

A *tRNA*^{Ala} mutation causing mitochondrial myopathy clinically resembling myotonic dystrophy

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More than 100 pathogenic mitochondrial (mt) DNA mutations have been described in the past decade in association with different neuromuscular disorders. Apart from large scale mtDNA rearrangements and common point mutations in mt tRNA genes, pathogenic mutations affecting structural genes of mtDNA encoded respiratory chain subunits have been reported as well.¹

Pathogenic tRNA mutations can affect translation of respiratory chain (RC) complexes that are partly encoded by the mtDNA, that is, complexes I, III, and IV. The most common tRNA mutation is associated with mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) and is located in the *tRNA*^{Leu(UUR)} gene. So far, 13 additional pathogenic mutations have been described in this gene in association with different neurodegenerative disorders. Similarly, mutations in *tRNA*^{Ile}, *tRNA*^{Lys}, and *tRNA*^{Ser(UCN)} are known to cause various mitochondrial encephalomyopathies or non-syndromic deafness (see Mitomap <http://www.mitomap.org/>). The remaining 18 mitochondrially encoded tRNAs are rarely reported to be mutated in mitochondrial disorders.² In *tRNA*^{Ala} only one pathogenic mutation is listed in Mitomap to date. This mutation was found in an Italian patient who presented with late onset progressive external ophthalmoplegia and dysphagia.³ Another heteroplasmic G>A mutation at position 5650 of mt *tRNA*^{Ala} was described in addition to a *Notch3* mutation in a 53 year old patient with typical symptoms of cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy (CADASIL) together with symptoms of a myopathy with numerous ragged red fibres.⁴ Therefore, the authors suggested a pathogenic role for both, the *Notch3* and the *tRNA*^{Ala} G5650A mutation. However, the G5650A mutation has not been reported to any mitochondrial database.

Here we report a patient suffering from a novel type of mitochondrial myopathy resembling muscular dystrophy associated with the heteroplasmic G5650A mutation in the mtDNA encoded *tRNA*^{Ala} gene.

PATIENTS AND METHODS

Case report

The patient is the first of two children born to non-consanguineous parents. He is currently 38 years old. His mother died at the age of 36 from a stroke of unknown aetiology. Clinical data about his father are not available. His 35 year old sister is clinically not affected by neuromuscular disease. She has three healthy children. The patient developed normally until the age of 14, when he first noticed weakness during regular exercise at school and difficulties in climbing stairs. Proximal muscle weakness and atrophy of the lower limbs progressed slowly. At the age of 32 he first noticed proximal weakness of the upper limbs. Twice, a pectoral lipomatosis was surgically removed.

Key points

- A heteroplasmic mutation, G5650A in *tRNA*^{Ala}, was found in a 38 year old patient suffering from a novel type of mitochondrial myopathy. The clinical presentation and the muscle histology suggested a dystrophic muscle disease on first sight. Myotonic discharges were repeatedly observed on EMG suggesting the diagnosis of myotonic dystrophy. Several cytochrome c oxidase negative ragged red fibres together with clinically observed exercise intolerance and lactic acidosis pointed to a mitochondrial origin.
- Single fibre PCR showed a significantly higher rate of mutant in ragged red fibres. Apoptosis as a possible underlying pathomechanism of the dystrophic features in muscle was excluded.

A detailed neurological investigation was carried out at the age of 36 owing to progressive weakness. On examination, proximal muscle weakness and atrophy of the lower limbs and, although less severe, the upper limbs was noted. He had a waddling gait, and walking on his heels was slightly impaired. Gower's sign was present. Deep tendon reflexes were weak, but obtainable. Clinically, there was no evidence of sensory deficits, myotonia, ophthalmoplegia, ptosis, or retinopathy. Electromyography was carried out independently by two experienced investigators and showed myopathic changes in three examined muscles (M tibialis anterior, M biceps brachii, M rectus femoris). In addition, typical myotonic runs, that is, amplitude and frequency modulated spontaneous activity, were detected in all three muscles. Motor conduction velocities of the median and peroneal nerve were normal. Clinically, a slowly progressive primary myopathy was suspected. The electrophysiological evidence of myotonia prompted us to consider myotonic dystrophy or glycogen storage disorder type 2 (Pompe's disease). Therefore, an open muscle biopsy from the M biceps brachii was carried out.

Neuroimaging including diffusion tensor imaging and localised proton spectroscopy (¹H-MRS) in cortical and subcortical grey and white matter was performed to assess subclinical CNS involvement. MRI showed bilateral symmetrical discrete hyperintensities on T2 weighted images in the medial aspect of the middle cerebellar peduncles without signs of restricted diffusion. ¹H-MRS showed normal metabolite profiles and no signs of raised lactate or alanine.

Echocardiography was normal. A 24 hour electrocardiography showed 854 polytopic ventricular extrasystoles and six couplets (Lown 4a). Creatine kinase levels were slightly raised (210 U/l, normal range max 80 U/l). Blood lactate was

increased at rest (3.9 mmol/l, normal range 0.7–2.0 mmol/l). A non-ischemic forearm exercise test showed a normal increase of lactate.

Morphology, immunohistochemistry, and biochemistry of skeletal muscle

Eight μm serial cross sections were obtained for histochemical stains according to standard procedures. A frozen part of the biopsy was used for biochemistry. RC complexes I-IV activities were determined in skeletal muscle as previously described.⁵ Immunohistochemistry and immunoblotting of skeletal muscle were performed using commercially available antibodies against dystrophin, calpain, dysferlin, -sarcoglycan, merosin, and caveolin (Novocastra, Newcastle, UK).

DNA analysis and single fibre PCR

DNA extraction from the patient's muscle, myoblasts (satellite cells from muscle), and blood, and from blood of his sister was done according to standard purification protocols (Qiagen, Hildesheim, Germany). Southern blot was performed to test for mtDNA deletions. The complete mitochondrial genome from skeletal muscle DNA was sequenced and compared to the revised Cambridge mtDNA sequence.⁶

The G5650A mutation was quantified in the above mentioned DNA samples by PCR and restriction digest. A reverse mismatch primer (5'-AGTCCCATTGGTCTAGTAAGCG-3') generating an additional *Hae*II restriction site was used together with a forward primer (np 5424–5447). The G5650A mutation leads to a loss of the *Hae*II restriction site.

Resulting RFLPs were separated on a 4% NuSieve agarose gel and analysed by densitometry after ethidium bromide staining.

Similarly, the G5650A mutation was quantified in single muscle fibres by PCR and restriction digest. Single fibre PCR analysis was performed on 30 μm thick muscle sections from the patient as described previously.⁷ The mutational load in single succinate dehydrogenase (SDH) hyper-reactive fibres ($n = 11$) was compared to SDH normal fibres ($n = 11$).

Myoblast cell lines and cell culture experiments: apoptosis

The phenotype in our patient resembling muscular dystrophy prompted us to investigate apoptosis as a possible underlying pathomechanism. Primary myoblasts were therefore established from the patient's skeletal muscle and from a normal control and were grown in skeletal muscle growth medium (PromoCell) supplemented with 10% FCS (Gibco).

To induce apoptosis, myoblasts from the patient and from a healthy control were treated with TNF- (25 ng/ml, Sigma) and actinomycin D (25 ng/ml, Calbiochem) together and separately, or three hours with camptothecin (4 $\mu\text{g}/\text{ml}$, Aldrich).^{8–10} To induce metabolic stress the cells were additionally grown in glucose free medium (RPMI, 10% FCS) supplemented with 5 mmol/l galactose and the supplement mix used for skeletal muscle growth (PromoCell).¹¹ Cells were used for the TUNEL (terminal deoxynucleotidyl transferase mediated dUTP nick end labelling) assay and for DNA apoptotic laddering (KITS, Firma). All studies were carried out on cells after a similar number of population doublings. Apoptosis detection studies were carried out after three and six days of culturing in glucose free galactose containing medium.

The TUNEL technique was applied to detect nuclear fragmentation in situ (DeadEndTM Colorimetric Apoptosis Detection System kit, Promega) in myoblast cells and frozen muscle sections of the patient and controls. Using this procedure, apoptotic nuclei are stained dark brown with DAB reaction or detected as a red fluorescence by streptavidin-Cy3

(1:1000, Dianova). In order to counterstain all nuclei, incubation of the same muscle section with bisbenzimidazole (Sigma, 12 $\mu\text{g}/\text{ml}$) was also performed.

As a confirmatory additional test we used the Apoptotic DNA Ladder kit (Roche) to test for DNA laddering in our cell samples of the patient and in control myoblasts. We performed this test in parallel on another aliquot of the same cell samples preincubated with different apoptosis inducing drugs (TNF- and/or actinomycin D or camptothecin) or culturing in glucose free, galactose containing medium.

RESULTS

Skeletal muscle shows myopathy with RRF and accumulation of abnormal mitochondria on electron microscopy

The muscle biopsy showed signs of a chronic myopathy with rounded fibres and increased fibre size variation. The number of central nuclei was slightly increased. There was a slight increase of perimysial and endomysial connective tissue, several necrotic muscle fibres, and numerous moth eaten fibres (fig 1A–D). Approximately 5–10% of all fibres showed ragged red and/or ragged blue appearance on Gomori trichrome and on SDH stains (fig 1F); these fibres were cytochrome c oxidase (COX) negative (fig 1E). In summary, the histological changes were typical in part of a degenerative myopathy and in part of a mitochondrial disorder. On electron microscopy, a marked proliferation of mitochondria was noted. Some of them showed enlargement and structural anomalies such as paracrystalline inclusions strongly suggestive of a mitochondrial aetiology (fig 2A, B).

Biochemically, the absolute activity of all RC complexes I–IV were normal when related to gram non-collagen protein (NCP) but were markedly reduced when related to the greatly increased mitochondrial marker enzyme citrate synthase (CS) (NADH-CoQ-oxidoreductase 0.08 U/U CS, normal range 0.17–0.56; succinate cytochrome c oxidoreductase 0.04 U/U CS, normal range 0.08–0.45; 0.08U COX/ U CS, normal range 0.90–4.70). The increased activity of the mitochondrial marker enzyme CS (202 U/g NCP, normal range 45–105) also indicated mitochondrial proliferation.

Immunohistochemistry and immunoblotting of skeletal muscle using antibodies for dystrophin, calpain, dysferlin, -sarcoglycan, merosin, and caveolin showed normal results.

DNA analysis shows a heteroplasmic mutation in the *tRNA^{Ala}* gene

Genetic testing for myotonic dystrophy type 1 and type 2 did not show any mutations. Southern blot for mtDNA deletions did not show any abnormalities. Mutational screening of the entire mtDNA showed a heteroplasmic mutation in *tRNA^{Ala}* at np 5650. This mutation was not found in 400 controls. In addition, a homoplasmic C to T base change was found at position 2259 in the mt16S rRNA, likely to be a single nucleotide polymorphism.¹² Electrophoresis showed a high rate (95%) of the G5650A point mutation in both skeletal muscle and myoblasts. A lower rate (60%) was detected in white blood cells (WBC) of the patient. In the patient's sister, the mutation was also present to a lower extent in WBC (25%) (fig 3).

On single fibre PCR the mutational load in RRF ($n = 11$) was virtually homoplasmic compared to an average of 62% in normal fibres ($n = 11$) (ANOVA $p < 0.01$) (fig 4). We sequenced all 22 tRNA genes in four additional patients presenting with similar signs of muscular dystrophy, electrophysiological evidence of myotonia, and RRF on muscle histology. No pathogenic tRNA mutations were found in these patients.

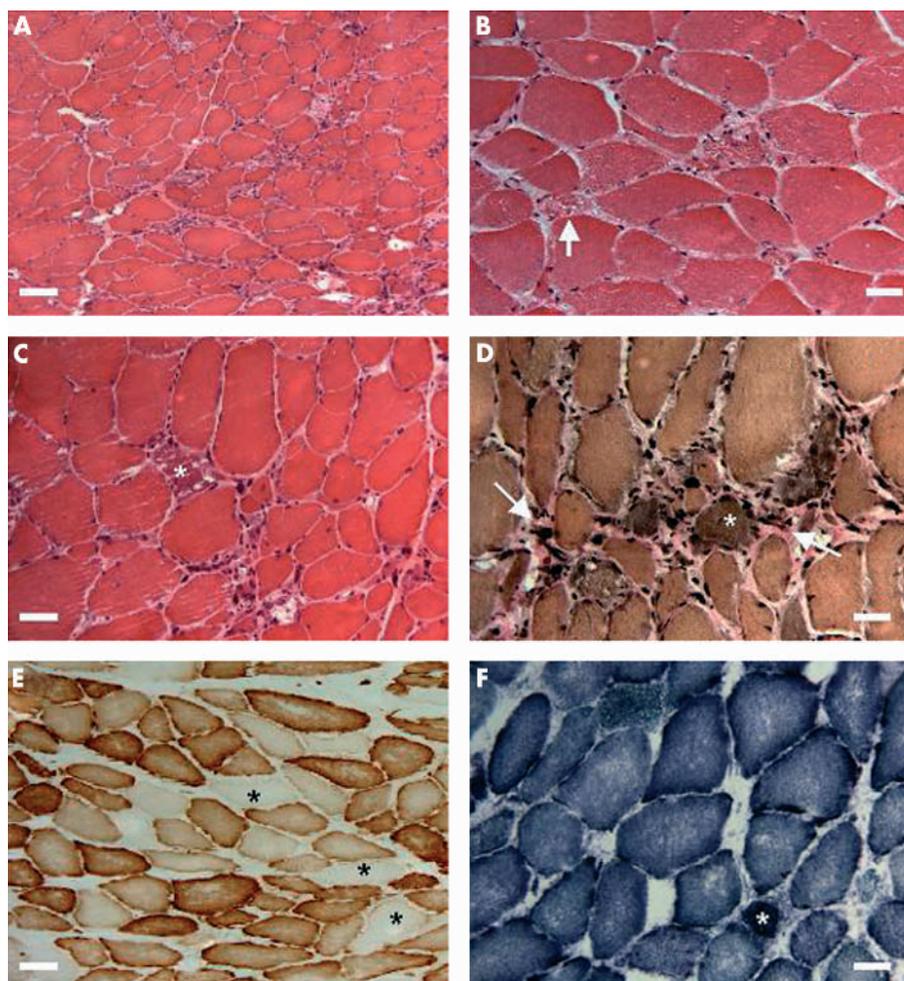


Figure 1 Muscle histology of the patient showed signs of a chronic degenerative myopathy with an increase of connective tissue (arrows in D), rounded fibres, necrotic fibres (arrow in B), increased fibre size variation, and central nuclei on HE (A-C) and Van Gieson (D) stains. On COX stain (E) 5–10% of all fibres were COX negative (black stars) and several of these fibres showed a “ragged” appearance on SDH (F) and other stains (white stars). Bars in A and E: 50 μ m. Bars in B, D and F: 30 μ m.

Apoptosis detection studies

TUNEL immunohistochemistry, DNA laddering

We did not detect a relevant number of apoptotic nuclei in the patient’s muscle and myoblasts when compared to a negative control (fig 2C-F). The positive control was preincubated with RNase free DNase. Repeated analyses with the patient’s myoblasts, harbouring 95% of the 5650 mutation, were done using different apoptosis inducers (camptothecin, actinomycin D, TNF- α and the combination of actinomycin D and TNF- α). No differences were seen when compared to normal control myoblasts. Similarly, glucose free medium supplemented with 5 mmol/l galactose, which has been recently shown to induce increased cell death and apoptosis in complex V deficient cells,¹¹ did not significantly induce apoptosis in our cells. No DNA laddering was seen (data not shown).

DISCUSSION

This is the second report on the G5650A mutation of the *tRNA^{Ala}* gene in a patient with mitochondrial myopathy and RRF. Our patient presented with a pure mitochondrial myopathy and with decreased respiratory chain enzyme activities in skeletal muscle. Interestingly, this myopathy was also associated with clinical and histological signs of a muscular dystrophy. Moreover, myotonic discharges were detected on electromyography which are very unusual in mitochondrial encephalomyopathies.

The G5650A mutation is heteroplasmic with a high rate of mutant in the affected organ, skeletal muscle. The mutation is not conserved between species, but lies within a

functionally highly conserved region in the amino acid acceptor stem of the *tRNA^{Ala}*. As has been previously shown,⁴ the G5650A mutation creates a mismatched base pair that can be designated U6:G67 according to the canonical tRNA structure, and it leads to an additional wobble which may distort the conformation of the acceptor stem causing instability of the molecule and possibly affecting the aminoacylation of the tRNA as well.¹³

Single fibre PCR showed a higher rate of the mutation in RRF than in non-RRF, further confirming the pathogenic role of the G5650A mutation.

Finnilä *et al*⁴ were the first to report a patient carrying the G5650A mutation. Since their patient carried in addition a pathogenic mutation in the *Notch3* gene causing CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy), the putative pathogenic role of the G5650A mutation was difficult to prove. CADASIL is characterised by cerebral symptoms only, and peripheral nerve and muscle involvement have not been reported in association with mutations in the *Notch3* gene. Therefore, the authors concluded that the additional G5650A mutation could lead to an exclusively myopathic phenotype, which was further confirmed in our patient by the absence of CNS symptoms and no signs of cerebral lactic acidosis or gross morphological abnormalities. The focally discrete hyperintensities in the cerebellar peduncles may still be a sign of minor subclinical CNS involvement as similar features have been reported in some patients with CPEO or Kearns-Sayre syndrome. In the Finnish patient the mutation load was similarly distributed with 99% mutant in skeletal muscle,

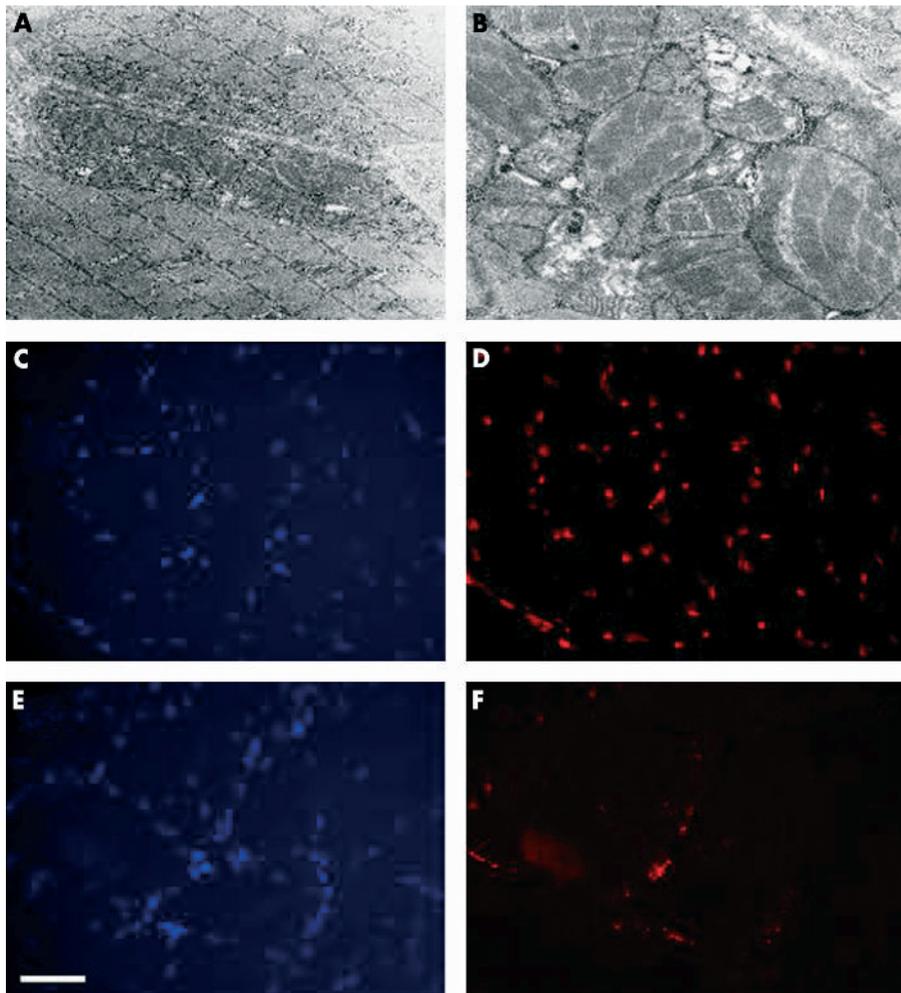


Figure 2 Accumulation of atypical mitochondria with paracrystalline inclusions in the subsarcolemmal zone of muscle fibres was observed on electron microscopy. Apoptotic nuclei showing red fluorescence were rarely detected in muscle sections of the patient (F) if compared to the total number of nuclei stained with bisbenzimidazole (E). As a control, a normal muscle treated with RNAase free DNAase shows numerous positive nuclei (C, D). Bar: 50 μ m.

96% in the buccal epithelium, 95% in skin, and 65% in blood DNA. On muscle histology numerous RRF were seen and electron microscopy showed an accumulation of mitochondria. As with our patient, moderate muscular atrophy was noted. These similarities in both patients further support the pathogenic role of the mutation.

Signs of muscular dystrophy are rare in mitochondrial myopathies. In early reports deletions of the mtDNA were found in small quantities,¹⁴⁻¹⁷ but further investigations did not confirm these findings.¹⁸⁻¹⁹ After discovering the disease causing mutations of myotonic dystrophy type 1²⁰ and type 2,²¹ mitochondrial changes were thought to be related to aging processes.²²

Specific associations between congenital myotonic dystrophy and mitochondrial DNA alterations have been reported in a single well documented patient presenting with muscle

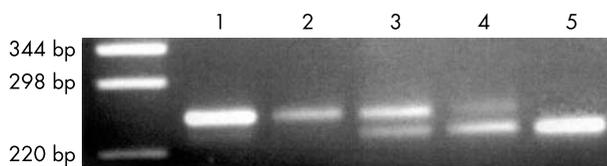


Figure 3 RFLP analysis for G5650A shows 95% mutant (247 bp band) in skeletal muscle (lane 1) and myoblasts (lane 2), 60% mutant in blood leucocytes (lane 3) of the patient, and 25% mutant in the sister's leucocytes (lane 4). Normal control (227 bp band) (lane 5).

weakness, severe exercise intolerance, and lactic acidosis. Morphologically dystrophic features were observed along with several RRF and a heteroplasmic U4409C mutation in the mitochondrial *tRNA^{Met}* gene was suspected to cause the disease.²³

In order to investigate more patients with a possible association of pathogenic mt tRNA mutations with dystrophic features in addition to typical mitochondrial

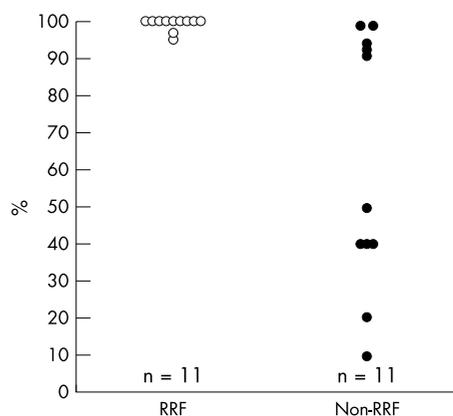


Figure 4 Single fibre PCR analysis for G5650A showed a significantly higher rate of mutant in ragged red fibres (RRF) comparing to non-ragged red fibres (non-RRF).

abnormalities like RRF, but negative for the DM1 and DM2 mutations, we sequenced all 22 tRNA genes in four patients fulfilling these criteria. An association, however, seems to be rare, as we did not identify any further mutation.

In addition to the muscular dystrophy, our patient showed myotonic discharges on EMG without clinical manifestation of myotonia. Myotonic discharges are rare EMG signs and highly characteristic for different forms of myotonia and myotonic dystrophy, but also found in Pompe's disease. Looking through the clinical presentation of patients with different types of mitochondrial myopathies, we found one case report only where rare myotonic discharges were mentioned on EMG without clinical signs of myotonia in a patient harbouring the G12315A mutation in mt *tRNA^{Leu}(CUN)*.²⁴ A previous report on the same mutation described a patient with mitochondrial myopathy but without any clinical or electrophysiological signs of myotonia.²⁵ Muscular dystrophy and myotonia together are the hallmarks of myotonic dystrophy. The molecular mechanism leading to dystrophic features in our patient is unknown. It has been shown that erroneous splicing of the chloride channel *CLC1* RNA may cause myotonia in DM1 and DM2.^{26, 27} It will be interesting to investigate which molecular mechanism may cause myotonic discharges in other disorders such as glycogen storage disease type II (Pompe's disease) or mitochondrial disorders. So far, no RNA disturbances have been described in these disorders.

Another possible mechanism is apoptosis as it has been repeatedly suggested in mitochondrial disorders.^{28–30} Apoptotic features, mainly localised to cytochrome c oxidase negative fibres, were observed in muscle fibres of patients carrying a high percentage of single mt DNA deletions (>40%) and of different tRNA point mutations (>70%).^{29, 30} Therefore, we investigated the possible role of apoptosis in skeletal muscle sections and myoblast cells of our patient. A relevant degree of apoptosis was not detected using different approaches. We conclude that despite the high mutational load in skeletal muscle and myoblasts (95%), apoptosis does not play a role in the development of dystrophic features along with mitochondrial abnormalities in our case.

In summary our patient carrying the G5650A *tRNA^{Ala}* mutation presented with a rare type of mitochondrial myopathy associated with muscular dystrophy and myotonic discharges and therefore widens the spectrum of clinical manifestations in mitochondrial disorders.

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ECHO.....

Finns with *CARD15/NOD2* variants have more familial and invasive coeliac disease



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A study of the gene frequencies of *CARD15/NOD2* gene variants has suggested that Finnish people with inflammatory bowel disease (IBD) may have different variants from those predisposing to coeliac disease (CD) or IBD in Westerners.

More research will be needed to locate the other gene polymorphisms. What the study has shown—despite low frequency in the genetically homogeneous Finnish population—is that heterozygosity for the three main variants R702W, G908R, and 1007fs overall is significantly higher for patients with CD than controls or patients with ulcerative colitis (UC) (8.7% *v* 3.5%). The 1007fs variant alone was more common in patients with CD than controls (4.8% *v* 1.7%) and more frequent in familial than sporadic CD (10.9% *v* 3.5%).

Patients with CD had one or more of the variants (15.5%) more commonly than patients with UC (9.1%) or controls (6.7%). Among these CD patients the ileum was more often affected than among the other CD patients (90% *v* 73%) and their disease was more invasive or complicated by strictures (88% *v* 56%). Significant differences in treatment between these two groups were not to blame.

The study screened patients and controls for the main *CARD15* variants. There were 198 patients with sporadic CD; 46 probands with familial CD and 27 probands with CD from mixed IBD families; 99 unrelated patients with UC; and 300 unrelated healthy controls.

The study was intended to determine any links between the main *CARD15* variants and familial IBD or complex forms of CD in Finnish patients.

▲ *Gut* 2003;**52**:558–562.