



## Short Communication

## Poly (A) tail length of human mitochondrial mRNAs is tissue-specific and a mutation in LRPPRC results in transcript-specific patterns of deadenylation

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## ABSTRACT

Mutations in LRPPRC cause Leigh Syndrome French Canadian (LSFC), an early onset neurodegenerative disease, with differential tissue involvement. The molecular basis for tissue specificity in this disease remains unknown. LRPPRC, an RNA binding protein, forms a stable complex with SLIRP, which binds to, and stabilizes mitochondrial mRNAs. In cell culture and animal models, loss of LRPPRC function results in transcript-specific alterations in the steady-state levels of mitochondrial mRNAs and poly (A) tail length, the mechanisms for which are not understood. The poly (A) tail length of mitochondrial mRNAs has not been investigated in human tissues from healthy subjects or LSFC patients. Here we have mapped the 3'-termini of mature mitochondrial mRNAs in three tissues (skeletal muscle, heart, and liver) from a healthy individual and an LSFC patient. We show that the poly (A) tail length of mitochondrial mRNAs varies amongst tissues, and that the missense mutation in LRPPRC that causes LSFC results in tissue- and transcript-specific deadenylation of a subset of mitochondrial mRNAs, likely contributing the nature and severity of the biochemical phenotype in different tissues. We also found a relatively large fraction of short transcripts lacking a stop codon, some with short poly (A) tails, in patient tissue, suggesting that mutations in LRPPRC may also impair proper 3' end processing of some mRNAs.

## 1. Introduction

Leigh Syndrome French Canadian (LSFC) is an autosomal recessive, neurodegenerative disorder with onset in infancy that is characterized by delayed psychomotor development, lesions in the brain stem and basal ganglia, and microvesicular steatosis in the liver [13]. The gene responsible for LSFC was identified in 2003 as *LRPPRC* [12], an RNA binding protein containing multiple pentatricopeptide (PPR) motifs. Proteins in the PPR family bind specific RNA targets in a modular fashion, and are involved in many different aspects of organellar RNA metabolism [19]. LSFC is a founder effect disease in the Saguenay-Lac-Saint-Jean region of Quebec [13], where the carrier frequency of the founder missense mutation is ~1/23. Affected individuals are frequently diagnosed during fatal metabolic crises, and mortality occurs as a result of acidosis, coma, and multi-organ failure [11,13].

LRPPRC forms a stable complex with SLIRP [16], a mitochondrial protein that harbors an RRM domain (RNA Recognition Motif), but which has limited ability to bind RNA on its own [20]. Rather, SLIRP functions to stabilize LRPPRC at a protein-protein interface. The LRPPRC/SLIRP complex acts as an RNA chaperone to stabilize

mitochondrial mRNAs, promoting polyadenylation and translation, and suppressing mRNA degradation [18], and recent structural studies show that it likely functions in delivery of mRNA to the ribosome [1]. In the eukaryotic cytosol polyadenylation stabilizes mRNA transcripts, and in bacteria, archaea, chloroplasts, and plant mitochondria this post-transcriptional modification is a signal for mRNA degradation [8,9,21]. In mammalian mitochondria, the precise role of polyadenylation in mitochondrial gene expression remains unclear.

Human mitochondrial mRNA poly (A) tail length is regulated by a mitochondrial poly (A) polymerase (MTPAP), which mediates mRNA polyadenylation, and by the polynucleotide phosphorylase (PNPase), which mediates mRNA degradation [5,6]. The polyadenylation profile of mitochondrial mRNAs following siRNA-mediated suppression of LRPPRC and SLIRP in HeLa cells showed a specific reduction in the poly (A) tail length of several mRNAs including COX1, COX3, ND2, ND3, ND5, and CYTB [6]; however, other studies of LRPPRC patient fibroblasts failed to identify significant changes in poly (A) tail length in the mRNAs analyzed (RNA14, COXI or ND3) [14]. Investigation of heart-specific LRPPRC knock-out mice showed a marked reduction in the length of the poly (A) tail in all mitochondrial mRNAs except ND6,

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which does not contain a terminal poly (A) tail [15]. We previously showed that the poly (A) tail length of a subset of mitochondrial mRNAs was decreased in the liver-specific LRPPRC knock-out mice; however, there was no clear correlation between the decrease in steady-state mRNA levels and poly (A) tail length [7]. It is not known how or if LRPPRC regulates the tail length of mt-mRNA poly (A), as no data have suggested an interaction of MTPAP with LRPPRC or SLIRP. Thus, the role of LRPPRC in polyadenylation may be linked to RNA chaperone activity of this protein that makes the 3' end sites of mitochondrial mRNAs recognizable by RNA binding proteins and MTPAP.

Although it is clear that mutations in *LRPPRC* or modulation of the level of LRPPRC protein are associated with alterations in poly (A) tail length and stability of mitochondrial mRNAs, different results have been obtained with different cell lines and tissues, and to date there is no animal model carrying a germline missense mutation as a model of the human disease. Marked tissue-specific differences in the nature and severity of biochemical defects in oxidative phosphorylation (OXPHOS) exist in LSFC patients [17]: complex I and IV defect in skeletal muscle, severe complex IV defect in liver, and a mild complex IV defect in heart, so we set out to investigate whether tissue-specific differences exist in poly (A) addition in the same tissues from an LSFC patient.

## 2. Methods

### 2.1. Subjects

Human tissues used were obtained from an LSFC patient homozygous for the common missense mutation c.1061C > T (p. A354V) in *LRPPRC* with typical clinical features of the syndrome, and from a control subject. The study was approved by the human ethics review panel of the Montreal Neurological Institute.

### 2.2. Total RNA isolation

Total RNA was isolated using the TRIzol reagent (Invitrogen). Approximately 100 mg of human tissue (liver, heart, skeletal muscle) was homogenized in 1 ml of TRIzol reagent and incubated at room temperature for 5 min. Then, 200  $\mu$ l of chloroform was added and the mixture was shaken vigorously for 15 s and incubated at room temperature for 2–3 min. The sample was centrifuged 15 min at 12,000g at 4 °C. The aqueous phase, the colorless upper phase that corresponds to 60% of the volume of TRIzol, was transferred to a fresh tube. The RNA was precipitated by mixing the aqueous phase with 500  $\mu$ l of isopropanol, incubated at room temperature for 10 min, and centrifuged for 10 min at 12,000g at 4 °C. The RNA pellet, which was visible on the side of the tube, was washed with 1 ml 75% ethanol, centrifuged at 7500g for 5 min at 4 °C, air dried, and solubilized in RNase free water.

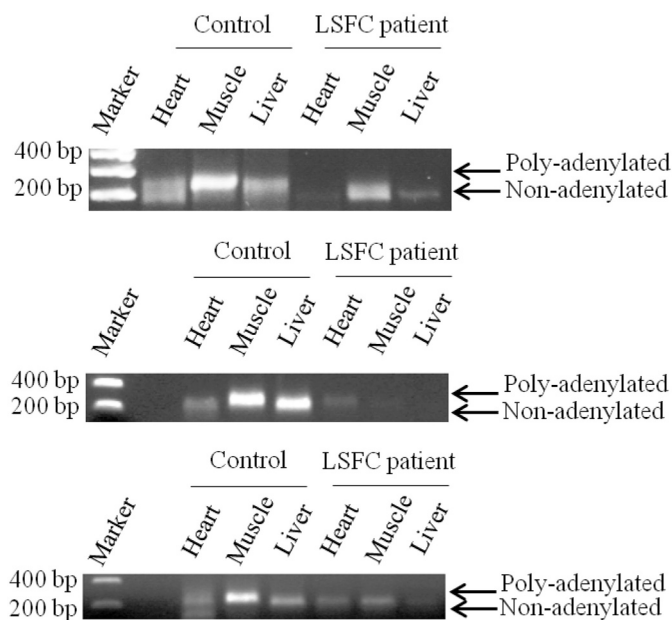
### 2.3. mRNA poly (A) tail length (MPAT) assay

This assay was based on the method described by Temperley *et al* [21]. A universal linker DNA oligonucleotide 5'-phospho-ATG TGA GAT CAT GCA CAG TCA TA-3'-NH<sub>2</sub> was ligated to the 3' termini of total RNA (2.5  $\mu$ g) by T4 RNA ligase (New England Biolabs) at 37 °C for 3 h. The ligated RNA was extracted with phenol/chloroform extract and precipitated with ammonium acetate/ethanol. Briefly, 1 ml of acidic phenol/chloroform was mixed with 40  $\mu$ l of chloroform/isoamylalcohol, 200  $\mu$ l of this mixture was added to the ligated RNA, vortexed for 1 min, and centrifuged for 2 min at room temperature at 12,000g. The aqueous phase was transferred to a fresh tube containing 200  $\mu$ l of chloroform/isoamylalcohol, vortexed for 1 min, and centrifuged for 2 min at room temperature at 12,000g. The aqueous phase was then mixed with 100  $\mu$ l of 10 M ammonium acetate and 500  $\mu$ l of 100% cold ethanol, vortexed and stored at –80 °C overnight. The sample was centrifuged for 10 min at 16,000g at 4 °C. After removing the supernatant, the pellet was washed with 70% cold ethanol and centrifuged for 10 min at 16,000g at

**Table 1**

List of oligonucleotides complementary to human mitochondrial genes used in the MPAT assay.

Primer	Sequence
Linker	5' TAT GAC TGT GCA TGA TCT CAC AT 3'
Upper COX1	5' AGA ACC CTC CAT AAA CCT GGA 3'
Lower COX1	5' CAC ATT CGA AGA ACC CGT AT 3'
COX2	5' ACG ACC GGG GGT ATA CTA CG 3'
COX3	5' AAC ATC ACT TTG GCT TCG 3'
ND1	5' GAA TTC GAA CAG CAT ACC 3'
ND2	5' ACC TCA ATC ACA CTA CTC 3'
ND3	5' AAT TGC CCT CCT TTT ACC C 3'
ND4	5' CTC CCT CTA CAT ATT TAC CAC 3'
ND5	5' CAT CAT ACT CTT TCA CCC AC 3'
ND6	5' AGT TGG AAT AGG TTG TTA GC 3'
CYTB	5' ATC ATT GGA CAA GTA GCA TC 3'
ATP6	5' CCC TCT ACA CTT ATC ATC TTC 3'



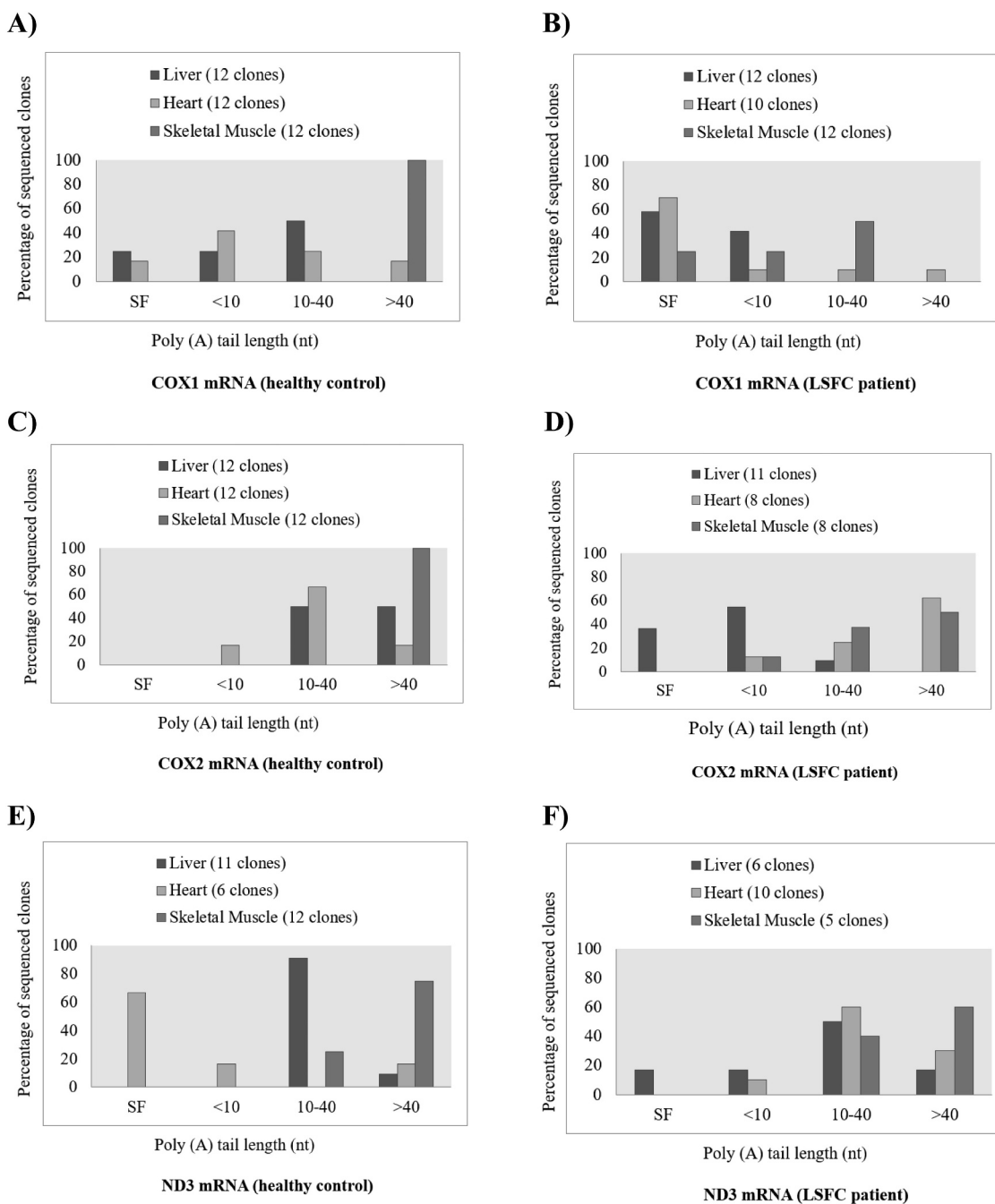
**Fig. 1.** LRPPRC deficiency alters the polyadenylation profile of a subset of mitochondrial mRNAs. Upper, middle, and lower panels show the amplicon from the MPAT assay for COX1, COX2, and ND3 mRNAs in liver, heart, and skeletal muscle from a healthy control and an LSFC patient, respectively. The arrowhead indicates non-adenylated and poly-adenylated mRNAs.

4 °C. The ligated RNA pellet was air dried, solubilized in RNase free water, and amplified using one-step RT-PCR kit (QIAGEN) with a primer complimentary to the linker. A first round of PCR (35 cycles) was applied using a gene-specific upper primer and the linker primer, followed by a second round of 10-cycle PCR using a gene-specific lower primer and the linker primer. Half of the reaction product was resolved by 10% polyacrylamide gel electrophoresis in TBE buffer, dyed with SYBR green (Invitrogen) and visualized by on a Molecular Dynamics PhosphorImager. The remaining half was cloned in the TOPO TA cloning vector (Invitrogen) and 183 clones (see Fig. 2) were subjected to Sanger sequencing. DNA oligonucleotides are listed in Table 1.

## 3. Results

### 3.1. Poly (A) tail length of mt-mRNAs is shorter in LSFC tissues

Analysis of liver tissue in mice with a liver-specific knockout of *Lrpprc*, showed that the steady-state levels of several mitochondrial mRNAs including COX1 and COX2 were reduced while the levels of others, such as ND3, remained unchanged [7]. We therefore selected



**Fig. 2.** The poly (A) tail length of COX1, COX2, ND3 mRNAs is tissue-specific and is reduced in an LSFC patient. Panels A and B show the relative proportion of poly (>40), oligo-(10–40), and non-adenylated (<10) COX1 mRNAs in tissues of a healthy control (A) and an LSFC patient (B). Panels C and D show the relative proportion of poly- (>40), oligo-(10–40), and non-adenylated (<10) COX2 mRNAs in tissues of a healthy control (C) and an LSFC patient (D). Panels E and F show the relative proportion of poly- (>40), oligo-(10–40), and non-adenylated (<10) ND3 mRNAs in tissues of a healthy control (E) and an LSFC patient (F). Short fragments (SF) refer to transcripts that terminate before the stop codon.

these three transcripts for further investigation in human tissues. Using the MPAT assay, we measured the polyadenylation profiles in liver, heart, and skeletal muscle from an LSFC patient homozygous for the common missense mutation c.1061C > T (p. A354V), and a control subject.

Visualization of the amplicons by SYBR green for the three transcripts showed a rather diffuse band from heart tissue compared to skeletal muscle and liver, and clear differences in size amongst the tissues. In addition, the amplicons were fainter in the tissues from the LSFC patient (Fig. 1), reflecting lower levels of these mRNAs in patient tissues. There were marked differences in the length of the poly (A) tails

amongst tissues that were transcript-specific. For instance, the poly (A) tail on the COX1 mRNA in skeletal muscle was much longer (>40A's) than in heart or liver, and even in the patient skeletal muscle about half of the COX1 transcripts were oligoadenylated (Fig. 2). Strikingly, there was a large proportion of short fragments, lacking a stop codon and the majority with a short and variable poly (A) chain length in patient tissues, especially liver and heart.

The pattern for COX2 mRNA was slightly different in that the poly (A) tails were on average longer in heart and liver than those for COX1, but still shorter than in skeletal muscle, and short fragments were only found in liver tissue in the patient (Fig. 2). These data suggest that the

**Table 2**

Features of the 3' ends of human liver mitochondrial mRNAs. All mRNAs except ND6 are polyadenylated. Only COX1, COX2, ND5, and ND6, have 3' UTRs.

mRNA	3' Trailer	Poly A tail length
COX1	63–71	35–62
COX2	25	30–68
COX3	0	33–45
ATP8/ATP6	0	39–53
CYTB	0	34–43
ND1	0	36
ND2	0	33
ND3	0	27–66
ND4L/ND4	0	37–49
ND5	300–362	40
ND6	630	0

large fraction of short fragments seen for COX1 is not an artifact due to degradation of the mRNA. The pattern for ND3 mRNA was different again: about 75% of the transcripts were polyadenylated (>40 A's) in skeletal muscle, 80% were oligoadenylated (10–40 A's) in liver, and, unexpectedly the majority of transcripts in the heart was either non-adenylated or appeared as short fragments. In contrast to the COX transcripts, the majority of ND3 transcripts remained oligo- or polyadenylated in all tissues examined (Fig. 2).

### 3.2. The 3' ends of liver mitochondrial mRNAs

The 3' ends of mitochondrial mRNAs have been previously determined in human Hep G2 cells [21], but to our knowledge not in human tissues, and the 3' end of ND6 mRNA remained controversial. We investigated the 3'-ends of mitochondrial mRNAs isolated from liver, and although our results generally reproduced what was reported in HepG2 cells, we observed some significant differences in the 3' UTRs of ND5, COX1 and we were able to define the 3' UTR of ND6 mRNA, which is not polyadenylated, as 630 nucleotides composed of ND5 sequences (Table 2). We also observed a range of poly (A) tail lengths for most of the mRNAs between ~30–70 A's.

## 4. Discussion

In this study we have characterized the poly (A) status of mitochondrial mRNAs in tissues from LSFC patients and controls. We demonstrate tissue- and transcript-specific differences in poly (A) tail length in control tissues, and that in general, the length of the poly (A) tail is reduced in LSFC tissues in agreement with previous studies on cell lines and animal models [6,15,17]. Despite the general reduction in polyadenylation, a significant proportion of transcripts remain oligo- or polyadenylated in the patient tissues, the only apparent exception being the COX2 transcript in patient liver tissue. We also demonstrate that a substantial proportion of transcripts have aberrant ends, with no stop codon and the majority with a short poly (A) tail, even in control tissues. Exactly what these transcripts represent, or how they arise, remains unclear, but they could be errors of processing the primary polycistronic transcript, and they are almost certainly are non-functional.

Mitochondrial gene expression is primarily regulated post-transcriptionally. The primary polycistronic RNAs transcribed from the mitochondrial genome are processed and matured in mitochondrial RNA granules (MRGs), non-membrane delimited structures that are juxtaposed to the mitochondrial nucleoid [2,3,10]. Transcripts punctuated by tRNAs are released by the activity of the processing enzymes RNase P and Z that cleave tRNAs at the 5' and 3' ends, respectively. Non-canonical mRNAs, not flanked by tRNAs, which include ATP8/6, COX1, COX3, CYTB, and ND5 are required FASTKD5 for maturation [3], and perhaps other proteins of the FASTKD family [4]. MRGs also contain the enzymes that regulate poly (A) tail length: MTPAP [22] and the SUV3-PNPase complex that comprises the RNA degradosome [5].

LRPPRC does not specifically localize to MRGs (but neither is it excluded), it seems likely that the LRPPRC/SLIRP complex is involved in stabilizing mature mitochondrial transcripts while, and after, they are processed in MRGs by acting as an RNA molecular chaperone [18]. Such a chaperone function might be essential for promoting mRNA polyadenylation, regulating poly (A) tail length, inhibiting the degradation of mRNA by SUV3-PNPase complex machinery, and presenting the 5'-UTR to the ribosome for translation. What is perplexing is the fact that alterations in poly (A) tail length caused by loss of LRPPRC or MTPAP function [22] have such variable effects on the stability of specific mRNAs and their translation in cell culture models. In contrast, in tissues of LSFC patients, the steady-state levels of all mRNAs are reduced, but to varying degrees depending on the tissue [17]. These reductions in mRNAs correlate with the alterations we report here in poly (A) tail length, but the biochemical consequences at the level of assembly of the OXPHOS complexes, which are quite distinct in the three tissues we examined, are not entirely explained by the post-transcriptional alterations in mRNAs [17]. It is however clear that all three tissues have a reduction in complex IV assembly, and all exhibit shortened polyA tails in COX I and COX II mRNAs. This is most pronounced in the liver, where there are no adenylated COX I mRNAs, and almost no detectable fully assembled Complex IV [17]. On the other hand Complex I is only reduced in patient skeletal muscle, though the level of ND3 mRNA and its polyA Tail are similarly affected in all tissues. Although we have not been able to directly assess mitochondrial translation in the tissues, it seem clear that translation of oligoadenylated mRNAs is sufficient to support assembly of some OXPHOS complexes. This problem requires future study, which would be greatly facilitated by the development of an animal model faithfully modeling the human disease.

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