

Mitochondrial DNA Alterations and Oxidative Stress in Acute Leukemia

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Abstract: Mitochondrial DNA (mtDNA) alterations were reported in many cancers but their roles in oncogenesis are still debated. We aimed to examine qualitative and quantitative mtDNA modifications and oxidative stress in normal and cancer cells.

23 leukemia patients and 18 healthy subjects were recruited from the hospital of Azziza Othmana (Tunisia). Mitochondrial D-loop was sequenced, mtDNA level was determined by Q-PCR, the oxidative stress was assessed by a fluorescent probe and the mitochondrial transcription factor A (mTFA) level was quantified.

No somatic mutation was evidenced in leukemia cells compared to non-malignant cells. However, a significant higher level of mtDNA associated with an increase of mTFA expression and reactive oxygen species (ROS) production were measured in patients' cells compared to non-malignant cells.

In conclusion, our results don't support the role of mtDNA mutations in leukemogenesis but increase of mtDNA and ROS levels would be molecular signatures of leukemia.

Keywords: Mitochondrial DNA, leukemia, reactive oxygen species (ROS), mitochondrial transcription factor A (mTFA).

INTRODUCTION

Since the pioneering works of Otto Warburg in 1920s' about differences in the mitochondrial metabolism between normal and tumoral tissue, an abundant literature has been published to date pointing out alterations of mitochondria in cancer and their potential roles in carcinogenesis (see for review [1]). The human mtDNA contains 16,569 bp [2] and plays an important role in energy metabolism. While the majority of mtDNA encodes ribosomal RNAs (rRNAs), transfer RNAs (tRNAs) and proteins, there is a small non-coding region of about 1.1 kb named D-loop which binds regulatory factors such as mitochondrial transcription factor A (mTFA) that plays a major role in the maintenance of mtDNA [3]. It's now well admitted that this region is a "hot spot" for mutations of mtDNA associated with various cancers [4] that would result from oxidative stress. Mitochondrion is the major site of reactive oxygen species (ROS) production. Under physiological conditions, ROS participate into various cell functions but their abnormal production or the loss of detoxifying enzyme would cause mtDNA damages and diseases [5].

Mitochondria were shown to be altered in hematological disorders. For instance, myelodysplastic syndromes are characterized by an ineffective hematopoiesis caused by mtDNA mutations that would directly be responsible for carcinogenesis [6]. Several laboratories also reported the presence of mtDNA mutations in chronic lymphocytic (CLL) and myeloid (CML) leukemia and in acute lymphoblastic (ALL) and myeloid (AML) leukemia [7-9]. However, a recent study challenged the potential role of mtDNA mutations in leukemogenesis [10].

Since the role of mitochondria in cancer is still a matter of debate, our study aimed to evaluate mtDNA instability within the D-loop and mtDNA copy number in Tunisian patients affected by acute leukemia. This was accomplished by PCR/sequencing and quantitative PCR of samples obtained from patients and healthy subjects. The significant increase in mtDNA content observed in tumor cells prompted us to examine mTFA and ROS, two factors known to regulate mitochondrial DNA replication. mTFA was quantified by real-time-RT-PCR and western-blot and ROS production was evaluated by hydroethidine/flow cytometry.

MATERIALS AND METHODOLOGY

Patients

Twenty three Tunisian children or young adults, with a mean age of 8.8 years and a sex ratio male/female of 1.87,

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affected either by ALL (n=16) or AML (n=7) were studied (Table 1) and compared to eighteen healthy children or young adults from Tunisia with a sex ratio male/female of 1.25 and a mean age of 12.3 years. All patients, except P5, were included without prior chemotherapy. The study was conducted according to the Tunisian biomedical research rules that was approved by the ethics committee of the hospital of Azziza Othmana (Tunisia) and according to the ethical standards formulated in the Helsinki Declaration. All biological samples were collected anonymously.

Table 1. Characteristics of Patients Affected by Leukemia

Samples	Sex	Age (Years)	Diagnosis	Evolution	Haplogroupe
P1	M	13	ALL	CR	H
P2	M	10	ALL	ABMT	H
P3	M	10	ALL	CR	H
P4	M	12	AML	CR	L3f3
P5	F	3	ALL	CR	L3f*
P6	F	7	ALL	CR	H
P7	F	5	ALL	CR	K
P8	F	4	ALL	Death	H11
P9	F	8	AML	Death	H
P10	M	4	ALL	CR	J1b
P11	F	10	AML	ABMT	H5
P12	F	2	ALL	CR	H
P13	F	15	AML	Death	H
P14	M	20	AML	Death	H
P15	M	13	ALL	CR	HV4a
P16	M	9	ALL	CR	H11
P17	M	7	AML	ABMT	H
P18	M	7	ALL	CR	H
P19	M	6	ALL	CR	H
P20	M	11	AML	RE	H
P21	M	17	ALL	CR	H
P22	M	3	ALL	CR	H1f
P23	M	8	ALL	RE	H

M: male; F: female; CR: complete remission; RE: Relapse; ABMT: allogenic bone marrow transplantation; ALL: acute lymphoblastic leukemia; AML: acute myeloid leukemia.

Peripheral Blood Mononuclear Cells Preparation

Peripheral blood mononuclear cells (PBMC) were obtained from patients and healthy donors using a gradient Ficoll-Paque (Amersham Biosciences, Saclay France) and then were frozen at -192°C into two vials for nucleic acid and protein extraction and measurement of superoxide anion ($\text{O}_2^{\cdot-}$) production.

Nucleic Acid Extraction

Total DNA was extracted from isolated PBMC and buccal cells by using the standard phenol/chloroform

extraction procedure. Total RNAs were extracted from frozen PBMC cells of patients and healthy donors with TRIzol® reagent (Invitrogen, Saint-Aubin France). 3 µg of total RNAs were reverse transcribed using the Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen) to obtain cDNAs.

mtDNA Sequencing

Detection of polymorphisms/mutations in the D-loop of the mtDNA was performed by PCR using four primer pairs L1R1, L2R2, L3R3 and L4R4 (SUPPLEMENTARY MATERIAL 1) followed by direct sequencing using the Genomelab DTCS (Beckman Coulter, France) and analyzed on a Beckman Coulter CEQ 8000 sequencer. Estimation of mitochondrial haplogroups was determined using the mtDNAmanager interface developed by Hwan Young Lee *et al.* (2008) using the control region sequence [11].

Determination of the mtDNA/nDNA Ratio by Real-Time PCR

Quantification of the mtDNA was performed by real-time PCR amplification on a Light Cycler 480 (Roche Diagnostics, Meylan France) using Cyler FastStart DNA master Sybr green I mix (Roche Diagnostics) according to the method developed by May-Panloup *et al.* [12] except that we amplified the 18S rRNA for nDNA quantification (SUPPLEMENTARY MATERIAL 1). All samples were analyzed in duplicate. Considering that 1 cell contains 10 pg of nDNA, results are expressed as mtDNA copy number per cell.

Real-Time PCR

Real-time quantification of mTFA expression relative to β -actin mRNAs was performed on the Light Cycler 480 system (Roche Diagnostics) using specific set of primers (SUPPLEMENTARY MATERIAL 1). The threshold cycle (Ct) was determined and the mTFA/ β -actin ratio was calculated according to the $2^{-\Delta\Delta\text{Ct}}$ method [13].

Western-Blot Analysis

mTFA and β -actin expression were studied from PBMC by western-blot with a 1:500 dilution of rabbit anti-human mTFA (Abcam, Paris France) and a 1:1.000 dilution of the monoclonal anti- β -actin (santa-Cruz, Le Perray en Yvelines France) antibodies, respectively. Protein concentration was determined by the BCA Protein assay (Bicinchoninic Acid).

Measurement of Superoxide Anion Production

Superoxide anion radical production was determined by using the fluorescent sensitive probe hydroethidine (HE) (Molecular Probes, Invitrogen, Saint-Aubin France). PBMC ($5 \cdot 10^4$ cells) obtained from controls or patients were incubated with $78\mu\text{M}$ HE for 15 min in PBS at 37°C in the dark. Then, fluorescence of oxidized HE was determined by flow cytometry (Becton Dickinson FACS CANTO II, FACS Diva Software version 6.1) with 485 and 510 nm wavelengths for excitation and emission, respectively.

Statistical Analysis

Data presented correspond to mean \pm S.E.M. The Fisher's exact test and the t-test were used to determine the statistical significance of differences between controls and

patients. Differences were considered significant when p values were less than 0.05. For multiple comparison tests, we applied the Bonferroni correction.

RESULTS

mtDNA Sequence Analysis

The D-loop region of the mtDNA was entirely sequenced in leukemia patients and healthy subjects. We observed several homoplasmic and heteroplasmic variations both in patients and controls compared to the rCRS (revised Cambridge Reference Sequence) (**SUPPLEMENTARY MATERIAL 2A**) but only the heteroplasmic m.343C>T substitution was found significantly under-represented in patients compared to controls (22% vs 89 %, $p < 0.001$ respectively) (**SUPPLEMENTARY MATERIAL 2B**). In order to distinguish between somatic and germinal mutation/polymorphism, the m.343C>T substitution was screened in buccal epithelial cells from 11 among 23 patients; an identical distribution of this polymorphism was found in both tissues.

Quantitative Real Time PCR Analysis

As the D-loop plays a major role in the regulation of mtDNA replication, quantification of mtDNA level was achieved from PBMC of patients and healthy donors by quantitative PCR using both mitochondrial and nuclear standards. As depicted in the Fig. (1A), a significant 3-fold

increase in the mtDNA/nDNA ratio was measured between leukemia patients and controls (t-test, $p = 0.012$). Values of mtDNA copy number from the control group were in the same range as previously reported in healthy donors with a different method and targeted genes [14].

mTFA Expression

mTFA was described as a major activator of mtDNA transcription and replication. So, we sought to study the expression of this regulatory factor by real-time PCR and western-blot. We observed a significant increase in mTFA mRNAs in leukemia patients compared to controls (t-test, $p = 0.009$) (Fig. 1B), that was correlated with the increase at the protein level (t-test, $p < 0.001$) (Fig. 1C, D). The specificity of anti-mTFA antibody was verified using the Ramos and Jurkat cells lines in which we were able to detect a single band at the predicted size (data not shown).

Measurement of ROS Production

We measured the superoxide anion production in PBMC isolated from 14/23 leukemia patients and 11/18 healthy donors using the fluorescent probe HE. The more ROS are produced, the more fluorescence is increased (**SUPPLEMENTARY MATERIAL 3**). As shown in the Fig. (2), the level of ROS production, reflected by the mean fluorescence intensity, was significantly increased by about 2-fold in leukemia patients compared to controls (t-test, $p = 0.04$).

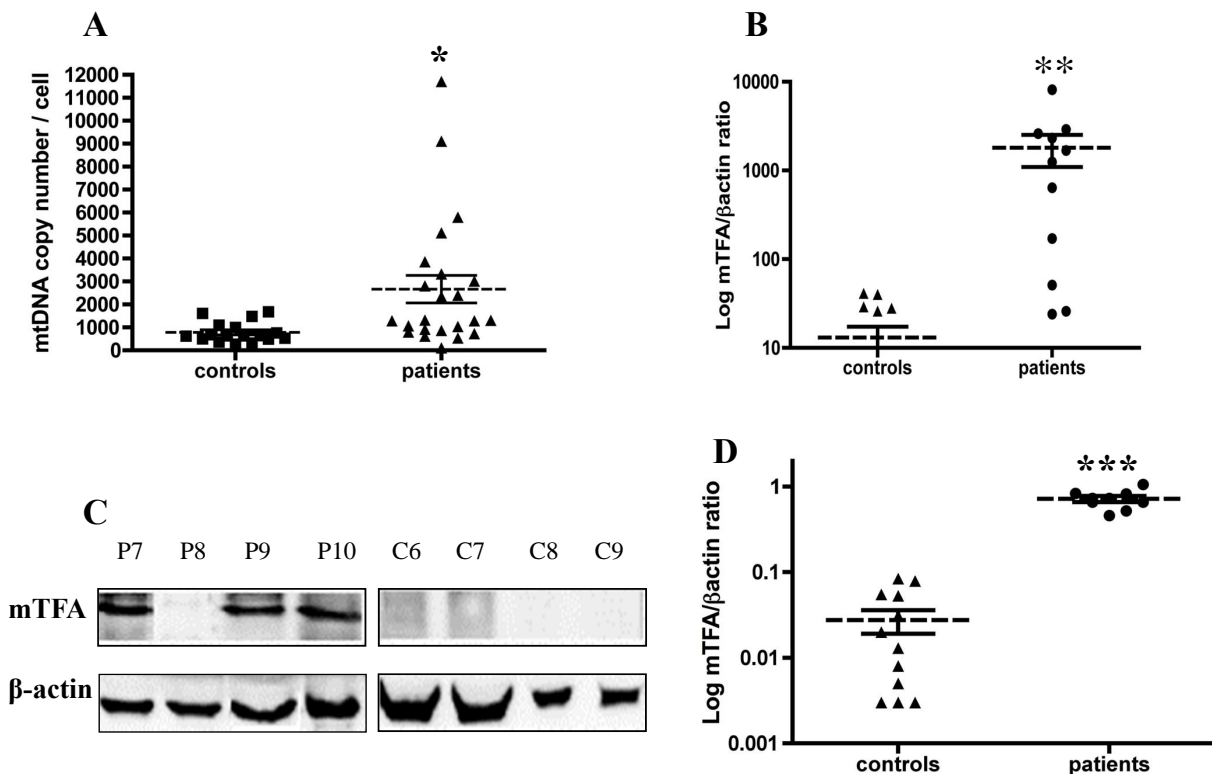


Fig. (1). Analysis of mtDNA and mTFA in leukemic patients and healthy subjects. (A) The mtDNA/nDNA ratio was determined by real-time PCR using primers directed against both mitochondrial and nuclear genes. Data are mean \pm S.E.M. *, $P < 0.05$. (B) mRNAs expression of mTFA was determined by Q-PCR in 11/23 leukemic patients and 9/18 healthy controls. Results are expressed as the mTFA/ β actin ratio and correspond to the mean \pm S.E.M. **, $P < 0.01$. (C, D) The mTFA level was determined by western-blot in 9/23 leukemic patients and 13/18 healthy controls. Results are expressed as the mTFA/ β actin ratio and correspond to the mean \pm S.E.M. ***, $P < 0.001$.

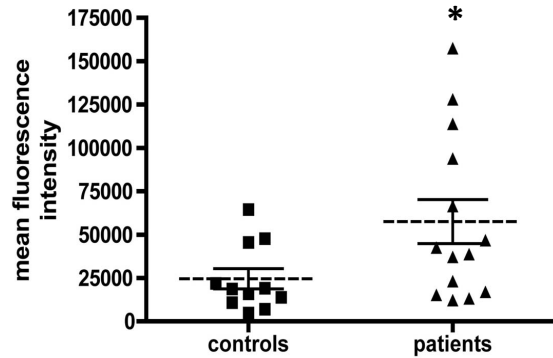


Fig. (2). Determination of ROS production in PBMC. ROS production was quantified using the fluorescent probe HE in cells extracted from leukemia patients and controls. ROS levels are reflected by the the mean fluorescence intensity. Data are mean \pm S.E.M. *, $P < 0.05$.

DISCUSSION

There is an abundant literature about mtDNA mutations and cancers, both in solid tumors and leukemia but the relevance of such mitochondrial abnormalities in tumor genesis is still unclear (see for review [15]). These mtDNA point mutations are somatic and detected mainly in the D-loop. Regarding leukemia, mitochondrial mutations were also reported in this region and were suggested to contribute to tumor growth [8]. In contrast, our data don't support the existence of any link between mtDNA mutations and acute leukemia. Such conclusions have also been recently drawn by Cerezo *et al.* (2009) [10] from their study on B-cell CLL. While we detected a single significant difference at the position 343 between patients and healthy controls in PBMC, the epithelial buccal cells of patients, a non-malignant tissue, also displayed the same C nucleotide in the homoplasmic state; this indicates that this mutation was not segregated in tumoral cells. There are different hypothesis to explain discrepancies between our data and those previously published:

- Our patients, except N°5, didn't receive neither chemotherapy nor platelets transfusion at the time of diagnosis that could lead to a false positive detection of polymorphism/mutation [16].
- D-loop is known to be a very polymorphic region and when using the rCRS as template, polymorphisms due to haplogroup can be mistaken as mutations. To avoid such pitfall and on the contrary of some studies [17] we rather compared D-loop sequences obtained from patients with controls coming from the same geographical region.
- We also checked that our PCR conditions did not cause amplification of nuclear pseudogenes closely related to mtDNA by using rho^o cells.
- In our study, we compared tumoral (blood) and non-malignant tissues (epithelial buccal cells) from same patients to determine the role of mtDNA mutation in cancer in contrast to some in which only the tumoral cells were studied [18].

We found that leukemia cells displayed a higher level of ROS production compared to non-malignant PBMC as recently reported in CML [19]. Indeed, alteration of the mitochondrial membrane potential was suggested to disturb electron flow through the mitochondrial respiratory chain and generate elevated level of ROS. While in our study we sequenced only the D-loop region, we can't rule out the presence of mutations or polymorphisms that would enhance the electron leakage and ROS production. Our data are also in good agreement with the study of Er *et al.* (2007) [20] who demonstrated higher superoxide anion production in AML compared to healthy controls. In such conditions, we would have expected to detect common oxidative damage signature mutations (C-T, CC-TT and G-T) of DNA in our patients as reported by Grist *et al.* (2004) [8]. However, in this study the authors selected a high proportion of patients who had been previously treated and relapsed; furthermore, data of mtDNA sequence analysis were compared to the rCRS. So, the mutations that were reported could be due to chemotherapy treatment or would correspond to polymorphisms. In addition to promote damages to cellular components and DNA mutations, ROS were suggested to play a role in intracellular signaling and to activate proliferative and/ or survival pathways (see for review [21]). ROS would be deleterious in two ways: they would promote DNA instability by promoting DNA breaks and would confer a proliferative advantage in cancer cells. Due to the difficulty to maintain patients' cells in culture, we didn't test the role of ROS in proliferation.

ROS were also suggested to regulate the mtDNA copy number as recently demonstrated in nondiabetic hemodialysis patients [22]. In this study, the authors showed a good and positive correlation between mtDNA content in peripheral blood leukocytes and the plasma thiobarbituric acid-reactive substances level used as an indicator of oxidative stress. In the present study, we observed a significant increase in the mtDNA/nDNA ratio in patients compared to controls. Such an increase was previously reported in blast cells of AML as well as during transformation of chronic granulocytic leukemia [23]. Aneuploidy is frequently observed in cancers including leukemia cells and could potentially lead to erroneous nDNA quantification. However this is unlikely in our study since we used the 18S rRNA which is a multicopy gene. mTFA, whose level is known to regulate the mtDNA copy number (see for review [3]), was increased in patients compared to healthy controls both at the levels of mRNA and protein. Very few data are available about the expression level of mTFA so it's difficult to compare our data. However, using a non-quantitative RT-PCR mTFA expression was shown to be absent in normal B-lymphocytes from 2 healthy donors while it was greatly expressed in CLL [24]. So it's not surprising to observe a strong increase in mTFA expression in patients compared to controls. Moreover, our molecular studies are in good agreement with western-blot experiments since in the control group mTFA was barely detectable while a strong immunoreactivity was measured in patients. We can question about signals that would trigger mTFA overexpression which consequently would induce increase in mtDNA level in leukemia patients. We can speculate that oxidative stress would damage mtDNA and compensatory mechanisms would be initiated to replace altered DNA

molecules by new ones *via* mTFA. Such relationship between ROS and mTFA expression was previously reported in yeast mitochondria subjected to oxidative stress [25]. More, in addition to its role in mtDNA maintenance, mTFA was recently demonstrated to be localized in nucleus and its over-expression, in the human prostate cancer cell line PC3, was shown to induce cellular proliferation [26].

In conclusion, while our data suggest no evidence for mtDNA instability in leukemogenesis, we observed an increase in oxidative stress, mtDNA and mTFA levels in leukemia patients' cells. Such mitochondrial alterations could be used a molecular signature of leukemia cells. Further studies would be necessary to determine any link between such abnormalities and cell proliferation.

ABBREVIATIONS

ROS	=	Reactive oxygen species
mTFA	=	Mitochondrial transcription factor A
CLL	=	Chronic lymphocytic leukemia
CML	=	Chronic myeloid leukemia
ALL	=	Acute lymphoblastic leukemia
AML	=	Acute myeloid leukemia
mtDNA	=	Mitochondrial DNA
PBMC	=	Peripheral blood mononuclear cells.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

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