

Mitochondrial DNA in pediatric leukemia patients

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Numerous studies of mitochondrial DNA (mtDNA) in cancer have shown differences between mtDNA sequences in tumor and normal tissue and at various stages of cancer treatment in the same patient. However, there is little data on acute lymphoblastic leukemia (ALL), the most common type of leukemia in children. In this study we compared mitochondrial sequence variation in the D-loop region and in 5 genes of mtDNA in bone marrow samples of 6 pediatric patients with ALL at various stages of therapy. We found several common polymorphisms and one variant at position 3688 whose level varied during leukemia treatment. Our results suggest that mitochondrial DNA mutations, whose levels change during patient treatment, could be potential biomarkers for monitoring treatment efficacy and disease progression.

Key words: pediatric ALL, mtDNA, chemotherapy

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Abbreviations: mtDNA, mitochondrial DNA; ALL, acute lymphoblastic leukemia; D-loop, displacement loop

INTRODUCTION

Mitochondria are responsible for energy production during cellular respiration. They contain their own circular DNA of about 16.5 kb. The mitochondrial DNA (mtDNA) consists of the coding region and the so-called displacement loop (D-loop) of 1124 bp, which contains transcription and replication initiation sites. mtDNA is more susceptible to damage than nuclear DNA mainly due to less efficient DNA repair systems, the lack of protection of genetic material by histone proteins and mutagenic effects of free radicals generated in the process of oxidative phosphorylation (OXPHOS) in mitochondria. So far, over 100 mtDNA point mutations causing human disease have been detected, and more mutations are discovered every year (Kim *et al.*, 2008).

Hematological malignancies represent 44.9% of all neoplasms in patients under 18 years of age. The term leukemia refers to a family of malignant neoplasms of the bone marrow characterized by uncontrolled proliferation of hematopoietic stem cells which do not differentiate into normally functioning blood cells and interfere with the production of other types of healthy cells in the bone marrow (Nordlund, 2007). In general leukemia can be divided into two types based on which white blood cells are affected: lymphoblastic leukemia affects cells of the lymphoid line and myeloblastic leu-

kemia affects cells of the myeloid line. Lymphoid neoplasms are assigned to two main categories: derived from B or T lineage lymphoid precursors. The most common in children is acute lymphoblastic leukemia (ALL) (de Rooij *et al.*, 2015). Approximately 80-85% childhood ALL are of a precursor B phenotype. Immunophenotype analysis allows subclassification of B-lineage ALL into pro-B ALL, common ALL (cALL), pre-B ALL and mature B-cell ALL (Chiaretti *et al.*, 2014). There are multiple genetic and environmental risk factors contributing to the development of this type of cancer. In some patients chromosomal alterations like translocations or hyperdiploidy as well as mutations or gene fusions (like *BCR/ABL* or *TEL/AML1*) occur in lymphoid or myeloid progenitor cells, causing their dysfunction (Rubnitz & Pui, 1997; Buffler *et al.*, 2005). Although most studies focus on the analysis of nuclear DNA alterations, an increased interest in the potential influence of mutations and polymorphisms in the mitochondrial genome on leukemogenesis is observed. Somatic mtDNA mutations were detected in 40% of adult leukemia patients (He *et al.*, 2003). Few studies on monitoring changes in mutation level at different cancer stages and at different levels of treatment have been conducted. In one study on adult ALL patients changes in mtDNA sequence in different leukemia stages and at different stages of the treatment were observed (He *et al.*, 2003). In one patient the mtDNA mutation in bone marrow cells (15296A>G in the cytochrome *b* gene) was lost in hematological remission and reappeared when the patient relapsed and played the role of a clonal marker for the leukemia. These investigations have not been reproduced. It is also possible that chemotherapy could contribute to cancer relapse by damaging DNA and generating new mutations. However, little is known about mtDNA damage caused by anticancer agents. Many chemotherapeutic agents are mutagenic to nuclear DNA, but their influence on mtDNA has not been widely explored. Despite the abundance of data about alterations in mtDNA in many types of cancers, so far little is known about the changes in mtDNA mutation levels in people, especially in children, suffering from proliferative disorders of the bone marrow. The described data about mitochondrial DNA variation in different types of leukemia mainly come from analyses of adult patient tissues and do not necessarily reflect changes in the mtDNA that occur during the development of ALL in children. In this study we examined alterations of the D-loop region and in 5 genes of mtDNA in bone marrow cells from pediatric patients with ALL.

Our aim was to investigate whether chemotherapy causes any changes in the mtDNA sequence. Another goal was to find polymorphisms or mutations with levels changing during chemotherapy, which could aid in monitoring treatment.

MATERIALS AND METHODS

Patients. Newly diagnosed ALL patients under 18 years of age were selected for the study in 2009 in the Department of Laboratory Diagnostics and Clinical Immunology of Developmental Age, Medical University of Warsaw. The material was 6 bone marrow samples (from 4 boys and 2 girls) remaining after routine biopsies taken at the time of diagnosis and at different times of treatment of the patients. The study was approved by the Independent Ethics Committee of the Medical University of Warsaw.

Diagnostic procedure and treatment. A diagnosis of leukemia was based on studies of bone marrow cells (BM), peripheral blood (PB) and cerebrospinal fluid (CSF). Obligatory diagnostics of ALL included: cytomorphological studies (smears stained by the May-Grünwald-Giemsa method in BM, PB and CSF), flow cytometry (immunophenotype, DNA index in BM/PB), cytogenetic analyses (G binding patterns of high resolution chromosomes in BM/PB) and molecular studies (RT-PCR for selected genes: BCR/ABL, MLL/AF4, TEL/MLL1 in BM/PB). Four patients were treated according to ALL IC-BFM 2002 protocol (Stary *et al.*, 2013), one according to ALL IC-BFM 2009 protocol (Campbell *et al.*, 2009) and one according to INTERFANT 06 protocol (Pieters *et al.*, 2006). Information about patients, the protocols which were used during treatment and the time when the second samples, collected during treatment, were received are shown in Table 1.

DNA isolation. The material was total DNA isolated from cryopreserved bone marrow cells. Isolation of DNA was performed by the phenol:chloroform method. Thawed cells were suspended in a lysis buffer comprising water, 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 10 mM EDTA, 5% SDS and 20 mg/ml Proteinase K and placed at 37°C for 24 hours. After digestion, lysates were mixed with an equal volume of phenol (Sigma) and centrifuged at 12000 rpm for 15 min at room temperature. The aqueous phase was transferred to new tubes and mixed with an equal volume of phenol:chloroform (1:1) and centrifuged again. The aqueous phase was collected and mixed with an equal volume of chloroform and centrifuged in the same conditions. After centrifugation the aqueous phase was collected and mixed with 0.1 volumes of 4M NaCl and 2.5 volumes of ice-cold ethanol and placed at -20°C overnight. DNA was precipitated by centrifugation at 13000 rpm for 15 min, the

pellet was washed once with 75% ethanol, dissolved in water and stored at -20°C.

PCR of the mtDNA and sequencing. The D-loop region and 5 mtDNA genes (*ATPase6*, *ATPase8*, *ND1*, *cytB*, *COXII*) were amplified by PCR. PCR was done in a final volume of 50 µl with M13-tagged primers to facilitate the direct sequencing of the PCR-amplified products (Table 2). PCR conditions for amplification were: initial denaturation at 94°C for 3 min; 35 cycles of denaturation at 94°C for 30s; annealing at 58°C for 30 s; elongation at 72°C for 1 min. The PCR reaction mixture contained 100 ng DNA template, 400 µM dNTP (A&A Biotechnology), 0.2 µM of each primer, 1x buffer for Walk polymerase (A&A Biotechnology) and 1.25 U Walk DNA polymerase (A&A Biotechnology). Following PCR amplification, samples were purified to remove unincorporated primers and sequenced directly on the ABI3730xl DNA Analyzer (Applied Biosystems). The obtained sequences were compared directly to the revised Cambridge reference sequence (rCRS) using Sequencher 4.1.4 software. Description of the primers used in amplification of different regions of mtDNA are presented in Table 2.

Analysis of the 3688G>A mutation. The heteroplasmy level of the 3688G>A mutation was determined by last cycle hot PCR-RFLP. The region spanning this mutation was PCR amplified. PCR was performed in a final volume of 25 µl using the following primers: forward, 3659-3687 (C/G mismatch at position 3687 introduces a restriction site for HaeIII endonuclease in the wild type sequence), reverse 3809-3790. PCR conditions for amplification were the same as previously described. The PCR product was radiolabeled with α -³²P-dATP in an additional PCR cycle. The 151bp product was cut with HaeIII restriction enzyme and separated on a 12% nondenaturing polyacrylamide gel. The wild type sequence is cut into two fragments of 121 bp and 30 bp. The sequence containing the 3688G>A transition was not cleaved. The radioactivity in each fragment was quantified using MultiGauge V3.0 software.

RESULTS

Mitochondrial DNA sequence variation in bone marrow cells prior to chemotherapy treatment

We compared sequences of the D-loop and 5 genes of the mitochondrial genome (*ATPase6*, *ATPase8*, *ND1*, *cytB*, *COXII*) from bone marrow cells collected at the time of diagnosis from 6 leukemic patients to the rCRS. This comparison revealed the presence of several changes. Almost all of found changes in the analyzed regions of the mtDNA were microsatellite variations, variants defining mitochondrial haplogroups and common population polymorphisms according to the Human Mitochondrial

Table 1. Clinical details of pediatric leukemia patients.

Patient	Diagnosis	Age (years)	Gender	Protocol of treatment	Second bone marrow sample (day of treatment)
P1	pro-B ALL/cALL	3	Female	ALL IC-BFM 2002	48
P2	pro-B ALL/cALL	14	Male	ALL IC-BFM 2002	41
P3	cALL + coexpression of CD33	3	Male	ALL IC-BFM 2002	96
P4	cALL	9	Female	ALL IC-BFM 2002	25
P5	cALL	17	Male	ALL IC-BFM 2009	80
P6	pro-B ALL + coexpression of CD65, CD15, NG2	8 months	Male	INTERFANT 06	15

Table 2. Primers for D-loop, *ATPase 6*, *ATPase 8*, *ND1*, *cytB*, *COXII* gene amplification and their nucleotide sequences.

Each starter has a M13 tagged tail. The sequence of the FM13 tail: 5'TAAAACGACGCCAGT3' and of the RM13 tail: 5'GAGGAAACAGCTATGACC3'.

primer name	primer sequence (5'-3')	nucleotide position (5'-3')	mtDNA region
M13.D1F-M13.D3R	D1F: AATGGGCTGTCTTGTAG D3R: GGTGTGGCTAGGCTAAGC	15879-803	D-loop
M13.5F-M13.5R M13.6F-M13.6R M13.7F-M13.7R	5F: GGATCAGGACATCCCGATG 5R: AACGGCTAGGCTAGAGGTG 6F: TAGCTCTACCCATCGCTC 6R: GATTGTTAATGGGTATGGAGAC 7F: TCCTACCACTCACCTAGC 7R: GTCATGTGAGAAGAAGCA	2995-4869	ND1
M13.12F-M13.12R M13.13F-M13.13R M13.14F-M13.14R	12F: CCTAATTAGTAGAAGAACCCTC 12R: CTCGATTGTCAACGTC AAGG 13F: ATTATTCCTAGAACCAGGCG 13R: TGATGAGATATTTGGAGGTGG 14F: ACAATCCTAGGCCTACCCG 14R: GATAGGCATGTGATTGGTGG	7349-9231	COXII, <i>ATPase8</i> , <i>ATPase6</i>
M13.23F-M13.23R M13.24F-M13.24R M13.25F-M13.25R	23F: CCCATAATCATACAAAGCCC 23R: GTTGAGGCGTCTGGTGAG 24F: ACTACAAGAACCAATGACC 24R: TGTAGTAAGGGTGGAAAGGTG 25F: TAGGAATCACCTCCATTCC 25R: GTC AACTTGGGTGGTACC	14227-16067	cytB

drial Genome Database (mtDB, Department of Genetics and Pathology Uppsala University, Sweden, updated 1st of March 2007, www.mtodb.igp.uu.se) and according to MITOMAP database (www.mitomap.org). The highest level of polymorphisms were found in D-loop region and in the *cytB* gene. Only in patient 2 a change at position 3688 in *ND1* gene was found. This change was not listed as a polymorphism in mtDNA databases. This transition results in substitution threonine for alanine (A128T) in a highly conserved residue of the protein (Fig. 1). All the detected changes were homoplasmic except the change at position 3688 which was found to be heteroplasmic. Mitochondrial DNA sequence variations in bone marrow cells in analyzed patients are shown in Table 3 in Appendix 1 (at www.actabp.pl).

Search for disease and treatment markers in mtDNA sequence

In order to explore the possibility that treatment can cause mtDNA mutations and to look for possible markers of the disease, the control region and 5 genes of mtDNA (*ATPase6*, *ATPase8*, *ND1*, *cytB*, *COXII*) were analyzed for 6 patients using DNA isolated from bone marrow collected at the time of ALL diagnosis and several days/months later when patients were undergoing chemotherapy. We did not find additional changes

	A128T
Patient	WSGWASNSNY T LIGALRAVAQ
<i>Homo sapiens</i>	WSGWASNSNY A LIGALRAVAQ
<i>Gorilla gorilla</i>	WSGWASNSNY A LIGALRAVAQ
<i>Pan paniscus</i>	WSGWASNSNY A LIGALRAVAQ
<i>Pongo pygmaeus</i>	WSGWASNSNY A LIGTLRAVAQ
<i>Danio rerio</i>	QSQWASNAKY A LIGALRAVAQ
<i>Drosophila melanogaster</i>	VAGWSSNSNY A LLGGLRAVAQ
<i>Canis lupus familiaris</i>	WSGWASNSKY A LIGALRAVAQ

Figure 1. Comparison of the ND1 polypeptide region encompassing the Ala128Thr change (A128T) (bolded) among several species.

in samples collected after chemotherapy, but in one of the patients we noticed the loss of the transition at position 3688. The level of the 3688G>A transition in DNA from patient's 2 bone marrow collected at the moment of ALL diagnosis was 17% \pm 0.23%, but this change was not found in the DNA isolated from this patient's blood and bone marrow collected at day 42 of treatment (Fig. 2 and Fig.3).

DISCUSSION

In the present study we sequenced the D-loop as well as 5 genes of mtDNA derived from bone marrow cells of 6 pediatric patients with ALL. Analysis of the mtDNA sequences showed a number of common polymorphisms, most of them located in the D-loop region. In one of the samples derived from a patient at the moment of ALL diagnosis a heteroplasmic transition at position 3688 of mtDNA was found. The 3688G>A transition is located in the highly conserved region of *ND1*

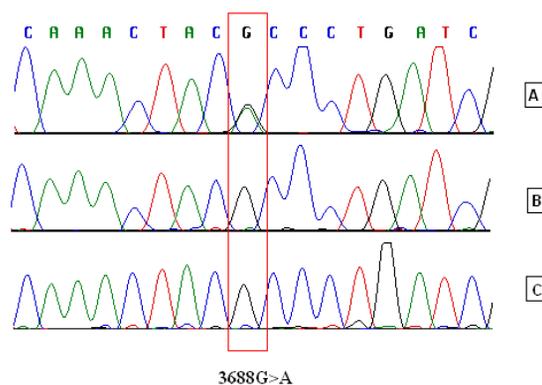


Figure 2. Molecular analysis of the *ND1* gene in an ALL patient. Electropherograms of the *ND1* gene showing the 3688G>A transition only in a bone marrow sample collected at the time of leukemia diagnosis (A). (A) DNA isolated from bone marrow cells collected from the patient at the time of diagnosis. (B) DNA isolated from bone marrow cells collected from the patient at day 42 of treatment. (C) DNA isolated from the patient's blood.

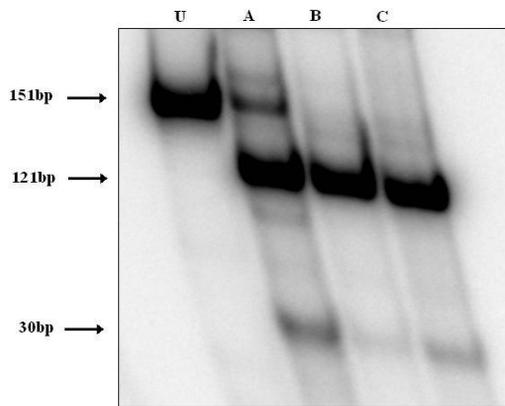


Figure 3. PCR-RFLP analysis for the transition 3688G>A.

The region spanning codon 128 in the *ND1* gene was PCR amplified and the 151bp PCR product was cut with *HaeIII*. The wild type sequence is cut into two fragments of 121bp and 30bp. In the presence of the 3688G>A mutation the *HaeIII* restriction site is lost, permitting discrimination between wild-type and mutated mtDNA. (A) DNA isolated from bone marrow cells collected from the patient at the time of diagnosis. (B) DNA isolated from bone marrow cells collected from the patient at day 42 of treatment. (C) DNA isolated from patient's blood. (U) uncut DNA

gene. This change, in a homoplasmic state, was previously described in one patient with Leigh syndrome, in whom it was associated with a strong reduction of complex I activity in both muscle and fibroblasts (Valente *et al.*, 2009). We do not know if the 3688G>A transition appeared in bone marrow cells of our patient as a consequence of malignant transformation because we did not have a sample collected before the patient was diagnosed with leukemia. However, we can speculate that, if the presence of this transition caused a reduction of complex I activity in a Leigh syndrome patient's tissues it could also cause dysfunction in bone marrow cells and can be considered as a pathogenic mutation. A decrease of the complex I activity could cause reactive oxygen species overproduction and lead to oxidative stress which could contribute to malignant transformation in bone marrow cells. Analyses of cell cultures derived from consecutive bone marrow specimens collected from our patient are required to obtain information about the influence of the 3688G>A transition on cell physiology and to confirm the pathogenicity of the mutation and whether it has any influence on the course of treatment.

In our research we also wanted to check whether the mutation load can change in different treatment stages, as was previously described by He *et al.* (2003). The aim of chemotherapy is the elimination of cancerous cells with mutations and aberrations potentially dangerous to cell physiology. The 3688G>A mutation, present in bone marrow cells collected from one patient with ALL, disappeared in the course of chemotherapy treatment (in the induction phase), therefore we can suspect that chemotherapy killed the cancerous cells and only healthy cells remained which were free of this mutation. In our considerations on the cause of the lack of detection of mutation in bone marrow samples taken at day 42 of treatment, we also have to take into account that bone marrow consists of heterogeneous populations of cells. In addition to hematopoietic stem cells we can also find endothelial, pluripotent and tissue committed stem cells as well as multipotential adult progenitor cells (Ratajczak *et al.*,

2004). This raises the question whether the first and second bone marrow sample collected from our patient contain the same proportions of cells from different populations? We cannot exclude that the differences in proportion of various cell types could be also responsible for the absence of mutation detection in the sample collected during treatment. Moreover, the heteroplasmy level of detected mutation was quite low so if in the second bone marrow sample there was a much lower level of cells of a specific type in which the mutation previously existed, its detection could be difficult or impossible. If the mutation would be nearly homoplasmic it would mean that it was present in almost all populations of cells in bone marrow and interpretation of such a result could be much easier. In this case the absence of mutation in the bone marrow sample after chemotherapy which was homoplasmic in the sample collected at the time of diagnosis could be more informative and we could suspect that its disappearance was caused by treatment. Mutations, whose levels change during patient treatment, could be potential biomarkers for monitoring treatment efficacy and disease in this patient.

In our study we were also interested whether chemotherapy causes mtDNA mutations. If mtDNA is sensitive to chemotherapeutics, then it might be possible to monitor changes in mtDNA sequence during treatment which could reflect treatment effectiveness. Comparative analyses revealed that there was no additional accumulation of polymorphisms or mutations in specimens collected after chemotherapy. It should be noted that in our study the examined group of pediatric patients with ALL is too small to draw conclusions about the lack of detrimental chemotherapeutic treatment effects on the mtDNA sequence. Moreover, only part of the mtDNA was analyzed and the potential mutations caused by chemotherapeutics could occur in the other coding regions of mtDNA. Such changes, if found, could be used as a genetic marker of DNA damage and might explain some of the adverse effects of treatment and also relapse of cancer.

It is conceivable that some mutations could contribute to the development of a cancer phenotype by changing cellular energy capacities, increasing free radical generation leading to further DNA damage and genetic instability and modulating apoptosis. Further studies to determine at what stage the somatic mtDNA mutation occurs could give an insight into the role of somatic mtDNA mutation in leukemia and carcinogenesis. The presence of a mutation does not prove that they play a causative role in carcinogenesis, however, it provides additional information about the contribution of mitochondria to this process. Moreover, if damage of the mitochondrial genome results in long term adverse effects, this knowledge may be helpful to develop agents which protect the mitochondrial genome during cancer.

Conflicts of interests

The authors report no conflicts of interest.

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