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ARTICLE

Mitochondrial dysfunction and organic aciduria in five patients carrying mutations in the Ras-MAPK pathway

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Various syndromes of the Ras-mitogen-activated protein kinase (MAPK) pathway, including the Noonan, Cardio-Facio-Cutaneous, LEOPARD and Costello syndromes, share the common features of craniofacial dysmorphisms, heart defect and short stature. In a subgroup of patients, severe muscle hypotonia, central nervous system involvement and failure to thrive occur as well. In this study we report on five children diagnosed initially with classic metabolic and clinical symptoms of an oxidative phosphorylation disorder. Later in the course of the disease, the children presented with characteristic features of Ras-MAPK pathway-related syndromes, leading to the reevaluation of the initial diagnosis. In the five patients, in addition to the oxidative phosphorylation disorder, disease-causing mutations were detected in the Ras-MAPK pathway. Three of the patients also carried a second, mitochondrial genetic alteration, which was asymptomatically present in their healthy relatives. Did we miss the correct diagnosis in the first place or is mitochondrial dysfunction directly related to Ras-MAPK pathway defects? The Ras-MAPK pathway is known to have various targets, including proteins in the mitochondrial membrane influencing mitochondrial morphology and dynamics. Prospective screening of 18 patients with various Ras-MAPK pathway defects detected biochemical signs of disturbed oxidative phosphorylation in three additional children. We concluded that only a specific, metabolically vulnerable sub-population of patients with Ras-MAPK pathway mutations presents with mitochondrial dysfunction and a more severe, early-onset disease. We postulate that patients with Ras-MAPK mutations have an increased susceptibility, but a second metabolic hit is needed to cause the clinical manifestation of mitochondrial dysfunction.
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INTRODUCTION

It is well established that heterozygous germline mutations in genes encoding participants of the Ras-mitogen-activated protein kinase (MAPK) pathway cause Noonan syndrome (NS) and the related disorders, Cardio-Facio-Cutaneous (CFC), LEOPARD and Costello (CS) syndromes.[1] These are clinically characterized by distinctive facial appearance, heart defects, ectodermal abnormalities, variable cognitive defects, motor delay and susceptibility to certain malignancies. Mutations in *PTPN11*, *RAF1* and *NF1* have been identified in patients with NS/LEOPARD syndrome and neurofibromatosis type 1. *HRAS* mutations have been identified in 80–90% of patients with CS, and *BRAF* and *MEK1/2* mutations were found in CFC patients. *KRAS* mutations have been identified in NS and CFC. *SOS*, *SHOC2*, *NRAS* and *RAF1* mutations were found in 10–30% of NS patients without *PTPN11* and *KRAS* mutations, respectively. Loss-of-function mutations in *SPRED1* were identified in neurofibromatosis type I-like syndrome. All molecules regulate the Ras-MAPK cascade.[2]

Mitochondrial dysfunction is the most common inborn error of metabolism in children, the diagnosis of which is based on characteristic clinical symptoms of multisystem involvement and on the presence of metabolic markers. Clinical diagnosis is possible using a validated scoring system (mitochondrial disease criteria, MDC score) considering clinical signs and symptoms, as well as biochemical abnormalities (eg, lactic acidemia, elevated serum alanine and urinary excretion of certain organic acids and Krebs' cycle intermediates).[3] The ultimate diagnosis is based on the measurement of abnormal ATP production from substrate oxidation and the presence of oxidative phosphorylation (OXPHOS) enzyme complex deficiency in muscle specimen or cultured fibroblasts.[4]

Recently, it was shown that the clinical presentation of congenital myopathy with excess of muscle spindles and hypertrophic cardiomyopathy is caused by *HRAS* germline mutations.[5] Moreover, mitochondrial dysfunction with multiple enzyme deficiencies of the OXPHOS system has been described in one patient with CFC syndrome carrying a *BRAF* mutation and showing muscular

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coenzyme Q deficiency. This suggests a functional connection between the Ras-MAPK pathway and mitochondrial function.[6]

Interestingly, the role of mitochondrial DNA (mtDNA) mutations has been implicated before in a patient with NS, carrying not *PTPN11* mutations but a heterozygous 3-bp deletion in the beta myosin heavy chain gene associated with seven mtDNA alterations. Unfortunately, familiality and functional assays in that patient were not available. The authors raised the possibility, however, that the mtDNA mutations might have a role in phenotypic presentation.[7] Another recent study underlines these findings by showing evidence that the mtDNA haplogroup 'R' is associated with NS in South India. In seven patients with the typical clinical picture of NS, mutation analysis of *PTPN11* failed to show alterations. In contrast, a total of 146 mtDNA mutations, five of which were novel and exclusively observed in NS patients, were found.[8] Furthermore, another study could show lower mitochondrial membrane potential and lower ATP content as well as higher levels of reactive oxygen species (ROS) in mouse fibroblast cell lines with constitutively active SHP2 (as found in NS patients) compared with wild type.[9]

We encountered several patients presenting with clinical and metabolic features of OXPHOS dysfunction, encephalomyopathy and lactic acidemia in infancy, who later developed clinical features suggesting defects in genes involved in the Ras-MAPK pathway. To collect information about the association of mitochondrial dysfunction and Ras-MAPK pathway defects, we systematically studied clinical, biochemical, histological and molecular features in five patients diagnosed with both an OXPHOS disorder and a Ras-MAPK gene defect. To further evaluate the effect of Ras-MAPK pathway mutations on mitochondrial function, we prospectively screened 18 additional patients carrying mutations in genes involved in the Ras-MAPK pathway for clinical and biochemical signs of OXPHOS dysfunction.

PATIENTS AND METHODS

Patients

We evaluated the clinical, biochemical and molecular genetic data of five children who were referred to our center for mitochondrial disorders under suspicion of a primary disorder of the OXPHOS. All of them underwent a standard diagnostic protocol, including the assessment of organ function, imaging and laboratory studies. The patients showed an MDC score of >6, indicating probable-to-definite mitochondrial disease, and therefore underwent a combined open muscle/skin biopsy.[3] During the further course of disease, all five children developed variable clinical features suggestive of a syndrome diagnosis caused by a mutation in a gene involved in the Ras-MAPK signaling pathway, leading to critical reassessment of the initial diagnosis and further molecular genetic investigations. Moreover, we prospectively screened 18 patients with known mutations of genes in the Ras-MAPK pathway, referred for additional metabolic investigations owing to feeding and swallowing difficulties, hypotonia, growth delay and failure to thrive (FTT). These routine clinical investigations were performed on the basis of standard protocols for patients with a suspected mitochondrial dysfunction with the approval of the regional ethical commission (CMO Number 2007/295).

Biochemical analysis in blood, fibroblasts and urine

Serum amino acids, serum acyl-carnitine profile and very-long-chain fatty acids analyses, as well as serum transferrin isoelectric focusing, were performed by standard methods.[3,10–15] Measurement of short-chain acyl-CoA dehydrogenase (SCAD) activity, 3-methylglutaconyl-CoA hydratase activity and cardiolipin analysis was taken in blood or fibroblasts by standard methods.[13,14] Organic acids in urine were analyzed by gas chromatography/mass spectrometry with standard methods or by using NMR spectroscopy as described elsewhere.[10,12]

Laboratory analysis in muscle and fibroblast samples

Both open muscle and skin biopsies were performed in all cases using standard methods. Musculus quadriceps samples were obtained by open muscle biopsy under general anesthesia and by obtaining the adjacent skin sample during biopsy.[3] The samples were used for histological, histochemical (hematoxin eosin, succinate dehydrogenase (SDH), COX, NADH immune staining and electron microscopy) and detailed biochemical investigations.

After biopsy, muscle tissue was immediately placed in ice-cold SETH buffer (0.25 mol/l sucrose, 2 mmol/l EDTA, 10 mmol/l Tris, 5×10^4 IU/l heparin, pH 7.4) and transported to the laboratory within 15 min. Fat and connective tissue samples were removed. Muscle tissue was minced with a Sorvall TC2 tissue chopper, homogenized in SETH buffer and centrifuged at 600 *g*. A portion of the 600 *g* supernatant was used for measuring oxidation rates and ATP production rates. The remaining 600 *g* supernatant was frozen in 100-μl aliquots in liquid nitrogen and kept at –80 °C for enzymatic measurements.[4]

OXPHOS was evaluated by determining the ATP production rate from pyruvate oxidation in fresh muscle and the activity of the enzyme complexes I–V of the respiratory chain by spectrophotometric assays in frozen muscle and under standard condition-cultured fibroblasts.[3,4,10]

Incubations were performed at 37 °C in 20-ml glass incubation vials for 20 min. The vials for measuring ^{14}C-labeled substrate oxidations contained a small plastic tube with 0.2 ml of hyamine. Incubation volume was 0.5 ml, containing 30 mmol/l potassium phosphate, 75 mmol/l potassium chloride, 8 mmol/l Tris, 1.6 mmol/l EDTA, 5 mmol/l $MgCl_2$ and 0.2 mmol/l p^1,p^5-di(adenosine-5′) pentaphosphate (myo-adenylate kinase inhibitor). To regenerate ADP by creatine kinase in the 600 *g* supernatant, 20 mmol/l creatine was added to all ADP-containing incubations.[4]

ATP and phosphocreatine were measured in the supernatant. Samples were thawed, placed on ice for 5 min and centrifuged (2 min at 14 000 *g* and 2 °C) in an Eppendorf 5402 centrifuge.

Oxidation rates of [U-^{14}C]malate were measured in the presence of malonate (inhibitor of SDH) to prevent the oxidation of [2,3-^{14}C]malate to proceed beyond one TCA cycle. ATP production was measured in incubations containing pyruvate, malate, creatine and ADP, in both the absence and presence (blank reaction) of arsenite. Incubations were started with 50 μl of 600 g supernatant and stopped by the addition of 0.2 ml of 3 mol/l perchloric acid through a rubber septum using a hypodermic syringe. Incubations were kept on ice for 1 h to trap the $^{14}CO_2$ in the hyamine.[4] Incubations for ATP production measurements were kept on ice for 15 min and then centrifuged (5 min at 14 000 *g* and 2 °C) in an Eppendorf 5402 centrifuge, after which 0.5 ml of the supernatant was neutralized with 0.6 ml of ice-cold 1 mol/l $KHCO_3$. The mixtures were kept on ice for 15 min and frozen at –20 °C.[3,4,10]

Genetic analysis

Mitochondrial and nuclear DNA samples were extracted from muscle. mtDNA mutations were evaluated using a standard mitochip analysis (GeneChip Human Mitochondrial Resequencing Array 2.0, Affymetrix Inc., Santa Clara, CA, USA). Deletions were analyzed by long template PCR. Sequencing of nuclear coded genes was performed on an ABI 3730 DNA analyzer using BigDye terminator chemistry (Applied Biosystems, Lekkerkerk a/d IJssel, The Netherlands). Molecular investigations included sequence analysis of the structural genes of the OXPHOS complex I (*NDUFS1*, *NDUFS2*, *NDUFS4*, *NDUFS7*, *NDUFS8*, *NDUFV1*) and of the *POLG1*, *DGUOK* and *OPA3* genes depending on the biochemical results from the muscle biopsy and fibroblasts.[3] Genes within the Ras-MAPK pathway comprising *PNTP11*, *BRAF*, *HRAS*, *RAF1*, *SOS1*, *MEK1* and *MEK2* were sequenced by standard methods. Molecular karyotyping (250k Affymetrix SNP array platform) was performed in all patients.

RESULTS

Patient characteristics of the five patients with proven OXPHOS dysfunction are presented in detail (see the following subsections); the data of the prospective screening of 18 patients with known Ras-MAPK disorders are summarized at the end of this section.

Clinical patient characteristics

The characteristic facial profiles and skin abnormalities of the patients are shown in Figures 1 and 2, and the MRI changes in patients 3–5 are shown in Figure 3. The clinical features are summarized in Table 1.

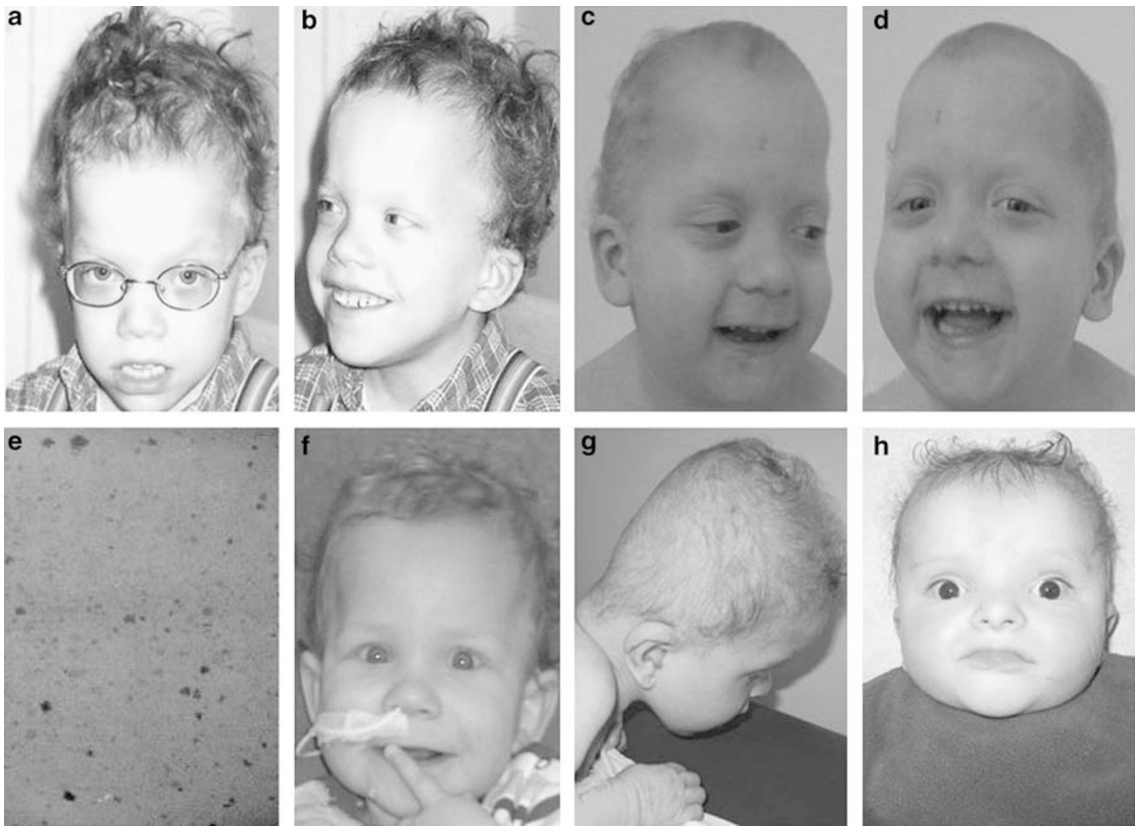

Figure 1 Clinical images of patient 2 (**a**, **b**; aged 3 years), patient 3 (**c**, **d**; aged 3 years), patient 4 (**f**; aged 3.5 years) and patient 5 (**g**, **h**; aged 1.5 years). In patient 1 (**e**; aged 14 years), note multiple lentiges in the clavicular region.

Patient 1. The female patient was observed for short stature, mild muscle hypotonia with muscle weakness, delayed motor and speech development and hypertophic cardiomyopathy with mild left ventricular outflow obstruction at the age of 14 months. Mild dysmorphic features, hypertelorism and prominent ears were noted. Metabolic investigations were initiated on the basis of a mixed conductive/sensorineural hearing loss and progressive ventricular septal hypertrophy, revealing a chronic ethylmalonic aciduria at the age of 4 years. On the basis of a slow clinical progression and the suspicion of a mitochondrial multisystem disorder (MDC score 8), the child underwent an open muscle biopsy during a diagnostic cardiac muscle biopsy session at the age of 14 years. Later in the disease course, at the age of 14–16 years, multiple lentiges appeared, leading to a reevaluation of the initial diagnosis.

The current state of the patient is stable; at 17 years of age, her speech development is age appropriate and she has an IQ of 72 (Figure 1e).

Patient 2. The male patient was followed up for muscular hypotonia, ptosis and delayed psychomotor development and FTT from the age of 4 months. Short stature, bifid uvula, cryptorchidism, long fingers and wrinkled skin of the hands were also noted. He developed seizures at the age of 8 months. A small ventriculoseptal heart defect closed spontaneously at the age of 17 months. Owing to a suspected encephalomyopathy, metabolic screening was initiated, revealing chronic lactic acidemia and lactic aciduria. Muscle biopsy was performed (MDC score 6). At the age of 3 years, the patient developed the characteristic facial features of CFC syndrome (Figures 1a, b and 2a).

The current state of the patient is stable; at the age of 8 years he is still being treated for ichthyosiform dermatitis and tonic–clonic seizures, and has an IQ of 69.

Patient 3. The male patient had severe muscle hypotonia from the age of 1 month, as well as swallowing problems, recurrent infections and West syndrome. By 10 months of age, pulmonic stenosis was detected with right ventricular hypertrophy. Owing to severe FTT and swallowing problems, he underwent a gastrostomy. Severe developmental delay (he could not roll over at the age of 18 months) and chronic lactic acidemia, together with multisystem involvement (MDC score 8), led to the patient undergoing a muscle biopsy. Skin biopsy conducted because of the appearance of wrinkled skin on the hands, around the knees and in the nuchal region showed no structural or histological elastin alterations. From the age of 3 years, the patient started to develop impressively, including eating on his own and reaching the motor milestones of sitting, standing and walking at the age of 4.5 years. His epilepsy came under control from the age of 4 years. Physical examination initially revealed mild and unspecific dysmorphic features, thick hair and very dry skin. At the age of 4.5 years, dysmorphic features characteristic of CFC syndrome became obvious (Figures 1c, d and 2d).

The current state of the patient is stable; at the age of 6 years he walks independently and has an IQ of 65.

Patient 4. In this male patient, generalized muscle hypotonia, swallowing problems and a lack of visual contact were apparent at birth. At the age of 3 months, he was diagnosed with West syndrome. Severe FTT and swallowing problems necessitated a gastrostomy.

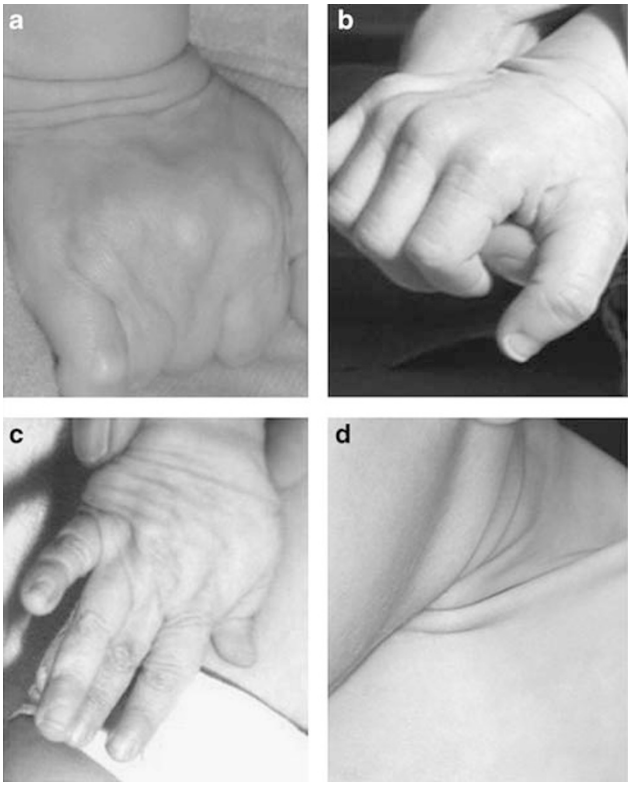

Figure 2 Wrinkled skin of the hands in patients 2 (**a**), 4 (**b**) and 5 (**c**). Wrinkled, loose skin in the nuchal region in patient 3 (**d**). Note dry, ichthyosiform skin abnormalities in patients 3 (**d**) and 5 (**c**). Skin histology in patients 3 and 5 showed no histological alterations of the elastin fibers.

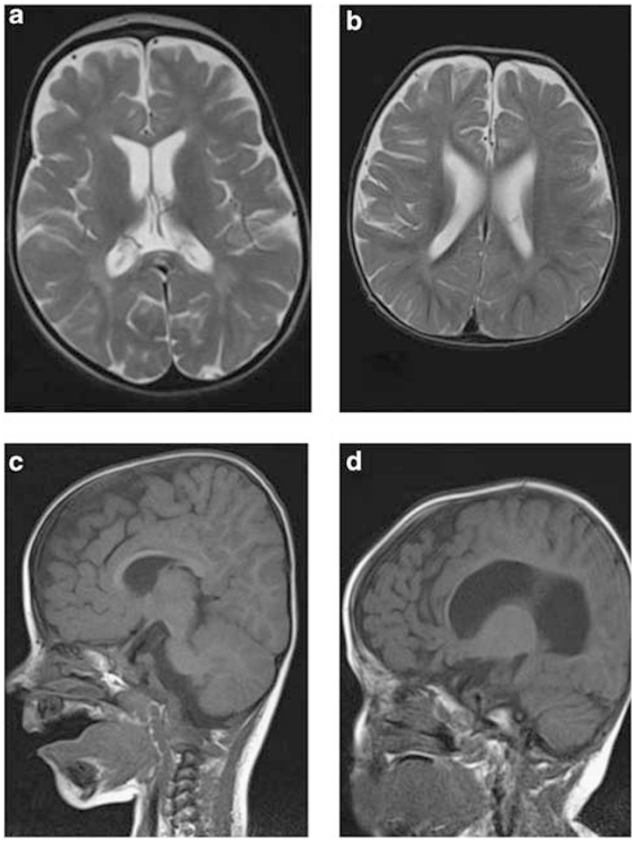

Figure 3 Mild abnormalities on MRI imaging of three patients. Cerebral MRI of patient 3 shows mild white matter changes on T1-weighed images (**a**; aged 4 years). Cerebral MRI of patient 5 shows delayed myelinization (**b**; aged 19 months) on T1-weighed images. Cerebral MRI of patients 4 (**c**; aged 2 years) and 5 (**d**; aged 15 months) shows hypoplasia of the corpus callosum and frontal atrophy on T2-weighed images.

Mild dysmorphic features comprising coarse facies, cutis laxa of the hands and feet and curly hair were noted (Figure 3). Owing to severe developmental delay (no head control at the age of 10 months), progressive spasticity, chronic lactic acidemia and 3-methylglutaconic aciduria, he underwent a muscle biopsy (MDC score 8). Physical reexamination a year later revealed evolving dysmorphic facial features, thick hair and erythema/ichthyosiform eczema of the face. From the age of 2.5 years, he started to develop faculties, including increasing visual contact, producing noises, starting to eat on his own and reaching the motor milestones of rolling over, head control and sitting with support (Figures 1f and 2b).

The current state of the patient is stable; at the age of 4 years his epilepsy is still difficult to control and his developmental quotient is appropriate for a child of 6 months of age.

Patient 5. The male patient was followed up for FTT, psychomotor developmental delay, muscular hypotonia, congenital sensorineural deafness, dysmorphic facial features, microcephaly, short stature, cutis laxa at the hands, ichthyosis and progerioid features from the age of 4 months. He was also diagnosed with left ventricular hypertrophy and mitral valve insufficiency without significant alterations in the cardiac function. Dietary intervention was started in order to correct severe malnutrition. Facial dysmorphic features at the age of 9 months encompassed a prematurely aged appearance, cutis laxa on the dorsal hand and feet, wrinkled skin axial and nuchal, broad and prominent forehead, hypertelorism, upslanting palpebral fissures, short nose, low-set and posteriorly rotated ears, short and webbed neck, thin and curly hair and thin and sparce eyebrows. Owing to the severity of the phenotype, elevated lactic acid levels in blood and mild elevation of methylmalonic acid excretion in urine, a muscle biopsy (MDC score 7) was performed in parallel with detailed genetic investigations at the age of 18 months (Figures 1g, h and 2c).

The current state of the patient is stable, but motor development is still severely delayed at the age of 22 months. Since starting to roll over at the age of 6 months, and sitting with support at 18 months, no additional developmental mile stones have been achieved. The patient received a cochlear implant at 21 months of age.

Neuroradiological patient characteristics

Mild abnormalities were observed by cerebral MRI imaging in three out of five patients (Figure 3). Patient 3 shows mild white matter changes at age 4 years (Figure 3a), and patient 5 shows delayed myelinization (Figure 3b; aged 19 months). Patient 4 (Figure 3c; aged 2 years) and patient 5 (Figure 3d; aged 15 months) show hypoplasia of the corpus callosum and frontal atrophy.

Biochemical patient characteristics

The biochemical features are summarized in Table 2.

All patients had elevated lactate and/or alanine levels in blood. Urine organic acid analysis with GC-MS or *in vitro* NMR spectroscopy showed an increased urinary excretion of ethylmalonic acid in patient 1, methylmalonic acid in patients 3 and 5 and increased excretion of

Table 1 Clinical patient characteristics

	Patient 1	*Patient 2*	*Patient 3*	*Patient 4*	*Patient 5*
Muscle hypotonia	+	+	+	+	+
Developmental/speech delay	+/+	+/+	+/+	+/+	+/+
Epilepsy	+	–	+	+	–
Deafness	–	+	–	+	+
Cardiomyopathy	–	+	–	–	+
Pulmonal stenosis	+	+	+	–	–
Short stature	+	+	+	+	+
Swallowing problems	+	–	+	+	+
Failure to thrive	+	–	+	+	+
Dysmorphic facial features (at the time of syndrome diagnosis)	+	+	+	+	+
Hair/skin abnormalities	+	+	+	+	+
MDC score before muscle biopsy[a]	6	8	8	8	7
Age at muscle biopsy	14 y	2 y	2.5 y	14 m	18 m
Syndrome diagnosis (age at diagnosis)	CFC (15 y)	LEOPARD (3 y)	CFC (4.5 y)	CFC (2 y)	NS (18 m)

Abbreviations: m, months; y, years; NS, not significant.
[a]MDC score 6: mitochondrial disease probable, MDC score 8: mitochondrial disease definite.[3]

Table 2 Biochemical patient characteristics and details on genetic findings.

	Patient 1	*Patient 2*	*Patient 3*	*Patient 4*	*Patient 5*
Serum lactate (0.4–2.0 mmol/l)	1.2–2.2	2.4–3.2	2.8–7.2	1.7–3.0	1.8–3.2
Blood alanine (120–460 mmol/l)	412–484	820	680	190–634	410
Urinary ethylmalonic acid (0–16 μmol/mmol creatinine)	120	N	N	N	N
Urinary methylmalonic acid (0–18 μmol/mmol creatinine)	N	N	62	N	42
Urinary 3-methylglutaconic acid (0–20 μmol/mmol creatinine)	N	N	N	45–46	N
ATP production (42–81 nmol/h/mU/CS)	38	ND	24	28.5	30
Complex I (70–250 mU/mU CS)	N	61	52	N	N
Complex II (335–749 mU/mU CS)	N	N	310	N	N
Complex III (2200–6610 mU/mU CS)	N	N	2039	N	N
Complex IV (810–3120 mU/mU CS)	N	N	N	N	N
Complex V (169–482 mU/mU CS)	N	N	N	N	N
Pathogenic mutation (*de novo*, heterozygous)	*PTPN11* c.836A>C	*BRAF* c.721A>C	*BRAF* c.721A>C	*BRAF* c.1914T>G	*PTPN11* c.124A>G
Second genetic alteration (asymptomatic maternally present)	*LHON* m.11778G>A	Not found	*POLG1* c.2492A>G	*MTCO1* m.6489C>A	1.4-Mb loss in Xp22.31 encompassing the *STS* gene[a]

Abbreviations: N, normal; ND, not determined.
[a]Present in mother with isolated lamellar ichthyosis.

3-methylglutaconic acid with slight increase of 3-methylglutaric acid and 3-hydroxy-isovaleric acid in patient 4. SCAD activity was normal in the blood and fibroblasts in patient 1. Urinary propionic acid excretion, blood homocystein and vitamin B12 and folate levels were normal in patients 3 and 5. The disorders including 3-methylglutaconic aciduria type I, type II (Barth syndrome) and type III (Costeff syndrome) were excluded in patient 4, as the activity of 3-methylglutaconyl-CoA hydratase, the cardiolipin levels and molecular analysis of the *OPA3* gene, respectively, showed no abnormalities.

As all our patients scored >6 on the clinical diagnostic scoring system for mitochondrial disorders (MDC; see Table 1), the possibility of a respiratory chain defect was further explored.[3] Indeed, as shown in Table 2, the biochemical analysis of mitochondrial function in the fresh muscle biopsy showed disturbed OXPHOS in four patients with an ATP production from pyruvate oxidation ranging between 24 and 38 nmol ATP/h/mU CS (control range 42–81 nmol/h/mU/CS). Patient 2 showed an associated complex I deficiency and patient 3 multiple complex deficiencies. No biochemical abnormality supporting coenzyme Q deficiency was found in either patient. No enzyme deficiency of complexes I–V was found in the fibroblast cell lines of the patients.

Muscle histology/electron microscopy

No alterations were found on histology by light microscopy in the patients, except for atrophy of type II fibers in patient 1 and fiber-type disproportion with a slight increase of internal nuclei (3%) in patient 4. Staining with hematoxin eosin, as well as SDH and COX immune staining, was normal. No ragged red fibers were detected. NADH immune staining was also within normal limits in patients 2 and 3. No significant abnormalities were observed by electronmicroscopy in patients 1, 2, 3 and 5. Histopathological evaluation in patient 4 detected two giant mitochondria with a somewhat abnormal, fragmented membrane structure. No specific alterations pointing to abnormal myogenic differentiation were found in patient 1.

Genetic studies

The common m.11778G>A *MT-ND1* (*LHON*) mutation was detected in homoplasmic form in patient 1 and in her 48-year-old mother without any ophthalmological alterations. No mtDNA alteration was found in patients 2 and 3. A heterozygous *POLG1* mutation was detected in patient 3 (c.2492A>G; p.Tyr831Cys in exon 16). The same heterozygous alteration was found in the healthy mother of the patient at the age of 38 years. Molecular analysis of mtDNA detected a homoplasmic alteration (m.6489C>A; pLeu196Ile in *MT-CO1*) in patient 4 without COX deficiency. The same alteration was present in homoplastic form in the healthy mother without any symptoms of epilepsy. Molecular karyotyping by SNP array revealed a 1.4-Mb loss in chromosome Xp22.31 (6.3–7.76 Mb) encompassing ~5 genes, including the steroid sulfatase (*STS*) gene causing X-linked recessive ichthyosis in patient 5. The same alteration was seen in the mother.

Several heterozygous *de novo* pathogenic mutations were identified in different genes of the Ras-MAPK pathway in the five children, encompassing c.836A>C (p.Tyr279Ser) in *PTPN11* in patient 1, c.721A>C (p.Thr241Pro) in *BRAF* in patients 2 and 3, c.1914T>G (p.Asp638Glu) in *BRAF* in patient 4 and c.124A>G (p.Thr42Ala) in *PTPN11* in patient 5.

Clinical, biochemical and genetic data of 18 patients with known Ras-MAPK gene mutations, prospectively screened for metabolic features of OXPHOS dysfunction

All patients were previously diagnosed because of suggestive clinical features with a molecularly proven Ras-MAPK defect. Seven children had *BRAF*, six *PTPN11*, three *HRAS* and two children carried *KRAS* mutations.

The majority of patients had the clinical presentation of NS; five children had CFC, one patient had LEOPARD syndrome and one patient had CS.

In 15 patients, metabolic screening did not show any abnormalities; two patients had recurrent lactic acidemia (one also elevated serum alanine levels) and one patient had elevated urinary excretion of ethylmalonic acid. Only one of these three patients had an MDC score >6, receiving nutritional intervention and awaiting an open muscle/skin biopsy. (The other two patients are under follow-up, but have not undergone muscle biospy because of ethical considerations.)

The three patients with biochemical abnormalities were screened with Mitochip analysis for possible mtDNA alterations in DNA extracted from leukocytes, but did not show abnormalities.

DISCUSSION

Here we describe five patients with the initial diagnosis of mitochondrial encephalomyopathy based on the early infantile presentation of multisystem disease, chronic lactic acidemia and the presence of abnormal organic acids in urine. The diagnosis was also supported by significant OXPHOS abnormalities measured in muscle biopsy of all children. Although three patients carried alterations in (or related to) the mitochondrial genome, the functional consequence of these alterations on mitochondrial function was not evident, as these segregated in healthy family members as well. During the clinical course of disease, evolving dysmorphic features led to reinvestigation of the initial diagnosis and investigation of the Ras-MAPK pathway. All five patients carried *de novo* Ras-MAPK pathway-related gene mutations.

One should note that, although the common m.11778G>A *LHON* mutation has no significant biochemical effect on muscular function in most carriers, it might have important clinical relevance in patient 1. This mutation has a highly variable penetrance and the patient might develop visual loss during young adulthood. The mother, who carries the same alteration, has no ophthalmological complaints at the age of 48 years.

In patient 3, a heterozygous missense change in *POLG1* (c.2492A>G; p.Tyr831Cys) was detected. Although this heterozygous alteration was described once as pathogenic in one adult patient, the same alteration was present in the healthy mother of that patient at the age of 38 years as well.[16] A homoplasmic alteration (m.6489C>A; pLeu196Ile in *MT-CO1*) was found in patient 4 without COX deficiency. This alteration has been described as pathogenic in severe epilepsy in association with fibrillar COX deficiency; however, the same alteration was homoplastic, present in the healthy mother without any symptoms of epilepsy.[17]

In patient 5, a 1.5-Mb deletion on Xp22 including the *STS* gene was identified. This deletion has been frequently reported in literature and occurs because of recombination between low-copy repeats. Although individuals with this deletion usually show normal development, some also present with intellectual disability and susceptibility to ADHD, autism and social communication deficits.[18,19] Therefore, we cannot exclude the fact that, besides ichthyosis, this deletion additionally contributes to the developmental and behavior phenotype. One should note that the mother of the patient carries the same deletion without features of ichthyosis.

Interestingly, parallel with a spontaneous clinical improvement in patients 2–4, there was an evolving syndromic appearance: early clinical presentation with severe neurological symptoms, including intractable epilepsy and the predominance of muscle disease, turning into an unexpected improvement from the age of 2–3 years, with the development of classic facial and skin features facilitating correct syndrome diagnosis in these patients. In patient 1, the occurrence of lentigines around the age of 15 years led to correct syndrome diagnosis. In patient 5, parallel with the diagnosis of NS, the severe phenotype with muscular hypotonia and cardiomyopathy, as well as lactic acidosis and methylmalonic aciduria, led to further evaluation and confirmation of mitochondrial disease. In patient 4, the severe phenotype with multisystem disease, the lactic acidemia and especially the 3-methylglutaconic aciduria, a known marker for mitochondrial dysfunction, led us to perform a muscle biopsy and, later on, on the basis of dysmorphic features, mutation analysis of the genes of the Ras-MAPK pathway.

Intrigued by these findings and the growing evidence for a functional connection between the Ras-MAPK pathway and mitochondria, we prospectively screened 18 patients with known mutations in the Ras-MAPK pathway for signs of OXPHOS dysfunction.[6] Metabolic abnormalities, comparable to possible mitochondrial dysfunction, were detected in three out of 18 patients. Only one patient who was severely malnourished had an MDC score >6, indicating probable mitochondrial dysfunction. No additional mtDNA alteration was found. One should also consider that the abnormal metabolic markers are secondary to malnutrition. Primary and secondary mitochondrial dysfunction remains a challenging issue in metabolic pediatrics; several patients have been previously reported with secondary mitochondrial dysfunction diagnosed with chromosomal abnormalities and various classic genetic syndromes.[3] We hypothesize that the OXPHOS dysfunction in our patients is a consequence of metabolic susceptibility due to mutations in Ras-MAPK pathway-related genes. This is of high importance for further counseling and disease management.

Recently, a study revealed a direct correlation of this pathway to mitochondrial function.[20] The control of mitochondria dynamics is regulated by A-kinase anchor protein (AKAP) complexes. Protein kinases and phosphatases are assembled by AKAPs within

transduction units, providing a mechanism to control signaling events at the mitochondria and other targeted organelles.[21] Interestingly, it was shown that a distinctive property of the RAF kinases is not only their ability to interact with cellular membranes but also their presence on mitochondria and subsequent involvement in these AKAPs. The existence of target proteins for RAF kinases on the surface of mitochondria suggests that these kinases could directly control some important aspects of the physiology of these membrane organelles. Indeed, activation of a specific isoform of the RAF family, C-RAF (RAF1), through its functional coupling with mitogen-activated protein kinase/ERK kinase (MEK), resulted in a dramatic change in morphology and subcellular distribution of the mitochondria.[20] The hypothesis that abnormal signaling of RAS/RAF/MEK can result in mitochondrial dysfunction or lead to an increased susceptibility is underlined by another study in mouse fibroblast cell lines with constitutively active SHP2 activity, showing decreased mitochondrial membrane potential and ATP, with an increase in ROS compared with wild type.[7] Another recent study underlines these findings by showing evidence that the mtDNA haplogroup 'R' is associated with NS in South India. In seven patients with the typical clinical picture of NS, mutation analysis of *PTPN11* failed to show mutations. In contrast, a total of 146 mtDNA mutations, five of which were novel and exclusively observed in NS patients, were found.[8]

Therefore, the severe mitochondrial dysfunction observed in our patients with NS, CFC and LEOPARD syndrome might be explained by this pathophysiological mechanism and merits the clinician to search for mutations in genes involved in the Ras-MAPK pathway in patients presenting with mitochondrial encephalomyopathy with additional features that could fit with CFC, NS, CS or LEOPARD syndrome. Mutations in the Ras-MAPK pathway could lead to increased vulnerability of the mitochondria to further mitochondria-related biochemical or genetic alterations. One might speculate that, although the single mitochondrial alterations in the three families do not appear to be significant in the pathogenesis, in association with a second alteration (MAPK mutation), they could lead to further deterioration of mitochondrial function. Unfortunately, the biochemical defects of OXPHOS were not expressed in the fibroblasts of the patients, delaying further functional studies on pathogenesis. One point has to be considered critically. The ATP production as a maker of overall mitochondrial function is measured in intact mitochondria of fresh muscle specimen. On extraction from the patient, the muscle specimen is directly cooled, brought to the laboratory and worked up for measurements within 10 min. It is possible that the phosphorylation state of the OXPHOS complexes is not maintained during transport of the tissue specimens and subsequent mitochondria isolation, because no phosphatase inhibitors were included in our buffers and isolation media. Therefore, possible changes due to alterations in cell signaling caused by mutations in the RAS-MAPK pathway may have been overlooked, and data presented in Table 2 relating to OXPHOS enzyme activities have to be viewed with some caution. In addition, the diminished activity of the OXPHOS complexes in patients 2 and 3, measured consecutively in frozen muscle, might be partly influenced by unspecified phosphorylation. This has to be investigated in the future in more detail.

On the basis of the obvious clinical variability in Ras-MAPK patients, especially with regard to muscle involvement, and the normal biochemical screening in 15 out of 18 prospectively screened patients with classic, molecularly characterized Ras-MAPK syndromes, we conclude that only a specific subgroup of these patients presents with a 'mitochondrial phenotype'. This could be expressed because of a second genetic hit (mitochondria genetic alteration/polymorphism), another polymorphism in a related metabolic pathway increasing mitochondrial vulnerability, or malnutrition, explaining the more severe clinical presentation in some of the patients of the Ras-MAPK spectrum.

Clinicians should continue to critically review their diagnosis during the course of disease, particularly if there are incongruities between the clinical severity and the genetic diagnosis obtained. The finding of mitochondrial dysfunction in syndromic, Ras-MAPK pathway related gene mutation carriers is also highly relevant, both for correct diagnostic procedure and for interpretation of results. The detection of an underlying signaling pathway mutation in children with mitochondrial dysfunction is essential for genetic counseling, future prognosis assessment and possible therapy.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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