

## Negative Effect of DNA Hypermethylation on the Outcome of Intensive Chemotherapy in Older Patients with High-Risk Myelodysplastic Syndromes and Acute Myeloid Leukemia following Myelodysplastic Syndrome

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**Abstract Purpose:** Promoter hypermethylation of, for example, tumor-suppressor genes, is considered to be an important step in cancerogenesis and a negative risk factor for survival in patients with myelodysplastic syndromes (MDS); however, its role for response to therapy has not been determined. This study was designed to assess the effect of methylation status on the outcome of conventional induction chemotherapy.

**Experimental Design:** Sixty patients with high-risk MDS or acute myeloid leukemia following MDS were treated with standard doses of daunorubicin and 1- $\beta$ -D-arabinofuranosylcytosine. Standard prognostic variables and methylation status of the *P15<sup>ink4b</sup>* (*P15*), *E-cadherin* (*CDH*), and *hypermethylated in cancer 1* (*HIC*) genes were analyzed before treatment.

**Results:** Forty percent of the patients achieved complete remission (CR). CR rate was lower in patients with high WBC counts ( $P = 0.03$ ) and high CD34 expression on bone marrow cells ( $P = 0.02$ ). Whereas *P15* status alone was not significantly associated with CR rate ( $P = 0.25$ ), no patient with hypermethylation of all three genes achieved CR ( $P = 0.03$ ). Moreover, patients with *CDH* methylation showed a significantly lower CR rate ( $P = 0.008$ ), and *CDH* methylation retained its prognostic value also in the multivariate analysis. Hypermethylation was associated with increased CD34 expression, but not with other known predictive factors for response, such as cytogenetic profile.

**Conclusions:** We show for the first time a significant effect of methylation status on the outcome of conventional chemotherapy in high-risk MDS and acute myelogenous leukemia following MDS. Provided confirmed in an independent study, our results should be used as a basis for therapeutic decision-making in this patient group.

Epigenetic modification of DNA is an important biological function regulating gene expression in normal and tumor cells. Promoter hypermethylation, and hence silencing of tumor suppressor and cell cycle control genes, is considered to be an important step in tumor development and has been shown in several hematologic malignancies, including myelodysplastic syndromes (MDS; refs. 1–6). In MDS, hypermethylation of the

cell cycle control gene *P15<sup>ink4b</sup>* (*P15*) has been reported to be associated with high-risk disease and to predict leukemic transformation and survival (5, 7).<sup>15</sup> Examples of other frequently hypermethylated genes in MDS include *E-cadherin* (*CDH*), which is involved in cell adhesion, and the tumor-suppressor gene *HIC* (*hypermethylated in cancer 1*). Given these findings, therapeutic agents with the potential to reverse

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hypermethylation, such as 5-azacytidine (azacytidine) and 5-aza-2'-deoxycytidine (decitabine), have gained much interest and proved effective when used in low doses in patients with high- and intermediate-risk MDS (8–12). Both drugs have been suggested to prolong time to death or leukemic transformation in randomized studies comparing active treatment versus supportive care only (9, 11).

The mechanisms of action of azacytidine are not known in detail, but it is clear that this drug can induce hypomethylation and reexpression of silenced genes; it can also induce a broad spectrum of apoptotic pathways (13–17).<sup>16</sup>

Unless allogeneic stem cell transplantation could be done, few patients with MDS will be cured. Patients with intermediate-2 and high-risk MDS (8) have a median survival of <1.5 years and are usually considered to be candidates for chemotherapy in lower or higher doses. Induction chemotherapy may lead to complete remission (CR) in 40% to 60% of patients; however, median remission duration is generally less than 1 year (18–25). We have previously shown a 43% CR rate in a cohort of 93 patients with RAEB-t and acute myelogenous leukemia (AML) following MDS with a median age of 72 years (range 35–90 years; ref. 24). Median duration of CR was 11.3 months. Our study showed that induction chemotherapy is potentially successful also in older patients, but that therapeutic approaches aiming to prolong remission are highly warranted.

Hypercellular bone marrow, high-risk karyotype, elevated lactate dehydrogenase (LDH), high WBC counts, and age have, in various combinations, been associated with a lower probability for CR (18, 20–22, 24). Interestingly, these variables did not predict duration of CR in the above-described Nordic study. The role of methylation status for outcome of induction chemotherapy in MDS and AML is yet to be determined.

In the present study, we show that methylation status has a significant effect on the outcome of induction chemotherapy, and, among known risk factors, only relates to the percentage blasts and of CD34<sup>+</sup> cells in the bone marrow.

## Materials and Methods

**Patients.** Consecutive patients with intermediate-2 and high-risk MDS, chronic myelomonocytic leukemia (CMML) with >10% marrow blasts, and patients with AML following an established MDS or mixed MDS/myeloproliferative disease (26), who were considered eligible for at least one cycle of standard induction chemotherapy, were included in the protocol. Patients were excluded if they were considered candidates for potentially curative regimens, including AML-like induction chemotherapy followed by intensive consolidation chemotherapy or allogeneic stem cell transplantation. Thus, the study was designed mainly for older patients or patients with multiple risk factors. The study was approved by the national ethical committees, and medical product agencies of Sweden, Norway, and Denmark. All patients gave their informed consent.

<sup>15</sup> L. Shen. DNA methylation in myelodysplastic syndrome: biomarkers to predict prognosis and responses to epigenetic therapy. EHA Scientific Workshop on the Role of Epigenetics in Hematological Malignancies; 2007 Feb 9–11, 2007; Mandelieu, France.

<sup>16</sup> R. Khan, et al. Mitochondrial involvement in 5-azacytidine-induced apoptosis. Abstract. 11th Congress of the EHA; 2006 June 15–18; Amsterdam, the Netherlands.

**Study design.** Initial diagnosis as well as verification of CR was assessed at a central hematopathology unit (Department of Pathology at Karolinska University Hospital Solna, Stockholm, Sweden). For diagnosis and classification, WHO criteria were applied (26). Bone marrow cellularity was assessed on bone marrow biopsies and percentage of blasts in bone marrow smears was determined by counting 500 cells in representative areas of the smears. Flow cytometry was done on bone marrow aspirates that were sent by overnight transport. Percentages of CD34<sup>+</sup> cells were determined in nonseparated bone marrow samples after four-color direct immunofluorescence staining with CD13FITC/CD11bPE/CD45PECy5/CD34APC and acquisition on FACSCalibur equipped with Cell Quest software program (Becton Dickinson). Cytogenetic analyses were done locally at each center by standard techniques and the findings were classified according to the International Prognostic Scoring System (IPSS) into good, intermediate, and poor prognostic subgroups (8). Induction chemotherapy consisted of a standard DA 2+7 regimen: daunorubicin 60 mg/m<sup>2</sup> i.v. days 1 to 2 and cytarabine 150 mg/m<sup>2</sup> s.c. days 1 to 7. Patients who did not respond to the first course were given a second course if judged medically appropriate. Patients obtaining CR were eligible for a maintenance study of low-dose azacytidine, but no patients received standard consolidation chemotherapy. The criteria for CR were <5% bone marrow blasts, stable hemoglobin >100 g/L, WBC >1.5 × 10<sup>9</sup>/L with normal differential count, and platelets >100 × 10<sup>9</sup>/L. These criteria were identical with those in the previous Nordic study (24) to allow a comparison of CR rates and effect of pretreatment variables.

**Bone marrow sampling.** Bone marrow for methylation analysis was sampled at registration. To avoid the effect of local separation and storage factors, bone marrow was shipped by DHL using a <24 h service to a central laboratory for isolation of mononuclear cells (MNC) and CD34<sup>+</sup> cells as previously described (27). Methylation analysis on all samples obtained before start of treatment was done during one serial experiment.

**Cell isolation.** MNC were isolated from bone marrow by density gradient technique through Lymphoprep (Axis Shield). Where the yield of MNC was sufficient, CD34<sup>+</sup> cells were purified by magnetic cell sorting using the CD34 Progenitor Cell Isolation kit (MACS; Miltenyi Biotec) according to the manufacturer's guidelines. The cells were stored as pellet at -80°C.

**DNA isolation and bisulfite modification.** Genomic DNA was isolated from MNC and CD34<sup>+</sup> cells using the QIAmp DNA mini kit (Qiagen) according to the manufacturer's guidelines. The amount of DNA obtained was measured by spectrophotometer (ND-1000, NanoDrop Technologies). The DNA was further modified with sodium bisulfite as previously described (28). In brief, 2.5 µg of DNA from each sample were denatured in NaOH for 15 min at 37°C followed by the incubation in sodium bisulfite at 55°C for 16 h. Thereafter, DNA were recovered using the GeneClean II kit (Qbiogene), desulfonated in NaOH, and precipitated in ethanol.

**Polymerase chain reaction.** PCR specific for bisulfite-reacted *P15*, *CDH*, and *HIC* promoters was carried out in a final volume of 25 µL containing 50 to 100 ng of bisulfite modified DNA, 1 × TEMPase PCR Buffer (Bie & Berntsen AS), 0.2 mmol/L deoxyribonucleotide triphosphate (ABgene), 0.5 µmol/L each of forward and reverse primers (5), and 0.75 unit of TEMPase Hot Start DNA polymerase (Bie & Berntsen). PCR was done in a PX2 Thermal cycler (Thermo Electron Corporation). The polymerase was activated by incubation at 95°C for 15 min followed by 40 cycles of 95°C for 30 s, 55°C (*P15* and *HIC*) or 48°C (*CDH*) for 30 s, 72°C for 30 s, and a final extension at 72°C for 5 min. PCR results were examined by electrophoresis in a 2.5% agarose gel.

**Denaturing gradient gel electrophoresis.** Fifteen to 20 µL of the PCR product were loaded onto a 10% denaturant/6% polyacrylamide-70% denaturant/12% polyacrylamide double gradient gel (29). A fully methylated control (SssI) and an unmethylated control (peripheral blood lymphocytes) were also loaded on each gel. Gels were run at 160 V for 270 min in 1 × Tris acetate/EDTA buffer kept at a constant temperature of 58°C (*P15*) or 55°C (*CDH* and *HIC*). After

electrophoresis, gels were stained in 1× Tris acetate/EDTA buffer containing ethidium bromide (2 µg/mL) and photographed under UV transillumination. Samples were scored as methylated when bands or smears were present on the gels in the area below the band corresponding to unmethylated DNA as shown in Fig. 1 and as reported by previous publications (5, 29).

**Statistics.** CR and non-CR patients were compared regarding all pretreatment variables using Student's *t* test and  $\chi^2$  analysis, when appropriate. The size of the patient material did not allow a logistic regression analysis starting with all variables. We therefore used logistic regression analysis to compare the relative importance of methylation status of the three studied genes, and the most significant variable in the univariate analysis, CD34 expression.

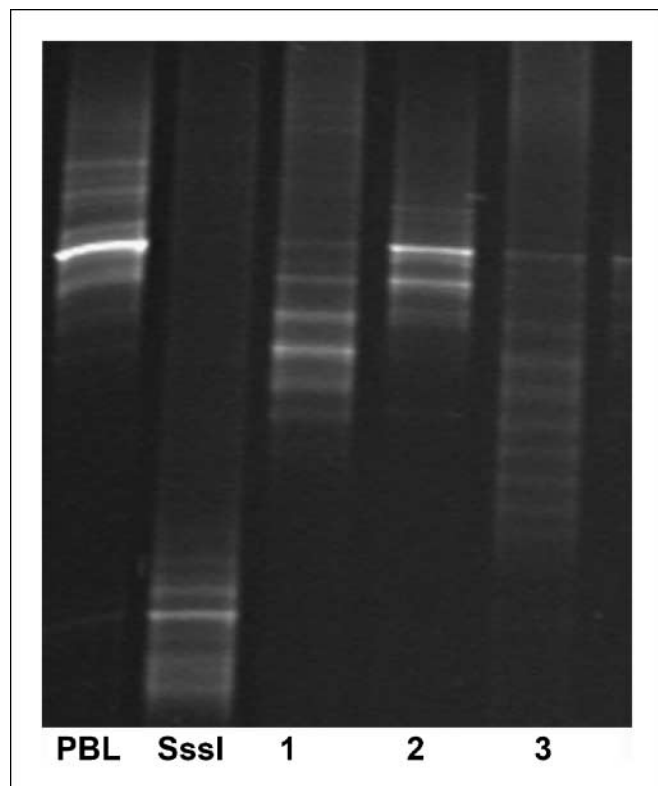
## Results

**Patients.** A total of 60 patients with a median age of 68 years (54-83 years) were enrolled in the clinical study. Baseline characteristics are shown in Table 1. Seventeen had MDS (RAEB-1 or RAEB-2), 6 had CMML, and 37 had AML following a well-documented phase of MDS or mixed MDS/myeloproliferative disease. All CMML patients had myelodysplastic rather than myeloproliferative features, and were scored according to IPSS. All patients with AML belonged to the WHO subcategory "AML with trilineage dysplasia with preceding MDS." Chromosome analysis was successful in 52 patients. Patients were divided into the three cytogenetic risk groups defined by IPSS (8). Twenty-five patients had a good prognosis karyotype

**Table 1.** Baseline characteristics of 60 enrolled patients

	<b>N = 60, n (%)</b>	<b>Median (range)</b>
Sex		
Male	40 (67)	
Female	20 (33)	
Age		68 (54-83)
Diagnosis WHO		
MDS	17 (28)	
MDS-AML	37 (62)	
CMML	6 (10)	
Cytogenetic risk group*		
Good (5 MDS, 6 CMML, 14 AML)	25 (42)	
Intermediate (4 MDS, 0 CMML, 6 AML)	10 (17)	
Poor (6 MDS, 0 CMML, 11 AML)	17 (28)	
Not done/unsuccessful	8 (13)	
Hemoglobin (g/L)		95 (62-122)
WBC ( $10^9$ /L)		4.3 (0.8-90.9)
Platelets ( $10^9$ /L)		65 (5-381)
S-LDH ( $\mu$ kat/L)		4.8 (1.9-27)
Bone marrow cellularity (%)		90 (30-100)
Blasts in bone marrow (%)		23 (6-98)
CD34 <sup>+</sup> in bone marrow (%)		10 (1-82)

\*Cytogenetic risk group according to the IPSS.



**Fig. 1.** Promoter methylation detected by bisulfite-denaturing gradient gel electrophoresis (DGGE). PBL, peripheral blood lymphocytes from a healthy individual (unmethylated); Sssl, *in vitro* methylated DNA (fully methylated); patient 1 (methylated); patient 2 (unmethylated); patient 3 (methylated). Patients were judged methylated when bands or smears were observed in the area below the band corresponding to unmethylated control.

(normal or isolated -Y, del5q or del20q), 17 had a poor prognosis karyotype ( $\geq 3$  aberrations or chromosome 7 abnormalities), and 10 had an intermediate karyotype (not fulfilling the criteria for good or poor). Table 1 shows cytogenetic risk groups in the different WHO subgroups. The percentage of patients with poor cytogenetics was 40%, 0%, and 35% in patients with MDS, CMML, and AML, respectively. All patients started induction chemotherapy and were evaluated according to intention to treat.

**Outcome of induction chemotherapy.** A CR was achieved in 24 patients (40%), 22 after one induction course and 2 after a second course. The CR rate in patients with MDS was 59%, versus 30% in patients with MDS-AML, and 50% in CMML (Table 2). The difference in CR rate between patients with AML and those with disease other than AML was not statistically significant ( $P = 0.07$ ). There was also no significant difference in CR rate observed between the cytogenetic risk groups. As in our previous study (24), a high WBC count was associated with a lower CR rate ( $P = 0.03$ ), whereas the predictive value of marrow cellularity and LDH levels could not be confirmed. This could probably be explained by the fact that the patients in this study had a higher median marrow cellularity (90% versus 70%) compared with the previous study. A new finding in this study was the association between CD34 expression, as measured by flow cytometry, and CR rate ( $P = 0.02$ ).

**Methylation status.** Bone marrow for methylation analysis was obtained from 50 patients. In 10 patients, there was either no yield due to fibrotic bone marrow, or no sample due to logistic reasons. In 23 of 50 samples, the yield was too low to allow further sorting for CD34<sup>+</sup> cells. Methylation analysis on MNC was successful in 45 of 50 (P15), 35 of 50 (CDH), and 39 of 50 (HIC), whereas the analysis of methylation on CD34<sup>+</sup>

**Table 2.** Results of treatment in subgroups of patients

	CR (n = 24)	No CR (n = 36)	CR %	P
Sex (M/F)	13/11	27/9		0.11
Age*	69 ± 5	68 ± 7		0.58
Diagnosis WHO				
RAEB-1/RAEB-2	10	7	59	
AML	11	26	30	
CMML	3	3	50	
AML vs. MDS	11/10	26/7		0.07
AML vs. CMML	11/3	26/3		0.37
Cytogenetic risk group				
Good	12	13	48	
Intermediate	5	5	50	
Poor	7	10	41	
Not done	0	8	0	
Poor vs. "not poor"	7/17	10/18		0.77
Hemoglobin (g/L)*	96 ± 11	93 ± 14		0.43
WBC (10 <sup>9</sup> /L)*	6.4 ± 14	17.6 ± 22		0.03
Platelets (10 <sup>9</sup> /L)*	117 ± 97.1	85.9 ± 89.7		0.22
S-LDH (μkat/L)*	5.8 ± 5.5	6.6 ± 4.5		0.59
Bone marrow cellularity (%)	76.7 ± 21.5	84.6 ± 23.5		0.24
Bone marrow blasts (%)	26.4 ± 23.7	35.2 ± 24.3		0.20
CD34 <sup>+</sup> (%)	10.7 ± 12.5	28.3 ± 28.01		0.02

\*Mean ± SD.

cells was successful in 24 of 27 (*P15*), 19 of 27 (*CDH*), and 20 of 27 (*HIC*), respectively. In a first step, we compared methylation status of MNC versus CD34<sup>+</sup> cells. None of the 22 patients evaluable for both MNC and CD34<sup>+</sup> cells and with negative *P15* methylation status in MNC showed hypermethylation in CD34<sup>+</sup> cells. Only one patient showed *P15* methylation in MNC but not in CD34<sup>+</sup> cells ( $P < 0.001$ ). This strong correlation between methylation patterns in MNC and CD34<sup>+</sup> cells was present also for *CDH* and *HIC*.

Twenty-six of 47 (55%) patients showed hypermethylation of *P15*; 9 of 41 (22%) showed hypermethylation of *HIC*; and 15 of 41 (37%) showed hypermethylation of *CDH*. Nineteen patients showed hypermethylation of *P15* plus one other gene, and six patients showed hypermethylation of all three genes. All three genes were evaluable for methylation status in 41 patients. Hypermethylation of *HIC* and *CDH* was mostly observed in patients with *P15* hypermethylation; only three *P15*-negative patients had *CDH* or *HIC* hypermethylation. Hence, hypermethylation of *P15* seems to be an earlier event than hypermethylation of *CDH* and *HIC* also in our study (5).

**Methylation status in relation to other prognostic factors.** Eighteen of 28 (64%) MDS-AML showed *P15* hypermethylation, compared with 6 of 13 (46%) and 2 of 6 (33%), respectively, in the RAEB and CMML subgroups. These differences were not statistically significant (Table 3). More patients with MDS-AML were methylated on *P15* plus at least one other of the genes than in the "non-AML group" (RAEB and CMML;  $P = 0.02$ ). Overall, there was no difference in methylation status between patients with good and intermediate cytogenetic risk group according to IPSS. Cases with hypermethylation of *P15* plus one other gene had slightly more often a high-risk karyotype ( $P = 0.13$ ). *P15* hypermethylation was significantly associated with CD34 expression ( $P = 0.04$ ). Patients with *P15* plus one other gene hypermethylated showed both higher number of CD34<sup>+</sup> cells and bone marrow blasts ( $P = 0.01$  and  $P = 0.007$ , respectively). There was no association between methylation status and other known risk factors such as WBC count, marrow cellularity, and LDH levels. Hypermethylation of all three genes was associated with higher age ( $P = 0.02$ ).

**Effect of methylation status on CR rate.** *P15* hypermethylation alone did not influence the outcome of induction chemotherapy ( $P = 0.25$ ; Fig. 2). However, only 3 of 15 patients (20%) with hypermethylation of *P15* plus one other gene achieved CR, versus 54% (15 of 28) in the group with no hypermethylated genes or only *P15* methylation ( $P = 0.05$ ). Importantly, none of the six patients with all genes hypermethylated

**Table 3.** Methylation status in relation to other pretreatment variables

	<i>P15</i> methylated			<i>P15</i> + one methylated gene			All three genes methylated		
	Yes	No	P	Yes	No	P	Yes	No	P
Sex (M/F)	19/7	12/9	0.36	11/4	16/12	0.34	5/1	24/15	0.40
Age*	70.3 ± 6.3	67.4 ± 5.6	0.12	71. ± 6.0	68.2 ± 5.6	0.07	74.2 ± 6.5	68.4 ± 5.4	0.02
Diagnosis †	18/8	10/11	0.15	13/2	13/15	0.02	5/1	21/18	0.22
Cytogenetics ‡	10/12	5/16	0.20	6/5	7/21	0.13	1/4	13/23	0.65
Hb*	97.3 ± 9.6	93.5 ± 16.0	0.33	96.1 ± 10.7	95.9 ± 14.6	0.97	96.8 ± 9.4	95.1 ± 13.3	0.76
WBC*	12.0 ± 13.7	11.3 ± 18.2	0.89	13.1 ± 14.5	9.9 ± 16.1	0.52	12.4 ± 10.9	11.8 ± 16.8	0.93
Platelets*	95.3 ± 84.6	104.9 ± 105.5	0.73	75.1 ± 70.8	106.8 ± 97.6	0.29	75.8 ± 50.1	98.4 ± 93.0	0.57
LDH*	5.7 ± 4.0	6.8 ± 0.5	0.50	4.8 ± 2.2	6.5 ± 5.0	0.31	5.1 ± 2.7	6.5 ± 5.0	0.59
Cellularity (%)*§	81.0 ± 21.3	80.0 ± 24.0	0.90	75.6 ± 6.0	80.8 ± 21.6	0.56	80.0 ± 33.7	80.0 ± 21.7	1.00
Blasts (%)*	37.2 ± 28.7	27.7 ± 22.5	0.24	49.0 ± 31.1	25.0 ± 20.3	0.007	49.0 ± 32.2	30.8 ± 25.7	0.16
CD34 <sup>+</sup> (%)*¶	28.0 ± 29.6	10.3 ± 7.8	0.04	33.8 ± 31.7	11.9 ± 11.2	0.01	36.8 ± 33.3	19.1 ± 23.3	0.16

\*Mean ± SD.  
† Diagnosis MDS-AML versus not AML, according to WHO.  
‡ Cytogenetic risk group, poor versus not poor, according to IPSS.  
§ Bone marrow cellularity.  
|| Blasts in bone marrow.  
¶ CD34<sup>+</sup> (immune phenotyping of bone marrow cells).

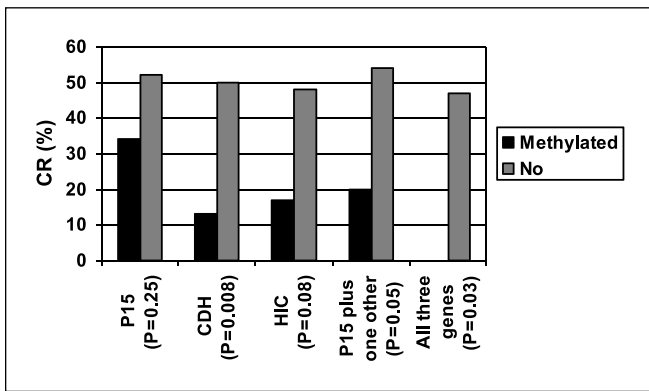


Fig. 2. Percentage of CR in patients with or without methylation of *P15*, *CDH*, *HIC*, *P15* plus at least one other gene, or of all three genes.

achieved CR ( $P = 0.03$ ). Patients with all three genes methylated did not display any particular cytogenetic pattern; three had a normal karyotype, one had a poor-risk karyotype, one had  $-7$ , and one was unsuccessful. Hypermethylation of *CDH* had a strong negative effect on CR rate; only 2 of 15 (13%) patients reached CR versus 58% in the non-*CDH*-hypermethylated group ( $P = 0.008$ ).

Logistic regression analysis was used to assess the relative effect of methylation of *P15*, *CDH*, *HIC*, and the combination of *P15* plus one other gene. Only *CDH* methylation retained significant effect on CR rate ( $P = 0.04$ ) in this analysis. Comparing the other pretreatment values, percentage of  $CD34^+$  marrow cells clearly outruled the importance of other risk factors, excluding methylation analysis. When percentage of  $CD34^+$  and *CDH* status was added, none of them retained statistical significance, confirming the covariation between these two variables.

## Discussion

The aim of this prospective clinical trial was to assess the effect of DNA hypermethylation on outcome of induction chemotherapy in patients with high-risk MDS and MDS-AML, and to investigate the covariation between methylation patterns and other known risk factors. Because hypomethylating agents are available for patients with MDS, it is important to address how these therapeutic options should be used in relation to conventional chemotherapy.

The observed frequencies of methylation of the three genes corresponded well with the findings of others, which suggest that the denaturing gradient gel electrophoresis method is stable and with acceptable interlaboratory variation (5). Confirming the results from a previous study by Aggerholm et al. (5), studying methylation of the same genes with the same method, there was no difference in methylation status between MNC and  $CD34^+$  cells. Furthermore, we conclude that only a few patients were methylated in *CDH* or *HIC* without showing *P15* methylation. Hence, *P15* methylation seem to be an earlier event in the course of the disease, but whether it is necessary for subsequent hypermethylation of the other genes in MDS remains to be shown.

The CR rate in the present study was 40%, which compares well with other studies on similar patient cohorts (18–22,

24, 25). In those studies, various combinations of pretreatment factors indicative of more advanced or proliferative disease had an effect on CR rate. Our study is first to define  $CD34$  expression, assessed by flow cytometry, as a more important predictor of response than stage of disease, marrow blast percentage, IPSS cytogenetic risk group, and LDH level ( $P = 0.02$ ). In fact,  $CD34$  expression was a stronger predictor of response than WBC in the multivariate analysis. The novelty of this finding could probably be explained by the fact that immunophenotyping, although suggested as a predictive test for survival (30), rarely had been included as a pretreatment variable in clinical trials of high-risk MDS and MDS-AML. Previous studies have suggested a negative prognostic effect of surface expression of  $CD34$  on survival in MDS and AML and also a relation between resistance to chemotherapy and  $CD34$  expression (30–33). Our data strongly supports the conclusion that immunophenotyping is an important predictive tool in MDS and MDS-AML undergoing induction chemotherapy.

Interestingly, and in line with the previous Nordic study (24), age did not affect the outcome of chemotherapy. It is clear that biological features of the tumor cell population have a greater effect on outcome than age in itself, and that age should not be used as a standard component to predict the response to moderate induction chemotherapy.

The present study is the first to investigate the effect of promoter methylation status on the outcome of chemotherapy in elderly high-risk MDS and MDS-AML. DNA hypermethylation was not related to peripheral blood values, LDH levels, or morphologic features, indicating that it is not associated to the degree of inefficient hematopoiesis. Instead, methylation of more than one gene was associated with  $CD34$  expression and, to a lesser extent, with percentage of marrow blasts. Clinical studies have suggested that azacytidine and decitabine have at least as good effect in patients with high-risk karyotype as in those with low- and intermediate-risk karyotypes (9, 10). In our material, patients with methylation of *P15* plus one other gene had a somewhat higher incidence of high-risk cytogenetics than other patients, which would support this observation. Interestingly, however, patients with three methylated genes showed no particular cytogenetic pattern, indicating that cytogenetics and methylation status are at least partly separated phenomenon.

Although *P15* methylation has been suggested as an important biological finding in MDS (5, 7, 34), hypermethylation of this gene alone showed no correlation with other variables and did not effect CR rate in our material. Instead, hypermethylation of more than one gene had a stronger effect on response rate than cytogenetics and blast percentage. These results correspond well with the findings of Aggerholm et al. (5), showing a prognostic effect on survival of multiple gene hypermethylations, but not *P15* methylation alone in low-risk MDS. *CDH* is a gene involved in cell adhesion and has an invasion-suppressor function (35). The specific finding of *CDH* hypermethylation as a particularly poor prognostic factor deserves follow-up in a larger patient material, but cannot, from the present study, be used to draw extensive conclusions. It is, however, difficult to neglect the fact that none of the six patients with all three genes hypermethylated and few of those with two hypermethylated genes responded to conventional chemotherapy. This finding corresponds well with the growing evidence that promoter hypermethylation plays an important

role for drug resistance in cancer treatment (36). On the other hand, CR was achieved in more than 50% of patients without hypermethylation, which suggests that induction chemotherapy indeed is a valuable therapeutic option in this patient group. A prospective study investigating the relation between methylation status, assessed by several methods, cDNA expression profiling, and outcome of therapy in MDS would certainly add to the scientific knowledge in this field.

Azacytidine and decitabine have been shown to prolong time to death or leukemic transformation in MDS (9, 11) and is widely used in countries where they have a label. However

likely that they will constitute an important addition to the therapeutic arsenal for MDS in the future, there is still a lot to learn about their mode of action and which patients are most likely to respond to therapy. Also, there is potentially much to gain by combining these agents with conventional chemotherapy. Achieving CR is usually a prerequisite for cure in high-risk myeloid disease. In this study, we show a strong negative effect of hypermethylation on the outcome of induction treatment. Whether pretreatment with hypomethylating agents could lead to a better response to conventional chemotherapy may be worth analyzing in a prospective study.

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