Tracking myeloid malignancies by targeted analysis of successive DNA methylation at neighboring CG dinucleotides

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Supplemental material

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Materials and Methods

Sample collection and DNA isolation

Blood samples of healthy controls and AML patients were taken at the University Hospital in Aachen, Germany. MDS samples were taken at Dresden University Medical School, Germany. The study was approved by the local ethics committees and conducted in accordance with the Helsinki Declaration. Each patient signed an approved informed consent. Genomic DNA was isolated with the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany).

Pyrosequencing

Genomic DNA was either isolated with the NucleoSpin Tissue (Macherey und Nagel, Düren, Germany) or the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Subsequently, 500 ng DNA were bisulfite-converted using the EZ DNA Methylation Kit (Zymo, Irvine, USA). DNA was amplified using the PyroMark PCR Kit (Qiagen, Hilden, Germany). Amplification products were immobilized to 2 µl Streptavidin Sepharose™ HP beads (GE Healthcare, Piscataway, NJ, USA) followed by annealing to 1.0 µl sequencing primer (5 µM) for 2 minutes at 80°C. Primers for pyrosequencing analysis are listed in Table S2. Samples for pyrosequencing were processed by Cygenia GmbH (Aachen, Germany; www.cygenia.com).

Next generation sequencing

Bisulfite converted DNA was amplified in a first PCR reaction (32 cycles) using gene-specific primers containing handle sequence overhangs using the PyroMark PCR Kit (Qiagen, Hilden, Germany). Primers were removed via clean up with Agencourt AMPure XP Beads (Beckmann Coulter, Indianapolis, USA). DNA was amplified in a second PCR (16 cycles) using primers binding to the handle sequence and containing donor-specific barcodes and Illumina adapter sequences. Samples were pooled and primer were removed by using the Select-a-Size DNA Clean & Concentrator (Zymo, Irvine, USA). 20% PhiX DNA was spiked in and the run was performed using a MiSeq Reagent Nano Kit v2 (500-cycles) and a MiSeq System (Illumina, San Diego, USA).

Statistics

Error bars indicate standard deviations (SD). Differences between groups were estimated by unpaired t-tests.

Supplemental Tables and Figures

Supplemental Table 1. Clinical data AML cohort.

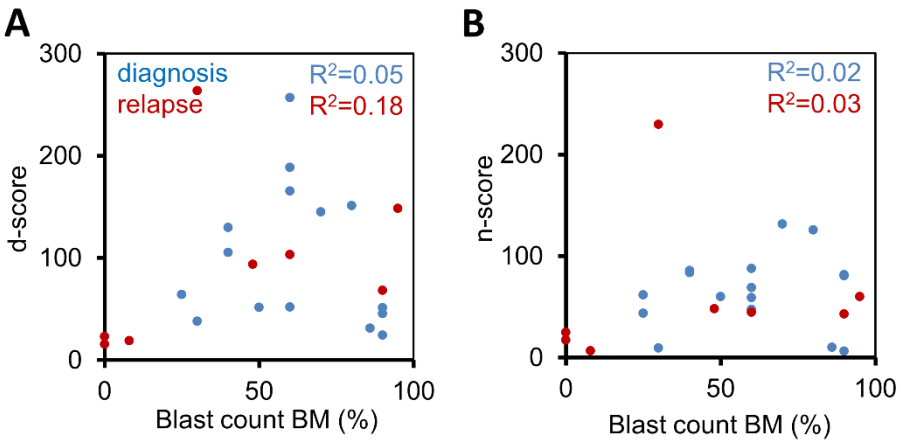
Disease	FAB Classification	Blast Count BM (%)	Material	Gender	Age	Predicted Age
First Diagnosis	M4	84	PB	m	41	95
First Diagnosis	M0	25	BM	f	52	145
First Diagnosis	M0	25	BM	m	53	100
First Diagnosis	M5	unclear	BM	f	70	87
First Diagnosis	M4	60	BM	m	55	27
First Diagnosis	M1	60	BM	f	46	68
First Diagnosis	M5	85	PB	f	70	163
First Diagnosis	M4	40	BM	m	43	31
First Diagnosis	M4	90	BM	f	51	52
First Diagnosis	M1	50	BM	m	68	54
First Diagnosis	M1	90	PB	f	36	54
First Diagnosis	M1	60	BM	f	75	125
First Diagnosis	M4	80	BM	m	55	138
First Diagnosis	M1	unclear	BM	f	49	117
First Diagnosis	M1	90	BM	f	27	26
First Diagnosis	N.A.	40	BM	f	58	41
First Diagnosis	M4	60	BM	f	33	92
First Diagnosis	M1	70	BM	m	72	136
First Diagnosis	M4	86	BM	f	58	34
First Diagnosis	M1	30	BM	f	66	121
Persistent	M1	0	BM	f	46	47
Persistent	M1	60	BM	f	75	92
Persistent	M4	48	BM	m	55	90
Persistent	N.A.	30	BM	f	58	143
Persistent	M4	8	BM	f	33	34
Relapse	M4	90	PB	m	41	138
Relapse	M4	unclear	BM	m	43	174
Relapse	M5	90	BM	f	70	86
Relapse	M4	95	BM	f	44	150
Relapse	M4	0	BM	f	72	74
CR	M4	-	PB	m	41	37
CR	M4	-	BM	m	43	48
CR	M4eo	-	BM	m	38	39
CR	M4eo	-	BM	m	38	40
CR	M0	-	BM	f	52	41
CR	M0	-	BM	f	52	42
CR	M1	-	BM	m	62	58
CR	M1	-	BM	m	62	66
CR	M4	-	BM	m	55	50
CR	M4	-	BM	m	55	41
CR	M5	-	BM	m	34	38
CR	M5	-	PB	f	70	57
CR	M5	-	PB	f	70	60
CR	M4	-	BM	m	43	54

CR	M4	-	BM	f	51	47
CR	M4	-	BM	f	51	46
CR	M1	-	BM	m	68	60
CR	M1	-	PB	f	36	43
CR	M0	-	PB	m	51	73
CR	M0	-	PB	m	51	82
CR	M4	-	PB	f	35	50
CR	M1	-	BM	f	63	63
CR	M1	-	BM	f	63	57
CR	M5	-	BM	f	53	55
CR	M5	-	BM	f	53	49
CR	M1	-	BM	f	75	70
CR	M1	-	BM	m	53	59
CR	M1	-	BM	f	49	56
CR	M1	-	BM	f	49	57
CR	M1	-	BM	f	61	50
CR	M1	-	BM	f	61	52
CR	M4	-	BM	f	44	47
CR	M1	-	BM	m	53	58
CR	M1	-	BM	m	53	54
CR	M4eo	-	BM	m	46	51
CR	M4eo	-	BM	m	46	49
CR	M2	-	BM	m	39	51
CR	M1	-	BM	m	72	70
CR	M1	-	BM	m	72	66
CR	M2	-	BM	m	47	56
CR	M2	-	BM	m	47	50
CR	M4	-	BM	f	72	105
CR	M2	-	BM	m	50	52
CR	M2	-	BM	m	50	53
CR	M4eo	-	BM	m	27	32
CR	M4eo	-	BM	m	27	31
CR	M1	-	BM	f	34	32
CR	M1	-	BM	f	34	37
CR	M2	-	BM	f	36	42
CR	M2	-	BM	f	36	39
CR	M1	-	BM	f	66	61
CR	M1	-	BM	f	66	55
CR	M4	-	BM	f	52	70
CR	M4	-	BM	f	52	57
CR	M2	-	BM	m	55	54
CR	M2	-	BM	m	55	54
CR	M1	-	BM	f	50	78
CR	M4	-	BM	m	65	84
CR	M4	-	BM	m	65	101
CR	M4eo	-	BM	m	21	28
CR	M4eo	-	BM	m	21	31

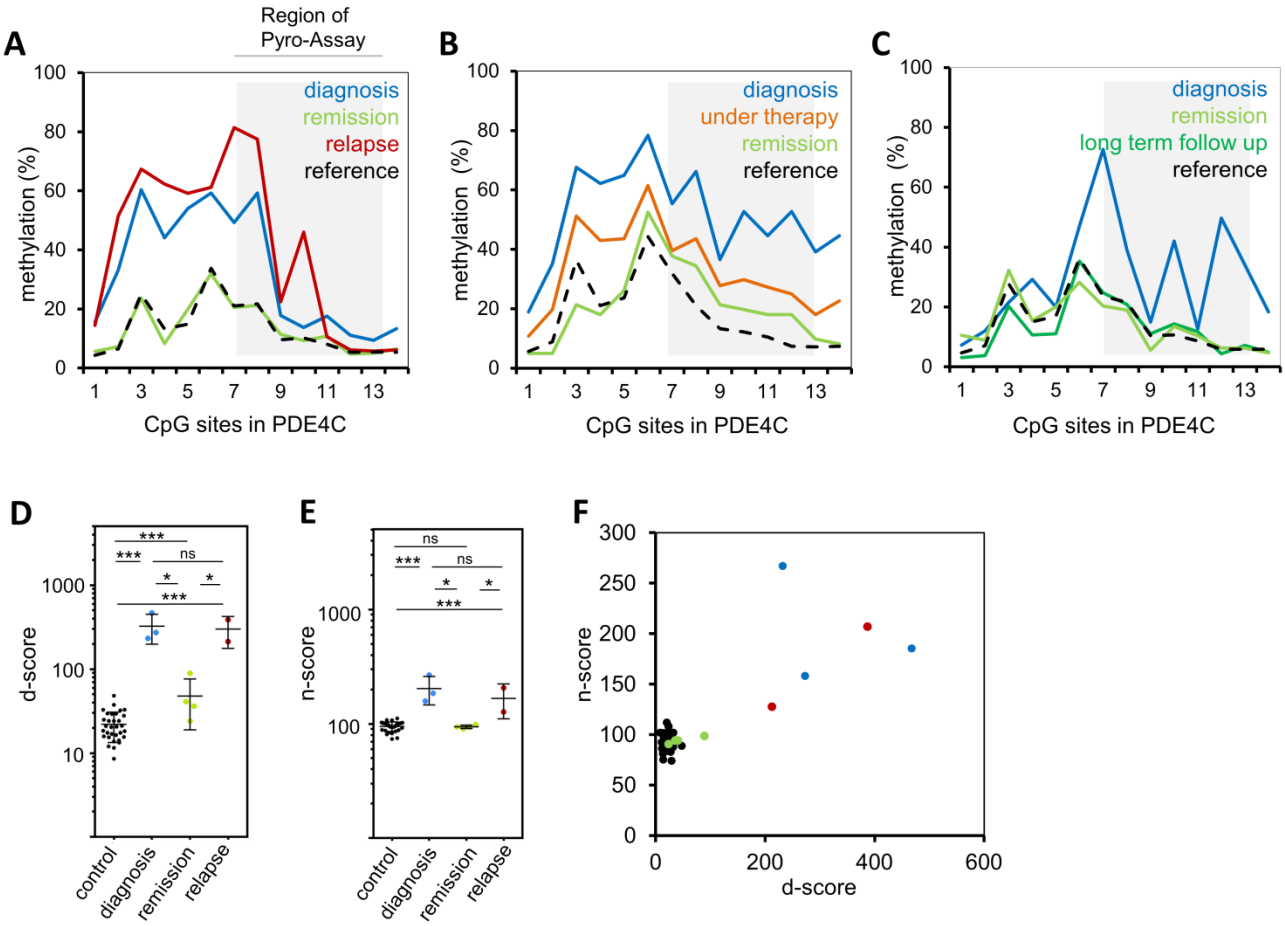
CR: Complete Remission; BM: Bone Marrow; PB: Peripheral Blood; FAB: French-American-British

Supplemental Table 2. Primers for pyrosequencing.

CpG site	Location	Sequence
cg17861230 (*PDE4C*)	Forward	AGGTTTGTAGTAGGTTGAG
	Reverse	Biotin-AACTCAAATCCCTCTC
	Sequencing	GTTATAGTATGATTAGAGTTT
cg02228185 (*ASPA*)	Forward	Biotin-ATTATTTGGTGAAATGATT
	Reverse	CAACCCTATTCTCTAAATCTC
	Sequencing	CCCTATTCTCTAAATCTCA
cg25809905 (*ITGA2B*)	Forward	Biotin-TAATTTTTTTTGGGTGATG
	Reverse	ACCAAAAATAAACAATATACTCAAT
	Sequencing	CAATATACTCAATACTATACCT

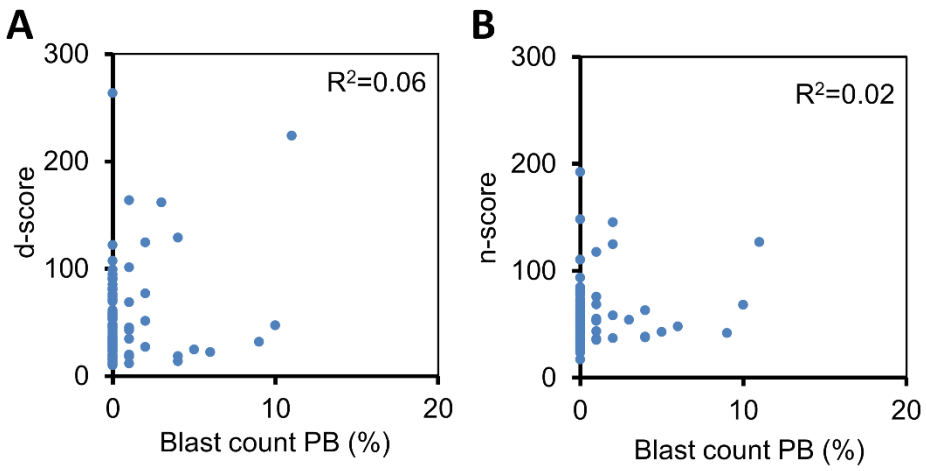


Supplemental Figure 1. Association of d-score and n-score with blast counts in AML.
Correlation of **A**) d-score and **B**) n-score with blast counts (bone marrow) of AML patients at first diagnosis (blue) and relapse (red). In tendency higher variability scores were observed at higher blast counts, albeit the correlation was very low.



Supplemental Figure 2. Barcoded bisulfite amplicon sequencing results of the *PDE4C* region.

A-C) AML samples were re-analyzed by barcoded bisulfite amplicon sequencing (BBA-seq). The depicted samples correspond to those depicted in main figure 1 D-F. The CpGs within the grey shaded region were also covered by the pyrosequencing assay. **D, E**) Delta-scores and neighborhood scores were calculated based on the BBA-seq measurements. Due to the higher number of CpGs within the amplicons the malignant and healthy samples are separated even more clearly. **F**) Correlation of n-score and d-score in BBA-seq analysis. * denotes p<0.05, ** denotes p<0.01, *** denotes p<0.001, ns denotes not significant



Supplemental Figure 3. Association of d-score and n-score with blast counts in MDS.

Correlation of the **A**) d-score and **B**) n-score with blast counts in peripheral blood (PB) of MDS patients. There was no clear association.