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Short Title: Lack of NSD in a nonstop mRNA mutation.

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ABSTRACT: The cellular quality control systems enable surveillance and selective degradation of nonsense, nonstop, and no-go mRNAs. In the case of nonstop mRNA, different mechanisms of nonstop-mediated decay (NSD) have been described for bacteria, yeast and mammals, but the molecular consequences of nonstop mutations have been examined in only few cases of human disease. We describe a novel homozygous nonstop mRNA mutation (c.1416delC) in the *TYMP* gene encoding thymidine phosphorylase, in a patient with mitochondrial neurogastrointestinal encephalomyopathy (MNGIE). In contrast to previous reports showing selective decay of pathogenic nonstop mRNAs, quantitative real-time PCR and 3'-RACE-RFLP analysis revealed unreduced nonstop mRNA levels in our patient and 2 heterozygous carriers of the mutation. The absence of thymidine phosphorylase protein in the homozygous patient, together with the partial decrease in levels of this protein in 2 carriers suggest that the main control system in this case resides at the translational or post-translational levels rather than through NSD. This is the first report showing an absence of NSD in a human disease, revealing that this surveillance mechanism has exceptions in vivo. ©2010 Wiley-Liss, Inc.

KEY WORDS: TYMP, Nonstop mutation, nonstop mediated decay, NSD, MNGIE, neuropathy

INTRODUCTION

Cells have developed surveillance systems to avert deleterious effects of premature stop, nonstop, or no-go mRNAs (Isken and Maquat, 2007). Several studies have described a translation-dependent surveillance step that detects and promotes degradation of mutant mRNA, which otherwise would result in formation of aberrant proteins. In mutations leading to mRNAs that lack in-frame stop codons (nonstop mRNAs), different mechanisms have been described for bacteria, yeast, and mammals (Akimitsu, 2008). In eubacteria and yeast, stalled ribosomes at the 3'end of nonstop mRNA trigger degradation of the messenger, an action mediated by several factors (nonstop-mediated decay, NSD). In mammalian cells, translation repression, sparing the nonstop mRNA, has been observed (Akimitsu, et al., 2007). However, studies of nonstop mRNAs in human disease have reported considerable reductions in steady state nonstop mRNA levels in all cases (Ameri, et al., 2007; Chatr-Aryamontri, et al., 2004; Lualdi, et al., 2006), indicating that NSD has occurred.

Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is an autosomal recessive disorder caused by mutations in the nuclear gene TYMP, encoding thymidine phosphorylase (TP) (Nishino, et al., 1999). This enzyme catalyzes a key step in degradation of thymidine (dThd) and deoxyuridine (dUrd), which reach micromolar concentrations in MNGIE patients, and are undetectable in controls (Marti, et al., 2004; Marti, et al., 2005). The main clinical features of MNGIE are gastrointestinal dysmotility, cachexia, peripheral neuropathy, progressive external ophthalmoplegia, diffuse leukoencephalopathy, and mitochondrial dysfunction (Hirano, et al., 2004). However, cases of mild or incomplete clinical symptoms have been reported (Bedlack, et al., 2004; Gamez, et al., 2002; Martin, et al., 2004; Needham, et al., 2007; Szigeti, et al., 2004).

To date, around 60 different TYMP mutations have been associated with MNGIE (Giordano, et al., 2008; Massa, et al., 2009; Poulton, et al., 2009). More than 50% are missense mutations, followed by splice site mutations (~20%), and frameshifts generating nonstop mRNA (~10%). Nonsense mutations or frameshifts generating premature stop codons (~10%), and mutations causing amino acid duplications or deletions (~4%) are less common. The consequences of TYMP mutations on transcripts have been experimentally characterized for some splice mutations (Nishino, et al., 1999; Szigeti, et al., 2004; Taanman, et al., 2009), but such studies have never been performed on nonstop mRNA mutations in MNGIE. We report the case of a MNGIE patient with marked peripheral neuropathy, harboring a novel nonstop mRNA mutation. Unlike the reported features of other nonstop mutations in human disease, we found that the nonstop mRNA is stable and coexists with wild-type mRNA at similar levels in carriers.

MATERIALS AND METHODS

Subjects

Informed consent for participation was obtained from all subjects included. A 16-year-old female was visited in the emergency department for weakness that had dramatically exacerbated in the previous 2 weeks, with hypoesthesia and hyperalgesia in hands and feet. Her parents were second cousins, and her mother had psoriatic arthritis and mild bilateral ptosis; her sister was asymptomatic. The pregnancy, neonatal period, and early neurological development were normal. She had a history of vomiting, flatulence, and abdominal pain after meals since the age of 7 years. She had been diagnosed of aorto-mesenteric compression syndrome that was surgically treated, with no improvement. She began to lose weight and was admitted to another hospital for suspected anorexia nervosa. The patient gradually developed generalized weakness, with difficulty standing up from a low chair and sitting up in bed, but she had an almost normal lifestyle, excluding sports. Her weakness had worsened before her first visit to our hospital, affecting predominantly the right arm and leg, and progressed to tetraparesis of distal predominance in a few weeks. She was able to walk only a few steps without help and became dependent for some everyday tasks with her hands.

Physical examination showed cachexia (35 kg), short stature (154 cm), generalized muscle atrophy, and high-arched feet. Neurological study revealed normal cognitive function, upgaze ophthalmoparesis, asymmetric generalized weakness with distal predominance, and absence of deep tendon reflexes. There was distal sensory loss for all modalities and hyperalgesia in the distal legs. She had a steppage gait and was unable to stand up from the floor without help. Fundoscopy findings were normal.

Laboratory exams showed high plasma lactate concentrations in 2 determinations (4.2 and 2.6 mmol/L) and CSF protein of 3.29 g/L (normal 0.2-0.5) with albumino-cytologic dissociation. Neurophysiologic studies demonstrated demyelinating, axonal polyneuropathy. These later findings prompted treatment with intravenous immunoglobulin administration, with no improvement. Cranial MRI showed diffuse leukoencephalopathy, sparing the U fibers and corpus callosum. Abdominal ultrasound examination and echocardiography showed no significant abnormalities. Gastrointestinal evaluation showed hypomotility in the distal esophagus, with normal lower esophageal sphincter. Gastric emptying scintigraphy study showed mild gastroparesis with 26% retention of radiolabeled meal at completion of the study (normal <10%). The small bowel was diffusely dilated and intestinal manometry showed neuropathic dysmotility with bacterial overgrowth. TP dysfunction was then assessed on the basis of suspected MNGIE.

Biochemical and Molecular Studies

Anticoagulated blood was collected from the proband, her parents, and her sister, and from 2 healthy controls, age- and sex-matched to the father (C1) and mother (C2). Plasma dThd and dUrd concentrations and TP activity in buffy coat were measured as reported (Marti, et al., 2004), with minor changes. Buffy coat DNA was isolated and TYMP coding regions and intronic boundaries were sequenced (Nishino, et al., 1999). Total RNA was extracted from buffy coat (Trizol, Invitrogen), and total random-primed cDNA (High-capacity cDNA reverse transcription kit, Applied Biosystems) and poly(A) RNA-derived cDNA (by 3'-RACE with the FirstChoice RLM-RACE kit, Applied Biosystems) were obtained. One random primed cDNA and 2 different 3'-RACE cDNAs from the same RNA extracts were used for TYMP mRNA real-time PCR quantification (Hs00157317_m1, Applied Biosystems). Results were normalized to beta-glucuronidase (GUSB) mRNA levels (human GUSB endogenous control probe 4333767F, Applied Biosystems). As a representative control of low TYMP expression, poly(A) RNA-derived cDNA, obtained from a control skeletal muscle biopsy following the above detailed procedure was included in the real-time PCR analysis, because skeletal muscle is konwn to express TYMP very poorly (Yoshimura, et al., 1990).

For TYMP mRNA sequencing and PCR-RFLP analysis, 3'-RACE cDNA was PCR-amplified with specific forward TYMP primer (cDNA-TYMP-F1, AGGCCCGCCAGACTTAAGGG), and reverse primer annealing the 3'-RACE adapter sequence (GCGAGCACAGAATTAATACGACT), followed by semi-nested PCR (forward, cDNA-TYMP-F1; reverse cDNA-TYMP-R1, CGCGGCAAAGGAGCTTTATT). The PCR product was sequenced using internal forward primer (cDNA-TYMP-F2, AGCAGGAGGAGCTGCTGGCG), and BssHII-digested for RFLP analysis. The digestion products were electrophoresed and stained with ethidium bromide, and bands were quantified (Quantity One, Bio-Rad). The wild-type 574-bp amplicon has a unique BssHII target resulting in 2 bands (502-bp+72-bp). This target disappears with the c.1393G>A change, cis-linked to the c.1416delC mutation.

Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence NM_001113755.1, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

Western-blot

Four µg of buffy coat protein extract, obtained as described (Marti, et al., 2004), were electrophoresed in 10% SDS-PAGE, transferred to PVDF membrane, and incubated with anti-TP (Calbiochem) and anti-β-actin (Sigma-Aldrich) mouse monoclonal antibodies, and then with peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). Bands were visualized (LumiGlo chemiluminiscent kit, Cell Signaling Technology) and quantified using Quantity One software. All TP-to-β-actin ratios were referred to the corresponding ratio obtained for the C2 sample.

Statistics

The association between the number of mutated alleles and TP activity, TP protein levels, percentage of mutated RNA and mRNA levels was tested with the nonparametric Spearman correlation test. For statistical purposes, undetectable values were considered as zero.

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RESULTS

Biochemical and Genetic Studies

The patient's TP activity was severely reduced, and plasma dThd and dUrd concentrations were increased, confirming the diagnosis of MNGIE (Table 1). TYMP gene sequencing revealed a homozygous single-nucleotide deletion in the last coding segment of exon 10 (c.1416delC; Ref Seq NM_001113755.1), predicting a frameshift that leads to a nonstop mRNA (p.F473SfsX41). Both parents and the sister were heterozygous mutation carriers. Analysis of the sequences revealed that the c.1416delC change was cis-linked to two previously reported non-pathogenic polymorphisms: c.1393G>A (Martin, et al., 2004), located in exon 10, 23 bases upstream of c.1416delC and absent in both parents' alleles lacking the deletion, and c.972C>T (rs131804), in exon 8, present in both alleles from the father but only in the c.1416delC-mutated allele of the mother (Figure 1). Buffy coat TP activity in the parents and sister was moderately reduced (62%-76% of control values). Plasma dThd and dUrd were undetectable in all carriers (Table 1).

TYMP mRNA and TP Protein Levels

Several studies have reported decay of nonstop mRNA species in other human diseases caused by nonstop mRNA mutations (Ameri, et al., 2007; Chatr-Aryamontri, et al., 2004; Lualdi, et al., 2006; Temperley, et al., 2003); hence, we investigated whether the c.1416delC mutation results in NSD. Quantitative real-time PCR was used to assess mRNA levels in buffy coat of the patient, parents, and 2 healthy controls. As is shown in Table 1, levels of TYMP poly(A) mRNA (analyzed from the 3'-RACE cDNA product) were similar in the patient (homozygous for the mutation), both parents, and both controls. Nor were differences detected when random-primed cDNA instead of 3'-RACE cDNA was used as template. A healthy control's skeletal muscle, tissue known to express TYMP poorly (Yoshimura, et al., 1990), expectedly showed very low poly(A) TYMP mRNA levels (around 4% - 8 % of those observed in buffy coat from controls, mutation carriers and patient).

Specific quantification of mutated and wild-type species by 3'-RACE-nested PCR-RFLP analysis of the c.1393G>A polymorphism (cis-linked to the mutated allele) revealed that more than 50% of the carriers' TYMP mRNA was mutant species (Figure 1, Table 1). This finding was consistent with the cDNA sequencing results, which showed coexistence of mutant and wild-type TYMP mRNA in both parents (Figure 1). Despite the similar levels of TYMP mRNA in the patient, parents, and controls, TP protein levels were undetectable in the patient and partially reduced in the carriers as compared to controls (Figure 1, Table 1), in accordance with the TP activity results.

DISCUSSION

The number of TYMP mutations associated with MNGIE has continuously increased since the genetic cause of this disorder was discovered (Nishino, et al., 1999; Poulton, et al., 2009). Although the clinical picture of MNGIE has been well defined (Hirano, et al., 2004), cases with missing or reduced symptoms are not rare, including presentations with predominant neuropathy and reduced or subclinical gastrointestinal involvement (Bedlack, et al., 2004; Gamez, et al., 2002; Hirano, et al., 2004; Martin, et al., 2004; Needham, et al., 2007; Szigeti, et al., 2004). Genotype differences do not account for these variations, as the only genotype-phenotype correlation observed in MNGIE was reported in patients with late-onset disease, harboring p.R202T, p.V208M, p.L285P, p.G311R or p.E379K mutations, which are associated with less severe TP dysfunction (Marti, et al., 2005; Massa, et al., 2009). In the case reported here, subacute worsening of weakness established peripheral neuropathy as the predominant clinical feature. This fact, together with the strikingly high CSF protein level, led us to consider a diagnosis of chronic inflammatory demyelinating polyneuropathy (CIDP) at one point. Nonetheless, TP dysfunction ruled out CIDP and confirmed MNGIE. This case further illustrates variants in the clinical presentation of this disease, and the need to test TP dysfunction to rule out MNGIE in patients whose clinical features resemble other conditions (Bedlack, et al., 2004).

We found a novel single-nucleotide deletion in the TYMP region encoding the C-terminal fraction of the protein. This change generates a frameshift that eliminates the termination codon, with no additional stop codons within the 3'-UTR (nonstop mRNA mutation). At least 5 different nonstop mRNA mutations have been associated with MNGIE (Poulton, et al., 2009), but the stability or steady-state levels of the nonstop mRNA species were not investigated. Study of the molecular consequences of the novel mutation reported here has provided singular

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results. Previous reports found that nonstop mRNA mutations associated with Diamond-Blackfan anemia (Chatr-Aryamontri, et al., 2004), mucopolysaccharidosis II (Lualdi, et al., 2006) and FX-coagulation deficiency (Ameri, et al., 2007) result in pronounced reduction of steady-state levels of mutant mRNA. Similarly, translation-dependent deadenylation decay was observed in nonstop mitochondrial mRNA as a consequence of a microdeletion (Temperley, et al., 2003). In contrast, TYMP mRNA levels were not reduced in the patient presented herein (homozygous for the c.1416delC mutation) or her parents, heterozygous carriers. Consistently, RFLP analysis showed similar amounts of mutant and wild-type transcripts in the carriers. Therefore, we conclude that c.1416delC nonstop mRNA molecules are as stable as those transcribed from the wild-type alleles in this family. The protein product was absent in the patient and reduced to ~50% in the carriers, as compared to the amounts detected in the controls, strongly suggesting that translation of mutant transcript is repressed and/or its protein product is not stable.

Cells surveillance systems result in post-transcription specific degradation of mRNAs harboring nonstop, premature stop, or no-go mRNA mutations (Isken and Maquat, 2007). Different NSD mechanisms have been described in bacteria, yeast and mammals (Akimitsu, 2008). All studies analyzing nonstop transcripts in human genetic disease have shown drastically reduced steady-state levels of nonstop mRNAs, but the case reported here demonstrates that this mechanism has exceptions. Since we only analyzed mRNA and protein from buffy coat samples, we cannot rule out reduced nonstop mRNA levels in other tissues. However, the lack of NSD in human platelets and leukocytes, where TYMP is highly expressed, indicates that, at least for this particular mutation, the main control system to avoid additional deleterious effects of an aberrant protein happens at the translational or post-translational levels rather than through NSD.

The reasons for the lack of NSD for this particular mutation are unknown. In nonsense-mediated decay, another mechanism that selectively eliminates mRNAs with premature stop codons, degradation occurs or not, depending on the relative position of the premature stop codon with respect to the exon-exon junctions of mRNA (Isken and Maquat, 2007). Similarly, unknown molecular restrictions might determine the occurrence or not of NSD, but this mechanism remains to be characterized. We speculate that this could account for the lack of NSD in the mutation studied herein. (Akimitsu, et al., 2007) examined translation of nonstop mRNA in vitro in mammalian cells and found no decreases in steady-state nonstop mRNA levels or reductions in protein stability. Instead, they observed that translation of nonstop mRNA is repressed, a mechanism that better fits with the observations reported here.

In summary, we report the first case of a patient with a pathogenic nonstop mRNA mutation and unreduced levels of nonstop mRNA, in contrast to previous reports which always show selective mRNA decay associated with this type of mutation in human disease. Determination of the reasons for these different responses to pathogenic nonstop mRNA mutations and the mechanisms involved will require further investigation.

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TABLE 1

	Genotype[1]	TYMP mRNA[2]			% mutant mRNA[3]	TP protein[4]	TP activity[5]	dThd[6]	dUrd[6]
		3'-RACE cDNA (a)	3'-RACE cDNA (b)	Random primed cDNA (c)					
Patient (II-1)	c.1416delC / c.1416delC	1.08 (0.87 - 1.32)	1.20 (1.03 - 1.40)	0.94 (0.48 – 1.81)	100	UND	3.0	9.0	10.0
Father (I-1)	c.1416delC / wt	0.95 (0.88 - 1.03)	0.71 (0.54 - 0.95)	0.79 (0.70 – 0.89)	63	0.28 (0.08)	602	UND	UND
Mother (I-2)	wt / c.1416delC	1.07 (0.85 - 1.35)	1.23 (1.02 - 1.49)	1.12 (1.01 – 1.24)	79	0.38 (0.06)	733	UND	UND
Sister (II-2)	wt / c.1416delC	ND	ND	ND	ND	ND	613	UND	UND
Control 1	wt / wt	0.90 (0.74 - 1.08)	0.74 (0.62 - 0.88)	0.94 (0.78 – 1.13)	UND	0.69 (0.08)	920	UND	UND
Control 2	wt / wt	1.00 (0.92 - 1.08)	1.00 (0.81 - 1.24)	1.00 (0.85 – 1.18)	UND	1.00	1,017	UND	UND
Control muscle	wt / wt	0.04 (0.04 - 0.05)	0.08 (0.04 - 0.19)	ND	ND	ND	ND	ND	ND

[1] Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence NM_001113755.1, according to journal guidelines (www.hgvs.org/mutnomen). [2] Real-time PCR relative quantification of 3 different

cDNAs obtained from two 3'-RACE reverse transcriptions (a and b) and one random primed reverse transcription (c) of the same buffy coat total RNA. The results indicate mean (minimum and maximum) of 3 replicates from each cDNA, normalized to *GUSB* mRNA levels and referred to the value obtained for control 2, used as calibrator. [3] Percentage of the mutant band (574 bp, Figure 1) over the total product (574 bp +502 bp bands) obtained from *Bss*HII digestion of the 3'-RACE – seminested PCR product of buffy coat RNA (see methods). [4] Relative quantification of the TP protein bands obtained by western blot, normalized by β-actin and referred to the ratio obtained for control 2, used as calibrator; results represent the mean (SD) of 3 different western blots of the same buffy coat homogenate.[5] Buffy coat TP activity in nmol thymine/hour/mg protein.[6] Plasma dThd and dUrd in µmol/L (lower limit of detection, 0.05 µmol/L). The number of mutated alleles (2 for the patient, 1 for the carriers, 0 for the controls) correlated positively with the percentage of mutated RNA (P=0.005), and negatively with TP activity (P=0.008) and TP protein (P=0.014), but did not correlate with 3'-RACE primed (P=0.306) or random primed (P=0.760) cDNA levels. wt: wild-type UND: undetectable. ND: not determined.

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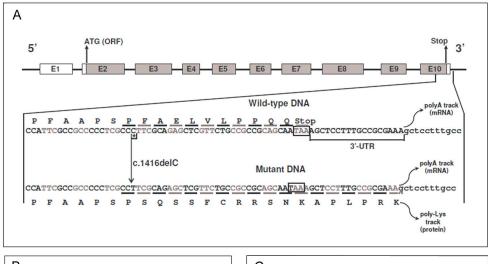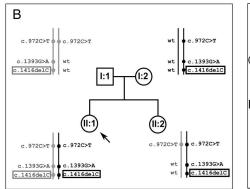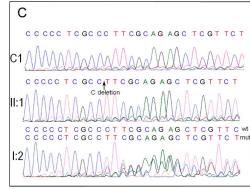
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$$\textbf{TYMP mRNA}^{2}$$

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1.00 (0.92-1.08)

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Reference sequence: GenBank # NM_001113755.1

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Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence NM_001113755.1, according to journal guidelines (www.hgvs.org/mutnomen). Panel A: Scheme of the TYMP gene. Boxes represent exons (white: 5'UTR and 3'UTR; grey: coding sequence). Intron lengths are not in scale. The 3'-end of the exon 10 (uppercase) plus a portion of flanking DNA (lowercase) are expanded, showing the wild-type and mutant (c.1416delC) sequences. The deleted 1416 C is labeled with an asterisk. The encoded amino acids are indicated in one-letter code, and the native stop codon is framed. The frame-shifted codons in the mutated sequence are underlined, showing the disruption of the native TAA stop codon, with no additional stop codons in the transcript. Panel B: pedigree of the family; the arrow indicates the patient. c.972C>T and c.1393G>A are previously reported, non-pathogenic polymorphisms. c.1416delC (framed) is the pathogenic mutation. Panel C: electropherograms showing the cDNA sequences from buffy coat polyA+ TYMP mRNA, obtained from a healthy control, showing the wild-type sequence (C1), the patient showing the c.1416delC mRNA (II:1) and a mutation carrier showing the coexistence of both mRNA species (I:2). A similar result was obtained for the other carrier analyzed (I:1; not shown). Panel D: PCR-RFLP analysis of cDNA obtained from buffy coat polyA+ TYMP mRNA. The wild type amplicon (574 bp) harbors a target for BssHII, which

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generates two digestion products of 502 bp and 72 bp (not shown); the c.1393G>A polymorphism (cis-linked to the c.1416delC mutation) destroys the restriction target. U, undigested sample; D, digested sample. Panel E: representative western-blot showing the TP and β-actin bands from buffy coat homogenates.
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