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Promoter methylation of DAPK1, E-cadherin and thrombospondin-1 in de novo and therapy-related myeloid neoplasms ☆

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ABSTRACT

DNA methylation is one of the major epigenetic changes in human cancers, leading to silencing of tumor suppressor genes, with a pathogenetic role in tumor development and progression in myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML). Methylation of key promoter regions, induced by cytotoxic therapy together with complex genetic changes, is important in the biology of therapy-related myeloid neoplasms (t-MN).

We were interested in the characterization of the methylation pattern of AML and MDS de novo and therapy-related. We studied 385 patients (179 females, 206 males), of a median age of 66 years (range 16-98 years). There were 105 MDS, 208 de novo AML and 72 t-MN (45 MDS and 27 AML). Using a methylation-specific PCR, we studied the promoter methylation status of E-cadherin (CDH1), TSP1 and DAP-Kinase 1. These genes have been shown to be involved in the malignant transformation, interfering with angiogenesis, interaction with micro-environment, apoptosis and xenobiotic detoxification.

We found no associations between promoter hypermethylation and gender or age at the time of initial diagnosis. In patients with MDS, there were no associations between hypermethylation and clinical characteristics, including IPSS score, WHO classification and cytogenetics. DAPK1 was more frequently methylated in t-MDS/AML when compared to de novo MDS and AML (39% vs 15.3% and 24.4%, $p = 0.0001$), while methylation of CDH1 was similar in t-MDS/AML and AML (51% and 53.4%), but less frequent in de novo MDS (29%) ($p = 0.003$).

In the t-MDS/AML group, we found that the methylation pattern appeared to be related to the primary tumor, with DAPK1 more frequently methylated in patients with a previous lymphoproliferative disease (75% vs 32%, $p = 0.006$). On the other hand, methylation of CDH1 was associated to radiotherapy for the primary malignancy (84.5% vs 38%, $p = 0.003$). TSP1 hypermethylation was rare and not characteristic of t-MDS/AML. In 177 patients studied for concurrent methylation of several promoters, t-MN and AML de novo were significantly more frequently hypermethylated in 2 or more promoter regions than de novo MDS (20% vs 12.4%, $p < 0.001$).

Chemotherapy and individual genetic predisposition have a role in t-MDS/AML development, the identification of specific epigenetic modifications may explain complexity and genomic instability of these diseases and give the basis for targeted-therapy. The significant association with previous malignancy subtypes may underlie a likely susceptibility to methylation of specific targets and a role for constitutional epimutations as predisposing factors for the development of therapy-related myeloid neoplasm.

Introduction

DNA methylation is a physiologic process involved in growth, differentiation and maturation of cells. Correct levels of DNA methylation, especially in the pericentromeric region of chromosomes, are necessary for their stability and the successful DNA replication. Pathologic changes in chromatin structure, including aberrant DNA methylation, are known to contribute to the development of diseases, cancer and aging. In cancer, two different methylation patterns may occur: global hypomethylation, resulting in loss of chromosomal stability and genetic instability, and promoter-specific hypermethylation, leading to silencing of tumor suppressor genes [1]. The "methylator" phenotype has been described in several hematological and solid neoplasms and involves pathways of DNA-repair, cell-cycle control, development, differentiation, apoptosis and

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detoxification and it is thought to be relevant for leukemogenesis [2,3]. Global hypermethylation is associated to disease progression, cooperating mutations and cytogenetic abnormalities [4,5].

So far, several studies have investigated aberrant promoter hypermethylation in myeloid malignancies and hypomethylating agents are currently used in the treatment of MDS and AML [3]. On the contrary, only few data are available on epigenetic changes in therapy-related MN.

We were interested in the characterization of the methylation pattern of de novo compared to therapy-related myeloid neoplasms and studied the methylation profile of genes involved in different carcinogenetic pathways, including Death-associated protein kinase-1 (DAPK1), E-cadherin (CDH1) and thrombospondin1 (TSP1). DAPK1 is a pro-apoptotic calcium/calmodulin-regulated serine/threonine kinase, participating to several apoptotic pathways initiated by IFN-γ, TNF-α, activated Fas, and detachment from extracellular matrix. DAPK-1 is abnormally methylated in a significant proportion of human tumors, including haematological malignancies. [6,7]. CDH1 is a member of membrane glycoproteins mediating interaction with cytoskeleton through β- and γ-catenin, critical for the formation of extracellular cell-cell adhesion. CDH1 functions as a tumor suppressor: its loss due to methylation enables disaggregation of cancer cells from one another and increases their metastatic potential [8,9]. CDH-1 has been reported to be methylated in 82% acute myeloid leukemias, 60% acute lymphoblastic leukemia and 60% chronic lymphocytic leukaemia [10,11]. TSP1 belongs to a family of glycosylated extracellular matrix adhesion proteins modulating interactions between cells and the micro-environment. TSP1 is a large, multifunctional protein binding to CD36 on endothelial cells and inhibits their ability to migrate while inducing apoptosis in these cells [12]. Therefore, TSP-1 is an effective inhibitor of angiogenesis. No data exist about its role in haematological malignancies.

Patients and methods

Patient's characteristics

We studied 385 patients (179 females, 206 males), of a median age of 66 years (range 16-98 years). The whole group of patients included 105 MDS, 208 de novo AML and 72 t- MN, consecutively admitted to our Institution between January 2000 and October 2008. This series includes the 47 patients previously reported for DAPK1 [7]. The diagnosis was established according to standard morphologic and immunophenotypic criteria and revised according to the WHO classification [13]. Karyotype was defined for 268 patients. The IPSS score [14] was calculated for 85 MDS patients. Patients gave informed consent and the study was conducted according to good clinical and laboratory practice rules and the principles of the Declaration of Helsinki.

Analysis of DAPK1, CDH1 and TSP1 methylation

Mononuclear cells (MNC) were separated from the bone marrow (BM) at the time of initial diagnosis, using Ficoll density centrifugation and CD34+ cells were freshly isolated from normal bone marrows using immunomagnetic beads (Minimacs, Milteny Biotec. GmbH, Germany), according to the manufacturer's instructions. DNA methylation pattern in the CpG island of DAPK1 and TSP1 promoter genes was determined by methylation-specific PCR (MS-PCR) (supplementary data) [15,16]. The methylation status of CDH1 was studied using a nested MS-PCR [17]. DNA extracted from Jurkatt, Raji and Hela cells was used as control for methylated DNA, and DNA from normal lymphocytes or from KG-1a cells (LGC-ATCC Middlesex, UK) was used as control for unmethylated DNA.

PCR was performed in a final volume of 25 μL, using the Hot Master Mix (Eppendorf AG), 0.1 g DNA, and 20 pmoles of oligonucleotides (Supplementary data). Ten microliters of each PCR reaction were directly loaded onto a 3% agarose gel, stained with ethidium bromide, and visualized under UV illumination.

The Fisher's exact test was used to examine for differences in patients' characteristics according to genes' hypermethylation. All computations were performed using the Stata 6.0 software (Stata Corp.).

Results

Methylation of tumor suppressor genes

The distribution of DAPK1, TSP1, and CDH1, hypermethylation of according to diagnosis is shown in Fig. 1. Confirming our previous data on a smaller series of patients, we found that DAPK1 was more frequently methylated in t-MN when compared to de novo MDS and AML (39% versus 15.3% and 24.4%, respectively, $p < 0.0001$) (Fig. 1) (Table 1). In this line we observed a higher frequency of DAPK1 methylation in higher risk MDS, comparing RAEB2 to other MDS subtypes (43% vs 15%), although the difference did not reach statistical significance, probably due to the small number of patients (Table 2).

Methylation of CDH1 was similar in t-MN and AML (51% and 53.4%, respectively) and less frequent in de novo MDS (29%, $p = 0.003$) (Fig. 1). Moreover, therapy-related MN patients with higher blast count (t-AML) were more frequently methylated for CDH1 than patients with lower blast count (t-MDS) (63% vs 31.8%, $p = 0.02$, O.R. 3.75, 95% C.I. 1.195 to 11.77)(Table 3).

TSP1 hypermethylation was rare and was not characteristic of MDS and AML (figure1). We found no further correlations between CDH1 and DAPK1 methylation and patients' characteristics at the time of initial diagnosis, including gender, age, WHO classification, cytogenetics and white blood cell counts in AML, and the IPSS score in MDS (Table 1).

In the t-MN group, we found that the methylation pattern was related to the primary tumor, with DAPK1 more frequently methylated in patients with a previous lymphoproliferative disease (75% vs 31%, $p = 0.006$, O.R. 6,37, 95% C.I. 1.517 to 26.79). No significant differences were observed in CDH1 and TSP1 methylation frequencies and previous malignancies, although CDH1 methylation seems to be more frequent in t-MN secondary to genitourinary apparatus neoplasms (75% vs 48%) without reaching the statistical significance, probably due to the small number of patients (Table 3). On the other hand, methylation of CDH1 was associated to radiotherapy for the primary malignancy (84.5% vs 38%, $p = 0.003$, O.R. 8.938, 95% C.I. 1.750 to 45.65) (Table 3).

Of 177 patients studied for concurrent methylation of the three genes, t-MN and AML were more frequently hypermethylated in 2

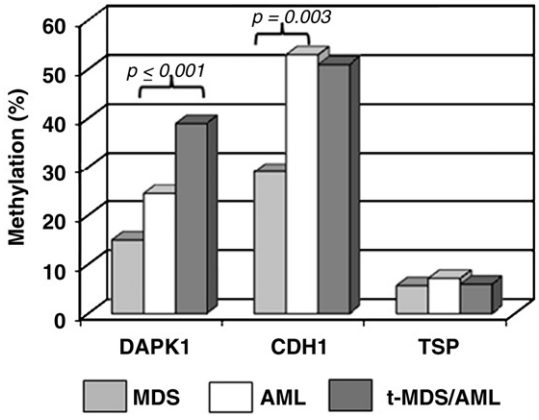

Fig. 1. Frequency of DAPK1, CDH1 and TPS1 promoter methylation in de novo MDS (105 patients), de novo AML (208 patients) and t-MN (72 patients).

Table 1
Promoter Methylation frequency in de novo AML and patients' characteristics.

	Patients'number (N=208)	DAPK1-m 43/176 (24.4%)	CDH1-m 47/88 (53.4%)	TSP-m 7/97 (7.2%)
Age (years)	< 60 (89)	15/70 (21.4%)	25/42 (59.5%)	2/48 (4.1%)
	>60 (119)	28/106 (26.4%)	22/46 (47.8%)	5/49 (10.2%)
Gender	F (90)	18/71 (25.3%)	19/44 (43.1%)	3/48 (6.2%)
	M (118)	25/105 (23.8%)	28/44 (63.6%)	4/49 (8.1%)
Morphology	Minimally differentiated (12)	2/10 (16.6%)	3/5 (60%)	0/4
(194)	Without maturation (21)	6/21 (28.5%)	2/7 (28.5%)	0/5
	With maturation (45)	9/37 (24.3%)	11/18 (61%)	1/18 (5.5%)
	Promyelocytic (24)	6/21 (28.5%)	4/10 (40%)	0/14
	myelomonocytic (20)	4/15 (26.6%)	8/10 (80%)	0/10
	Monoblastic (19)	3/18 (16.6%)	3/7 (42.8%)	3/13 (23%)
	With dysplasia (43)	9/35 (25.7%)	10/19 (52%)	3/21 (14%)
	other* (10)	1/9 (11%)	3/5 (60%)	0/7
Citogenetyc risk	Favourable (31)	6/28 (21.4%)	8/15 (53.3%)	0/16
group ** (141)	Intermediate (86)	16/71 (22.5%)	22/39(56.4%)	2/39 (5%)
	Unfavourable (24)	4/22 (18%)	2/7 (28.5%)	4/13 (30%)
WBC (178)	< 10.000/mmc (88)	20/75 (26.6%)	21/40 (52.5%)	3/41 (7.3%)
	> 10000/mmc (90)	16/75 (21.3%)	20/40 (40%)	4/48 (8.3%)

* "Other" include 8 erythroid leukaemia, 1 megakaryoblastic leukaemia and 1bifenotipic leukemia.
**Citogenetyc risk group:
➢Favourable: t(8;21); inv(16); t(15;17).
➢Intermediate: Normal +8 +21 +22, del(9q), Abnormal 11q23 All other structural/numerical abnormalities.
➢Unfavourable: del(5q); del (7q); -7;-5; complex caryotype.

promoters than de novo MDS (20% versus 12.4%, $p<0.001$, O.R. 4.27, 95% C.I. 2.256-8.103).

Hypermethylation and disease evolution

We looked at the dynamics of DAPK1 and CDH1 hypermethylation during disease evolution in MDS. We examined sequential samples from 11 patients at diagnosis and after a median of 9.6 months (range 1.8-36.5 months), either during routine follow-up ($n=3$), or in the presence of disease progression ($n=8$), with blast counts increasing from 6.9 ± 1.7% to 31.7 ± 7.5% (mean ± SEM, $p=0.014$). We found that the methylation status of DAPK1 did not change. On the other hand CDH1 hypermethylation occurred at the time of AML transformation in 4 of 6 patients (66%).

Since MDS bone marrow samples may be heterogeneous for the proportion between normal and abnormal cells, and between mature and immature cells, we freshly isolated CD34+ cells from 11 consecutive MDS samples. DAPK1 methylation status was concordant in the CD34+ and CD34- cell fractions (7 unmethylated and 4 methylated). On the other hand, CDH1 hypermethylation seems to be more characteristic of mature cells, since it was absent in CD34+ progenitors in 7 patients, 3 of them hypermethylated in CD34- cells.

Discussion

In this study, we assessed the frequencies of aberrant promoter methylation of genes involved in apoptosis, interaction with the microenvironment and angiogenesis in de novo and therapy-related myeloid neoplasms. All the studied genes were already reported to be common targets of aberrant hypermethylation in several tumors, including hematological malignancies. We found DAPK1 and CDH1

Table 2
Promoter Methylation frequency in de novo MDS and patients' characteristics.

	Patients' number (n=105)	DAP-K1-m 15/98 (15.3%)	CDH1-m 30/103 (29%)	TSP1-m 6/104 (5.7%)
Age (years)	< 60 (19)	3/17 (17.6%)	5/19(26.3%)	0/19
	> 60 (86)	12/81(14.8%)	25/86(29%)	6/85 (7%)
Gender	F (44)	5/43 (11.6%)	14/44(31.8%)	3/44 (6.8%)
	M (61)	10/55 (18%)	16/61(26.2%)	3/60 (5%)
Type*	RA (27)	2/25 (8%)	8/27 (29.6%)	1/27 (3.8%)
	RARS (15)	3/14 (21.4%)	6/15 (40%)	0/15
	RCMD (10)	1/10 (10%)	4/10 (40%)	1/10 (10%)
	RAEB-1 (20)	3/19 (15.7%)	5/19 (26.3%)	1/20 (5%)
	RAEB-2 (10)	3/7 (42.8%)	1/10 (10%)	1/10 (10%)
	Syndrome 5q- (4)	0/4	1/4 (25%)	1/4 (25%)
	SMP/SMD (19)	3/19 (15.7%)	5/18 (27.7%)	1/18 (5.5%)
Cytogenetic risk group**	Favourable(63)	7/62 (9.7%)	19/62(30.6%)	2/62 (3.2%)
(85)	Intermediate (8)	3/7 (42.8%)	2/8 (25%)	1/8 (12.5%)
	Unfavourable(14)	2/12 (16.6%)	1/13 (7.6)	2/14(14.2%)
		p = 0.03		
PLTs (103)	>50000/mmc (82)	9/82 (11%)	16/81(19%)	4/82 (4.8%)
	<50000/mmc(21)	4/19 (21%)	10/20 (50%)	1/20 (5%)
HB (103)	> 10 g/dl (41)	6/41 (14%)	7/40 (17.5%)	0/41
	< 10 g/dl (62)	9/57 (15%)	21/61(34.4%)	5/61 (8%)

*RA: refractory anemia; RARS: refractory anemia with ringed sideroblast; RCMD: refractory cytopenia with multilineage dysplasia; RAEB-1: refractory anemia with excess blast-1; RAEB-2: refractory anemia with excess blast-2; SMP/SMD: myeloproliferative/myelodysplastic syndromes.
**Cytogenetic risk group includes:
➢Favourable: normal caryotype; del (5q); del (20q); -Y.
➢Intermediate: tris 8, other.
➢Unfavourable: del (7q); -7; complex caryotype.

Table 3
Promoter Methylation frequency in t-MN and patients' characteristics.

	Patients'number (n = 72)	DAPK1-m 25/64 (39%)	CDH1-m 28/55 (51%)	TSP1 3/49 (6%)
Age (years)	< 60 (28)	7/26 (26.9%)	10/19 (52.6%)	2/18 (11%)
	> 60 (44)	18/38 (47.3%)	18/36 (50%)	1/31 (3%)
Gender	F (45)	13/40 (32.5%)	19/35 (54%)	2/30 (6.4%)
	M (27)	12/24 (50%)	9/20 (45%)	1/19 (5%)
Morphology	t- MDS (25)	7/22 (31.8%)	7/22 (31.8%)	1/20 (5%)
	t-AML (47)	18/42 (42.8%)	21/33 (63%)	2/29 (6.8%)
			p = 0.02	
Primary tumor (71)	Hodgkin's lymphoma (7)	1/7 (14.2%)	1/4 (25%)	0/4
	Non Hodgkin lymphoma (15)	9/12 (75%)	3/10 (30%)	0/11
	APL and MPN (6)	3/6 (50%)	3/6 (50%)	1/5 (20%)
	Breast (23)	4/20 (20%)	10/18 (55.5%)	0/14
	Genital-Urinary apparatus* (11)	5/10 (50%)	6/8 (75%)	1/7 (14.2%)
	Lung, colorectal and thyroid** (9)	3/8 (37.5%)	5/8 (62.5%)	1/8 (12.5%)
		p = 0.006		
Citogenetyc risk group (42)	Favourable (2)	0/2	1/2 (50%)	1/1(100%)
	Intermediate (19)	6/18 (33.3%)	7/14 (50%)	0/13
	Unfavourable (21)	7/18 (38.8%)	8/18 (44.4%)	2/16 (12.5%)
Treatment (66)	Alkylating Agents (20)	8/17 (47%)	4/13 (30.7%)	1/14 (7%)
	Chemo/Radiotherapy (16)	6/15 (40%)	5/16 (31%)	1/12 (8.3%)
	Radiotherapy. Alone (17)	6/14 (42.8%)	11/13(84.5%)	0/10
	Other*** (13)	3/12 (25%)	7/13 (53%)	0/11
			p = 0.003	
WBC (58)	< 10000/mmc (45)	14/39 (35.8%)	15/36 (41.6%)	2/32 (6.2%)
	> 10000/mmc (13)	5/13 (38.4%)	7/9 (77%)	0/8

*Genito-urinary cancers include testicular, ovary, uterus, urothelial cancer (1 patient each), 2 bladder cancers and 5 patients with prostate cancer.
**This group includes: 5 patients with thyroid cancer, 3 patients with lung cancer and another one with colorectal cancer.
***This group includes: hydroxyurea, fludarabine, cytarabine, methotrexate, 5-fluorouracil, idarubicin, etoposide, bleomycin, carboplatin.

more commonly methylated in AML than MDS while TSP1 methylation was present in a small number of patients without any difference among different disease groups.

Promoter hypermethylation may affect disease biology and progression. Genome-wide methylation has been shown to cooperate with chromosome deletions to silence tumor suppressor genes and is actually considered a dominant mechanism for clonal variation in MDS evolution to AML [4]. Methylation of the p15 promoter has been found as late event in therapy-related leukemogenesis particularly in cases with abnormalities of chromosome 7, and was shown to increase during progression from refractory anemia to refractory anemia with excess of blasts in transformation [18,19]. In this line, we found more frequent CDH1 and DAPK1 methylation in acute myeloid leukemia, when compared to myelodysplastic syndromes. Since expression of CDH1 by stromal and mononuclear cells in the bone marrow was shown to affect intercellular interactions, sustaining hematopoiesis [20], CDH1 methylation in AML may lead to the impaired maturation and infiltrative phenotype during leukemic transformation. In this line, CDH1 methylation was acquired ex novo in 4 of 6 MDS patients at the time of AML transformation. No changes in DAPK1 methylation occurred during MDS progression, suggesting that other pathways may be involved in the switch from apoptosis to proliferation found in AML transformation.

Looking at DAPK1 and CDH1 methylation in purified CD34+ and CD34- bone marrow populations, CDH1 methylation was absent in the CD34+ cell fraction, while concordant data between the two fractions were found for DAPK1. The difference between CDH1 methylation pattern in CD34+ and CD34- cell fractions might be related to a dynamic control of methylation during differentiation. Studying in vitro differentiation of normal and myelodysplastic CD34+ cells, Hopfer et al. [21] described an increasing frequency of abnormal promoter hypermethylation during differentiation of hematopoietic progenitors and a lineage specific pattern of methylation for several genes, as p15, CHK2, and survivin.

We were particularly interested in therapy-related myeloid neoplasms, which are now classified according to the WHO as a distinct nosographic category [22]. Some reports have suggested a possible link between DNA methylation and DNA damage induced by environmental carcinogens, including benzene, tobacco and reactive oxygen species (ROS), as well as cytotoxic drugs and radiation. [18,23–27].

In our study population, DAPK1 was more commonly methylated in t-MN compared to de novo MDS and AML, as previously reported in a smaller patient series [7]. By analyzing methylation frequencies and patients characteristics, significant associations emerged between gene promoter methylation, previous neoplasm and type of treatment. Interestingly, we found higher methylation frequency of DAPK1 in patients with therapy-related MN following treatment of lymphoproliferative diseases, and of CDH1 in t-MN secondary to genitourinary apparatus neoplasms (75% vs 48%). Constitutional promoter hypermethylation of tumor suppressor genes has been observed in normal tissues from cancer patients as well as in different neoplasms arising in the same patient [28]. The association between the methylation pattern of the primary tumor and the subsequent therapy-related MN might be related to an individual susceptibility for methylation of specific targets. In this line, we previously described the association between BRCA1 promoter hypermethylation and t-AML following breast cancer [29]. In this context a predisposition to epimutations of commonly methylated tumor suppressor genes might contribute to the pathogenesis of both primary tumor and therapy-related MN.

In our t-MN patients CDH1 methylation was significantly associated with previous exposure to radiotherapy. Using an engineered experimental model, O'Hagan et coll. [30] showed as a defined double strand break in an exogenous CDH1 promoter construct is able to recruit the SIRT1 histone deacetylase along with PRC2, DNMT1, and DNMT3B at the site of DNA damage, leading to DNA repair, as well as heritable silencing by chromatin modifications including deacetylation at H4K16, methylation at H3K27, and DNA hypermethylation. According to these data and our findings, we hypothesize that exposure to DNA damage agents as radiation or cytotoxic drugs, may contribute to the induction of aberrant promoter hypermethylation in hematopoietic tissue by eliciting DNA damage response and repressive chromatin modifications and creating the epigenetic background for development of therapy-related myeloid malignancies.

In conclusion our findings suggest that promoter hypermethylation in genes involved in apoptosis, interaction with the microenvironment and angiogenesis, especially if associated, may be involved in the pathogenesis of myeloid malignancies and in particular in therapy-related MN. The significant association with previous malignancy subtypes may underlie a susceptibility to methylation of specific targets and a role for constitutional epimutations as predisposing factors for the development of therapy-related myeloid neoplasm.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcmd.2010.05.008.

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