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Methylation patterns in CD34 positive chronic myeloid leukemia blast crisis cells

Treatment results of chronic myeloid leukemia blast crisis are unsatisfactory, even with second generation tyrosine kinase inhibitors and allogeneic stem cell transplantation. Loss of tumor suppressor gene activity is one of the events that is associated with progression to blast crisis. Next to genetic alterations, this may be related to hypermethylation of the promoter regions of these genes. Demethylating drugs, like decitabine and 5-azacytidine, have recently become available for clinical purposes and may act by reversing abnormal methylation of chronic myeloid leukemia blast crisis. To estimate their potential value for treatment of chronic myeloid leukemia blast crisis, we investigated the methylation status of the promoters of tumor suppressor genes in clinical samples, using methylation specific multiplex ligation-dependent probe amplification (MS-MLPA), a technique that has been demonstrated to give reliable results, comparable to classical sodium bisulphite sequencing based assays or methylation specific polymerase chain reaction (MSP).[1-3] Methylation specific multiplex ligation-dependent probe amplification enables simultaneous assessment of a large number of chronic myeloid leukemia blast crisis, requires low amounts of DNA (unlike MSP) and is relatively inexpensive.

After informed consent, DNA was isolated from peripheral blood and bone marrow CD34 positive cells (selected by AutoMACS, Miltenyi Biotec, Bergisch Gladbach, Germany) of 19 blast crisis patients (Table 1) and 15 newly diagnosed, untreated chronic myeloid leukemia patients as controls. MS-MLPA was subsequently performed as described previously using probe mixes ME001-Tumor suppressor-1 and ME002-Tumor suppressor-2, which include probes targeted to the CpG islands within the promoter regions of 35 candidate tumor suppressor genes (Online Supplementary Table S1).[1,3]

Using this technique, we observed methylation of at least one tumor suppressor gene promoter CpG island in all 19 blast crisis samples, with a total number of 69 methylated genes. *CDH13*, *ESR1*, *IGSF4*, *MGMT* and *CDKN2B* were the genes that were most frequently

Table 1. Clinical characteristics and results of MS-MLPA. Only genes that were methylated in at least one patient sample are shown. Blanks indicate unmethylated promoter regions.

Pt n.	Age (yr)	Myeloid/ Lymphoid BC	Interval D/-BC (mos)	Previous T/	BRCA 1 1/2	CD 44 1/2	CDH 13 1/2	CDKN 2A 1/2	CDKN 2B	ESR 1 1/2	FHIT	GATA 5	IGSF 4 1/2	MGMT 1/2	MSH 6	PAX 5	PAX 6	RARB 11 1/2	STK 1	THBS 3	TIMP 73 1/2	TP 1	WT	total*
				probe set number																				
1	38	my	26	IM		U/M	M/M[2]		M	M/M	M	M	M/M	M/M			M	M/M	M	M	M	M/M		14
2	44	my	43	HU		U/M									M									2
3	17	my	3	HU			U/M							U/M				U/M						3
4	26	my	0	none					M												M	U/M		3
5	71	bi[3]	0	none		U/M	U/M			U/M	M		M/M	U/M										6
6	53	my	0	none			M/M		M				M/U										M	4
7	66	ly	4	IM			M/M			M/M			M/M					U/M				U/M		5
8	56	my	1	none			M/M															U/M		2
9	65	my	84	IM			U/M			U/M			M/U											3
10	46	my	156	IFN/LD-AraC			U/M			U/M														2
11	39	my	13	IM/ID-AraC						U/M				U/M			M							3
12	54	my	7	IM/ID-AraC; Ida/AraC			U/M			U/M			M/U	U/M										4
13	66	my	40	IM			U/M			U/M				U/M										3
14	59	my	46	HU/IFN			U/M									M								2
15	52	ly	0	none																		U/M		1
16	46	my	0	none			U/M	U/M																2
17	62	my	3	IM/ID-AraC	U/M		U/M		M															3
18	61	my	3	HU/IFN			U/M																	1
19	79	u	119	u			M/M		M		M	M	M					U/M						6
total					1	3	15	1	5	8	3	2	7	6	1	1	2	4	1	1	2	5	1	

D/: diagnosis; BC: blast crisis; U: unknown; T/: treatment; IM: imatinib; HU: hydroxyurea; IFN: interferon-alpha; LD-AraC: low-dose cytarabine; ID-AraC: intermediate dose cytarabine; Ida: idarubicin 1: blanks in this row indicate that only one probe set was used as shown in Online Supplementary Table S1. *2: M: methylated; U: unmethylated; 3: biphenotypic *: In case two probe sets for a single gene promoter were used, the gene was scored positive when at least one probe set showed methylation; if two CpG islands tested in a single gene promoter were methylated, then counted only once .*

methylated (Table 1). In the 15 control CML-CP samples, only 3 individual patients had a single methylated gene, *CDH13*, *MGMT* and *MSH1*, respectively. All other tested genes were non-methylated ($P<0.001$ for difference in number of methylated genes between CP and blast crisis, Mann-Whitney U-test).

Thus, aberrant methylation of tumor suppressor genes seems a common event in chronic myeloid leukemia blast crisis. In clinical chronic myeloid leukemia samples and chronic myeloid leukemia cell lines, methylation of *BRCA1*, *CDH13*, *CDKN2B*, *ESR1*, *FHIT* and *PAX6* was demonstrated before.[4-8] However, to our knowledge, aberrant methylation of *CD44*, *GATA5*, *IGSF4*, *MGMT*, *MSH6*, *PAX5*, *RARB*, *STK11*, *THBS1*, *TP73* and *WT1* in chronic myeloid leukemia blast crisis has not been reported previously. Importantly, as we used CD34 positive progenitor cells in all samples (blast crisis as well as CP), methylation appears to be an acquired event during or preceding transformation of the disease and not just the result of increasing numbers of progenitor cells that had methylated genes from the start in CP, but that were obscured by the admixture of more differentiated cells with non-methylated promoters. As we did not have at our disposal CD34 positive samples from diagnosis and blast crisis of the same patients, this hypothesis cannot, however, be proven.

The high number of methylated genes in blast crisis may suggest that aberrant methylation is a non-specific, global process that is a feature of advanced phase chronic myeloid leukemia, at least in a subset of patients. Possibly, this may be related to increased expression of dimethyltransferases, as was demonstrated for DNMT1, 3A and 3B in chronic myeloid leukemia blast crisis samples when compared with normal bone marrow.[9] In the mononuclear cell fraction of chronic myeloid leukemia CP samples, no increase in DNMT levels was observed. The mechanism behind the upregulation of these enzymes is unknown.

Restoring tumor suppressor gene expression by demethylating treatment may be a rational therapeutic strategy. A few clinical studies assessing the efficacy of demethylating treatment in chronic myeloid leukemia have been published. Kantarjian *et al.* investigated treatment of chronic myeloid leukemia patients with decitabine in different phases of the disease.[10] Responses were superior in early phase chronic myeloid leukemia compared to more advanced phases, while myelosuppression was the most important adverse effect. The combination of decitabine with imatinib seemed to have only modest activity in a second, phase II study.[11] In patients who responded to the combination, there was a lesser decrease of global DNA methylation than in the non-responders. The results of both studies call into question whether the observed clinical effect relates to reduction of methylation. Further studies are warranted to determine if, indeed, hypomethylating activity is their main mechanism of action.

In conclusion, the progenitor cell fraction in chronic myeloid leukemia blast crisis clearly shows more extensive methylation of putative tumor suppressor genes than in CP, where methylation is almost absent. Aberrant methylation of several of the tumor suppressor genes that we detected has not been previously reported. Our results suggest that hypermethylation of tumor suppressor genes may be a therapeutic target. However, more extensive pre-clinical and clinical studies incorporating demethylating agents are needed to assess their therapeutic role in chronic myeloid leukemia blast crisis.

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Key words: CML, methylation, blast crisis.

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Citation: Janssen JJWM, Denkers F, Valk P, Cornelissen JJ, Schuurhuis G-J, and Ossenkoppele GJ. Methylation patterns in CD34 positive chronic myeloid leukemia blast crisis cells. Haematologica. 2010;95(6):1036-1037. doi:10.3324/haematol.2009.015693

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