

DNA damage in acute myeloid leukemia patients of Northern Mexico

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damage characterized by a variety of chromosomal and molecular changes,⁴ which can be useful for associations with pathogenesis, progression, and chemotherapy response or resistance.⁵ The few studies that assessed genomic damage in patients with leukemia used approaches such as the micronucleus (MN) technique,^{6,7} sister chromatid exchange (SCE),⁸⁻¹² or the alkaline comet assay.^{13,14} The findings of those studies were contradictory. DNA damage in Mexican patients with AML has not been explored. Currently, the DNA breakage detection-fluorescent *in situ* hybridization (DBD-FISH) technique *ex vivo* that allows the quantification of single-stranded DNA breaks (SSBs) and alkali-labile sites in the whole genome or specific sequences.¹⁵

The purpose of this preliminary study was to evaluate DNA damage in the whole genome of peripheral blood leukocytes (PBL) from Mexican patients with newly diagnosed AML compared with a control group using DBD-FISH.

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Abstract

The purpose of this study was to evaluate DNA damage in the whole genome of peripheral blood leukocytes from patients with acute myeloid leukemia (AML) compared with a control group using DNA breakage detection-fluorescent *in situ* hybridization (DBD-FISH). Our results suggest that the DNA damage detected in patients with newly diagnosed AML was similar to that observed for the controls; this might be explained by the stimulation of a repair pathway by the pathogenesis itself. These findings indicate that inhibiting the repair pathway could be proposed to enhance the efficacy of chemotherapy.

Introduction

Acute myeloid leukemia (AML) is a very heterogeneous disorder in which hemopoietic progenitor cells (blasts) lose their ability to differentiate normally and respond to normal regulators of proliferation. AML is the most common form of acute leukemia in adults, with a median age at diagnosis of 65-70 years.¹ The prognosis of patients with AML is poor. In patients younger than 60 years, the complete remission (CR) rate is 60-70%, whereas the overall cure rate is only 35-40%.^{2,3} Elderly patients and those with adverse karyotypes exhibit a CR rate of 35-50% and a cure rate of 10% or less.² This pathology is associated with genomic

Materials and Methods

Study population

We enrolled patients with AML from the Unidad Medica de Alta Especialidad No. 25 (UMAE 25), Instituto Mexicano del Seguro Social (IMSS). The clinical diagnosis was based on the morphological characteristics of Wright-Giemsa-stained smears of bone marrow aspirates and on immune-phenotyping analyses of leukemic cells. Hematological data of peripheral blood revealed that all patients registered high percentage of leukemia blasts (67-96%). Controls showed only normal leucocytes. The study participants were classified as newly diagnosed patients [n=22; age (average \pm SD) = 41 \pm 18.87 years (range 17-65 years)] and healthy volunteers, who served as controls [n=13; age 37 \pm 14.38 years (range 19-64 years)].

Written informed consent was obtained from all patients. The protocol was approved by the Research and Ethics Committee of IMSS (R-2015-785-033).

DBD-FISH technique

DBD-FISH was performed in PBL samples from patients and controls. The protein-depletion procedure followed by treatment with an alkaline solution to produce SSBs was performed according to Fernández and Gosálvez.¹⁵

To determine the total DNA damage, a whole-genome DNA probe was produced

from leukocyte pellets by using a DNA isolation kit for mammalian blood (Roche Diagnostics Co., Indianapolis, IN, USA). The hybridized DNA probe was detected by incubation for 30 min with fluorescein isothiocyanate-labeled avidin (1:400; Roche Diagnostics Co.). Finally, the slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (1 mg/mL) in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA).

Comet assay

To confirm the results obtained with DBD-FISH, the alkaline comet assay was performed using the basic rationale of Singh *et al.*,¹⁶ with modifications.¹⁷ The quantification of DNA damage was performed by protein-depletion, treatment with an alkaline solution and SSBs migration in an electric field.

Treatment with H₂O₂ (positive control)

Each experiment included two positive controls; a sample of healthy volunteers and one of AML patient. Slides were exposed to H₂O₂ (20 mM), previously to DBD-FISH technique.

Repair experiment *in vitro*

PBL from a 37-year-old female who was newly diagnosed with AML were used to

perform three cultures on RPMI-1640 medium supplemented with fetal calf serum (Gibco-Life Technologies, Waltham, MA, USA): to evaluate DNA damage, cells were treated with mitomycin C (0.2 mg/mL) for 48 h; to evaluate DNA repair, cells were treated with mitomycin C for 72 h; and cells was used as the negative control because it was not treated with any mutagenic agent. All cultures were harvested at 72 h for processing through the DBD-FISH technique.

The same strategy was applied to a sample of control PBL from an age- and sex-matched healthy individual. Two replicates for each experiment were performed.

Image analysis

The slides were analyzed using a digital image analysis platform based on a Zeiss Axiophot fluorescence microscope (Carl Zeiss, Göttingen, Germany). The Images were recorded using an Axiocam 16-bit black-and-white charge-coupled device in a 12-bit TIFF format. The integrated density (ID) values obtained after background subtraction were calculated using Image J 1.4.3.6.7. (<http://rsb.info.nih.gov/ij/>). For this purpose, ID (area \times gray scale) after background subtraction was calculated in pixels (px).¹⁸ Fifty cells were analyzed for each sample.

Statistical analysis

Data were entered a database for statistical analysis. All analyses were performed using the package IBM-SPSS for Windows 20.0 (IBM Corp., Armonk, NY, USA). The ID in the four groups studied was transformed using \log_{10} . The mean values for transformed data and for not transformed data were analyzed by one-way ANOVA. A P-value less than 0.05 was considered significant. The sample size was estimated using the Minitab software (ver. 15) for one-way ANOVA ($\alpha = 0.05$, $1 - \beta = 0.80$, corrected sum of the squares of means = 2534102586, and Sigma = 54400.5). A minimum sample size of 13 individual per group was obtained. Significance was set at $P < 0.05$ for all tests.

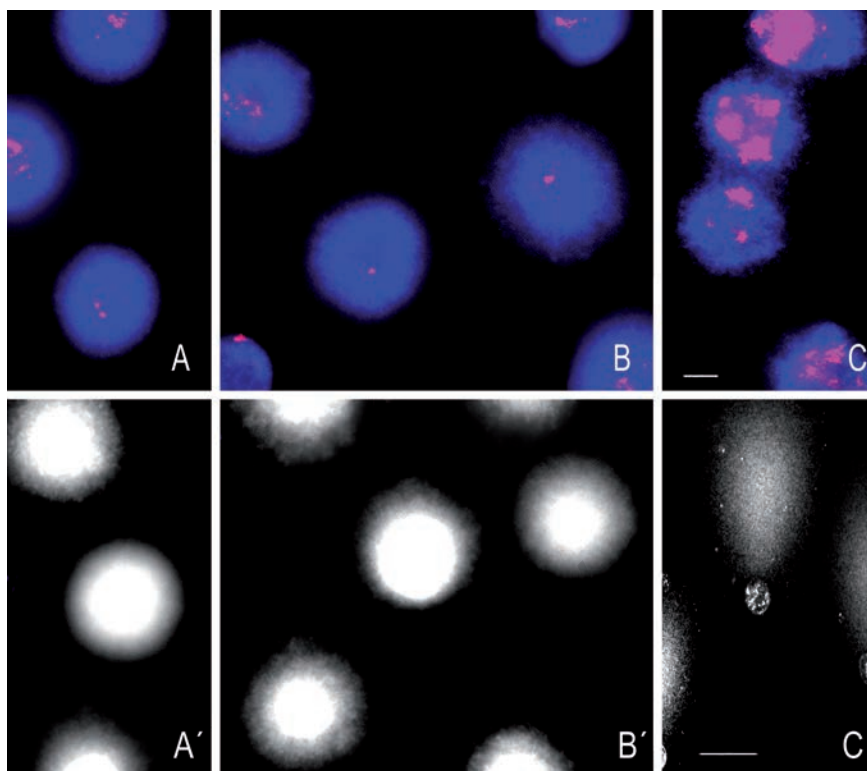


Figure 1. Analysis of DNA damage in peripheral blood leukocytes after DBD-FISH using whole probe labeled with biotin (red) and counterstained with DAPI (blue). A) Healthy individual; B) patient with AML; C) positive control healthy (H_2O_2). Analysis by comet assay (Comet tail μm): A') 42.7 ± 6.1 ; B') 47.4 ± 8.3 ; C') 130 ± 11.8 . Scale bars: A-C) $5 \mu m$; A'-C') $10 \mu m$.

Table 1. Integrated density values in peripheral blood leukocytes of acute myeloid leukemia patients after DBD-FISH using probe whole genome.

Group	n	Fluorescence analysis	
		ID (average \pm SE*) ^a	ID (average \pm SE*) ^b
Healthy (A)	13	$18.99 \pm 4.39^{3,4}$	$4.17 \pm 0.93^{3,4}$
AML (B)	22	$17.34 \pm 3.83^{3,4}$	$4.08 \pm 0.09^{3,4}$
Positive control-healthy (C)	13	$79.37 \pm 24.97^{1,2}$	$4.63 \pm 0.14^{1,2}$
Positive control-AML (D)	22	$126.71 \pm 28.49^{1,2}$	$4.71 \pm 0.16^{1,2}$

ID, integrated density; *average \pm SE values represent 103; ^anot transformed ID values; ^btransformed ID values; AML, acute myeloid leukemia; Test ANOVA: ¹different from group A, $P < 0.05$; ²different from group B, $P < 0.05$; ³different from group C, $P < 0.05$; ⁴different from group D, $P < 0.05$.

Table 2. Experiments for evaluate DNA damage/repairment by DBD-FISH in peripheral blood leukocytes cultures of a patients with acute myeloid leukemia and a healthy individual after exposition at mitomycin-C.

Time	Acute myeloid leukemia sample			Healthy sample		
	Control	Damage	Reparation	Control	Damage	Reparation
T0			MMC			MMC
24 h		MMC			MMC	
72 h	Harvest	Harvest	Harvest	Harvest	Harvest	Harvest
ID	18.39	287.13	60.05	16.48	123.80	30.80

Time, time of culture; damage, culture with exposition to mitomycin-C for 48 h for evaluating DNA damage; reparation, culture with exposition to mitomycin-C for 72 h for evaluating DNA damage; control, represent DNA damage and reparation levels in a culture without the exposition mutagenic agent; ID, integrated density average value in pixels; MMC, mitomycin-C.

Results

The average age was similar among the groups ($P>0.05$). Cells that were treated with hydrogen peroxide exhibited elevated ID values, which confirmed the accessibility of the cells to the tested chemicals (Figure 1C). Conversely, cells without treatment showed a slight fluorescent signal that corresponded to the basal or constitutive damage for this type of cells (Figure 1A). Patients with AML demonstrated similar results to the negative control group (Figure 1). The results presented above were confirmed by the comet assay (Figure 1A'-C').

The analysis of the ID in the group of patients with AML (Figure 1B) yielded results that were similar ($P>0.05$) to those obtained for the control group (Figure 1A). Leukocytes of positive controls (treated with H_2O_2) of healthy and AML, showed a significant increase in ID compared with the group of AML patients and healthy group ($P<0.05$) (Table 1).

The repair experiment performed on leukocytes from a patient with AML showed an ID value that suggest a sensitivity higher to damage (287.13 px) than control cells (123.8 px) and repair efficiency similar in the two groups of cells (21% and 24%, respectively). Finally, ID values of AML and healthy control were similar (Table 2).

Discussion

Our results demonstrated that the DNA damage detected in patients with newly diagnosed AML was similar to that observed for the controls. Previous studies of DNA damage in patients with newly diagnosed leukemia *vs* controls used *in vitro* techniques, such as SCE^{8-10,19} and the MN^{6,7} tests showed findings contradictory. To our knowledge, only a study of DNA damage *ex vivo* in PBL of patients with newly diagnosed leukemia *vs* controls used the alkaline comet assay to determinate DNA damage in patients with AML from Cairo, Egypt, showed that the tail moment was significantly higher in patients compared with the control group.¹³ This result disagrees with our findings from Mexican patients, ethnic differences could be one of the causes underlie this discrepancy.^{20,21}

Our results may be attributed to repair-induced DNA lesions. This capacity to repair was reported previously by Müller *et al.*²² for lymphocytes from patients with Chronic lymphocytic leukemia who were nonresponsive to alkylating agents. Those

cells exhibited faster processing repair-induced SSBs compared with control cells, which could be explained by the presence of cytotoxic DNA lesions induced by the alkylating agents. Recently, Portich *et al.*¹⁴ assessed DNA damage in pediatric patients with acute lymphoid leukemia using the comet assay and found no increase in damage during induction therapy (diagnosis, 15 days, and 35 days), which suggests that this repair pathway may be stimulated in the following scenarios: i) as a tumor response; ii) as a defense from chemotherapy; or iii) both (pathogenesis of the disease and chemotherapy).

Our findings support the first scenario because we did not find increased damage in patients with newly diagnosed AML compared with the control group. In addition, the repair experiment performed on leukocytes from a patient with AML are indicative that the sensitivity to damage in cells from the patients was greater that of the control cells, and the percentage of repair was similar in the two groups.

To confirm our findings, it will be necessary to perform studies using DBD-FISH with probes for specific chromosomal regions^{8,23} with significance in leukemogenesis or for genes involved in DNA damage and repair, which have been identified in AML patients.^{5,13,24-26} This information may be useful for the measurement of cellular interindividual heterogeneity³ and to perform associations with sensitivity to the pathology itself or to chemotherapy.^{24,26}

In conclusion, in this study preliminary we reported absence of an increase in DNA damage in patients with newly diagnosed AML compared with controls and suggested that this may be explained by the stimulation of a repair pathway by the pathogenesis itself. Future studies are needed to confirm our findings

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