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CONCURRENT METHYLATION OF PROMOTERS FROM TUMOUR ASSOCIATED GENES PREDICTS OUTCOME IN ACUTE MYELOID LEUKEMIA

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ABSTRACT

By assessment of the methylation status of 25 candidate Tumour Suppressor Genes (TSGs) in 119 acute myeloid leukemia (AML) patients and 5 controls, we aimed to determine whether simultaneous methylation of multiple TSGs exerts prognostic impact.

Methylation-Specific Multiplex Ligation Probe Amplification (MS-MLPA) revealed methylation of at least one TSG in 59/119 patients, while no methylation was found in controls.

Methylation of different TSGs within patients was substantially correlated (intra-class correlation; 0.38).

ESR1 methylation (34/119) strongly predicted concurrent methylation of other genes, OR 7.33 (95%CI 4.13-12.99).

A Cox regression model that included the three most frequently methylated TSGs ESR1, CDKN2B/p15 and IGSF4, showed ESR1 to have opposite effects on overall survival (OS) compared to the other two, HR 0.22 (95% CI 0.09-0.53) and HR 1.66 (95% CI 0.73-3.79), HR 1.61 (95%CI 0.66-3.93).

By assessment of CDKN2B/p15 and IGSF4 methylation, patients with methylation at multiple loci can be identified.

Accumulation of methylation aberrancies is much more pronounced in ESR1 methylated patients. When combined, the methylation status of ESR1, CDKN2B/p15 and IGSF4 enable identification of patient subgroups with large differences in OS ($p<.0001$).

This study shows that methylation profiling allows risk stratification in AML.

In addition, ESR1 methylation may reflect a biological pathway that leads to hypermethylation of multiple genes, which is reflected by methylation of IGSF4 and/or CDKN2B/p15.

INTRODUCTION

Despite its uniform clinical presentation, the clonal hematopoietic malignancy acute myeloid leukemia (AML) is characterized by a wide variety of cytogenetic abnormalities[1-3]. The molecular heterogeneity of the disease translates into mixed responses to standard chemotherapy[4].

Although for some molecular aberrancies a direct involvement in leukemogenesis has been shown, animal models have demonstrated that AML-specific translocations and/or inversions alone are not sufficient to cause overt leukemia[5-8]. Classically, subsequent occurrences of other genetic alterations are thought to play an important role in the leukemogenic process by disrupting tumour suppressor genes (TSGs).

In the past decades transcriptional silencing by hypermethylation of the CpG rich 5' regions of TSGs has emerged as an alternative mechanism for inactivating mutations[9,10]. Previous studies in AML showed promoter hypermethylation of several genes; for example ESR1[11-13], IGSF4[14] and CDKN2B/p15[15,16]. Moreover, promoter methylation resulted in suppressed gene expression for ESR1[13,17,18], IGSF4[19,20] and CDKN2B/p15[21,22].

In vitro experiments have shown that promotor methylation induced inactivation is reversible in most cases. This is especially important since treatment opportunities including 5-aza-2'-deoxycitidine (decitabine) and 5-azacytidine (azacitidine) are emerging. Although initially not always intended to target methylation, these strategies have demonstrated efficacy in clinical trials that included MDS[23] and AML patients[24,25].

Equivalent to genetic alterations, the occurrence of epigenetic aberrations is likely to have (indirect) functional effects.

For this reason, epigenetic changes have been suggested as biomarkers for risk assessment and indicators of treatment susceptibility[26]. However, direct functional significance of methylation status of individual genes for the malignant AML phenotype is not always substantiated.

Based on concomitant methylation of different genes/sites[12,27,28] these individual aberrancies are hypothesized to be part of general methylation defects in a subset of AML patients, rather than random events.

This study for the first time describes such associated methylation aberrancies in AML and identifies patient subgroups with profound differences in clinical outcome.

MATERIALS AND METHODS

Study definitions

Patients <60 years of age were treated according to HOVON-29 (during 1998-2000) and HOVON-42 (during 2001-2006) protocols, which have essentially the same design, combining cytarabine and an anthracycline for induction.

Consolidation chemotherapy consisted of one cycle of etoposide and mitoxantrone or, for eligible patients, a myeloablative regimen of cyclophosphamide and busulfan, followed by autologous or allogeneic stem cell transplantation.

Patients ≥60 years were either treated according to the HOVON-32 (during 1996-1999), or according to the HOVON-43 protocol (during 2000-2006).

The HOVON-32 regimen consisted of cytarabine alone (arm A) or the combination of cytarabine and fludarabine (arm B) in two induction cycles, supplemented with granulocyte colony stimulating factor (filgrastim, 5μg/kg, day 0 until neutrophil recovery $>0.5 \star 10^9$/l) in both arms.

In HOVON-43 cytarabine and daunorubicine were combined in the first cycle and followed by cytarabine as single agent in the second cycle. Details regarding protocol contents can be found at http://www.hovon.nl.

Complete remission (CR) or was defined as a bone marrow with fewer than 5% blast cells and normal haematopoiesis of all cell types. Early/toxic deaths included all deaths occurring within 7 days after completion of the first induction cycle or death during therapy-induced bone marrow hypoplasia.
Overall survival (OS) was defined as the time period from inclusion to death. OS data were available for 100 patients, while information on achievement of CR was present for 93 patients.

Sample characteristics
Quick frozen cell pellets of 119 bone marrow samples obtained at diagnosis were selected from a tissue bank.
Five control bone marrow samples were obtained from healthy individuals.
All samples were acquired after informed consent, according to institutional guidelines.
In the control samples the $CD34^+$ subpopulation was considered to represent the normal counterpart of the leukemic blasts. This subpopulation was isolated by means of FACS sorting using 7-AAD 1:20 (Via Probe, Pharmingen, San Diego) and APC labelled CD34 1:20 (Becton Dickinson, Alphen aan den Rijn, the Netherlands).
Genomic DNA from cell pellets was isolated using QIAamp DNA Blood Minikit (Qiagen, Valencia, CA) according to the manufacturer's recommendations.

MS-MLPA procedure
As outlined in figure 1A and previously described[29], 100 ng genomic DNA of each samples was denatured for 10 min at 98°C.
After denaturation, SALSA MLPA buffer and MS-MLPA probe mix was added to the DNA. The mixture was incubated for 1 min at 95°C, followed by hybridisation for 16h at 60°C.
After hybridisation samples were diluted at room temperature with H2O and 3µl ligase buffer to a final volume of 20µl.
The samples were divided over two tubes and heated to 49°C. To the first series of tubes a volume of 10µl of a mixture containing 0.25 µl ligase, 1.5µl ligase buffer B and 5 U Hha1 was added. To the second series of tubes, an identical mixture was added in which Hha1 was replaced by 50% glycerol.
Ligation and digestion were performed simultaneously for 45 min at 49°C, followed by 5 min incubation at 95°C to inactivate the enzymes.
For amplification of the ligation products, 5µl was added to a PCR mix containing 10 x SALSA PCR buffer, 1U SALSA polymerase and SALSA FAM PCR primer-dNTP mix (provided by MRC Holland).
PCR consisted of 1 min at 95°C; 35 cycles (30 s at 95°C, 30s at 60°C, 1 min at 72°C); 20 min at 72°C.
For fragment analysis, 8.75 µl Formamide, 0.25 µl Gene Scan-500 ROX size standard and 1µl PCR product was mixed. Analysis of PCR products was performed on the ABI 3100 Avant using GeneScan software for data analysis (both, Applied Biosystems, Warrington, UK).

MS-MLPA data processing
This study was performed using MS-MLPA probe mix P041A (MRC-Holland, Amsterdam, The Netherlands), which contained 25 probes directed towards a Hha1 recognition site in the promoter region of the target gene. These probes detect aberrant methylation in the presence of Hha1.

The remaining control probes lacked the Hha1 recognition site and were used as for quantification.
Most of the 25 selected cancer-related target genes have been described to be methylated in hematopoietic malignancies[11,12,28,30]. Functions and chromosomal localisation of the selected genes are outlined in Table 1, while probe sequences can be found at www.mlpa.com.
Methylation of individual genes was defined by the presence of a signal peak in a patient DNA sample in the absence of such signal in normal control DNA samples, Figure 1B. The methylation percentage was calculated by dividing the peak area of the target probe by the peak area of the mean peak area of the flanking control probes. This signal percentage was then divided by the blast percentage of the sample under studies. Final signal percentages of less than 10% were considered background.
Methylated DNA was used positive control for methylation of all assessed genes.

Table 1. Function, chromosomal localisation and methylation frequencies of the selected genes in AML

Gene		Function	Chromosomal localisation	Samples with methylated genes	
				N	%
TIMP3	Tissue inhibitor of metalloproteinase 3	Apoptosis regulation Tyrosine kinase signalling	22q12.3	4	<10
APC	Adenomatosis polyposis coli	Wnt signalling Cell adhesion	5q21-q22	0	0
CDKN2A/ p14-ARF	Cyclin-dependent kinase inhibitor 2A	Cell cycle control Apoptosis regulation	9p21	0	0
MLH1	MutL protein homolog 1	Cell cycle control Mismatch repair	3p22.1	0	0
ATM	Ataxia teleangiectasia mutated	DNA repair Apoptosis regulation Cell cycle control Toll-like receptor signalling	11q23	0	0
RARβ	Retinoic acid receptor beta	Transcription regulation Signal transduction	3p24	9	<10
CDKN2B/p15	Cyclin-dependent kinase inhibitor 2B	Cell cycle control	9p21	43	36
HIC1	Hypermethylated in cancer 1	Cell cycle control Transcription regulation	17p13.3	0	0
CHFR	Checkpoint with forkhead and ring finger domains	Cell cycle control Ubiquitination	12q24.33	1	<10
PAHα	Phenylalanine hydroxylase family member	L-phenylalanine catabolism Amino acid biosynthesis	12q23	1	<10
CASP8	Caspase 8	Apoptosis regulation	2q33.2	0	0
CDKN1B/p27	Cyclin-dependent kinase inhibitor 1B	Cell cycle control	12p13.2	0	0
PTEN	Phosphatase and tensin homolog	Cell adhesion Apoptosis regulation Cell cycle control	10q23.3	0	0
BRCA2	Breast cancer 2	DNA repair Cell cycle control	13q12.3	0	0

Gene *(Continued)*		Function	Chromosomal localisation	Samples with methylated genes	
				N	%
CD44	CD44 molecule	Cell adhesion	11p212	0	0
DAPK1	Death associated protein kinase 1	Apoptosis regulation	9q34.1	1	<10
TP73	Tumor protein p73	Cell cycle control	1p36	2	<10
VHL	Von hippel-lindau tumor suppressor	Transcription factor binding Apoptosis regulation Cell cycle control	3p25.3	0	0
ESR1	Estrogen receptor 1	Transcription regulation Signal transduction Cell growth regulation	6q25.1	34	29
RASSF1	Ras association (RalGDS/AF-6) domain family 1	Cell cycle control Ras signalling	3p21.3	0	0
FHIT	Fragile histidine triad gene	Cell cycle control Nucleotide metabolism	3p14.2	4	<10
IGSF4	Immunoglobulin superfamily, member 4	Cell adhesion	11q23	39	33
CDH13	Cadherin 13	Cell adhesion	16q24.2	11	<10
GSTP1	Glutathione S-transferase pi	Apoptosis regulation	11q23	3	<10
BRCA1	Breast cancer 1	DNA repair Cell cycle control	17q21	0	0

Statistical analysis

The association between methylation of multiple genes within a patient sample was measured by the intra-class correlation coefficient[31].

Associations between methylation status and clinical/pathological characteristic were determined using two-sided T-tests or Chi-square tests.

These characteristics included white blood cell count (WBC) at diagnosis (expressed as logarithm), age at diagnosis (continuous variable), presence or absence of internal tandem duplication (ITD) in the FLT3 gene (FLT3-ITD or FLT3-WT), FAB subtype (categorical variable; AML secondary to myelodysplastic syndrome (MDS) and RAEB-T were included as separate subtype) and cytogenetics[32]. The prognostic impact of entering a particular treatment protocol was included both as categorical variable (4 administered HOVON protocols) as well as dichotomous variable (protocol for younger AML patients i.e. <60 yrs versus protocols for elderly AML patients i.e. ≥60 yrs).

The effects of methylation at individual and combined sites on OS were assessed by Cox regression and visualised by Kaplan-Meier analyses.

Only sites that were frequently methylated, i.e. in more than 10% of the patients, were included in combined analysis.

All observed effects were adjusted for the impact of significant clinical/pathological parameters.

Log-rank testing assessed differences in survival times between groups.

The distribution of clinical parameters across patient subgroups was assessed using Fisher's exact test and the two-sided t-test or Mann-Whitney U-test.

The significance level was set at .05 and reported p-values are two-sided. Calculations were performed using SPSS 12.0.1 (SPSS, Chicago, IL) software and STATA8.0.

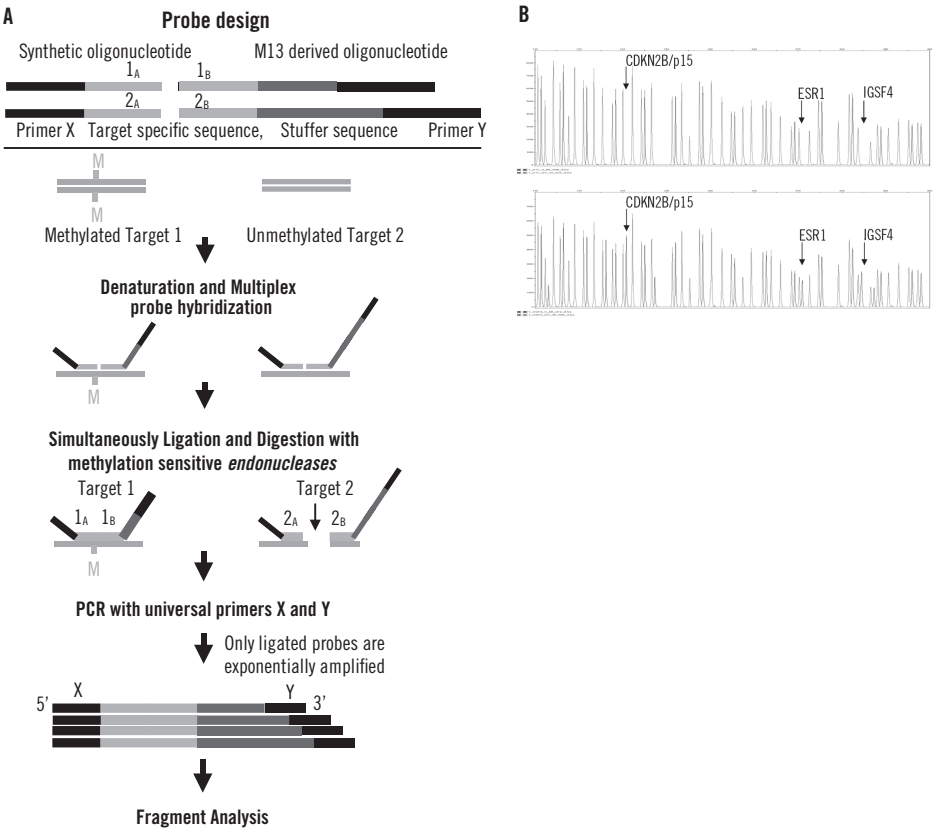

Figure 1. MS-MLPA overview and data processing. **A.** Overview of the MS-MLPA procedure (Nuygren et al, 2005). **B.** Detection of abberant methylation by MS-MLPA. MS-MLPA products were analysed by capillary electrophoresis. Profiles in black correspond with the undigested samples and gray with digested samples. The three frequently methylated genes ESR1, CDKN2B/P15 and IGSF4 are depicted by arrows. MS-MLPA profiles are from (upper figure) a control genomic DNA sample without aberrant methylation and (lower figure) genomic DNA of an AML patient sample showing methylation.

RESULTS

Methylation status of TSGs in normal and AML bone marrow

The MS-MLPA technique was used for to analyse of aberrant methylation in 25 different genes. Of all 119 AML patient samples analysed, 59 showed methylation of at least one gene; of these, 39 showed methylation of more than one gene. Methylation was not observed for 13 of the analysed TSGs in our patient cohort, as is indicated in table 1. All analysed TSG showed methylation in artificially methylated DNA, used as positive control (data not shown). Frequent methylation was observed for CDKN2B/p15 (n=43; 36% of all patients), ESR1 (n=34, 29%) and IGFS4 (n=39; 33%); all other TSGs were found methylated in less than 10% of all patients. None of the 5 control $CD34^+$ samples showed methylation of any of the analysed sites (figure 1B).

When the methylation status of all analysed TSG was taken into consideration an intra-class correlation coefficient of 0.38 (95%CI 0.27-0.50) was found. Of all analysed genes, ESR1 methylation was the strongest predictor for concomitant methylation of the other analysed sites, OR 7.33 (95%CI 4.13-12.99). In patients with methylated ESR1 26/34 patients showed 3 or more TSG methylated. Only 2/85 patients in which ESR1 was unmethylated showed 3 or more TSG methylated.

TSG methylation and the prediction of Overall Survival

Only the frequently methylated genes (>10% of all patients) were included on individual bases in survival analysis. In univariate Cox regression, *ESR1* methylation was associated with prolonged OS: HR 0.43 (95%CI 0.21-0.85; p=.01). Neither the frequently methylated TSGs, IGSF4 (39/119) and CDKN2B/p15 (43/119), nor the methylation index. (i.e. the number of genes other than *ESR1*) yielded significant effects on OS: HR 0.90 (95%CI 0.51-1.60; p=.73), HR 1.04 (95%CI 0.60-1.83; p=.88) and HR 1.06 (95%CI 0.86-1.29; p=.60), respectively.

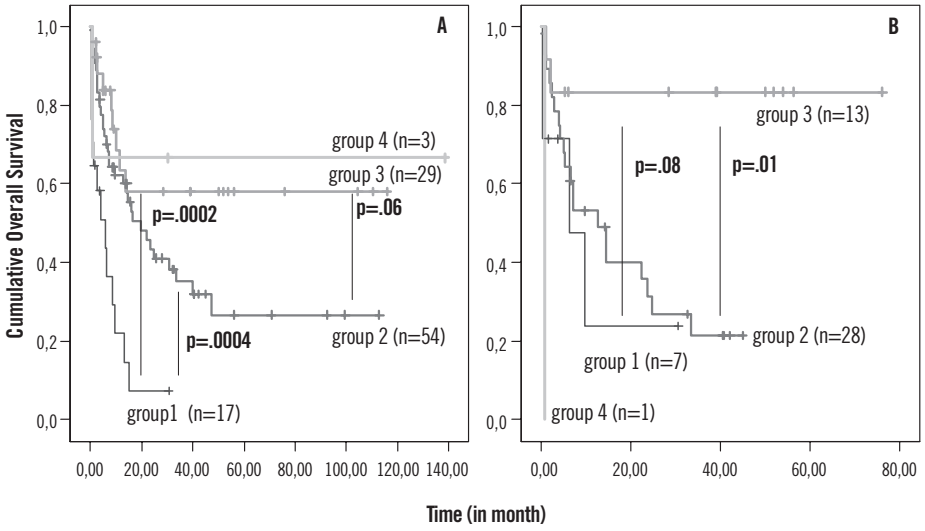

Figure 2. A. Kaplan Meier survival curves based on the methylation status of ESR1 combined with the absence or presence of IGSF4 and/or CDKN2B/p15 methylation
Prognostic stratification of AML patients at diagnosis for which OS times were available, based on the methylation status of ESR1 combined with the absence or presence of methylation of IGSF4 and/or CDKN2B/p15.
This classification was used to stratify patients in four prognostic groups;
ESR1 unmethylated, IGSF4 and CDKN2B/p15 methylated (group 1),
ESR1 unmethylated, IGSF4 and CDKN2B/p15 unmethylated (group 2),
ESR1 methylated, IGSF4 and CDKN2B/p15 methylated (group 3),
ESR1 methylated, IGSF4 and CDKN2B/p15 unmethylated (group 4).
B. As described in A, now confined to the intermediate cytogenetic risk group, OS data were available in 48 cases.

Table 2. Patient characteristics according to the four group division

	Total study population (n=119)	ESR1$^+$/TSG$^-$ (n=3)	ESR1$^+$/TSG$^+$ (n=31)	ESR1$^-$/TSG$^-$ (n=60)	ESR1$^-$/TSG$^+$ (n=25)	p-value[§]
Blast percentage Median, (range)	62 (4-97)	50 (10-88)	62 (10-95)	66 (8-97)	64 (4-97)	.12
log WBC 1x109/L Median, (range)	1.58 (0.15-2.54)	1.37 (0.78-1.52)	1.30 (0.08-2.24)	1.67 (0.15-2.30)	1.33 (0.18-2.54)	.26
Age, years Median, (range)	55 (16-77)	46.0 (40-56)	50.0 (16-72)	59.0 (18-77)	59.5 (19-74)	.04
FAB classification						.58
M0	5	0	1	2	2	
M1	14	0	3	9	2	
M2	20	0	7	10	3	
M3	4	0	0	2	2	
M4	22	2	7	9	4	
M5	34	0	6	20	8	
M6	6	0	3	2	1	
M7	0	0	0	0	0	
RAEB-T/AML secondary to MDS	8	1	3	3	1	
Unknown	6	0	1	3	2	
Cytogenetics						.06
Favourable	12	2	1	5	4	
Intermediate	54	1	15	28	10	
Adverse	7*	0	3	1	3	
Unknown	46	0	12	26	8	
FLT3 status						.94
FLT3-WT	62	2	15	32	13	
FLT3-ITD	23	0	7	10	6	
Unknown	34	1	9	18	6	
CR status						.01
CR achieved	81	2	26	43	10	
no CR achieved	12	1	3	5	3	
Unknown	5	0	0	3	2	
Early/toxic death	21	0	2	9	10	.03
Treatment protocol						.08
HOVON 29	27	1	5	15	6	
HOVON 42	40	2	18	15	5	
HOVON 32	17	0	3	9	5	
HOVON 43	30	0	4	20	6	
Unknown	5	0	1	1	3	

* All based on monosomy or deletion of the long arm of chromosomes 5 and/or 7, except for one patient in group 2
[§] Differences in the distribution of patient characteristics across the four prognostic groups, significance is indicated by p-values <.05

In multivariate Cox regressions with ESR1 methylation and one other covariate (IGSF4 methylation, CDKN2B/p15 methylation or methylation index), we found significant effects for IGSF4: HR 2.24 (95%CI 1.08-4.62; p=.03), CDKN2B/p15 HR: 2.14 (95%CI 1.10-4.15; p=.02) and methylation index: HR 1.92 (95%CI 1.19-3.10; p=.007).
We also constructed a binary covariate with a positive score if IGSF4 and/or CDKN2B/p15 was present. The IGSF4 / CDKN2B/p15 methylation variable was highly indicative for methylation in the regions other than ESR1 (a methylation index>1).
In the ESR1 methylated group, 30/31 patients had a methylation index of >1, all 30 patients were either IGSF4 (n=22) or CDKN2B/p15 methylated (n=18). Also in the ESR1 unmethylated group in which 25/85 had a methylation index of >1, 22/25 patients were either IGSF4 (n=12) or CDKN2B/p15 methylated (n=18).
In a multivariate Cox regression with ESR1 and IGSF4 / CDKN2B/p15 methylation, strong independent and opposing effects were found for ESR1 HR 0.21 (95%CI.089-.459, p<.0001) and IGSF4 / CDKN2B/p15 methylation HR 2.73 (95%CI 1.44-5.15, p=.002).

For visualisation in Kaplan Meier curves, the IGSF4 / CDKN2B/p15 methylation status was combined with the absence or presence of ESR1 methylation, figure 2A.
By definition, the latter scoring system divided the patients in four subgroups, defined in the legends of figure 2. This 4-group division enabled identification of patient subgroups with different OS times, HR 0.44 (95%CI 0.29-0.68; p<.0001).
If only the intermediate cytogenetic risk group was selected, ESR1 methylation (n=13) in the patients of which survival times were available (n=48 out of 54 patients) predicted prolonged OS: HR 0.24 (95%CI 0.07-0.82; p=.022). Again, the methylation index as sole parameter did not reveal impact on OS: HR 0.89 (95%CI 0.61-1.30; p=.56). However, analogous to the total patient population, the impact of ESR1 and methylation index on OS strongly increased when they were combined, HR 0.06 (95%CI 0.01-0.33; p=.001) and HR 1.92 (95%CI 1.19-3.10; p=.007), respectively.
In the intermediate cytogenetic risk group the 4-group division was also predictive for OS HR 0.51 (95%CI 0.27-0.95; p=.03). This is shown in figure 2B, group 4 containing 1 patient does not suit the distribution of the total patient group.

Clinical/pathological features of TSG methylation
Assessment of relations between the methylation status of individual TSGs and clinical/pathological parameters, revealed an association between ESR1 methylation and a younger age at diagnosis (p=.04). CDKN2B/p15 and IGSF4 promoter methylation correlated with adverse cytogenetics (p=.02 and p=.03, respectively). IGSF4 methylation was associated with a borderline lower WBC (p=.07). Achievement of CR was not associated with the methylation status of these three individual TSGs. However, patients that experienced early/toxic death were frequently ESR1 unmethylated (19/21; p=.03).
A detailed summary of the distribution of clinical/ pathological characteristics of four subgroups, is provided in table 2.

Predictive impact of concurrent TSG methylation on Overall Survival independent of clinical/pathological features
Assessment of the impact of the clinical/pathological parameters on patient OS yielded significance for FLT3-status: HR 1.84 (95%CI 0.99-3.43; p=.05), age at diagnosis: HR 1.01 (95%CI 1.00-1.03; p=.02), cytogenetics: HR 2.62 (95%CI 1.21-5.63; p=.01) and achievement of CR: HR 0.13 (95%CI 0.06-0.32; p<.0001).

Treatment protocol both as categorical as well as dichotomous variable did not reveal prognostic impact on OS (p=.49 and p=.30, respectively).
After adjustment for the clinical/pathological parameters which had predictive impact on OS, the methylation status of ESR1 remained significantly or predictive for OS: HR 0.21 (95%CI 0.04-0.93; p=.04).
No predictive power was observed for the methylation index HR 0.26 (95%CI 0.01-4.98; p=.38). Yet, the 4-group division maintained predictive for patient OS, independent of clinical/pathological parameters, HR 0.48 (95%CI 0.24-0.83; p=.04).

DISCUSSION

In the current investigation the methylation status of twenty-five candidate TSGs was examined in a large (n=119) unselected cohort of patients, newly diagnosed with AML. Associations between methylation of cancer related genes, as well as correlations between these epigenetic aberrancies and clinical features or patient survival were explored.
The methylation status of these cancer related genes was assessed by MS-MLPA. Although there are limitations to the use of only MS-MLPA in our study for the analysis of aberrant methylation, as discussed below, this approach enabled the rapid analysis of a relative large number of genes in a large number of samples.
In addition, the predictive power of concurrent methylation as assessed by MS-MLPA on patient survival, as well as the strength of the associations between methylated genes was eminent and sufficient to fit our enquiries.
One of the limitations of this study is that samples were non-purified and blast percentages ranged from 4-97%. Because of this, methylation of TSGs may be underestimated. This may partly explain the observed low methylation incidences for a number TSGs when compared to the incidences reported by others, e.g. for HIC-1[33] and for RARß[28].
It is also not formally proven that the aberrant methylation observed by MS-MLPA results in transcriptional silencing of the target genes in all samples. However, for a selection of the studied genes observed aberrancies by MS-MLPA were previously confirmed by methylation specific PCR (MSP)[29,34,35] and MS-SNuPE[36]. In addition, sufficient evidence has been provided by others for methylation induced transcriptional silencing of genes that are found to be frequently methylated in this study e.g. ESR1[13,17,18], IGSF4[19,20] and CDKN2B/p15[21,22].
Recent studies have focused on the identification of TSGs that exhibit concurrent methylation, to discover a pattern that may render a pathophysiological explanation and affect patient survival in AML[11,12]. In most of these studies, limited numbers of TSGs[11,12,28] were assessed in non-selected patient cohorts, ranging from 20 to 60 AML patients. The analysed subsets of methylated TSGs varied but these were found not to be significantly associated with patient survival.
A positive correlation between ESR1 methylation density and methylation density of TSGs was reported in a study that included 36 AML patients samples, of which for this purpose 16 were selected for either high (n=8) or low ESR1 (n=8) methylation density and resulted in the formulation of a CpG island methylation phenotype, analogous to other cancers[37].
Whether such methylation phenotype exerts prognostic impact via transcriptional deregulation of specific target genes with prognostic impact, or whether the phenotype itself represents a distinct oncogenic pathway remains to be elucidated.

Our results indicate that concomitant methylation of genes in AML is common. The observed association between the methylation statuses was strong (0.38) and best pre-

dicted by ESR1 methylation.
Transcriptional targets of the selected and associated TSGs in this study were part of a distinct number of overlapping cellular pathways that, when impaired, are known to contribute to leukemogenesis.
The observed methylation of TIMP3[38], DAPK[39] and GSTP1[40] may for instance, impair execution of apoptosis, whereas inactivating IGSF4[41] and CDH13[42] function inflicts proper cell adhesion. In the case of CDKN2B/p15[43], CHFR[44], TP73[45] and FHIT[46] promoter methylation can affect the cell cycle, while epigenetic changes in RARß[47] could negatively influences transcription regulation (for a detailed summary see table 1).
ESR1, being strongly associated with TSG methylation, is involved in transcriptional regulation[48], cell growth[49] and signal transduction[50]. Unmethylated ESR1 has been observed to have growth and metastasis suppressing activity and function as negative regulator of human haematopoiesis[17].
We have shown in this study that the frequently methylated genes ESR1 and CDKN2B/p15 or IGSF4 have independent oppositely directed predictivity with respect to patient survival. Moreover, combining methylation of CDKN2B/p15 and IGSF4 enables identification of patient with methylation at more that one TSG promoter region (e.g. a methylation index of >1).
A higher methylation index yielded significant negative effects on patient OS in both $ESR1^+$ and $ESR1^-$ subgroups.
Although methylation at multiple promoter regions takes place in both subgroups, the frequency in the $ESR1^+$ subgroup as compared to $ESR1^-$ subgroup is much higher.
ESR1 methylation may thereby reflect a biological mechanism/pathway that leads to hypermethylation of multiple genes. Yet, by its negative regulation of haematopoiesis, methylated ESR1 conveys a favourable prognosis.
The main clinical/pathological characteristic that could render a biological explanation for this phenomenon was the association between an ESR1 methylation and age. ESR1 methylation was found to be associated with increasing age for a number of epithelial malignancies like colon[51] and prostate cancer[52].
However, inverse relations between ESR1 methylation status and age have been previously described in haematological malignancies, by comparing juvenile and adult AML[11], as well as within a relatively young (median age 39 yrs) adult AML patient cohort[27].
Analogous to these studies, we established promoter methylation of ESR1 to be significantly associated with a younger age in a patient cohort that is more representative for adult AML (median age 55 yrs). It has been hypothesized that the association between younger age and an ESR1 methylated status, reflects differences in disease characteristics between younger and elderly patients that are possibly based on different leukemogenic events and reflected by different cytogenetic changes and clinical courses[27].
A potential pitfall in this premise is the fact that reduction of treatment intensity is a characteristic of most studies designed for elderly patients and which can partly explain their inferior outcome.
Indeed in our patient cohort, patients with $ESR1^+$ were more frequently treated according to the HOVON 29 or 42 protocols (26 out of 34 $ESR1^+$, table 2) as a consequence of their younger age. However, no significant effect of treatment protocol on patient survival was observed. Furthermore, no significant association was observed between ESR1 methylation status and cytogenetics that could substantiate the idea of age related cytogenetic characteristics reflected by ESR1 methylation status.
Yet, we did observe a significant association between methylation of both CDKN2B/p15 and IGFS4 and adverse cytogenetics; mainly including monosomy or deletion of the

long arm of chromosomes 5 and/or 7. Associations between CDKN2B/p15 methylation, 7q deletions and poor OS have previously been reported[53]. It should be emphasized that associations including small cytogenetic subgroups should be readdressed in studies that include larger number of patients.

Based on our results we would strongly advise to include screening for ESR1 methylation in future investigations on the prognostic impact of methylation status of individual or combined candidate TSGs in AML.

The absence of this parameter in a number of previously conducted investigations may explain the relatively low observed predictive power for hypermethylated genes on patient survival in AML.

In summary, we have demonstrated the age associated methylation status of the ESR1 gene to be a predictor for concurrent methylation of TSGs. Patients with concurrently methylated genes can be identified by a methylated status of CDKN2B/p15 and/or IGSF4. Moreover, methylation of ESR1 and methylation of CDKN2B/p15 or IGSF4 represent processes with independent and opposing predictivity with respect to patient overall survival.

When combined, they constitute a unique and powerful factor for predicting clinical outcome, both in the total AML population as well as within the intermediate cytogenetic risk group.

The identification of patients with methylation aberrancies that translate into survival prognosis, offers opportunities for application of therapeutic approaches that include demethylating agents in a subgroup of AML patients.

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