Brief report

Aberrant methylation of DAP-kinase in therapy-related acute myeloid leukemia and myelodysplastic syndromes

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Death-associated protein kinase (DAP-kinase), a proapoptotic serine/threonine kinase, is a candidate tumor suppressor gene. We studied the methylation status of DAP-kinase of 194 bone marrow samples from 160 patients with acute myeloid leukemia (AML) and 34 with a myelodysplastic syndrome (MDS) at the time of initial diagnosis by polymerase chain reaction (PCR). Hypermethylation of DAP-kinase was present in 27.5% (44 of 160) of AML and in 47% (16 of 34) of MDS specimens and significantly correlated to loss of DAP-kinase expression (*P* = .008). It was significantly more frequent in AML secondary to therapy for other malignancies (s-AML; 14 of 29, 48.3%), as compared to de novo AML (30 of 131, 22.9%, *P* = .01). DAP-kinase hypermethylation in AML was associated with myelodysplastic changes in the bone marrow at the time of the initial diagnosis (*P* = .002) and with the presence of cytogenetic abnormalities (*P* = .02). Alteration in the apoptotic response due to the loss of DAP-kinase function may be an early event in the transformation pathway to secondary leukemia via myelodysplasia. (Blood. 2004;103:698-700)

Introduction

Aberrant DNA hypermethylation plays an important role in tumor development and progression.[1-6] A hypermethylator phenotype has been described in several neoplasms, as in breast, prostate, or colorectal cancer. In acute myeloid leukemias (AMLs), Rush and colleagues found a wide variation in the amount of aberrant methylation among patients.[7] Other authors reported high levels of methylation in a subset of genes, including the estrogen receptor (ER), the myogenic transcription factor MyoD, and the epithelial adhesion molecule E-cadherin, whereas hypermethylation of other genes was relatively infrequent.[3,8] Because many of these hypermethylated genes are not expressed in hematopoietic cells, it is most probable that only a few have truly functional significance.[3]

Among genes identified as deregulated through hypermethylation and that could be of functional significance as tumor suppressor gene is death-associated protein kinase (DAP-kinase).[9] DAP-kinase is a proapoptotic calcium/calmodulin-regulated serine/threonine kinase that participates in several apoptotic pathways initiated by interferon γ (IFN-γ), tumor necrosis factor α (TNF-α), activated Fas, and detachment from extracellular matrix.[9,10] DAP-kinase is abnormally methylated in a significant proportion of human tumors, providing evidence that inactivation of this gene is critical for cancer development.[1,11-13] Among hematologic malignancies, all Burkitt lymphomas, 84% of B-cell lymphomas, 70% of multiple myelomas, 62% natural killer (NK)/T-cell lymphomas, and 10% to 30% AMLs show DAP-kinase hypemethylation.[1,11-13]

We studied the frequency of cytosine phosphate guanine (CpG) hypermethylation in adult AML and myelodysplastic syndromes (MDSs) and analyzed associations of DAP-kinase methylation status with patient characteristics.

Study design

Our retrospective analysis included 160 patients with AML (Table 1) and 34 patients with an MDS (19 with refractory anemia [RA], 9 with RA with blast excess [RAEB], 3 with RA with ring sideroblasts, and 3 with a 5q− syndrome), consecutively diagnosed between February 1992 and April 2003. The diagnosis was established according to standard morphologic and immunophenotypic criteria.[14] The presence of myelodysplasia was defined following the evaluation by 2 distinct expert observers of 300 marrow nucleated cells and 20 megakaryocytes on bone marrow smears stained with May-Grünwald-Giemsa panoptical stain. The diagnosis of AML "with myelodysplasia" was established when myelodysplastic features involved 2 or more cell lineages and were present in more than 10% of marrow cells.[14,15]

Twenty-nine patients had a leukemia secondary to a cytotoxic treatment for a previous malignancy (10 breast cancer, 4 Hodgkin disease, 4 non-Hodgkin lymphoma, 2 multiple myeloma, 1 essential thrombocythemia, 2 prostate, 2 thyroid, 1 bladder, 1 colon, 1 lung, and 1 ovarian cancer). Karyotype was available for 19 patients with secondary AML (s-AML; Table 2). Three patients had a partial or total chromosome 7 deletion, 1 of chromosome 5, and 1 of chromosome 16. Two patients had a trisomy of chromosome 8, 2 had a t(15;17) and 1 had a t(9;16) translocation, 1 had an inversion of chromosome 16, 4 had a normal karyotype, and 4 patients had a complex karyotype with ring chromosomes and aneuploidy. Six patients with a history of a previous malignancy treated with surgery alone were not included in the s-AML group and were included in the de novo AML group.

Mononuclear cells (MNCs) were separated from the bone marrow of AML patients at the time of initial diagnosis, using Ficoll density centrifugation, and DNA was extracted using DNAzol (Gibco BRL, Eggenstein, Germany). DAP-kinase methylation pattern was determined

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Table 1. Methylation status and patient characteristics

	No. of patients	DAP-kinase methylated, no. (%)	*P*	OR (95% CI)
Age			.1	—
Younger than 59 y	66	14 (21)		
60 y or older	94	30 (31.9)		
Sex			.5	—
Female	75	23 (30.7)		
Male	85	21 (24.7)		
Type			.01	3.1 (1.4-7.2)
De novo	131	30 (22.9)		
Therapy-related	29	14 (48.3)		
Cytogenetics			.02	3.1 (1.1-8.5)
Normal	47	6 (12.8)		
Abnormal	64	20 (31.5)		
Cytogenetic risk group			.02	—
Favorable	28	10 (35.7)		
Intermediate	65	9 (13.8)		
Unfavorable	18	7 (38.9)		
Myelodysplastic changes			.0004	3.7 (1.8-7.5)
No	114	25 (21.9)		
Yes	26	13 (50)		

The percentages shown represent the percentage of the total number of patients analyzed in each group. ORs are not given for nonsignificant differences (—; $P > .05$).

by methylation-specific polymerase chain reaction (MSP), using the primers specific for methylated or unmethylated DNA described by Katzenellenbogen et al.[11,16] DNA extracted from HeLa cells was used as positive control for methylated DNA, and DNA from normal lymphocytes was used as control for unmethylated DNA. Controls without DNA were performed for each polymerase chain reaction (PCR). Then, 10 μL of each PCR reaction was directly loaded onto a 3% agarose gel, stained with ethidium bromide, and visualized under UV illumination. The presence of a distinct methylated band on MSP defined hypermethylation. In a dilution curve we determined the sensitivity to clearly detect a methylated allele to be 6.75%.

To evaluate the functional significance of DAP-kinase hypermethylation, we studied its expression by reverse transcription–PCR (RT-PCR). Total RNA available from 37 patients was reverse transcribed using random hexamer as reaction primers.[11] DAP-kinase expression was compared to that of the housekeeping gene glyceraldehydes-3-phosphate dehydrogenase *(GAPDH)*. PCR products were analyzed on a 2% agarose gel, stained with ethidium bromide.

The statistical significance of the differences between groups was calculated using the Fisher exact test (2-sided). A multivariate analysis using the Cox regression model was also performed. Odds ratios (ORs) are given within 95% confidence intervals (CIs). Cytogenetic risk groups were defined according to Grimwade et al.[17] All computations were performed using the Stata 7.0 software (Stata, College Station, TX).

Results and discussion

We found aberrant DAP-kinase hypermethylation in 44 of 160 samples from patients with acute leukemia (27.5%; Table 1). Esteller et al[1] reported DAP-kinase hypermethylation in 8 of 86 AML cases and Katzenellenbogen et al in 2 of 6 cases.[11] DAP-kinase was not methylated in MNCs isolated from 13 bone marrow and 15 peripheral blood samples from elderly controls (median age, 66 and 76 years, respectively), as reported by other authors.[11,12] The functional role of hypermethylation was confirmed in 37 patients whose RNA was available, where hypermethylation correlated to loss of expression. Eleven of 17 methylated samples were negative for DAP-kinase on RT-PCR, whereas only 4 of 20 unmethylated samples were negative ($P = .008$).

We then analyzed for associations between DAP-kinase methylation status and patients' characteristics. A significantly higher frequency of methylated samples was found in therapy-related leukemias, when compared to de novo leukemias ($P = .01$; Table 1) and both patients with an MDS secondary to treatment for a solid tumor presented DAP-kinase hypermethylation. DAP-kinase hypermethylation was also significantly more frequent in AML with abnormal karyotype and in AML with associated MDS ($P = .02$ and .006, respectively; Table 1). In the multivariate analysis, including presence of MDS, treatment for a previous malignancy and karyotype, the presence of MDS proved to be an independent factor for DAP-kinase hypermethylation ($P = .03$).

The induction of myelodysplastic changes and hypermethylation in hematopoietic stem cells may be an early epigenetic change during cytotoxic therapy.[18] Radiation exposure induces a high rate of ER methylation in rodent models of lung cancer.[19] In the "multistep model" of carcinogenesis of colorectal tumors, hypermethylation of different genes can already be detected in precancerous lesions, arguing for a role for hypermethylation during the initiation of tumorigenesis.[20] Along this line, in our patients, DAP-kinase hypermethylation was a frequent finding in AML with associated MDS and in primary MDS (47%), although no differences became evident when grouping patients according to the type of MDS. Accordingly, a common feature of MDSs is a decreased apoptosis rate in bone marrow progenitor cells.[21] It will be interesting to study changes in the methylation profile in a longitudinal way from cytotoxic therapy to s-AML in high-risk patients.

We found a significant correlation between DAP-kinase hypermethylation and older age in s-AML (Table 2). Studies on the methylation status in the normal colonic mucosa revealed that methylation density of CpG islands steadily increases with age.[22] The mechanisms causing altered DNA methylation during aging are incompletely understood but may include exposure to environmental agents and drugs or altered methyltransferase abilities.

Interestingly, 6 of 10 patients with a history of a B-lymphoproliferative disorder and both patients with a previous

Table 2. DAP-kinase and therapy-related AML

	Unmethylated, n = 15	Methylated, n = 14	*P*
Age, y, mean ± SEM	51.9 ± 3.6	65.6 ± 2.2	.003
Sex, F/M	12/3	8/6	.2
Morphology			.7
No MDS	8	6	
With MDS	4	5	
Primary tumor, n (%)			.3
Breast	7 (70)	3 (30)	
Lymphoproliferative	4 (40)	6 (60)	
Others	4 (44.4)	5 (55.6)	
Time from primary tumor, y, mean ± SEM	7.9 ± 1.8	4.4 ± 0.7	.09
Treatment, n (%)			—
Chemotherapy	11 (52)	10 (48)	
Alkylating agents	7 (50)	7 (50)	
Topo inhibitors	6 (66.6)	3 (33.3)	
Alkylating agents + topo inhibitors	5 (71.4)	2 (28.6)	
Chemotherapy + radiotherapy	2 (40)	3 (60)	
Radiotherapy alone	3 (43)	4 (57)	

Morphology subpopulations were as follows: no MDS, n = 14; and with MDS, n = 9. Primary tumor subpopulations evaluated were as follows: breast, n = 10; lymphoproliferative, n = 10; and others, n = 9.

— indicates not calculated.

prostate cancer presented DAP-kinase hypermethylation. These malignancies have frequent hypermethylation in the DAP-kinase gene,[1,11,12] which may indicate an individually increased methyltransferase ability that could increase the susceptibility to the development of multiple malignancies.

In immortalized cell lines, which frequently lack DAP-kinase expression, the latter is induced by treatment with demethylating agents, including 5-aza-2′-deoxycytidine, restoring the normal apoptotic activity.[11] These data point to a role for the inclusion of demethylating agents into treatment protocols for AML, in particular for therapy-related forms.[23,24]

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