Prognostic DNA methylation patterns in cytogenetically normal acute myeloid leukemia are predefined by stem cell chromatin marks

**Short title:** Profile of prognostic DNA methylation in AML

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Abstract

Cytogenetically normal acute myeloid leukemia (CN-AML) comprise between forty and fifty percent of all adult acute myeloid leukemia (AML) cases. In this clinically diverse group molecular aberrations such as FLT3-ITD, NPM1 and CEBPA mutations recently have added to the prognostic accuracy. Aberrant DNA methylation is a hallmark of cancer including AML. We investigated in total 118 CN-AML samples in a test and a validation cohort for genome-wide promoter DNA methylation with Illumina Methylation Bead arrays and compared them to normal myeloid precursors and global gene expression. IDH and NPM1 mutations were associated with different methylation patterns (p=0.0004 and 0.04, respectively). Genome-wide methylation levels were elevated in IDH mutated samples (p=0.006). We observed a negative impact of DNA methylation on transcription. Genes targeted by Polycomb group (PcG) proteins and genes associated with bivalent histone marks in stem cells showed increased aberrant methylation in AML (p<0.0001). Furthermore, high methylation levels of PcG target genes were independently associated with better progression free (OR 0.47, p=0.01) and overall survival (OR 0.36, p=0.001). In summary, genome wide methylation patterns show preferential methylation of PcG targets with prognostic impact in CN-AML.

Introduction

Malignant transformation is a complex process, partly driven by genetic lesions, facilitated by environmental, inheritable and most likely also immunological factors. In addition, it is now widely recognized that epigenetic changes are an integral part of the process of neoplastic transformation and clonal evolution, causing aberrant gene expression. Epigenetic regulation include mRNA modulation by noncoding RNAs, various chromatin modifications such as histone tail acetylation, methylation, phosphorylation, ubiquination and sumoylation as well as promoter DNA methylation and global demethylation.

Acute myeloid leukemia (AML) is the most prevalent type of acute leukemia in adults. The mainstay of treatment ever since the 1970s is a combination of cytarabine and anthracyclines. The major reason for the improved treatment results in AML seen over the last 40 years is the refined use of allogeneic transplantation by both better selection of candidate patients and better treatment protocols including reduced intensity conditioning regimens and improved supportive care. Risk stratification of AML patients as a selection tool for intensified treatment including allogeneic stem cell transplantation is thus crucial. Current stratification protocols include chromosomal lesions validated in large cohorts of patients, and recently, the emergence of molecular diagnostics such as NPM1, FLT3 and CEBPA mutational analysis. Molecular diagnostics are especially important in the large group of AML with normal
karyotype comprising around 45% of all adult AML with a very heterogeneous prognosis. Furthermore, understanding the molecular mechanisms behind hematological malignancies facilitates the development of new classes of targeted drugs exemplified by FLT3-inhibitors in leukemia and JAK2 inhibitors in myeloproliferative diseases.

Epigenetic changes in AML, although extensively studied on individual gene level and to a much lesser extent on global level, have not yet found their place in risk stratification and prognostication. In part this may be explained by the lack of standardized methodology in the epigenetic field. Even so, clinical studies with presumably epigenetically acting drugs such as DNA methyl transferase inhibitors and histone deacetylase inhibitors are ongoing.

Thus, the need for increased knowledge of epigenetic mechanisms in AML is substantial, especially in CN-AML. In this study we used a state of the art platform to evaluate a test cohort of 58 well characterized CN-AML patients for DNA methylation, correlating our findings with molecular parameters, clinical outcomes, gene-expression and genome-wide studies of chromatin marks. Furthermore we validated our findings in a separate cohort of 60 CN-AML. We show that aberrant promoter methylation is of prognostic relevance and, importantly, correlate this to genes associated with Polycomb group proteins (PcG) and bivalent chromatin marks in stem cells.

Patients and methods

Patients, samples and molecular analyses
Primary bone marrow samples from 58 de-novo CN-AML patients were obtained at diagnosis from patients at the Karolinska University Hospital, Huddinge, Uppsala University Hospital and Örebro University Hospital. Informed consent was obtained in accordance with the Declaration of Helsinki and the study was approved by the regional institutional review boards. All patients were treated with standard induction regimens containing Cytarabine and anthracycline, according to the national AML guidelines. All patients were eligible for consolidation treatment including allogeneic stem cell transplantation. Clinical characteristics of all patients are shown in table 1. A validation cohort of 60 de-novo CN-AML patients was set up. Thirty-one of these patients were diagnosed and analyzed in Angers, France and the remaining 29 were diagnosed Ulm, Germany and in the Swedish centra mentioned above. The clinical characteristics are shown in table S1. DNA was extracted after mononuclear cell isolation (Lymphoprep; Axis-Shield PoC, Oslo, Norway). Chromosome banding was done using standard laboratory techniques. Mutation analyses of FLT3 (internal tandem duplications [FLT3ITD] and
tyrosine kinase domain [FLT3TKD] mutations at codon D835 and I836), NPM1, CEBPA, IDH1 and IDH2 (exon 4) were performed as previously described.7,8 Standard diagnostic and remission criteria were used.9 Bone marrow from healthy donors (n=9) was separated for mononuclear cells by Lymphoprep™ (Axis-Shield, UK). Bone marrow CD34+ cells were further separated by MACS, indirect CD34 microbead kit (Miltenyi Biotec, Auburn, CA, USA), according to the manufacturer’s instructions. In five cases the Common Myeloid progenitor (CMP) and Granulocyte-Macrophage progenitor (GMP) cells were purified from the CD34+ pool with FACS, using antibodies specified in supplementary table S2 as previously described10, and CD34 positive cells were used in four.

Methylation analysis

Genome-wide DNA methylation profiling was performed using the Illumina Infinium HumanMethylation27 BeadChip (Illumina Inc, San Diego, CA, USA) in the whole test cohort and in 31 of 60 cases in the validation cohort. Twenty-nine cases were analyzed with the Illumina HumanMethylation 450K array where only the 25978 CpG sites overlapping with the 27k array were considered. To exclude sources of technical bias we excluded all CpG sites with detection p-values >0.01 in more than five samples; n=126. Furthermore, a systematic bias dependent on gender was found, why only probes on autosomes were used for further analyses except for validation purposes. The analyses were performed at the BEA core facility at Karolinska Institute and the genomics core facility in Angers, France. The EZ DNA methylation kit (Zymo Research) was used for bisulfite conversion of 500ng of DNA, the remaining assay steps were performed as previously published, using Illumina-supplied reagents and conditions.11 The readout from the array is a beta-value, which is defined as the ratio between the fluorescent signal from the methylated allele to the sum of both methylated and unmethylated allele and thus correlates to the level of DNA methylation. A beta-value of 1.0 corresponds to complete methylation and 0 is equal to no DNA methylation. The array data in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE32252.

Bisulfite pyrosequencing was performed for the CDKN2A, CDH1, HIC1 and CDKN2B promoter. After bisulfite conversion pyrosequencing was performed on the PyroMark12 platform (Qiagen, Sollentuna, Sweden) as previously described.12 Primer sequences are available at: http://techsupport.pyrosequencing.com. Samples were considered methylated at mean levels of >15% methylation.

Global DNA methylation was quantified by luminometric assay (LUMA) as previously described.13 500 ng genomic DNA was cleaved with HpaII + EcoRI or MspI + EcoRI in separate reactions. After the digestion step, the extent of cleavage was quantified by pyrosequencing. HpaII
andMspIaremethylation-sensitiveisoschizomers. DNA methylation was defined as 1-(HpaII/MspI ratio); fully methylated DNA gives a ratio that approaches 1 whereas if methylation is completely absent the ratio approaches 0. The assay was carried out in duplicate.

**Gene expression**

Preparation of mRNA was performed using the RNeasy kit (Qiagen, Sollentuna, Sweden) according to instructions and quality was controlled using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Whole genome expression arrays were performed by the BEA core facility using the Illumina HumanHT-12 v4 Expression BeadChip using the Direct Hybridization Assay and iScan system (Illumina Inc. San Diego, CA, USA).

**Statistics**

Statistical analysis of the methylation data was carried out in the statistical computing language R (http://www.r-project.org). In order to search for the differentially methylated genes between the different prognostic subgroups the data was arcsin transformed and an empirical Bayes moderated t-test was then applied, using the ‘limma’ package. The p-values were adjusted using the method of Benjamini and Hochberg and a level of p<0.05 was used as a cutoff. An additional filter for the average beta geometric difference was also applied to assure only genes with absolute differences of more than 0.10 Beta between the groups remained. Integration of gene expression array data with methylation array data and integration of previously published ChIP-chip results was made with the BeadStudio3.2 software (Illumina Inc, San Diego, CA, USA).

Unsupervised hierarchical clustering analysis (HCL) and Principal Components Analysis (PCA) were performed with the Genesis software package. HCL distance was measured using Pearson correlation with a complete clustering algorithm. Samples were normalised using a global mean centering method. Methylation levels of different molecular subgroups were measured as continuous variables. Hypermethylation of individual CpG sites was defined as Beta values >0.7, unmethylated as Beta <0.3 and values in between were called “methylated”.

Survival data is presented using the Kaplan-Meier method and compared with the log-rank test. Logistic regression was used for univariate and multivariate analysis of complete remission (CR) rates and Cox regression for survival. For multivariate analyses both the test and validation cohorts were pooled. Odds ratios are shown with 95% confidence intervals. Correlations were calculated using Spearman’s $\rho$ or Pearson correlation as indicated. Comparisons between two groups of continuous parameters were made using the Students T-test or Mann-Whitney as appropriate and categorical data compared with Chi-square or Fishers Exact test. ANOVA was used for multiple group comparisons with the Tukey post-hoc test if not
otherwise indicated. Statistical calculations were performed with the PASW 18.0 software (SPSS Inc, Chicago, IL, USA). Gene ontological analyses were performed using the David Functional annotation tool (http://david.abcc.ncifcrf.gov/). All p-values are two-sided.

Results

Validation of the Illumina Methylation array

Bisulfite pyrosequencing of the HIC1, CDH1, CDKN2B and CDKN2A promoter was performed in 14-17 samples. The beta values were higher for methylated (>15%) than unmethylated genes in all samples, p=0.01, 0.06, 0.03 for HIC1, CDH1 and CDKN2B respectively (fig 1A). For CDKN2A all AML samples were unmethylated by pyrosequencing with matching low methylation levels on the array.

Two technical replicates were run on the 27k array and two replicates on both the 27k and 450k array. They were compared prior to normalization. All were highly correlated (p<0.0001) with Pearson correlations coefficients of r=0.99, 0.94 and r=0.98, 0.98, respectively (fig 1B). Even though the Illumina HumanMethylation27 array covers 27 578 CpG sites in 14 495 genes the global CpG content is not necessarily reflected as the array is biased towards promoter CpG island methylation. In fact, there is often an inverse relationship between promoter methylation and global CpG methylation in tumors.\textsuperscript{12,18} To investigate this in the current cohort we measured a surrogate of the global 5-methyl-cytosine content with LUMA, a non-bisulfite, unbiased method utilizing the methylation sensitive isoschizomeric restriction enzymes MspI and HpaII. These enzymes target CCGG sites which are distributed throughout the genome with around 20% in CpG islands and the rest reflecting inter- and intra-genomic DNA as well as repetitive sequences.\textsuperscript{19,20} Twenty-nine samples were investigated and we found an inverse relationship between promoter methylation assessed by the Illumina HumanMethylation27 array and global CpG methylation as measured by LUMA (Pearson r=-0.45, p=0.02, fig 1C), indicating that promoter and non-promoter methylation are inversely correlated in AML as in many solid tumors. Since one of the X-chromosomes is epigenetically silenced and hypermethylated in females we further validated the arrays by calculating average beta values for the 1084 X-chromosomal CpG sites common in the 27k and 450k array. There was a significant increase in methylation of female samples compared to male, average beta 0.51 vs 0.38, p=2E-16 (Supplementary figure S1). In a logistic regression model, the X-chromosome methylation level was able to predict gender. The hazard ratio (HR) for male sex was 0.01 when methylation levels were high (beta >0.42), p=2.2E-9.
Methylation in relation to molecular subtypes, age and morphology

Differences in average beta values were investigated for DNMT3A, NPM1, FLT3ITD, IDH1 and IDH2, FLT3TKD and CEBPA mutated samples compared to their wild type counterparts. The associations between age and methylation as well as differences in CpG-island vs. non-CpG-island methylation in the different sub-groups were investigated. Results are shown in supplementary table S3. In short, a mutation of any IDH gene was associated with increased Beta values (p=0.006). Interestingly, in IDH1 mutated samples, only CpG-island annotated sites showed increased methylation (p=0.02) whereas in IDH2 mutated samples, only non-CpG island sites were hypermethylated (p=0.0002). Average methylation was lower in FLT3TKD mutated samples compared to unmutated (p=0.01), however, only twelve samples had a FLT3TKD mutation. Age and morphological French-American-British class were not significantly associated with differences in beta values, nor were DNMT3A, FLT3ITD, NPM1 or CEBPA mutational status.

Unsupervised hierarchical clustering of the samples (Pearson complete clustering) was performed (fig 2). Six clusters were identified and two outliers. The normal progenitor samples clustered together within cluster one. Differences in frequencies of common molecular aberrations between clusters were assessed with the Fisher-Freeman-Halton test. There was an unequal distribution of NPM1 and IDH mutations (p=0.01 and p=0.0001 respectively), with concordantly high frequencies in cluster two and six and low in clusters three and four.

Promoter methylation is inversely correlated to gene expression

It is well known that promoter DNA methylation does not always correlate with gene expression.21 To examine this in our cohort we performed gene expression arrays for ten of the samples. There was a moderate but highly significant inverse correlation between average beta values and average gene expression (Spearman´s Rho -0.17, p=2E-88), verifying an association between promoter methylation and transcription (fig 3A). Plots for individual genes are also shown in figure 3B. Methylation of CpG islands versus non-CpG islands affected gene expression to a similar extent (Spearman´s Rho -0.14, p<0.0001 and -0.14, p<0.0001 respectively). To further investigate this, Spearman correlation coefficients were calculated for every CpG site annotated to valid expression data for the quartile of genes with the highest standard deviation between samples (n=1979 genes annotated to 3851 CpGs). Overall, 29.2% of genes had a moderate to strong inverse correlation (i.e less than -0.3 Rho) between methylation and expression, while only 11.9% had a moderate to strong positive correlation (i.e more than +0.3 Rho), corroborating our findings.
Different methylation in normal myeloid progenitors and AML

Myeloid progenitor cells from the bone marrow CD34+ pool from nine healthy donors were FACS separated and cells from the CMP and GMP-stages were selected in five since their gene expression signatures are most similar to AML gene expression signatures (Bo Porse, BRIC/University of Copenhagen, personal communication). Differences in average methylation levels between the CD34+, CMP and GMP stages were negligible (fig 4E) and they were thus pooled together for further comparisons. To identify groups of genes that were differentially methylated between progenitors and CN-AML while keeping false positives to a minimum we applied a Bayes modified, Benjamini-Hochberg adjusted t-test for difference set to <0.05 and a minimum Geometric Average distance between groups of 0.10 beta. With these criteria 2764 CpG residues corresponding to 2304 genes were found to be differentially methylated. Using unsupervised hierarchical clustering four clusters of differentially methylated CpGs (DMCs) were defined (fig 4A). DMC 1 and 2 were less methylated in normal progenitors than in AML samples. DMC 3 and DMC 4 were more methylated in progenitors than in AML (fig 4B). A principle components analysis (PCA) of the same CpGs clearly separated clusters one and two from three and four, confirming their relevance. However, DMC 1 and 2 separated only on the Z-axis as did DMC 3 and 4 (fig 4C). On the whole array, 72.5% of all CpG residues are located in CpG islands. In comparison, DMC 1 consisted overwhelmingly of CpG residues within CpG islands, 95% (p=1E-40), whereas in DMC 3 and 4 only 24% and 29% of the CpG residues were located in CpG islands respectively (p<1E-65 for both). DMC 2 had a slight increase in the proportion of CpG island-annotated sites, 80% (figure 4D). Gene ontological analyses showed that DMC 3 and 4 were enriched for genes involved in defense responses, DMC 1 and 2 for genes related to cell-cell signaling, neuron differentiation, embryonic morphogenesis and cell fate commitment; mainly homeobox (HOX) genes (table S4). The average beta values of the differentially methylated CpG sites were higher in most AML samples compared to normal progenitors (p=0.0003) (fig 4E). However, the individual AML samples showed a great variability from close to normal to extreme hypermethylation. To confirm our findings we repeated the hierarchical clustering and gene ontological analyses in the validation AML cohort and normal controls with similar results as in the test cohort; four major clusters with similar gene ontological enrichment as in the test cohort (data not shown).

Polycomb target genes and genes with bivalent histone marks in stem cells are preferably methylated in CN-AML

It has been reported that genes in stem cells associated with Polycomb group (PcG) transcriptional repressor proteins become hypermethylated in various solid tumors22,23. To investigate this on a genome-wide scale in AML we integrated chromatin immunoprecipitation sequencing (ChIP-seq) data of Polycomb repressor complex 2 (PRC2)
proteins in human embryonic fibroblasts (HEF) by Bracken et al with our own methylation data.\textsuperscript{22} We recorded the difference in beta values between AML samples in the test cohort (\(n=58\)) and normal progenitor cells (\(\Delta\beta\)) for every CpG residue present on the Illumina array and compared \(\Delta\beta\) for all CpGs annotated to the genes implicated as PcG targets in the ChIP-seq dataset to those that were not. Indeed, PcG target genes in the Bracken study had a greater increase in methylation (\(\Delta\beta 0.09\) compared to 0.05, \(p<0.0001\)). The difference was restricted to CpG islands (\(\Delta\beta 0.08\) vs 0.02, \(p=1.9E-144\)) whereas in non-CpG island residues, there was actually a slight but significant decrease of methylation in PcG associated genes (\(\Delta\beta 0.12\) vs 0.13, \(p=0.005\)) (fig 5A). Furthermore, PcG target genes in HEFs were two to fourfold more common in DMC:s 1 and 2 (OR 3.5(3.0-4.1) and OR 2.1(1.7-2.4), respectively, \(p<1E-20\) for both, fig 5B). Interestingly, PcG targeted genes were significantly more common among hypermethylated genes in \textit{IDH1}-mutated vs nonmutated samples (33% vs 17%, \(p=3E-8\)) whereas in \textit{IDH2}-mutated samples they were not (19 vs 17%, \(p=0.10\)). There was a clear association between DNA methylation and silencing also in PcG targeted genes (fig 3C).

The Polycomb repressor complex 2 catalyzes the trimethylation of lysine 27 on histone H3 (H3K27Me3). Concurrent methylation of lysine 4 on histone H3 (H3K4Me) coincide with H3K27Me3 at transcription factors important for developmental control in stem cells.\textsuperscript{24} The relevance of such “bivalent marks” were investigated by applying ChIP-seq data of bivalently marked genes from a study in hematopoetic stem cells (HSC) by Cui et al in a similar fashion as described for PcG targets above.\textsuperscript{25} \(\Delta\beta\) was greater among genes with bivalent marks (\(\Delta\beta 0.08\) contra 0.05, \(p<0.0001\)), again restricted to CpG islands (\(\Delta\beta 0.08\) vs 0.01, \(p<0.0001\)) (fig 5C). Among non-CpG island residues there was a slight decrease of methylation of bivalently marked genes (\(\Delta\beta 0.11\) vs 0.13, \(p=0.006\)). As for PcG targets, genes with bivalent marks were enriched in DMC:s 1 and 2 (OR 5.3(4.6-6.2), 2.3(2.0-2.6), respectively, \(p<1E-20\) for both, fig 5B). Similar results were attained when using gene sets from LIN1-HSCs with bivalent marks\textsuperscript{26} as well as ChIP for the PRC 2 protein SUZ12\textsuperscript{27} (data not shown). PcG target genes and genes with bivalent marks were significantly overlapping. Overlapping genes and genes that were PcG targets but not bivalently marked had similar \(\Delta\beta\) values (\(p=0.8\)), which was higher than for genes with bivalent marks who were not PcG targets (\(p<0.0001\), fig 5D).

**Promoter methylation and prognosis**

The CpG sites that were most significantly coupled to CR and two year survival were selected by a Bayes moderated T-test (adjusted \(p<0.05\), geometric difference \(>0.10\) beta). Of the 42 CpG sites most significantly coupled to CR in this supervised analysis, 38% were hypermethylated (i.e beta values \(>0.6\)) in patients achieving CR and 18% among patients who did not (\(p=0.0002\)) (fig 6A and table S5).
Similarly, of the 62 CpG sites most significantly correlated to two year survival, 64% were hypermethylated in the group of patients that achieved two year survival compared to 30% in the group who did not (p=6E-11) (fig 6B). Interestingly, 33 of the selected 62 CpG residues were located in the PcG target enriched DMC 2, which is 31 times more than would be expected by chance (p=1.4E-97).

The methylation levels of Polycomb target genes and bivalently marked genes are associated with outcome

Since there was a relative enrichment of PcG-associated genes in DMC 2, where also the most predictive CpG residues for OS and PFS were found, we further investigated the potential of the methylation status of all PcG targets to predict prognosis. In fact, 20 of the 62 CpG sites most predictive of two year survival were annotated to genes targeted by PcG, a significant enrichment (p=0.001). Average Beta values of PcG-annotated CpG residues were calculated for every sample (betaPcG) in the test cohort. Construction of Kaplan Meier diagrams for the tertiles of betaPcG showed that increasing betaPcG was significantly associated with OS and PFS, p(trend)=0.001 and 0.002, respectively (fig 6C, 6D; upper panel). The analysis was repeated in the validation cohort with similar results; p=0.009 for OS and p=0.035 for DFS (fig 6C, 6D; middle panel), and highly significant for pooled samples, p=0.00009 and 0.0002 for OS and DFS, respectively (fig 6C, 6D; lower panel).

There was a trend towards increased CR rates for samples with high betaPcG, OR 0.36(0.12-1.06), p=0.06 in univariate analysis, which however was lost in logistic regression analysis correcting for age, WBC count, FLT3ITD and NPM1 status (OR=0.7, p=0.48). A Cox regression analysis of PFS and OS, entering the same variables at baseline, showed an independent association of high betaPcG with better PFS; OR 0.47(0.26-0.85), p=0.01, and OS; OR 0.36(0.19-0.68), p=0.001 (table 2). Both cohorts were pooled prior to multivariate analyses to maximize statistical strength.

Discussion

This study investigates 118 CN-AML samples using the Illumina HumanMethylation Bead arrays. Novel findings are that PcG target genes and genes with bivalent marks are preferentially methylated compared to other genes and that the level of this aberrant methylation is an independent prognostic factor for clinical outcome in CN-AML.

Furthermore, we describe that differentially methylated CpG sites between normal progenitors and AML cluster in groups with distinct ontological functions, inferring that aberrant DNA methylation and demethylation are not random events in CN-AML. We also show associations between NPM1 and IDH mutations and clustering of samples. The clustering of AML samples according to various genetic
and molecular aberrations has been reported previously, in most detail by Figueroa et al who found 16 different clusters in a mixed cohort of AML samples.\textsuperscript{28} Nine of the 16 clusters primarily consisted of CN-AML samples. Of these, cluster 12, 13, 14 and 16 were characterized by frequent \textit{NPM1} mutations as in our cluster two and six. Cluster 4, 5, 9 and 15 by Figueroa et al. to the contrary had a low frequency of \textit{NPM1} mutations as in our clusters three and four, highlighting similarities of our findings. A notable difference is that we cannot detect any sample clustering according to \textit{CEBPA} mutational status as noted in that study. However, there were only six \textit{CEBPA} mutated samples in our cohort, which may explain the absence of clustering. It is also important to stress the difference in methylation analysis techniques used in our studies with substantial differences in sensitivity and specificity for different types and areas of methylation between the methods. The clusters in this study differed from each other particularly by the different rates of \textit{IDH} and \textit{NPM1} mutations which overlapped. Interestingly, sample cluster two is characterized by \textit{IDH2} mutations and cluster six by \textit{IDH1} mutations, indicating that they have different epigenetic profiles, differing not least in the proportion of PcG targets that were preferably methylated in \textit{IDH1} mutated compared to \textit{IDH2} mutated samples. The mechanistic link behind this is not clear. However, \textit{IDH1} is a cytosolic enzyme and \textit{IDH2} mitochondrial, making it feasible that their influence on DNA methylation would differ. The link between DNA methylation and \textit{IDH} mutations has recently been explored by others. Mutant \textit{IDH} enzymes produce 2-hydroxyglutarate which in turn is an inhibitor of \textit{TET2} activity, affecting the conversion of 5-methylcytosine to 5-hydroxymethylcytosine with implications on DNA methylation homeostasis.\textsuperscript{29-31} Our findings confirm the presence of generally increased DNA methylation and a specific epigenetic signature in \textit{IDH} mutated samples, in line with what was recently shown by Figueroa et al.\textsuperscript{31}

In this study we show that Polycomb target genes implicated in human embryonic fibroblasts are generally more hypermethylated than other genes as compared with normal progenitor cells. The Polycomb group of transcriptional repressors is central for stem cell maintenance and differentiation and is also implicated in cancer. Polycomb proteins are also critical regulators of both benign and malignant hematopoiesis, as reviewed recently by Martin-Perez.\textsuperscript{32} It has been suggested that Polycomb associated genes in stem cells are silenced by aberrant DNA hypermethylation in cancer\textsuperscript{33}, and this has also been shown for several hematopoietic neoplasms such as follicular lymphoma, acute lymphatic leukemia and chronic myeloid leukemia, but not previously in AML.\textsuperscript{34} The association between genes with bivalent marks in stem cells and hypermethylation in cancer may in part, but not completely, be due to the overlap with PcG target genes. There are few investigations of this in the literature and, to our knowledge, none in AML; Ohm et al. showed that several tumor
suppressor genes hypermethylated in various malignancies had bivalent marks in stem cells. Rodriguez et al had similar findings in CpG islands at a region of chromosome 5q in colon cancer and McGarvey et al showed it functionally on a global scale using a DNMT1 and 3 double knock-out cell line of colon cancer. Our results are in agreement with these findings, strengthening the hypothesis that there is an association of bivalent marks in stem cells and DNA hypermethylation in multiple malignancies.

Furthermore, we show an association between clinical outcome and the degree of promoter methylation among the PcG target genes. Increased methylation of PcG targets was independently associated with disease free and overall survival in multivariate analyses. One plausible explanation of this finding could be the central role of Homeobox (Hox) gene methylation in AML pathogenesis. Hox genes encode DNA binding proteins central in embryonic development and hematopoesis and their expression is epigenetically regulated by PcG and Trithorax proteins. They are highly enriched in our cohort in DMC 1 and 2 (table S4). Hox genes are overexpressed in AML with Mixed Lineage Leukemia (MLL)-translocations, which is a marker of poor prognosis. Several authors have found that high expression of Hox genes correlate with poor prognosis and low expression with favorable prognosis, compatible with our results. Other possible explanations are offered when looking at the most significant genes associated with outcome (table 2), many which are PcG targets. Interestingly, several potassium channel encoding, PcG targeted, genes were on the list of methylated genes associated with two year survival. Potassium channels have been associated with AML prognosis, with low expression being a favorable prognostic factor.

The genome wide approach applied in a well defined cohort of CN-AML enabled us to show an association between PcG targets and the AML methylome, with increased aberrant methylation of targeted genes, a finding that has not previously been reported. We also found possible prognostic implications of PcG target methylation that could be reproduced in a separate validation cohort. These findings suggest that there is a “CpG island methylator phenotype” among CN-AML with separate clinical characteristics. However, further investigation of the relationship between PcG target silencing, molecular mutations and outcome would be warranted in order to define such an entity for use in clinical practice.

In conclusion, we show that NPM1 and IDH mutations associate with specific clusters of samples, that PcG target genes have an increase of aberrant methylation compared to other genes and that the level of PcG target methylation may be an independent prognostic factor for clinical outcome in CN-AML.
Authorship
Contribution: S.D., P.G, A.L., Y.Q., O.B, S.B. and M.K. performed research. S.D, P.G, H.N, B.U., O.B, U.T., M.H. collected clinical data and samples from the AML patients. A.L and K.E. separated and analyzed the myeloid progenitors. V.G. and K.D. characterized the AML samples. S.D, A.L., S.L. and K.E. designed and interpreted the experiments. S.D wrote the manuscript, with contribution from all co-authors.

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References


Captions

Table 1. Clinical characteristics of 58 de-novo CN-AML samples in the test cohort.

Table 2. Factors retained at the last step in a Logistic Regression analysis of two year survival and achievement of CR. Age at diagnosis (older or younger than 40 years), NPM1, FLT3ITD mutational status and log(WBC-count) was entered together with PcG methylation levels (high or low, divided at median) at the first step. At each step all factors with p-values above 0.1 were disregarded.

Figure legends

Figure 1: (A) Average beta levels for 15-17 samples (Y-axis) according to bisulfite pyrosequencing results. Bisulfite pyrosequencing was considered methylated at a cutoff of 15%. P-values are p=0.03, 0.01, 0.06 for CDKN2B, HIC1, and CDH1 respectively. There were no methylated samples of CDKN2A according to bisulfite pyrosequencing. (B) Technical replicates; two samples were replicated on the 27k array (upper panel) and two replicates on both the 27k and 450k array (lower panel). All were highly correlated (p<0.0001) with Pearson correlations coefficients of r=0.99, 0.94 and r=0.98, 0.98, clockwise from upper left. (C) There is an inverse relationship between promoter methylation assessed by the Illumina HumanMethylation27 array and global CpG methylation as measured by LUMA (Pearson r=-0.45, p=0.02), n=29.

Figure 2: Unsupervised complete linkage hierarchical clustering of samples according to methylation of the 2764 differentially methylated CpG sites. There are two outliers and six major clusters of samples. The normal samples clustered together within cluster one (grey) and are marked by grey boxes below the dendrogram. Mutational status is indicated below; white boxes for wild type and colored boxes for mutations. P-values are given for unequal distribution between clusters for each mutation using the Fisher-Freeman-Halton test (outliers were disregarded). The frequency of NPM1 mutations are increased in cluster two and six, IDH1 in cluster six and IDH2 in cluster two.

Figure 3: (A) Log average gene expression is plotted on the Y-axis and corresponding average beta values on the X-axis for seven samples. Data for 6541 CpG sites corresponding to 3395 genes had significant signals on both expression and methylation arrays in more than six of the samples. Spearman correlation Rho= -0.17, p=2E-88. (B) Examples of correlation between expression (Y-axis) and beta values (x-axis) for twelve genes. (C) Average gene expression of Polycomb associated genes according to average methylation levels in ten samples, showing an inverse relation in this subgroup.
Figure 4: (A) Two-way unsupervised hierarchical analysis (Pearson correlation, complete clustering) of differentially methylated CpG residues \((n=2764)\) between CN-AML and myeloid progenitor cells. Each column represents a sample and each row a CpG site. CpG sites are divided into four major differentially methylated clusters (DMC:s) which are color coded. DMC 1=red, DMC 2=blue, DMC 3=green, DMC 4=purple. (B) An average plot showing differentially methylated CpGs. Each autosomal CpG residue is represented with its average beta value for myeloid progenitors on the Y-axis and the corresponding average beta value of all CN-AML samples on the X-axis. Color codes mark the clustered CpG:s defined in 3A and beige dots are non differentially methylated CpG residues. (C) Principal components analysis of the differentially methylated CpG sites shows separation of the DMC 1+2 from DMC 3+4 whereas only Z-axis differences separate DMC 1 from DMC 2 and DMC 3 from DMC 4. (D) The DMC:s have significantly different precentages of CpG sites within CpG islands shown here in a Venn diagram. On the whole array 72.5% of CpG sites are within CpG islands. (E) Scatterplot showing the average beta values of the differentially methylated genes on the Y-axis for all CN-AML samples (blue) and the myeloid progenitors from normal bone marrow (green), highlighting the variability among AML samples who have increased average beta values compared to the normal controls \((p=0.0003)\).

Figure 5: The difference between average Beta values for AML minus the average Beta values in normal CD34+, CMP and GMP progenitors is defined as \(\Delta \) Beta. (A) \(\Delta \) Beta for PcG targeted genes; overall and for CpG residues within and outside CpG islands. (B) Venn diagram showing the overlap of PcG targets, bivalently marked genes and DMC 1+2 in relation to all measured CpG sites. The odds ratio for overlap between PcG targets and DMC 1+2 is 2.8. p<1E-20 and for bivalently marked genes and DMC1+2 4.4. p<1E-20. (C) \(\Delta \) Beta for genes with Bivalent chromatin marks; overall and for CpG residues within and outside CpG islands. (D) \(\Delta \) Beta for genes both bivalently marked and PcG targeted (middle, dark blue, \(n=694\)), PcG targeted only (left, light green, \(n=1662\)) and bivalently marked only (right, light blue, \(n=1180\)). Error bars=95% CI.

Figure 6: Using a Bayes moderated T-test the most significant CpG sites for achieving CR after one induction \((n=42)\) and for two year survival \((n=62)\) were selected. Red indicates more methylation and green less. (A) Heat map showing a one-way hierarchical clustering analysis of CpG residues (columns) according to CR after first induction (rows). (B) Left panel: Two way hierarchical clustering of CpG residues (columns) and samples (rows) according to the most significant residues for two year survival. Right panel: Kaplan-Meier diagrams demonstrate the discriminating ability of the selected CpG sites on OS. (C, D) Kaplan-Meier diagram showing the impact of the methylation levels of polycomb group (PcG) marked
genes on overall and progression free survival. The samples were divided in tertiles according to the average methylation levels of PcG marked genes. The tertile with most methylation (tertile 3), marked with a solid line, had significantly better overall survival (C) and progression free survival (D) than the less methylated tertiles; in the test cohort (upper panel): p(trend)=0.001, 0.002, respectively. In the validation cohort (lower panel): p(trend)=0.009 and p(trend)=0.035 (middle panel), and for pooled samples: p(trend)=0.00009 and 0.0002, respectively.
### Clinical characteristics n=58

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<tr>
<th>Clinical characteristic</th>
<th>Value</th>
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<tr>
<td>Age at diagnosis</td>
<td>years (range) 47 (18-68)</td>
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<tr>
<td>Gender</td>
<td>n female 37 (64%)</td>
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<td>FAB class</td>
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<tr>
<td>M0</td>
<td>5</td>
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<tr>
<td>M1</td>
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</tr>
<tr>
<td>M2</td>
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<tr>
<td>M4</td>
<td>10</td>
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<tr>
<td>M5</td>
<td>9</td>
</tr>
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<td>M6</td>
<td>2</td>
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<tr>
<td>Blast percentage at diagnosis</td>
<td>(range) 67% (20-98)</td>
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<td>Leukocyte count at diagnosis</td>
<td>xE9/ml (range) 66 (0.9-312)</td>
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<td>NPM1 mutation</td>
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<td>FLT3-ITD</td>
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<td>FLT3-TKD</td>
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<tr>
<td>CEBPA mutation</td>
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<td>IDH2 mutation</td>
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<td>Any IDH mutation</td>
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<tr>
<td>DNMT3A mutation</td>
<td>n/analyzed 13/47 (28%)</td>
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<tr>
<td>CR after 1 induction</td>
<td>n 23 (39%)</td>
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<tr>
<td>Allogeneic sct</td>
<td>n 24 (41%)</td>
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<tr>
<td>Alive at 2 years</td>
<td>n/elegible 23/51 (45%)</td>
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**Table 1.**
<table>
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<th>Retained at last step</th>
<th>Odds ratio</th>
<th>95% confidence interval</th>
<th>p-value</th>
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<td>Overall survival</td>
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<td>PcG target methylation</td>
<td>0.36</td>
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<td>Log WBC-count</td>
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<td>Progression-free survival</td>
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<td>0.26-0.85</td>
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<tr>
<td>CR</td>
<td>0.7</td>
<td>0.22-2.04</td>
<td>0.48</td>
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</tbody>
</table>

Table 2.
Figure 1
Figure 2
Gene expression (Log)

Beta values for gene A

Beta: <0.3 >0.7

Figure 3
Figure 4

A

DMC 1
DMC 2
DMC 3
DMC 4

B

CD34+, CMP & GMP average Beta values

C

Y

Z

X

D

CpG island residues

DMC 1

95%

80%

29%

24%

DMC 2

DMC 3

DMC 4

E

Average Beta

AML

Normal controls
Figure 5
Test cohort
n=58

Validation cohort
n=60

Pooled samples
n=118

Figure 6
Overall survival (months)  Progression free survival (months)
Prognostic DNA methylation patterns in cytogenetically normal acute myeloid leukemia are predefined by stem cell chromatin marks

Stefan Deneberg, Philippe Guardiola, Andreas Lennartsson, Ying Qu, Verena Gaidzik, Odile Blanchet, Mohsen Karimi, Sofia Bengtzén, Hareth Nahi, Bertil Uggla, Ulf Tidefelt, Martin Höglund, Christer Paul, Karl Ekwall, Konstanze Döhner and Sören Lehmann