EUROPEAN
JOURNAL OF HAEMATOLOGY

Promoter hypermethylation of $p15^{INK4B}$, $HIC1$, $CDH1$, and ER is frequent in myelodysplastic syndrome and predicts poor prognosis in early-stage patients

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Eur J Haematol 2006: 76: 23–32. © Blackwell Munksgaard 2005.

Abstract: The propensity of myelodysplastic syndrome (MDS) to transform into acute myeloid leukemia (AML) suggests the existence of common pathogenic components for these malignancies. Here, four genes implicated in the development of AML were examined for promoter CpG island hypermethylation in cells from 37 patients with different stages of MDS. Aberrant methylation was detected by polymerase chain reaction amplification of bisulfite-treated DNA followed by denaturing gradient gel electrophoresis. The highest rate of methylation was found for $p15^{INK4B}$ (51%), followed by $HIC1$ (32%), $CDH1$ (27%), and ER (19%). Concurrent hypermethylation of ≥3 genes was more frequent in advanced compared with early-stage MDS ($P \leq 0.05$), and hypermethylation of $p15^{INK4B}$ was associated with leukemic transformation in early MDS ($P \leq 0.05$). The median overall survival was 17 months for cases showing hypermethylation of ≥1 genes vs. 67 months for cases without hypermethylation ($P = 0.002$). Specifically, promoter hypermethylation identified a subgroup of early MDS with a particularly poor prognosis (median overall survival 20 months vs. 102 months; $P = 0.004$). In multivariate analysis including stage and thrombocyte count, hypermethylation of ≥1 genes was an independent negative prognostic factor ($P < 0.05$). These data suggest that hypermethylation of $p15^{INK4B}$, $HIC1$, $CDH1$, and ER contribute to the development and outcome of MDS.

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Myelodysplastic syndrome (MDS) comprises a heterogeneous group of clonal disorders with varying degrees of bone marrow (BM) failure, characterized by hematopoietic progenitors that fail to fully differentiate. Approximately 30% of the cases will eventually transform into acute myeloid leukemia (AML). The MDS diagnosis is usually based on the exclusion of other reasons for BM failure. According to the French-American-British (FAB) classification (1), the disease complex can be divided into five subgroups on the basis of cell morphology and blast counts in BM aspirates: refractory anemia (RA), refractory anemia with ringed sideroblasts (RARS), refractory anemia with excess blasts (RAEB), chronic myelomonocytic leukemia (CMML), and refractory anemia with excess blasts in transformation (RAEB-t). Despite the indubitable clinical value of the FAB classification, each subgroup is highly heterogeneous with respect to pathogenesis and clinical outcome. Other scoring systems encompassing clinical variables and karyotypic analyses have been proposed (2), and recently the World Health Organization (WHO) published refined classification schemes to enhance the prognostic utility of MDS classification (3). Still, prognostic

discrimination is challenging, particularly in low-grade patients.

Several lines of evidence have suggested that classification of MDS may be improved by including data on molecular changes, which represent the underlying leukemogenic mechanisms in MDS (for a recent review, see 4). A number of structural and cytogenetic changes occur with high frequencies in MDS, including trisomy 8 and deletions of chromosomes 5q and 7. Specifically, deletion of the long arm of chromosome 5 is associated with a better prognosis, and the 5q− syndrome has been included as a clinically distinct entity in the WHO classification. However, the genes targeted by this and other cytogenetic aberrations have not been identified, and hence the pathogenesis remains obscure. In recent years, transcriptional inactivation of tumor suppressor genes by promoter CpG island hypermethylation has been a subject of intense interest as a causal factor in hematologic malignancies (reviewed in 5). One of the most frequent and best studied epigenetic events in MDS is silencing of the cyclin-dependent kinase inhibitor $p15^{INK4B}$, which controls the progression of cells from G1 to S phase. Hypermethylation of the $p15^{INK4B}$ promoter region occurs in approximately 50% of MDS cases (6), most frequently in high-risk cases (i.e. RAEB and RAEB-t), and has been reported to be acquired during disease progression (7, 8), and to be associated with leukemic transformation (9) and poor prognosis (7, 8).

Given the preliminary indications that promoter hypermethylation may play a role in the development of MDS, we undertook an investigation aiming at identifying more genes targeted by this mechanism. Specifically, we wished to examine the methylation status of genes previously implicated in the development of AML, and to address the possible diagnostic and prognostic implications of promoter hypermethylation in MDS. Several methods for demonstrating promoter methylation have been developed (10–14). For this study, we have used bisulfite-denaturing gradient gel electrophoresis (bisulfite-DGGE) (13), which has the advantage of providing a detailed methylation band pattern in heterogeneous partially methylated samples (13). By analyzing CD34$^+$ cell populations and/or BM from 37 patients with MDS, we show that promoter hypermethylation of the $p15^{INK4B}$, E-cadherin (*CDH1*), hypermethylated in cancer 1 (*HIC1*), and estrogen receptor (*ER*) genes occurs frequently in MDS, similar to the situation in AML (5, 15). Furthermore, our data show that hypermethylation of $p15^{INK4B}$, *CDH1* or *HIC1* is associated with leukemic transformation and poor prognosis in low-risk MDS.

Materials and methods

Patient material

Cryopreserved mononuclear cells (MNC) were obtained from BM from 37 patients with MDS, including 14 RA, three RARS, 11 RAEB, four RAEB-t, and five AML secondary to MDS (MDS/AML) (Table 1). All material was obtained from patients referred to the Department of Hematology, Aarhus University Hospital. As successful karyotypic analysis was not available in all patients and blast counts were based on evaluation of only 100 nucleated cells in most of the patients, clinical data did not allow us to employ the International Prognostic Scoring System (IPSS) for all. Instead patients were stratified into early (RA and RARS) and advanced (RAEB, RAEB-t and MDS/AML) MDS according to the FAB classification. None of the patients had received cytoreductive therapy prior to initial methylation status sampling. Cytoreduction was instituted in two early stage patients (one low-dose Ara-C, one hydroxyurea) and in eight with advanced MDS (low-dose Ara-C in three, five with anthracycline containing regimens). Only one patient (UPN 12) received an allogeneic transplantation due to severe BM failure. Patients were sampled at scheduled follow-up depending on clinical status, meaning that the sequential analyses presented were performed when enough cells had been available for cryopreservation after routine examination. The patient material was collected according to the principles in the Helsinki II Declaration and protocols were approved by the Local Ethical Committee for the County of Aarhus. In addition, the Danish Board of Registries approved the biobase from which samples were obtained. BM-MNC from seven normal donors served as non-malignant controls for CD34 isolation.

CD34$^+$ cell isolation

CD34 positive cells from BM-MNC were isolated by magnetic cell sorting, using the CD34 Progenitor Cell Isolation Kit (MACS; Miltenyi Biotech GmbH, Bergisch Gladbach, Germany), according to the manufacturer's guidelines. The purity of the CD34$^+$ cells was determined by flow cytometry (median 87% and 83% CD34$^+$ in MDS and normal, respectively).

DNA isolation and bisulfite modification

Genomic DNA was isolated from 10^5–10^6 cells on a MagNAPure Robot (Roche Diagnostics GmbH, Mannheim, Germany) and modified with sodium bisulfite as described previously (16). Briefly, 0.5–

Table 1. Demographic, cytogenetic and molecular data in 37 MDS patients

UPN	Sex/age	FAB[1]	Sampling[2]	Cytogenetics	Promoter hypermethylation				Transformation to AML	Survival[3] (months)
					$p15^{INK4B}$	CDH1	HIC1	ER		
1	M/50	RAEB	0	−5,+8	+	+	+	+	−	8
2	F/58	RAEB	NA	−7,16q−,17p−	+	−	+	−	−	NA
3	F/46	RA	0	+8	+	−	−	−	−	12
4	M/55	RAEB	3	Normal	−	−	−	−	+	7
5	M/68	RAEB-t	0	NM	−	−	−	−	−	7
6	M/63	MDS/AML	3	Complex	+	−	−	−	+	4
7	M/82	RAEB-t	9	Normal	+	−	+	+	+	11
8	M/65	RA	34	17p−	−	−	−	−	−	102
9	F/69	RAEB-t	12	12q−	+	+	+	+	+	29
10	M/54	RAEB	2	−7	+	+	+	+	−	3
11	F/69	RARS	32	Normal	−	−	−	−	−	53
12	M/47	RARS	0	5q−	+	+	−	−	+	50[4]
13	F/79	RA	6	Normal	−	−	−	+	−	23
14	M/72	RA	0	Normal	+	−	−	+	−	7
15	M/66	MDS/AML	62	Normal	−	−	−	−	+	86
16	F/67	RA	0	Normal	−	−	−	−	−	63[4]
17	F/74	RA	55	NA	−	−	−	−	−	59
18	F/76	RA	49	16q−,17p−,20p−	−	−	−	−	−	240[4]
19	F/56	MDS/AML	17	Complex	+	+	+	+	+	36
20	M/72	RAEB	0	+8	−	−	−	−	+	10
21	M/69	RAEB	24	Complex	−	−	−	−	−	67
22	F/47	RA	27	Complex	−	−	−	−	−	61
23	M/75	RAEB-t	14	17p−	+	+	+	−	+	24
24	M/74	RAEB	65	Normal	−	−	−	−	−	75
25	M/71	RARS	62	NA	−	−	−	−	−	131
26	M/46	MDS/AML	12	der(1)t(1;7),−7,+21	+	+	+	−	+	17
27	F/77	MDS/AML	NA	+6	−	−	+	−	+	NA
28	F/65	RA	3	Complex	+	+	+	−	+	24
29	F/68	RAEB	37	5q−,17p−,20q−	+	−	−	−	−	82
30	M/69	RA	0	−Y,17p−	−	−	−	−	−	8
31	M/71	RAEB	NA	Normal	+	+	−	−	−	NA
32	F/61	RA	NA	Complex	−	−	−	−	NA	NA
33	F/85	RAEB-t	17	11p−	+	+	−	−	−	23
34	F/50	RAEB-t	0	Complex	+	−	+	−	−	2
35[5]	M/61	RA	5	Complex	+	−	+	−	+	20
36	M/60	RA	1	NM	+	−	−	−	−	9
37	M/23	RA	51	16q−,17p−	−	−	−	−	−	223

FAB type and methylation status is shown for the first sample in those patients, in whom more samples were analyzed.

UPN, unique patient number; NM, no metaphases; NA, not available; Complex, more than three clonal aberrations.

[1]FAB type at the time of the first analyzed sample.

[2]Time (in months) from date of diagnosis to sample date.

[3]Survival is given as time between diagnosis and time of death or end of follow-up.

[4]Alive at the end of follow-up.

[5]RA and $p15^{INK4B}$ hypermethylated at diagnosis 5 months earlier, but not tested for methylation in the other genes due to lack of material.

2 µg of DNA were denatured in 0.3 M NaOH for 15 min at 37°C, followed by the addition of sodium bisulfite to a final concentration of 3.1 M and hydroquinone to a final concentration of 2.5 mM. After incubation at 55°C for 16 h, the DNA samples were recovered using the GeneClean II kit (Bio 101 Inc., Vista, CA, USA), desulfonated in 0.3 M NaOH, and ethanol precipitated. DNA was resuspended in Tris–EDTA and used immediately or stored at −80°C until use.

PCR and bisulfite-DGGE

Primers specific for bisulfite-reacted DNA were designed using the OLIGO software version 6.76 (Molecular Biology Insights, Plymouth, MN, USA). Generation of melt maps was performed using MACMELT software (Bio-Rad Laboratories, Hercules, CA, USA). Polymerase chain reaction (PCR) was carried out in a final volume of 25 µL containing 15–100 ng of bisulfite-modified DNA, 1x HotStarTaq PCR buffer with 1.5 mM MgCl$_2$, 0.2 mM cresol red, 12% sucrose, 0.2 mM each dNTP, 0.5 µM each primer and 0.75 U of HotStarTaq enzyme (Qiagen, Hilden, Germany). The enzyme was activated by incubation at 95°C for 10 min followed by 40 cycles of 95°C for 30 s, 55°C (48°C for CDH1) for 30 s and 72°C for 30 s, and a final extension at 72°C for 5 min. PCR was performed in a GeneAmp PCR System 9600 (Applied Biosystems, Foster City, CA, USA). Primer sequences were: [CCGCC]-GTTAGGAGTTTTTTTTAGA-

AGTAATTT (*p15*^{*INK4B*}: F), [GC3]-AAACTAAA-
CTCAACTTCATTACCCTC (*p15*^{*INK4B*}: R),
[GC1]-GGGTTTATTTGGTTGTAGTTA(*CDH1*:
F), CTCCAAAAACCCATAACTAAC (*CDH1*:
R), [GC1]-ATAATTAGAGTATTAAGGGTTTT-
TTGTG (*HIC1*: F), [CGCCCGCCGC]-
CACCCAAAAACTTAAAATAAACACTACTA
(*HIC1*: R), [GGCGCGGC]-GTTTTGGGATTG-
TATTTGTTTT (*ER*: F), [GC1]-AACCACCTA-
AAAAAAAAACACAA (*ER*: R). Nucleotides
in brackets represent GC-clamps; [GC1] =
CGCCCGCCGCGCCCCGCGCCCGTCCCGCC-
GCCCCCGCCCG [GC3] = CCCGCCGCCCG-
CCGCTCGCCCGCCGCGCCCCGCGCCCGTC-
CCGCCGCCCCCGCCCG. Fifteen microliters of
the GC-clamped PCR-product were loaded onto
a 10% denaturant/6% polyacrylamide – 70%
denaturant/12% polyacrylamide double gradient
gel (17). Gels were run at 160 V for 4.5 h in 1x Tris
acetate/EDTA buffer kept at a constant temperature
of 58°C (*p15*^{*INK4B*} and *ER*) or 55°C (*CDH1* and
HIC1). After electrophoresis, gels were stained in 1x
Tris acetate/EDTA buffer containing ethidium bro-
mide (2 μg/mL) and photographed under UV trans-
illumination. A fully methylated (SssI) and an
unmethylated (peripheral blood lymphocytes) con-
trol were loaded on all gels. Samples were scored as
methylated when bands or smears were present on
the gels in the area below the band corresponding to
unmethylated DNA.

Statistics

Fisher's exact test was used to compare methylation
status between patient groups. Survival curves were
plotted using the Kaplan–Meier method; differ-
ences between curves were analyzed using the
log-rank test. Cox regression models were used
for uni- and multivariate analyses.

Results

Hypermethylation of the *p15*^{*INK4B*}, *HIC1*, *CDH1*, and *ER* promoter CpG islands in MDS

To examine the methylation status of the
p15^{*INK4B*}, *CDH1*, *HIC1*, and *ER* promoter CpG
islands in MDS, we initially analyzed paired
samples of separated CD34⁺ cells and total BM-
MNC from 17 patients with MDS and seven
normal controls. Figure 1 shows representative
bisulfite-DGGE experiments. While the normal
controls consistently showed unmethylated gene
promoters, all four genes were found to be
hypermethylated at variable rates in the MDS
samples. In general, the DGGE band patterns
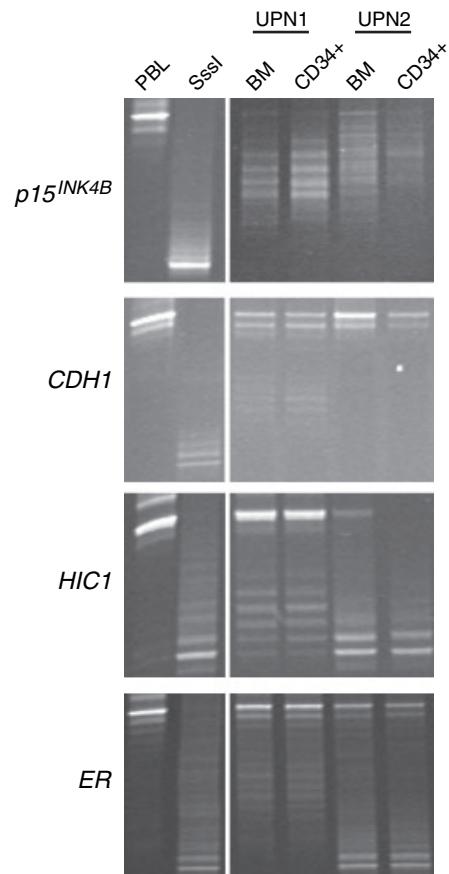were complex, showing extensive intra- and inter-

Fig. 1. Promoter hypermethylation in MDS detected by
bisulfite-DGGE. Bisulfite-modified DNA was amplified
with primers specific for *p15*^{*INK4B*}, *CDH1*, *HIC1* or *ER* and
analyzed in denaturing gradient gels. PBL, peripheral blood
lymphocytes from an healthy individual (unmethylated);
SssI, *in vitro* methylated DNA (fully methylated); UPN 1
and UPN 2; MDS patients analyzed for promoter hyper-
methylation in unseparated BM-MNC (BM) and MACS-
isolated CD34⁺ cells (CD34⁺). Hypermethylation was
observed as distinct bands or smears below the band
corresponding to the unmethylated control.

individual heterogeneity with respect to promoter
methylation density, as previously described in
AML (13, 15, 18). The same patterns of promoter
hypermethylation were found in CD34⁺ cells and
total BM-MNC (Fig. 1). For three of the patients,
the fraction of methylated alleles was larger in the
CD34⁺ cells than in total BM-MNC (e.g. Fig. 1;
UPN 2, *HIC1*). Nevertheless, these data suggest
that information on promoter methylation status
could be reliably obtained by analysis of DNA
from total BM-MNC.

Methylation analysis was subsequently per-
formed on BM-MNC DNA from 20 additional
patients, for whom isolation of CD34⁺ cells was
not possible due to an insufficient number of
banked cells. Data on promoter methylation status
for all 37 patients are given in Table 1. The highest
rate of methylation was found for *p15*^{*INK4B*} (19/37;

51%), followed by *HIC1* (12/37; 32%), *CDH1* (10/37; 27%), and *ER* (7/37; 19%). Notably, hypermethylation of *CDH1*, *HIC1*, and *ER* coincided with $p15^{INK4B}$ hypermethylation in all cases except for UPN 13 and UPN 27, which showed hypermethylation of *ER* and *HIC1*, respectively.

The distribution of hypermethylation events in early (RA and RARS) vs. advanced (RAEB, RAEB-t, and MDS/AML) MDS is shown in Fig. 2. For all four genes, hypermethylation was more frequent in advanced stages than in early stages (Fig. 2A), although the difference was only statistically significant for *HIC1* ($P < 0.05$). Concurrent hypermethylation of ≥3 genes was more frequent in advanced than in early stages of MDS ($P < 0.05$; Fig. 2B).
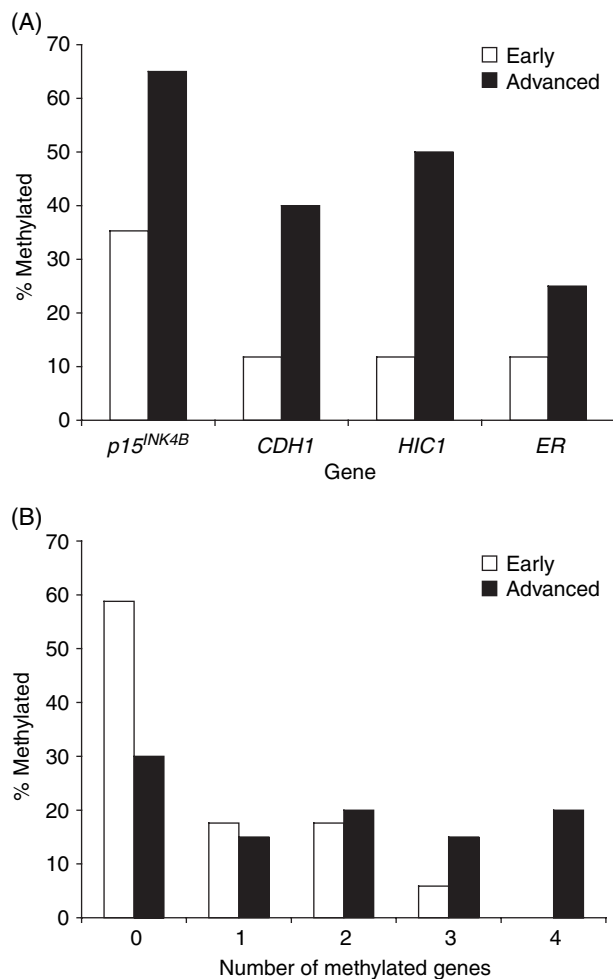

(A)

(B)

Fig. 2. Distribution of hypermethylation events in early and advanced MDS. (A) Frequencies of promoter hypermethylation. *HIC1* was more frequently hypermethylated in advanced compared with early MDS ($P < 0.05$). For $p15^{INK4B}$ and *CDH1*, similar trends were observed ($P = 0.1$ and 0.07, respectively). (B) Number of hypermethylated genes. Concurrent hypermethylation of ≥3 genes occurred more frequently in advanced than in early MDS ($P < 0.05$).

Sequential analysis of methylation status

To determine the dynamics of promoter methylation during the course of disease, BM-MNC were analyzed from 11 MDS cases from which sequential samples were available, with observation times ranging from 43 to 1132 days (median 284). These analyses showed that both the profiles of hypermethylated genes (Fig. 3A) and the methylation patterns of individual promoters (Fig. 3B) remained stable over time. Thus, the number of methylated genes changed in only three of the patients. One of these cases (UPN 2) showed hypermethylation of $p15^{INK4B}$ and *HIC1* in the initial sample, but in addition had acquired *ER* hypermethylation when sampled again 25 months later. All three genes remained hypermethylated in the following two samples from this patient, who had been diagnosed as RAEB at the two first samplings and as RAEB-t at the two latest sampling dates. The second case (UPN 11), which had RARS at all three samples dates, was unmethylated at all four genes in the two first samples, but had acquired $p15^{INK4B}$ hypermethylation in the third sample. Finally, UPN 3 showed $p15^{INK4B}$ hypermethylation at diagnosis and displayed the same methylation status 8 months later while still RA. However, three months later, when the patient had progressed to RAEB, $p15^{INK4B}$ hypermethylation could no longer be detected. The remaining eight cases showed the same profiles of hypermethylated genes ($N = 5$) or lack of promoter hypermethylation ($N = 3$) at all sample dates.

Correlation of promoter hypermethylation with transformation to MDS/AML and median survival time

In the group of 16 early-stage MDS patients for whom information on transformation was available, six showed hypermethylation of the $p15^{INK4B}$ promoter (Table 2). Three of these cases later progressed to MDS/AML, while none of the 10 cases without $p15^{INK4B}$ hypermethylation transformed to AML ($P < 0.05$). Hypermethylation of *CDH1* or *HIC1* was also suggested to be associated with leukemic transformation in early MDS, but subgroups were too small to allow for statistical evaluation (data not shown). In contrast, no statistically significant correlations between specific methylation events and transformation were found in the group of patients with advanced disease (Table 2).

Overall survival of the patients was also found to be dependent on methylation status (Fig. 4 and Table 3). Patients showing hypermethylation of ≥1 genes had a significantly shorter survival than patients without methylation of any of the four
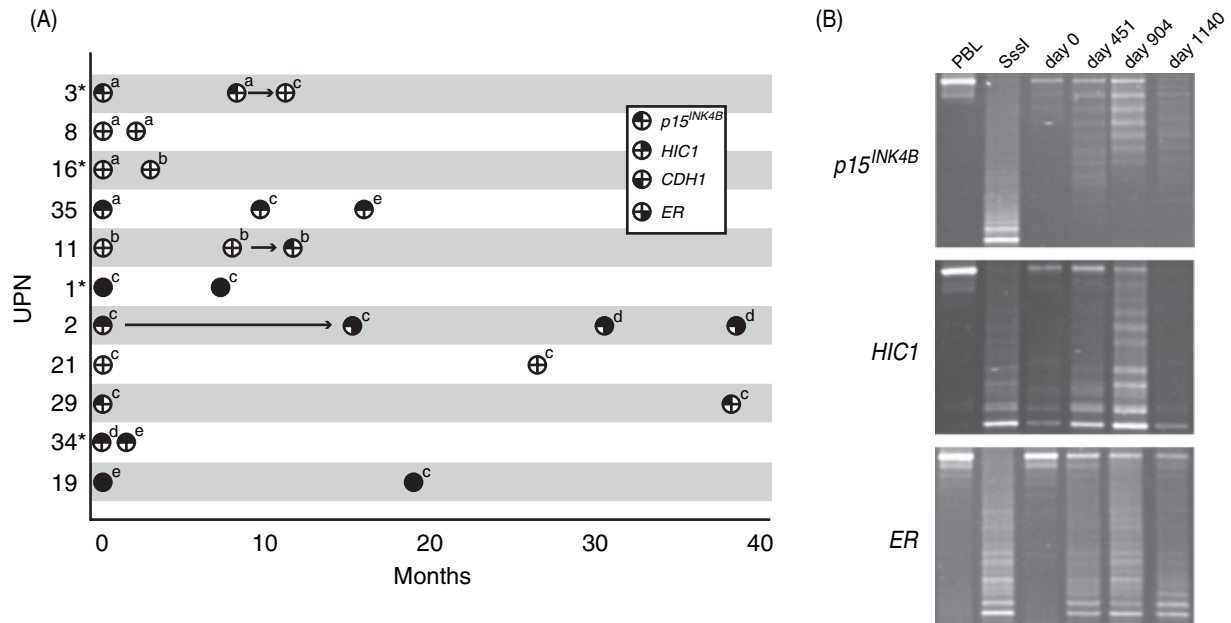
Fig. 3. Promoter hypermethylation status over time. (A) Development in methylation status in 11 patients. Arrows indicate patients where methylation status changes over time. a, RA; b, RARS; c, RAEB; d, RAEB-t; e, MDS/AML. Day 0 is the time of the first analyzed sample. *Patients where the first sampling was taken at time of diagnosis. Time intervals between samples are given as months from the first sample. Black quarters: methylated; white quarters: unmethylated. (B) Bisulfite-DGGE experiments for methylation of the $p15^{INK4B}$, *HIC1* and *ER* promoters in UPN 2. The FAB type at day 0 and 451 was RAEB and had progressed to RAEB-t at day 904 and 1140. At day 0 hypermethylation was observed in the $p15^{INK4B}$ and *HIC1* genes. From day 451 hypermethylation was also observed in the *ER* promoter.

Table 2. Association between transformation and promoter methylation status

Gene	Number of transformed/untransformed cases		P
	Unmethylated	Methylated	
All patients (N = 36)			
≥1 genes	3/12	10/11	0.16
$p15^{INK4B}$	4/13	9/10	0.18
HIC1	5/19	8/4	0.01
CDH1	7/19	6/4	0.12
ER	10/19	3/4	0.69
Low-risk MDS (N = 16)			
≥1 genes	0/9	3/4	0.06
$p15^{INK4B}$	0/10	3/3	0.04
High-risk MDS (N = 20)			
≥1 genes	3/3	7/7	1
$p15^{INK4B}$	4/3	6/7	1

genes (median 17 months vs. 67 months; $P = 0.002$). This difference in survival was even more pronounced when the analysis was confined to patients with early MDS (20 months vs. 102 months; $P = 0.004$), whereas there was no statistically significant difference in the group of advanced MDS patients. When evaluating course of disease in the subgroups of early-stage patients, we initially observed that two patients received cytoreduction, both due to progression, and both in the methylated group. Conversely, none of the patients unmethylated at primary sampling subse-quently received cytoreduction. The methylation status of some individual gene promoters also correlated negatively with survival, with the highest level of significance for $p15^{INK4B}$ (Table 3).

In univariate analyses, hypermethylation of ≥1 genes, hypermethylation of $p15^{INK4B}$, hypermethy-lation of *HIC1*, hypermethylation of *ER*, stage (early/advanced) and thrombocyte count showed prognostic value for survival (Table 4). In a Cox three-variate analysis of the prognostic implications of these variables, only hypermethylation of ≥1 genes remained a prognostic factor ($P < 0.05$; Table 4).

Discussion

It is generally assumed that MDS is a stem cell disorder and that most, but not necessarily all, myelopoiesis in patients with MDS is defective, resulting in the accumulation of immature cells in the BM and varying degrees of cytopenias in the peripheral blood. While this mechanistic approach is useful in the management of MDS, it does not address the etiology behind these disorders, which is generally assumed to be the result of multi-step hits at the genomic level. While cytogenetic analysis is clearly central to the diagnosis, aberrations not detectable by karyotypic analysis are likely to play additional roles.
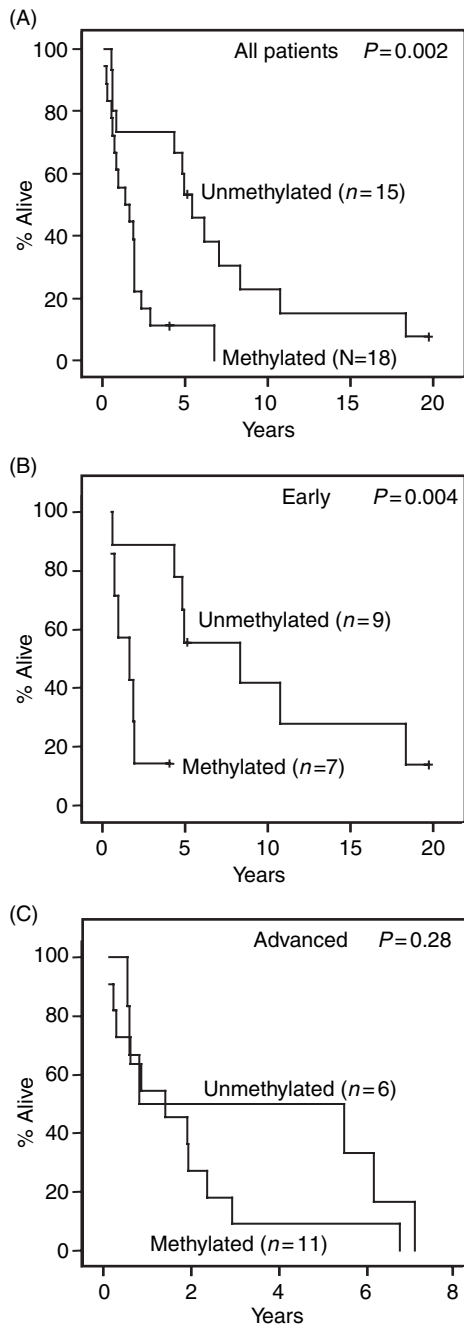
Fig. 4. Kaplan–Meier survival curves according to methylation status in ≥1 genes. (A) All patients; (B) early MDS patients; and (C) advanced MDS patients.

Table 3. Association between median overall survival and promoter methylation status

Gene(s)	Survival in months (M)		P
	Unmethylated	Methylated	
All patients (N = 33)			
≥1 genes	67 (15)	17 (18)	0.002
$p15^{INK4B}$	61 (16)	17 (17)	0.003
HIC1	59 (23)	17 (10)	0.01
ER	24 (26)	11 (7)	0.02
CDH1	59 (24)	24 (9)	0.23
Early MDS (N = 16)			
≥1 genes	102 (9)	20 (7)	0.004
$p15^{INK4B}$	61 (10)	12 (6)	0.01
Advanced MDS (N = 17)			
≥1 genes	10 (6)	17 (11)	0.28
$p15^{INK4B}$	10 (6)	17 (11)	0.28

Table 4. Univariate and multivariate analyses of prognostic factors for survival in MDS

Variable	P value	HR	95% CI for HR
Univariate analyses			
Hypermethylation			
≥1 gene	0.003	3.81	1.58–9.23
$p15^{INK4B}$	0.005	3.41	1.45–8.00
HIC	0.01	3.08	1.26–7.51
ER	0.03	2.87	1.14–7.25
CDH1	NS	1.72	0.70–4.23
Thrombocyte count	0.04	2.43	1.06–5.57
Stage (early/advanced)	0.03	2.38	1.08–5.24
Leukocyte count	NS	1.09	0.47–2.53
FAB			
RA	NS	0.58	0.18–1.92
RARS	NS	0.35	0.06–1.99
RAEB	NS	1.12	0.32–3.91
RAEB-t	NS	1.93	0.52–7.18
MDS/AML	NS	1.00	
Age	NS	1.00	0.98–1.03
Karyotype by G-banding			
Good risk	NS	1.00	
Intermediate risk	NS	0.94	0.27–3.30
Adverse risk	NS	1.65	0.49–5.51
NA/NM	NS	0.77	0.23–2.56
Cox multivariate analyses			
Hypermethylation			
≥1 gene	0.03		
$p15^{INK4B}$	0.06		
HIC	0.15		
ER	0.07		
Thrombocyte count	0.20*		
Stage (low risk/high risk)	0.32*		

HR, hazard ratio (relative risk); CI, confidence interval; NS, not significant (P > 0.05); NA; not available, NM; no metaphases.
*P values from multivariate analysis with ≥1 genes.

The data presented here strongly suggest that aberrant promoter hypermethylation events could be important features of the malignant phenotype in MDS. Specifically, we have shown that the $p15^{INK4B}$, HIC1, CDH1, and ER promoter CpG islands are targeted by hypermethylation at appreciable rates in MDS (19–51%), and that these events have prognostic significance. Hypermethylation of $p15^{INK4B}$ was observed in 35% of patients with RA or RARS, which is more frequent than in some earlier studies (7–9), but in accordance with the 32% found by compilation of available data in the literature (6). Among the four genes studied here, $p15^{INK4B}$ showed the highest rates of hypermethylation in both early MDS and more advanced stages. It should be mentioned that our patient material was derived from cases where sufficient

material was available after routine immunophen-otyping for cryopreservation. Thus, it cannot be excluded that a banking bias may have affected the above frequencies, as indeed may be the case in other studies.

The characteristics and profiles of $p15^{INK4B}$, *HIC1*, *CDH1*, and *ER* promoter methylation in MDS are similar to AML in several aspects. First, these four genes are also targeted by hypermethyla-tion in AML, at frequencies $> 50\%$ for $p15^{INK4B}$, *CDH1*, and *ER*, and $> 80\%$ for *HIC1* (15). Secondly, a subgroup of MDS (15 of 37 cases) showed simultaneous hypermethylation of two or more genes, suggesting the existence of a 'methylator phenotype' in MDS, as previously described in AML (19) and other cancers (20). In the majority of these patients, hypermethylation of *HIC1*, *CDH1* or *ER* was found in combination with $p15^{INK4B}$ hypermethylation, indicating that $p15^{INK4B}$ hyper-methylation was acquired first in MDS development. Thirdly, for all four genes, bisulfite-DGGE analysis revealed a high number of different bands, suggest-ing a high variability in the content and distribution of 5-methylcytosine within each gene promoter. Intra- and interindividual heterogeneity of $p15^{INK4B}$ methylation has previously been described in AML (13, 15, 18, 21) as well as in CMML (22). Although the mechanism(s) responsible for aberrant promoter methylation and the cause of extensive methylation heterogeneity remain elusive, the similarities in methylation patterns between AML and MDS suggest that promoter hypermethylation events rep-resent a common etiological component in these malignancies.

Hypermethylation of promoter CpG islands could be detected in both immunoseparated CD34$^+$ precursor cells and total BM-MNC, and the methylation band patterns in these sample types were similar. Together with a previous study showing that $p15^{INK4B}$ hypermethylation is not restricted to blast cells but to cells from the MDS clone in general (23), these observations suggest that promoter hypermethylation encompasses both immature and end-stage hematopoietic cells and provide independent support for the conten-tion that MDS is essentially a stem cell disorder (24). As hypomethylating agents are being increas-ingly used in clinical trials of treatment for MDS (25, 26), it may be helpful to know that clinical monitoring of promoter methylation status can be reliably accomplished directly on unseparated BM-MNC with no need for purification of CD34$^+$ precursor cells.

An important parameter of laboratory-based studies of MDS is the longitudinal approach, which the often protracted course of disease allows for. Unexpectedly, sequential analyses showed that the number of methylated genes changed in only three of 11 patients, suggesting that the methylation profiles of individual patients remain relatively stable during disease progression. These data may be in contrast to the data published by two other groups (8, 9), showing that $p15^{INK4B}$ hypermethy-lation is acquired with disease progression. In the study by Tien *et al.* (9), five of 22 patients (22%) changed methylation status over time, which is not significantly different from our study (3/11 cases; 27%). However, in the study by Tien *et al.* (9), all patients who acquired $p15^{INK4B}$ methylation also experienced progression of their disease (one RA to RAEB, two RAEB-t to AML, one AML to AML and one CMML to AML), whereas in our series, progression was associated with a hypermethyla-tion event in only one case (UPN 2; hypermethy-lation of *ER*). The reason for this discrepancy between our and previous studies (8, 9) is presently unknown.

Although promoter hypermethylation was not found to be acquired during disease progression in our study, concurrent methylation in ≥ 3 genes occurred more frequently in advanced than in early-stage MDS ($P \leq 0.05$), suggesting that patients with concurrent hypermethylation of mul-tiple genes might present with more advanced MDS. Furthermore, hypermethylation of $p15^{INK4B}$ was associated with leukemic transformation in early stages of MDS. Specifically, three of six patients with early MDS and $p15^{INK4B}$ gene methylation later progressed to MDS/AML, while none of the early-stage cases without $p15^{INK4B}$ methylation showed transformation. These data support the strong association between $p15^{INK4B}$ methylation and transformation to AML previ-ously reported by Tien *et al.* (9).

Among our patients with MDS, the median survival time for those with hypermethylation of ≥ 1 genes was significantly shortened (up to fourfold) compared with that for patients with no hypermethylation events. In univariate analy-ses, hypermethylation of $p15^{INK4B}$, *HIC1* or *ER*, hypermethylation of ≥ 1 genes, stage (early/ad-vanced) and thrombocyte count were all signifi-cant prognostic factors, whereas age, karyotype, FAB, and leukocyte count were not. That cyto-gentics and FAB did not appear to influence survival was surprising and we suspect the limited number of samples to be the reason. In a three-variable Cox model including stage, thrombocyte count and the four specific methylation profiles, hypermethylation of ≥ 1 genes remained an inde-pendent prognostic marker. Most interestingly, promoter hypermethylation identified a subgroup of our early-stage MDS patients with a partic-ularly poor prognosis (median survival $>$ fivefold

shorter). Our data are in agreement with the studies by Quesnel *et al.* (8) and Tien *et al.* (9), who found that hypermethylation of $p15^{INK4B}$ had negative prognostic significance. However, in both of these studies, hypermethylation of $p15^{INK4B}$ did not show prognostic significance factor in multivariate analysis. Specifically, in the study by Quesnel *et al.* (8), $p15^{INK4B}$ hypermethylation showed strong correlation with marrow blast percentage. As only 100 nucleated cells were counted in most of the patients in the present study, blast counts would give the same information as the FAB classification and was hence not included in the multivariate analysis. Nevertheless, on the basis of our data and the data of Quesnel *et al.* (8) and Tien *et al.* (9), we propose that the methylation status of a limited number of tumor suppressor genes may be used to identify MDS patients with stable disease and those at risk of progression. Further longitudinal studies as well as studies of additional tumor suppressor genes will finally establish the role of promoter hypermethylation in the development and outcome of MDS.

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