Comprehensive Scanning of Somatic Mitochondrial DNA Alterations in Acute Leukemia Developing from Myelodysplastic Syndromes

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ABSTRACT

Myelodysplastic syndromes (MDS) are clonal myeloid disorders characterized by ineffective hematopoiesis resulting in refractory cytopenias. Transformation resulting in acute myeloblastic leukemia is the final stage in the multistep process of MDS evolution. Functional relevant mutations of mitochondrial DNA (mtDNA) have been related to sideroblastic anemia and MDS. To investigate the role of mtDNA in malignant transformation to acute leukemia, we used high-resolution techniques such as single-strand conformational polymorphism and fluorescence sequencing for investigation of the whole mitochondrial genome from blood cells of 10 patients with MDS. Functionally relevant point mutations in mitochondrial RNA and polypeptide-encoding genes were detected in 50% of patients with MDS. Their increasing mutation load connects MDS and the developing acute myeloid leukemias. Several point mutations of mtDNA, including secondary point mutations for Leber’s hereditary optic neuropathy, occur in one bone marrow and may synergically affect bone marrow stem cells by an apoptotic pathway.

INTRODUCTION

Mitochondrial defects have been associated with both severe neurodegenerative disorders and primary human cancers. Hereditary nuclear mutations in succinate dehydrogenase and fumarase genes cause a predisposition to paraganglioma and kidney cancers (1). Mutations of mitochondrial DNA (mtDNA), including intragenic deletions (2), missense and chain terminating point mutations (3), and alterations of homopolymeric sequences (4), have also been identified in various human cancers. A variety of disorders with predominantly neurological symptoms have been linked to alterations of mtDNA. Various point mutations of mtDNA are located in tRNA genes (5). Hereditary missense mutations of mtDNA can result in one of three clinical presentations: dystonia; Leigh’s disease; and Leber’s hereditary optic neuropathy (LHON) (6). Rearrangements of mtDNA cause Pearson’s marrow-pancreas syndrome. This is a congenital mitochondrial cytopathy, which clinically presents as sideroblastic anemia (7). The same dysplastic changes of the bone marrow including ringed sideroblasts (8, 9) can also be observed in refractory anemia with ring sideroblasts belonging to myelodysplastic syndromes (MDS).

These are clonal myeloid disorders characterized by ineffective hematopoiesis and bone marrow cell dysplasia (10). Multiple combinations of lesions affect pluripotent hematopoietic progenitors and therefore affect myeloid, monocytic, erythroid, and megakaryocytic lineages (11). Transformation resulting in acute myeloid leukemia is the final stage in the multistep process of MDS evolution (12). Sideroblastic anemia and MDS have been shown to be associated with the final stage in the multistep process of MDS evolution (12). Functional relevant mutations in mtDNA from myelopoietic cell lineages (11, 13) in MDS and extend these observations to the developing acute leukemias. Furthermore, we monitor the temporal development of the mutations during progression of MDS toward acute leukemia.

MATERIALS AND METHODS

Case Reports

Patient 1. This 58-year-old female presented with anemia and thrombocytopenia in April 1996 and was diagnosed with MDS subtype refractory anemia (RA). In June 1996, a severe leukopenia developed. Bone marrow analysis revealed MDS subtype RAEBt according to the French-American-British classification. No cyogenetical aberrations were found. In addition, she had a hysterectomy in 1976 and suffered from cystic hepatic insufficiency and arterial hypertension.

When a malignant transformation to acute myeloid leukemia type M4 occurred in October 1996, this patient’s blood showed 10,000 leukocytes/μl with increasing blasts up to 95%. One cycle of chemotherapy with idarubicine and five cycles of chemotherapy with etoposide were administered. She died from leukemic infiltration of the bowel and pneumonia.

Patient 2. This 54-year-old female with a previous history of anemia and thrombocytopenia was diagnosed with MDS in 1994. In October 1996, she developed acute leukemia type M1, and blood analysis revealed 76,000 leukocytes/μl with 99% blasts. Cytogenetic analysis revealed translocation t(2;5) and a trisomy 19.

During her clinical course period, she suffered from cerebral bleeding and multiple infections. In addition, the patient suffered from arterial hypertension and had a previous history of tuberculosis. The patient had undergone surgery for treatment of rectal carcinoma and removal of the gallbladder. One cycle of chemotherapy with cytosinarabinoside and idarubicine, one cycle of chemotherapy with mitoxantrone and etoposide, one cycle of chemotherapy with cytosinarabinoside and idarubicine, and four cycles of chemotherapy with mitoxantrone were administered before the patient died from cerebral bleeding.

Patient 3. This 75-year-old-female had a previous history of anemia since 1986 and leukopenia since 1987. In February 1996, she presented with pancytopenia. A bone marrow biopsy showed dysplastic changes and ring sideroblasts. A diagnosis of refractory anemia with excess of blasts (RAEB) was made.

Cytogenetic analysis revealed multiple abnormalities such as 5q−, −7, −21, and 11/8ND. The patient was treated with radioactive iodine in 1978 for hyperthyroidism. In addition, she suffered from peripheral arterial occlusive disease, osteoporosis, and impaired liver function and had a remote history of tuberculosis.

A transformation to acute myeloid leukemia type M4 according to the French-American-British classification was observed shortly after the initial diagnosis of RAEB. After 13 days of therapy with low dose 1-Beta-arabinofuranosylcytosine and 6 days of chemotherapy with etoposide and mitoxantrone, the patient died from a rapid increase of blasts up to 200,000/μl in the peripheral blood.

Samples. These patients are part of a series of 10 patients. After obtaining informed consent, patient blood and bone marrow samples were subjected to PCR-single-strand conformational polymorphism (SSCP (Table 1)). Seven were diagnosed according to the criteria of the French-American-British classification with acute myeloid leukemia developing from MDS, one was diagnosed with refractory anemia with ring sideroblasts (RARS), one was diagnosed with refractory anemia with excess of blasts in transformation (RAEBt),...
and one was diagnosed with 5q− syndrome. Peripheral blood from age-matched, healthy volunteers was used as control. 

Preparation of mtDNA

Mitochondria and, subsequently, their mtDNA were extracted from peripheral blood cells of different lineages and from unfractionated bone marrow according to the protocol from Wallace (14) to exclude nuclear pseudogenes or double minutes from analysis. Lymphocytes were separated using Dynabeads (Dynal, Oslo, Norway) according to recommendations of the manufacturer.

<table>
<thead>
<tr>
<th>Patient</th>
<th>FAB type</th>
<th>Mitochondrial gene</th>
<th>Nucleotide position</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>Disease relevance</th>
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<tbody>
<tr>
<td>1</td>
<td>AML-M4 (RAEBi)</td>
<td>16 S rRNA</td>
<td>1.721</td>
<td>C→T</td>
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<td></td>
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<tr>
<td></td>
<td></td>
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<td>12.196</td>
<td>C→T</td>
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<td>RHON</td>
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<td></td>
<td></td>
<td>ND1</td>
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<td>T→C</td>
<td>Y→H</td>
<td>LHON</td>
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<tr>
<td></td>
<td></td>
<td>ND5</td>
<td>13.708</td>
<td>G→A</td>
<td>A→T</td>
<td>LHON</td>
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<td>3.394</td>
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<td>Y→H</td>
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<td>4.216</td>
<td>T→C</td>
<td>Y→H</td>
<td>LHON</td>
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<td></td>
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<td>ND5</td>
<td>13.708</td>
<td>G→A</td>
<td>A→T</td>
<td>LHON</td>
</tr>
</tbody>
</table>

mtDNA Amplification

A long-distance PCR was performed to screen for deletions and duplications. The XL PCR was carried out in a reaction volume of 50 μl with 20–50 ng of mitochondrial template DNA. The primer pair H1 and L14570 (Table 2) was used to amplify the 14,570-bp-long fragment. Amplification was performed in a thermocycler constructed in our institute as follows: first denaturation, 94°C, 60 s; following denaturations, 98°C, 20 s; no annealing; extension, 68°C, 600 s; last extension, 68°C, 1200 s (polymerization kit provided by Takara Biomedicals, Gennevilliers, France).

As a second step, the entire mitochondrial genome was amplified in 14 radioactively labeled PCR products (Table 2) using the XL amplicon as template. These PCRs were performed in 100 μl using 1–10 ng of template, 50 mm KCl, 1.5 mm MgCl2, 10 mm Tris-HCl (pH 9.0), 0.4 mm forward and backward primer, 200 μmol of each nucleotide, 2.5 units of Taq polymerase (Pharmacia Biotech, Uppsala, Sweden), and 5–20 μCi of [α-32P]dATP. The time of denaturation was 94°C, the time of annealing was 300 s in the first circle and 30 s in the following. The time of annealing was 60 s, and extension temperature was 72°C. The extension time varied between 40 s and 60 s, and the annealing temperature varied between 53°C and 62°C according to the length of the fragment and the primers used (Table 2).

PCR-SSCP and RFLP

Each amplified mtDNA fragment was digested with one or several of these restrictions enzymes: AluI, DdeI, MspI (Promega, Heidelberg, Germany); and BsaI and BsmBI (New England Biolabs, Frankfurt, Germany). Restriction reactions were performed according to the manufacturer’s recommendations, using approximately 800 ng of amplicon, 20 units of restriction enzyme in a 50 μl volume, and incubation for 24 h at 37°C in a 50 unit/min shaking incubator. The restriction digests were heat denatured and loaded on a 5–7% native acrylamide gel containing 29:1 bisacrylamide and 7.5% glycerin. Two runs per digest were performed in Tris-borate buffer: one at 7°C and 1000 V; and one on a temperature gradient containing periods at 10°C, 7°C, 6°C, and 4°C at 2500 V. Restriction digests with MnlI (New England Biolabs) for estimation of mutation loads were quantified by the beta scanner Storm using its program ImageQuant (Molecular Dynamics, Krefeld, Germany).

Sequencing

Fragments showing a bandshift in SSCP analysis were amplified using the patient’s mtDNA as template and gel purified, and then both strands were automatically sequenced using the ABI Prism 310 Genetic Analyzer (Perkin-Elmer, PE Applied Biosystems) according to the manufacturer’s instructions. Sequencing primers have also been used as primers for PCR (Table 2). Sequence data were edited by the Sequence Navigator Version 1.0.1 for ABI Prism (Perkin-Elmer, PE Applied Biosystems), permitting the detection of ambiguous bases in heteroplasy.
RESULTS

Mitochondrial Genome Analysis. SSCP analysis revealed heteroplasmic band shifts in the BfaI digest of fragment 2, the BsaI digest of fragment 3, the AluI, MspI, and BsaI digests of fragment 5, and the AluI digest of fragment 10 (Fig. 1). Subsequent sequence analysis of these shifted fragments and fragment 7 revealed two 16S rRNA mutations, one C to T transition at nucleotide position 1721 and one G to A transition at nucleotide position 2056; two secondary LHON mutations, one T to C transition at nucleotide position 3394 and another A to G transition at nucleotide position 4216 in the ND1 gene; a T to C transition at nucleotide position 5843 in the mitochondrial tRNA tyrosine gene; an A to G transition at nucleotide position 8617 in the ATPase 6 gene; and a G to A transition at nucleotide position 12196 in the mitochondrial tRNA histidine gene (Fig. 1). Analyzing mtDNA from the blood of patients with acute myeloid leukemia, all these mutations appeared to be homoplasmic (Fig. 1). The pathogenic potential of these mutations is supported by high-resolution screening of human populations involving thousands of individuals, in which these base changes have never been observed (15). As expected from mitochondrial, interindividual sequence differences, we found many known polymorphisms and three unknown polymorphisms at positions 6554 (C to T) and 6824 (C to G) in cytochrome c oxidase (COX) I and position 7768 (A to G) in COX II. These mutations do not lead to any amino acid substitution.

Long PCR screening for large deletions or duplications of mtDNA did not reveal any large-scale rearrangements like those seen in
Table 3 Interspecies comparison of mitochondrial tRNA tyrosine and histidine

<table>
<thead>
<tr>
<th>tRNA tyrosine</th>
<th>Stem</th>
<th>Loop</th>
<th>TGC</th>
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<tr>
<td>Patient</td>
<td>AAGG</td>
<td>GCTT</td>
<td>AGG</td>
</tr>
<tr>
<td>Human</td>
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<td>GCTT</td>
<td>AGG</td>
</tr>
<tr>
<td>Bovine</td>
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<td>GCTT</td>
<td>AGG</td>
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<tr>
<td>Mouse</td>
<td>AAGG</td>
<td>GCTT</td>
<td>AGG</td>
</tr>
<tr>
<td>Chicken</td>
<td>AAGG</td>
<td>GCTT</td>
<td>AGG</td>
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<td>Frog</td>
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<td>GCTT</td>
<td>AGG</td>
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<td>Carp</td>
<td>AAGG</td>
<td>GCTT</td>
<td>AGG</td>
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<tr>
<td>S. urchin</td>
<td>GCAG</td>
<td>TAAA</td>
<td>CTCT</td>
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</table>

* Nucleotides in bold represent evolutionary highly conserved bases. S. urchin, sea urchin.

Table 4 Interspecies comparison of amino acid sequences in the start region of ATP synthase 6

<table>
<thead>
<tr>
<th>Patient</th>
<th>MNENLFAFSI</th>
<th>APTILGLPAA</th>
<th>VLLILFPLP</th>
<th>P---TSKYL</th>
</tr>
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<tbody>
<tr>
<td>Human</td>
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<td>VLLILFPLP</td>
<td>P---TSKYL</td>
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<tr>
<td>Bovine</td>
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<td>P---TSKYL</td>
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<tr>
<td>Mouse</td>
<td>MNENLFAFSI</td>
<td>APTILGLPAA</td>
<td>VLLILFPLP</td>
<td>P---TSKYL</td>
</tr>
<tr>
<td>Frog</td>
<td>MNENLFAFSI</td>
<td>APTILGLPAA</td>
<td>VLLILFPLP</td>
<td>P---TSKYL</td>
</tr>
<tr>
<td>Shark</td>
<td>MNENLFAFSI</td>
<td>APTILGLPAA</td>
<td>VLLILFPLP</td>
<td>P---TSKYL</td>
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</table>

* Nucleotides in bold represent evolutionary highly conserved amino acids.

MITOCHONDRIAL DNA ALTERATIONS IN LEUKEMIA

Pearson’s syndrome. All patients were analyzed by RFLP for mutations of mitochondrial encephalomyopathies including mitochondrial encephalopathy, lactate acidosis and stroke-like episodes (MELAS), myoclonic epilepsy and red ragged fibers (MERRF), and neurogenic muscle weakness, ataxia, retinitis pigmentosa (NARP) and the primary mutations of LHON. None of these mutations, including the MERRF mutation described by Wang et al., were detected (16). Also, our patients did not show any mutations in the COX subunit I and II genes (17, 18), which were thought to disturb the process of mitochondrial iron reduction (data not shown; Ref. 19). Although we used a PCR-SSCP variant with a high sensitivity of mutation screening, we cannot exclude the possibility that we missed some point mutations in our study.

**Functional Significance of Somatic Mutations.** Using the DNA-sis program (Hitachi, Japan) to determine the secondary structures of nuclear tRNAs, the mutations in mitochondrial tRNA Tyr and tRNA His in patients 3 and 1 will both affect the T/P arm of the tRNA. Changing a U to C in the T/P arm of tRNA Tyr and stabilizing the T/P C stem of tRNA His by a C to U mutation, which will introduce a fifth bp in the stem, could both lead to impaired binding of the tRNA to the ribosome during the translational process (Fig. 2). During evolution, the T mutated in the mitochondrial tRNA Tyr gene has been highly conserved among a wide range of species. The base mutated in the mitochondrial tRNA His gene is mildly conserved: at this position, there is also a T in cow and mouse (Table 3).

The point mutation detected in the ATP synthase subunit 6 gene is found at nucleotide position 8617 in leukocytes (Fig. 1; Table 1) and platelets of patient 1 in the leukemic state. The resulting amino acid change converts an isoleucine to valine in position 31 of ATPase subunit 6 (Table 1). The change takes place in the NH₂-terminal start region of subunit 6, which represents a sequence of hydrophobic amino acids and may be placed in the membrane. During evolution, besides isoleucine, only phenylalanine is observed at this amino acid position (Table 4).

Interspecies comparison showed that the 16S ρRNA mutations at nucleotide positions 1721 and 2056 in patients 1 and 2 (Fig. 1; Table 1) are not part of conserved sequences of this RNA. Sequence comparison with the 23S ρRNA of Escherichia coli revealed that the position corresponding to mitochondrial nucleotide 2056 is a binding site for the antibiotic drug viomycin in E. coli.

**Stage-Dependent Development of Mutation Load.** Assuming that mitochondrial MDS mutations are best able to be detected in the final stages of this disease, sequence analysis was performed from mtDNA of leukocytes, platelets, and bone marrow in the leukemic stage. Fluorescence sequencing showed all point mutations homoplasmic in acute leukemia (Fig. 1). However, subsequent RFLP analysis of these mutations revealed their absence in patient lymphocytes and heteroplasmy in earlier MDS subtypes. The latter phenomenon is illustrated by a restriction test using restriction endonuclease MnlI for the tRNA His mutation at nucleotide position 12,196 of patient 1 (Fig. 3). A 240-bp amplicon is synthesized flanking the latter mutation. The wild-type amplicon is cut into a 26-bp fragment and a 214-bp fragment. The tRNA His mutation creates an additional restriction site for MnlI, and the 214-bp fragment is cut again into a 29-bp fragment and an 185-bp fragment. As shown in Fig. 3, the mutation load represented by the 29-bp fragment is continuously increasing in comparison with the 26-bp wild-type fragment to near homoplasmy during 6 months of transformation to leukemia. The mutation load estimated by quantification using a beta scanner was 14% for thrombocytes in January 1997.
1997, 37% in February 1997, 73% in March 1997, and 96% in June 1997. Leukocytes showed a mutation load of 9% in February 1997, 48% in March 1997, and 83% in June 1997. In January, the patient’s state was RAEBt and progressed to acute leukemia. In June, the patient died from acute leukemia.

DISCUSSION

Functional Relevance of Somatic Mutations. The discovery of novel point mutations in rRNA, tRNA, and polypeptide-encoding genes of blood mtDNA as well as secondary LHON mutations (Table 1) in 50% of patients with MDS (5) and developing leukemias shows that the role for mtDNA mutations in the development of malignancies is not restricted to various human cancers (20).

tRNA and missense mutations have the potential to be functionally relevant in carcinogenesis. The point mutation observed in the ATP synthase subunit 6 gene leads to an NH₂-terminal amino acid change and may affect membrane integration of this proton channel forming subunit. Missense mutations in ATPase genes have been reported in MDS (11, 13) as well as in ovarian carcinomas (21), and missense mutations have been reported for all mitochondrial polypeptide-encoding genes in a variety of tumors (20). Functional studies have only been performed for mutations in COX in MDS, and it has been shown in the rho₀ cell system that they do not impair energy metabolism severely (19). Recently, the presence of missense mutation hot spots in COX I and COX II genes has been described (17). This is in striking contrast to the results of our study and other studies (11, 13), and one explanation could be the analysis of nuclear-embedded pseudogenes, where these mutations have been described previously (22).

Mitochondrial tRNA histidine and tyrosine mutations may diminish mitochondrial protein synthesis by different pathways, and there may be even more to tRNA mutations than their role in mtDNA translation; they also seem to be important in regulating transcription termination or tissue specificity of respiratory chain complexes (23). Gattermann et al. (24) also described heteroplasmic point mutations of mitochondrial tRNA genes in MDS subtypes RA and RARS.

Stage-Dependent Development of Mutation Load. In our patients, we found heteroplasmic point mutations in CMML, RAEB, and RAEBt. We show that these MDS subtypes are characterized by an increasing mtDNA mutation load using the tRNA histidine gene as an example for our RFLP results on these novel mutations. This mutation is present in low heteroplasmy in subtype RAEBt. We show that these MDS subtypes are characterized by an increasing mtDNA mutation load, their increasing amounts of reactive oxygen species by the Fenton reaction (32). The mutational spectrum of our patients encompassed half G to A transitions (Table 1) and therefore represents oxidative damage (33, 34). Mitochondria with iron overload accumulate perinuclear in sideroblasts and, depending on the mtDNA mutation load, their increasing amounts of reactive oxygen species may lead to oxidative damage of oncogenes and tumor suppressor genes of the neighboring nuclear genome. These nuclear mutations may exclusively drive clonal expansion toward leukemia. Therefore, functionally relevant mutations of mtDNA, found in neoplasms, may contribute to carcinogenesis in a “pseudoclonal” way (1, 25), because they represent a growth handicap to the proliferating cells (35). Clonal expansion may now lead in an epiphenomenal, stochastic way (36) to mitochondrial outgrowth and may even explain why polymorphisms evolve by random drift to homoplasy.

MDS is an excellent model disease to study the stepwise effects of mtDNA mutations in cancer because it develops over five stages to acute leukemia. We detected mutations in MDS that at least influence the sideroblastic phenotype of the cells, as exemplified on Pearson’s syndrome, and may play a role in apoptosis of MDS. The study of secondary MDS, where, for example, 16S rRNA mutations can be related to the mode of drug operation, should give insights with regard to the pathomechanism. The issue of whether an increasing mutation load, as observed in RAEBt, can overcome apoptotic control and promote carcinogenesis should be clarified by future biochemical investigations.

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