***ECEL1* gene related contractural syndrome: long-term follow-up and update on clinical and pathological aspects**

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Abstract

Autosomal recessive mutations in the *ECEL1* gene have recently been associated with a wide phenotypic spectrum including severe congenital contractural syndromes and distal arthrogryposis type 5D (DA5D). Here, we describe four novel families with *ECEL1* gene mutations, reporting 15 years of follow-up for five patients and detailed muscle pathological description for three individuals. In particular, we observed mild myopathic features, prominent core-like areas in one individual, and presence of nCAM positive fibres in three patients from 2 unrelated families suggesting a possible problem with innervation. Our findings expand current knowledge concerning the phenotypic and pathological spectrum associated with *ECEL1* gene mutations and may suggest novel insights regarding the underlying pathomechanism of the disease.

Key words: ECEL1 gene, distal arthrogryposis, neuromuscular junction

Highlights:

- Recessive mutations in the *ECEL1* gene cause heterogeneous contractural phenotypes

- We describe 7 novel patients from 4 unrelated families with *ECEL1* gene mutations

- We provide long-term clinical follow-up for 5 patients with *ECEL1* gene mutations

- We observed myopathic features, core-like areas and nCAM positive fibres on histopathology

- We provide novel pathomechanical insights into *ECEL1* gene related conditions

Introduction

Distal Arthrogryposis (DA) is a congenital limb malformation disorder characterized by multiple joint contractures, including camptodactily, overriding fingers, clenched fists, ulnar deviation of the wrist and/or clubfoot [1, 2]. DAs are genetically and phenotypically heterogeneous, and to date 13 distinct OMIM-listed DAs subtypes have been defined on the basis of phenotypic features. Known DA genes typically encode for sarcomeric proteins of the contractile apparatus: *MYH3* (DA2A, 2B and DA8), *MYH8* (DA7), *MYBPC1* (DA1B), *TPM2* (DA1A), *TNNI2* (DA2B), *TNNT3* (DA2B), *PIEZO2* (DA3 and DA5), all transmitted with autosomal dominant inheritance with the exception of rare families segregating recessive pathogenic *PIEZO2* variants [3]. There is a considerable genetic and clinical overlap with the congenital myopathy spectrum, in particular nemaline myopathies and myosinopathies [4].

In 2013, recessive mutations in the Endothelin-converting Enzyme-Like 1 (*ECEL1*) gene were identified in patients presenting with features suggestive of DA (DA5D), characterized by congenital limited knee flexion, hip dislocation, scoliosis and ptosis [5, 6]. Since then, 41 patients from 23 families with pathogenic *ECEL1* gene variants have been reported [5-13]. The phenotype reported so far is characterised by congenital contractures affecting mostly the distal joints of upper and lower limbs, in particular fingers and knees, club feet, hip dislocation, short stature and scoliosis. Ocular findings, such as ptosis, severe ophthalmoplegia and astigmatism, as well as micrognathia, tongue groove and cleft palate are also common. An even more severe presentation with multiple pterygia and foetal arthrogryposis has also been described [7, 9].

The *ECEL1* gene encodes for the endothelin-converting enzyme-like 1 (ECEL1), a type II integral transmembrane zinc metalloprotease [14]. Although the exact substrate of ECEL1 remains unknown, *in vitro* studies of transfected cells suggested it localizes to the

endoplasmic reticulum and to a lesser extent to the cell surface [15]. The ECEL1 protein is thought to play a role in neural and subsequently neuromuscular junction (NMJ) development during foetal life in mice and humans [14, 16, 17]. Indeed, mice deficient in the homologous murine gene, *Ecel1*, die of respiratory failure immediately after birth [17, 18], and the rodent homologue knock-in animal model shows significant decrease of peripheral motor axons, arborization and failure of NMJs formation, suggesting this could be a key pathophysiological mechanism of *ECEL1*-related conditions [19].

Herein, we report 7 novel patients from 4 unrelated families with pathogenic *ECEL1* variants and provide long-term clinical follow-up for 5 patients. We also provide novel insights into the pathological features associated with *ECEL1* gene mutations, further expanding our knowledge concerning this rare condition and its pathogenesis.

Materials and Methods

The study was approved by the Institutional Ethical Review Board of the University College London Institute of Child Health and Great Ormond Street Hospital in the UK. Families were recruited among the children attending the neuromuscular centres in London, Oswestry and Glasgow, UK. Informed consent was obtained from all individuals included in this study or from their parents or legal guardians. The consent and local review board approval were in accordance with the UK10K project ethical framework (http://www.uk10k.org/ethics.html). Genomic DNA was extracted from blood using standard procedures. Whole exome sequencing (WES) was performed in probands from family A and B (Fig.1). Details for WES analysis and filtering process are described elsewhere [20]. Variants identified by exome sequencing were confirmed by Sanger sequencing. *ECEL1* gene analysis in family C and D was performed as part of a next generation sequencing analysis of a panel of genes implicated in congenital myopathies, at the Viapath laboratory, Guys and St Thomas Hospital in

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London, UK. Assessment of pathogenicity was carried out using bioinformatics software Alamut (http://www.interactive-biosoftware.com). Further details for panel analysis and Sanger sequencing are available on request.

Muscle biopsies were available from individuals from families A and B. Histological, histochemical and immunohistochemical studies were performed using standard techniques [21] with additional immunohistochemical labelling performed using the Menarini kit or on a Ventana Discovery Ultra automated machine.

Conventional T_1- weighted Muscle MRI images of lower limbs were obtained for one proband from family A.

Electromyography (EMG) was performed using standardised techniques [22]. Jitter measurements were made by the technique of SPACE, which is a modification of stimulated single fibre EMG [23].

Results

Clinical features

Family A: Patient A1 and A2 were born to healthy first degree cousin parents of Irish origin (Fig. 1, Table 1). Pregnancy for patient A1 was complicated by poor foetal movements, premature rupture of membranes at 30 weeks and prolonged breech position. At birth, she presented with bilateral contractures of fingers, knees and feet, and a cleft palate. Slow feeding and choking persisted during childhood despite cleft repair at 10 months. She sat and walked at 1 and 3 years, respectively. At age 4 years, she was able to hop and slowly run, but required a handrail to climb stairs. At age 8 years, she had mild facial weakness with temporomandibular joint contractures, and deep central groove in the tongue. She showed severe bilateral upper and lower limb contractures, digital webbing, calcaneo-cavus posture of the feet and flexion deformity of the toes (Table 1, Fig. 2e, f). Spine was straight but rigid. She presented mild proximal upper limb weakness. Speech was nasal in tone. Mild learning

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difficulties were diagnosed at 8 years. She remained under follow up until age 26 years. Over the years, joint contractures remained fixed. In her teens, she developed exercise intolerance with declining walking distance, and progressive weakness in neck, shoulders, and hips flexors. At age 21 years, forced vital capacity (FVC) was 48% of the predicted value and remained stable over the following years. She had tendon release of toes (at 13 years), fingers (17 years) and feet (24 years). Creatine kinase (CK) levels were normal. EMG of the peroneus muscle at age 21 years was myopathic and jitter measurement was normal, indicating normal NMJ function.

Patient A2, born at 37 weeks by caesarean section (CS) for breech presentation, presented at birth with extension contractures of elbows and knees, flexion contractures of fingers, bilateral ptosis and undescended testes. He walked at 12 months, and was able to climb stairs on all fours at 3 years. At age 8 years, he showed mild facial weakness, high-arched palate, central tongue groove and bilateral upper and lower limb contractures (Fig. 2a-d). Spine was rigid with a mild thoracic scoliosis. He had surgical correction of left tibial torsion (at 6 years), orchidopexy and ptosis surgery (at 8 years). CK was normal. FVC was 70% at age 18 years and remained stable. At age of 22 years, he was able to walk up to about one kilometre. EMG at age 18 years was myopathic and jitter measurement was normal, excluding a NMJ dysfunction.

Patient A3, first degree cousin of patient A1 and A2, born from first degree cousin parents, showed bilateral fixed adducted thumbs and flexion contractures of proximal interphalangeal joints at birth (Fig. 2g-h). She had limitation in hip abduction, fixed flexion contractures of knees and vertical talus with mild pterygia of fingers and knees. Psychomotor development was within normal limits. At age 7 years, she had mild facial weakness with ptosis, mild proximal limb and neck extension weakness, and fine motor skills difficulties due to finger

contractures. She had multiple orthopaedic tendon-release surgeries from age 8 to 12 years. CK levels were normal.

Family B: Patient B1, the second child of unrelated healthy parents from Scotland, was born at 39 weeks of gestation by elective CS for breech position and reduced foetal movements, partly due to fixed knee joint contractures (Fig. 1, Table 1). At birth, hip flexors and knee extensors contractures and reduced shoulder movements were noted. He had bilateral ptosis, epicanthic folds with limited ability to close the eyes, neck webbing and bilateral cryptorchidism. He walked at 18 months (Fig. 2i). At age 5 years, he was able to walk a few miles, but had difficulties climbing stairs due to knee contractures. At age 14 years, he developed scoliosis and had corrective surgery at age 17 years. Restrictive pulmonary syndrome was diagnosed at age 15 years and he started overnight non-invasive ventilation at age 17 years. At age 20 years, he had a genetic diagnosis of myotonic dystrophy type 1 (DM1), after identification of a pathogenic CTG repeats expansion in the *DMPK* gene, following clinical and genetic diagnosis of DM1 in the patient's father. At age 24 years, he remained ambulant. He showed facial weakness, bilateral ptosis, central furrowing of the tongue, upper and lower limbs proximally weakness but no myotonia (Fig. 2j-k, m and o). CK levels were normal.

Patient B2, the younger sister of patient B1, was born at 37 weeks of gestation by elective CS for extended breech presentation. Multiple joints contractures were detected on antenatal scans. At birth, she presented with cleft palate, bilateral hip luxation, knee contractures in extension and dorsiflexed feet. Psychomotor development was normal, but fine motor skills were affected by fingers contractures. Scoliosis was diagnosed at age 13 years. At age 22 years, she presented with incomplete eye closure and a convergent strabismus, central furrowing of the tongue, neck and fingers webbing, finger flexion contractures and proximal weakness at upper and lower limbs (Fig.2l and 2n). She was ambulant only for short

distances, using a wheelchair outdoors. She had a cleft repair at 6 months, strabismus correction at 7 years and spinal surgery at 17 years. CK level was normal. Genetic testing excluded DM1.

Family C: patient C1 was born to healthy first-cousin parents from India (Fig. 1, Table 1). Decreased foetal movements were noted in pregnancy and she was born by CS at term for breech position. At birth, she presented with extension contractures at the knees and fixed adducted right thumb but no finger contractures. Feet were in dorsiflexion and abduction, but flexible. She sat at 6 months and walked with support at 12 months. At age 2 years and 4 months, she was ambulant, but unable to run or jump. She had limited knee flexion and calcaneovalgus feet deformities. Thumbs were held in a partially reducible adducted position. She had no muscle weakness. CK, muscle ultrasound and EMG at 16 months was normal.

Family D: patient D1 was born to unrelated parents from Somalia (Fig. 1, Table 1). An older brother was reported to have congenital contractures of lower limbs, but no further information was available. Flexed elbows, clenched hands, extended knees and clubfeet were noted on antenatal scans from 20th weeks. CS was performed at 39 weeks for breech position. At birth she showed bilateral flexion contractures of elbows, fingers and hips, bilateral extension contractures of the knees and right talipes equino varus deformities. She walked independently at 3 years and at age 4 years, she was unable to run or jump because of fixed knee contractures. Speech and language was mildly delayed. Ankle tendon release was performed at 4 months of age, followed by right hip osteotomy at 17 months. CK levels and a muscle ultrasound were normal. EMG at 1 month of age was mildly myopathic and jitter measurement was normal.

Muscle MRI Imaging

Muscle MRI scan of the lower limbs from patient A1 at age 22 years showed symmetrical reduction of thigh and calf muscle bulk, particularly of *biceps femoris*, posterior calf

compartment and *extensor digitorum longus* muscles (Fig. 3). There was also some increase in abnormal signal within the *sartorius* and the posterior compartment in the thigh (Fig. 3).

Muscle pathology

Muscle biopsies were available from patient A1 (*vastus lateralis*, taken at age 12 years), A2 (peroneal muscles, taken at age 6 years when tibial torsion was surgically corrected) and B2 (*vastus lateralis*, taken at age 14 years). Variation in fibre size was minimal in patient A1 but was mildly abnormal in A2 and B2 (Fig. 4a and 4d). A few internal nuclei and predominance of type1/slow fibres were observed, particularly in B2 (Fig. 4b and 4e). Several small core-like areas, particularly in type 1 fibres, were observed in patient A2 (Fig. 4b) and electron microscopy suggested that these may have been related to abnormal mitochondrial distribution with only minimal myofibrillar disruption. Fibres positive for developmental and foetal myosin were not a feature indicating no fibre degeneration/regeneration. Immunolabelling of neuronal cell adhesion molecule (nCAM) of all 3 biopsies showed clusters of positive fibres with abnormal sarcolemmal and internal labelling suggesting an abnormality of innervation of some fibres (Fig. 4c and 4f).

Genetics

Whole exome sequencing (WES) analysis, performed in patient A1, identified a novel homozygous missense variant (c.589G>A; p.(Gly197Ser)) in exon 2 of the *ECEL1* gene. Pathogenicity prediction software Alamut predicted this variant to be deleterious. WES analysis of patient B1 revealed a homozygous frameshift variant (c.2005_2006delAC; p.(Thr669fs)) in exon 15 of the *ECEL1* gene. This variant has not been reported previously and pathogenicity prediction software considered it to be deleterious. Segregation studies confirmed the presence of the novel *ECEL1* homozygous variants in all affected individuals from Family A and B, respectively, and in heterozygosity in healthy parents and siblings, further supporting their pathogenicity (Fig.1). Next generation panel gene sequencing showed

the presence of previously identified homozygous *ECEL1* gene mutations (c.1210C>T; p.(Arg404Cys) and c.1470G>A; p.(Trp490Ter)) in patient C1 and D1, respectively [5, 13]. Parents were confirmed to be heterozygous carriers.

Discussion

In this study, we provide novel clinical, pathological and genetic insights into a recessive form of contractural syndrome associated with *ECEL1* gene mutations. In particular, we describe 7 novel patients with *ECEL1* gene mutations in addition to the 41 individuals previously reported [5-13]. The clinical analysis of our cohort indicates that the most common clinical findings are knee (7/7) and finger contractures with adducted thumb (7/7), ankle contractures (6/7), clubfeet (6/7) and hip contractures (5/7), similarly to previous reports (Table 1). Limitation of shoulder movements was more common than previously reported in the literature (4/7, 57% versus 34%) suggesting that the upper limbs are also often affected. Facial weakness and central tongue groove were more often observed in our cohort (5/7, 71% versus 35% and 4/5, 80% versus 43%, respectively). We also report for the first time temporomandibular contractures in one patient. Bilateral ptosis was the main ophthalmological finding in our cohort (5/7, 71% versus 76% in the literature), followed by strabismus (1/7, 14% versus 15% in the literature). Ophthalmoplegia, previously reported in 9% of patients [5, 12], wasn't observed in any of our patients. Few published data were available to date on antenatal findings in *ECEL1* gene patients. One report indicated AMC in two pregnancies terminated around 1st trimester [9]. In our cohort, we observed a prevalent prenatal presentation, with reduced foetal movements (4/7), antenatal contractures (3/7) and breech presentation necessitating CS in most patients (6/7), further emphasising the foetal onset of the condition. Interestingly, both male patients from this cohort also had cryptorchidism.

We also report on long term follow-up of 4 adults with *ECEL1* gene related condition (A1, A2 and B1, B2), providing valuable information on evolution and long term complications of this rare condition not available so far. Proximal weakness of upper and lower limbs was reported in about half of our patients (4/7), with progression over time. Ambulation was preserved in all patients, but we noted limitation of walking distance from the second decade of life in all older patients (4/4). Patient B1 had a coincidental diagnosis of DM1 but at last examination at age 24 years, he did not show complications typical of this coexisting condition. Progressive scoliosis was relatively common both in our cohort and in the literature (3/7, 43% versus 42%), and was associated with respiratory compromise in about two of our patients. Previously, Shaheen et al. reported severe respiratory complication secondary to thoracic deformity, leading to death in a 17-year-old patient [13]. Of note, cardiac function was normal in all patients. It is unclear if the severity of the clinical picture at birth is determinant of the severity of the disease course and later complications, but standardized follow-up and analysis of further patients might help to clarify this point. Overall, these clinical findings highlight the complex clinical course of *ECEL1* gene related conditions and advocate for an appropriate, multidisciplinary follow-up of affected patients with in particular neuromuscular, orthopaedics and respiratory specialist inputs.

To date, skeletal muscle biopsy has been reported only from a minority of ECEL1 patients (6/36) and histopathological findings were variable [5, 9]. Features suggestive of centronuclear myopathy were observed in two foetuses with severe AMC but the foetuses were only 13-14 weeks of gestation and these findings could be related to development [9]. In our cases, the lack of developmental and foetal myosin not only suggested an absence of muscle fibre regeneration but that maturation, at least in terms of myosin expression, had occurred normally. In common with published cases, type 1/slow fibre predominance and mild fibre size variation were predominant features. Abundant intracellular lipid was

observed in a few reported patients, with no evidence of structural abnormalities or features of denervation [5]. Excess lipid was not seen in our cases. In our series, we observed mild myopathic features and for the first time, presence of prominent core-like areas in one individual which initially raised the possibility of a core myopathy (Fig. 4). We also found occasional internal nuclei in three biopsies but not the appearance of a centronuclear myopathy (Fig. 4). Furthermore, in three patients (A1, A2 and B2), we noted the presence of fibres positive for nCAM suggesting a possible problem with innervation of some fibres (Fig.4c and 4f). This finding supports the hypothesis, gathered from the Ecel1 mouse model, that the lack of peripheral motor axon arborisation contributes to the pathogenesis of DAD5 [19]. Analysis of muscle biopsies of further patients with *ECEL1* gene mutations might help to clarify this point and to characterize further the histopathological spectrum of this condition, assessing if the condition is neurological rather than muscular in origin. Interestingly, we observed patchy MRI pattern of involvement in selected muscles (Fig. 3), a feature more in keeping with a neurogenic rather than a myopathic aetiology [24]. Nevertheless, the result of the EMG examination was more in keeping with a myopathic process in three out of four patients tested, similarly to that reported by Dieterich et al., who however also indicated neurogenic signs in one patient at a second, more extensive investigation [5]. The jitter measurement was normal in three patients of our series examined by this means, effectively ruling out a neuromuscular junction defect. This is relevant in view of the suggestion, from the Ecel1 mouse model, of a contribution of the neuromuscular junction in the disease pathogenesis [19]. From a clinical diagnostic point of view, in view of the major clinical and pathological overlap with similar myopathic contractural syndromes, we strongly suggest adding *ECEL1* gene into panel of genes responsible for congenital myopathies and / or distal arthrogryposis.

The allelic spectrum of *ECEL1* gene related conditions is wide and no clear genotype phenotype correlations have been reported to date. Interestingly, homozygous or compound *ECEL1* gene mutations leading to truncated protein do not associate with a severe presentation [5, 6, 13]. Conversely, a homozygous missense variant affecting a highly conserved amino acid of the catalytic domain of the protein (c.2023G>A p.(Ala675Thr)) caused severe foetal presentation with AMC [9]. We identified two novel *ECEL1* mutations, a missense change (c.589G>A; p.(Gly197Ser) in family A) affecting a highly conserved residue in exon 2, and a frame shift variant (c.2005_2006delAC; p.(Thr669fs) in family B) in exon 15 involved in the catalytic function of the protein. Patients from our cohort with homozygous missense variants (families A and C) did not significantly differ in severity compared to those with homozygous truncating mutations (families B and D). Coexisting mutations in other genes were potentially implicated in the increased severity of the phenotypes of some patients and the intrafamiliar variability described here and in previous reports could potentially support this suggestion [5, 8].

In summary, our findings further expand the knowledge on *ECEL1* gene related contractural disorders, confirming these as variable phenotypes, ranging from severe AMC to milder forms of DA5D. The long-term follow up data as well as the novel clinical and pathological findings reported here will be of diagnostic and prognostic value for clinicians and families. Further investigations are warranted to characterize better the function of the ECEL1 protein and the effect of the mutations on nerve and muscle function, in order to recognise the full spectrum of associated clinical and pathological features.

Acknowledgements

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