

## Cytogenetic effect of 5-azacytidine in patients with hematological malignancies

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**Background:** Recently, the importance of cytogenetics has grown in the diagnosis, prognosis and treatment of leukemias and myelodysplastic syndromes. 5-azacytidine is a drug that has well-known cytogenetical effects and is approved in the treatment of myelodysplastic syndromes. To date, no studies have been performed to evaluate the impact of 5-azacytidine on the chromosomes of patients with hematological neoplasias. This study aimed to investigate the effects of 5-azacytidine on chromosomes of patients with different hematological malignancies using G-band analyses to identify possible cytogenetical alterations.

**Methods:** The peripheral blood of 18 patients with hematological malignancies and 18 controls was collected in heparinized tubes. 5-azacytidine was added, at a final concentration of 10-5M, to cultures 7 hours prior to harvest.

**Results:** Uncoiled centromeric/pericentromeric heterochromatin of chromosomes-1, 9 and 16 occurred more frequently in the patients than in controls. This higher frequency of uncoiled heterochromatin was statistically significant ( $p$ -value = 0.004) for chromosome-9. Conversely, we observed that the fragile site at 19q13 was more frequent in controls ( $p$ -value = 0.0468).

**Conclusions:** The results of this study suggest that satellite sequences, located in the heterochromatin of chromosome-9, are hypomethylated in hematological malignancies. This hypomethylation may contribute to the disease, activating transposable elements and/or promoting genomic instability, enabling the loss of heterozygosity of important tumor suppressor genes. An investigation of the 19q13 region may help to understand whether or not the predominant occurrence of the fragile site at 19q13 in controls is due to hypermethylation of this region.

**Keywords:** Cytogenetics; Azacitidine; Heterochromatin

## Introduction

Leukemias and myelodysplastic syndromes (MDS) are both clonal hematopoietic stem cell disorders, but while MDS is characterized by ineffective hematopoiesis, peripheral cytopenias and cytological atypia in one or more myeloid cell lineages, leukemias involve overproliferation of hematopoietic stem cells which when added to their failure to differentiate leads to an accumulation of non-functional cells, known as blasts, in the bone marrow and the peripheral blood.<sup>(1-4)</sup> About 30% of MDS cases evolve to acute myeloid leukemia (AML), thus MDS is considered a pre-leukemia disease.<sup>(5)</sup> Aberrant karyotypes are found in 30-50% of MDS, 40% of AML, 64-85% of adult acute lymphoid leukemia (ALL), 60-69% of pediatric ALL and in 95% of chronic myeloid leukemia (CML).<sup>(2-5)</sup>

Pretreatment cytogenetic findings have repeatedly proved to be among the most important independent prognostic factors for AML and ALL.<sup>(4)</sup> Chromosomal analyses are important prognostic markers; this is one of the parameters evaluated by the International Prognostic Scoring System (IPSS), the gold standard for risk assessment of patients with de novo MDS.<sup>(1)</sup>

DNA methylation is an epigenetic process, meaning it has heritable information other than the DNA sequence involved in gene expression.<sup>(6,7)</sup> This process is essential for embryonic development, tissue differentiation, X chromosome inactivation in females and imprinting.<sup>(7,8)</sup> Epigenetic alterations have gained increasing recognition as important participants in tumor development and progression.<sup>(9)</sup> Abnormal DNA methylation patterns have been recognized in cancer cells for over two decades.<sup>(10)</sup> It is well established that although the genome as a whole is hypomethylated in cancer, CpG islands located in promoter regions of certain genes are found hypermethylated.<sup>(9,11-16)</sup> In experimental settings, demethylating agents are capable of reactivating tumor suppressor genes silenced by promoter hypermethylation.<sup>(14)</sup> 5-azacytidine (5-AZC) is a cytidine analog, in which a nitrogen atom replaces the carbon in the 5 position of the pyrimidine ring. As a

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result, when 5-AZC is incorporated into DNA, it cannot be methylated.<sup>(17)</sup> Low doses of 5-AZC induce DNA demethylation in malignant myeloid cells. The use of 5-AZC in clinical trials yielded positive results and so this was the first drug to be approved by the US Food and Drug Administration (FDA) for the treatment of MDS.<sup>(18)</sup>

When added into human lymphocyte cultures, 5-AZC is known to cause uncoiling of the centromeric/pericentromeric heterochromatic regions (Figure 1A) of chromosomes 1, 9, 15, 16 and Y, allowing associations between these under-condensed regions (Figure 1B), which occurs during the interphase.<sup>(19,20)</sup> Cytogenetically, this demethylating agent also induces the formation of the fragile sites at 1q42 and 19q13 (Figure 1C) and, after 2 cycles of replication, it provokes differential under-condensation of sister-chromatids.<sup>(21,22)</sup>

In spite of the central role played by cytogenetics in the diagnosis, prognosis and treatment choice of leukemias and MDS, and of 5-AZC being a drug of well reported cytogenetic effects used to treat these diseases, no studies have been performed to evaluate the impact of 5-AZC on chromosomes of patients diagnosed with hematological neoplasias.

This study aimed to investigate the effects of the demethylating agent, 5-AZC, on chromosomes of patients with different hematological malignancies. In addition, G-band analyses were performed to detect possible chromosomal rearrangements in the peripheral lymphocytes of patients.

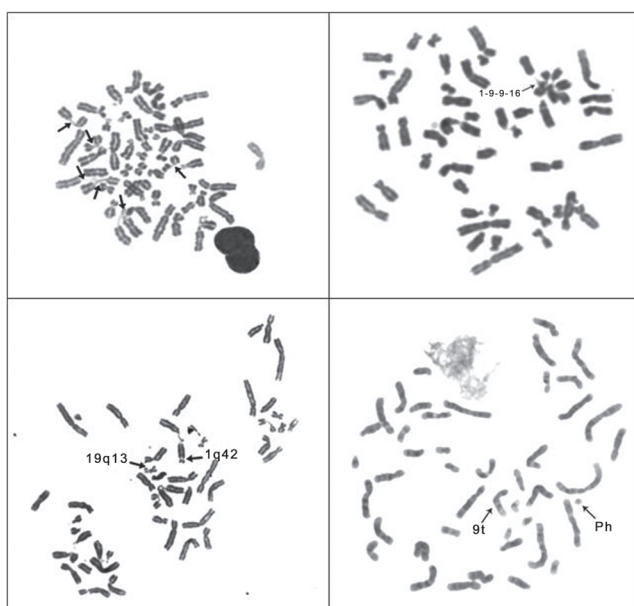


Figure 1 – Metaphases exhibiting: (A) uncoiled heterochromatic regions of chromosomes 1, 9 and 16: (B) an association between uncoiled heterochromatic of chromosomes 1, 9 and 16: (C) the 1q42 and 19q13 fragile sites: (D) Philadelphia (Ph) and derivative 9 (9t) chromosome

## Methods

### Samples

Heparinized peripheral blood samples from 13 female and 5 male patients with hematological malignancies were obtained at diagnosis of malignancies in the Hematology Department of FAMEMA (Faculdade de Medicina de Marília, SP, Brazil). The peripheral blood of 18 individuals, without history of neoplasias, matched for age ( $\pm 5$  years) and gender to the patients was also collected and used as a control group. All subjects gave their informed consent to participate and this study which was approved by the local Ethics Committee.

### Blood culture and 5-azacytidine treatment

Blood cells were seeded in RPMI 1640 medium supplemented with 10% fetal calf serum and incubated for 72 hours at 37°C in a 5% CO<sub>2</sub> atmosphere. 5-AZC was added to the culture 7 hours prior to harvest at a final concentration of 10<sup>-5</sup> M. Harvesting was performed as described by the AGT Cytogenetics Laboratory Manual.<sup>(23)</sup>

### Slide preparation and staining techniques

Slides were prepared following conventional techniques. Slides of 5-AZC-treated cultures were stained with 5% Giemsa for 8 minutes. Non-treated cultures of the patients were stained according to the G-band technique as described by the AGT Cytogenetics Laboratory Manual.<sup>(23)</sup>

### Cytogenetic analysis

One hundred Giemsa-stained 5-AZC-treated metaphases were analyzed for each subject. The parameters evaluated were: 1) the number of under-condensations of the heterochromatin of chromosomes 1, 9 and 16, 2) the occurrence of the fragile sites at 1q42 and 19q13 or other chromosomal lesions and 3) the presence of associations between uncoiled regions. Analyses were performed in a blind manner. Ten G-band metaphases of each patient were analyzed in order to investigate possible chromosomal alterations in these individuals.

The Mann-Whitney test was used to analyze the number of uncoiled heterochromatic regions of the human chromosomes 1, 9 and 16 and the number of associations between uncoiled regions and the Poisson test was used to analyze the fragile sites at 1q42 and 19q13. A level of significance was adopted for a p-value = 0.05 (Bioestat 5 software).

## Results

Through G-band analyses, the only consistent chromosomal abnormality found in patients was the

Table 1 - Age, gender and hematological malignancy of the 18 patients and the number of chromosomes 1, 9 and 16 presenting uncoiled heterochromatin in 100 metaphases of lymphocyte cultures treated with 5-AZC for patients and controls

Patient	Disease	Age	Gender	Chromosome 1		Chromosome 9		Chromosome 16		Total	
				Patient	Control	Patient	Control	Patient	Control	Patient	Control
1	ALL	9	M	83	69	47	10	35	28	179	107
2	ALL	10	F	125	51	50	22	91	50	266	123
3	ALL	16	F	87	95	87	19	61	51	235	165
4	ALL	24	M	52	54	11	22	44	29	107	105
5	AML	49	M	132	83	57	32	92	45	281	160
6	AML	65	F	65	69	33	30	35	48	133	147
7	AML	86	F	100	55	95	14	78	14	273	83
8	CML	42	F	60	56	34	32	34	41	128	129
9	CML	53	F	68	58	22	21	40	29	130	108
10	CML	73	F	51	76	54	20	24	51	129	147
11	MDS	50	F	73	55	41	24	32	44	146	123
12	MDS	64	F	57	69	24	34	29	41	110	145
13	MDS	66	F	61	61	22	13	41	33	124	107
14	MDS	76	F	66	32	16	19	20	36	102	87
15	MDS	78	F	36	73	59	18	39	38	134	129
16	MDS	80	M	73	79	27	34	43	54	143	167
17	MDS	80	M	57	51	30	21	39	24	126	96
18	MDS	84	F	99	81	78	45	76	40	253	166
Mean				74.72	64.83	43.72	23.89	47.39	38.67	166.61	127.44
standard deviation				25.54	15.06	24.37	8.91	22.31	10.85	63.42	27.61
Mann-Whitney Test (p-value)				0.3110		0.0040 [‡]		0.6460		0.0847	

[‡] statistically significant

ALL: acute lymphoid leukemia; AML: acute myeloid leukemia; CML: chronic myeloid leukemia; MDS: myelodysplastic syndrome; F: female; M: male

Philadelphia chromosome in all 3 patients diagnosed with CML.

Patients 4 and 9 had Down's syndrome and therefore an extra chromosome 21 was observed in all metaphases of these individuals.

Our results showed that the centromeric/pericentromeric heterochromatin of chromosomes 1, 9 and 16 were more frequently uncoiled in the group of patients compared to the control group and this higher frequency of uncoiled heterochromatin was statistically significant (p-value = 0.004) for chromosome 9 (Table 1).

## Discussion

The cytogenetical effects of 5-AZC were first described in 1976<sup>(19-22)</sup> and its use in the treatment of MDS was approved by the US FDA in 2004.<sup>(24,25)</sup> Nevertheless, to date, studies evaluating the effects of 5-AZC on chromosomes of patients with hematological malignancies are unavailable in the literature.

Recently, cytogenetic analyses have gained importance in the diagnosis, prognosis and treatment choice for hematological malignancies. In the present study, the effects of exposure to 5-AZC were observed on the chromosomes of patients newly diagnosed with hematological malignancies.

The low incidence of cytogenetical abnormalities in the patients could be due to the fact that the samples were collected from peripheral blood instead of bone marrow. This finding suggests that the heterochromatic region of chromosome 9 is hypomethylated in patients, because it is more sensitive to the drug. The results are in accordance to what is described in the literature regarding neoplasias and hypomethylation.

Heterochromatin located in the centromeric/pericentromeric region of chromosomes 1, 9 and 16 is mainly constituted by repetitive sequences known as satellites. The centromeric regions are rich in satellite a (Sat a), whereas the pericentromeric regions of chromosomes 1 and 16 are mainly constituted by satellite 2 (Sat 2) and of chromosome 9 by satellite 3 (Sat 3). In normal tissue, those regions are found heavily methylated.<sup>(26,27)</sup> It is possible that the higher frequency of uncoiled centromeric/pericentromeric heterochromatin of chromosome 9, observed in the patients, reflects alterations in the patterns of Sat 3 methylation in these individuals. On studying urothelial carcinoma, Nakagawa et al.<sup>(28)</sup> observed loss of heterozygosity (LOH) of chromosome 9 in more than half of the cases (52%) and, in the cases where LOH of chromosome 9 was detected, rather large regions of 9p and/or 9q were lost. DNA hypomethylation of Sat 2 and Sat 3 was significantly correlated with LOH of

chromosome 9 in this neoplasia. Thus, the study concluded that DNA hypomethylation of pericentromeric satellite regions may participate in the development and progression of urothelial carcinomas by inducing loss of heterozygosity of chromosome 9. It is possible that hypomethylation of satellite sequences can contribute to the occurrence and progression of hematological malignancies as well. Fragile sites at 1q42 and 19q13 were more common in the control group than in patients, but the difference was only statistically significant (p-value = 0.0468) for 19q13 (Table 2).

Table 2 - Number of fragile sites at 1q42 and 19q13 in 100 metaphases of lymphocyte cultures treated with 5-AZC of patients and controls

	Fragile Site 1q42		Fragile Site 19q13	
	Patient	Control	Patient	Control
1	2	2	2	6
2	1	1	3	0
3	0	8	0	6
4	1	0	0	1
5	0	1	1	1
6	0	2	1	4
7	0	0	0	0
8	0	1	2	0
9	0	0	0	3
10	0	1	1	3
11	0	0	0	0
12	2	1	0	0
13	0	0	1	1
14	2	0	2	0
15	0	0	0	1
16	0	0	2	1
17	1	0	0	0
18	0	0	0	1
mean	0.50	0.94	0.83	1.56
Standard deviation	0.79	1.89	0.99	2.01
Poisson test (p-value)	0.1146		0.0468[‡]	

[‡] statistically significant

One possible explanation for this finding is that the 19q13 region is hypermethylated in patients and, therefore, is less sensitive to the action of 5-AZC. We can speculate that, in the 19q13 region, there is a tumor suppressor gene whose silencing through methylation favors the development of the neoplasia. The LOH of the 19q13 region is commonly found in neuroblastomas and gliomas, suggesting the existence of an important tumor suppressor gene in this region involved in the development of these cancers. One candidate gene is the epithelial membrane protein 3 (EMP3).<sup>(29)</sup>

Nevertheless, studies associating hypermethylation of either the EMP3 gene or the 19q13 band and hematological malignancies were not found in the literature. The induction of hypomethylation using 5-AZC provokes translocations between satellite regions in the second cell generation,

Table 3 - Number of associations between uncoiled heterochromatin of chromosomes 1, 9 and 16 in 100 metaphases of lymphocyte cultures treated with 5-AZC of patients and controls

	Associations	
	Patient	Control
1	1	2
2	5	2
3	16	3
4	3	1
5	9	3
6	0	4
7	14	5
8	5	4
9	4	0
10	4	4
11	3	1
12	2	1
13	0	5
14	1	6
15	4	5
16	1	4
17	10	5
18	5	3
mean	4.83	3.22
Standard deviation	4.60	1.73

Mann-Whitney test p-value = 0.597

probably resulting from the somatic associations observed between regions in the first cell generation.<sup>(30)</sup> Associations involving the uncoiled regions of chromosomes 1, 9 and 16 did not statistically differ between patients and the control group. However, patients 3, 5, 7 and 17 presented respectively 16, 9, 14 and 10 associations whereas the highest number of associations found in the control group was 6 (Table 3). The high frequency of associations exhibited by these 4 patients could be indicative that the satellite regions of these individuals are more unstable.

As previously mentioned, in cancer, the genome as a whole is hypomethylated and the loss of methyl groups occurs mainly in the body of genes and in the repetitive sequences, such as the sequences present in the heterochromatic region of chromosomes 1, 9 and 16. Overall hypomethylation has the potential to contribute to a malignant phenotype through chromosomal instability, reactivation of transposable elements, and loss of normal gene imprinting patterns.<sup>(14,26)</sup> The results of this study showed that the centromeric/pericentromeric heterochromatin of chromosomes 1, 9 and 16 appeared more frequently uncoiled in the group of patients compared to the control group and this higher frequency of uncoiled heterochromatin was statistically significant (p-value = 0.004) for chromosome 9 (Table 1). This finding suggests that the heterochromatic region of chromosome 9 is hypomethylated in patients because it is more sensitive to the drug. Results suggest that

the satellite DNA of chromosome 9 is hypomethylated in patients with different hematological malignancies. It is possible that this hypomethylation contributes to the pathogenesis of these neoplasias by activating parasite sequences and/or promoting chromosomal instability. In addition, the higher frequency of the fragile site at 19q13 in the control group may indicate that that region is hypermethylated in patients; nonetheless molecular studies searching for candidate tumor suppressor genes involved in the development and/or progression of hematological malignancies located in the 19q13 band are necessary in order to prove this finding. The increased occurrence of associations between the uncoiled regions of chromosomes 1, 9 and 16 observed in 4 patients could be a sign that the satellite DNA in these individuals is unstable.

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