

GENETIC INVESTIGATIONS AND COUNSELLING ON NEUROGENETIC DISORDERS

Summary of Ph.D. thesis

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1. INTRODUCTION

1.1. Introduction into neurogenetics

The field of neurogenetics emerged from advances made in molecular biology, genetics and a desire to understand the link between genes, behavior, the brain, and neurological disorders and diseases. The field started to expand in the 1960s through the research of Seymour Benzer, considered by some to be the father of neurogenetics (Benzer, 1967). His pioneering work with *Drosophila* helped to elucidate the link between circadian rhythms and genes, which lead to further investigations into other behavior traits (Konopka and Benzer, 1971). Currently neurogenetic is emerging field that might yield a causative connection and provide the basis of the development of future novel therapeutic modalities.

In this study, I have summarized the genetic investigations and the difficulties of the genetic counselling on family planning in three neurogenetic disorders – Angelman syndrome, nemaline myopathy and hyperekplexia – presented in three Hungarian families.

1.2. Angelman syndrome

Angelman syndrome (OMIM 105830) is a rare neurodevelopmental disorder. Angelman syndrome has been reported throughout the world among different ethnic groups. Its prevalence is estimated between 1:10000 and 1:20000 (Petersen *et al.*, 1995; Steffenburg *et al.*, 1996).

Angelman syndrome is characterized by severe mental and physical delay, limited speech, fine tremor, ataxia, excessive mouthing behavior, fascination with water, jerky limb movements, seizures, craniofacial abnormalities and unusually happy sociable behavior characterized by frequent episodes of inappropriate smiling (Clayton-Smith and Laan, 2003).

Seventy percent of Angelman syndrome cases investigated with molecular genetics methods are the result of a small deletion in the 11-13 region of the maternal chromosome 15. A deletion in the same region of the paternal chromosome 15 results in the sister disorder Prader-Willi syndrome. Expression of the genes in the 11-13 region is regulated by the imprinting center, which differentially silences the paternal copy of the ubiquitin protein ligase E3A (*UBE3A*) gene in the hippocampus and in the cerebellum.

Other genetic abnormalities resulting in Angelman syndrome include uniparental disomy (UPD; 5%), mutations of the imprinting center (5%), mutations of the *UBE3A* gene (10%), and other mechanisms (10%; Poyatos *et al.*, 2002; Tan *et al.*, 2011).

1.3. Nemaline myopathy

Nemaline myopathy belongs to the group of congenital myopathies, which are a group of genetic muscle disorders with clinical, pathological and molecular heterogeneity (Nance *et al.*, 2012). Nemaline myopathy is a pathologically well-defined subgroup of congenital myopathies characterized by the presence of rod-like structures in the muscle fibers (Wallgren-Pettersson *et al.*, 2011). Annual incidence has been estimated at 1:50000 live births in a Finnish study.

The nemaline myopathy presents a wide range of clinical variability and severity, although generalized hypotonia, muscle weakness, feeding difficulties and delay of motor milestones are almost always present (Wallgren-Pettersson *et al.*, 2011). Its most common form is nemaline myopathy type 2, which is characterized by early onset muscle weakness, which is most pronounced in the axial muscles and proximal limb-girdles. Weakness in facial and bulbar muscles commonly results in dysarthria (Wallgren-Pettersson *et al.*, 1989). The course of the disease is non-progressive or slowly progressive and life-expectancy depends on the severity of respiratory-muscle

weakness. Mechanical ventilation is necessary for some patients and orthopedic complications are frequent (Wallgren-Pettersson *et al.*, 1999).

All molecularly characterized forms of nemaline myopathy are autosomal, but inheritance can be recessive or dominant, and singleton cases may arise from de novo dominant mutations (North *et al.*, 2011). To date, eight genes have been linked to this condition: α -tropomyosin (*TPM3* gene, 1q22-q23), nebulin (*NEB* gene, 2q22), skeletal muscle α -actin (*ACTA1* gene, 1q42.1), β -tropomyosin (*TPM2* gene, 9p13.2-p13.1), muscle troponin T1 (*TNNT1* gene, 19q13.4), cofilin-2 (*CFL2* gene, 14q12) and the recently identified *KBTD13* gene, 15q22.31 gene, and *KLHL40* gene, 3p22.1 whose function is still unknown (Labeit *et al.*, 2011). Six of the genes encode components of skeletal muscle sarcomeric thin filaments.

Its most common form, the nemaline myopathy type 2 is a rare autosomal recessive condition and develops as the consequence of mutations in the *NEB* gene (OMIM 256030). The *NEB* gene is located at 2q22 and encodes nebulin, which is an actin-binding protein (McElhinny *et al.*, 2003). It binds approximately 200 actin monomers (McElhinny *et al.*, 2003). Its length is proportional to thin filament length and it acts as a thin filament regulator and influences thin filament length during sarcomere assembly.

1.4. Hereditary hyperekplexia

Hereditary hyperekplexia also known as Stiff baby syndrome (ORPHA3197) is an early-onset neurological disorder characterized by excessive startle responses with violent jerking to sudden, unexpected auditory or tactile stimuli (Kirstein and Silfverskiöld, 1958). The exact prevalence of hereditary hyperekplexia is unknown. This condition has been identified in more than 70 families worldwide (Lapunzina *et al.*, 2003).

Hereditary hyperekplexia usually develops shortly after birth: neonates have prolonged periods of stiffness, clenching fists and attacks of a high-frequency trembling (Kirstein and Silfverskiöld, 1958; Ryan *et al.*, 1992). Hereditary hyperekplexia can have severe consequences such as sudden infant death due to laryngospasm and cardiorespiratory failure (Ryan *et al.*, 1992). The symptoms tend to resolve after infancy; however adults may have increased startle-induced falls or nocturnal muscle jerks (Ryan *et al.*, 1992).

Hereditary hyperekplexia has a heterogeneous genetic background (Harvey *et al.*, 2008; James *et al.*, 2012). Different mutations in several genes involved in glycinergic neurotransmission can lead to hereditary hyperekplexia, and the disease exhibits both autosomal recessive and dominant inheritance (Harvey *et al.*, 2008; James *et al.*, 2012). Mutations in the glycine receptor alpha 1 subunit gene (*GLRA1* gene, 5q32) result in hereditary hyperekplexia type 1 (OMIM149400) and occur in about 30% of the cases (Rees *et al.*, 1994). Mutations in other genes such as the glycine receptor beta subunit gene (*GLRB*; OMIM614619; Al-Owain *et al.*, 2012), the glycine transporter solute carrier family 6 member 5 gene (*SLC6A5*; OMIM614618; Rees *et al.*, 2006), the glycine receptor locator gephyrin gene (*GPHN*; OMIM603930; Reiss *et al.*, 2001) and the postsynaptic glycine enhancer collybistin gene (*ARHGEF9*; OMIM300429; Harvey *et al.*, 2004) have also been associated with this clinical condition.

The most common form of hereditary hyperekplexia is a rare autosomal dominant or autosomal recessive condition and develops as the consequence of mutations in the *GLRA1* gene. The *GLRA1* gene is located at 5q32 and encodes the alpha-1 subunit of the glycine receptor, a ligand-gated chloride channel (Grenningloh *et al.*, 1990). This inhibitory glycine receptor mediates postsynaptic inhibition in the spinal cord and other regions of the central nervous system (Grenningloh *et al.*, 1990).

1.5 Aims

In this study, my aim was to summarize the genetic investigations and the difficulties of the genetic counselling on family planning in Hungarian families

affected by three different neurogenetic disorders: My aim was to investigate the genotype-phenotype correlation in a 16-month-old Hungarian child, in whom the cytogenetic and molecular genetic analyses revealed Angelman syndrome and to investigate the parents and help the family in further family planning.

It was also among my goals to investigate the genetic background in a 4-year-old Hungarian boy presenting with the clinical and histological findings of nemaline myopathy type 2. When I started my investigations the mother of the affected child was already expecting her next baby therefore I aimed to investigate the available family members and help the parents with prenatal genetic diagnostic investigations.

I have also aimed to identify the causative genetic abnormalities in a 1-year-old Hungarian child with the clinical symptoms of hereditary hyperekplexia type 1 and to investigate the parents and help the family in further family planning.

My investigations have great importance for the affected families since they help family planning. Hopefully, these findings might also provide the basis of future studies for the development of novel therapeutic modalities in neurogenetic disorders.

2. PATIENTS AND METHODS

2.1. Patients

2.1.1. Patient affected by Angelman syndrome

A 16-month-old Hungarian child was referred to our genetic counselling unit with delayed psychomotor and speech development and dysmorphic features, including wide nasal bridge, low set ears, thick lips, wide mouth with protuberant tongue. Tongue thrusts were observed. Head circumference was 47cm (25 percentile).

The affected child was born at term after an uneventful first pregnancy with normal weight (3260 g) and head circumference (33 cm). The Apgar scores were 9, 10 and 10 at 1, 5 and 10 minutes, respectively. No signs of decreased fetal movement, neonatal hypotonia or feeding difficulties were reported.

The investigated patient was the only affected family member, all the others including the patient's parents were healthy and showed no symptoms of the Angelman syndrome.

2.1.2. Patient affected by nemaline myopathy type 2

The patient, a boy was born at term from the fourth uneventful pregnancy. Birth weight was 3450 g and Apgar scores were 9 and 10 at 5 and 10 minutes, respectively. Floppiness and weak cry were noticed in the neonatal period. Generalized hypotonia, weakness and delayed acquisition of motor milestones were observed in early childhood. He began to walk with support at the age of 2 years. Deep tendon reflexes were reduced. There was no fasciculation in the tongue. He suffered also from recurrent respiratory tract infections. The serum creatine kinase activity was normal. The optic fundus and brain MRI were also normal. Tandem mass spectrometry and urine gas chromatography screening showed no evidence of inborn error of metabolism. Nerve conduction velocity was normal, and electromyography did not show any abnormalities.

The possibility of a congenital myopathy emerged and muscle biopsy was performed at the age of 4 years. Severe respiratory tract infection and pneumonia led to respiratory failure later requiring tracheostomy and mechanical ventilation. His condition improved, however he needs ventilatory support during sleep. Cardiological examination did not show any abnormalities. The patient's cognitive abilities were appropriate for his age.

The histological work up of the muscle biopsy tissue followed standard procedures. The majority of the muscle fibers were hypoplastic or atrophic with large variation in fiber size. Fiber necrosis, regeneration, phagocytosis, or inflammatory cell infiltration were not noted. Gomori's trichrome technique detected prominent red-

stained inclusion bodies in the fibers with variation in number and distribution. Gomori trichrome stained sections showed rod-shaped particles in the fibers.

Electronmicroscopy revealed that the rods appeared as electron-dense structures localized mainly along the thickened Z-lines. These rod-shaped particles were identified as nemaline rods/bodies, and these findings were consistent with the diagnosis of nemaline myopathy.

The patient's parents and his three siblings - one brother and two sisters - showed no symptoms of nemaline myopathy type 2.

2.1.3. Patient affected by hereditary hyperekplexia

A male neonate was born on term at the 40th week of gestation by cesarean section delivery after an uneventful pregnancy. His birth weight was 3990 g and Apgar score was 9/10. At day 1 post-term, he developed pneumothorax and was admitted to the perinatal intensive care unit for extra oxygen and parenteral fluid therapy. At day 4 post-term, abnormal movements, stiffness of the muscles and convulsions were observed, and phenobarbital therapy was initiated. Neurological investigation suggested developmental disturbances of the basal moving circle. At day 11 post-term, he was hospitalized in a developmental neurology ward. Examination did not identify hypoxia-induced regulatory abnormalities. The observed recurrent muscular hypertonia was attributed to a suspected ion channel disorder and carbamazepine therapy was initiated.

The investigated patient was the only affected family member, the patient's parents were healthy and showed no symptoms of the hereditary hyperekplexia.

2.2. Methods

2.2.1. Cytogenetic analysis of chromosomes

For cytogenetic analysis whole peripheral blood samples of the investigated individuals were used. Blood samples were collected in sterile tubes containing sodium heparin. Whole blood leucocytes separated from red blood cells were placed in culture medium supplemented with serum and antibiotics. Then, mitogen was added to induce mitosis. The cultures were incubated at 37°C for 72 hours in an incubator. The cultures have to be shaken at least twice daily which significantly increases mitosis. Then, colchicine was added to the cultures few hours before harvesting to arrest the cells in metaphase. After 72 hours, the centrifuge tubes, in which the cultures were set were centrifuged at 1000 rpm for 10 minutes. Then, the supernatant was discarded and the cells were gently suspended in freshly prepared potassium chloride solution and incubated in an incubator at 37°C for about 30 minutes. Then centrifugation was repeated followed by addition of 3:1 methanol and acetic acid, which acted as a fixative. The fixative was washed out to obtain a clear cell button at the bottom of the tube. Then, the chromosomes were prepared by dropping the cell suspension on a clean, grease free slide, where, the drop was spreaded out and the chromosomes got fixed on the slides. Once the slides were prepared, standard G-banding staining technique was carried out and the Cytovision imaging system was used to analysis the staining results.

2.2.2. Chromosome segregation analysis

Genomic DNA was extracted from venous blood samples of the investigated individuals. Chromosome 15 segregation analysis with intragenic and extragenic markers for the fibrillin gene was performed for all family members using amplified fragment length polymorphism analysis on an ALFexpress instrument (Judge et al., 2001). To determine the molecular background and the recurrence risk, primers for the following microsatellite markers were used in the analysis: D15S119, D15S1028 and MTS2.

2.2.3. Mutation screening in nemaline myopathy

In view of the morphological findings a search was initiated in collaboration with the commercial diagnostic company, Centogene GmbH (Rostock, Germany) to find the molecular genetic etiology of nemaline myopathy in the affected child. Tests for ACTA1 and TPM3 were negative. Eventually testing the NEB gene revealed two previously unreported heterozygous mutations: a deletion (c.24527_24528delCT p.P8176fsX8179) in exon 174 and a duplication (c.24250_24253dupGTCA p.T8085fsX8100) in exon 171. These mutations result in a frameshift and a premature termination codon, respectively, presumably leading to truncated nebulin protein. Further testing of the parents and their unaffected children was carried out by us in the molecular biology laboratory of the Department of Medical Genetics Institute (Szeged).

Peripheral blood samples were taken from all investigated individuals. Genomic DNA was isolated using a BioRobot EZ1 DSP Workstation (QIAGEN; Hilden, Germany). The exon 171 and 174 of the NEB gene and the flanking introns were amplified by PCR reaction using specific primers.

The efficacy of the PCR reaction was checked by gel electrophoresis. PCR products were sequenced using ABI Prism 7000 Sequence Detection System (Life Technologies Magyarország Kft; Budapest).

2.2.4. Mutation screening in hereditary hyperekplexia type 1

Peripheral blood samples were taken from all investigated individuals. Genomic DNA was isolated using a BioRobot EZ1 DSP Workstation (QIAGEN; Hilden, Germany). The coding regions and the flanking introns of the GLRA1 gene were amplified by PCR reaction using specific primers. The efficacy of the PCR reaction was checked by gel electrophoresis. PCR products were sequenced using ABI Prism 7000 Sequence Detection System (Life Technologies Magyarország Kft; Budapest).

3. RESULTS

3.1. Results in Angelman syndrome

3.1.1. Pre-test counselling in Angelman syndrome

A 16-month-old Hungarian child was referred with the clinical symptoms of delayed psychomotor, speech development and dysmorphic features to our institute by the child neurologist for cytogenetic investigation. The aim of the cytogenetic investigation was to detect any numerical and/or structural abnormalities in the chromosome set of the cells. After the identification of the rearrangement of chromosomes 15 in the patient, further genetic investigations were performed to detect the paternal or maternal origin of the alteration. These investigations included the analysis of the segregation of chromosome 15.

During pre-test genetic counselling the parents were informed about the purpose of the cytogenetic and genetic studies. The parents were also informed about the putative causative alteration of the chromosomes 15 in the background of Prader-Willi/Angelman syndrome. The significance of this investigation was to identify the underlying cytogenetic abnormality, which would strongly support the clinical diagnosis and would help to estimate the course and the prognosis of the disease. The parents were also informed about the method of the cytogenetic and molecular genetic investigation and about the time it requires.

Besides these details, the parents were also informed about the legal regulations on the cytogenetic and genetic investigations and they gave their written informed consent before the investigations were started.

3.1.2. Genetic investigations

Cytogenetic analysis demonstrated a 45,XY,der(15;15)(q10;q10) karyotype in all analyzed cells from the 16-month-old patient. All metaphase cells displayed 45 chromosomes, suggesting a balanced homologous rearrangement of the long arms of chromosomes 15. The parent's karyotype was found to be normal, indicating a *de novo* chromosome rearrangement in the patient.

Analysis of polymorphic STR markers of the fibrillin-1 gene, which is located in 15q21.1, revealed that both long arms of the aberrant chromosome 15 were inherited from the father, allowing a diagnosis of Angelman syndrome caused by paternal UPD. The patient was homozygous at all loci for which his father was heterozygous, indicating that the rearrangement resulted from an isochromosome 15q.

3.1.3. Post-test counselling in Angelman syndrome

During the post-test genetic counselling, the parents were informed about the balanced structural chromosomal rearrangement 45,XY,der(15;15)(q10;q10) identified at the affected child. The performed investigations also revealed that the parents are not carriers of the balanced translocation of chromosomes 15, it emerged *de novo* in the infant. In order to prove the causative relationship between the carrier status of the balanced translocation and the clinical symptoms of the patient further genetic study was performed to identify the parental origin of chromosome 15. It was explained in details for the parents that the paternal origin of chromosome 15 would suggest Angelman syndrome, while its maternal origin would indicate Prader-Willi syndrome and its biparental origin would not likely to cause any clinical symptoms. The performed segregation analysis of chromosome 15 suggested paternal origin and thus Angelman syndrome.

The cytogenetic and genetic finding correlated well with the clinical symptoms of the affected child such as the detected minor anomalies, the developmental delay in speech and the sleeping abnormality. According to the literature, Angelman syndrome caused by paternal UPD is less severe in its course and prognosis compared to Angelman syndrome with other causative abnormalities. The importance of the appropriate diet was emphasized for the parents, since this form is associated with increased risk of obesity. Regarding family planning, the parents were informed about the very low risk - new mutation rate equivalent - of recurrence, since the identified abnormality emerged *de novo* in the affected child.

3.2. Results in nemaline myopathy

3.2.1. Pre-test counselling in nemaline myopathy

The 4-year-old Hungarian child with nemaline myopathy type 2 and his family were sent for genetic counselling and the cytogenetic and genetic investigations to our institute by the child neurologist. The affected child was previously investigated in abroad (Centogene GmbH Rostock, Germany) and two compound heterozygous mutations were identified in the *NEB* gene (c.24527_24528delCT, p.P8176fsX8179 and c.24250_24253dupGTCA, p.T8085fsX8100), which were considered as causative abnormalities for nemaline myopathy type 2. He was referred with his family for genetic counselling and further genetic investigations, since his mother was 9-week-old pregnant and was expecting her fifth baby. Previously the parents and the clinically unaffected brother and sisters of the patient were not investigated.

During pre-test genetic counselling the parents were informed about the purposes of the genetic studies, which were the followings: To verify the presence of the two heterozygous mutations of the *NEB* gene in the affected child, to identify the possible carrier status of the parents and the unaffected siblings and to provide the option of the prenatal diagnostic testing for the pregnant mother. The parents were also informed about the method of the genetic investigation and about the time it requires.

Besides these details, the parents were also informed about the legal regulations on the genetic investigations and they gave their written informed consent before the investigations were started.

3.2.2. Genetic investigations

Using direct sequencing the performed genetic investigations confirmed the presence of the two compound heterozygous mutations in the *NEB* gene in the affected child. Further testing of the parents revealed that the father carries the deletion and the mother has the heterozygous duplication. One of the two sisters of the patient carries wild type alleles, while another sister and the brother are heterozygous for the duplication. Thus, the clinically unaffected family members carry either wild type alleles or only one of the mutant alleles; either the duplication or the deletion. The patient however is a compound heterozygous carrier of both mutations.

3.2.3. Post-test counselling in nemaline myopathy

During the post-test genetic counselling, the parents were informed about their carrier status. In details, the performed investigations proved the presence of both previously identified mutation in the affected child and revealed that the deletion has paternal and the duplication has maternal origin. The performed investigations also revealed that two of the clinically unaffected siblings are also carrier of the maternal duplication and none of the clinically unaffected siblings are carrier of the paternal deletion.

These results had high impact on family planning, since they suggested autosomal recessive inheritance of the disease and proved that there is a 25% chance of expecting an affected child from the 5th pregnancy of the mother.

Based on the above findings, chorion villus sampling, fetal DNA isolation and fetal genetic testing were offered for the pregnant mother to detect the two investigated mutations of the *NEB* gene and to ensure the delivery of a healthy newborn. However, after detailed discussion with the genetic counsellor and with the family members and other relatives, the mother did not chose to undergo prenatal genetic testing, but referring to social causes she requested the termination of pregnancy before the 12th gestational week.

3.3. Results in hereditary hyperekplexia

3.3.1. Pre-test counselling in hereditary hyperekplexia

A 27-year-old pregnant woman on the 10th gestational week arrived for genetic counselling to assess recurrence risk of hereditary hyperekplexia and discuss prenatal genetic testing possibilities. She reported on her second child who suffers from hereditary hyperekplexia. Genetic testing was not performed previously.

During pre-test genetic counselling the purposes of the genetic studies were set and were the followings: To identify the causative abnormality of hereditary hyperekplexia in the affected child, to determine inheritance and recurrence risk with the identification of the possible paternal origin of the alteration and to provide the option of the prenatal diagnostic testing for the pregnant mother. The mother was also informed about the method of the genetic investigation and about the time it requires.

Besides these details, the mother was also informed about the legal regulations on the genetic investigations. After discussing this topic with the whole family, both parents gave their written informed consents and the genetic investigations were initiated.

3.3.2. Genetic investigations

The most common form of hereditary hyperekplexia develops as the consequence of mutations in the *GLRA1* gene. Therefore, direct sequencing of the coding regions and the flanking introns of the *GLRA1* gene was performed and revealed a novel heterozygous missense mutation (c.211A/T, p.Ile71Phe) in exon 3.

Comparison of GLRA1 protein sequences in the region of the mutation (p.Ile71Phe) from different species indicates that the region is highly conserved.

Genetic screening of the affected family revealed that the clinically unaffected parents and the unaffected sister did not carry the mutation, suggesting that the identified novel sequence alteration is a de novo mutation in the patient.

3.3.3. Post-test counselling in hereditary hyperekplexia

During the post-test genetic counselling, the parents were informed about the identified novel heterozygous de novo missense mutation in the affected child. It was emphasized that the parents did not carry the identified mutation therefore the risk of disease recurrence is very low and correlate with the risk of new mutation appearance rate. Based on the results prenatal diagnostic testing for hereditary hyperekplexia is not required. The results of the performed genetic testing suggest autosomal dominant inheritance, which will be an important issue when the affected child will grow up and will decide family planning.

4. DISCUSSION

4.1. Angelman syndrome

In this study the case of a 16-month-old Hungarian boy affected by delayed psychomotor development and dysmorphic features is reported. Cytological and molecular genetic investigation revealed UPD suggesting a Robertsonian-like translocation 45,XY,der(15;15)(q10;q10). A similar balanced 15;15 translocation resulting from paternal UPD in a child with Angelman syndrome was reported by Freeman et al. (1993). Results from polymorphic marker analysis for the fibrillin-1 gene, located in 15q21.1, indicated that both arms of the aberrant chromosome 15 were inherited from the father, allowing a diagnosis of Angelman syndrome caused by paternal UPD. DNA polymorphic markers demonstrated that the patient was homozygous at all loci for which the father was heterozygous, suggesting that the structural rearrangement was an isochromosome 15q and not a Robertsonian translocation.

The severity of Angelman syndrome varies significantly. The mildest symptoms have been reported for mutations of the UBE3A gene, whereas the most severe symptoms are reported for large deletions on chromosome 15 (Bottani et al., 1994; Tonk et al., 1996; Smith et al., 1997; Prasad et al., 1997; Moncla et al., 1999). Previous studies suggested that patients with Angelman syndrome caused by UPD may remain undiagnosed because of their milder or less typical phenotype, leading to an overall under-diagnosis of the disease (Lossie et al., 2001; Varela et al., 2004). In the investigated patient, we observed dysmorphic features, developmental delay, speech impairment and sleep disturbances, excessive mouthing behavior, short attention span, hand flapping, fascinating with water, and characteristic EEG and MRI results. The clinical features of our patient are similar to previously published results. The symptoms of the patient are relatively mild, which correlates well with the previous observations that Angelman syndrome patients with UPD usually have less severe clinical symptoms.

The patient was diagnosed with Angelman syndrome at the age of 16 months, earlier than in previous reports of UPD, allowing the parents to be given a correct prognosis and an explanation of delayed neurological developmental as well as the possibility of early interventional therapy. In addition, the parents were counselled that the child is at risk for obesity and its associated complications, which could be managed with lifestyle adjustments. As the aberration was the result of a de novo occurrence, the parents were not counselled on the risk of recurrence for further pregnancies.

4.2. Nemaline myopathy type 2

Nemaline myopathies are a clinically and molecularly heterogeneous group of congenital myopathies (Wallgren-Pettersson et al., 2011; Nance et al., 2012). The combination of characteristic clinical and histopathologic features are diagnostic for the disorder in most cases (North et al., 2011). The presence of red inclusions detected with Gomori trichrome staining and of rod-shaped particles in toluidine blue stained tissue from the patient strongly suggest nemaline myopathy (Wallgren-Pettersson et al., 2011; Nance et al., 2012). Ultrastructural studies reveal nemaline bodies as electron-dense, rod-shaped structures appearing as thickened Z-disks (Wallgren-Pettersson et al., 2011; Nance et al., 2012). Muscle imaging by MR can be helpful to visualize the pattern of selective muscle involvement and guide in localizing the site of the biopsy (Fischer et al., 2006; Mercuri et al., 2007).

The work up of a case with nemaline myopathy is further complicated by its heterogeneous genetic background: eight known causative genes have been linked to this condition and both autosomal dominant and recessive inheritance has been observed (Wallgren-Pettersson et al., 1999 and 2011). Six of these genes encode proteins associated with sarcomeric thin filaments (Wallgren-Pettersson et al., 1999 and 2011). Recessive mutations in the NEB gene, located on chromosome 2q22-23 are the most commonly recognized cause of the disease (Labeit et al., 2011). This gene has 182 exons and missense, nonsense and frameshift mutations have been reported (Wallgren-Pettersson et al., 2002). Hotspots have not been found and many patients proved to be compound heterozygotes for two mutations within the gene (Wallgren-Pettersson et al., 2002). The nebulin protein has a wide range of functions, including thin filament length specification and regulation of muscle contraction (Labeit et al., 2011).

The clinical, histological and molecular genetic findings in our patient are consistent with the typical congenital form of nemaline myopathy type 2, caused by mutations in the NEB gene (Wallgren-Pettersson et al., 2002). Compound heterozygosity for two novel mutations was found. A 2-base deletion (c.24527_24528delCT, p.P8176fsX8179) was inherited from the father and a 4-base duplication (c.24250_24253dupGTCA, p.T8085fsX8100) from the mother. These novel mutations led to a translational frameshift and a premature termination codon in the respective translated sequences and thus, presumably, to truncated nebulin protein.

Nemaline myopathy is a debilitating condition and further research is warranted in order to explore the details of the molecular pathology of this disorder. These efforts are complicated by the heterogenic molecular background of the disease and the fact that certain genes encode very large proteins, like nebulin. Molecular diagnosis however becomes available for more and more patients supporting preimplantation or prenatal diagnosis for subsequent pregnancies. This case report extends the genetic profile of nemaline myopathy with two previously unreported mutations in the NEB gene.

4.3. Hereditary hyperekplexia

In this study, a Hungarian family with one affected patient with hereditary hyperekplexia, a potentially fatal neurological disorder characterized by pronounced startle responses, was investigated. Abnormal movements, stiffness and convulsions were first noted in the patient at day 4 post-term, which correlates well with the early-onset of the disease. The initial severe symptoms of the patient were attenuated by carbamazepine therapy and physiotherapy and subsequently diminished with age.

Hereditary hyperekplexia has been linked to genetic alterations in genes involved in an inhibitory neurotransmitter, glycine neurotransmission (Harvey et al., 2008; James et al., 2012). GLRA1 mutations account for approximately 30% of all cases with hereditary hyperekplexia (Rees et al., 1994). Both compound heterozygous patients and homozygous mutation carriers have been described in the literature for recessive forms of the disease (Humeny et al., 2002). A heterozygous missense mutation (c.211A/T, p.Ile71Phe) was detected in the patient in exon 3 of the GLRA1

gene, establishing the diagnosis of hereditary hyperekplexia type 1 and suggesting that the mutation is an autosomal dominant form of the disease. As the non-coding regions of the GLRA1 gene were not examined, we cannot exclude the possibility of second mutation that represents a recessive form in our patient.

The GLRA1 gene encodes a neurotransmitter-gated ion channel transmembrane protein with three transmembrane segments (Lynch et al., 2004; Becker et al., 2006). Binding of glycine to its receptor increases the chloride conductance, produces hyperpolarization and, thus, the inhibition of neuronal firing (Lynch et al., 2004; Becker et al., 2006). Previous studies have attributed dominant forms of hereditary hyperekplexia type 1 to mutations within the pore-lining transmembrane segment (No.: 2) and adjacent regions, recessive forms to mutations within the other transmembrane segments (No.: 1 and 3), and the null allele of the GLRA1 gene to the deletion of exons 1–7 (Lynch et al., 2004; Becker et al., 2006).

The novel heterozygous missense mutation (p.Ile71Phe) reported here is located close to the NH2-terminal of the GLRA1 protein outside the transmembrane segments, in a highly conserved region (Figure 12). The mutation is located in a region that is not predicted or known to be a functional domain of the GLRA1 protein. Other missense mutations have been detected in this region in patients with hereditary hyperekplexia type 1 (p.Trp68Cys and p.Arg72His; Harvey et al., 2008), and in spasmodic mouse (p.Ala52Ser; Saul et al., 1994). The functional analysis performed on the spasmodic mouse model suggested that the p.Ala52Ser missense mutation results in reduced glycine sensitivity (Saul et al., 1994). Based on these previous studies, we hypothesize that the reported novel missense mutation detected in our Hungarian patient might lead to reduced glycine sensitivity as well.

It is also interesting to note, that previously reported missense mutations in this region (human p.Trp68Cys and p.Arg72His and murine p.Ala52Ser) are all associated with the recessive form of hereditary hyperekplexia type 1, indicating the possibility that other undetected mutations might contribute to the clinical symptoms of our Hungarian patient.

Having identified the putative causative mutation in the affected patient, clinically unaffected family members were also screened and shown to carry only wildtype sequence of the GLRA1 gene. Our results suggest that this novel missense sequence change (c.211A/T, p.Ile71Phe) identified in the affected patient is a *de novo* mutation. Our results correlate well with the data of the literature indicating that approximately 70% of the disease-causing GLRA1 mutations arise *de novo* (Kang et al., 2008).

The consequences of the hereditary hyperekplexia type 1 can be severe, warranting further efforts to elucidate the nature of the disease despite the complications implicit with the heterogenic genetic background. With the identification of the underlying genetic abnormalities, prenatal screening is available for affected families and allows informed family planning. In the future, knowledge of the genetic causes of this life-threatening disease may also contribute to the development of novel therapeutic alternatives.

4.4. Difficulties of genetic counselling in neurogenetic disorders

Genetic counselling is primarily dependent upon precise diagnosis and upon accurate pedigree. The genetic counsellor always needs to be careful with the diagnosis even if there is a clinical suspicion of certain diseases, the counsellor has to re-assess the symptoms of the affected patients, consider the appearance of the disease throughout the generations and determine the possible manners of inheritance.

This accuracy of the counsellor is shown in the presented first case with Angelman syndrome, since the correct genetic diagnosis was established at the age of 16 months. To my knowledge, this is the earliest diagnosed Angelman syndrome compared to previous reports of UPD, allowing the parents to be given a correct prognosis and an explanation of delayed neurological development as well as the possibility of early interventional therapy. In addition, the parents were counselled that

the child is at risk for obesity and its associated complications, which could be managed with lifestyle adjustments. As the aberration was the result of a de novo occurrence, the parents were not counselled on the risk of recurrence for further pregnancies.

Even if the diagnosis can suggest a group of monogenic neurogenetic diseases, which occurred in the case of nemaline myopathy establishing the accurate genetic diagnosis is still difficult. Since mutations in several genes can lead to the same phenotype such as the mutations of the following genes TPM3, NEB, ACTA1, TPM2, TNNT1, CFL, KBTBD13 and KLHL40 can lead to the development of nemaline myopathy. In this case, after screening several genes, the causative compound heterozygous mutations were finally found in the NEB gene suggesting nemaline myopathy type 2. After the causative mutations were identified, investigation of the whole family helped to assess carrier statuses and the recurrence risk of the disease.

Sometimes the identification of the putative causative mutation and establishing the correct genetic diagnosis are not enough for determining the mode of inheritance and the recurrence risk of the disease. This was the case with the Hungarian family having a child affected by hereditary hyperekplexia. With the identification of the novel missense heterozygous mutation of the GLRA1 gene, the established genetic diagnosis was hereditary hyperekplexia type 1. However, this type of the disease can have autosomal dominant or recessive inheritance as well. In case of a homozygous mutation the recessive inheritance is clear. In case of a heterozygous mutation the dominant inheritance can only be supposed, since there is a chance of an unidentified second mutation in the uninvestigated, non-coding regions of the GLRA1 gene.

The development of new techniques for antenatal and prenatal diagnosis of chromosomal anomalies, monogenic or multifactorial disorders and an increased ability to recognize carriers of recessive genes greatly improve the quality of genetic diagnosis. The Human Genome Project also helped the discoveries of candidate genes and novel gene variations in the background of monogenic diseases such as neurogenic disorders. There is still a strong need of further developments, since several neurogenic disorders have no causative therapy. Hopefully in the future genetic counselling will not only help family planning, but also the initiation of personalized therapy.

Another important aspect of genetic counselling is empathy of the counsellor. In counselling, simple statements of risk are rarely sufficient. Clinical acumen is required to perceive the unspoken fears and feelings of guilt, which are usually associated with inherited disease. Sometimes it is really difficult to eliminate unwanted and unappropriate feelings of the parents. In the family affected by nemaline myopathy type 2, the mother was pregnant at the time of the genetic counselling and investigations. Even though, the performed investigations elucidated the carrier statuses of the parents, accurately determined the recurrence risk (25%) and provided the possibility of prenatal screening, the family decided to terminate the pregnancy and not to undergo chorionic villus sampling and prenatal screening. Behind this decision, there are several rational reason and spoken and unspoken feelings. As a genetic counsellor, I can help the individual or the family with accurate and detailed informations about the disease, prognosis, outcomes, therapeutic modalities and family planning, but all the counsellors must accept that the final decision is made by the patient or the family.

5. SUMMARY

In this study, my aim was to summarize the genetic investigations and the difficulties of the genetic counselling on family planning in Hungarian families affected by three different neurogenetic disorders such as Angelman syndrome, nemaline myopathy and hereditary hyperekplexia.

Angelman syndrome is a rare neurogenetic disorder that results in intellectual and developmental disturbances, seizures, jerky movements and frequent smiling. Angelman syndrome is caused by two genetic disturbances: genes on the maternally inherited chromosome 15 are deleted or inactivated or two paternal copies of the corresponding genes are inherited. The results of this study allowed the earliest reported diagnosis of Angelman syndrome in a 16-month-old child with clinical symptoms. The patient was referred with minor facial anomalies, neurodevelopmental delay and speech impairment. Cytogenetic results suggested a de novo Robertsonian-like translocation involving both q arms of chromosome 15: 45,XY,der(15;15)(q10;q10). Molecular genetic studies with polymorphic markers of the fibrillin-1 gene, located in the 15q21.1, revealed that both arms of the translocated chromosome were derived from a single paternal chromosome 15 (isodisomy) and led to the diagnosis of Angelman syndrome caused by UPD. Angelman syndrome resulting from UPD caused by de novo balanced translocation t(15q;15q) of a single paternal chromosome has been reported by other groups. The detection of this rare UPD cause of Angelman syndrome contributes to the deeper understanding of the phenotype-genotype correlation in Angelman syndrome for non-deletion subclasses.

Nemaline myopathy is a type of the heterogeneous group of congenital myopathies. Generalized hypotonia, weakness and delayed motor development are the main clinical features of the typical congenital form. Histopathology shows characteristic nemaline rods in the muscle biopsy. Mutations in at least eight genes proved to be responsible for this muscle disease. Here I have investigated a Hungarian family with a child affected by nemaline myopathy type 2 caused by compound heterozygosity for two novel mutations, a deletion and a duplication in the NEB gene. The deletion was inherited from the father and the duplication from the mother. Since nemaline myopathy is a debilitating condition, further research is warranted in order to explore the details of the molecular pathology of this disorder. These efforts are complicated by the heterogenic molecular background of the disease. Molecular diagnosis however becomes available for more and more patients supporting preimplantation or prenatal diagnosis for subsequent pregnancies. Here I report extends the genetic profile of nemaline myopathy with two previously unreported mutations in the NEB gene.

Hereditary hyperekplexia is a neurological disorder characterized by excessive startle responses with violent jerking to noise or touch, stiffening of the trunk and limbs, clenching fists and attacks of a high-frequency trembling. Hereditary hyperekplexia has a heterogeneous genetic background with several identified causative genes and demonstrates both dominant and recessive inheritance. Mutations in the GLRA1 gene occur in about 30% of the cases. In this study, I report a Hungarian pedigree with one affected boy whose abnormal movements, muscle stiffness and convulsions were first noted when he was 4 days old. Neurological and electrophysiological investigation suggested the clinical diagnosis of hereditary hyperekplexia. Direct sequencing of the coding regions and the flanking introns of the GLRA1 gene revealed a novel heterozygous missense mutation (c.211A/T, p.Ile71Phe). Genetic screening of the patient's family revealed that the clinically unaffected parents and sister do not carry the mutation suggesting that the identified sequence change is a de novo mutation. Since hereditary hyperekplexia can have severe consequences, including sudden infant death due to laryngospasm and cardiorespiratory failure, identification of the causative genetic alteration(s) of the disease is high priority. Such knowledge is necessary for prenatal diagnosis, which would allow informed family planning and greater parental sensitivity to hereditary hyperekplexia-associated risks.

My investigations have great importance for the affected families since they help family planning. Hopefully, these findings might also provide the basis of future studies for the development of novel therapeutic modalities in neurogenetic disorders.

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7. LIST OF PUBLICATIONS

Publications directly related to the subject of the dissertation

- I. Emese Horváth, Zsuzsanna Horváth, Dóra Isaszegi, Gyurgyinka Gergev, Nikolett Nagy, János Szabó, László Sztriha, Márta Széll, Emőke Endreffy: Early detection of Angelman syndrome resulting from de novo paternal isodisomic 15q UPD and review of comparable cases. *Mol Cytogenet.* 2013;6:35. IPF: 2.360
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