

# Frequent Death-associated Protein Kinase Promoter Hypermethylation in Multiple Myeloma<sup>1</sup>

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## ABSTRACT

*Death-associated protein (DAP) kinase is a novel gene regulating apoptosis induced by IFN- $\gamma$ . In B-cell malignancies, loss of DAP kinase expression is commonly associated with promoter hypermethylation. These characteristics of DAP kinase may be of particular relevance in multiple myeloma (MM), a B-lineage malignancy in which prolonged survival capacity of the malignant plasma cells may be critical in the induction and maintenance of tumor cells.*

*Purpose:* The involvement and potential role of DAP kinase in MM pathogenesis was examined.

*Experimental Design:* In this investigation, methylation-specific PCR was conducted on primary MM and MM cell lines. Methylation status findings were correlated with clinical parameters.

*Results:* We first demonstrated frequent DAP kinase hypermethylation in 24 of 36 primary MMs (20 of 26 at diagnosis and 4 of 10 with relapse/residual MM after treatment), 1 of 2 patients with monoclonal gammopathy of undetermined significance, and 1 of 3 MM cell lines studied. The high frequency of DAP kinase hypermethylation was similarly observed in MM of different stages, immunoglobulin isotypes, and histological grades, with or without plasmacytomas. Although not statistically significant, the overall survival of patients with DAP kinase methylation was notably shortened among 23 MM patients followed prospectively ( $P = 0.38$  by Kaplan-Meier method and log-rank test). This preliminary finding suggests prognostic implications of DAP kinase in MM that may deserve further investigation.

*Conclusions:* Our data suggest an important role for DAP kinase in MM tumorigenesis.

## INTRODUCTION

Programmed cell death or apoptosis is a critical process in normal B-cell function in the immune system (1). Increasing evidence supports the argument that apoptosis and its regulation play a role in tumorigenesis (2, 3). These features may be of particular relevance in MM,<sup>3</sup> a low proliferative B-cell malignancy characterized by prolonged survival capacity of the malignant plasma cells. It is possible that protective mechanisms, which inhibit or suppress apoptosis, may participate in induction or maintenance of the malignant MM clone (4).

A number of cytokines are involved in MM pathogenesis (5–8). Interleukin 6 inhibits apoptosis and supports the growth of MM cells (5). IFNs mediate MM growth inhibition via modification of the cell cycle and the interleukin 6 signaling mechanism (6). IFN- $\alpha$  has been used in the maintenance therapy of some MM patients (7). Because occasional growth stimulation by IFN- $\alpha$  was also observed in some MM cell lines (8), IFN- $\gamma$ , which shows consistent antiproliferative activity, has been suggested as a potential alternative in MM management (6).

DAP kinase is a novel calcium/calmodulin-dependent and cytoskeletal-associated serine/threonine kinase with death-inducing functions (9). Overexpression of DAP kinase killed HeLa cells in the absence of any external stimuli (9). Mapped to chromosome 9q34.1, the DAP kinase gene was initially isolated as a positive mediator of apoptosis induced by IFN- $\gamma$ , using a strategy of functional cloning (10, 11). Loss of expression of DAP kinase was frequently found in B-cell lymphoma and some carcinoma cell lines, which highlights its potential role as a tumor suppressor (12). In B-cell malignancies, the loss of expression was commonly associated with hypermethylation of the DAP kinase CpG island (13). Recently, DAP kinase has also been found to mediate apoptosis induced by TNF- $\alpha$  and Fas, whose expression is found in some MM cell lines and patient-derived primary cells (14–17). However, a poor response to Fas-induced apoptosis was observed in a majority of the MM cases and a correlation between Fas antigen expression and susceptibility to Fas-mediated apoptosis in MM could not be established (15–17).

These findings have prompted us to examine the methylation status of DAP kinase in MM, which may have potential implications in our understanding of its pathogenesis and prognosis.

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<sup>3</sup> The abbreviations used are: MM, multiple myeloma; DAP, death-associated protein; MSP, methylation-specific PCR; MGUS, monoclonal gammopathy of undetermined significance; TNF, tumor necrosis factor; BM, bone marrow; ATCC, American Type Culture Collection; PB, peripheral blood.

## MATERIALS AND METHODS

**Patients and MM Cell Lines.** After obtaining informed consent, BM materials were taken from 38 patients recruited for the study (36 patients with MM and 2 patients with MGUS) in the Chinese University of Hong Kong, the Prince of Wales Hospital between March 1996 and June 2000. The diagnosis and staging classification were made in accordance with the major and minor criteria of Durie (18) and Durie and Salmon (19). Morphologically, the MMs were classified into three histological subtypes, namely, mature, intermediate, and blastic, as described previously (20). Three human MM-derived cell lines, HS-Sultan, NCI-H929, and U266 (ATCC, Manassas, VA), were also analyzed.

**Normal Controls and Positive Control.** Thirty normal PB samples were obtained from healthy volunteer staff and students of the Chinese University of Hong Kong to serve as normal controls. Raji (ATCC), a Burkitt's lymphoma cell line, was used as a methylated control (12, 13).

**DNA Extraction and MSP.** The buffy coat fraction was isolated from BM aspirate, and total genomic DNA was extracted using standard SDS-proteinase K treatment, followed by phenol/chloroform/isoamylalcohol extraction. DNA methylation patterns in the CpG island of *DAP kinase* were determined by chemical treatment with sodium bisulfite (CpGenome DNA Modification Kit; Intergen) and subsequent use of the previously described PCR procedure (13, 21, 22). Bisulfite treatment allows differentiation between methylated and unmethylated cytosine residues to be distinguished and detected by sequence-specific PCR primers (13, 21, 22). Bisulfite-treated buffy coat DNA (1  $\mu$ g) was amplified using primers DAPUF (5'-GGA-GGA-TAG-TTG-GAT-TGA-GTT-AAT-GTT-3') and DAPUR (5'-CAA-ATC-CCT-CCC-AAA-CAC-CAA-3') for the unmethylated sequence and primers DAPMF (5'-GGA-TAG-TCC-GAT-CGA-GTT-AAC-GTC-3') and DAPMR (5'-CCC-TCC-CAA-ACG-CCG-A-3') for the methylated sequence (13, 23). PCR was conducted using the GeneAmp DNA Amplification Kit and AmpliTaq Gold polymerase (Perkin-Elmer, Foster City, CA) according to the conditions described previously (13). In brief, 25  $\mu$ l of PCR mixture contained 1 $\times$  PCR buffer [10 mM Tris-HCl (pH 8.3) and 50 mM KCl], 2 mM MgCl<sub>2</sub>, deoxynucleotide triphosphates (each at 250  $\mu$ M), primers (1  $\mu$ M each per reaction), bisulfite-modified DNA (80 ng) or unmodified DNA (80 ng), and 1 unit of AmpliTaq Gold polymerase. Reactions were hot started at 95°C for 10 min, and the annealing temperature was 58°C. Amplification was carried out in a Thermal Cycler 480 (Perkin-Elmer) for 35 cycles. Ten  $\mu$ l of the PCR reaction were electrophoresed onto 10% polyacrylamide gels, stained with ethidium bromide, and visualized under UV light. Methylated control (Raji), unmethylated control (normal blood sample), and negative control (water blank) were included in each experiment.

**Culture of Cell Lines.** HS-Sultan, NCI-H929, and U266 human MM-derived cell lines and the Raji (Burkitt's lymphoma) cell line were purchased from ATCC. These cell lines were cultured in RPMI 1640 supplemented with 10% or 15% heat-inactivated fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD).

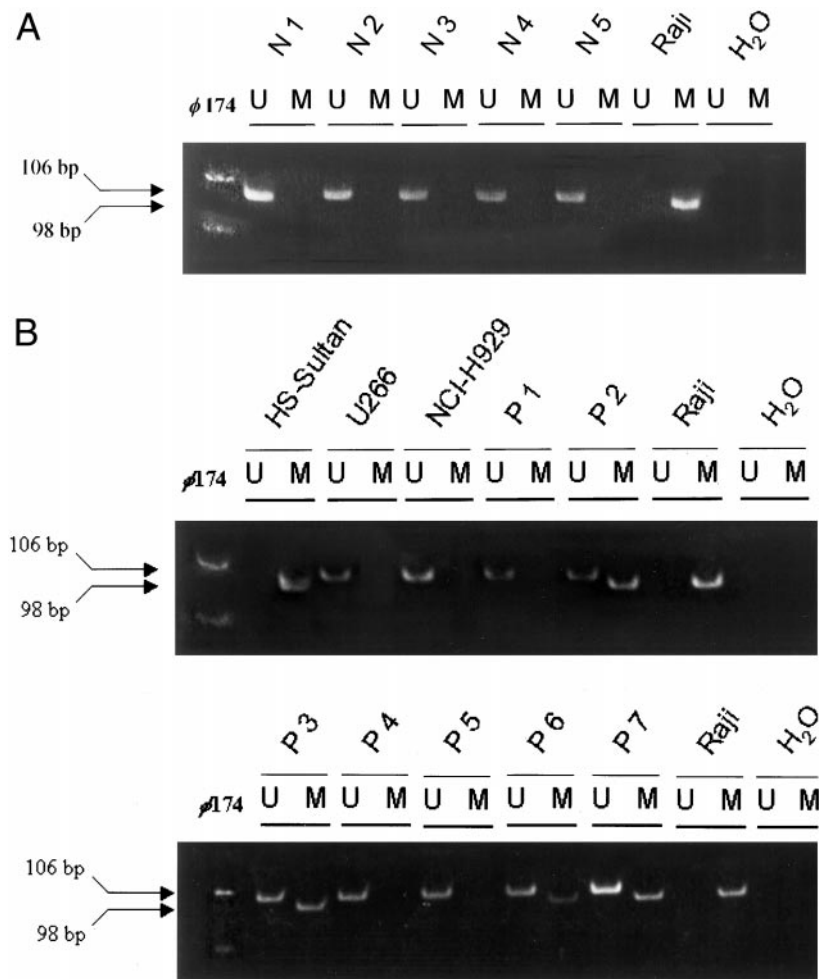
Table 1 DAP kinase methylation in 36 MM patients

	DAP kinase methylation		
	Pretreatment group (N = 26)	Posttreatment group (N = 10)	Total (N = 36)
Stage			
I	1/1	2/2	3/3
II	2/2	1/3	3/5
III	17/23	1/5	18/28
Immunoglobulin isotype			
G $\kappa$	7/9	4/7	11/16
G $\lambda$	6/7	0/2	6/9
A $\kappa$	4/5		4/5
A $\lambda$	1/1	0/1	1/2
D $\lambda$	1/1		1/1
$\kappa$	1/2		1/2
$\lambda$	0/1		0/1
Histology			
Mature	5/7	1/4 <sup>a</sup>	6/11 <sup>a</sup>
Intermediate	9/12	3/4	12/16
Blastic	6/7	0/2	6/9
Plasmacytoma			
Present	3/4	2/4	5/8
Absent	17/22	2/6	19/28
Total	20/26 (77%)	4/10 (40%)	24/36 (67%)

<sup>a</sup> One MM patient had no evidence of MM in BM (<5% of mature plasma cells) but had DAP kinase methylation.

## RESULTS

**Patient Characteristics.** A total of 38 patients, 36 MM patients (16 G $\kappa$ , 9 G $\lambda$ , 5 A $\kappa$ , 2 A $\lambda$ , 1 D $\lambda$ , 2 BJP $\kappa$ , and 1 BJP $\lambda$ ) and 2 patients with MGUS (G $\lambda$  and G $\kappa$ ), were analyzed for *DAP kinase* hypermethylation (Table 1). The male:female ratio was 1.7:1, with a median age of 66 years (range, 29–89 years). Five patients defaulted, 15 patients died (median survival, 7 months), and 18 were still alive after a median follow-up time of 23 months. In the MM group, 26 patients were analyzed at diagnosis, and 10 patients were analyzed at variable times after treatment (median treatment duration, 12 months). Twenty-eight patients had stage III disease, five patients had stage II disease, and three patients had stage I disease. Eight patients had plasmacytomas, as documented by histology. Six plasmacytomas involved bone, one plasmacytoma was found in chest wall muscle, and one plasmacytoma was found in pleura. Histologically, 10 MMs were classified as mature, 16 MMs were classified as intermediate, and 9 MMs were classified as blastic. One patient who fulfilled the other criteria of MM had less than 5% of mature plasma cells in the BM at both diagnosis and relapse. Excluding this patient, the mean percentage of plasma cell infiltration was 46  $\pm$  31% (range, 10–100%). The complete blood count revealed a mean hemoglobin of 9.1  $\pm$  2.2 g/dl (range, 4.5–15 g/dl), WBC count of 6.5  $\pm$  2.6  $\times$  10<sup>9</sup>/liter (range, 2.7–15  $\times$  10<sup>9</sup>/liter), and platelet count of 200.8  $\pm$  102.9  $\times$  10<sup>9</sup>/liter (range, 9–467  $\times$  10<sup>9</sup>/liter). Most MM patients were treated with melphalan and prednisone or dexamethasone with or without radiotherapy. A few patients were treated with vincristine, adriamycin, and dexamethasone or with cyclophosphamide, etoposide, vincristine, adriamycin, and dexamethasone. One patient with stable MM was given supportive blood transfusions only.



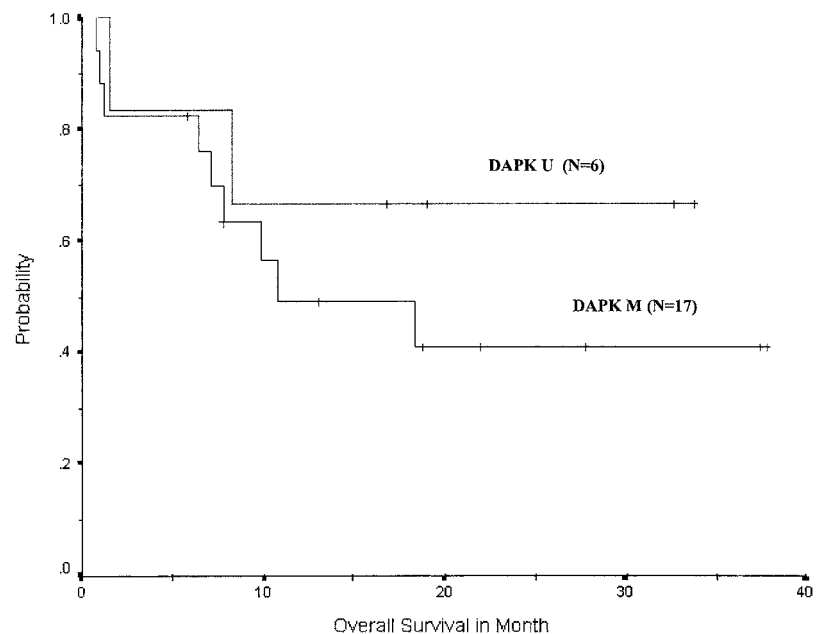
**Fig. 1** Methylation of the CpG island of the *DAP kinase* gene in primary MM and in MM cell lines. Examples of the MSP analysis on (A) normal controls (N1–N5) and (B) MM cell lines (HS-Sultan, U266, and NCI-H929) and primary MM samples (P1–P7) are shown. The presence of a visible PCR product in the Lanes U (size, 106 bp) or M (size, 98 bp) indicates the presence of unmethylated and methylated *DAP kinase*, respectively. Raji served as the methylated control, whereas water was used as a negative control. The molecular weight marker ( $\phi$ X174 RF DNA/*Hae*III fragments) is indicated on the left. A, the *DAP kinase* gene is unmethylated in all of the PB samples from healthy donors. B, HS-Sultan contains only methylated sequence, whereas U266 and NCI-H929 contain only unmethylated *DAP kinase*. In primary MM, P2, P3, P6, and P7 contain both unmethylated and methylated *DAP kinase*, whereas P1, P4, and P5 contain only unmethylated *DAP kinase*.

**Hypermethylation of *DAP Kinase* in Primary MM and MM Cell Lines.** The presence of a CpG island in the region of the first 525 bp of the *DAP kinase* transcription start site and transcriptional silencing associated with *DAP kinase* hypermethylation have been documented previously (12, 13). Using MSP with primer sequences as described previously (13, 23), we found no promoter hypermethylation of the *DAP kinase* gene in the 30 PB samples from normal healthy controls tested. Five of these samples are shown as N1–N5 in Fig. 1A. These data are consistent with previous studies, which showed no evidence of methylation of the gene in both normal controls and EBV-immortalized lymphoblastoid cell lines (12, 13). In contrast, a high frequency of hypermethylation of *DAP kinase* was found in the primary MM (67%; Table 1; Fig. 1B). Among the three MM cell lines tested, HS-Sultan demonstrated *DAP kinase* methylation. In all of the *DAP kinase*-methylated primary MM cases, unmethylated DNA bands were also observed as a result of the presence of normal BM cellular elements in all these samples (Fig. 1B). On the contrary, the HS-Sultan MM cell line revealed a complete methylation pattern with absence of the unmethylated band (Fig. 1B). One patient was diagnosed with MM because of the presence of bone plasmacytomas and significant G $\kappa$  paraproteinemia with immunoparesis. Despite re-

peated normal morphological findings on BM examination (<5% plasma cells) during diagnosis and relapse, *DAP kinase* hypermethylation was detected in this patient's BM sample at relapse. It is also noteworthy that of the two patients with MGUS tested, one demonstrated *DAP kinase* hypermethylation.

**Clinical Correlation of *DAP Kinase* Methylation.** The incidence of *DAP kinase* methylation in the pretreatment and posttreatment MM group was 77% and 40%, respectively. As a whole, *DAP kinase* methylation was observed at similar frequencies in MM of all stages (60–100%), paraprotein isotypes (50–100%), and histological grades (50–75%) and in MM with or without plasmacytoma (63–68%), findings that suggest that *DAP kinase* methylation is an early event during MM pathogenesis. This suggestion is further supported by the presence of *DAP kinase* methylation in one of the two patients with MGUS, which may represent a pre-MM condition. In terms of survival, among 23 MM patients followed prospectively, the median survival time for 17 patients with *DAP kinase* methylation at diagnosis was 11.3 months, which represents a 3-fold reduction compared with the 34-month survival time for 6 patients with unmethylated *DAP kinase* ( $P = 0.38$  by the Kaplan-Meier method and log-rank test; Fig. 2). Moreover, a generally poorer treatment response was observed in patients with methylated

**Fig. 2** Kaplan-Meier survival curves for 23 MM patients according to DAP kinase methylation category ( $P = 0.38$  by the Kaplan-Meier method and log-rank test). Probability refers to proportion surviving among MM patients. Seventeen MM patients had methylated DAP kinase (*DAPK M*), and six MM patients had unmethylated DAP kinase (*DAPK U*).



*DAP kinase* as compared with patients with unmethylated *DAP kinase*. In the methylated *DAP kinase* group, 2 patients were treated at the plateau phase, and the remaining 15 patients with active disease showed variable responses to chemotherapy that were considered good (rapidly decreasing paraprotein levels with improvement of symptoms), fair (slowly settling response), or poor (progressive disease) for 5 patients in each category. In contrast, among the six patients with unmethylated *DAP kinase*, one was in plateau phase, one demonstrated a fair response to chemotherapy, and the other four patients demonstrated good responses to chemotherapy, which were also more sustainable compared with the responses of patients with methylated *DAP kinase* who also had a good initial response.

## DISCUSSION

MM is an invariably fatal malignancy of clonal plasma cells with a wide variability in clinical features, responses to treatment, and survival times among patients, which suggests a high biological heterogeneity. The disease course is characterized by different phases: (a) an inactive phase; (b) an active phase; and (c) a fulminant phase with the frequent occurrence of extramedullary proliferation and an expansion of proliferative plasmablastic cells. The role of apoptosis and its regulation has become increasingly important in tumorigenesis (2, 3). *DAP kinase*, which was identified as a positive mediator of apoptosis induced by IFN- $\gamma$  (9, 10), has been shown to be commonly inactivated by promoter hypermethylation in B-cell malignancies (12, 13). In the present investigation, using MSP, we first demonstrated a high incidence of *DAP kinase* hypermethylation in primary MM and in MM cell lines. Our finding is consistent with the type of *DAP kinase* alteration found previously in B-cell malignancies and may extend its role in the pathogenesis of this spectrum of diseases. In the HS-Sultan MM cell line, it was also observed that *DAP kinase* methylation was associated

with loss of transcription, which was restored after demethylation treatment with 5-aza-2'-deoxycytidine concomitant with the reappearance of the unmethylated alleles detected by MSP.<sup>4</sup>

It has been postulated that loss of *DAP kinase* may confer a selective advantage during the multiple stages of metastasis for tumor cells with resistance to various apoptotic stimuli encountered after detachment from the original tumor and transport in the circulation (24). In lung carcinoma, loss of *DAP kinase* has been associated with a more aggressive highly metastatic phenotype. Thus, loss of *DAP kinase* expression provides a unique mechanism that links suppression of apoptosis to metastasis (24). Although slowly proliferating MM cells are localized predominantly in the BM, the disease manifests itself in a disseminated form with the consistent presence of a circulatory pool of MM precursors (25). Failure to eradicate and control this proliferative circulatory pool of MM cells may be the cause of the high fatality and poor treatment outcomes in this malignancy. It is possible that the selective advantage conferred by the inactivation of *DAP kinase* may play a role in the induction and maintenance of the circulatory MM tumor pool. The high frequency of *DAP kinase* hypermethylation in MM suggests that it may play a critical role in the etiology of MM. Furthermore, its frequent occurrence in MM of all stages, immunoglobulin isotypes, and histological grades and in MM with or without plasmacytomas may indicate that it is an early event in MM pathogenesis. This is further supported by the presence of *DAP kinase* hypermethylation in MGUS, which may represent a pre-MM condition.

There was apparently a shorter (3-fold) survival duration in the methylated *DAP kinase* group than in the unmethylated *DAP*

<sup>4</sup> Unpublished data.

kinase MM group (Fig. 2). In a recent study, a shorter 5-year survival rate has also been observed in patients with non-small cell lung cancer with *DAP kinase* hypermethylation (26). Whether this poor prognosis for patients with *DAP kinase* methylation in MM is related to enhanced survival of the circulatory MM precursor cells requires further study in the future.

The Fas antigen is a member of the TNF receptor family of proteins and is expressed in many neoplastic and normal cells including hematopoietic cell lines, lymphoma cells, and activated normal T and B lymphocytes (27, 28). Although Fas expression was observed on some MM cell lines and patient-derived primary cells, a poor response to Fas-induced apoptosis was demonstrated in a majority of the MM cases (15–17). The high expression of Fas antigen and the low number of cells induced to apoptose may suggest a defect in the Fas signaling pathway. Consistent with these observations, it has been shown recently that expression of *DAP kinase* antisense RNA protected HeLa cells from killing by anti-Fas/APO-1 agonistic antibodies (14). Thus, *DAP kinase* not only mediates cell deaths induced by IFN- $\gamma$  but also by TNF- $\alpha$  and Fas activation (14). The high frequency of *DAP kinase* hypermethylation (thus downstream signaling defect) in primary MM found in this study may explain, in part, the poor response of apoptosis induced by Fas activation. However, it was also observed that pretreatment with IFN- $\gamma$  augmented Fas-induced apoptosis in the MM cells (29). This suggests that a *DAP kinase*-independent pathway may operate in this latter death scenario.

We have demonstrated previously that there is a frequent hypermethylation of *p16* and *p15* genes in MM (20, 30, 31). Taken together, these data support the argument that multiple epigenetic events may be a common and important mode of gene inactivation that may coexist with other genetic alterations in the constellation of changes associated with MM transformation. The therapeutic potential of IFNs acts via their effects on growth inhibition and induction of apoptosis (6). In MM, IFN- $\alpha$  has been used in maintenance therapy. Because occasional growth-stimulatory effects on MM cells by IFN- $\alpha$  were also observed (8), it has been suggested that IFN- $\gamma$  may be an alternative (6). However, the treatment potential of IFN- $\gamma$  in MM may likely be limited in the presence of a high incidence of *DAP kinase* methylation, such as that found in this study. In contrast, clinical evaluation of the use of a demethylating agent, alone or in combination with other therapeutic agents, may be worthwhile in MM, where concurrent methylation of multiple genes is involved (20, 30, 31).

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