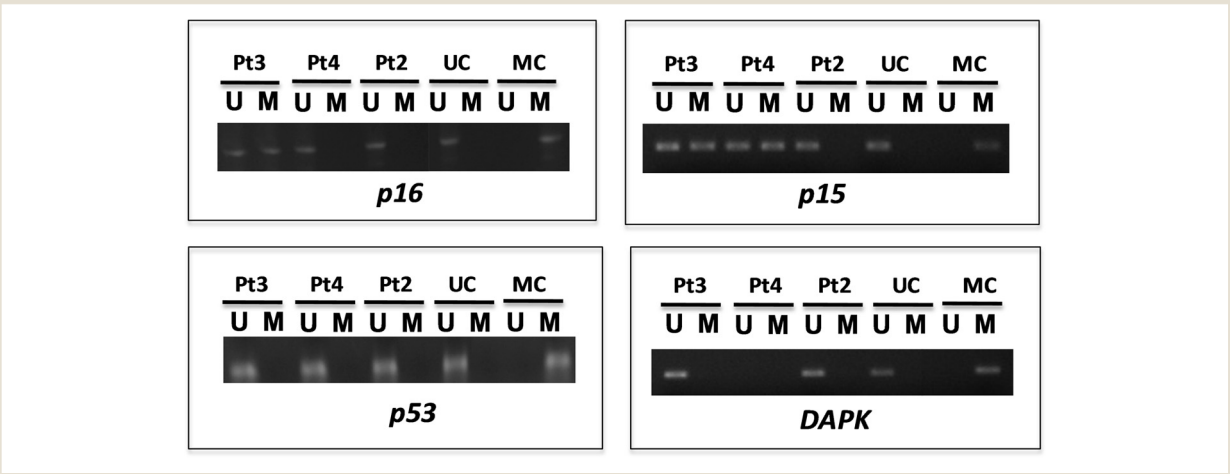


Abbreviation: *DAPK* = death-associated protein kinase.

creatinine levels, presence of bone disease, cytogenetic abnormalities, and aberrant expression of immunophenotypic markers (CD27, CD28, CD56, and CD117).

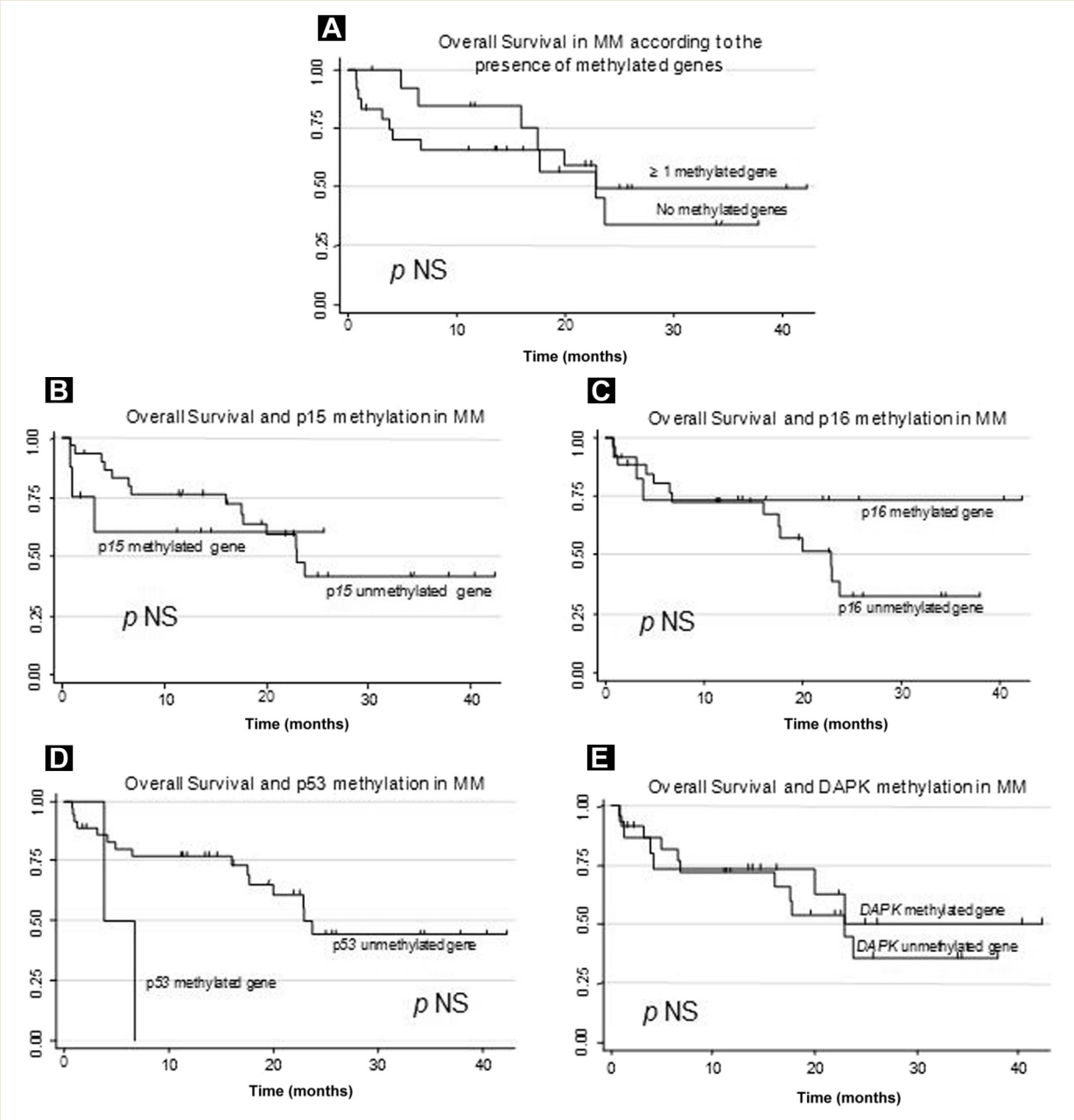
Patients with *p16* gene methylation were more likely to have stage III disease (ISS) ($P = .03$) and an increased serum creatinine level >2 mg/dL ($P = .016$). There was a statistically significant association between *p15* and *p53* gene methylation and elevated lactate dehydrogenase ($P = .04$ and $P = .028$, respectively). Patients with *DAPK* gene methylation were more likely to have immunoglobulin-A subtype ($P = .04$).

Figure 2 Examples of the Electrophoresis Analysis of the Methylation-Specific Polymerase Chain Reaction (PCR) Products of *p15*, *p16*, *p53*, and *DAPK* Genes in Bone Marrow Samples Collected at Diagnosis of Patients With MM (Pt3), SMM (Pt4), and MGUS (Pt2)



Abbreviations: MC = universal methylated control; Pt = patient; UC = universal unmethylated control.

Figure 3 Survival Curves According to Gene Methylation Profile in Symptomatic Patients With MM. Kaplan–Meier Overall Survival Curves Stratified by the Absence or Presence of 1 or More Methylated Genes (A) and According to *p15* (B), *p16* (C), *p53* (D), and *DAPK* (E) Gene Methylation Profile



Abbreviations: MM = multiple myeloma; NS = not significant.

No correlation was found between the methylation status of any of the studied genes and the ploidy or cytogenetic aberrations, namely, t(14q32), t(4;11), t(11;14), and del(17p), in patients with MM, SMM, and MGUS. Furthermore, no statistically significant associations were observed between the methylation status of the evaluated genes, individually or aggregated, with any of the analyzed immunophenotypic features in the 3 groups of patients with plasma cell dyscrasia.

The median follow-up was 28.5, 25, and 31.7 months in the MM, SMM, and MGUS groups, respectively. According to our results, the presence of methylated genes did not influence the overall survival in the MM group. The overall survival curves in the MM subgroup are shown in Figure 3.

Discussion

Epigenetic modifications have been recognized as playing a relevant role in the critical points of cancer initiation and progression.[29] One of the most common and well-defined epigenetic modifications involves methylation of CpG dinucleotides in the

promoter regions of tumor suppressor genes associated with gene silencing.[30]

Although it is known that MM is a highly heterogeneous disease resulting from the accumulation of several genetic and epigenetic events,[8] published data regarding epigenetic events in monoclonal gammopathies, namely, the methylation profile of tumor suppressor genes, are limited and controversial.

In this study, we examined the role of aberrant methylation of promoter regions of tumor suppressor genes (*p15*, *p16*, *p53,* and *DAPK*) and their methylation status in the clonal evolution from MGUS to MM. In all cases, promoter hypermethylation of these genes was previously found to be associated with transcriptional silencing.[4,15,31,32] We used methylation-specific PCR analysis because it is a sensitive and quick method, better suited to the analysis of large numbers of samples.[27,33,34]

MSP has been used to detect methylation in both unsorted bone marrow mononuclear cells and sorted CD138-positive cells (which includes the tumor clone).[15,18,35] Several authors have studied the methylation of a number of genes in unsorted mononuclear cells[18,33,36] and sorted CD138-positive cells[37] in MM and other B-cell malignancies, with comparable methylation frequencies of *p15* and *p16*,[15] which indicates that the technique can be performed in mononuclear cells.

Overall, we identified at least 1 hypermethylated gene in 52% of patients with monoclonal gammopathy, with patients with MM presenting a significantly higher frequency of hypermethylation (63%) compared with those with MGUS (39%) and no aberrant methylation status in controls. These results confirm that aberrant methylation of tumor suppressor genes is a frequently observed event in patients with monoclonal gammopathies and involves a number of genes that control different pathways in the development and progression of MGUS to a more aggressive symptomatic phase, in agreement with previous studies.[14-17,26]

Our study also showed that aberrant methylation of *p15*, *p16*, *p53*, and *DAPK* is already present in patients with MGUS (15%, 15%, 2%, and 19%, respectively) and increases in symptomatic MM (21%, 32%, 5%, and 39%, respectively). However, only *DAPK* methylation showed a statistically significant increase in patients with MM compared with patients with MGUS ($P < .05$).

These observations support the hypothesis that aberrant methylation of *p15*, *p16 p53*, and *DAPK* constitutes an early event in the pathogenesis and development of plasma cell disorders, consistent with the results previously published by Stanganelli et al[16] and Ng et al.[18]

A wide range of frequencies (5.9%-77%) of *DAPK* methylation was reported in MM at diagnosis[15,16,18,26] and in other neoplasms (16%-81%),[38-40] with some studies reporting that the loss of *DAPK* may confer a selective advantage during the multistep process of metastasis.[30] Thus, the loss of *DAPK* expression provides a unique mechanism that links suppression of apoptosis to metastasis[41] and may be involved in the clonal progression from MGUS to MM.

One of the most frequent gene alterations in MM is methylation of the *p15* and *p16* genes in the 5′ upstream region. P15 and p16 proteins are cell cycle regulators involved in the inhibition of transition from G1 to S phase.[37]

Frequencies of *p15* or *p16* gene methylation up to 32% and 53%, respectively, have been reported in MM cases.[14-17,26,37] Previous investigations have demonstrated that *p16* methylation is more frequent in MM than in MGUS,[13,17,42] whereas others reported identical frequencies.[16,26] Guillerm et al[37] described frequencies of methylation of the *p15* and *p16* genes not significantly different among MGUS and MM samples and also between stage I/II and stage III, suggesting a lack of association with malignant transformation from MGUS to MM and a possible contribution to plasma cells immortalization.

In line with these authors, we showed that *p15* and *p16* gene methylation is present at similar rates in MGUS and MM cases, supporting the concept that an alteration of the regulation of cell cycle, namely, in the transition of G1 to S phase, is a very early event in the history of MM.

A reduced number of reports compared the methylation status of tumor suppressor genes among MGUS, SMM, and MM. As in the current study, Seidl et al[26] considered SMM as an individual subgroup, but the number of patients included was also low ($n = 5$), resulting in nonsignificant differences when compared with MGUS.

The *p53* tumor suppressor gene is the most frequently mutated gene in human cancer (~50% of human tumors), resulting in a decreased apoptosis in response to DNA damage.[4,43] However, this is rare in MM, but increases with disease progression[8,9,44,45] being relatively infrequent in MM at diagnosis, occurring in 0% to 3% of patients, and reaching up to 20% of patients with advanced disease.[8,46]

P53 gene methylation was reported in 32% to 55% of the samples from myeloma cell lines.[9] This finding is in contrast with our results concerning *p53* methylation, which was observed in a low number of MM and MGUS cases (2% and 5%, respectively). These discrepancies may be related to the sample type (cell lines vs. patients' samples).

Another objective of the current study was to examine possible correlations between clinical and laboratorial features of MGUS, SMM, and MM and the methylation pattern of the 4 evaluated genes at diagnosis. We found that patients with *p16* gene methylation were more likely to have advanced ISS stage and an increased serum creatinine level. We also observed a significant association between *p15* methylation and elevated lactate dehydrogenase, as well as between *DAPK* gene methylation and an immunoglobulin-A subtype. These findings may suggest that hypermethylation of the studied genes is associated with a more aggressive disease phenotype.

Looking at cytogenetic abnormalities, we did not find any association between the methylation pattern of any of the 4 evaluated genes and the presence of chromosome 13 deletion, t(4;14), t(11;14), or 17p deletion by fluorescence in situ hybridization. These results are in accordance with those of Braggio et al[15] and Walker et al[11] in all mentioned cytogenetic subgroups, except for t(4;14), which was associated with a poor prognosis.

We also searched for associations between immunophenotypic markers with a demonstrated impact on outcome (CD27, CD28, CD56, and CD117) and the methylation profile of the studied genes in patients with MM.

The loss of CD27 in MGUS has been linked to MM progression,[47] and the loss of CD56 expression was associated with extramedullary spreading, aggressive disease, and inferior progression-free survival, but not overall survival.[48] CD117 expression in clonal plasma cells is associated with favorable

biological features and a lower frequency of adverse cytogenetic characteristics.[49] MM plasma cell expression of CD28 in newly diagnosed patients is a major prognostic predictor of poor clinical outcome, allowing MM cells to better survive to treatment and resulting in their selective outgrowth.[50]

In a multivariate analysis, we evaluated possible correlations involving methylation profile of *p15*, *p16*, *p53*, and *DAPK* and each specific immunophenotypic marker (CD27, CD28, CD56, and CD117), and no statistically significant associations were found. To the best of our knowledge, this is the first study to investigate possible associations between immunophenotypic features and methylation status of tumor suppressor genes in plasma cell dyscrasia.

A third aim of our investigation was to evaluate the impact of the methylation profile of *p15*, *p16*, *p53*, and *DAPK*, individually or combined, on progression-free and overall survival. We did not find any statistically significant difference in progression-free survival and overall survival in MM cases for any individual studied gene or combination of genes, controlling for age and treatment approaches. Our findings are in agreement with the reports of Guillerm et al,[37] who observed that methylation of *p15* and p16 was not associated with shorter survival in patients with MM. In contrast, some studies noted that *DAPK* methylation was related to inferior overall survival and reported that *p16* methylation also was associated with a worse prognosis in patients with MM.[17,42]

Conclusions

Aberrant methylation of CpG islands in promoter regions of crucial genes involved in cell cycle regulation, apoptosis, and tumor suppression is frequent and early events in plasma cell immortalization, leading to the development of monoclonal gammopathies. However, the relevance of methylation in the clonal evolution from MGUS to MM and the associations between cytogenetic and epigenetic events in this malignant transformation remain unclear. According to our investigation, *DAPK* methylation may be a marker of disease progression in monoclonal gammopathies. Another unsolved question is the prognostic impact of methylation of individual and combined genes in MM. Although several authors have reported a poor prognosis of *p16* and *DAPK* methylation in patients with MM, this negative impact on outcome was not confirmed in more recent reports, namely, in the current study. To answer all of the questions about the role of epigenetic mechanism during myelomagenesis, a larger study with a longer follow-up and uniform treatment approaches for patients with MM will be necessary.

Clinical Practice Points

- Multiple myeloma (MM) is associated with genetic, epigenetic, and bone marrow microenvironment alterations, and currently remains incurable.
- Increasing evidence supports that epigenetic aberrant modifications play a relevant role in carcinogenesis and in the progression from monoclonal gammopathy of uncertain significance (MGUS) to MM.
- Although it was previously demonstrated that hypermethylation of the cell cycle inhibitors *p15* and p16 of the apoptosis regulator death-associated protein kinase and of the tumor suppressor gene *p53* occurs in plasma cell disorders, its role in myelomagenesis is not yet clarified.
- As measured by methylation-specific polymerase chain reaction analysis of bone marrow of patients with MGUS and MM and controls, our study revealed that a large percentage of patients with MM and MGUS (63% and 39%, respectively) presented at least 1 hypermethylated gene ($P < .05$), with no alterations in normal bone marrow.
- We found no correlation between aberrant methylation and immunophenotypic markers, cytogenetic features, progression-free survival, and overall survival in patients with MM. Death-associated protein kinase methylation also was found to be a marker of disease progression from MGUS to MM.
- Our study contributed in clarifying the role of the methylation status of a number of tumor suppressor genes in myelomagenesis and in the diagnosis, prognosis, and therapeutic approach of patients with monoclonal gammopathies.

Disclosure

C.G. has received honoraria from and serves on the advisory boards of Janssen, Celgene, Amgen and Bristol-Myers Squibb. The other authors report no declarations of interest.

Supplemental Data

Supplemental table and figure accompanying this article can be found in the online version at http://dx.doi.org/10.1016/j.clml.2016.08.016.

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Supplemental Table 1 Methylation-Specific Polymerase Chain Reaction: Primer Sequences and Reaction Conditions

Gene	Forward Primer (5′-3′)	Reverse Primer (3′-5′)	Ta/$MgCl_2$	Reference
P15				
M-MSP	GCGTTCGTATTTTGCGGTT	CGTACAATAACCGAACGACCGA	60°C/6.7 mM	Herman et al[27]
U-MSP	TGTGATGTGTTTGTATTTTGTGGTT	CCATACAATAACCAAACAACCAA	60°C/6.7 mM	
P16				
M-MSP	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	65°C/6.7 mM	Herman et al[27]
U-MSP	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	60°C/6.7 mM	
P53				
M-MSP	TTCGGTAGGCGGATTATTTG	AAATATCCCCGAAACCCAAC	60°C/6.7 mM	Yeh et al[28]
U-MSP	TTGGTAGGTGGATTATTTGTTT	CCAATCCAAAAAAACATATCAC	58°C/6.7 mM	
DAPK				
M-MSP	GGATAGTCGGAT CGAGTTAACGTC	CCCTCCCAAACGCCGA	63°C/2 Mm	Chan et al[51]
U-MSP	GGAGGATAGTTG GATTGAGTTAATGTT	CAAATCCCTCCCAAACACCAA	63°C/2 mM	

Abbreviations: *DAPK* = *DAP* kinase; M-MSP = methylation-specific polymerase chain reaction for the methylated allele; MSP = methylation-specific polymerase chain reaction; Ta = annealing temperature; U-MSP = MSP for the unmethylated allele.

Supplemental Figure 1 Methylation Status of *p15*, *p16*, *p53*, and *DAPK* Genes in Monoclonal Gammopathy of Uncertain Significance (MGUS) (A), Multiple Myeloma (MM) (B), Smoldering Multiple Myeloma (SMM) (C), and Healthy Controls (D). White Boxes Represent Unmethylated Samples, and Grey Boxes Represent Methylated Samples.