CLINICAL AND GENETIC EXAMINATION OF LIMB DEVELOPMENTAL DEFECTS

Ph.D. Thesis

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ABBREVIATIONS

bp – basepair
CGH – comparative genomic hybridisation
Chr – chromosome
DNA – deoxy-ribonucleic-acid
FISH – fluorescent in situ hybridisation
HOS – Holt Oram syndrome
Kb – kilo basepair
Mb – mega basepair
NIT DHM – National Institute of Traumatology, Department of Hand and Microsurgery
pc - percentile
PCR – polymerase chain reaction
UP DMG – University of Pécs (Medical Faculty), Department of Medical Genetics
SRPD – short rib polydactylia
STS – short tandem segments
TBX – T-box
TF – transcription factors

I. INTRODUCTION

The fundamental biological question is how the body plan is laid down during embryonic development. In humans, the specialized cells, tissues and organs arise from one cell, the fertilized oocyte. But how this complex development can be controlled and what are the factors that explain changes and rare abnormalities in this well conserved process?

Limb developmental defects are well-known, conspicuous abnormalities. Their significance cannot be negligible, although they are known to be rare conditions. Based upon international studies, limb abnormalities occur in about 10 / 10 000 live births. The development of scientific methods provides more and more opportunities which may help to understand developmental processes and defects of this compound system. Limbs are useful model systems to study the genetic control of morphogenesis because, beside their complex anatomy and development, their abnormalities are easy to recognize and verify.

Despite the latest techniques, genetic background of limb development and its defects still belongs to an unrevealed part of science. Characteristic limb malformations together with associated symptoms may help to find the diagnosis and give opportunity for genetic examinations in chromosomal or monogenic syndromes. In isolated limb defects the number of target genes is around ten and can be detected only in low proportion of the cases.

The role of transcription factors, among others, is well known during normal embryogenesis, including limb development. They regulate protein formation and function by binding to DNA and controlling gene expression. Mutations are generally lethal, but contribute to endocrine disorders, tumor genesis and developmental defects. They are necessary for cell growth, proliferation, differentiation and are required in tissue and organ development. Consequently transcription factors are essential in developmental processes and homeostasis. Mutations of genes encoding transcriptional regulators have been shown to be involved in various genetic diseases, particularly in malformations of the skeletal system, cranium, limbs (e.g.: hand-foot-genital syndrome), as well as in congenital syndromes presenting anomalies of these organs combined with somatic/mental retardation (e.g.: Feingold syndrome) . Evidences support the need of the identification of transcription factor mutation in clinical practice. Determination of transcription factors in congenital malformations and genetic syndromes might be of clinical significance, even in the near future, for exact causative diagnosis and genetic counseling in affected families. In addition, studies of transcription factors may lead to a better understanding of human embryogenesis.

The determination of the background responsible for the developmental defect is very complex. **Exact clinical diagnosis**, based upon detailed examination and classification is needed; **patient database**, collection of similar cases may help to find the common origin. With the **classical and latest methods of genetics** extended examinations have become possible, revealing either bigger chromosomal abnormalities or smaller mutations affecting the level of the base-pairs.

II. OBJECTIVES

The aim of understanding the development of the limb and their abnormalities has leaded us to begin our comprehensive study which may help to reveal the background of these abnormalities. The development of diagnostic and prognostic methods can help prevention and hopefully, in the near future, the substantive treatment as well.

The following aims were set in our study:

1. Patient collection, database

The first objective was to create a comprehensive database which may help to handle and augment our patient files and give opportunity to compare similar cases and follow examinations.

2. Clinical examination protocol

We aimed to establish a clinical examination protocol, based upon the contemporary literature. A standard protocol was needed to ensure that symptoms are described and patients are sorted on common aspects. It may also help to recognize associated symptoms and provide enough data for phenotype – genotype correlation analysis as well.

3. Sorting, classification

A standardized classification system was required to support exact clinical diagnosis and genetic examination plans. Handling and sorting cases can help to select patients for genetic examinations.

4. Genetic examination protocol

Our aim was to work out a genetic examination protocol that can help to find the most effective way to reveal the background of limb developmental defects. To this end we planned to apply conventional, classical cytogenetic and molecular methods as well as the latest technologies.

5. Examination of some specific genes (transcription factors)

In our study we wanted to focus on a group of genes playing an important role in human embryogenesis. It seemed to be worth to focus on the transcription factor gene family from the whole genomic system for more detailed examination, due to their role in isolated limb defects as well as in compound syndrome.

6. Phenotype – genotype correlation

We aimed to define and publish phenotype – genotype correlation data gained in our studies.

III. METHODS

1. Creation of a patient database

Our study for collecting patients with limb developmental defects, and referring them for detailed clinical and genetic examination, was initiated in 2005. In the beginning we sorted patients from our institute (University of Pécs, Department of Medical Genetics). Cases with limb defects were selected from patients who had visited our department in the last 14 years (1993-2007).

In 2006 we got in touch with the National Institute of Traumatology, Department of Hand and Microsurgery, where patients requiring correctional operation due to inherited or acquired limb defects are treated. Based upon their medical records and X-ray we chose cases from the last 4 year (2003-2007).

The information was summarized in a database.

2. Clinical examination, questionnaire

A questionnaire and a clinical examination protocol for patients with inherited limb developmental abnormalities were constructed by the candidate. Detailed anamnesis, physical examination (including collaboration with other professionals if needed) seemed to be essential for exact diagnosis.

3. Sorting, classification

Before genetic examinations are performed classification of the abnormality is needed. Clinical (morphological, topological) and developmental aspects were also considered during analysis of individual cases.

All limbs were examined in available cases to determine the affected ones. Abnormal number of bones, growth aberration or formal variances may appear in different axis of the hands and feet. It is also important to determine what kind of tissue is involved in the developmental defect. Furthermore we examined some other organs, especially heart, kidneys and central nervous system to reveal previously non-described additional symptoms.

We found several references for sorting limb developmental defects with different aspects. We tried to create our own classification system which was based upon clinical signs, but considered the latest knowledge about developmental processes and could help to plan genetic tests. As a basic we chose the classification system of Swanson, accepted worldwide

in hand surgery. On the basis of our experiences we modified this classification table, while also creating novel groups that can help to categorize all the patients we have examined, on a simple way.

4. Genetic methods

Following detailed clinical inspection, genetic examinations were performed in selected cases. Firstly, we usually indicated chromosomal analysis that was followed by FISH in negative cases. Beside cytogenetic methods we used state of the art molecular techniques as well, such as arrayCGH that is a useful method to detect submicroscopic deletions or duplications. Candidate attended a training course in Belgium to acquire the technique of arrayCGH next applied in her study in Denmark. In some cases we used other molecular techniques as well. We sequenced candidate genes based upon the latest literature. STS marker analysis was performed before sequencing in familial cases. We could indicate whole genome linkage analysis and homozygote mapping altogether in two cases.

IV. RESULTS

1. Patient database

202 patients were collected in our database which helped to summarize the most important information. Patients haved been sorted based upon various aspects and summarized in a table that provides the most important information at a glance (Table 1).

Table 1: Summarized data, based upon different aspects

Source of the patients	NIT DHM	**118**	**Proportion of sexes**	♂			**109**
	UP DMG	**84**		♀			**93**
Source of information	Personal clinical examination	**34**	**Familial anamnesis**	Positive (for limb developmental defects)			**43**
	Questionnaire	**32**		Negative			**73**
	Previous medical documents	**136**		Negative for close relatives (no more data available)			**86**
Other symptoms	Can be detected	**58**	Known syndrome				**44**
			Unknown association / syndrome				**14**
	Cannot be detected	**144**					
Affected limbs	Left upper	**125**	**How many limbs are affected**	Four	**31**	Three	**5**
	Right upper	**132**		2 upper	**53**	2 lower	**7**
	Left lower	**51**		1 upper	**89**	1 lower	**9**
	Right lower	**44**		1 upper and 1 lower			**8**

2. Sorting and classification of the patients

Based upon the classification system of Swanson we created a table that can be useful for patient classification in everyday practice (Table 2). Developmental and topological sorting was separated in order to simplify the system. Additionally, the candidate added a column that may help in clinical terminology.

3. Genetic examination protocol

A genetic examination protocol has been established that includes the opportunity of the latest techniques beside the classic cytogenetic and molecular methods (Figure 1). We could perform some of these genetic test altogether in 35 cases.

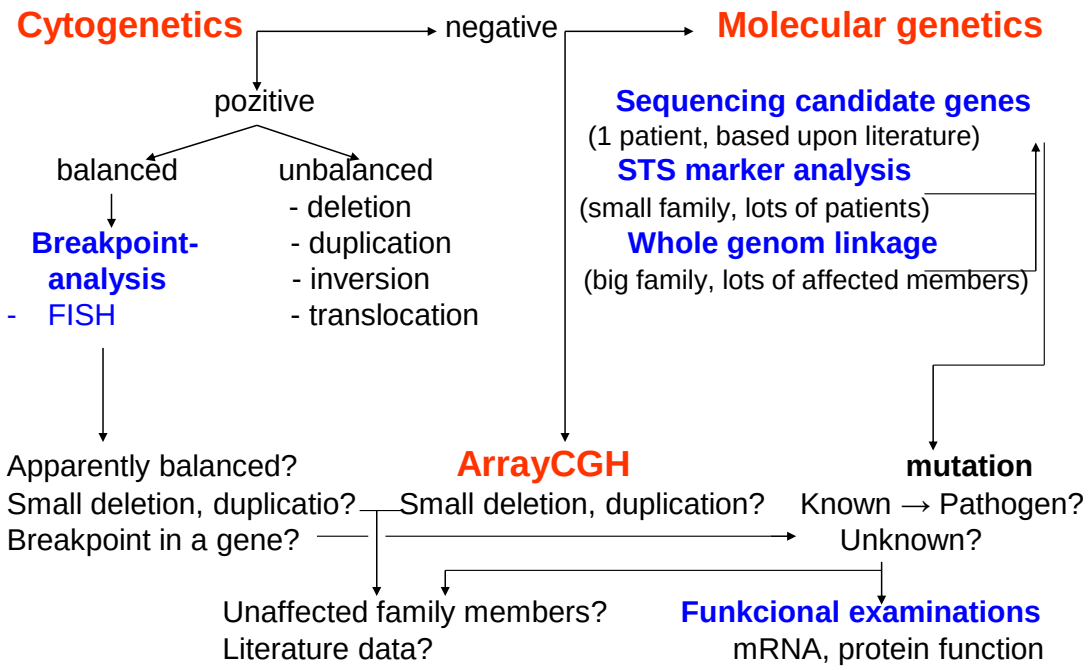

Figure 1: The procession of genetic examination in limb developmental defects

Table 2: Classification of limb defects, based upon clinical and developmental aspects

Pathomechanism	Topology	Clinical terminology
I. Congenital construction deformity	*Transvers defect:* - shoulder, arm - forearm - hand - fingers *Longitudinal defect:* - radial (preaxial) - middle (central) - ulnar (postaxial)	Amelia Hemimelia Phocomelia Acheiria Oligodactyly Ectrodactyly Brachydactyly Synbrachydactyly
II. Abrupted development		
III. Hypoplasia	- whole limb - whole hand - carpi / metacarpi - fingers	
IV. Duplication	- whole limb - humeral segment radial / ulnar segment - fingers preaxial, central, postaxial - phalanges	Mirror hand Polydactyly Synpolydactyly Triphalangeal thumb
V. Hyperplasia	- whole limb - whole hand - carpi / metacarpi - fingers	Hemihypertrophy Macrodactyly
VI. Disturbed differentiation	*Soft tissuesaffected:* - disseminated - shoulder - elbow / forearm - wrist / hand *Bone system affected:* - shoulder - elbow - forearm - wrist / hand	Arthrogryposis Camptodactyly „Thumb in palm” Syndactyly Synostosis Symphalangia Clinodactyly
VII. Generalized bone developmental defect	Generalized	Achondroplasia Marfan syndrome …

4. Case reports

Our work plans were built with the purpose to assess general definitions based upon the clinical and genetic analysis of unique cases. Some illustrative cases will serve to present the results detailed above.

4.1. Isolated limb defects

The background of isolated limb defects is still an unidentified part of genetics. About ten genes are known to be responsible for these kinds of anomalies. We examined 144 cases with isolated defects, but only 20-25 patients showed symptoms that can be connected with any of the ten genes. Due to the rare incidence we could perform genetic examination in only a few cases. Among isolated limb defects a tetramelic, postaxial, familial case was highlighted during our study.

Isolated dominant tetramelic postaxial oligodactyly

To our knowledge isolated dominant tetramelic postaxial oligodactyly characterized by reduction defects affecting the postaxial ray of both upper and lower limbs was reported only once previously. Here we describe a second family with this kind of malformation, affecting the mother and her two children, a boy and a girl (Figure 2).

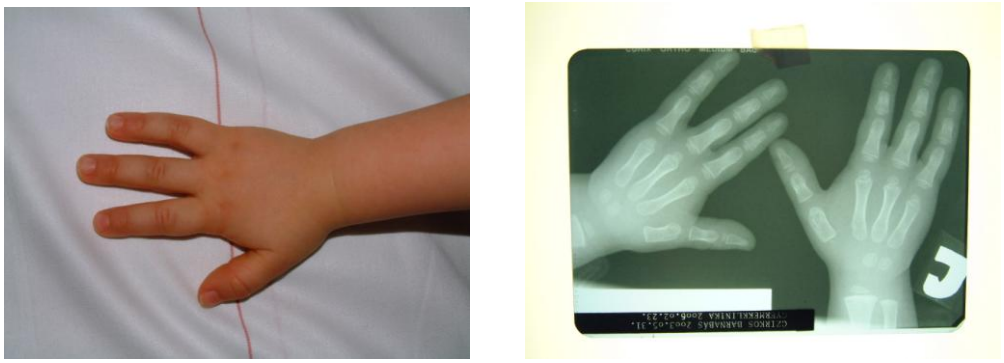

Figure 2: Photo and X-ray of the boy (3-year-old) hands

The chromosomal analysis and molecular examinations have not revealed any causative abnormality. We indicated array which was performed on genomic DNA from peripheral blood using a previously described whole-genome tiling path BAC array comprising 36.000 BAC clones. ArrayCGH revealed a small duplication affecting 7q36.3 in each patient. This variation has not been reported as a normal polymorphism in the Database of Genomic Variants and it has not been observed repeatedly in >800 controls analysed on the same platform. The region includes two genes that are known to play an important role in limb development (*LMBR1 – „limb region 1 homolog”*, *HLXB9 – „homeobox HB9”*) and may be responsible for the limb defect inherited in the family.

4.2. Compound syndromes

Dysmorphic features, connected symptoms may help to find exact diagnosis in compound syndromes and give chance for genetic examinations.

We would like to feature some of our cases – with compound syndrome including limb developmental defects – where genetic examinations were available.

4q deletion syndrome

Deletion of the terminal region of the long arm of chromosome 4 results in a series of clinic features including developmental delay, cleft palate, micrognathia and limb defects, mainly reduction of the ulnar ray.

We have examined two patients presented with the 4q-deletion syndrome and mapped the breakpoints using G-banded chromosome analysis, FISH and array CGH. The overlapping deleted regions of the patients may be responsible for the common features, while the differences in the site of the deletion may explain the variance in the clinical spectrum (Table 3). Our study has helped to restrict the region that is responsible for the limb abnormalities and define new phenotype-genotype correlations related to the terminal 4q region.

Table 3: Characteristic and other symptoms may relate to 4q del syndrome

Characteristic features of 4q del syndrome	**1. case**	**2. case**
Cleft lip and palate	+	+
Micrognathia	+	+
Epicanthus	-	-
Upslanting palpebral fissures	-	-
Small, anteverted nostrils	-	-
Mental retardation	-	+
Heart developmental defect	+	-
Limb developmental defect (ulnar ray)	+	+
Other symptoms (may relate to 4q del syndrome)		
Frequent infections (otitis media)	+	+
Sacral deformity	+	-
Ankyloglossia	+	-
Epilepsy	-	+

Feingold syndrome

Feingold syndrome is an autosomal dominant disorder characterized by variable combinations of microcephaly, limb malformations, oesophageal and duodenal atresias and learning disability/mental retardation. A causative gene in Feingold syndrome is the gene coding MYCN transcription factor. We reported two unrelated patient with Feingold syndrome. The first patient is a 2-year-old boy, showing milder phenotype. The other case includes a family, a 4-year old boy with classical features of the syndrome and his mother and grandmother, only possessing the clinical phenotype of "microcephaly-digital abnormalities-normal intelligence".

Sequencing revealed the mutation of MYCN gene in every case (Figure 3).

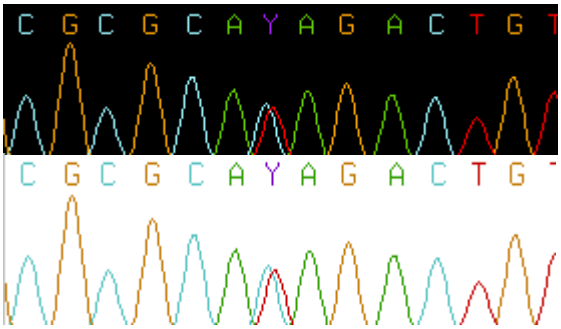

Figure 3: Result of sequencing in the family with Feingold syndrome

Our cases highlight the significantly variable expressivity of *MYCN* mutations in Feingold syndrome and support evidence that „microcephaly-digital abnormalities- normal intelligence" syndrome represents a mild form if this genetic entity.

Short rib – polydactyly syndrome (SRPD)

SRPD is a rare, lethal, autosomal recessively inherited disorder. The genetic background has not been revealed yet. In a period of 8 years a total of 9 propositi of 7 different family were diagnosed with the syndrome SRPD type IV (Beemer-Langer) based upon X-ray examination. All met the criteria of SRPD (lethality, hypoplastic thorax, short limbs, and short tubular bones) (Figure 4).

Each case was lethal (the patients died within 1.5 months following delivery). All patients were born in gipsy families living in the South Transdanubian region (the number of roma ethnicity in this area is estimated to be around 50-60 000). Although, consanguinity could not be detected among the patients' families, it is well known that roma usually marry amongst each other and have had limited geographical mobility in the last 1-2 hundred years. It may suggest that all SRPD cases are likely a consequence of homozygosity for a single founder allele occurring fairly frequently in a small, relatively isolated human population.

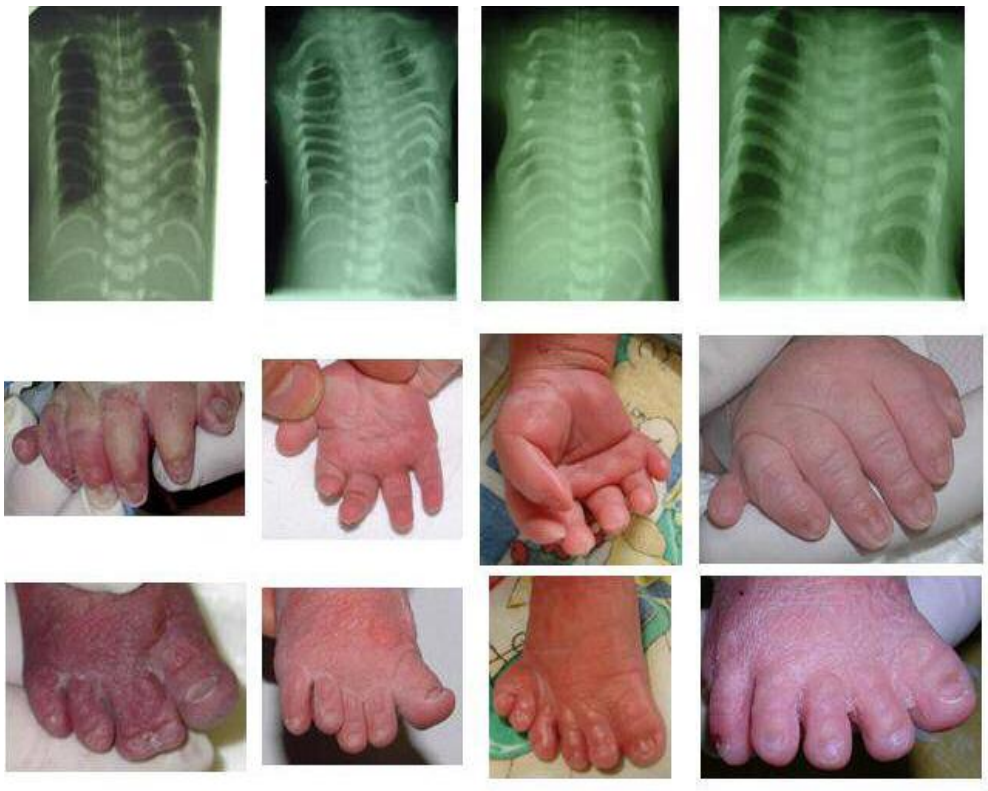

Figure 4: Thoracic X-ray pictures and photos of the limbs of the SRPD patients

DNA was obtained from 4 patients (in 2 cases from the parents as well). Homozygote mapping examination could be performed to find alleles that are homozygous in all the affected patients, showing the same haplotype. The examination was performed with a SNP based (16 000 SNP) Affymetrix chip (resolution of 50Kb) that revealed an allele sized about 4Mb on chromosome 14. 40 genes known to be involved in this region based upon Human Genome Browser. We have verified the results with STS marker analysis (Chr 14 bp: 56222543 – 61770134) that was completely overlapped by the "mapping" examination. Further examinations, sequence of the target genes and functional examinations of the encoded proteins are needed to reveal the exact background of this lethal syndrome.

Brachydactyly E, ulnar-fibular ray defect

Brachydactyly E is characterized by the shortening of the metacarpi and phalanges in the ulnar ray (mainly 4^{th}). We examined a family showing autosomal dominant inherited brachydactyly E in some cases combined with ulnar and fibular ray defect. The anomaly affected four generation of the family (Figure 5). In the youngest affected member of the family, a 7-

year-old girl, the clinical status was complicated with heart defect. Small stature (<5pc) was observed in the half of the cases.

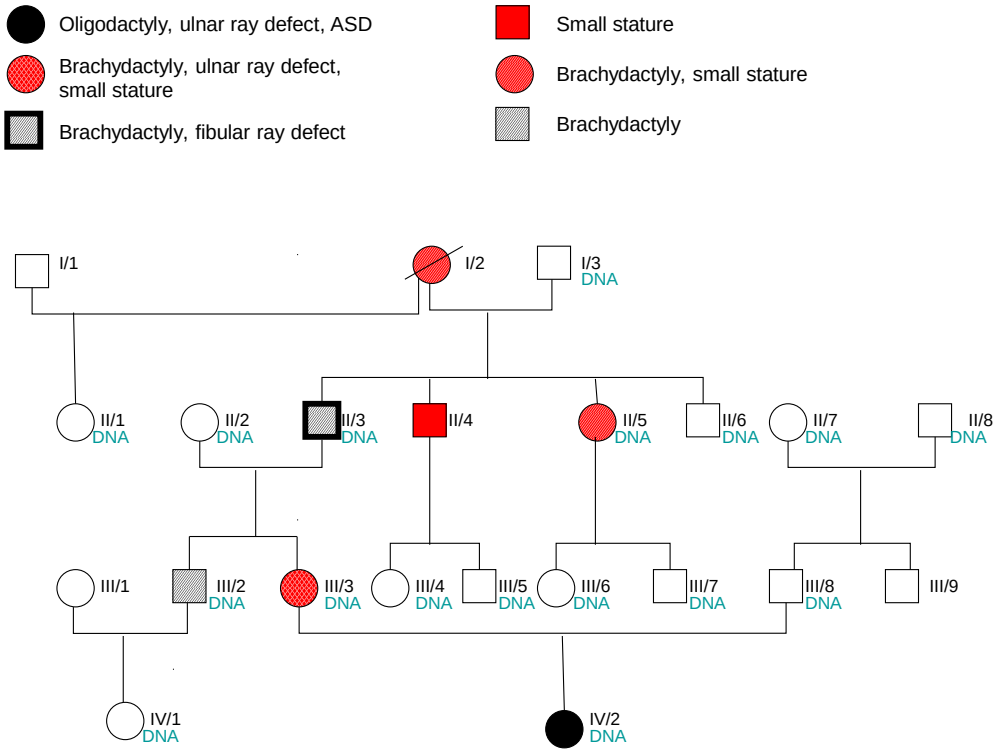

Figure 5: Pedigree, showing the variable symptoms

Based upon the chromosome analysis (showing no abnormality), the pedigree and the clinical signs an autosomal dominant inherited, monogenic mutation was suggested, showing complete penetrance and variable expressivity.

Microsatellite marker based linkage examination had been performed previously in our department, excluding affection of TBX3 or TBX5. Apropos of our study we examined all family members again, based upon our clinical examination protocol. DNA was observed from affected and non-affected family members who were not examined previously. Therefore, DNA was available in 17 cases, including 5 affected patients. Whole genome linkage examination could be performed. 8 meiosis were examined, but was not enough to find tight connection between any suspected allele and the inherited anomalies. The advanced techniques of the near future may help to reveal target genes responsible for the symptoms.

V. CONCLUSIONS

I summarized our observations and emphasized our scientific conclusions related to individual cases.

1. Our study brought up new aspects and information related to limb developmental defects and accompanied symptoms. Our achievements were summarized in publications that may raise attention of other professionals who can meet these kinds of abnormalities.

2. We have constructed and now propose a novel classification system that was created to sort and classify limb developmental defects based upon Swanson's recommendation.

3. Our study highlights the importance of the examination of transcription factors in limb abnormalities, especially in compound syndrome.

4. New results, scientific conclusions:

- We reported on a family with apparentely autosomal dominant inherited, *isolated tetramelic postaxial oligodactyly*. We tried to attract attention for this rare condition that was described to our knowledge only once before. Additionally we mapped a microduplication that can be found only in affected family members. Some genes in that region are known to be involved in limb development. Our findings can highlight the connection between the genes and this rare developmental abnormality.

- Analysis of two cases with *4q deletion* helped us to define some new phenotype-genotype correlations related to regions involved in the deletions.

- Detailed clinical examination and results of sequencing in the family of a *Feingold syndrome* patient supported evidence that *"microcephaly – digital abnormalities – normal intelligence"* syndrome represents a mild form of Feingold syndrome.

- Analysis and genetic examination of *short rib polydactyly* cases has given opportunity to get closer in revealing the background of this rare, lethal syndrome with unknown origin.

Finally it could be stated that study of genetic background of limb developmental defects is still in its infancy. Despite of the development in scientific methods and techniques, declaring exact diagnosis, unravelling causative mutations is a complicated or – in most of the cases – impossible task. To this end in view the candidate feels necessary – and is committed to continue and extend this study to approve cognition of developmental processes and their abnormalities.

LIST OF PUBLICATIONS

Publications related to the thesis:

1. **Tészás A**, Kárteszi J, Kosztolányi Gy. Új lehetőségek a fejlődési rendellenességek hátterének feltárására: transzkripciós faktorok klinikai genetikája. Orv Hetilap 2006; 147: 697-702.
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2. ***Tészás A***. Genetests in limb developmental defects/ Génvizsgálatok végtagfejlődési rendellenességekben. Genetikai Műhelyek Magyarországon (5^{th} miniconference) (Szeged, 2006. September).
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Cummulative impact factor (without citated abstracts): 25,286.

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