Molecular Pathogenesis of Genetic and Inherited Diseases

Gastrointestinal Dysmotility in Mitochondrial Neurogastrointestinal Encephalomyopathy Is Caused by Mitochondrial DNA Depletion

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Chronic intestinal pseudo-obstruction is a life-threatening condition of unknown pathogenic mechanisms. Chronic intestinal pseudo-obstruction can be a feature of mitochondrial disorders, such as mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), a rare autosomal-recessive syndrome, resulting from mutations in the thymidine phosphorylase gene. MNGIE patients show elevated circulating levels of thymidine and deoxyuridine, and accumulate somatic mitochondrial DNA (mtDNA) defects. The present study aimed to clarify the molecular basis of chronic intestinal pseudo-obstruction in MNGIE. Using laser capture microdissection, we correlated the histopathological features with mtDNA defects in different tissues from the gastrointestinal wall of five MNGIE and ten control patients. We found mtDNA depletion, mitochondrial proliferation, and smooth cell atrophy in the external layer of the muscularis propria, in the stomach and in the small intestine of MNGIE patients. In controls, the lowest amounts of mtDNA were present at the same sites, as compared with other layers of the gastrointestinal wall. We also observed mitochondrial proliferation and mtDNA depletion in small vessel endothelial and smooth muscle cells. Thus, visceral mitochondrial myopathy likely causes gastrointestinal dysmotility in MNGIE patients. The low baseline abundance of mtDNA

molecules may predispose smooth muscle cells of the muscularis propria external layer to the toxic effects of thymidine and deoxyuridine, and exposure to high circulating levels of nucleosides may account for the mtDNA depletion observed in the small vessel wall. (*Am J Pathol* 2008, 173:1120–1128; DOI: 10.2353/ajpath.2008.080252)

Chronic intestinal pseudo-obstruction (CIPO) is a highly morbid and often life-threatening condition characterized by marked dysfunction of gut propulsive motility, which results in a clinical picture mimicking mechanical obstruction.^{1,2,3} Patients with CIPO usually complain of severe symptoms including abdominal pain and distension, early satiety, bloating, and vomiting, as well as constipation and/or diarrhea. CIPO is an important cause of chronic intestinal failure, because affected individuals become unable to maintain normal nutrition and body weight. Concerning etiologies, CIPO may be primary or secondary to a variety of systemic diseases.^{2,3} Primary CIPO may be due to abnormalities of smooth muscle cells of muscularis propria (ie, visceral myopathy) and/or enteric neuronal supplies of gastrointestinal (GI) wall (ie, visceral neuropathy).³ In addition, abnormalities of the GI pacemaker cells, the interstitial cells of Cajal have been reported.⁴

CIPO is an increasingly recognized clinical feature of mitochondrial encephalomyopathies.⁵ This heterogeneous group of genetic disorders is caused by dysfunction of the mitochondrial respiratory chain that usually affects highly energy dependent tissues such as brain and muscle.⁶ Among mitochondrial encephalomyopathies, one most frequently associated with GI dysmotility and CIPO is mitochondrial neurogastrointestinal en-

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cephalomyopathy (MNGIE), an autosomal recessive syndrome due to mutations in the thymidine phosphorylase gene *TYMP*.⁷ MNGIE is defined clinically by severe GI dysmotility, cachexia, ptosis, ophthalmoparesis, peripheral neuropathy, white matter changes in brain magnetic resonance imaging, and mitochondrial abnormalities.⁷ GI dysmotility leads to progressive weight loss and cachexia of MNGIE patients, and diverticulosis of small intestine complicated by inflammation and perforation often causes their death in early adulthood. Biochemical abnormalities in MNGIE include drastically reduced thymidine phosphorylase activity leading to accumulation of thymidine (dThd) and deoxyuridine (dUrd) in blood and tissues.^{8,9} Toxic levels of dThd and dUrd induce nucleotide pool imbalances that in turn lead to mtDNA abnormalities (point mutations, multiple deletions, and depletion).^{8,10–12} The pathogenic mechanisms causing GI dysmotility in MNGIE are still unclear. We recently showed atrophy, mitochondrial proliferation, and mtDNA depletion in muscularis propria of small intestine in one patient.¹³ In the present study, we provide a detailed morphological and molecular investigation of the entire GI tract in five MNGIE patients, hence establishing a link between marked mtDNA depletion and myopathic changes of the external layer of muscularis propria in this syndrome.

Materials and Methods

Patients

A summary of clinical features of the five MNGIE patients investigated is reported in Table 1.

Tissue Sample Preparation

All studies conformed to Sapienza, University of Rome, Ethical Committee protocols. At autopsy, after informed consent was provided from relatives, multiple tissue sam-

ples were obtained from esophagus, stomach, small intestine, and colon from five MNGIE patients. We used as controls, ten age-matched sudden cardiac death cases whose autopsies were performed in the Department of Pathology, Sapienza, University of Rome. Autopsies from both MNGIE patients and controls were performed after 24 to 30 hours after death. For molecular analysis of whole tissue homogenate, samples were snap-frozen in liquid nitrogen-chilled isopentane. For histological analysis, tissue sections obtained from formalin-fixed, paraffin-embedded samples, were stained with H&E and Masson's trichrome. Immunohistochemistry for S-100, synaptophysin, neuronal-specific enolase, glial fibrillary acidic protein (DAKO Glostrup, Denmark), and mitochondrial antigens (Clone MTC, UCS Diagnostic, Morlupo, Italy) was also performed. Combined cytochrome c oxidase/succinate dehydrogenase (COX/SDH) stain was performed on frozen sections of proximal esophagus and small intestine obtained from patients 3 and 4 (see Table 1). Combining the histochemical mitochondrial activities of COX (orange) and SDH (blue) results in a brown stain. Single cells bearing mtDNA defects commonly lose their COX activity; however, leaving intact the nuclear-encoded SDH activity, thus becoming highlighted in blue on the brown background. For ultrastructural analysis, samples were fixed in 4% paraformaldehyde-phosphate buffered saline and postfixed in osmium tetroxide. Thin sections were stained with uracyl acetate and lead citrate and examined with a CM10 Philips electron microscope (Eindhoven, the Netherlands).

Laser Capture Microdissection

Paraffin sections from patients and controls were subjected to laser capture microdissection with the MMI NIKON UV-CUT System (Molecular Machines & Industries, Glattbrugg, Switzerland) as previously described.¹³ Briefly, serial 5- μ m-thick cut sections were mounted on a

Table 1. Summary of the Clinical Data from MNGIE Patients

Patient (reference no.)	Gender	Onset	Age at diagnosis	Age at death	TYMP mutation	Exon/intron	Blood thymidine levels
1 (13)	M	5 years epilepsy and childhood-onset bilateral ptosis, 25 years onset GI symptoms	35 years	38 years	c.457 G > A homozygous	Exon 4	7.6 μ mol/L
2	M	Childhood-onset GI symptoms, 37 years onset bilateral ptosis	38 years	39 years	c.522T > A homozygous	Exon 5	7.0 μ mol/L
3 (patient 3 in reference 7)	M	18 years foot numbness (neuropathy)	34 years	37 years	c.433 G > A homozygous	Exon 4	Not determined
4 (patient 4 in reference 7)	F	15 years borborygmi, abdominal pain, diarrhea	26 years	28 years	c.665 A > G c.1406 insC	Exon 6 exon 10	Not determined
5 (patient 7 in reference 7)	F	26 PEO	36 years	39 years	c.866 A > C IVS9-1 G > C	Exon 7 intron 8	5.7 μ mol/L

(Continued)

polyethylene foil slide and stained with H&E. Sections were observed under light microscope with a $\times 40$ objective. Selected tissue areas were microdissected by an UV laser, which performs circumferential dissection, following precisely a drawn incision path. The microdissected tissue areas were measured, documented, and collected on an adhesive cap of nanotubes for nucleic acid extraction. All microdissection experiments were performed in triplicate. The following cell types were separately microdissected: 1) smooth muscle cells from tunica muscularis of esophagus; 2) skeletal fibers of cricopharyngeal muscle; 3) smooth muscle cells from internal and external layers of muscularis propria; 4) myenteric ganglion cells from stomach, small intestine, and colon; and 5) smooth muscle and endothelial cells from the wall of small arteries and arterioles from submucosal layer of the GI tract and from liver, kidney, and pancreas parenchyma. Histological recognition of cell types for laser capture microdissection was based on strict morphological criteria. In selected cases, to confirm the detection of smooth muscle cells within fibrous tissue, slides were stained with Masson's trichrome (not shown). Between 50 and 100 cells were collected for each cell population and pooled for analyses. In addition, frozen sections of proximal esophagus stained with combined COX/SDH were subjected to laser capture microdissection to isolate single COX-positive and COX-negative skeletal muscle cells from cricopharyngeal muscle.

Molecular Analysis

Total DNA was extracted from whole tissue homogenates by phenol-chloroform standard procedures and from dissected samples with Picopure DNA extraction Kit (Arcturus, Los Altos, CA).

Amounts of mtDNA were measured in both homogenate and microdissected tissues by real-time quantitative

PCR (RT-PCR) assays using a previously described method.¹⁴ Briefly, a mtDNA fragment (nt 4625 to 4714) and a nuclear DNA fragment corresponding to FasL gene were co-amplified by multiplex polymerase chain reaction using TaqMan probe system and Platinum Quantitative PCR SuperMix-UDG (Invitrogen, Life Technologies, Parsippany, NJ). PCR conditions, primers, and probes were as previously detailed.¹⁴ With each assay, a standard curve for mtDNA and nDNA was generated using serial known dilutions of a vector (provided by Genomere, Modena, Italy) in which the regions used as template for the two amplifications were cloned tail to tail, to have a ratio of 1:1 of the reference molecules. The absolute mtDNA copy number per cell was obtained by the ratio of mtDNA to nDNA values multiplied by 2 (as two copies of the nuclear gene are present in a cell). PCR was performed in an "iCycler" Thermal cycler (BioRad, Hercules, CA) and at least three measurements were obtained for each sample.

Evaluation of mtDNA deletions (Δ mtDNA) on tissue homogenates was performed by Southern blot analysis.¹⁰ Several PCR reactions with shifted primers were performed to detect the mtDNA deletions on microdissected tissues, as described.¹⁰ To evaluate the deletion junctions of mtDNA molecules, a series of PCR experiments, with the following set of oligonucleotide primers, were performed as described¹⁰: forward primer, nt 8287 to 8306 and reverse primer, nt 13590 to 13571, for the Δ 5 kb fragment; forward primer, nt 6229 to 6249 and reverse primer, nt 14268 to 14249, for the Δ 7.7 Kb fragment; forward primer, nt 5651 to 5671 and reverse primer, nt 14268 to 14249 for the Δ 8.1 Kb fragment; and forward primer, nt 4370 to 4390 and reverse primer, nt 14268 to 14249 for the Δ 9.5 Kb fragment. PCR-amplified fragments were visualized by electrophoresis in a 2% agarose gel, extracted using the QIA quick gel extraction kit (Qiagen, Valencia, CA), and sequenced in an ABI Prism

Table 1. (Continued)

GI symptoms	Neuromuscular	CNS	Other
Borborygmi, diarrhea, abdominal pain, dysphagia, diverticulosis, acute intestinal pseudo-obstruction	PEO, demyelinating sensorimotor polyneuropathy and myopathy (EMG), COX-negative fibers at muscle biopsy	Epilepsy, sensorineural deafness, diffuse white matter hyperintensity (MRI)	Markedly cachectic BMI = 12.9
Borborygmi, diarrhea, abdominal pain, dysphagia	PEO, demyelinating sensorimotor polyneuropathy and signs of myopathy (EMG), COX-negative fibers at muscle biopsy	Sensorineural deafness, diffuse white matter hyperintensity (MRI)	Markedly cachectic BMI = 15.7
Borborygmi, diarrhea, abdominal cramps and pain, intestinal pseudo-obstruction, diverticulosis	PEO, demyelinating sensorimotor polyneuropathy and signs of myopathy (EMG), COX-negative fibers and RRF at muscle biopsy	Diffuse white matter hyperintensity (MRI)	Markedly cachectic BMI = 13.8
Borborygmi, abdominal pain, diarrhea, intestinal pseudo-obstruction, diverticulosis	PEO, demyelinating sensorimotor polyneuropathy, epilepsy, COX-negative fibers and RRF at muscle biopsy	Diffuse white matter hyperintensity (MRI)	Markedly cachectic BMI = 13.8
Borborygmi, early satiety, abdominal cramps and pain	PEO, demyelinating sensorimotor polyneuropathy, COX-negative fibers and RRF at muscle biopsy	Diffuse white matter hyperintensity (MRI)	Markedly cachectic BMI = 14.8

310 Genetic analyzer (Applied-Biosystem, Foster City, CA) following standard procedures. Since it was not possible to define the breakpoint of the $\Delta 8.1$ -Kb and of the $\Delta 9.5$ -Kb deletions by direct sequencing of the PCR product, PCR-amplified fragments were ligated into pGEM-T Easy Vector and subcloned using pGEM-T Easy Vector System (Promega, Madison, WI). Approximately 10 cloned plasmids of each PCR product were purified using Wizard *Plus* SV Minipreps DNA Purification Systems (Promega, Madison, WI) and then sequenced.

To screen for the presence of mtDNA point mutation in microdissected tissues, we amplified by PCR and sequenced three selected mtDNA regions corresponding to nt 5651 to 6022, nt 9917 to 10568, and nt 15756 to 16119. Within these mtDNA segments, point mutations have been identified in most MNGIE patients.¹⁵

Statistical Analysis

Statistical analysis was performed on the data with a mixed effect model. This model can explain the various values of mtDNA in different tissues taking account of patient heterogeneity.¹⁶ Numerical estimate have been obtained by the statistical software R Foundation for Statistical Computing, Vienna, Austria with the package nlme (www.r-project.org last accessed November 19, 2007).

Simple linear regression was performed with mtDNA copy number/cells means in different tissue components of GI in MNGIE patients and controls.

Results

Histological Findings

All five MNGIE patients showed similar morphological features in the GI wall. The most remarkable abnormalities were observed in the external longitudinal layer of muscularis propria of stomach and small intestine, which showed atrophy and vacuolization of smooth muscle cells, and interstitial fibrosis. These features were more prominent in the small intestine, which showed patchy areas with only a few residual muscle cells within the fibrous tissue (Figure 1, A and B). In contrast, the external layer of muscularis propria from the large bowel (not shown) and the internal layer of muscularis propria of the entire GI tract appeared normal. The myenteric and submucosal nervous plexi appeared well preserved and showed a normal distribution with immunostains for S-100, synaptophysin, neuronal-specific enolase, and glial fibrillar acidic protein (not shown). Immunostain with anti-mitochondria antibodies showed marked mitochondrial proliferation at the level of muscularis propria of GI tract in all patients, as compared with controls (Figure 1, C and D). Electron microscopy confirmed this finding (Figure 1E). The combined COX/SDH stain revealed clusters of COX-negative smooth muscle cells in the muscularis propria of small intestine and focally, COX deficient ganglion cells in the myenteric plexus in two patients (patients 3 and 4) (Figure 1, F and H). These abnormalities were absent in control tissues (Figure 1G). A marked

mitochondrial proliferation was observed also in smooth muscle and endothelial cells from the wall of small arteries and arterioles in the GI tract and other visceral organs (liver, kidney, heart, and pancreas, not shown) of MNGIE patients (Figure 2A), as compared with controls (Figure 2B). These vessels appeared COX-negative with combined COX/SDH stain (Figure 2C). Electron microscopy confirmed the presence of abundant mitochondria in endothelial cells from small vessels (Figure 2D). Tunica muscularis of proximal esophagus did not show morphological alterations (Figure 3A). However, combined COX/SDH stain revealed numerous COX-negative fibers with increased SDH intensity in the cricopharyngeal muscle (Figure 3B).

Molecular Analysis

mtDNA Deletions

Analyses of tissue homogenates from GI tract of MNGIE patients demonstrated mtDNA deletions only in the upper esophagus. The most abundant deletions were the same as those reported in skeletal muscle of MNGIE patients,¹⁰ and corresponded to the "common deletion," $\Delta 5.0$ kb, $\Delta 7.7$ kb, $\Delta 8.1$ kb, and $\Delta 9.5$ kb (Figure 3C). The deletion junctions were as described.^{10,13}

Analysis of microdissected smooth muscle cells from tunica muscularis and striated muscle cells from cricopharyngeal muscle revealed detectable levels of deleted mtDNA molecules only in striated muscle fibers (Figure 3D). Attempts to detect mtDNA deletions in single COX-positive and COX-negative cricopharyngeal fibers failed, due to the inability to amplify mtDNA from COX-negative fibers. The likely explanation for this phenomenon is the marked mtDNA depletion at this site, as described below. However, we confirmed the presence of mtDNA deletions in COX-positive fibers as reported¹⁷ (not shown).

Analyses of microdissected smooth muscle cells from muscularis propria and ganglion cells from myenteric plexi of stomach, small intestine, and colon, and from smooth muscle and endothelial cells of small arteries and arterioles did not show mtDNA deletions in MNGIE patients (not shown).

mtDNA Depletion in Tissue Homogenates

Analyses of tissue homogenates revealed, in all MNGIE patients, a marked mtDNA depletion confined to the small intestine (91% decreased compared to controls). A milder reduction in mtDNA amount was observed also in the stomach (43% decrease), whereas esophagus and colon did not show differences between patients and controls (Figure 4A). Noteworthy, even in the control group, we observed a non-homogeneous distribution of mtDNA within the different segments of GI tract; the relative amount of mtDNA in the small intestine was 55% of the level in esophagus, 49% of stomach, and 61% of colon (Figure 4A), confirming our previous report.¹³

Figure 1. Morphological features of the GI tract in MNGIE. **A:** The wall of small intestine shows marked atrophy and fibrosis of the external layer (EL) of muscularis propria. Few residual smooth muscle cells are evident within the fibrous tissue (**arrows**). The internal layer (IL) is unremarkable (patient 4, Masson trichrome stain, original magnification $\times 10$). **B:** Residual smooth muscle cells surrounded by fibrous tissue in the external layer (EL) of muscularis propria show extensive vacuolation and pyknotic nuclei. A morphologically normal myenteric plexus is marked by the **asterisk**. (patient 1, H&E stain, original magnification $\times 20$). **C:** Immunostain for mitochondrial antigens shows marked mitochondrial proliferation in smooth muscle cells of internal (IL) and external (EL) layers of muscularis propria from small intestine of MNGIE patient 2 (**C**) as compared with one control (**D**). Of note, the density of mitochondria in ganglion cells (**asterisk**) is high as compared with smooth muscle cells of the internal (IL) and external (EL) layer of tunica muscularis (anti-mitochondrial antigens antibody, clone MTC, UCS Diagnostic, original magnification $\times 10$). **E:** Ultrastructural features of smooth muscle cells in the small intestine of MNGIE patient 1. Smooth muscle cells are identified by the presence of "focal adhesions" (square boxes). The cell cytoplasm shows numerous mitochondria (**arrows**) with postmortem artifactual swelling. **F:** The combined COX/SDH stain shows numerous COX-negative smooth muscle cells in the internal layer (IL) of muscularis propria of small intestine. These cells appear blue since they are devoid of the COX activity (orange) but retain the SDH activity (blue). Two blue COX-negative ganglion cells are marked by the **asterisk** (patient 3, COX/SDH, original magnification $\times 10$). **G:** Combined COX/SDH stain in the small intestinal wall of a control subject. Note the absence of blue COX-negative muscle fibers. Ganglion cells stained with COX are marked by an **asterisk** (IL, internal layer; EL, external layer; COX/SDH, original magnification $\times 10$). **H:** The combined COX/SDH stain of the muscularis propria of small intestine shows patchy areas with COX-negative smooth muscle cells in blue marked by **arrows**. (patient 3, COX/SDH, original magnification $\times 20$).

Figure 2. Mitochondrial proliferation in smooth muscle and endothelial cells of small vessels in MNGIE. **A:** Immunostain for mitochondrial antigens shows mitochondrial proliferation in the tunica media of a small artery from a MNGIE patient (patient 1, anti-mitochondrial antigens antibody, clone MTC, UCS Diagnostic, original magnification $\times 10$). **B:** Immunostain for mitochondrial antigens in a small artery from a control subject (Anti-mitochondrial antigens antibody, clone MTC, UCS Diagnostic, original magnification $\times 10$). **C:** The combined COX/SDH stain shows a blue COX-negative vessel from the small bowel surrounded by brown COX-positive smooth muscle cells of muscularis propria (patient 3, COX/SDH, original magnification $\times 40$). **D:** Ultrastructural features of endothelial cells from a small vessel of MNGIE patient 2. Note the numerous mitochondria (arrows) with postmortem artifactual swelling. A red blood cell is marked by the asterisk.

mtDNA Depletion in Microdissected Tissues from Esophagus Wall

Microdissected tissues from the esophageal wall revealed marked mtDNA depletion limited to the skeletal muscle fibers from cricopharyngeal muscle. In fact, in MNGIE patients, the mtDNA/nuclear DNA ratio was less than normal controls, both in single COX-positive (decreased by 67% as compared to controls) and COX-negative fibers (decreased by 96% as compared to controls) (Figure 3E). In contrast, mtDNA amount in smooth

muscle cells from tunica muscularis was comparable in patients and controls (not shown).

mtDNA Depletion in Microdissected Tissues from GI Tract

A marked mtDNA depletion (93% decrease) was detected in the external layer of muscularis propria of small intestine in patients as compared with controls, whereas a milder reduction was observed in its internal layer (48%

Figure 3. Histopathology and molecular analysis of esophagus in MNGIE. **A:** The wall of proximal esophagus has a normal appearance. The arrow indicates smooth muscle cells of tunica muscularis and the asterisk marks the striated fibers of the cricopharyngeal muscle (patient 2, H&E, original magnification $\times 4$). **B:** The combined COX/SDH stain shows frequent COX-negative fibers in blue (patient 3, COX/SDH, original magnification $\times 40$). **C:** Southern blot analysis of DNA from tissue homogenates of patient 1 shows multiple deletions only in the esophagus and skeletal muscle. In addition to the signal from the 16.6-kb mtDNA molecule (WT), signals of lower molecular weight, corresponding to mtDNA deleted molecules (arrows), are evident. C, colon; S, stomach; I, ileum; E, esophagus; and M, skeletal muscle. M+ is a positive control with multiple deletion. M- is a control subject. U, uncut sample. **D:** The $\Delta 5$ -Kb, $\Delta 7.7$ -Kb, $\Delta 8.1$ -Kb, and $\Delta 9.5$ -Kb deletions were investigated by PCR assays using oligonucleotide primers flanking the regions upstream and downstream breakpoints in the parental molecule (see Materials and Methods). Representative 2% agarose gels from patient 3. The bands corresponding to the mtDNA deletions were detected in esophagus homogenate (E) and in the microdissected striated fibers from cricopharyngeal muscle (Sk). No deletions were detected in smooth muscle cells from tunica muscularis (Sm). (Negative control, C-; the arrow indicate 500 bp). **E:** MtDNA content was evaluated in single COX-positive (blue bar) and COX-negative (green bar) skeletal muscle fibers from cricopharyngeal muscle from MNGIE patients 3 and 4 versus three controls (red bar). In MNGIE patients, mtDNA/nuclear DNA ratio is less than normal controls, in both COX-positive and COX-negative fibers. Severely COX-deficient fibers have lower mtDNA amount than fibers with residual COX activity.

Figure 4. Assessment of mtDNA content in the different GI segments. **A:** Interaction plot of mtDNA copies/cells means (after transformation to the natural logarithm) $\pm$ 2 standard errors (SE) versus GI tissue segment homogenates for patients (red line) and controls (black lines). MNGIE patients shows a striking reduction in mtDNA content limited to the small intestine ($P < 0.001$ vs controls). A milder, although significant, decrease is observed in the stomach ($P < 0.01$ vs controls). In control subjects, the small intestine has a lower amount of mtDNA relative to esophagus, stomach, and colon. **B:** Interaction plot of mtDNA copies/cells means (after transformation to the natural logarithm) $\pm$ 2 SE versus microdissected tissues of stomach, small intestine and colon, for MNGIE patients (green line) and controls (red line). In MNGIE patients the external (EL) layers of muscularis propria from all segments of the GI wall show significant reductions in mtDNA content as compared to controls ($P < 0.001$). The lowest mtDNA content is observed in the EL of small intestine (93% decrease). A milder although significant decrease is observed in the internal layer (IL) from the entire GI tract and in the myenteric plexus (MP) of small intestine ($P < 0.001$). In control subjects, the EL of muscularis propria shows a lower amount of mtDNA as compared to the IL (internal/external layer ratio 2:1). **C:** Regression analysis between the mtDNA amount in different tissue components of GI wall, (ie, internal and external layer of muscularis propria and myenteric plexus from stomach, small intestine, and colon) from MNGIE patients and control subjects (black dots) ($r^2 = 0.89$; $P < 0.0001$). In contrast, microdissected small vessels (red dot) show marked mtDNA depletion in MNGIE patients, despite the high mtDNA levels in controls. S = stomach; I = small intestine; C = colon; 1 = myenteric plexus; 2 = internal, and 3 = external, layers of muscularis propria.

decrease) and in the ganglion cells of the myenteric plexus (79% decrease). Significant reductions of mtDNA were also observed in the external layer of muscularis propria of stomach (65% decrease) and colon (76% decrease), whereas their internal layer and the ganglion cells of the myenteric plexus showed only mild decreases of mtDNA (less than 45%) (Figure 4B). The control group also demonstrated a non-homogeneous distribution of mtDNA within different tissues of GI wall, with the external layer of muscularis propria showing the lowest amount (internal/external layer ratio 2:1) (Figure 4B). Intriguingly, regression analysis showed a significant linear relationship between the residual mtDNA level in different segments and tissue types of the GI tract of MNGIE patients and the mtDNA levels in the corresponding sections of control subjects (Figure 4C). A marked reduction of mtDNA (87% decrease) was observed also in microdissected vascular smooth muscle and endothelial cells from MNGIE patients (Figure 4C), despite the high mtDNA content in controls at this site.

mtDNA Point Mutations

Direct sequencing of the candidate regions screened for point mutation detection, based on the previously reported analysis,¹⁵ identified similar heteroplasmic nucle-

otide changes in all microdissected tissues from the entire GI tract. None of the mutations studied showed segregation to high mutant loads in any of the tissues analyzed (data not shown).

Discussion

To determine the pathogenic mechanism of CIPO in MNGIE, we performed a systematic correlation of morphological and molecular features of the entire GI tract, in autopsy samples of patients and controls. We demonstrated, in five MNGIE patients, that visceral mitochondrial myopathy is likely to cause GI dysmotility. This is due to mtDNA depletion and is characterized by patchy reactivity of smooth muscle cells to cytochrome c oxidase. As usually occurs in mitochondrial disorders, the mtDNA depletion is associated with marked mitochondrial proliferation, a well known compensatory strategy for the reduced respiratory chain function.¹⁸ The mtDNA depletion involves the muscularis propria of the entire GI tract and is most dramatically prominent in its external layer. In stomach and small intestine, the profound mtDNA depletion at this site correlates well with the observed atrophy and interstitial fibrosis. In addition, only in the small intestine, we reported significant mtDNA deple-

tion (79% reduction of mtDNA in patients as compared with controls) with evidence of COX-deficient ganglion cells in the myenteric plexus. Notably, we observed a linear relationship between the residual mtDNA content in different segments and tissues of the GI tract in MNGIE patients and the mtDNA amount in the corresponding sections in normal controls. Thus, the baseline low abundance of mtDNA molecules may predispose smooth muscle cells of the external layer of muscularis propria to the toxic effects of circulating dThd and dUrd, with resultant mtDNA depletion. Based on our findings, the physiological abundance of mtDNA copies in different tissues may be one of the factors accounting for the selective threshold effect and the phenotypic expression of mtDNA abnormalities.

A novel interesting observation of our study is the ubiquitous finding of mtDNA depletion, COX deficiency, and marked mitochondrial proliferation, within small vessels in all MNGIE patients. In contrast to the external layer of muscularis propria, vascular endothelial and smooth muscle cells of vessels wall normally contain high numbers of mtDNAs to support their high energy requirements.¹⁹ A possible explanation for our findings is that small vessels, because of their anatomical position at the blood-tissue interface, play a key role in regulation of extracellular and vascular nucleoside levels. In fact, they possess high-affinity nucleoside transporters²⁰ and have a high capacity for nucleoside accumulation.²¹ Thus, we hypothesize that in MNGIE, deoxynucleotides accumulate in small vessels wall, where they exert their toxic effect. The observation of a marked mtDNA depletion in small vessel wall supports the hypothesis that the edematous features shown at cerebral magnetic resonance imaging in MNGIE patients could be due to breakdown of the blood-brain barrier.²²

Regarding the other types of mtDNA abnormalities described in MNGIE (ie, mtDNA deletion and point mutations), our results confirm the finding of very low levels of site-specific mtDNA point mutations in the different tissues of the GI wall. In addition, we show that mtDNA deletions are limited to the skeletal muscle component of the upper esophagus, in combination with partial mtDNA depletion, which is virtually complete in the COX-negative fibers as demonstrated in our single-fiber analysis. The morphological and molecular features of cricopharyngeal muscle account for the oropharyngeal dysfunction (dysphagia) observed in MNGIE patients. The selective localization of mtDNA deletions confirms our previous findings in a single MNGIE patient¹³ and strengthens the hypothesis that deleted mtDNA molecules accumulate over the years only in postmitotic tissues such as skeletal muscle.¹⁰ In contrast, the load of deleted mtDNA molecules may be reduced in replicating cells because of negative selection.²³

In summary, our study demonstrates that severe depletion of mtDNA is the most striking molecular defect in smooth muscle of the GI tract and vascular wall of MNGIE patients. The depletion of mtDNA correlates with histopathological abnormalities and is likely due to toxic levels of dThd and dUrd disrupting mitochondrial nucle-

otide pool, as confirmed by *in vitro* studies.^{12,23} The mechanism for this effect remains unknown.

The constitutive low abundance of mtDNA within smooth muscle cells of the external layer of muscularis propria may account for the marked mtDNA depletion at this site. Exposure of vascular smooth muscle to the high circulating levels of nucleosides may contribute to the selective vulnerability of these cells. Because mtDNA depletion is more apt to correction than point mutations or deletions, the severe CIPO in MNGIE may be amenable to therapies that reduce circulating dThd and dUrd as recently reported.²⁴

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