Genetic investigations on rare monogenic diseases

Ph.D. Thesis

Adrienn Sulák M.Sc.

Graduate School of Clinical Medicine

University of Szeged

Supervisor:

Nikoletta Nagy MD, Ph.D.

Department of Medical Genetics

University of Szeged

Szeged

2017.

TABLE OF CONTENTS

LIST OF PUBLICATIONS
Publications providing the basis of the dissertation

I. **Sulák A**, Tóth L, Farkas K, Tripolszki K, Fábos B, Kemény L, Vályi P, Nagy K, Nagy N, Széll M. One mutation, two phenotypes: a single nonsense mutation of the *CTSC* gene causes two clinically distinct phenotypes. *Clin Exp Dermatol* 2016; 41(2):190-195. **IF: 1.315**

II. Tripolszki K, Knox R, Parker V, Semple R, Farkas K, **Sulák A**, Horváth E, Széll M, Nagy N. Somatic mosaicism of the *PIK3CA* gene identified in a Hungarian girl with macrodactyly and syndactyly. *Eur J Med Genet* 2016; 59(4):223-226. **IF:1.81**

III. Tripolszki K, Farkas K, **Sulák A**, Szolnoky G, Duga B, Melegh B, Knox RG, Parker VER, Semple RK, Kemény L, Széll M, Nagy N. Atypical neurofibromatosis type 1 with unilateral limb hypertrophy mimicking overgrowth syndrome. *Clin Exp Dermatol* 2017; doi: 10.1111/ced.13154. [Epub ahead of print] **IF: 1.315**

Publications directly related to the subject of the dissertation

I. Nagy N, Valyi P, Csoma Z, **Sulak A**, Tripolszki K, Farkas K, Paschali E, Papp F, Toth L, Fabos B, Kemeny L, Nagy K, Szell M. *CTSC* and Papillon–Lefèvre syndrome: detection of recurrent mutations in Hungarian patients, a review of published variants and database update. *Molecular Genetics & Genomic Medicine* 2014; 2:(3) 217-228.

II. Vályi P, Farkas K, Tripolszki K, **Sulák A**, Széll M, Nagy N, Nagy K. Rekurrens európai misszensz mutáció egy magyar Papillon-Lefévre szindrómában szenvedő családban. *Fogorvosi Szemle* 2014; 107:(3) 87-92.

Cumulative impact factor of the scientific publications providing the basis of the dissertation: 4.44

Publications indirectly related to the subject of the dissertation

I.   Nagy N, Farkas K, Tripolszki K, **Sulák A**, Kemény L, Széll M. A cylindromatosis gén mutációi által okozott genodermatosisok. *Bőr Vener Szemle* 2014; 90:(5) 185-193.

II. Nemes E, Farkas K, Kocsis-Deák B, Drubi A, **Sulák A**, Tripolszki K, Dósa P, Ferenc L, Nagy N, Széll M. Phenotypical diversity of patients with LEOPARD syndrome carrying the worldwide recurrent p.Tyr279Cys *PTPN11* mutation. *Arch Dermatol Res* 2015; 307(10):891-895. **IF: 2.327**

III. Tóth L, Fábos B, Farkas K, **Sulák A**, Tripolszki K, Széll M, Nagy N. Identification of two novel mutations in the *SLC45A2* gene in a Hungarian pedigree affected by unusual OCA type 4. *BMC Med Genet* 2017; 18(1):27. **IF: 2.198**

IV. Tripolszki K, Török D, Goudenège D, Farkas K, **Sulák A**, Török N, Engelhardt JI, Klivényi P, Procaccio V, Nagy N, Széll M. High-throughput sequencing revealed a novel *SETX* mutation in a Hungarian patient with amyotrophic lateral sclerosis. *Brain Behav* 2017; 7(4):e00669. **IF: 2.157**

V.   Fábos B, Farkas K, Tóth L, **Sulák A**, Tripolszki K, Tihanyi M, Németh R, Vas K, Csoma Z, Kemény L, Széll M, Nagy N. Delineating the genetic heterogeneity of *OCA* in Hungarian patients. *Eur J Med Res* 2017; 19;22(1):20. **IF: 1.414**

1. INTRODUCTION

1.1. Rare diseases

Rare diseases (RDs) are defined by the European Union as life-threatening or chronically debilitating conditions whose prevalence is less than 5 in 10 000 of the general population. Nowadays, there are more than 8000 distinct RDs and 6-8% of the European population is affected by a rare disease at some point in their lives (Baldovino *et al.*, 2016). In other words, 27 - 36 million persons are affected by thousands of different types of rare disease in the European Union (Aronson, 2006).

In opposite with common diseases, RDs are usually monogenic disorders and they are mostly determined by the presence or the absence of any causative genetic alteration which can cause the consequential failure of the certain protein and can lead to the development of the disease.

RDs represent a major challenge for health care organizations due to the small number of patients and the lack of the relevant knowledge and expertise of the specific rare disease. Nowadays, the attention is significantly directed to RDs: to improving the quality and traceability of RDs in health information systems, the European Commission aids the Orphanet approach by using Orphacodes. The Orphacode is a nomenclature based on published expert classifications and peer-reviewed papers (Aymé S *et al.,* 2015).

Investigations on RDs are essential for the development of the knowledge about the genetic background on these diseases and the research can provide a novel therapeutic modality for the affected patients in the future.

In my thesis, I have summarized the results of my genetic investigations in very stigmatizing, rare monogenic diseases: the clinical variants of the *cathepsin C* gene (*CTSC*) mutation-caused disease spectrum such as Papillon-Lefévre syndrome and Haim-Munk syndrome, atypical neurofibromatosis type 1 mimicking overgrowth syndrome and unilateral overgrowth of two fingers (macrodactyly).

1.2. The *CTSC* mutation-caused disease spectrum

1.2.1. Papillon-Lefévre syndrome

Papillon-Lefévre (PLS; OMIM 245000) syndrome is a rare, autosomal recessive disorder, characterized by symmetrical palmoplantar hyperkeratosis and severe periodontitis leading to the loss of both the primary and permanent teeth. Skin symptoms include transgrediens spread with hyperkeratosis of palms and soles. Patients with PLS can also develop mild mental retardation, calcification of the dura mater, hyperhidrosis and increased susceptibility to infections (Dalgic *et al.*, 2011; Gorlin *et al.*, 1964; Haneke, 1979). PLS was first decribed by Paul-Henri Papillon and Paul Lefévre in 1924 (Papillon and Lefévre, 1924).

The prevalence of PLS is estimated four cases per million and to date, approximately 300 cases have been reported worldwide. Parental consanguinity has been noted in more than 50% of these cases (Gorlin *et al.*, 1964; Hewitt *et al.*, 2004).

1.2.2. Haim-Munk syndrome

Haim-Munk syndrome (HMS; OMIM 245010) and PLS are characterized by overlapping dermatological and dental symptoms such as hyperkeratosis of the palms and soles as well as severe periodontitis (Nagy *et al.*, 2014; Selvaraju *et al.*, 2003). Besides these symptoms, specific features of HMS include pes planus, arachnodactyly, acroosteolysis and onychogryphosis (Haim and Munk, 1965; Hart *et al.*, 1999). HMS is named after Salim Haim and J. Munk who described for the first time the disease entity among a few members of an extended Jewish family from Cochin, India in 1965 (Haim and Munk, 1965). HMS is inherited as an autosomal recessive trait and the prevalence of the disease is approximately one case per million. Fewer than 100 HMS cases have been reported in the literature to date. The majority of these reported cases are descendants of a few consanguineous families from a religious isolate in Cochin, India. One unrelated Brazilian patient has also been reported (Haim and Munk, 1965; Hart *et al.*, 1999).

1.2.3. Aggressive periodontitis type 1

Aggressive periodontitis type 1 (AP1; OMIM 170650) is an autosomal recessive condition characterized by severe periodontal inflammation leading to tooth loss. The prevalence of the disease is approximately less than one case per million individuals and so far only a few cases were described in the literature (Hart *et al.,* 2000c; Hewitt *et al.*, 2004).

1.2.4. Genetic background

PLS, HMS and AP1 are both inherited in an autosomal recessive manner and develop as a consequence of mutations of the *cathepsin C* (*CTSC;* OMIM 602365) gene (Adkison *et al.*, 2002; Toomes *et al.*, 1999). The *CTSC* gene has been mapped to human chromosome 11q14-q21, spans over 44.2 kb and contains seven exons and six introns (Toomes *et al.*, 1999) (Figure 1).
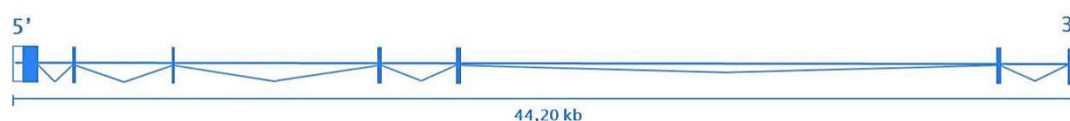
Figure 1. *Schematic drawing of the cathepsin C (CTSC) gene (Ensembl Genome Browser). The horizontal line illustrates the gene and the vertical lines indicate the exons.*

The *CTSC* gene encodes the cathepsin C protein, a 200 kDa lysosomal exo-cysteine proteinase belonging to the peptidase C1 family. The cathepsin C protein is a tetrameric enzyme consisting of four subunits linked together by non-covalent bonds (Dolenc *et al.*, 1995; Turk *et al.*, 2001). There are three polypeptide chains in each subunit: an N-terminal fragment or exclusion domain (∼13 kDa), a heavy chain (∼23 kDa), and a light chain (∼7 kDa) (Turk *et al.*, 2001) (Figure 2).
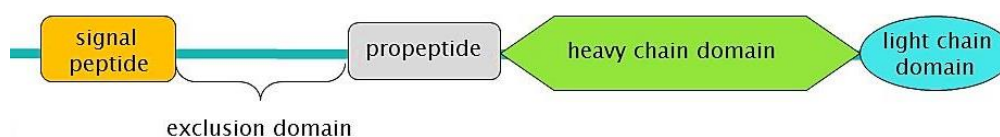
Figure 2. *Schematic drawing of the cathepsin C protein structure*

Cathepsin C plays an important role in various immune reactions and inflammatory reactions via activation of various granular serine proteinases from neutrophils (proteinase 3, elastase, cathepsin G and NSP-4) (Adkison *et al*., 2002; Perera *et al.*, 2013), mast cells (Wolters *et al.*, 2001), cytotoxic T lymphocytes and natural killer cells (Meade *et al.*, 2006). Cathepsin C was proved as the activator of the serine proteinases elastase, cathepsin G and proteinase 3 in polymorphonuclear leukocytes by Adkison *et al.* in a *cathepsin C* knockout mice model (Adkison *et al.,* 2002).

Previous studies have been reported 79 different *CTSC* gene mutations from ethnically diverse populations, which mainly include point mutations (Kobayashi *et al.*, 2013; Li *et al*., 2014; Nagy *et al.*, 2014; Tekin *et al*., 2016). However a large, 110 kb deletion (Chr11: 88032292:88142997 [NC_000011]) also have been identified by Wu *et al.* in 2016 (Wu *et al.*, 2016). The majority of the 79 mutations (78 of 79, 99%), have been detected in PLS patients, whereas only 4% (three of 79), have been associated with HMS (Hart *et al*., 1999; Kobayashi *et al.*, 2013; Li *et al*., 2014; Nagy *et al.*, 2014; Papillon and Lefévre, 1924; Selvaraju *et al.*, 2003; Tekin *et al*., 2016). Two mutations (c.145C/T p.Gln49X and c.857A/G p.Gln286Arg) were present in patients exhibiting both PLS and HMS phenotypes. Only one mutation (c.587T/C p.Leu196Pro) has been associated exclusively with HMS (Cury *et al.*, 2002; Papillon and Lefévre, 1924; Selvaraju *et al.*, 2003; Turk *et al.*, 2001)

1.3. *PIK3CA*-related overgrowth spectrum

Somatic mutations in the *phosphatidylinositol 3-kinase catalytic alpha* (*PIK3CA*, OMIM 171834) gene cause segmental overgrowth disorders. These *PIK3CA*-related overgrowth diseases include fibroadipose hyperplasia and non-classifiable conditions characterized by muscular, boney and fatty tissue overgrowth, congenital lipomatous overgrowth, vascular malformations, epidermal nevi and skeletal abnormalities (CLOVES) syndrome, macrodactyly, hemihyperplasia multiple lipomatosis, and the brain overgrowth conditions megalencephaly capillary malformation and megalencephaly-polymicrogyria-hydrocephalus syndrome (MPPH) (Kurek *et al.*, 2012; Lee *et al.*, 2012; Lindhurst *et al.*, 2012; Riviere *et al.*, 2012; Mirzaa *et al.*, 2013; Rasmussen *et al.*, 2014). These previously described

disease entities have overlapping clinical symptoms and represent a phenotypic spectrum (Keppler-Noreuil *et al.*, 2014). The phenotypic heterogeneity in these syndromes is attributed to the location of the cells bearing the mutation and to the proportion of the affected cells in the patient's tissues (Cohen *et al.*, 2014).

1.3.1. Macrodactyly

Macrodactyly (OMIM 155500) refers to a rare congenital malformation occurring in approximately 1 in 100,000 live births and is characterized by an increase in the size of all the structures of the limbs, including soft tissues, bones, vessels, nerves and skin (Rios *et al.*, 2013). It typically affects the terminal portions of the limb within a "nerve territory" and the individual peripheral nerve is both enlarged and elongated (Rios *et al.*, 2013). Recently macrodactyly has been added to the growing list of overgrowth syndromes (Kurek *et al*., 2012; Rios *et al.*, 2013; Emrick *et al.*, 2014).

1.3.2. Genetic background

Figure 3. *Schematic drawing of the phosphatidylinositol 3-kinase catalytic alpha (PIK3CA) gene (Ensembl Genome Browser). The horizontal line illustrates the gene and the vertical lines indicate the exons.*

The *PIK3CA* gene has been mapped to human chromosome 3q26.32, spans over 91,57 kb and comprises 20 coding exons (ENST00000263967.3, Ensembl Genome Browser) (Figure 3). The *PIK3CA* gene encodes the p110 alpha catalytic subunit of the phosphatidylinositol 3-kinase protein (PI3K). PI3K contains two subunits: an 85 kDa regulatory, and a 110 kDa catalytic subunit. This protein phosphorylates phosphatidylinositol (PtdIns), phosphatidylinositol 4-phosphate (PtdIns4P) and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) to generate phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 plays an important role via activating signaling cascades involved in cell growth, survival, proliferation, motility

and morphology by recruiting AKT1 and PDPK1 proteins to the cell membrane. Therefore, PI3K is an upstream regulator of the AKT-mTOR cell-signaling pathway (Rios *et al*., 2013) (Figure 4).

Cell cycle/apoptosis regulation, metabolism, angiogenesis
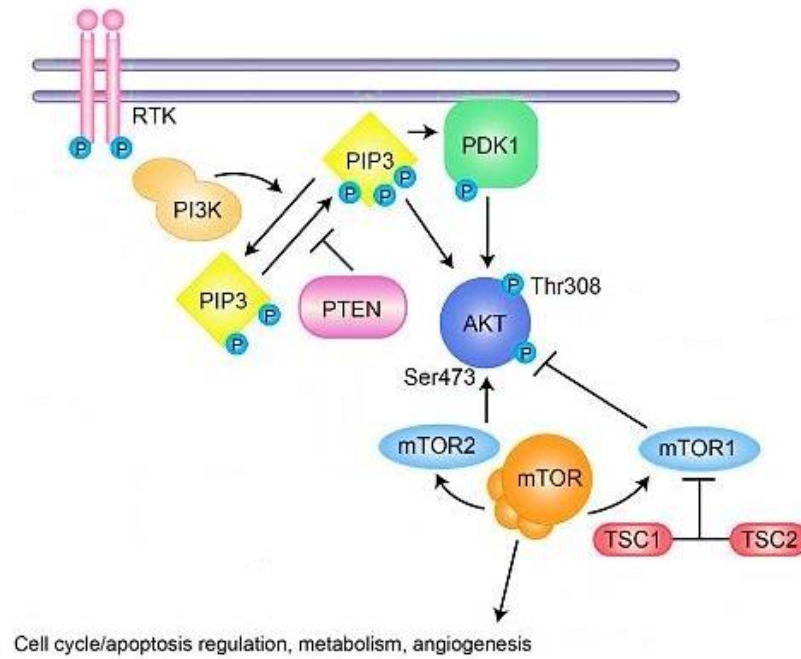
Figure 4. *PI3K-AKT signaling pathway. PI3K protein is an upstream regulator of the AKT-mTOR cell-signaling pathway. (Keppler-Noreuil et al., 2015)*

PIK3CA gene has been found the most commonly mutated proto-oncogene and has been implicated in human somatic cancers (Kandoth *et al.,* 2013). In the majority of patients with CLOVES, Klippel-Trenaunay syndrome, fibroadipose hyperplasia, hemimegalencephaly and macrodactyly have been reported three cancer hotspot mutations (p.Glu542Lys, p.Glu545Lys, and p.His1047Arg) (Cancer Genome Atlas Network, 2012; Hoadley *et al.,* 2014; Kandoth *et al.*, 2013). Previous studies have shown that postzygotic *PIK3CA* mutations are usually not detectable in blood samples of affected individuals, except in patients with megalencephaly capillary malformation syndrome (Keppler-Noreuil *et al.*, 2015; Mirzaa *et al*., 2016; Rivière *et al.*, 2012). The mutation screening of the *PIK3CA* gene requires specimens from an affected region, such as a skin biopsy or a surgical sample from an overgrown tissue (Kuentz *et al*., 2017).

1.4. Neurofibromatosis type 1

1.4.1. Clinical symptoms

Neurofibromatosis type 1 (NF1; OMIM 162200) is a rare monogenic disease with autosomal dominant inheritance due to mutations in the *neurofibromin* gene (*NF1;* OMIM 613113) (Huson, 2008; Mukonoweshuro *et al*., 1999). The clinical features of NF1 involve pigmentary changes such as café-au-lait macules and axillary freckling, the development of cutaneous fibromatous tumors and the development of hamartomas of the iris known as Lisch nodules (Huson, 2008; Mukonoweshuro *et al*., 1999). Besides these symptoms, central nervous system and skeletal abnormalities (mainly scoliosis, pseudoarthrosis of the tibia, macrocephaly and short stature) can occur (Stevenson *et al*., 1999; Pasmant *et al*., 2010; Spiegel *et al*., 2005). Frequently reported vascular symptoms are the vascular dysplasia with cerebral, gastro intestinal and/or renal involvement and the renovascular hypertension (Stevenson *et al*., 1999; Pasmant *et al*., 2010; Spiegel *et al*., 2005).
NF1 was firstly described by Professor Von Recklinghausen, a German pathologist in 1882 (Brosius, 2010). NF1 is one of the most common monogenic disorders worldwide, the prevalence of the disease is approximately 1 in 3000 live births (Yap *et al.*, 2014; Williams *et al.*, 2009).

1.4.2. Genetic background

The *NF1* gene has been localized to the human chromosome 17q11.2, contains 58 coding exons and spans over 282,75 kb (ENST00000358273.8, Ensembl Genome Browser). This gene encodes the neurofibromin, a cytoplasmic protein that is mainly expressed in neurons, Schwann cells, oligodendrocytes, and leukocytes. It is a multidomain molecule which is capable of regulating several intracellular processes, such as the RAS-cyclic AMP pathway, the ERK/MAP kinase cascade, adenylyl cyclase and cytoskeletal assembly (Trovó-Marqui and Tajara, 2006).

NF1 may be caused by many different loss-of-function mutations of the *NF1* gene (Huson, 2008; Mukonoweshuro *et al*., 1999). In about 5% of patients with NF1, microdeletions of the *NF1* gene and/or the surrounding genomic region are responsible for the disease (Pasmant *et al.,* 2010; Ning *et al.,* 2015). In patients with

NF1 microdeletion, somatic overgrowth has been frequently observed (Spiegel *et al.*, 2005). Mosaic overgrowth affecting a well-defined region of the body have only been rarely reported in NF1. In these previous reports, unilateral or bilateral breast enlargement (Sharma and Jain*,* 2009; Friedrich *et al.*, 2015), gingival overgrowth (Mahajan *et al.*, 2010) and clitoromegaly (Karabouta *et al*., 2015) were observed.

1.5. Aims

RDs represent a major challenge for health care organizations due to the small number of patients and the lack of the relevant knowledge and expertise of the specific rare disease. Therefore, my thesis focuses on the molecular genetic investigations of the RDs. The investigated RDs belong to the groups of the *CTSC*, *PIK3CA* and *NF1* mutations-caused disease spectrum. With the performed genetic and haplotype studies, my aim was to identify the underlying molecular mechanisms in patients affected by Papillon-Lefévre syndrome, Haim-Munk syndrome, atypical neurofibromatosis type 1, and unilateral macrodactyly. Although the investigated diseases involve rare phenotypic variants, their symptoms result in a huge life-long burden physically and mentally. Revealing the mechanism of the observed atypical phenotypes and phenotypic diversity in the investigated RDs contribute to the understanding of these diseases. The proposed genetic investigations might also lead to the identification of novel therapeutic target molecules and, eventually, to the development of novel therapeutic modalities for patients with RDs. These investigations have been performed in accordance with the current trends of biomedical research of the European Union, which supports the investigation of rare, so-called "neglected" diseases, since the mechanisms revealed in RDs would also lead to the further understanding of the mechanisms of common diseases. As my investigation on Papillon-Lefévre syndrome, Haim-Munk syndrome, atypical neurofibromatosis type 1, and unilateral macrodactyly might provide further insights into common mechanisms of palmoplantar hyperkeratosis and overgrowth of certain body parts.

2. PATIENTS AND METHODS

2.1. Patients

2.1.1. Hungarian patient affected by Haim-Munk syndrome

A 39-year-old Hungarian woman (Patient I) presented with a typical HMS phenotype. Mild hyperkeratotic plaques were observed symmetrically on her palms (Figure 5a) and soles (Figure 5c, 5d). Onychogryphosis and arachnodactyly were noted on her fingers (Figure 5b) and pes planus on her soles (Figure 5c, 5d). The patient lost all permanent teeth and uses a permanent dental prosthesis. She was brought up in state care without knowing her parents and has no husband or child. She was not aware of any known relatives.

Figure 5. *The clinical pictures of the HMS patient. Patient I, presenting with HMS, exhibited mild hyperkeratosis on her palms (a), onychogryphosis and arachnodactly of her fingers (b) and mild hyperkeratosis and pes planus on her soles (c,d). (Sulák et al., 2016)*

2.1.2. Hungarian patient affected by Papillon-Lefévre syndrome

A 25-year-old Hungarian man (Patient II) presented with the classical PLS phenotype. The hyperkeratosis on his palms (Figure 6b) and soles (Figure 6d) was more severe than the symptoms of Patient I. Onychogryphosis, arachnodactyly and pes planus were not present (Figure 6a, 6c). He was also missing all permanent teeth and using a permanent dental prosthesis. His parents and his wife were clinically unaffected. He had no siblings or children. He was not aware of any family members that are clinically affected.

Figure 6. *The clinical pictures of the PLS patient. Patient II, presenting with PLS, was affected by moderate hyperkeratosis of his palms (b) and soles (d) and exhibited no specific symptoms of HMS. (Sulák et al., 2016)*

Both patients were referred to the out-patient clinic of the Mór Kaposi Teaching Hospital (Kaposvár; Hungary).

2.1.3. Hungarian girl affected by isolated macrodactyly and syndactyly

A 4-year-old Hungarian girl was referred to the University of Szeged Department of Medical Genetics with isolated macrodactyly on the third and fourth fingers of the left hand (Figure 7a). X-ray imaging proved that the disease is characterized not only by the overgrowth of the soft tissues but also by the overgrowth of the bones of the affected fingers (Figure 7b). In addition to macrodactyly, syndactyly was associated with concrescence of the fingers and was constrained to the soft tissues of the affected fingers and not to the bones (Figure 7a, 7b). The macrodactyly and syndactyly of the left hand was present at birth and slowly progressed with the growth of the child. On examination, no vascular abnormality was present. There was no associated abnormality of the internal organs. Other body parts were symmetric and equally developed. The patient's parents were clinically unaffected, and they were not aware of any other family members with either macrodactyly or syndactyly (Figure 7c).
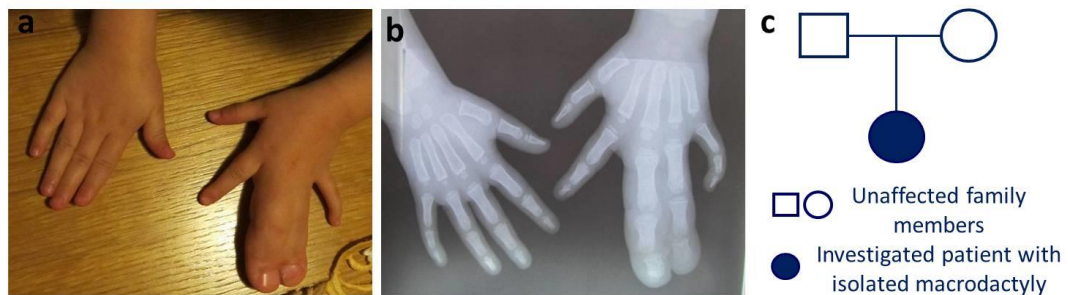

Figure 7. *Macrodactyly and syndactyly in a 4-year-old girl (a). Clinical features and bone radiographs of the patient (b). The patient's family is clinically asymptomatic (c). (Tripolszki K, Knox R, Parker V, Semple R, Farkas K, Sulák A et al., 2016)*

2.1.4. Hungarian patient affected by neurofibromatosis type 1

A 52-year-old Hungarian woman was referred to our out-patient clinic with an unusual phenotype exhibiting the clinical features of NF1. The patient was presented with the typical skin symptoms of NF1, including neurofibromas and cafe-au-lait macules on her body (Figure 8a, 8b) and axillary freckling. Ophthalmological examination determined the presence of Lisch nodules. Imaging studies did not find any indication of central nervous system malignancies. The skeletal abnormalities scoliosis, tibial pseudoarthrosis, short stature and macrocephaly were not present. Based on the clinical symptoms, the diagnosis of NF1 was established. In addition to the above described clinical features, the patient was also noted to have hypertrophy of the left leg, resulting in significant differences in the circumference and length of the legs (Figure 8c, 8d). This abnormality of the left leg was already present at birth. Imaging studies verified unilateral osteohypertrophy affecting the left leg.

When questioned about her family history, the patient was not aware of any relevant chronic diseases or other family members with NF1 or with overgrowth syndromes (Figure 8e).
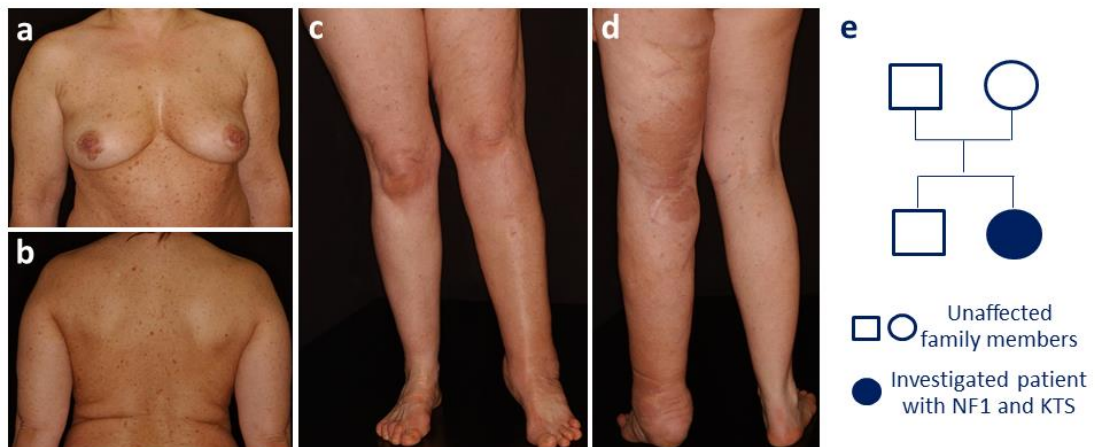

Figure 8. *The clinical symptoms of the affected Hungarian woman. The patient presenting the clinical phenotype of NF1 over her whole body, including her chest (a) and back (b). Unilateral osteohypertrophy affects her left leg (c, d). The rest of the patient's family are clinically asymptomatic (e). (Tripolszki K, Farkas K, Sulák A et al., 2017)*

2. 2. Methods

2. 2. 1. DNA isolation

The performed genetic investigations were approved by the Internal Review Board of the University of Szeged, which was conducted according to the Principles of the Declaration of Helsinki. After written informed consent was obtained from all investigated individuals, peripheral blood samples, tissue biopsy and deep surgical excision of the affected tissues were collected from the investigated patients and from unrelated controls for genetic analysis. Genomic DNA was extracted from the whole blood samples by a BioRobot EZ1 DSP Workstation (QIAGEN, Hilden, Germany). DNA extraction from tissues was isolated with the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). During the isolation, after proteinase K digestion, washings with alcohol were done following the instructions. Genomic DNA was dissolved in 100 µl distilled water.

2. 2. 2. Polymerase chain reaction (PCR) amplification

The coding regions and flanking introns of the investigated genes were amplified by PCR with specific primers. Amplifications were carried out in 20 µl volumes containing 4 µl sample DNA (50ng/µL), 9 µl Dream Taq Green PCR Master Mix (Fermentas), 4 µl distilled water and 1,5 - 1,5 µl of each primers (10 pmol/µL). The using primer sequences were obtained from the UCSC Genome Browser (www.genome.ucsc.edu) and Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/). The PCR conditions were the following: after an initial denaturation step at 95 °C for 10 min, 40 cycles of amplification was performed consisting of 30 sec at 95 °C (denaturation), 30 sec at the optimal annealing temperatures of the primers (~59°C) and 45 sec at 72°C (synthesis). The annealing temperature and the number of the cycles were depended on the primers, the synthesis reaction time was determined according to the length of the reaction product.

Finally a 10 min terminal elongation step was followed at 72°C in a MyCycler PCR machine (BioRad).

2. 2. 3. Gel electrophoresis and gel documentation

The PCR products were checked on 2% agarose gel (SeaKem LE agarose, Lonza) using TBE buffer (Lonza) and visualized by 2,5 µl GelRed (Biotium) staining. The gel was analyzed by BioRad Molecular Imager® GelDoc™ XR gel documentation system with QuantityOne software.

2. 2. 4. Sequencing

After amplifying the coding regions and flanking introns of the investigated genes, DNA sequencing was performed on the purified amplification products. The suitable PCR products were sequenced by a traditional capillary sequencer in an ABI Prism 7000 (Applied Biosystems) sequencing machine with Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Bio systems). The service of the sequencing was offered by Delta Bio 2000 Kft.

2. 2. 5. In-house PCR-based restriction fragment assay

The mutational hotspots of the *PIK3CA* gene were screened using an in-house PCR-based restriction fragment assay which has been previously described by Keppler-Noreuil et al. in 2014 (Cambridge, UK). Examining the region surrounding codon 542, a PCR product of 180 base pairs in length was amplified and digested with the *XbaI* restriction enzyme for 2 hours at 37°C (Figure 9). The resulting fragments were detected by fluorescent read-out using GeneMapper® software with GeneScan LIZ 500 (Life Technologies Corporation) as the size standard.
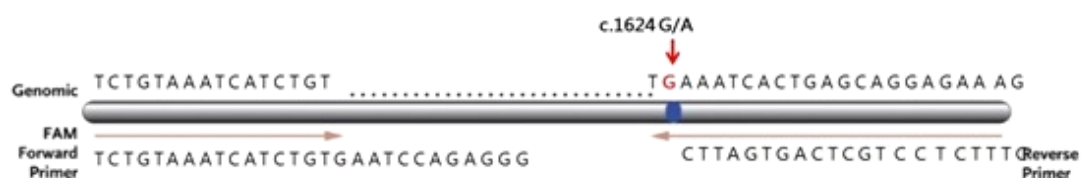
Figure 9. *The localization of the investigated mutation on the PIK3CA gene and the primer sequences used for PCR-based restriction fragment assay*

2. 2. 6. Haplotype analysis

For the haplotype analysis, common polymorphisms located in the 3' and 5' prime region of the identified mutation were genotyped using direct sequencing of the flanking coding and non-coding regions of the *CTSC* gene. Sequencing data was analyzed to screen for any additional mutations and to genotype all the common polymorphisms of the *CTSC* gene.

Based on the polymorphism data, haplotype analysis was performed to elucidate any familial relationship between the two investigated patients.

3. RESULTS

3.1. Genetic and haplotype investigations of the *cathepsin C* (*CTSC*) gene

3.1.1. Genetic investigation of the *CTSC* gene

After optimizing the PCR conditions, the coding regions and the flanking introns of the *CTSC* gene were amplified. Primers were designed on the UCSC Genome Browser (www.genome.ucsc.edu) (Table 1).

Primer	5'-3' sequence
CTSC-X1-Fw	CTC GGC TTC CTG GTA ATT CTT
CTSC-X1-Rev	GAA GCG GTA GTT GGC GTG
CTSC-X2-Fw	CAA ACT GGG TAG CAT GAA AGG
CTSC-X2-Rev	GAG TGG TGT CAA TTC CGG TC
CTSC-X3-Fw	GCC ATG GAA ATG GAC CTG
CTSC-X3-Rev	TGG TCC ATT ACT TTT GGA ACA CT
CTSC-X4-Fw	GCA CAG AGT GTG AAT GCC TG
CTSC-X4-Rev	AGG ACT GCT TAG GAG GGA GG
CTSC-X5-Fw	GGA AAT CAT CCT CAA AGG AAA G
CTSC-X5-Rev	GTA TCC CCG AAA TCC ATC AC
CTSC-X6-Fw	TGC ATG ATT CTC TGT GAG GC
CTSC-X6-Rev	GGC CAG ACT TGC ACT CAG AT
CTSC-X7a-Fw	TTC AGG GGT AAC ATG CAA AG
CTSC-X7a-Rev	CAT AGC CCA CAA GCA GAA CA
CTSC-X7b-Fw	CTG CAA TGA AGC CCT GAT G
CTSC-X7b-Rev	GAT TGC TGC TGA AAG TCT ACA GTC

Table 1. *The list of the CTSC primers used for genetic investigations.*

The results of PCR were checked by agarose gel electrophoresis (Figure 10) and DNA sequencing was performed.
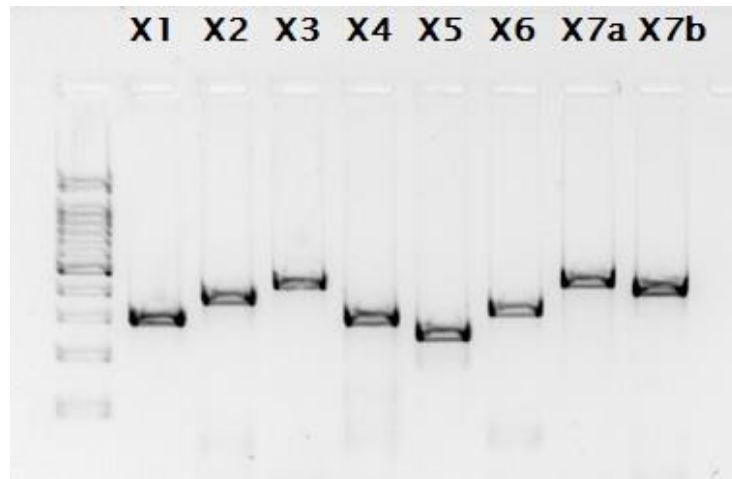
Figure 10. *The amplified products of the PCR were checked with gel electrophoresis. The 100bp DNA Ladder Molecular Weight Marker has eleven fragments that range in size from 100bp to 1000bp in 100bp increments with an additional band at 1500bp (Promega).*

Direct sequencing of the coding regions and the flanking introns of the *CTSC* gene from Patient I and Patient II revealed a nonsense mutation in the fifth exon (c.748C/T, p.Arg250X). The patients carried the mutation in homozygous form (Figure 11a), while the unrelated controls (n=100) carried the wild type sequence (Figure 11b).

Figure 11. *Mutation screening of the CTSC gene using direct sequencing revealed a nonsense mutation (c.748C/T, p.Arg250STOP) in the fifth exon (a). Both patients were homozygous for this mutation (a), wherease all the unrelated healthy controls (n=100) carried the wild-type sequence (b). (Sulák et al.,2016)*

3.1.2. Haplotype analysis of the patients diagnosed with PLS and HMS

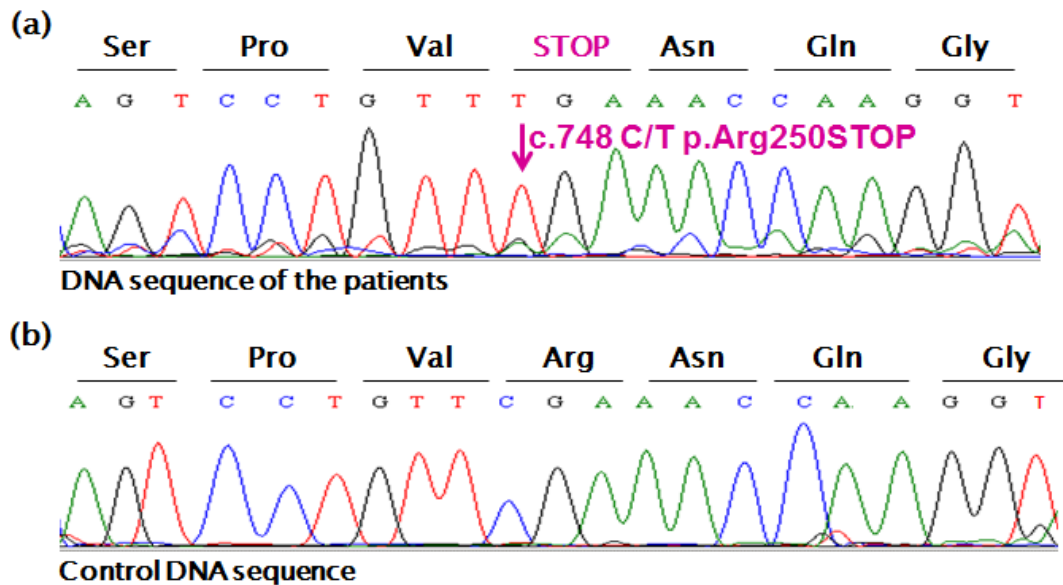The presence of the same homozygous nonsense mutation in both patients raised the possibility of familial relationship between them. To address this, the polymorphisms located in the 3' and 5' regions of the identified mutation were genotyped and the haplotypes were determined. The patients were homozygous for all the genotyped polymorphisms, and all genotypes were the same for both patients, indicating they carried exactly the same haplotype (Table 2).

Since the patients presented different phenotypes (i.e., HMS and PLS), but carried the same homozygous nonsense mutation, further screening was performed to identify a putative second genetic modifier variant within the *CTSC* gene. A comparison of all polymorphism and genotypes of the coding regions and flanking introns of the *CTSC* gene did not reveal any genetic difference between the two patients: they both carried exactly the same genotypes regarding all the investigated polymorphisms (Table 2).

Polymorphisms	Common allele	Rare allele	Patient I.	Patient II.	Unrelated control I.	Unrelated control II.	Unrelated control III.	Unrelated control IV.	Unrelated control V.	Unrelated control VI.	Unrelated control VII.	Unrelated control VIII.	Unrelated control IX.	Unrelated control X.
rs370927192	CTTT	-	CTTT/CTTT	CTTT/CTTT	-/-	CTTT/CTTT	CTTT/CTTT	CTTT/CTTT	CTTT/CTTT	CTTT/CTTT	CTTT/CTTT	CTTT/CTTT	CTTT/CTTT	CTTT/CTTT
CM002938*	C	T	TT	TT	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC
rs217116	A	G	AA	AA	GG	GG	GG	GG	AG	GG	GG	AG	AG	AA
rs217115	C	T	TT	TT	CC	CC	CT	CC	CC	CT	CT	CC	CC	CT
rs74325198	A	T	TT	TT	TT	TA	TT	TT	TT	TT	TA	TT	TT	TA

Table 2. *The results of the haplotype analysis. Patient I and II exhibited the same haplotype. (Sulák et al., 2016)*

3.2. Genetic investigation of the *phosphatidylinositol 3-kinase catalytic alpha* (*PIK3CA*) gene

After optimizing the PCR conditions, the coding regions and the flanking introns of the *PIK3CA* gene were amplified. Primers were designed on the UCSC Genome Browser (www.genome.ucsc.edu) (Table 3).

Primer	5'-3' sequence
PIK3CA-X2-Fw	GCT TTG GGA CAA CCA TAC ATC
PIK3CA-X2-Rev	ACG AAG GTA TTG GTT TAG ACA GAA A
PIK3CA-X3-Fw	AAT CTA CAG AGT TCC CTG TTT GC
PIK3CA-X3-Rev	TCA GTA TAA GCA GTC CCT GCC
PIK3CA-X4-Fw	GCA GCC CGC TCA GAT ATA A
PIK3CA-X4-Rev	CAG GAG AAT GGC ATG AAC C
PIK3CA-X5-Fw	TCT TGT GCT TCA ACG TAA ATC C
PIK3CA-X5-Rev	TTC GGA GAT TTG GAT GTT CTC
PIK3CA-X6-Fw	GTG GAT GAA GGC AGC AAC TA
PIK3CA-X6-Rev	CTG GCC AGT GCC TAG CTA AT
PIK3CA-X7-Fw	TGG TTG ATC TTT GTC TTC GTG
PIK3CA-X7-Rev	TTC AAT CAG CGG TAT AAT CAG G
PIK3CA-X8-Fw	TCT CAT GCT TGC TTT GGT TC
PIK3CA-X8-Rev	AGA CCA ATT GGC ATG CTC TT
PIK3CA-X9-Fw	TGG CAG TCA AAC CTT CTC TCT
PIK3CA-X9-Rev	GCC TTT CCC AGT GCT TTA CA
PIK3CA-X10-Fw	GTG AAT CCA GAG GGG AAA AAT
PIK3CA-X10-Rev	GCT GAG ATC AGC CAA ATT CA
PIK3CA-X11-Fw	CCT TTT GTG TTC TTT GCC ATT
PIK3CA-X11-Rev	AGC CAT CTA AAA CAC TGC CTT AAC
PIK3CA-X12-Fw	GCA GTG TTT TAG ATG GCT CAT T
PIK3CA-X12-Rev	CAA ATC AGG GTC AGT TTC TGC
PIK3CA-X13-Fw	CAT GCA GAA ACT GAC CCT GAT
PIK3CA-X13-Rev	TCT TCC AGC CAA ACA TAA ACA A
PIK3CA-X14-Fw	CTG AAA CTC ATG GTG GTT TTG T
PIK3CA-X14-Rev	CAC AAT GGC CTT ATG AAG CA
PIK3CA-X15-Fw	GGT ACA CAG TGC TGC CAG TC
PIK3CA-X15-Rev	GAA ATT CTG GCC ATA GAA GGT T

PIK3CA-X16-Fw	GGA TTC CTA AAT AAA AAT TGA GGT G
PIK3CA-X16-Rev	GCA GGG AAC AGG AAA AAG AA
PIK3CA-X17-Fw	CTG GGC AAC TTA GCA AGA CC
PIK3CA-X17-Rev	CAC TCC AGA GGC AGT AGC AG
PIK3CA-X18-Fw	TGG GGA AAG GCA GTA AAG G
PIK3CA-X18-Rev	TCT CTG TTC TAA CTC AGA GGA ATA CAC
PIK3CA-X19-20-Fw	ATG GAA ACT TGC ACC CTG TT
PIK3CA-X19-20-Rev	GAG TTG GGA TTA CAG GCA TGA
PIK3CA-X21-Fw	GAC ATT TGA GCA AGA CCT GA AG
PIK3CA-X21-Rev	ATG CTG TTC ATG GAT TGT GC

Table 3. *The list of the PIK3CA primers used for the genetic investigations.*

The results of the PCR were checked by agarose gel electrophoresis (Figure 12) and DNA sequencing was performed.
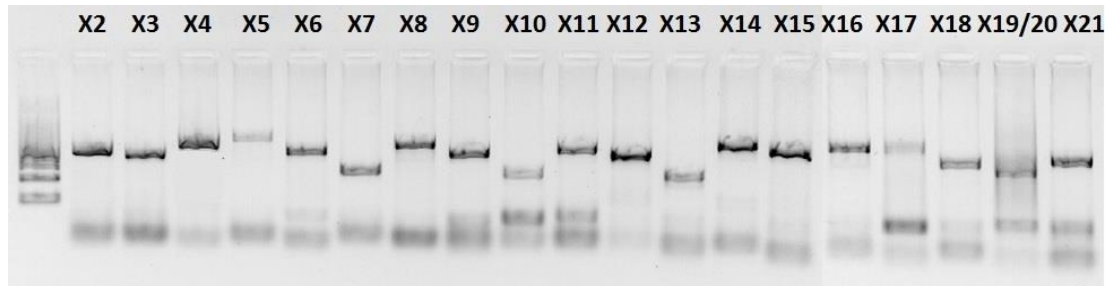

Figure 12. *The amplified products of the PCR were checked with gel electrophoresis. The 100bp DNA Ladder Molecular Weight Marker has eleven fragments that range in size from 100bp to 1000bp in 100bp increments with an additional band at 1500bp (Promega).*

Mutation analysis was performed from blood sample as well from the affected tissue sample, but the presence of any germline or somatic *PIK3CA* mutation was not detected by traditional Sanger sequencing.

Since traditional sequencing did not identify any putative causative variant of the *PIK3CA* gene, mutational hotspots were screened using an in-house PCR-based restriction fragment assay which has been previously described (Keppler-Noreuil *et al.*, 2014) (Cambridge, UK).

A somatic mosaic mutation at codon 542 (c.1624 G/A, p.Glu542Lys) was identified in the affected tissue with 4% mutation burden (Figure 13a). This mutation was not present in the genomic DNA sample isolated from the peripheral blood of

the patient (Figure 13b). This genetic analysis confirmed that the development of macrodactyly and syndactyly of the third and fourth fingers of the left hand are the consequence of the mosaicism of the p.Glu542Lys mutation in the *PIK3CA* gene.
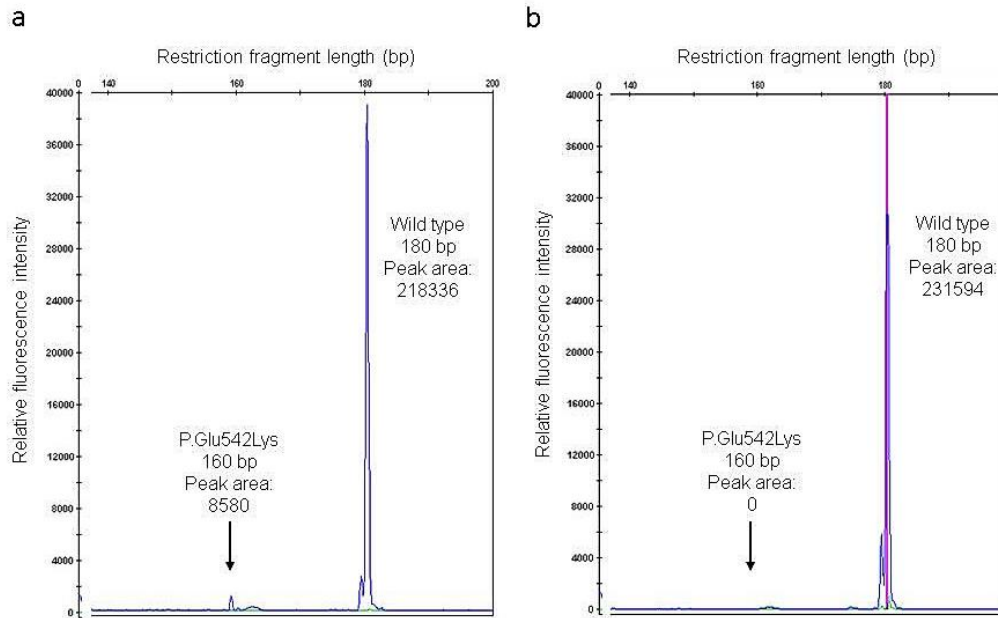

Figure 13. *Genetic investigation of the Hungarian patient with macrodactyly and syndactyly (a). DNA was extracted from the affected left hand and subjected to a PCR restriction assay, which identified the PIK3CA p.Glu542Lys mutation at 4% mutation burden (b). In DNA extracted from the peripheral blood, the peak indicating the presence of the mutation was absent in the digested PCR product, indicating the mosaicism of the mutation. (Tripolszki K, Knox R, Parker V, Semple R, Farkas K, Sulák A et al., 2016)*

3.3. Genetic investigation of the neurofibromin (*NF1*) gene

Direct sequencing of genomic DNA isolated from the peripheral blood of the patient using primers for the coding regions and the flanking introns of the *NF1* gene revealed a novel frameshift mutation (c.5727insT, p.V1909fsX1912; Figure 14) in exon 39. The patient carried the mutation in heterozygous form (Figure 14a), while the unrelated controls (n=50) carried the wild type sequence (Figure 14b).
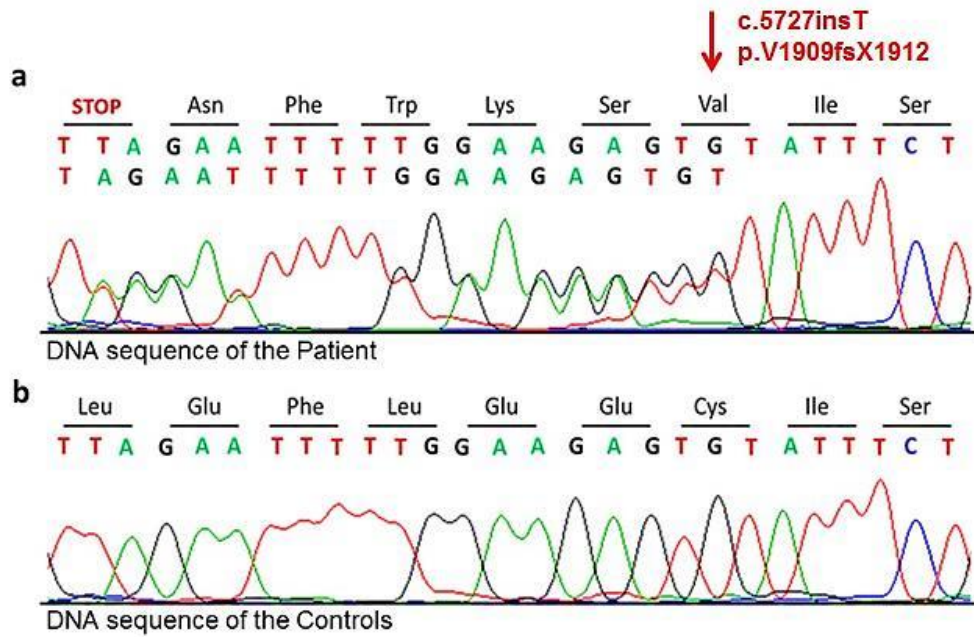
Figure 14. *Direct sequencing of the NF1 gene revealed a novel single-nucleotide insertion resulting in frameshift and the formation of premature termination codon (c.5727insT, p.V1909fsX1912). The patient carried the mutation in heterozygous form (a). Unrelated, healthy controls (n=50) carried only wild-type sequences (b). (Tripolszki K, Farkas K, Sulák A et al., 2017)*

To verify overgrowth syndrome, the coding regions and flanking introns of the *PIK3CA* gene were also amplified and sequenced from the peripheral blood and from the tissue sample of the left leg of the patient. Direct sequencing of the *PIK3CA* gene resulted in wild type sequence.

As traditional sequencing did not identify any putative causative variant of the *PIK3CA* gene, mutational hotspots were screened using an in-house PCR-based restriction fragment assay. Since these also did not identify any overgrowth-causing mutation, next-generation sequencing of approximately 2800 mutations from 50 oncogenes and tumor suppressor genes recorded in the Catalogue of Somatic Mutations in Cancer (COSMIC) was performed (Ion AmpliSeq[TMv] Cancer Hotspot Panel v2, Thermo Fisher Scientific) (Table 4), but also did not identify any putative causative variant that could be responsible for the overgrowth of the left leg.

ABL1	*EGFR*	*GNAS*	*KRAS*	*PTPN11*
AKT1	*ERBB2*	*GNAQ*	*MET*	*RB1*
ALK	*ERBB4*	*HNF1A*	*MLH1*	*RET*
APC	*EZH2*	*HRAS*	*MPL*	*SMAD4*
ATM	*FBXW7*	*IDH1*	*NOTCH1*	*SMARCB1*
BRAF	*FGFR1*	*IDH2*	*NPM1*	*SMO*
CDH1	*FGFR2*	*JAK2*	*NRAS*	*SRC*
CDKN2A	*FGFR3*	*JAK3*	*PDGFRA*	*STK11*
CSF1R	*FLT3*	*KDR*	*PIK3CA*	*TP53*
CTNNB1	*GNA11*	*KIT*	*PTEN*	*VHL*

Table 4. *The Ion AmpliSeq Cancer Hotspot Panel v2 targets these 50 genes (Thermo Fisher Scientific)*

4. DISCUSSION

4.1. A single nonsense mutation of the *CTSC* gene causes two clinically distinct phenotypes

I investigated two Hungarian patients affected by different phenotypic variants, one with PLS and one with HMS, who nonetheless carry the same homozygous nonsense mutation (c.748C/T; p.Arg250X) of the *CTSC* gene. Polymorphisms surrounding the mutation were investigated to determine whether these patients are relatives and to possibly identify a genetic modifier factor within the *CTSC* gene, which could be responsible for the development of the different phenotypes.

Currently, 79 different mutations have been reported worldwide for the *CTSC* gene (Kobayashi *et al.*, 2013; Li *et al.*, 2014; Nagy *et al.*, 2014; Tekin *et al.*, 2016). Nonsense mutations, accounting for 23% (n=18) of pathogenic *CTSC* mutations, occur in all coding regions of the gene; however the majority is located in exons 5–7, which encodes the heavy chain region of the cathepsin C protein (Kobayashi *et al.*, 2013; Nagy *et al.*, 2014; Hart *et al.*, 2000a). This protein is a lysosomal cysteine protease, and the heavy chain region is important for enzyme activity (Hart *et al.*, 2000a; Korkmaz *et al.*, 2010).
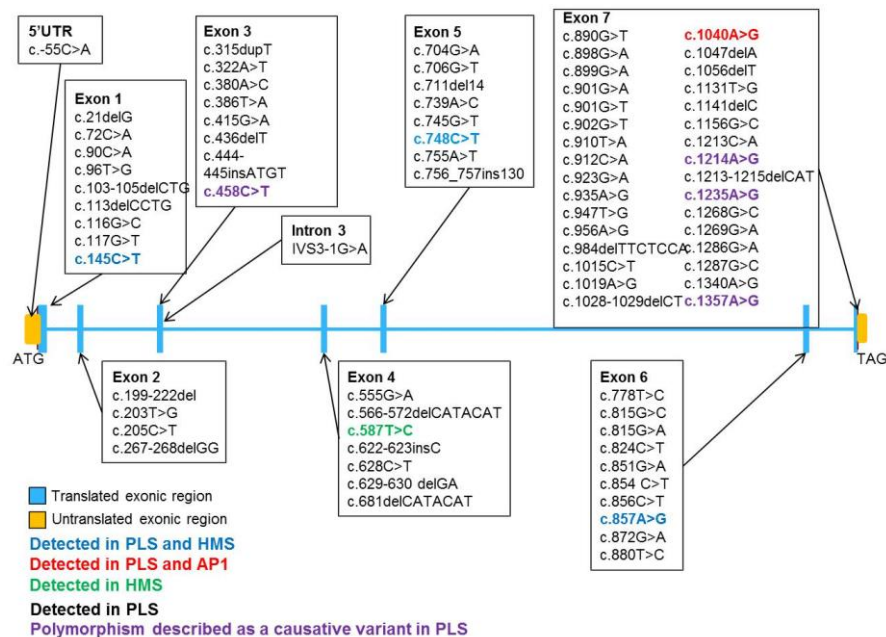

Figure 15. *CTSC gene mutations so far detected in PLS, HMS and AP1.*

The p.Arg250X homozygous nonsense mutation detected in the investigated patients is also located in this region. The p.Arg250X mutation leads to the formation of a truncated protein and may significantly impair enzyme activity. This hypothesis correlates well with previous studies demonstrating that pathological changes in the *CTSC* gene are loss-of-function mutations resulting in the inactivation of enzymatic activity and altered regulation of the immune response, which increase the susceptibility to periodontal inflammation and skin infections (Hart *et al.*, 2000b; Rai *et al.*, 2010).

The p.Arg250X nonsense mutation has already been previously reported in patients with PLS (Cury *et al.*, 2005; Pham *et al.*, 2004); however, this is the first report of its association with HMS. The mutation has been previously detected in homozygous (Cury *et al.*, 2005) and in compound heterozygous forms (Pham *et al.*, 2004). The latter mutation was suggested to be associated with an unidentified heterozygous mutation of the *CTSC* gene (Pham *et al.*, 2004). A previous investigation detected this mutation in homozygous form in Turkish patients (Cury *et al.*, 2005). Based on the common history of the two ethnic groups, we can not exclude that the identified mutation is the consequence of a single founder effect.

The two investigated Hungarian patients were affected by different variants (PLS and HMS) of the phenotypic spectrum caused by *CTSC* mutations. Clinical differences between the PLS and HMS symptoms of the patients were striking, although, surprisingly, genetic screening identified the presence of the same nonsense mutation (p.Arg250X) in homozygous form in both patients. Haplotype analysis revealed that the two patients exhibit the same haplotype (Table 2), indicating a strong likelihood of relatedness. The patients were not aware of any such relationship. Patient I was brought up in state care and did not know any of her relatives. Patient II was not aware of consanguinity within his family. However, our results and the fact that they share a common family name strongly suggest familial relationship between the two investigated patients.

As the patients had the same homozygous disease-causing mutation as well as the same haplotype, it was possible to examine genetic variations in the *CTSC* gene to identify any differences that could account for the development of the phenotypic differences. Our investigation could not identify any such genetic variant with the *CTSC* gene and flanking regions. Therefore, we hypothesize that the putative modifier factor, which results in the development of different phenotypic variants for

this *CTSC* mutation, is not located in the region of *CTSC*, but in another region. Moreover, we cannot exclude the possibility that the reason for the phenotypic variation is a non-genetic influence, such as environmental or life style factors.

Our results further support the accepted viewpoint that PLS and HMS are not different disease entities (Table 5), but that they are phenotypic variants of the same disease and their development is influenced by other factors. This phenomenon has also been observed in another group of rare monogenic diseases caused by mutations in the *CYLD* gene. Initially, Brooke-Spiegler syndrome (BSS; OMIM 605041), multiple familial trichoepithelioma type 1 (MFT1; OMIM 601606) and familial cylindromatosis (FC; OMIM 132700) were considered different entities, until genetic investigation revealed that they were allelic variants of the same disease (Nagy *et al*., 2013; Rajan *et al*., 2011). These observations highlight the importance of genetic investigation and the establishment of genotype–phenotype associations.

	Papillon-Lefévre syndrome (PLS)	**Haim-Munk syndrome (HMS)**	**Aggressive periodontitis type 1 (AP1)**
OMIM ID	245000	245010	170650
Clinical symptoms	Symmetrical palmoplantar hyperkeratosis, periodontitis	Symmetrical palmoplantar hyperkeratosis, periodontitis, arachnodactyly, acroosteolysis, onychogryphosis, pes planus	Severe periodontitis, tooth loss
CTSC **mutations**	All type of mutation	All type of mutation	All type of mutation

Table 5. *PLS, HMS and AP1 are phenotypic variants of the same rare disease.*

4.2. The somatic p.Glu542Lys *PIK3CA* mutation causes high phenotypic diversity in patients suffering from segmental overgrowth syndromes

Here we investigated a 4-year-old Hungarian patient with isolated macrodactyly and syndactyly of the third and fourth fingers of the left hand. Genetic investigation identified a somatic missense mutation (p.Glu542Lys) of the *PIK3CA* gene. This mutation affects the helical domain of the p110α catalytic subunit of the PI3K protein (Samuels *et al.*, 2004). Functional studies have previously shown that this p.Glu542Lys variant caused hyperactivation of AKT, a down-stream target of PI3K in the nerve cells of a patient with macrodactyly (Rios *et al.*, 2013). This particular mutation has been reported in eight patients with different forms of segmental overgrowth (Table 6) (Kurek *et al.*, 2012; Rios *et al.*, 2013; Maclellan *et al.*, 2013; Emrick *et al.*, 2014).

Patients	Index Patient	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9
Clinical diagnosis	Macrodactyly, syndactyly	Macrodactyly	Macrodactyly, Muscular hemihypertrophy	Facial infiltrating lipomatosis	Facial infiltrating lipomatosis	Facial infiltrating lipomatosis	CLOVES syndrome	CLOVES syndrome	CLOVES syndrome
Publication	This study	Rios et al., 2013	Rios et al., 2013	Maciellan et al., 2014	Maclellan et al., 2014	Maclellan et al., 2014	Kurek et al., 2012	Kurek et al., 2012	Emrick et al., 2014
Percentage (%) of somatic mosaicism	4	NR	NR	23	30	16-18	8	6-13	38
Lipomatous overgrowth									
Face	NR	NR	NR	+	+	+	NR	NR	NR
Trunk	NR	NR	NR	NR	NR	NR	+	+	+
Limb(s)	+	+	+	NR	NR	NR	+	+	+
Vascular anomalies									
Lymphatic malformation	NR	NR	NR	NR	NR	NR	+	+	+
Capillary malformation	NR	NR	NR	NR	NR	NR	+	+	+
Venous malformation	NR	NR	NR	NR	NR	NR	+	+	+
Musculoskeletal									
Macrodactyly	+	+	+	NR	NR	NR	+	+	+
Limb asymmetry	NR	NR	NR	NR	NR	NR	+	+	
Scoliosis	NR	NR	NR	NR	NR	NR	+		
True muscular hemihypertrophy	NR	NR	+	NR	NR	NR	NR	NR	NR
Other findings	NR	NR	NR	NR	NR	NR	Hypoplastic right kidney	NR	Epidermal nevi

Table 6. *Summary of the phenotypic diversity of the patients with somatic p.Glu542Lys PIK3CA mutation.(NR=not reported) (Tripolszki et al., 2016)*

Patients with somatic p.Glu542Lys mutation of the *PIK3CA* gene show high phenotypic diversity, Kurek *et al.* (2012) for example, described a female and a male patient affected by CLOVES syndrome. In addition to macrodactyly, both patients developed lipomatous overgrowth of the trunk and the limbs and vascular anomalies including lymphatic, capillary and venous malformations (Kurek *et al.*, 2012). The affected female patient also had a hypoplastic right kidney (Kurek *et al.*, 2012). A subsequent study reported the prenatal diagnosis of CLOVES syndrome in a 27-week-old fetus carrying the same somatic mutation of the *PIK3CA* gene (Emrick *et al.*, 2014). The observed clinical symptoms at birth were asymmetric chest and abdomen, bilateral multicystic malformations and asymmetric growth of the left leg with macrodactyly of the left foot and a sandal gap between the first and second toes (Emrick *et al.*, 2014). Rios *et al.* (2013) reported two patients with the same somatic mutation; both were affected by macrodactyly. However, one of the patients was also affected by true muscular hemihypertrophy, which was also attributed to the presence of the somatic p.Glu542Lys *PIK3CA* mutation. A subsequent study has also reported three further patients with facial infiltrating lipomatosis, which was attributed to the presence of the somatic p.Glu542Lys mutation (Maclellan *et al.*, 2013). These patients did not exhibit macrodactyly.

The high phenotypic diversity associated with the somatic p.Glu542Lys mutation might be explained by the different time points during embryogenesis of the mutational events. Patients with CLOVES syndrome might have developed the same somatic p.Glu542Lys mutation earlier during the embryogenesis than the ones with regional overgrowth (macrodactyly or facial infiltrating lipomatosis).

Genetic analysis has a huge significance for these patients, as once the genetic cause is determined, pharmacological intervention could be considered as a therapeutic option. In the interim, there are no clinically approved therapies for this condition; however there is a theoretical possibility that small molecule inhibitors of the PI3K-AKT-mTOR signaling pathway could be effective therapies for these patients (Segerstrom *et al.*, 2011; Iacobas *et al.*, 2011). Rapamycin (sirolimus) indirectly targets PI3K and may also be useful in treating macrodactyly (Segerstrom *et al.*, 2011; Iacobas *et al.*, 2011). In addition, rapamycin has been reported to be effective in isolated cases of allied conditions and may promote a breakthrough in the treatment of macrodactyly and overgrowth syndromes. However, long-term safety data of this treatment in PIK3CA-related overgrowth is currently lacking and, thus,

indicates the need for formal clinical trials to evaluate safety and efficacy. In light of the current situation, detailed genetic investigation and publication of these isolated cases is essential.

4.3. A novel mutation of the *NF1* gene affects an atypical manifestation in neurofibromatosis type 1

Recently we investigated a Hungarian woman presenting with the clinical features of both NF1 and left leg overgrowth. Such an atypical clinical form of NF1 has not been reported previously. However, the phenomenon of 'vascular neurofibromatosis phenotype' and the relatively frequent association of NF1 with vascular dysplasia have been frequently reported in the literature (Reubi, 1945). NF1 can also be accompanied by skeletal abnormalities, such as sphenoid wing dysplasia, macrocephaly, scoliosis, vertebral disc dysplasia, pseudoarthrosis of tibia and short stature (Ferner *et al.*, 2007; Williams *et al.*, 2009). Therefore, it was important to distinguish whether the patient was affected by two independent rare diseases or whether the symptoms of the left leg were the results of the atypical vascular and skeletal manifestations of NF1.

Our results demonstrated a novel heterozygous single-nucleotide insertion in the *NF1* gene, leading to frameshift and the formation of a premature termination codon (c.5727insT, p.V1909fsX1912). Because this mutation was present in the genomic DNA isolated from the peripheral blood of the patient, we suggest that this sequence change is a germline mutation. Considering that there is no other clinically affected member in the patient's family and that NF1exhibits autosomal dominant inheritance, we hypothesize that this is a *de novo* mutation of the *NF1* gene. The genotype–phenotype correlation is generally poor in NF1, with the exception that patients with large deletions in the *NF1* gene tend to have severe phenotypes (Heim *et al.*, 1994). *NF1* mutations usually result in loss of tumor suppressor function by disrupting the neurofibromin protein's ability to maintain the proto-oncogene RAS in an inactive form (Yohay, 2006). We suggest that this novel *NF1* mutation is the causative mutation for the development of NF1, and the presence of the unilateral limb hypertrophy is highly possible also the consequence of this mutation.

In conclusion, we investigated a patient affected with both the clinical features of NF1 and overgrowth of the left leg. Our results demonstrate that the patient is not suffering from two independent rare diseases, but the unusual clinical phenotype of NF1. To our knowledge, our study is the first to clearly elucidate the genetic background of such a complex case, and further confirms the causative role of the somatic mutations of the *NF1* gene in the development of overgrowth.

5. SUMMARY

In my PhD dissertation, my aim was to summarize the genetic and haplotype investigations in Hungarian patients affected by different rare diseases, such as Papillon-Lefévre and Haim-Munk syndromes, unilateral macrodactyly and atypical neurofibromatosis type 1.

Mutation screening of the *CTSC* gene from two Hungarian patients revealed the presence of the same homozygous nonsense mutation (c.748C/T; p.Arg250X). However, one patient exhibited the PLS phenotype and the other the HMS phenotype. Although these patients were not aware that they were related, haplotype analysis revealed that they carry the same haplotype, and the possibility that they are related cannot be excluded.
Our results support the hypothesis that PLS and HMS are the phenotypic variants of the same disease and, additionally, exclude the presence of a putative genetic modifier factor within the *CTSC* gene that is responsible for the development of the two phenotypes. We hypothesize that this putative genetic modifier factor is located outside the *CTSC* gene or, alternatively, that the development of the different phenotypes is the consequence of different environmental or life style factors.

Isolated macrodactyly belongs to a heterogeneous group of overgrowth syndromes. It is a congenital anomaly resulting in enlargement of all tissues localized to the terminal portions of a limb and caused by somatic mutations in the *PIK3CA* gene. Here we investigated a Hungarian girl with macrodactyly and syndactyly. Genetic screening at hotspots in the *PIK3CA* gene identified a mosaic mutation (c.1624 G/A, p.Glu542Lys) in the affected tissue, but not in the peripheral blood. To date, this somatic mutation has been reported in eight patients affected by different forms of segmental overgrowth syndromes. Detailed analysis of the Hungarian child and previously reported cases suggests high phenotypic diversity associated with the p.Glu542Lys somatic mutation. The identification of the mutation provides a novel therapeutic modality for the affected patients: those who carry somatic mutations in the *PIK3CA* gene are potential recipients of a novel "repurposing" approach of rapamycin treatment.

Neurofibromatosis type 1, a dominantly inherited multitumor syndrome, results from mutations in the *NF1* gene. Recently we investigated a Hungarian woman with the clinical phenotype of NF1 over her whole body and the clinical features of unilateral overgrowth involving her entire left leg. This unusual phenotype suggested either the atypical form of NF1 or the coexistence of NF1 and overgrowth syndrome. Direct sequencing of the genomic DNA isolated from peripheral blood revealed a novel frameshift mutation (c.5727insT, p.V1909fsX1912) in the *NF1* gene. Next-generation sequencing of 50 oncogenes and tumor suppressor genes, performed on the genomic DNAs isolated from tissue samples and peripheral blood, detected only wild-type sequences. Based on these results, we concluded that the patient is affected by an unusual phenotype of NF1, and the observed unilateral overgrowth of the left leg might be a rare consequence of the identified c.5727insT mutation.

6. ACKNOWLEDGEMENT

7. ELECTRONIC DATABASE INFORMATION

Ensemble Genome Browser (for the wild type sequencing data of the human genome, for the gene variation database regarding disease-causing and non-causing alterations and for the taxonomy analysis of the identified mutation) www.ensemble.org

Online Mendelian Inheritance in Man (for the detailed informations on the genetics, inheritance, clinical features and identified mutations in monogenic disorders) www.omim.org

Orphanet Database (for the collection and detailed description of rare diseases) www.orpha.net

UCSC Genome Browser, Primer3 (for the design of specific primers used to amplify the sequenced regions of the genes) http://genome.ucsc.edu/; http://bioinfo.ut.ee/primer3-0.4.0/

PubMed (for the literature search to identify the previously published cases) http://www.ncbi.nlm.nih.gov/pubmed

UniProt, SMART (for the detailed information of protein sequences and functional information) www.uniprot.org; smart.embl-heidelberg.de/

8. REFERENCES

1. Adkison AM, Raptis SZ, Kelley DG, *et al*. Dipeptidyl peptidase I activates neutrophil- derived serine proteases and regulates the development of acute experimental arthritis. J Clin Invest 2002; 109: 363-71.

2. Aronson JK. Rare diseases and orphan drugs. Br J Clin Pharmacol 2006; 61 (3): 243-5.

3. Aymé S, Bellet B, Rath A. Rare diseases in ICD11: making rare diseases visible in health information systems through appropriate coding. Orphanet J Rare Dis 2015; 10: 35.

4. Baldovino S, Moliner AM, Taruscio D, et al. Rare Diseases in Europe: from a Wide to a Local Perspective. Isr Med Assoc J. 2016; 18(6): 359-63.

5. Brosius S. A history of von Recklinghausen's NF1. J Hist Neurosci. 2010; 19(4): 333-48.

6. Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. Nature 2012; 490: 61–70.

7. Cohen AS, Townsend KN, Xiang QS, *et al*. Somatic mosaicism for the p.His1047Arg mutation in PIK3CA in a girl with mesenteric lipomatosis. Am J Med Genet A 2014; 164A: 2360-4.

8. Cury VF, Costa JE, Gomez RS, *et al*. A novel mutation of the cathepsin C gene in Papillon-Lefevre syndrome. J Periodontol 2002; 75: 307-12.

9. Cury VF, Gomez RS, Costa JE, *et al*. A homozygous cathepsin C mutation associated with Haim-Munk syndrome. Br J Dermatol 2005; 152: 353-6.

10. Dalgic B, Bukulmez A, Sari S. Eponym: Papillon-Lefevre syndrome. Eur J Pediatr 2011; 170: 689-91.

11. Dolenc I, Turk B, Pungercic G, *et al*. Oligomeric structure and substrate induced inhibition of human cathepsin C. J Biol Chem 1995; 270(37): 21626-31.

12. Emrick LT, Murphy L, Shamshirsaz AA, *et al*. Prenatal diagnosis of CLOVES syndrome confirmed by detection of a mosaic PIK3CA mutation in cultured amniocytes. Am J Med Genet A 2014; 164A: 2633-7.

13. Ferner RE, Huson SM, Thomas N, *et al*. Guidelines for the diagnosis and management of individuals with neurofibromatosis 1. J Med Genet 2007; 44: 81–8.

14. Friedrich RE, Hagel C, Mautner VF. Unilateral gynaecomastia in a 16-month-old boy with neurofibromatosis type 1 – case report and brief review of the literature. GMS Interdiscip Plast Reconstr Surg DGPW. 2015; 4: Doc11.

15. Gorlin RJ, Sedano H, Anderson VE. The syndrome of palmar-plantar hyperkeratosis and premature periodontal destruction of the teeth: a clinical and genetic analysis of the Papillon-Lefevre syndrome. J Pediat 1964; 65: 895-908.

16. Haim S, Munk J. Keratosis palmo-plantaris congenita, with periodontosis, arachnodactyly and a peculiar deformity of the terminal phalanges. Br J Dermatol 1965; 77: 42-54.

17. Haneke E. The Papillon-Lefevre syndrome: keratosis palmoplantaris with periodontopathy: report of a case and review of the cases in the literature. Hum Genet 1979; 51: 1-35.

18. Hart PS, Zhang Y, Firatli E, *et al*. Identification of cathepsin C mutations in ethnically diverse Papillon-Lefevre syndrome patients. J Med Genet 2000a; 37: 927-32.

19. Hart TC, Hart PS, Bowden DW, *et al.* Mutations of the cathepsin C gene are responsible for Papillon-Lefevre syndrome. J Med Genet 1999; 36: 881-7.

20. Hart TC, Hart PS, Michalec MD, *et al*. Haim-Munk syndrome and Papillon-Lefevre syndrome are allelic mutations in cathepsin C. J Med Genet 2000b; 37: 88-94.

21. Hart TC, Hart PS, Michalec MD, *et al.* Localisation of a gene for prepubertal periodontitis to chromosome 11q14 and identification of a cathepsin C gene mutation. J Med Genet 2000c; 37: 95-101.

22. Heim RA, Silverman LM, Farber RA, *et al.* Screening for truncated NF1 proteins. Nat Genet 1994; 8: 218–9.

23. Hewitt C, McCormick D, Linden G, *et al.* The role of cathepsin C in Papillon-Lefevre syndrome, prepubertal periodontitis, and aggressive periodontitis. Hum Mutat 2004; 23: 222-8.

24. Hoadley KA, Yau C, Wolf DM, *et al*.; Cancer Genome Atlas Research Network. Multiplatform analysis of 12 cancer types reveals molecular classification within and across tissues of origin. Cell 2014; 158: 929–44.

25. Huson SM. The neurofibromatosis: classification, clinical features and genetic counselling. In:Neurofibromatoses (Kaufmann D, ed). Basel: Karger, 2008; 1–20.

26. Iacobas I, Burrows PE, Adams DM, *et al*. Oral rapamycin in the treatment of patients with hamartoma syndromes and PTEN mutation. Pediatr Blood Cancer 2011; 57: 321-3.

27. Kandoth C, McLellan MD, Vandin F, *et al.* Mutational landscape and significance across 12 major cancer types. Nature 2013; 502: 333–9.

28. Karabouta Z, Rousso I, Athanassiadou-Piperopoulou F. Clitoromegaly as first presentation of a neurocutaneous syndrome in a 3-year-old girl. Clin Case Rep. 2015; 3(9): 767-8.

29. Keppler-Noreuil KM, Rios JJ, Parker VE, *et al.* PIK3CA-related overgrowth spectrum (PROS): diagnostic and testing eligibility criteria, differential diagnosis, and evaluation. Am J Med Genet A 2015; 167A: 287–95.

30. Keppler-Noreuil KM, Sapp JC, Lindhurst MJ, *et al*. Clinical delineation and natural history of the PIK3CA-related overgrowth spectrum. Am J Med Genet A 2014; 164A(7): 1713-33.

31. Kobayashi T, Sugiura K, Takeichi T, *et al*. The novel CTSC homozygous nonsense mutation p.Lys106X in a patient with Papillon-Lefèvre syndrome with all permanent teeth remaining at over 40 years of age. Br J Dermatol 2013; 169(4): 948-50.

32. Korkmaz B, Horwitz MS, Jenne DE, *et al*. Neutrophil elastase, proteinase 3, and cathepsin G as therapeutic targets in human diseases. Pharmacol Rev 2010; 62: 726-59.

33. Kuentz P, St-Onge J, Duffourd Y, *et al*. Molecular diagnosis of PIK3CA-related overgrowth spectrum (PROS) in 162 patients and recommendations for genetic testing. Genet Med. 2017 Feb 2. doi: 10.1038/gim.2016.220.

34. Kurek KC, Luks VL, Ayturk UM, *et al.* Somatic mosaic activating mutations in PIK3CA cause CLOVES syndrome. Am J Hum Genet 2012; 90: 1108-15.

35. Lee JH, Huynh M, Silhavy JL, *et al.* De novo somatic mutations in components of the PI3K-AKT3-mTOR pathway cause hemimegalencephaly. Nat Genet 2012.; 44: 941-5.

36. Li Z, Liu J, Fang S, *et al.* Novel compound heterozygous mutations in CTSC gene cause Papillon-Lefèvre syndrome with high serum immunoglobulin E. J Dermatol Sci 2014; 76(3) :258-60.

37. Lindhurst MJ, Parker VE, Payne F, *et al.* Mosaic overgrowth with fibroadipose hyperplasia is caused by somatic activating mutations in PIK3CA. Nat Genet 2012; 44: 928-33.

38. Maclellan RA, Luks VL, Vivero MP, *et al.* PIK3CA activating mutations in facial infiltrating lipomatosis. Plast Reconstr Surg 2014; 133: 12e-19e.

39. Mahajan A, Dixit J, Bhardwaj A. Gingival enlargement in neurofibromatosis type 1: a case report and literature review. J Contemp Dent Pract. 2010; 11(2): 057-63.

40. Meade JL, de Wynter EA, Brett P, *et al*. A family with Papillon-Lefevre syndrome reveals a requirement for cathepsin C in granzyme B activation and NK cell cytolytic activity. Blood 2006; 107: 3665-8.

41. Mirzaa G, Conway R, Graham JM, *et al.* PIK3CA-Related Segmental Overgrowth. In: Pagon RA, Adam MP, Ardinger HH, Bird TD, Dolan CR, Fong CT, Smith RJH, Stephens K, editors. GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2014. Available from://www.ncbi.nlm.nih.gov/books/NBK153722/.

42. Mirzaa G, Timms AE, Conti V, *et al*. PIK3CA-associated developmental disorders exhibit distinct classes of mutations with variable expression and tissue distribution. JCI Insight 2016; 1:e87623.

43. Mukonoweshuro W, Griffiths PD, Blaser S. Neurofibromatosis type 1: the role of neuroradiology. Neuropediatrics 1999; 30: 111–9.

44. Nagy N, Rajan N, Farkas K, *et al*. A mutational hotspot in CYLD causing cylindromas: a comparison of phenotypes arising in different genetic backgrounds. Acta Derm Venereol 2013; 93: 743-5.

45. Nagy N, Vályi P, Csoma Zs, *et al.* CTSC and Papillon-Lefèvre syndrome: detection of recurrent mutations in Hungarian patients, a review of published variants and database update. Mol Gen & Genom Med 2014; 2: 217-28.

46. Ning X, Farschtschi S, Jones A, *et al*. Growth in neurofibromatosis 1 microdeletion patients. Clin Genet. 2016; 89(3): 351-4.

47. Papillon MM, Lefèvre P. Deux cas de kératodermie palmaire et plantaire symétrique familiale (maladie de Meleda) chez le frère et la soeur. Coexistence dans les deux cas d'altérations dentaires graves. Bulletin de la Société française de dermatologie et de vénéorologie, Paris 1924; 31: 82-7.

48. Pasmant E, Sabbagh A, Spurlock G, *et al*. NF1 microdeletions in neurofibromatosis type 1: from genotype to phenotype. Hum Mutat 2010; 31: 1506–18.

49. Perera NC, Wiesmuller KH, Larsen MT, *et al.* NSP4 is stored in azurophil granules and released by activated neutrophils as active endoprotease with restricted specificity. J Immunol 2013; 191: 2700-7.

50. Pham CT, Ivanovich JL, Raptis SZ, *et al*. Papillon-Lefevre syndrome: correlating the molecular, cellular, and clinical consequences of cathepsin C ⁄ dipeptidyl peptidase I deficiency in humans. J Immunol 2004; 175: 7277–81.

51. Rai R, Thiagarajan S, Mohandas S, *et al*. HaimMunk syndrome and Papillon Lefevre syndrome--allelic mutations in cathepsin C with variation in phenotype. Int J Dermatol 2010; 49: 541-3.

52. Rajan N, Burn J, Langtry J, *et al*. Transition from cylindroma to spiradenoma in CYLD-defective tumours is associated with reduced DKK2 expression. J Pathol 2011; 224: 309-21.

53. Rasmussen M, Sunde L, Weigert KP, *et al.* Segmental overgrowth syndrome due to an activating PIK3CA mutation identified in affected muscle tissue by exome sequencing. Am J Med Genet A 2014; 164A: 1318-21.

54. Reubi F. Neurofibromatose et lesions vasculaires. Schweiz Med Wochenschr 1945; 75: 463–5.

55. Rios JJ, Paria N, Burns DK, *et al.* Somatic gain-of-function mutations in PIK3CA in patients with macrodactyly. Hum Mol Genet 2013; 22: 444-51.

56. Rivière JB, Mirzaa GM, O'Roak BJ, *et al*. De novo germline and postzygotic mutations in AKT3, PIK3R2 and PIK3CA cause a spectrum of related megalencephaly syndromes. Nat Genet 2012; 44: 934-40.

57. Samuels Y, Wang Z, Bardelli A, *et al*. High frequency of mutations of the PIK3CA gene in human cancers. Science 2004; 304(5670): 554.

58. Segerström L, Baryawno N, Sveinbjörnsson B, *et al.* Effects of small molecule inhibitors of PI3K/Akt/mTOR signaling on neuroblastoma growth in vitro and in vivo. Int J Cancer 2011; 129: 2958-65.

59. Selvaraju V, Markandaya M, Prasad PV, *et al*. Mutation analysis of the cathepsin C gene in Indian families with Papillon-Lefèvre syndrome. BMC Med Genet 2003; 4: 5.

60. Sharma R, Jain V. Bilateral breast enlargement in a male toddler: an unusual cause. Indian J Pediatr. 2009; 76(11): 1164–6.

61. Spiegel M, Oexle K, Horn D, *et al*. Childhood overgrowth in patients with common NF1 microdeletions. Eur J Hum Genet 2005; 13: 883–8.

62. Stevenson DA, Birch PH, Friedman JM, *et al*. Descriptive analysis of tibial pseudarthrosis in patients with neurofibromatosis 1. Am J Med Genet 1999; 84: 413–9.

63. Tekin B, Yucelten D, Beleggia F, *et al.* Papillon-Lefèvre syndrome: report of six patients and identification of a novel mutation. Int J Dermatol 2016; 55(8): 898-902.

64. Toomes C, James J, Wood AJ, *et al.* Loss-of-function mutations in the cathepsin C gene result in periodontal disease and palmoplantar keratosis. Nat Genet 1999; 23: 421-4.

65. Trovó-Marqui AB, Tajara EH. Neurofibromin: a general outlook. Clin Genet. 2006; 70(1): 1-13.

66. Turk D, Janjic V, Stern I, *et al*. Structure of human dipeptidyl peptidase I (cathepsin C): exclusion domain added to an endopeptidase framework creates the machine for activation of granular serine proteases. EMBO J 2001; 20(23): 6570-82.

67. Williams VC, Lucas J, Babcock MA, *et al.* Neurofibromatosis type 1 revisited. Pediatrics 2009; 123:124–33.

68. Wolters PJ, Pham CT, Muilenburg DJ, *et al*. Dipeptidyl peptidase I is essential for activation of mast cell chymases, but not tryptases, in mice. J Biol Chem 2001; 276: 18551-6.

69. Wu W, Chen B, Chen X, *et al*. A novel large deletion combined with a nonsense mutation in a Chinese child with Papillon-Lefèvre syndrome. J Periodontal Res 2016; 51(3): 376-80.

70. Yap YS, McPherson JR, Ong CK, *et al*. The NF1 gene revisited - from bench to bedside. Oncotarget. 2014; 5(15): 5873-92.

71. Yohay KH. The genetic and molecular pathogenesis of NF1 and NF2. Semin Pediatr Neurol 2006; 13: 21–6.

9. APPENDIX

Abbreviations of the investigated 50 oncogenes and tumor suppressor genes

ABL1: ABL proto-oncogene 1, non-receptor tyrosine kinase

AKT1: AKT serine/threonine kinase 1

ALK: ALK receptor tyrosine kinase

APC: APC, WNT signaling pathway regulator

ATM: ATM serine/threonine kinase

BRAF: B-Raf proto-oncogene, serine/threonine kinase

CDH1: Cadherin 1

CDKN2A: Cyclin dependent kinase inhibitor 2A

CSF1R: Colony stimulating factor 1 receptor

CTNNB1: Catenin beta 1

EGFR: Epidermal growth factor receptor

ERBB2: Erb-b2 receptor tyrosine kinase 2

ERBB4: Erb-b2 receptor tyrosine kinase 4

EZH2: Enhancer of zeste 2 polycomb repressive complex 2 subunit

FBXW7: F-box and WD repeat domain containing 7

FGFR1: Fibroblast growth factor receptor 1

FGFR2: Fibroblast growth factor receptor 2

FGFR3: Fibroblast growth factor receptor 3

FLT3: Fms related tyrosine kinase 3

GNA11: G protein subunit alpha 11

GNAS: GNAS complex locus

GNAQ: G protein subunit alpha q

HNF1A: HNF1 homeobox A

HRAS: HRas proto-oncogene, GTPase

IDH1: Isocitrate dehydrogenase (NADP(+)) 1, cytosolic

IDH2: Isocitrate dehydrogenase (NADP(+)) 2, mitochondrial

JAK2: Janus kinase 2

JAK3: Janus kinase 3

KDR: Kinase insert domain receptor

KIT: KIT proto-oncogene receptor tyrosine kinase

KRAS: KRAS proto-oncogene, GTPase

MET: MET proto-oncogene, receptor tyrosine kinase

MLH1: MutL homolog 1

MPL: MPL proto-oncogene, thrombopoietin receptor

NOTCH1: Notch 1

NPM1: Nucleophosmin

NRAS: NRAS proto-oncogene, GTPase

PDGFRA: Platelet derived growth factor receptor alpha

PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha

PTEN: Phosphatase and tensin homolog

PTPN11: Protein tyrosine phosphatase, non-receptor type 11

RB1: RB transcriptional corepressor 1

RET: Ret proto-oncogene

SMAD4: SMAD family member 4

SMARCB1: SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1

SMO: Smoothened, frizzled class receptor

SRC: SRC proto-oncogene, non-receptor tyrosine kinase

STK11: Serine/threonine kinase 11

TP53: Tumor protein p53

VHL: Von Hippel-Lindau tumor suppressor

I.

One mutation, two phenotypes: a single nonsense mutation of the *CTSC* gene causes two clinically distinct phenotypes

A. Sulák,[1] L. Tóth,[1] K. Farkas,[2] K. Tripolszki,[1] B. Fábos,[3] L. Kemény,[2,4] P. Vályi,[5] K. Nagy,[5] N. Nagy[1,2,4] and M. Széll[1,2]

[1]Department of Medical Genetics; [2]MTA SZTE Dermatological Research Group and [4]Department of Dermatology and Allergology, University of Szeged, Szeged, Hungary; [3]Mór Kaposi Teaching Hospital, Kaposvár, Hungary; and [5]Department of Parodontology, Faculty of Dentistry, University of Szeged, Szeged, Hungary

Summary

Background. Papillon–Lefévre syndrome (PLS; OMIM 245000) and Haim–Munk syndromes (HMS; OMIM 245010) are phenotypic variants of the same rare disease caused by mutations of the *cathepsin C* (*CTSC*) gene, and they exhibit autosomal recessive inheritance.

Aims. To identify diseases caused by mutations of the *CTSC* gene in two Hungarian patients and to perform haplotype analysis to elucidate any familial relationship between them.

Methods. Mutation screening and polymorphism analysis were performed by direct sequencing of the *CTSC* gene.

Results. Mutation screening of the *CTSC* gene from the two patients revealed the presence of the same homozygous nonsense mutation (c.748C/T; p.Arg250X). However, one patient exhibited the PLS phenotype and the other the HMS phenotype. Although these patients were not aware that they were related, haplotype analysis, especially the genotypes of the rs217116 and the rs217115 polymorphisms, clearly indicated that the patients carry the same haplotype, whereas the unrelated healthy controls carried several different haplotypes.

Conclusions. Our results demonstrate that PLS and HMS are phenotypic variants of the same disease and, additionally, exclude the presence of a putative genetic modifier factor within the *CTSC* gene that is responsible for the development of the two phenotypes. We suggest that this putative genetic modifier factor is located outside the *CTSC* gene, or alternatively, that the development of the different phenotypes is the consequence of different environmental or lifestyle factors.

Introduction

Papillon–Lefévre syndrome (PLS; OMIM 245000) and Haim–Munk syndromes (HMS; OMIM 245010) are characterized by overlapping dermatological and dental

symptoms such as hyperkeratosis of the palms and soles and severe periodontitis.[1,2] Patients with PLS can also develop mild mental retardation, calcification of the dura mater, hyperhidrosis and increased susceptibility to infections,[3–5] while the specific features of HMS include pes planus, arachnodactyly, acroosteolysis and onychogryphosis.[6–8] The prevalence of PLS is approximately four cases per million, and to date, approximately 300 cases have been reported worldwide. Parental consanguinity has been noted in more than 50% of these cases.[4,9] The prevalence of HMS is approximately one case per million, and the majority of reported cases are descendants of a few consanguineous

families from a religious isolate in Cochin, India. One unrelated Brazilian patient has also been reported. There have been >100 cases of HMS reported in the literature to date.[6–8] There is an equal sex ratio in both phenotypic variants. PLS and HMS are both inherited in an autosomal recessive manner and develop as a consequence of mutations of the *cathepsin C* (*CTSC*) gene.[10,11] Currently, 75 *CTSC* gene mutations have been identified.[1] The majority of these mutations (74 of 75; 99%), have been detected in patients with PLS, whereas only 4% (3 of 75), have been associated with HMS.[1,2,7,8] Three mutations (c.145C/T, p.Gln49X, c.857A/G p.Gln286Arg and c.1357A/G p.Ile453Val) have been found in patients exhibiting both the PLS and the HMS phenotypes.[1,12] However one of these, the p.Ile453Val (rs3888798) missense variant, is considered a polymorphism, and its eventual pathogenic role needs to be confirmed or excluded by further studies.[13] Only one mutation (c.587T/C p.Leu196Pro) has been associated exclusively with HMS.[2,8,14,15]

We report two Hungarian patients, one with PLS and one with HMS, who nonetheless carry the same homozygous nonsense mutation (c.748C/T; p.Arg250X) in the *CTSC* gene. In this study, we aimed to investigate the underlying mechanism by which these two patients with the same homozygous nonsense mutation have developed two different phenotypes. Therefore, polymorphism analysis was carried out in order to identify any genetic modifier factor within the *CTSC* gene that could be responsible for the development of the different phenotypes.

Methods

The Internal Review Board of the University of Szeged gave ethics approval for the study, which was conducted according to the Principles of the Declaration of Helsinki. Written informed consent was obtained from all investigated individuals.

Patients

Both patients were referred to the outpatient clinic of the Mór Kaposi Teaching Hospital (Kaposvár; Hungary).

Patient 1 was a 39-year-old Hungarian woman, who presented with a typical HMS phenotype. Mild hyperkeratotic plaques were present symmetrically on her palms (Fig. 1a) and soles (Fig. 1c). Onychogryphosis and arachnodactyly were noted on her fingers (Fig. 1b), and pes planus on her soles (Fig. 1b). The patient had lost all permanent teeth and uses a permanent dental

prosthesis. She was brought up in state care without knowing her parents, and has no husband or child. She was not aware of any known relatives.

Patient 2 was a 25-year-old Hungarian man, who presented with the classic PLS phenotype. The hyperkeratosis on his palms (Fig. 1d) and soles (Fig. 1e) was more severe than those of Patient 1. Onychogryphosis, arachnodactyly and pes planus were not present. He was also missing all permanent teeth and using a permanent dental prosthesis. His parents and his wife were clinically unaffected. He had no siblings or children. He was not aware of any family members who are clinically affected (Fig. 1f).

Genetic investigations

Blood samples were taken from the two investigated patients and from unrelated healthy controls (HCs) for genetic investigation. Genomic DNA was isolated (Bio-Robot EZ1 DSP Workstation; Qiagen, Godollo, Hungary). After the coding regions and the introns of the *CTSC* gene were amplifeied (using primer sequences displayed on the UCSC Genome Browser, http://www. genome.ucsc.edu), DNA sequencing was performed on the amplification products. Sequencing data was analysed to screen for any additional mutations and to genotype all the common polymorphisms of the *CTSC* gene. The polymorphism data of the patients were compared with each other in an attempt to identify a putative genetic modifier variant within the *CTSC* gene that could be responsible for the observed differences in the phenotypes of the patients.

Results

Direct sequencing of the regions of the *CTSC* gene revealed a nonsense mutation in the fifth exon (c.748C/T, p.Arg250X) in both patients. The patients were both homozygous for the mutation (Fig. 2a), whereas the unrelated Hungarian HCs ($n = 100$) carried the wild-type sequence (Fig. 2b).

To investigate the underlying mechanism by which these two patients carrying the same mutation developed different phenotypes, haplotype analysis was carried out. In total, 24 polymorphisms were genotyped on the *CTSC* gene, both upstream and downstream of the identified mutation, in the patients and in the unrelated Hungarian HCs ($n = 10$). The patients were found to be homozygous for all the genotyped polymorphisms, and all genotypes were the same for both patients, indicating that they carried exactly the same haplotype. The haplotype of the patients was compared with those
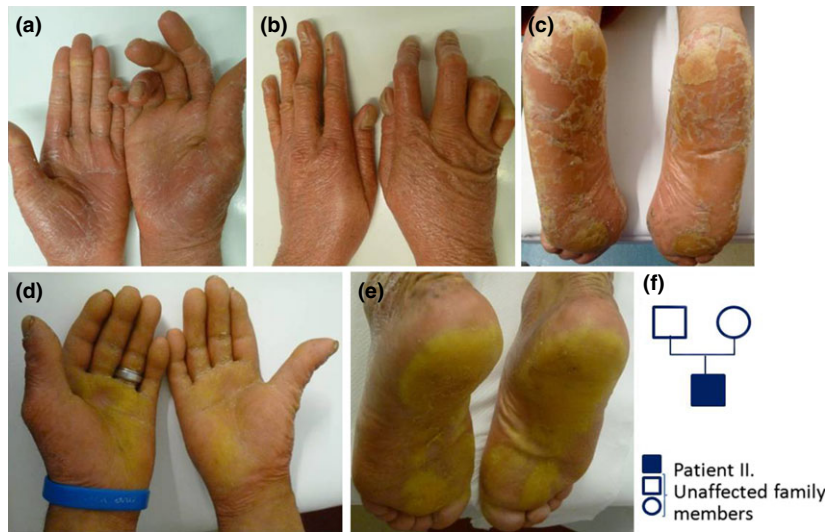
Figure 1 (a–c) Patient 1, presenting with Haim–Munk syndrome (HMS), exhibited (a) mild hyperkeratosis on her palms, (b) onychogryphosis and arachnodactly of her fingers and mild hyperkeratosis and (c) pes planus on her soles (d–f) Patient 2, presenting with Papillon–Lefévre syndrome, was affected by moderate hyperkeratosis of his (d) palms and (f) soles, and exhibited no specific symptoms of HMS. (e) No other affected individuals are known in the family of Patient 2, and Patient 1 was brought up in state care without knowing her relatives.
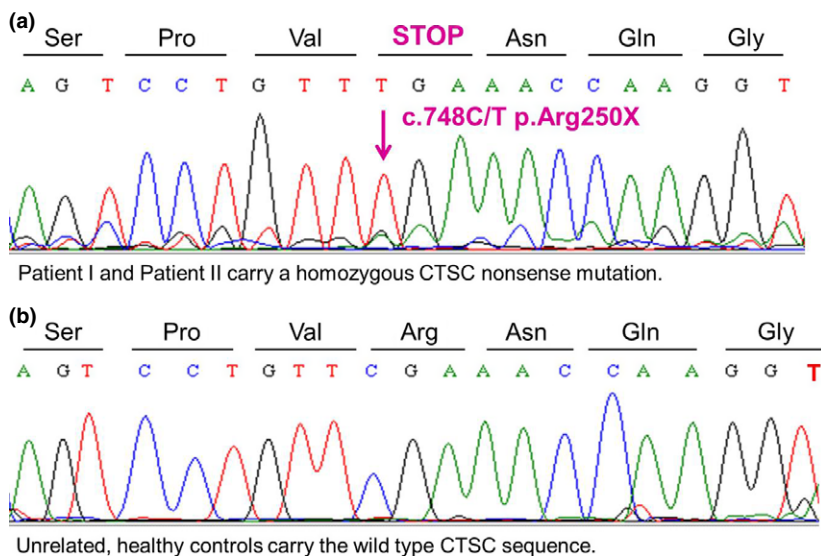

Figure 2 (a,b) Mutation screening of the CTSC gene using direct sequencing revealed a nonsense mutation (c.748C/T, p.Arg250STOP) in the fifth exon. (a) Both patients were homozygous for this mutation, wherease (b) all the unrelated healthy controls carried the wild-type sequence (b).

detected in the unrelated Hungarian HCs. For 20 of the 24 polymorphisms, the same genotype was present in all investigated individuals (patients and HCs), but for the other 4 polymorphisms, there were differences among the study subjects. In Table 1, we report the genotypes of those four polymorphisms (rs370927192, rs217116, rs217115 and rs74325198). The results, especially for rs217116 and rs217115, clearly indicate that the two independent patients, one with HMS and one with PLS, carry the same haplotype, whereas the unrelated HCs carry several different haplotypes.

Our results could not identify any genetic modifier factor within the CTSC gene that could explain the phenotypic differences between the patients. Moreover, our results demonstrated a familial relationship between the two investigated patients.

Discussion

Currently, 75 different mutations in the CTSC gene have been reported worldwide.[1] Nonsense mutations, accounting for 23% (n = 17) of pathogenic CTSC mutations, occur in all coding regions of the gene; however, most are located in exons 5–7, which encode the heavy chain region of the cathepsin C protein.[1,16] This protein is a lysosomal cysteine protease, and the heavy chain region is important for enzyme activity.[16,17] The p.Arg250X homozygous nonsense mutation detected in the investigated patients is also located in this region. This mutation leads to the formation of a truncated protein, and may impair enzyme activity significantly. This hypothesis correlates well with previous studies demonstrating that pathological changes in the CTSC

Table 1 Haplotype analysis. Patient I and II exhibited the same haplotype.

Polymorphisms	Common allele	Rare allele	Patient I	Patient II	Unrelated control I	Unrelated control II	Unrelated control III	Unrelated control IV	Unrelated control V	Unrelated control VI	Unrelated control VII	Unrelated control VIII	Unrelated control IX	Unrelated control X
rs370927192	CTTT	-	CTTT/CTTT	CTTT/CTTT	-/-	CTTT/CTTT	CTTT/CTTT	CTTT/CTTT	CTTT/CTTT	CTTT/CTTT	CTTT/CTTT	CTTT/CTTT	CTTT/CTTT	CTTT/CTTT
CM002938*	C	T	TT	TT	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC
rs217116	A	G	AA	AA	GG	GG	GG	GG	AG	GG	GG	AG	AG	AA
rs217115	C	T	TT	TT	CC	CC	CT	CC	CC	CT	CT	CC	CC	CT
rs74325198	A	T	TT	TT	TT	TA	TT	TT	TT	TT	TA	TT	TT	TA

*The identified homozygous nonsense mutation (c.748C/T p.Arg250X).

gene are loss-of-function mutations, resulting in the inactivation of enzymatic activity.[18,19] These mutations also promote protease degradation in mature haematopoietic subsets, but do not affect protease production in progenitor cells.[20] Biochemical analysis has demonstrated low levels of antioxidant markers and anti-inflammatory fatty acids and high levels of oxidative markers in patients with PLS.[21] The altered regulation of the immune response increases susceptibility to periodontal inflammation and skin infections.[18,19]

The p.Arg250X nonsense mutation has already been reported previously in patients with PLS,[22,23] but this is the first report of its association with HMS. The mutation has been previously detected in both homozygous[24] and compound heterozygous[22] forms. The latter mutation was suggested to be associated with an unidentified heterozygous mutation of the *CTSC* gene.[22] A previous investigation detected this mutation in the homozygous form in Turkish patients.[24] It is likely that these Turkish and Hungarian patients with PLS have a common haplotype (Table 1) and that the identified mutation is the consequence of a single founder effect.

The two investigated Hungarian patients were affected by different variants (PLS and HMS) of the phenotypic spectrum caused by *CTSC* mutations. Clinical differences between the PLS and HMS symptoms of the patients were striking, although unexpectedly, genetic screening identified the presence of the same nonsense mutation (p.Arg250X) in the homozygous form in both patients. Haplotype analysis revealed that the two patients exhibited the same haplotype (Table 1), indicating a strong likelihood of relatedness. The patients were not aware of any such relationship. Patient 1 was brought up in state care and did not know any of her relatives, while Patient 2 was not aware of consanguinity within his family. However, our results and the fact that they share a common family name and also a common haplotype strongly suggest a familial relationship between the two investigated patients.

As the patients had the same homozygous disease-causing mutation as well as the same haplotype, it was possible to examine genetic variations in the *CTSC* gene to identify any differences that could account for the development of the phenotypic differences. Our investigation could not identify any such genetic variant with the *CTSC* gene and flanking regions. Therefore, we suggest that the putative modifier factor, which would result in the development of different phenotypic variants for this *CTSC* mutation, is not located in the region of *CTSC*, but in another region. Moreover, we cannot exclude the possibility that the reason for the pheno-

typic variation is a nongenetic influence, such as environmental or lifestyle factors.

Our results further support the accepted viewpoint that PLS and HMS are not different disease entities, but rather phenotypic variants of the same disease, and that their development is influenced by other factors. This phenomenon has also been observed in another group of rare monogenic diseases caused by mutations in the *CYLD* gene. Initially, Brooke–Spiegler syndrome (BSS; OMIM 605041), multiple familial trichoepithelioma type 1 (MFT1; OMIM 601606) and familial cylindromatosis (FC; OMIM 132700) were considered different entities, until genetic investigation revealed that they were all allelic variants of the same disease.[23,25]

These observations highlight the importance of genetic investigation and the establishment of genotype–phenotype associations.

Acknowledgements

What's already known about this topic?

- Currently, 75 different mutations have been reported worldwide for the *CTSC* gene.
- The p.Arg250X nonsense mutation has already been previously reported in patients with PLS.

What does this study add?

- This is the first report on the association of the p.Arg250X nonsense mutation with HMS.
- The two investigated Hungarian patients were affected by different variants (PLS and HMS) of the phenotypic spectrum caused by the same *CTSC* mutation.

References

1 Nagy N, Vályi P, Csoma Z et al. CTSC and Papillon-Lefèvre syndrome: detection of recurrent mutations in Hungarian patients, a review of published variants and database update. *Mol Gen & Genom Med* 2014; **2**: 217–28.

2 Selvaraju V, Markandaya M, Prasad PV et al. Mutation analysis of the cathepsin C gene in Indian families with Papillon-Lefèvre syndrome. *BMC Med Genet* 2003; **4**: 5.

3 Dalgic B, Bukulmez A, Sari S. Eponym: Papillon-Lefèvre syndrome. *Eur J Pediatr* 2011; **170**: 689–91.

4 Gorlin RJ, Sedano H, Anderson VE. The syndrome of palmar-plantar hyperkeratosis and premature periodontal destruction of the teeth: a clinical and genetic analysis of the Papillon-Lefevre syndrome. *J Pediat* 1964; **65**: 895–908.

5 Haneke E. The Papillon-Lefevre syndrome: keratosis palmoplantaris with periodontopathy: report of a case and review of the cases in the literature. *Hum Genet* 1979; **51**: 1–35.

6 Haim S, Munk J. Keratosis palmo-plantaris congenita, with periodontosis, arachnodactyly and a peculiar deform1ity of the terminal phalanges. *Br J Dermatol* 1965; **77**: 42–54.

7 Hart TC, Hart PS, Bowden DW et al. Mutations of the cathepsin C gene are responsible for Papillon-Lefevre syndrome. *J Med Genet* 1999; **36**: 881–7.

8 Papillon PH, Lefèvre P. Deuxcas de kératodermiepalmaire et plantairesymétriquefamiliale (maladie de Meleda) chez le frère et la soeur. Coexistence dans les deux cas d'altérations dentaires graves. *Bull Soc Fr Dermatol Venereol* 1924; **31**: 82–7.

9 Hewitt C, McCormick D, Linden G et al. The role of cathepsin C in Papillon-Lefevre syndrome, prepubertal periodontitis, and aggressive periodontitis. *Hum Mutat* 2004; **23**: 222–8.

10 Adkison AM, Raptis SZ, Kelley DG et al. Dipeptidyl peptidase I activates neutrophil-derived serine proteases and regulates the development of acute experimental arthritis. *J Clin Invest* 2002; **109**: 363–71.

11 Toomes C, James J, Wood AJ et al. Loss-of-function mutations in the cathepsin C gene result in periodontal disease and palmoplantar keratosis. *Nat Genet* 1999; **23**: 421–4.

12 Aswath N, Swamikannu B, Ramakrishnan SN et al. Heterozygous Ile453Val codon mutation in exon 7, homozygous single nucleotide polymorphisms in entron 2 and 5 of cathepsin C are associated with Haim-Munk syndrome. *Eur J Dent* 2014; **8**: 79–84.

13 Nakano A, Nomura K, Nakano H et al. Papillon-Lefèvre syndrome: mutations and polymorphisms in the cathepsin C gene. *J Invest Dermatol* 2001; **116**: 339–43.

14 Cury VF, Costa JE, Gomez RS et al. A novel mutation of the cathepsin C gene in Papillon-Lefevre syndrome. *J Periodontol* 2002; **75**: 307–12.

15 Turk D, Janjic V, Sitern I *et al.* Structure of human dipeptidyl peptidase I (cathepsin C): exclusion domain added to an endopeptidase framework creates the machine for activation of granular serine proteases. *EMBO J* 2011; **20**: 6570–82.

16 Hart PS, Zhang Y, Firatli E *et al.* Identification of cathepsin C mutations in ethnically diverse Papillon-Lefevre syndrome patients. *J Med Genet* 2000; **37**: 927–32.

17 Korkmaz B, Horwitz MS, Jenne DE *et al.* Neutrophil elastase, proteinase 3, and cathepsin G as therapeutic targets in human diseases. *Pharmacol Rev* 2010; **62**: 726–59.

18 Hart TC, Hart PS, Michalec MD *et al.* Haim-Munk syndrome and Papillon-Lefevre syndrome are allelic mutations in cathepsin C. *J Med Genet* 2000; **37**: 88–94.

19 Rai R, Thiagarajan S, Mohandas S *et al.* Haim-Munk syndrome and Papillon. Lefevre syndrome–allelic mutations in cathepsin C with variation in phenotype. *Int J Dermatol* 2010; **49**: 541–3.

20 Sørensen OE, Clemmensen SN, Dahl SL *et al.* Papillon-Lefèvre syndrome patient reveals species-dependent requirements for neutrophil defenses. *J Clin Invest* 2014; **124**: 4539–48.

21 Bullón P, Morillo JM, Thakker N *et al.* Confirmation of oxidative stress and fatty acid disturbances in two further Papillon-Lefèvre syndrome families with identification of a new mutation. *J Eur Acad Dermatol Venereol* 2014; **28**: 1049–56.

22 Pham CT, Ivanovich JL, Raptis SZ *et al.* Papillon-Lefevre syndrome: correlating the molecular, cellular, and clinical consequences of cathepsin C/dipeptidyl peptidase I deficiency in humans. *J Immunol* 2004; **175**: 7277–81.

23 Nagy N, Rajan N, Farkas K *et al.* A mutational hotspot in CYLD causing cylindromas: a comparison of phenotypes arising in different genetic backgrounds. *Acta Derm Venereol* 2013; **93**: 743–5.

24 Cury VF, Gomez RS, Costa JE *et al.* A homozygous cathepsin C mutation associated with Haim-Munk syndrome. *Br J Dermatol* 2005; **152**: 353–6.

25 Rajan N, Burn J, Langtry J *et al.* Transition from cylindroma to spiradenoma in CYLD-defective tumours is associated with reduced DKK2 expression. *J Pathol* 2011; **224**: 309–21.

II.

Somatic mosaicism of the *PIK3CA* gene identified in a Hungarian girl with macrodactyly and syndactyly

Kornélia Tripolszki [a], Rachel Knox [b], Victoria Parker [b], Robert Semple [b], Katalin Farkas [c], Adrien Sulák [a], Emese Horváth [a], Márta Széll [a, c], Nikoletta Nagy [a, c, *]

[a] *Department of Medical Genetics, University of Szeged, Szeged, Hungary*
[b] *Metabolic Research Laboratories, Institute of Metabolic Science, Addenbrooke's Hospital Cambridge, Cambridge, UK*
[c] *MTA-SZTE Dermatological Research Group, University of Szeged, Szeged, Hungary*

ARTICLE INFO

ABSTRACT

Isolated macrodactyly (OMIM 155500) belongs to a heterogeneous group of overgrowth syndromes. It is a congenital anomaly resulting in enlargement of all tissues localized to the terminal portions of a limb and caused by somatic mutations in the *phosphatidylinositol 3-kinase catalytic alpha* (*PIK3CA*, OMIM 171834) gene. Here we report a Hungarian girl with macrodactyly and syndactyly. Genetic screening at hotspots in the *PIK3CA* gene identified a mosaic mutation (c.1624G > A, p.Glu542Lys) in the affected tissue, but not in the peripheral blood. To date, this somatic mutation has been reported in eight patients affected by different forms of segmental overgrowth syndromes. Detailed analysis of the Hungarian child and previously reported cases suggests high phenotypic diversity associated with the p.Glu542Lys somatic mutation. The identification of the mutation provides a novel therapeutic modality for the affected patients: those who carry somatic mutations in the *PIK3CA* gene are potential recipients of a novel "repurposing" approach of rapamycin treatment.

© 2016 Elsevier Masson SAS. All rights reserved.

1. Introduction

Macrodactyly (OMIM 155500) refers to a rare congenital malformation occurring in approximately 1 in 100,000 live births and is characterized by an increase in the size of all the structures of the limbs, including soft tissues, bones, vessels, nerves and skin (Rios et al., 2013). It typically affects the terminal portions of the limb within a "nerve territory" and the individual peripheral nerve is both enlarged and elongated (Rios et al., 2013). Recently macrodactyly has been added to the growing list of overgrowth syndromes caused by somatic mutations of the *phosphatidylinositol 3-kinase catalytic alpha* (*PIK3CA*, OMIM 171834) gene (Rios et al., 2013; Kurek et al., 2012; Emrick et al., 2014).

Further somatic overgrowth diseases associated with mosaic *PIK3CA* mutations include fibroadipose hyperplasia and nonclassifiable conditions characterized by muscular, boney and fatty tissue overgrowth, congenital lipomatous overgrowth, vascular malformations, epidermal nevi and skeletal abnormalities

(CLOVES) syndrome, hemihyperplasia multiple lipomatosis, and the brain overgrowth conditions megalencephaly capillary malformation and megalencephaly-polymicrogyria-hydrocephalus syndrome (MPPH) (Kurek et al., 2012; Lee et al., 2012; Lindhurst et al., 2012; Rivière et al., 2012; Mirzaa et al., 1993-2011; Rasmussen et al., 2014). The phenotypic heterogeneity in these syndromes is attributed to the location of the cells bearing the mutation and to the proportion of the affected cells in the patient's tissues (Cohen et al., 2014).

Here we describe a 4-year-old Hungarian patient with isolated macrodactyly and syndactyly caused by the c.1624G > A, p.Glu542Lys somatic mutation of the *PIK3CA* gene. We also provide detailed comparison of the clinical symptoms of the Hungarian patient with previously reported cases having the same p.Glu542-Lys somatic *PIK3CA* mutation.

2. Clinical report

A 4-year-old Hungarian girl was referred to the Department of Medical Genetics (University of Szeged, Szeged, HUNGARY) with isolated macrodactyly on the third and fourth fingers of the left hand (Fig. 1a). X-ray imaging proved that the disease is

---

* Corresponding author. Department of Medical Genetics, University of Szeged, 4 Somogyi utca, H-6720, Szeged, Hungary.
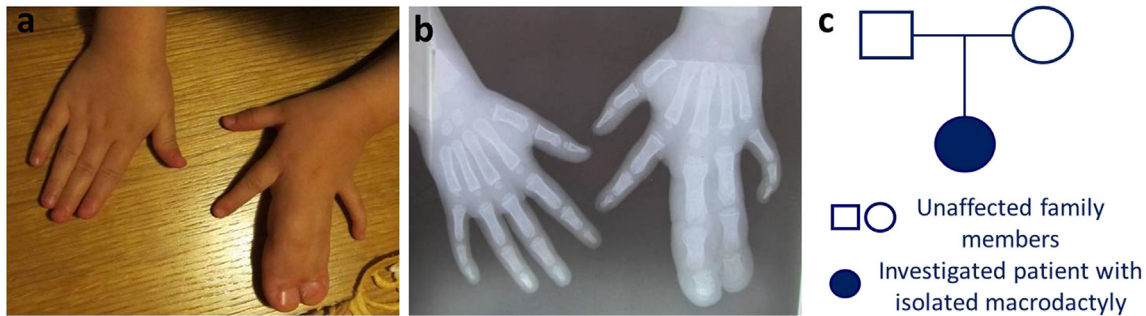 *E-mail address:* nikoletta.nagy@gmail.com (N. Nagy).

Fig. 1. Macrodactyly and syndactyly in a 4-year-old girl. (a) Clinical features and (b) bone radiographs of the patient. (c) The patient's family is clinically asymptomatic.

characterized not only by the overgrowth of the soft tissues but also by the overgrowth of the bones of the affected fingers (Fig. 1b). In addition to macrodactyly, syndactyly was associated with concrescence of the fingers and was constrained to the soft tissues of the affected fingers and not to the bones (Fig. 1a,b). The macrodactyly and syndactyly of the left hand was present at birth and slowly progressed with the growth of the child. On examination, no vascular abnormality was present. There was no associated abnormality of the internal organs. Other body parts were symmetric and equally developed. The patient's parents were clinically unaffected, and they were not aware of any other family members with either macrodactyly or syndactyly (Fig. 1c).

The performed genetic investigation was approved by the Internal Review Board of the University of Szeged. The study was conducted according to the Principles of the Declaration of Helsinki. After informed consent was obtained from the parents, peripheral blood sample and deep surgical excision of the affected left hand were taken in order to perform genetic analysis. Genomic DNA was isolated with the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). Primer sequences were obtained from the UCSC Genome Browser. The coding regions and flanking introns of the *PIK3CA* gene were amplified and sequenced with a traditional capillary sequencer (ABI Prism 7000). Since traditional sequencing did not identify any putative causative variant of the *PIK3CA* gene, mutational hotspots were screened using an in-house PCR-based restriction fragment assay which has been previously described (Keppler-Noreuil et al., 2014) (Cambridge, UK). Examining the region surrounding codon 542, a PCR product of 180 base pairs in length was amplified and digested with the *XbaI* restriction enzyme for 2 h at 37 °C. The resulting fragments were detected by fluorescent read-out using GeneMapper® software with GeneScan LIZ 500 (Life Technologies Corporation) as the size standard. A mosaic mutation at codon 542 (c.1624G > A, p.Glu542Lys) was identified in the affected tissue with 4% mutation burden (Fig. 2a). This mutation was not present in the genomic DNA sample isolated from the peripheral blood of the patient (Fig. 2b). This genetic analysis confirmed that the development of macrodactyly and syndactyly of the third and fourth fingers of the left hand are the consequence of the mosaicism of the p.Glu542Lys mutation in the *PIK3CA* gene.

3. Discussion

The case reported in this study presented with macrodactyly and syndactyly of the third and fourth fingers of the left hand. Genetic investigation identified a somatic missense gain-of-function mutation (p.Glu542Lys) of the *PIK3CA* gene (ClinVar database, http://www.ncbi.nlm.nih.gov/clinvar/accession number: SCV000258982). This mutation affects the helical domain of the p110α catalytic subunit of the PI3K protein (Samuels et al., 2004). Functional studies have previously shown that this p.Glu542Lys

variant caused hyperactivation of AKT, a down-stream target of PI3K in the nerve cells of a patient with macrodactyly (Rios et al., 2013). This particular mutation has been reported in eight patients with different forms of segmental overgrowth (Table 1) (Rios et al., 2013; Kurek et al., 2012; Emrick et al., 2014; Maclellan et al., 2014).

Patients with somatic p.Glu542Lys mutation of the *PIK3CA* gene show high phenotypic diversity; Kurek et al. (2012), for example, described a female and a male patient affected by CLOVES syndrome. In addition to macrodactyly, both patients developed lipomatous overgrowth of the trunk and the limbs and vascular anomalies including lymphatic, capillary and venous malformations. The affected female patient also had a hypoplastic right kidney (Kurek et al., 2012). A subsequent study reported the prenatal diagnosis of CLOVES syndrome in a 27-week-old fetus carrying the same somatic mutation of the *PIK3CA* gene. The observed clinical symptoms at birth were asymmetric chest and abdomen, bilateral multicystic malformations and asymmetric growth of the left leg with macrodactyly of the left foot and a sandal gap between the first and second toes (Emrick et al., 2014). Rios et al. (2013) reported two patients with the same somatic mutation; both were affected by macrodactyly. However, one of the patients was also affected by true muscular hemihypertrophy, which was also attributed to the presence of the somatic p.Glu542Lys *PIK3CA* mutation. A subsequent study has also reported three further patients with facial infiltrating lipomatosis, which was attributed to the presence of the somatic p.Glu542Lys mutation (Maclellan et al., 2014). These patients did not exhibit macrodactyly.

The high phenotypic diversity associated with the somatic p.Glu542Lys mutation might be explained by the different time points, in which the mutational events occurred during embryogenesis. Patients with CLOVES syndrome might have developed the same somatic p.Glu542Lys mutation earlier during the embryogenesis than the ones with regional overgrowth (macrodactyly or facial infiltrating lipomatosis).

Genetic analysis has a huge significance for these patients, as once the genetic cause is determined, pharmacological intervention could be considered as a therapeutic option. In the interim, there are no clinically approved therapies for this condition; however there is a theoretical possibility that small molecule inhibitors of the PI3K-AKT-mTOR signaling pathway could be effective therapies for these patients (Segerström et al., 2011; Iacobas et al., 2011). Rapamycin (sirolimus) indirectly targets PI3K and may also be useful in treating macrodactyly (Segerström et al., 2011; Iacobas et al., 2011). In addition, rapamycin has been reported to be effective in isolated cases of allied conditions and may promote a breakthrough in the treatment of macrodactyly and overgrowth syndromes. However, long-term safety data of this treatment in PIK3CA-related overgrowth is currently lacking and, thus, indicates the need for formal clinical trials to evaluate safety
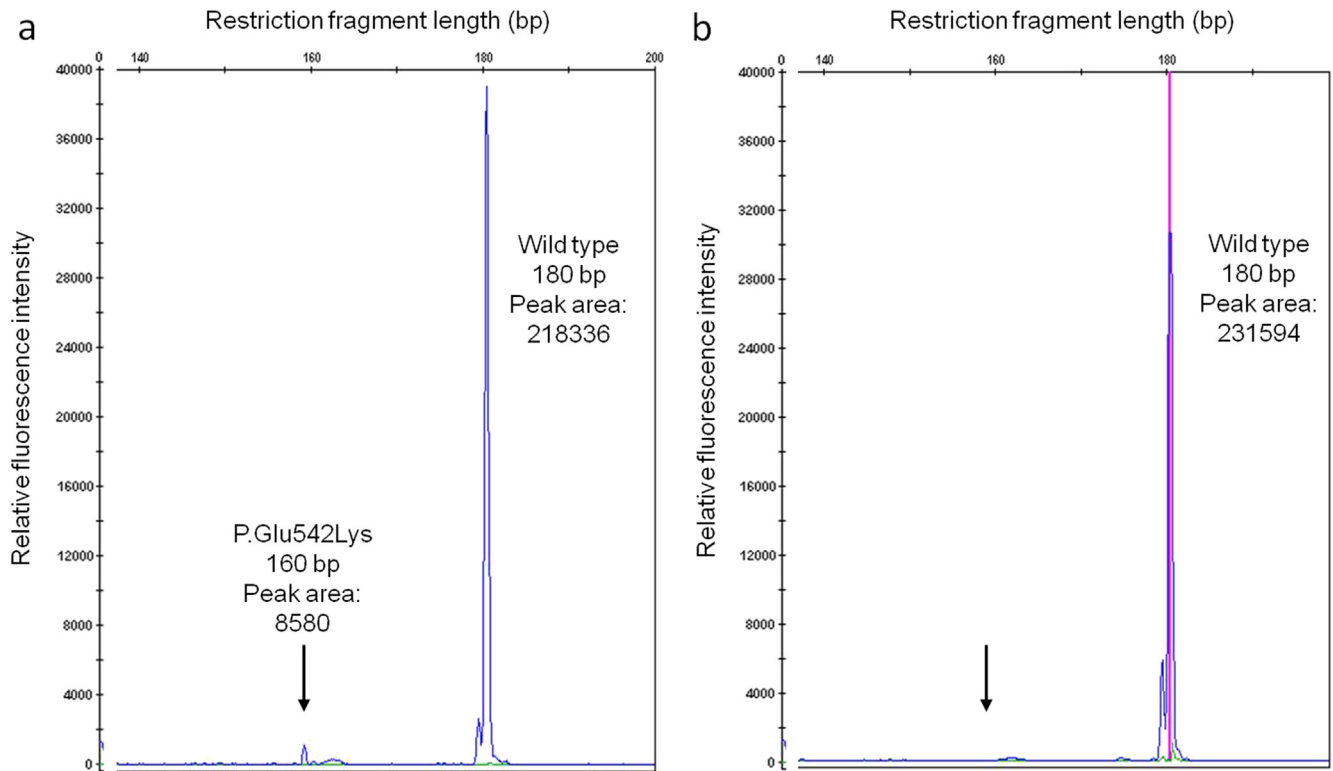
Fig. 2. Genetic investigation of the Hungarian patient with macrodactyly and syndactyly. (a) DNA was extracted from the affected left hand and subjected to a PCR restriction assay, which identified the PIK3CA p.Glu542Lys mutation at 4% mutation burden. (b) In DNA extracted from the peripheral blood, the peak indicating the presence of the mutation was absent in the digested PCR product, indicating the mosaicism of the mutation.

Table 1
Summary of the phenotypic diversity of the patients with somatic p.Glu542Lys PIK3CA mutation.

Patients	Index Patient	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9
Clinical diagnosis	Macro-dactyly, Syndactyly	Macro-dactyly	Macro-dactyly, Muscular hemi-hypert-rophy	Facial infiltrating lipo-matosis	Facial infiltrating lipo-matosis	Facial infiltrating lipo-matosis	CLOVES syndrome	CLOVES syndrome	CLOVES syndrome
Publication	This report	Rios et al., 2013	Rios et al., 2013	Maclellan et al., 2014	Maclellan et al., 2014	Maclellan et al., 2014	Kurek et al., 2012	Kurek et al., 2012	Emrick et al., 2014
Percentage (%) of somatic mosaicism	4	NR	NR	23	30	16–18	8	6–13	38
Lipomatous overgrowth									
Face	NR	NR	NR	+	+	+	NR	NR	NR
Trunk	NR	NR	NR	NR	NR	NR	+	+	+
Limb(s)	+	+	+	NR	NR	NR	+	+	+
Vascular anomalies									
Lymphatic malformation	NR	NR	NR	NR	NR	NR	+	+	+
Capillary malformation	NR	NR	NR	NR	NR	NR	+	+	+
Venous malformation	NR	NR	NR	NR	NR	NR	+	+	+
Musculoskeletal									
Macrodactyly	+	+	+	NR	NR	NR	+	+	+
Limb asymmetry	NR	NR	NR	NR	NR	NR	+	+	
Scoliosis	NR	NR	NR	NR	NR	NR	+		
True muscular hemihypertrophy	NR	NR	+	NR	NR	NR	NR	NR	NR
Other findings	NR	NR	NR	NR	NR	NR	Hypo-plastic right kidney	NR	Epidermal nevi

NR = not reported.

and efficacy. In light of the current situation, detailed genetic investigation and publication of these isolated cases is essential.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgments

References

Cohen, A.S., Townsend, K.N., Xiang, Q.S., Attariwala, R., Borchers, C., Senger, C., et al., 2014. Somatic mosaicism for the p.His1047Arg mutation in PIK3CA in a girl with mesenteric lipomatosis. Am. J. Med. Genet. 164A, 2360–2364.

Emrick, L.T., Murphy, L., Shamshirsaz, A.A., Ruano, R., Cassady, C.I., Liu, L., et al., 2014. Prenatal diagnosis of CLOVES syndrome confirmed by detection of a mosaic PIK3CA mutation in cultured amniocytes. Am. J. Med. Genet. 164A, 2633–2637.

Iacobas, I., Burrows, P.E., Adams, D.M., Sutton, V.R., Hollier, L.H., Chintagumpala, M.M., 2011. Oral rapamycin in the treatment of patients with hamartoma syndromes and PTEN mutation. Pediatr. Blood Cancer 57, 321–323.

Keppler-Noreuil, K.M., Sapp, J.C., Lindhurst, M.J., Parker, V.E., Blumhorst, C., Darling, T., et al., 2014. Clinical delineation and natural history of the PIK3CA-related overgrowth spectrum. Am. J. Med. Genet. 164A, 1713–1733.

Kurek, K.C., Luks, V.L., Ayturk, U.M., Alomari, A.I., Fishman, S.J., Spencer, S.A., et al., 2012. Somatic mosaic activating mutations in PIK3CA cause CLOVES syndrome. Am. J. Hum. Genet. 90, 1108–1115.

Lee, J.H., Huynh, M., Silhavy, J.L., Kim, S., Dixon-Salazar, T., Heiberg, A., et al., 2012. De novo somatic mutations in components of the PI3K-AKT3-mTOR pathway cause hemimegalencephaly. Nat. Genet. 44, 941–945.

Lindhurst, M.J., Parker, V.E., Payne, F., Sapp, J.C., Rudge, S., Harris, J., et al., 2012. Mosaic overgrowth with fibroadipose hyperplasia is caused by somatic activating mutations in PIK3CA. Nat. Genet. 44, 928–933.

Maclellan, R.A., Luks, V.L., Vivero, M.P., Mulliken, J.B., Zurakowski, D., Padwa, B.L., et al., 2014. PIK3CA activating mutations in facial infiltrating lipomatosis. Plast. Reconstr. Surg. 133, 12–19.

Mirzaa, G., Conway, R., Graham, J.M., Dobyns, W.B., 1993–2014. PIK3CA-Related segmental overgrowth. In: Pagon, R.A., Adam, M.P., Ardinger, H.H., et al. (Eds.), GeneReviews® [Internet]. Seattle (WA). University of Washington, Seattle. Available from: www.ncbi.nlm.nih.gov/books/NBK153722/.

Rasmussen, M., Sunde, L., Weigert, K.P., Bogaard, P.W., Lildballe, D.L., 2014. Segmental overgrowth syndrome due to an activating PIK3CA mutation identified in affected muscle tissue by exome sequencing. Am. J. Med. Genet. 164A, 1318–1321.

Rios, J.J., Paria, N., Burns, D.K., Israel, B.A., Cornelia, R., Wise, C.A., Ezaki, M., 2013. Somatic gain-of-function mutations in PIK3CA in patients with macrodactyly. Hum. Mol. Genet. 22, 444–451.

Rivière, J.B., Mirzaa, G.M., O'Roak, B.J., Beddaoui, M., Alcantara, D., Conway, R.L., et al., 2012. De novo germline and postzygotic mutations in AKT3, PIK3R2 and PIK3CA cause a spectrum of related megalencephaly syndromes. Nat. Genet. 44, 934–940.

Samuels, Y., Wang, Z., Bardelli, A., Silliman, N., Ptak, J., Szabo, S., et al., 2004. High frequency of mutations of the PIK3CA gene in human cancers. Science 304, 554.

Segerström, L., Baryawno, N., Sveinbjörnsson, B., Wickström, M., Elfman, L., Kogner, P., Johnsen, J.I., 2011. Effects of small molecule inhibitors of PI3K/Akt/mTOR signaling on neuroblastoma growth in vitro and in vivo. Int. J. Cancer 129, 2958–2965.

III.

Atypical neurofibromatosis type 1 with unilateral limb hypertrophy mimicking overgrowth syndrome

K. Tripolszki,[1] K. Farkas,[2] A. Sulák,[1] G. Szolnoky,[3] B. Duga,[4] B. Melegh,[4] R. G. Knox,[5] V. E. R. Parker,[5] R. K. Semple,[5] L. Kemény,[2,3] M. Széll[1,2] and N. Nagy[1,2,3]

[1]Department of Medical Genetics, [2]MTA-SZTE Dermatological Research Group and [3]Department of Dermatology and Allergology, University of Szeged, Szeged, Hungary; [4]Department of Medical Genetics, University of Pécs, Pécs, Hungary; and [5]Metabolic Research Laboratories, Institute of Metabolic Science, Addenbrooke's Hospital Cambridge, Cambridge, UK

Summary

Neurofibromatosis type 1 (NF1; OMIM 162200), a dominantly inherited multitumor syndrome, results from mutations in the *Neurofibromin 1* (*NF1*) gene. We present the case of a Hungarian woman with the clinical phenotype of NF1 over her whole body and the clinical features of unilateral overgrowth involving her entire left leg. This unusual phenotype suggested either the atypical form of NF1 or the coexistence of NF1 and overgrowth syndrome. Direct sequencing of the genomic DNA isolated from peripheral blood revealed a novel frameshift mutation (c.5727insT, p.V1909fsX1912) in the *NF1* gene. Next-generation sequencing of 50 oncogenes and tumour suppressor genes, performed on the genomic DNAs isolated from tissue samples and peripheral blood, detected only wild-type sequences. Based on these results, we concluded that the patient is affected by an unusual phenotype of NF1, and that the observed unilateral overgrowth of the left leg might be a rare consequence of the identified c.5727insT mutation.

Neurofibromatosis type 1 (NF1; OMIM 162200) is a rare monogenic disease with autosomal dominant inheritance, caused by mutations in the *Neurofibromin 1* (*NF1*) gene.[1] The clinical features of NF1 involve pigmentary changes such as café-au-lait macules and axillary freckling, development of cutaneous fibromatous tumours, and development of hamartomas of the iris known as Lisch nodules.[1] Besides these symptoms, central nervous system and skeletal abnormalities (scoliosis, pseudoarthrosis of the tibia, macrocephaly and short stature) can occur.[2,3] Frequently reported vascular symptoms include vascular dysplasia with cerebral, gastrointestinal and/or renal involvement, and renovascular hypertension.[2,3]

NF1 may be caused by many different loss-of-function mutations of the *NF1* gene.[1] In about 5% of

Correspondence: Dr Nikoletta Nagy, Department of Medical Genetics, University of Szeged, 4 Somogyi utca, H-6720 Szeged, Hungary
E-mail: nikoletta.nagy@gmail.com

Conflict of interest: the authors declare that they have no conflicts of interest.

patients with NF1, microdeletions of the *NF1* gene and/or the surrounding genomic region are responsible for the disease.[4] In patients with *NF1* microdeletion, somatic overgrowth has been frequently observed.[5] Mosaic overgrowth affecting a well-defined region of the body have only been rarely reported in NF1.

We report the case of a Hungarian woman presenting with the classic clinical features of NF1 and with unusual unilateral overgrowth of her left leg. The symptoms of NF1 were visible over her whole body, while the overgrowth was present only in her left leg. Our aim was to identify the underlying genetic abnormality of this unusual case and determine whether the patient is affected by two independent diseases or whether the symptoms of the left leg are an unusual manifestation of NF1.

Report

A 52-year-old Hungarian woman presented with an unusual phenotype, exhibiting the clinical features of

NF1. The typical skin symptoms of NF1 were present in the patient, including neurofibromas and cafe-au-lait macules on her body (Fig. 1a,b), and axillary freckling. Ophthalmological examination determined the presence of Lisch nodules. Imaging studies did not find any indication of central nervous system malignancies. Skeletal abnormalities such as scoliosis, tibial pseudoarthrosis, short stature and macrocephaly, were not present. Based on the clinical symptoms, the diagnosis of NF1 was established.

However, in addition to the aforementioned clinical features, the patient was also noted to have hypertrophy of the left leg, resulting in significant differences in the circumference and length of the legs (Fig. 1c,d). This abnormality of the left leg was already present at birth. Imaging studies verified unilateral osteohypertrophy affecting the left leg. The patient was not aware of any relevant chronic diseases or other family members with NF1 or with overgrowth syndromes (Fig. 1e).

Following ethics approved by the internal review board of the University of Szeged (Szeged, Hungary), genetic studies were carried out, conducted according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all participants. Tissue biopsy of the left leg and blood sample were taken from the patient, and genomic DNA was isolated (BioRobot EZ1 DSP Workstation; QIAGEN, Hilden, Germany). Her family members were unwilling to provide material for genetic investigation.

To confirm NF1, the coding regions and flanking introns of the NF1 gene were amplified and sequenced. Direct sequencing of genomic DNA isolated from the peripheral blood sample revealed a novel frameshift mutation (c.5727insT, p.V1909fsX1912) in exon 39 of the NF1 gene (Fig. 2). The patient carried the mutation in heterozygous form (Fig. 2a), while unrelated controls (n=50) carried the wild-type sequence (Fig. 2b).

To verify overgrowth syndrome, the coding regions and flanking introns of the PIK3CA gene were amplified and sequenced. As traditional sequencing of genomic DNA isolated from the peripheral blood and from the tissue sample of the left leg did not identify any putative causative variant of the PIK3CA gene, mutational hotspots were screened using an in-house PCR-based restriction fragment assay.[6] As these also did not identify any overgrowth-causing mutation, next-generation sequencing of approximately 2800 mutations from 50 oncogenes and tumour suppressor genes recorded in the Catalogue of Somatic Mutations in Cancer (COSMIC) was performed (Ion AmpliSeq™ Cancer Hotspot Panel v2; Thermo Fisher Scientific, Waltham, MA, USA), but also did not identify any putative causative variant that could be responsible for the overgrowth of the left leg.

This patient presented with the clinical features of both NF1 and left leg overgrowth. Such an atypical clinical form of NF1 has not been reported previously. However, the phenomenon of 'vascular neurofibromatosis phenotype' and the relatively frequent
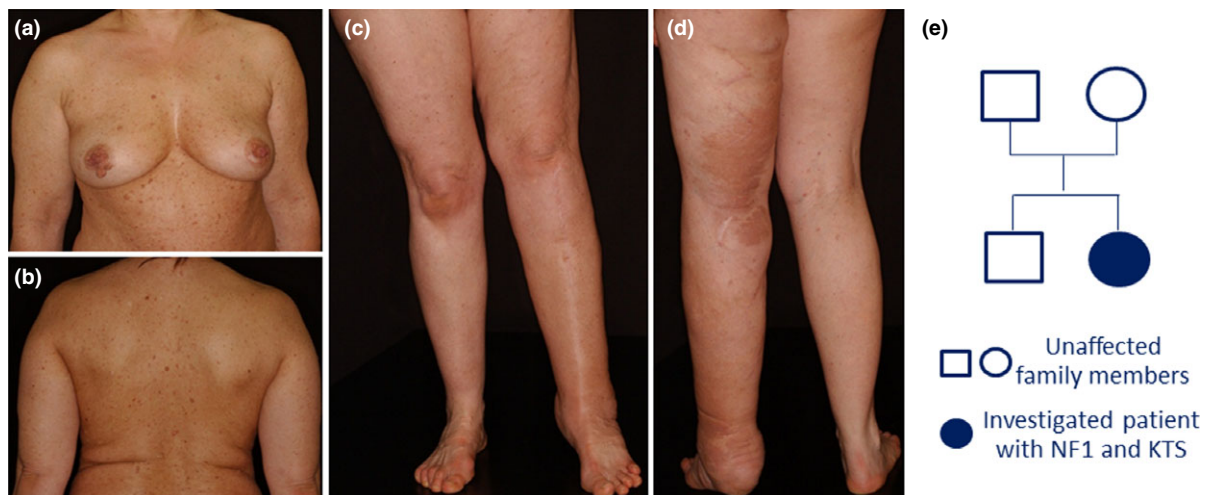

Figure 1 An unusual phenotype of neurofibromatosis (NF)1 in a 52-year-old Hungarian woman. The patient presented with the clinical phenotype of NF1 over her whole body, including (a) her chest and (b) back. (c,d) Unilateral osteohypertrophy affected the left leg. (e) The rest of the patient's family were clinically asymptomatic.
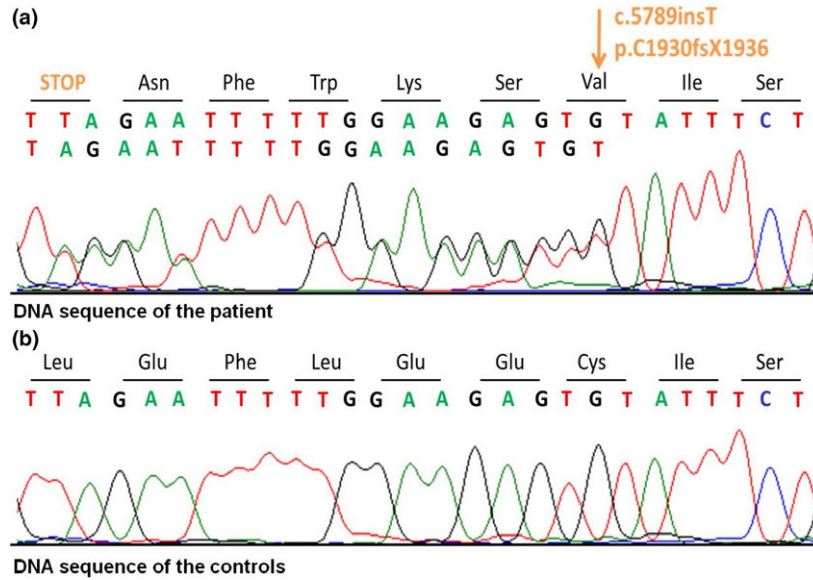
Figure 2 (a,b) Direct sequencing of the *NF1* gene. Direct sequencing revealed a novel single-nucleotide insertion resulting in a frame-shift and the formation of premature termination codon (c.5727insT, p.V1909fsX1912). (a) The patient carried the mutation in heterozygous form. (b) Unrelated, healthy controls carried only wild-type sequences.

association of NF1 with vascular dysplasia have been frequently reported in the literature.[7] NF1 can also be accompanied by skeletal abnormalities, such as sphenoid wing dysplasia, macrocephaly, scoliosis, vertebral disc dysplasia, pseudoarthrosis of tibia and short stature.[8,9] Therefore, it was important to distinguish whether the patient was affected by two independent rare diseases or the symptoms of the left leg were the results of the atypical vascular and skeletal manifestations of NF1.

Our results demonstrated a novel heterozygous single-nucleotide insertion in the *NF1* gene, leading to a frameshift and the formation of a premature termination codon (c.5727insT, p.V1909fsX1912). Because this mutation was present in the genomic DNA isolated from the peripheral blood of the patient, we suggest that this sequence change is a germline mutation. Considering that there is no other clinically affected member in the patient's family and that NF1 exhibits autosomal dominant inheritance, we hypothesize that this is a *de novo* mutation of the *NF1* gene. The genotype–phenotype correlation is generally poor in NF1, with the exception that patients with large deletions in the *NF1* gene tend to have severe phenotypes.[10] *NF1* mutations usually result in loss of tumour suppressor function by disrupting the NF1 protein's ability to maintain the proto-oncogene *RAS* in an inactive form.[11] We suggest that this novel *NF1* mutation is the causative mutation for the development of NF1, and that it is highly possible that the presence of the unilateral limb hypertrophy is also the consequence of this mutation.

In conclusion, we report a patient with both the clinical features of NF1 and overgrowth of the left leg. To our knowledge, our study is the first to clearly elucidate the genetic background of such a complex case, and further confirms the causative role of the somatic mutations of the *NF1* gene in the development of overgrowth.

Acknowledgements

Learning points

• The case reported in this study was presented with the clinical features of both NF1 and left leg overgrowth.

• The unusual clinical phenotype including the left leg overgrowth is the atypical manifestation of NF1.
• The patient carries a disease-causing germline mutation of the *NF1* gene.
• The patient does not carry any somatic mutation of *PIK3CA* or other genes.
• Our study elucidates the genetic background of the investigated case with unusal phenotype, and confirms that the observed left leg overgrowth is the consequence of NF1.

References

1 Huson SM. The neurofibromatosis: classification, clinical features and genetic counselling. In: *Neurofibromatoses* (Kaufmann D, ed). Basel: Karger, 2008; 1–20.

2 Mukonoweshuro W, Griffiths PD, Blaser S. Neurofibromatosis type 1: the role of neuroradiology. *Neuropediatrics* 1999; **30**: 111–19.

3 Stevenson DA, Birch PH, Friedman JM *et al.* Descriptive analysis of tibial pseudarthrosis in patients with neurofibromatosis 1. *Am J Med Genet* 1999; **84**: 413–19.

4 Pasmant E, Sabbagh A, Spurlock G *et al.* NF1 microdeletions in neurofibromatosis type 1: from genotype to phenotype. *Hum Mutat* 2010; **31**: 1506–18.

5 Spiegel M, Oexle K, Horn D *et al.* Childhood overgrowth in patients with common NF1 microdeletions. *Eur J Hum Genet* 2005; **13**: 883–8.

6 Keppler-Noreuil KM, Sapp JC, Lindhurst MJ, et al. Clinical delineation and natural history of the PIK3CA-related overgrowth spectrum. *Am J Med Genet A* 2014; **164A**: 1713–33.

7 Reubi F. Neurofibromatose et lesions vasculaires. *Schweiz Med Wochenschr* 1945; **75**: 463–5.

8 Ferner RE, Huson SM, Thomas N *et al.* Guidelines for the diagnosis and management of individuals with neurofibromatosis 1. *J Med Genet* 2007; **44**: 81–8.

9 Williams VC, Lucas J, Babcock MA *et al.* Neurofibromatosis type 1 revisited. *Pediatrics* 2009; **123**: 124–33.

10 Heim RA, Silverman LM, Farber RA *et al.* Screening for truncated NF1 proteins. *Nat Genet* 1994; **8**: 218–19.

11 Yohay KH. The genetic and molecular pathogenesis of NF1 and NF2. *Semin Pediatr Neurol* 2006; **13**: 21–6.