Investigation of copy number variations in the development of rare Mendelian diseases

DOCTORAL (PHD) THESIS

Gergely Büki

Supervisor: Dr. Judit Bene

Head of the Doctoral School: Prof. Dr. Ferenc Gallyas

Head of the Doctoral Program: Prof. Dr. Béla Melegh

Doctoral School of Interdisciplinary Medicine, Medical School, University of Pécs

Pécs, 2022

List of abbreviations

aCGH - array Comparative Genomic Hybridization

ADHD - Attention Deficit Hyperactivity Disorder

BIR - Break-Induced Replication

CALs - Café-Au-Lait spots

cb-EGF - Calcium-Binding EGF

CMT1A - Charcot-Marie-Tooth disease type 1A

CNV - Copy Number Variations

CT - Cutaneous

CV - Cardiovascular symptoms

DSB - Double-Strand Breaks

DNA - Deoxyribonucleic Acid

dsDNA - double-strand DNA

EGF - Epidermal Growth Factor

FBN1 - Fibrillin-1

FISH - Fluorescence In Situ Hybridization

FoSTeS - Fork Stalling and Template Switching

GRCh37 - Genome Reference Consortium Human genome build version 37

HCP - Health Care Provider

HR - Homologous Recombination

IF - In-Frame

LCR - Low Copy Repeats

LTBP - Latent TGF-β–Binding Protein

MASS syndrome - Mitral valve prolapse, Aortic enlargement, Skin and Skeletal findings

ME - Mobil Elements

MFS - Marfan syndrome

MLPA - Multiplex Ligation-Dependent Probe Amplification

MMBIR - Microhomology-Mediated Break-Induced Replication

MMEJ - Microhomology-Mediated End Joining

MPNST - Malignant Peripheral Nerve Sheath Tumours

MVP - Mitral Valve Prolapse

NAHR - Non-Allelic Homologous Recombination

NF1 - Neurofibromin

NGS - Next Generation Sequencing

NHEJ - Non-Homologous End Joining

OF - Out-of-Frame

OPG - Optic Pathway Glioma

PCR - Polymerase Chain Reaction

PL - Plexiform

PTC - Premature Termination Codon

SBC - Subcutaneous

SD - Segmental Duplications

SDiCD - Significant Delay in Cognitive Development

SNP - Single Nucleotide Polymorphisms

SRO - Smallest Region of Overlap

SRS - Serial Replication Slippage

SV - Structural Variations

TAAD - Thoracic Aortic Aneurysm Dissection

TB - TGF-ß1 Binding Protein

TE - Transposable Elements

TFBS - Transcription Factor Binding Sites

TGFBR1/2 - Transforming Growth Factor β Receptors 1 and 2

TMEJ - Theta-Mediated End Joining

TR - Tandem Repeats

1. Introduction

Technological innovations in the second half of the 20th century and the early 21st century have contributed greatly to the development of many areas of science, including human genetics. The study of genes, variations and heredity has come a long way. Recent major advances in molecular biology methods have enabled us to expand our knowledge of common and rare disorders. Due to the limitations of technology, the primary discoveries have focused attention on cytogenetically visible segments and single nucleotide variations and their effects. Structural variations (SV) have been significantly understudied due to the challenges of identification. The discoveries have contributed to unravelling our genomes and understanding the many mechanisms behind the development of rare diseases. The improvements expanded the detection of various forms of genetic variations. Nowadays, an increasing number of studies highlight the importance and consequence of SVs, especially copy number variations (CNV), in molecular biology and medicine, as well. SVs often affect important genomic regions and their effect manifests itself in a more complex, specific phenotype, repeatedly characterized as a genomic disorder. These disorders can be distinguished from Mendelian disorders by affecting a different size range and are usually associated with a more complex phenotype. The number of genomic disorders are expanding over time.

In order to characterize copy number variations in patients suffering from neurofibromatosis or Marfan syndrome, modern methodologies, including multiplex ligation-dependent probe amplification (MLPA) and array comparative genomic hybridization (aCGH) were applied in our research work. Furthermore, genotype-phenotype analysis was set up based on the demonstrated results and clinical data collection. During our thesis, examination of the correlation between the course and severity of the disease and the presented genetic variations was carried out. Moreover, an investigation of the association between the detected large CNVs and the severity of the cardiovascular manifestations in Marfan syndrome was performed and presented in our research. Exploration of the role of regulatory elements, especially focusing on transcription factor binding sites located within the *FBN1* gene was applied. In addition, breakpoints of a large *de novo* deletion in this gene were investigated and a molecular mechanism behind the formation of this non-recurrent CNV was proposed.

1.1. Genetic Variations

Although deoxyribonucleic acid (DNA) stores the code of life, therefore properly protected, maintained and regulated, variations occur quite frequently. Examination of genome-wide sequence variations facilitated the determination of the relationships between genetic variations and a variety of their consequences including cellular dysfunctions, phenotypic traits, or diseases. Individual susceptibility is influenced by numerous factors (for instance age, sex, genetic, epigenetic and environmental factors etc.) (1).

Genetic variations occur in various forms. They can arise at a single position (2), affect small stretches of nucleotide sequences (3, 4), and influence large segments and even entire chromosomes (5, 6). The type and/or location of a variation highly influence its consequences.

The following types of the known variations can be distinguished: nucleic acid substitutions (transition, transversion), insertion and/or deletions of nucleotides (from 1 base to hundreds of base pairs), differences in repeated sequences (dinucleotide, trinucleotide), balanced and unbalanced alterations of large genomic segments including changes in the copy numbers of DNA segments (deletion, duplication, insertion) and chromosomal rearrangements (inversion, translocation). They supply a wide range of effects on an organism, mostly based on the function of the affected genes.

First and foremost, modifications on DNA level can occur in protein coding or non-coding sequences. The amount of non-coding DNA varies greatly among species (7) and ca. 99% of the human genome is non-coding (8). Introns, special non-coding DNA elements and regulatory sequences build up most of the genome. Most observations of the functions of these regions are related to regulatory elements such as promoters, silencers, enhancers, and insulators. Among others, they essentially coordinate gene expression according to environmental conditions, and determine appropriate cell types at the correspondent developmental stages (9).

The genetic variations localized in the coding sequences have the most unequivocal consequences on an individual’s phenotype. Looking at the medical point of view, DNA sequence variations can contribute to the development of certain diseases, influence susceptibility and shape the responses to medications. Thereby the most straightforward classification is based on the effect on the appearing phenotype. A modification can be

beneficial, neutral or harmful generated by external factors (for example chemical agents) or occur by malfunction of biological or chemical processes (10).

In order to describe further the effect of variations, we have to differentiate and characterize variations. According to current knowledge, there are various ways to classify and organize variations, meaning there can be overlapping definitions. Hereinafter the characterization is based on the number of affected base pair(s). Andrew J. Sharp et al. (11) demonstrated a straightforward and completely understandable determination and summary of the different mutation types based on their affected size range. The characterization and the following train of thought are essentially based on their classification (Figure 1).

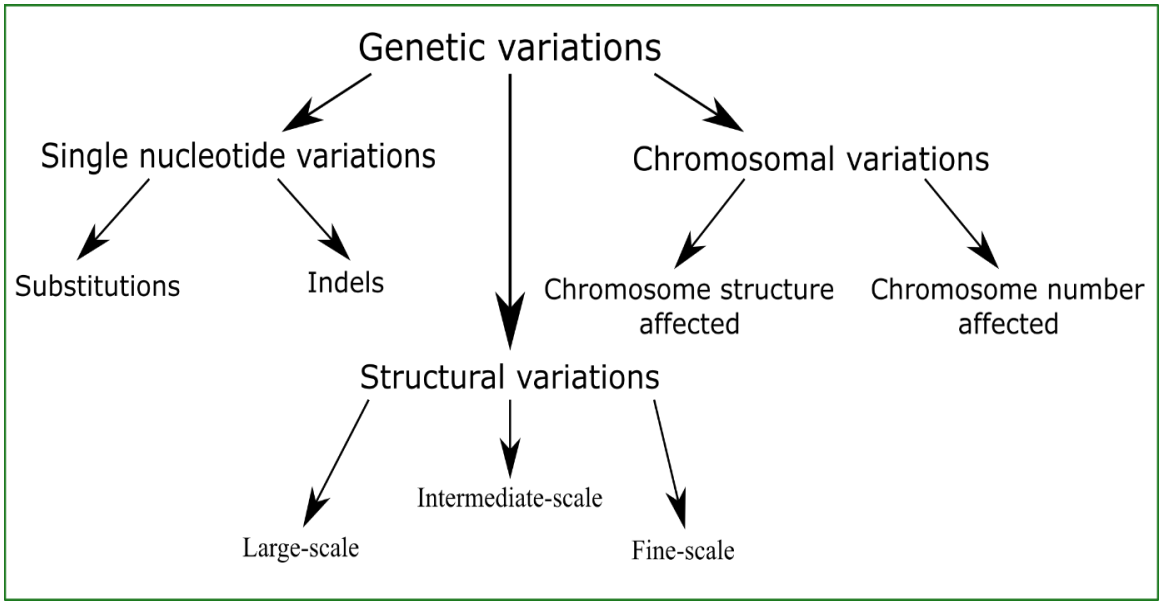


Figure 1. *Types of genetic variations*

First of all, it is essential to separate mutations and polymorphisms. They are often used interchangeably. The main distinction between the two is the frequency of the variant in the population. Although rare mutations tend to have a functional impact, it is not always that straightforward. Polymorphisms are fundamentally any kind of genetic variation found in at least 1% of the population. The term is usually referred to as common variations that do not directly cause disease. The phrase does not differentiate between the affected base pairs, meaning a modification in a larger genomic range (such as copy number polymorphism) can be a polymorphism just as single nucleotide changes in one base pair (such as single nucleotide polymorphism) (12). On the contrary, mutations are

rare variants, appearing in less than 1% in a population, and usually resulting in significant consequences to the individual (13).

For the sake of clarity, the following sections will draw a picture of the different types of mutations as displayed broadly in Figure 1. In addition to the shown genetic variations, repetitive elements will be mentioned in a non-exhaustive manner because of their wide range of presence in different sizes demonstrated all around the genome. Besides, more and more cases show the contribution of such elements in generating copy number variations (CNVs). Intermediate-scale SVs, specifically CNVs will be discussed in more detail.

1.1.1. Single nucleotide variations and short indels

Single nucleotide variant (SNV) is a general term for single nucleotide change in DNA sequence. They are the most frequent variations in the genome, they can be rare or common, germline or somatic. In case of a single base substitution present in more than 1% of the population, the variant is called single nucleotide polymorphism. The relatively high frequency of the variation suggests either neutral or beneficial effects. SNPs are used as important markers when looking at different populations. These modifications are the most common forms of genetic variations, they appear ca. by every 1,000 base pairs. Mutations at a single nucleotide position can be substitutions, insertions and deletions, and indels as well (1).

1.1.2. Repetitive elements

Patterns of nucleic acids occurring in multiple copies in the genome are called repeated sequences or repetitive elements. Repeats are widely dispersed among many organisms. Most of them are in non-coding regions of the genome. These identical segments many times serve as mutational hotspots for rearrangements of various size. It is believed that repetitive elements have regulatory roles (14), and might shape the 3D folding of the genome (15). Although the proposed hypotheses are only supported by limited experimental evidence.

Different types of repetitive elements are known, mainly based on the length of the affected nucleotides. Without completeness, repetitive DNA can be classified into five broad categories, where transposable elements account for most of the genomic DNA and are primarily composed of retrotransposons. The other classes include segmental duplications, simple sequence repeats, tandem repeats and satellite DNA sequences and processed pseudogenes (16).

Tandem repeats (TR) are frequently observed in genomes across all domains of life and are primarily a pattern of two or more nucleotides repeated adjacent to each other. The determination of a TR is based on the number and similarity of units and the length of the minimal repeating motif (17). TRs are essential components of genome biology through their functional and evolutionary roles (18). Tandem repeats can occur through replication slippage and form long stretches of nucleotides. Repeat units of less than 10 base pairs are microsatellites. The most known examples of these are telomeres which typically have 6 to 7 base pair repeat units. Microsatellites include a variety of simple di-, tri-, tetra-, and pentanucleotide tandem repeats. Prominent types are the di-, and trinucleotide repeats, where due to their name two or three nucleotides are repeated. Repeat units from about 10 to 60 base pairs are called minisatellites, which are found in many places in the genome including centromeres. Satellite DNAs are typically found in centromeres and heterochromatin. More and more studies (19, 20), which are focusing on the determination of the exact breakpoints of CNVs, and display that tandem repeats might play an essential role in the formation of non-recurrent CNVs (17).

Repetitive elements which might play a role in the formation of CNVs will be discussed in more detail later on (segmental duplications/low copy repeats).

1.1.3. Structural variations

Structural variations (SVs) are a group of genomic rearrangements affecting long stretches of a nucleotide sequence. By definition, the affected size range spans from 50 to thousands of base pairs (21, 22). They can be characterized into fine-, intermediate-, and large-scale SVs (Figure 2). It is estimated that altogether roughly 5-10% of the human genome is structurally variable (11, 23). Based exclusively on SNVs, the genomic variation is estimated to be about 0.1%, however, with the presence of SVs, this number is approximately 1.5% (24).

The latest research-based on short-read sequencing data suggests that around 7000-9000 SVs are present in the human genome. However, these numbers can be significantly higher (25-27). Another intriguing observation is that SVs distribution across the genome is nonrandom they often cluster into hotspots (17, 28). For instance, SVs show a higher rate at telomeric regions (29).

SVs are classified as balanced (inversions, translocations), unbalanced (insertions, deletions, duplications) or a complex combination of SVs. Genomic imbalances, such as copy number variations are the most frequent forms of SVs, thus most deeply determined and characterized (25, 30). The various types of SVs contribute diversely to dysfunction, including removing or adding copies of entire genes (amplification often leads to overexpression), truncating genes with intergenic rearrangements, influencing gene expression by altering regulatory sequences, gene fusions and so on (31). Altogether, they contribute to the appearing phenotype. Considering that SVs affect larger sizes, they potentially provide a higher impact on the phenotype compared to SNVs (32).

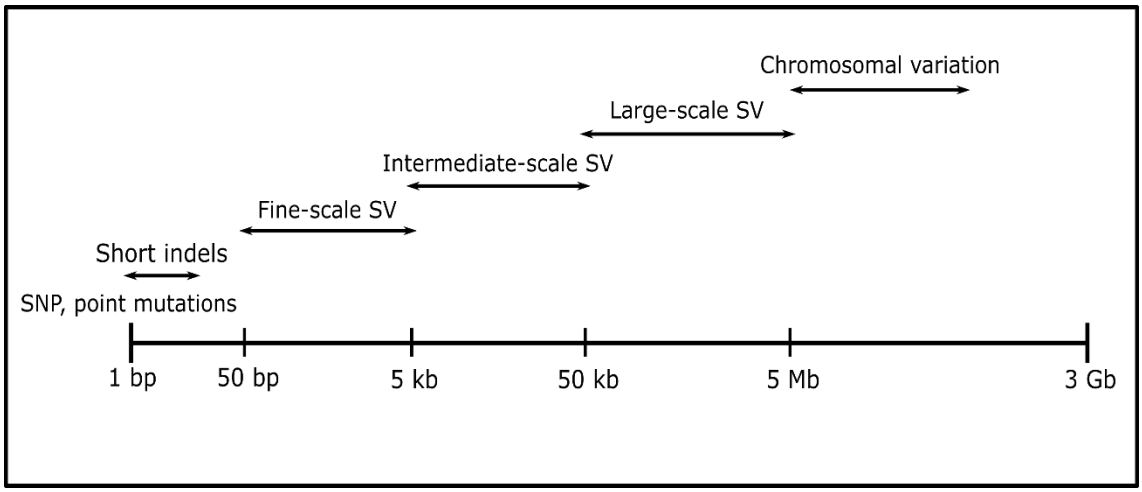


Figure 2. *Size characterization of the different groups of structural variants aligned with the spectrum of main genetic variations*

The characterization of structural variations is difficult to grasp, thus classifications and definitions often overlap with each other. Size ranges are not definitive. Altogether, insertion, deletion, duplication/amplifications, tandem repeat changes and inversions appear among the groups demonstrated in Figure 2.

In the following sections, fine-scale and large-scale structural variations will be explained further without claiming completeness, and then intermediate-scale structural variations, specifically copy number variations will be discussed more thoroughly.

Fine-scale structural variations

Fine-scale structural (50bp to 5kb) (11) variations include small insertions, inversions and deletions affecting several nucleotides, di-, and trinucleotide repeats and other short tandem repeats, usually referred to as microsatellites. Most of the cases, the addition or removal of several nucleotides results in the change of the open reading frame, which often leads to truncated proteins. However, a great number of fine-scale SVs localize into non-coding regions.

Tandem repeat sequences, particularly trinucleotide repeats, underlie several human disease conditions. Trinucleotide repeats may expand in the germline over successive generations leading to increasingly severe manifestations of the disease. The disease conditions in which expansion occurs include Huntington's disease, fragile X syndrome, several spinocerebellar ataxias, myotonic dystrophy and Friedrich ataxia. For instance, Huntington's disease is a trinucleotide repeat disorder, where the 'CAG' trinucleotide codon repeat increases (17). Trinucleotide repeat expansions may occur through strand slippage during DNA replication or during DNA repair synthesis (33, 34).

Large-scale structural variations and chromosomal variations

Large-scale structural variations (50kb to 5Mb) (11) include insertion, deletion, duplication and amplification, inversion and translocation (both reciprocal and Robertsonian) of large chromosome regions and broad tandem repeats. Chromosome variations affect either the structure or the number of chromosomes (such as aneuploidy).

The most known examples of chromosome number changes either affect sex chromosomes, such as Klinefelter's syndrome (35) or autosomes, for instance, Patau (36), Edwards (37) and Down (38) syndrome. Each carries an additional chromosome thus severely altering the appearing phenotype. Chromosome abnormalities have a significant impact on miscarriage risk and fertility (39). Around 0.5% of newborn infants carry chromosome rearrangements (40).

Initially conventional cytogenetic techniques were applied for the examination of these large-scale SVs. As a result of advances in molecular genetic diagnostic methods the detection resolution improved and more complex SVs became observable. Complex

forms involve more than two breakpoints and/or multiple chromosomes. However, according to a previous study presented in 2011 (41), these SVs are rare cases, until that time approximately less than 300 cases have been reported and most of them occur *de novo* (39, 42).

Although next-generation sequencing technologies are capable of sequencing thousands of base pairs, the detection and characterization of large-scale SVs, especially complex forms, remain a challenging task.

Intermediate-scale structural variations

Intermediate-scale structural variations (5kb to 50kb) (11) also include insertion, deletion, duplication/amplification, tandem repeats and inversion. The importance of these SVs was heavily underestimated due to the misinterpretation of previously existing results. The advances in technology made it available to detect submicroscopic, intermediate-sized structural variations, which were not observable before with light microscopes or sequencing-based methodologies. Around 20 years ago, a couple of studies (5, 43) triggered an "avalanche" by indicating that SVs potentially represent a significant portion of genetic variations (44).

1.1.4. Copy number variations

Abnormal copy number variation is a type of structural variant appearing quite often in an individual's genome. The exact determination of a CNV is challenging, resulting in many slightly different definitions. The most straightforward and used definition is that CNVs are large DNA segments that are present at variable copy numbers compared to the reference genome (resulting in the deletion, duplication or amplification of a certain DNA region) (45). The determination of the size range is still controversial and unclear. Generally speaking, the range varies from 50 base pairs to millions of bases (45, 46). However, CNVs are mostly acknowledged if the affected genomic segment is larger than 1kb (47).

The consequences of CNVs can range from beneficial to deleterious as well. The majority of CNVs occur in non-functioning, non-coding intergenic regions thus predicted to have

neutral or minor effects on an individual's phenotype (45, 47, 48). Although many times CNVs affect functioning regions of the genome and lead to dosage imbalances. A remarkable example of gene dosage effect is the 1.4 Mb long microduplication displayed in human 17p12, which involves the gene *PMP22* and results in Charcot-Marie-Tooth disease type 1A (CMT1A) (49). These CNVs are known risk factors in developing a variety of human disorders (50-53).

The first association between Mendelian traits and submicroscopic genomic duplications and deletions dates back to the early 1990s (49, 54, 55). Certain diseases are connected to CNVs, for example, Prader-Willi/Angelman syndrome (56), DiGeorge syndrome (57), Smith-Magenis syndrome (58), Williams-Beuren syndrome (59) and so on. In addition, CNVs have been associated with other complex traits, such as susceptibility to autism (50), schizophrenia (60, 61) and HIV infection (62, 63). CNVs may also be responsible for advantageous human-specific traits, for instance, cognition and endurance running (64, 65).

Evaluation of locus-specific mutation rates of *de novo* CNVs was estimated by pooled sperm PCR assays, prevalence calculations, aCGH analyses of trios and studying a single X-linked gene (DMD). The results suggested that the mutation rates appear much higher for CNVs than for SNPs (66-68). The mutation rates widely vary between different loci, which is potentially a result of the differences in genome architecture (69).

CNVs contribute to genomic diversity between individuals and play a significant role in evolution. Altogether, they have a more determining role in genetic variations than previously thought.

1.1.4.1. Types of copy number variations

Specific genomic architecture takes part in creating CNVs. Previous studies demonstrated that repeats in the genome play a considerable role in CNV formation and human evolution (70, 71).

Two major groups can be differentiated by breakpoint analyses of CNVs known as recurrent and non-recurrent CNVs (Figure 3). Although breakpoints can be located all over the genome, they are more frequent in subtelomeric and pericentromeric regions (69).

Recurrent CNVs are more or less located on the same genomic location with identical breakpoints, thus affecting similar sizes of DNA segments among unrelated individuals. Highly homologous sequences set up the possibility for the creation of various outcomes of CNVs, even complex rearrangements. Low copy repeats (LCR) are one of these highly homologous regions and many CNVs are associated with these. They are a subset of the so-called segmental duplications (SD) although most of the time the two definitions are used synonymously. SDs are roughly >1kb in size, exist in two or more genomic locations, occurring interspersed or tandem at multiple locations including subtelomeric, pericentromeric and even interstitial regions. These highly homologous sequences (more than 90%) can result in inter- or intrachromosomal segmental duplications. They are reported in 5% of the genome. SDs generate instability in the genome with the creation of diverse rearrangements. They have a great impact on genome evolution and the differences in various organisms (72).

In contrast, non-recurrent CNVs are detected at different locations with an observable difference in their breakpoints and sizes. A third group, called non-recurrent CNVs with grouping (Figure 3), may supplement the aforementioned forms. In this case, one side of the CNVs is localized into a broadly similar genome location, while the breakpoint at the other side varies, thus the size of the CNVs differs as well. The “fixed” breakpoints are usually localized adjoining to complex genomic architectural elements, such as palindromes or cruciforms. More complex chromosomal structural changes can be also seen at the breakpoints of non-recurrent CNVs, including the addition of short sequences from elsewhere. Most non-recurrent CNVs occur at sites of very limited homology of 2 to 15 base pairs. Thereby presence of complex DNA sequence architecture contributes to genomic instability, and indirectly to the formation of CNVs (69).

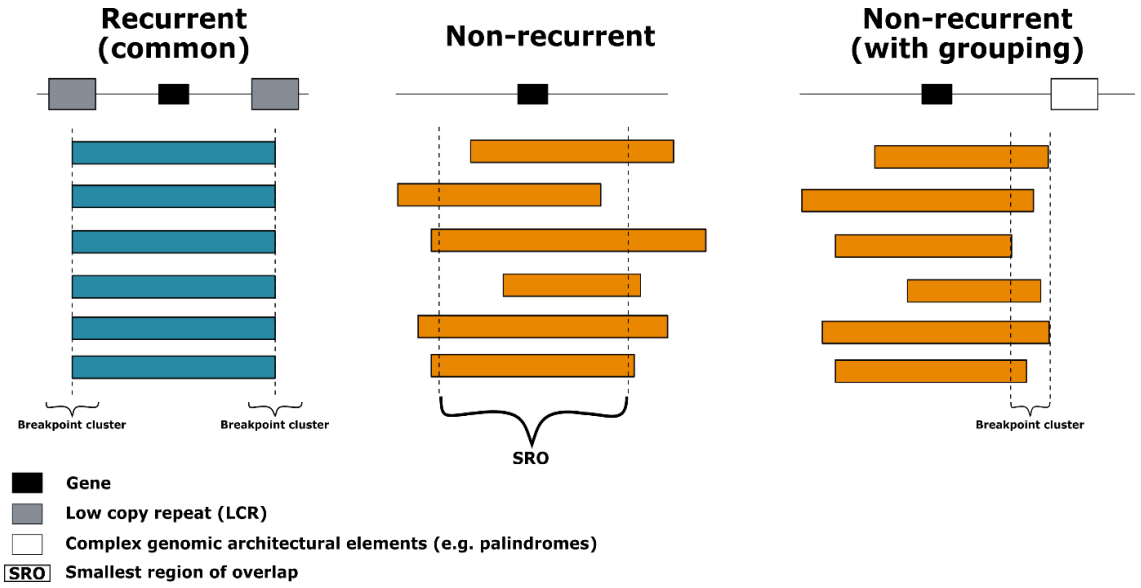


Figure 3. *Types of copy number variations (73)*

In the case of non-recurrent CNVs, a smallest region of overlap (SRO) can be identified, the definition of which depends on the chosen CNVs and their size range. In general, this may be helpful for the interpretation of different CNVs affecting a functional gene corresponding to a predicted phenotype. Appropriately, determined SRO might help to set up a better genotype-phenotype correlation between unrelated individuals.

1.1.4.2. Mechanism of copy number variation formation

The formation of CNVs can happen during recombination- and replication-based mechanisms, as well. Many cases show the contribution of transposable elements in the formation of numerous CNVs (74). Unequal meiotic recombination-based mechanisms are non-allelic homologous recombination (NAHR), non-homologous end joining (NHEJ) and microhomology-mediated end joining (MMEJ). Proposed mechanisms based on replication errors are serial replication slippage (SRS), fork stalling and template switching (FoSTeS) and microhomology-mediated break-induced replication (MMBIR). From previous studies (75, 76), it seems that certain conditions or agents lead to replications stress, which could potentially form harmful CNVs.

Non-Allelic Homologous Recombination

One of the most known mechanisms is NAHR, which contributes to most of the recurrent CNVs (73). The mechanism requires the misalignment of highly identical sequences and strand exchange. This non-allelic recombination event could occur during mitosis or meiosis as well. The CNVs are often found in close proximity to segmental duplications or LCRs (77), although other long stretches of homology can also be responsible for NAHR, such as Alu or L1 elements. Interestingly, smaller homology regions (200 base pairs to 1 kb) might serve as NAHR "hotspots" as well (78). For instance, the 22q11.2 deletion syndrome is a well-defined example of the involvement of LCRs and NAHR in the formation of a genomic disorder (57). It seems that the length of the SD and the inter-SD distance influence NAHR frequency (79).

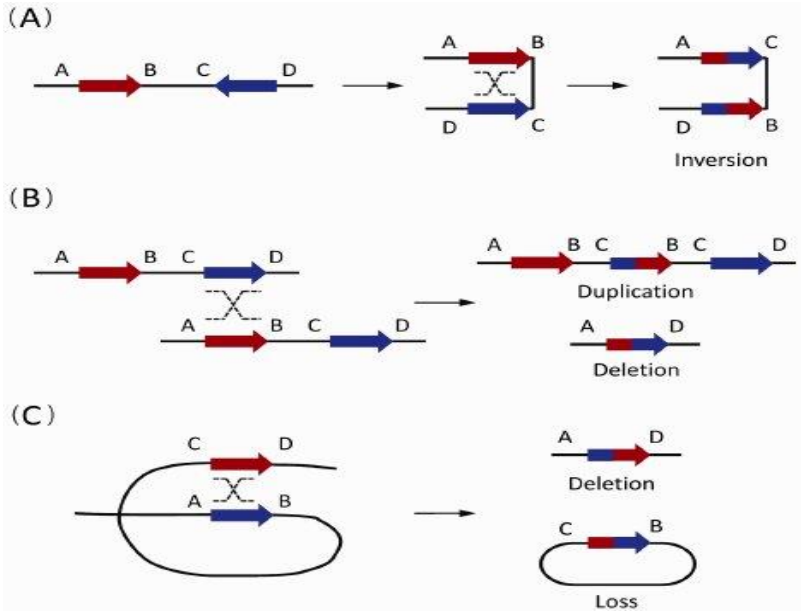


Figure 4. *Examples of NAHR mechanism (80)*

The genomic localization (inter- or intra-chromatid) and relative orientation (direct or reverse) of the repetitive elements determine the type of the created rearrangements. (Figure 4) (80) Deletions and duplications will be generated if the element is in direct orientation (Figure 4/B), while the opposite orientation will create inversions (Figure 4/A). SDs on the same chromosome with direct orientation can generate ring-shaped DNA segments or deletions shown in Figure 4/C (80). If homologous regions are located on different chromosomes reciprocal translocations can be generated (11, 80-82).

Non-Homologous End Joining mechanisms

NHEJ is one of the main repair mechanisms to restore double-strand breaks (DSBs), especially in G0 and G1 phases. The other mechanism is homologous recombination (HR), which predominates in G2 and S phases. Furthermore, it seems that HR and NHEJ compete with each other to repair DSBs. The presence of resection highly influences which pathway will be applied. Without resection, direct ligation will occur, therefore NHEJ is going to repair the DSB. Extensive resection will promote the HR pathway to repair the DSB, by generating an ssDNA tail, which might invade a homologous sequence. Maintaining proper continuity of the genome is essential since unrepaired DSB can lead to disease progression, cancer promoting initiation, and therapy resistance (83).

Fundamentally, NHEJ recognizes DNA DSBs and ligates the double-strand DNA (dsDNA) ends together with little (<4bp) or no sequence homology at all (Figure 5). This is a very fast, relatively accurate process where often the ligated dsDNA segments are from different genomic regions. In many cases the breakpoint junctions show short insertions or deletions of a few nucleotides. In addition, breakpoints often localize into certain repetitive elements (LTR, LINE, Alu etc.). In contrast to NAHR, it does not rely on the presence of highly homologous regions, thus it can occur anywhere in the genome (73, 84). Regularly deletions (73) and chromosomal translocations (85) have been associated with NHEJ; however, duplications (86) were also connected to it (84).

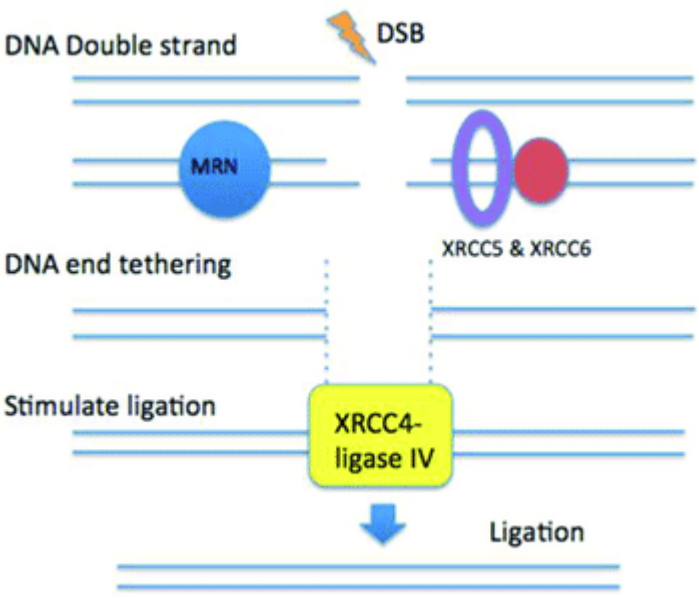


Figure 5. *NHEJ mechanism (73)*

After DSB many factors will participate in the process of NHEJ. First of all, the XRCC5 (also known as Ku80), XRCC6 (Ku70) and MRN complex (MRE11-RAD50-NBS1) will initiate the direct ligation of the DNA ends. The DNA ligation will be completed by XRCC4-ligase IV.

Microhomology-mediated end joining

MMEJ (Figure 6) is a more error-prone, independent, alternative form of NHEJ. Generally, DSBs are repaired by MMEJ if homologous replication or NHEJ repair mechanism are repressed. MMEJ is considered a major source of genomic instability and most cases generate deletions. However, it is associated with other types of genomic rearrangements as well, including inversions, translocations and other complex forms (87-89). It uses 5 to 25 bp long homologies to anneal the dsDNA ends together (90). Polymerase theta-mediated end joining (TMEJ) is a special form of MMEJ, which is capable to repair DSBs using really short microhomology (91, 92).

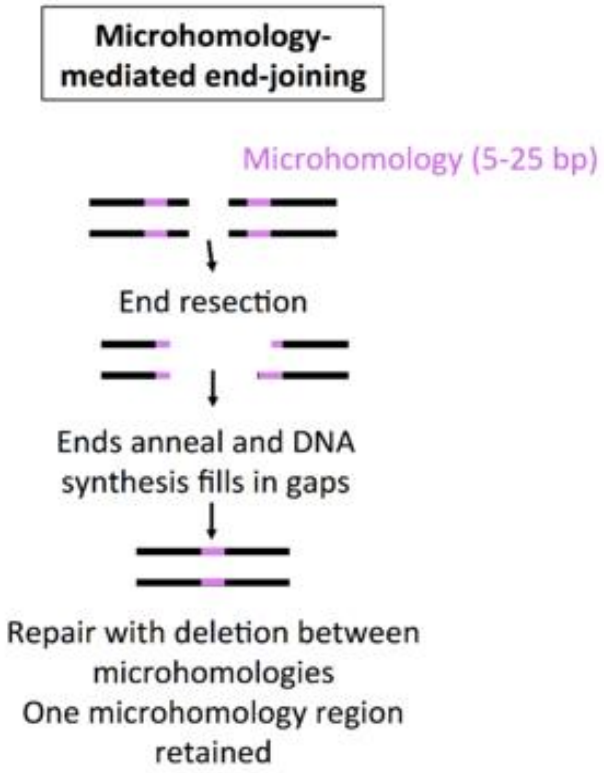


Figure 6. *MMEJ mechanism (https://blog.addgene.org/pitching-mmej-as-an-alternative-route-for-gene-editing)*

Fork Stalling and Template Switching

According to the model, the replication fork stops due to some event, and then switches to a different template by annealing to a complementary microhomologous region on a replication fork in close proximity and consequently continues replication (Figure 7). This DNA replication-based mechanism is proposed to take place during the S phase of the cell cycle (67, 84). The model was first introduced to explain gene amplification induced under stress in *E. coli* (93). Previous studies stated that replication fork collapse and reassembly might be more common than originally thought (94, 95). Besides chemical changes, certain genomic architecture including specific nucleotide motifs and repeat sequences contribute to the stalling of the replication (96). The generated rearrangements range from few kilobases to several megabases (84).

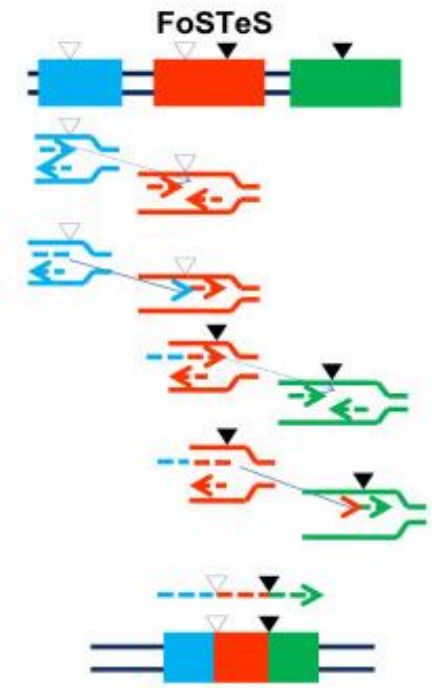


Figure 7. *FoSTeS mechanism (73)*

Figure 7 shows a complex deletion involving two DNA fragments between three different genomic regions (indicated by blue, orange and green rectangles). Microhomologous regions in the replication fork (orange) provides an environment for the other replication fork (blue) to invade this site bearing microhomology (2 to base pairs). Then the leading nascent strand at the second replication fork (orange) invades the third fork (green) via microhomology, and primes its own further synthesis using the right side fork as template. Thereby a complex rearrangements will be created by this mechanism.

Microhomology-mediated break induced replication

MMBIR (Figure 8) is a proposed, specific form of break-induced replication (BIR) that repairs single dsDNA ends coming from collapsed replication forks. When long DNA sequences (50 or more nucleotides in eukaryotes) are not available for homologous alignment (seen in BIR), MMBIR might be a potential explanation for CNV formation (97, 98). MMBIR uses short microhomology coming from another replication fork in close proximity for template switching. A couple of experimental observations come from multiple organisms (yeast, E. coli), including the efficiency of Rad51-independent BIR, reestablishment of replication forks, and microhomology-mediated SD formation (99, 100). As proposed earlier, polymerase eta is efficiently able to restart synthesis in a replication fork from really short primers (2-3bp) as well (100-102).

Considering the aforementioned, MMBIR is a potential mechanism for the formation of many non-recurrent copy number variations. The possibility of microhomology-mediated connection of sequences far away from each other would explain many *de novo* non-recurrent CNVs occurring throughout the genome. However, many factors, conditions and genetic environment influence the occurrence of such events leading to chromosomal structural changes. The consequences of structural changes could be deletions, inversions, translocations, rolling circle, duplications and amplification based on the position of fork breakage, the orientation and the chromosome (detailed in (100) more thoroughly). MMBIR could also potentially generate LCRs or LCR-like sequences which provide identical sequences for homology, thus inducing genomic disorders.

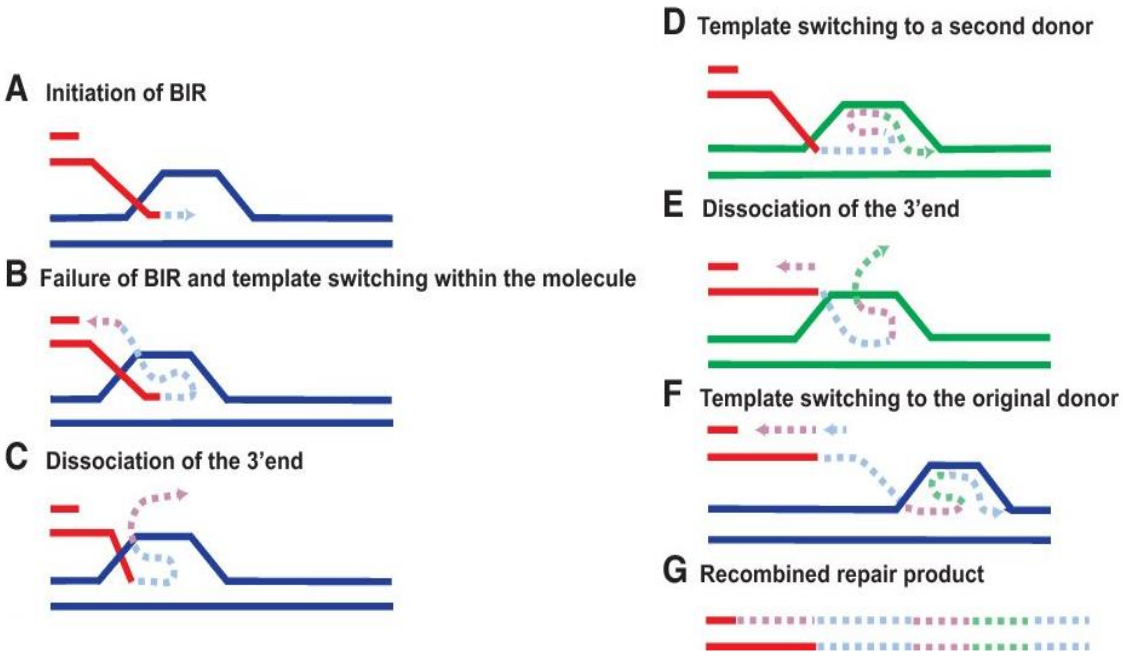


Figure 8. *MMBIR mechanism (103)*

MMBIR mechanism will be induced if the repair of collapsed replication forks by BIR mechanism fails (A and B). The 3' end dissociated from the original donor template (C) and can anneal to other microhomologous sequences in close proximity or at a second donor template (D, E). The 3 ′ strand extension proceeds and involves multiple template switching before annealing back to the original template (F), resulting in complex genomic rearrangements (G).

Serial replication slippage

SRS are basically multiple rounds of forward and backward replication slippage, which often generate smaller complex rearrangements (104). It involves slipped strand mispairing at the replication fork and can generate both tandem duplications with short direct repeats and simple deletions (104, 105). Forward slippage generates simple deletions, while backward slippage leads to tandem duplications (104, 106).

Mobile element insertion

Mobile elements (ME) make up a significant part of the human genome (107). MEs, also known as transposable elements (TEs), are a type of genetic material, which is capable of

relocating themself in and across genomes (Figure 9). According to their mechanism of action, MEs in humans can be classified as DNA transposons or retrotransposons (108).

Several studies (77, 109) demonstrated the importance of TEs in the evolution and shaping of the genome through constructing correlation between certain SVs and mobile elements. While, most elements are inactive, some retrotransposons remained active (mostly Alu, L1 and SVA families), influencing genome diversity. Most SVs caused by mobile elements are neutral, although some have been associated with certain human diseases including Pelizaeus-Merzbacher disease, Tay-Sachs disease, Lesch-Nyhan syndrome, Hemophilia A and Hunter syndrome (108, 110, 111). Four types of structural variants associated to mobile elements can be differentiated, such as classical retrotransposon insertion, non-classical insertions, non-allelic homologous recombination-mediated insertion/deletion, and non-homologous end joining-mediated deletion (110).

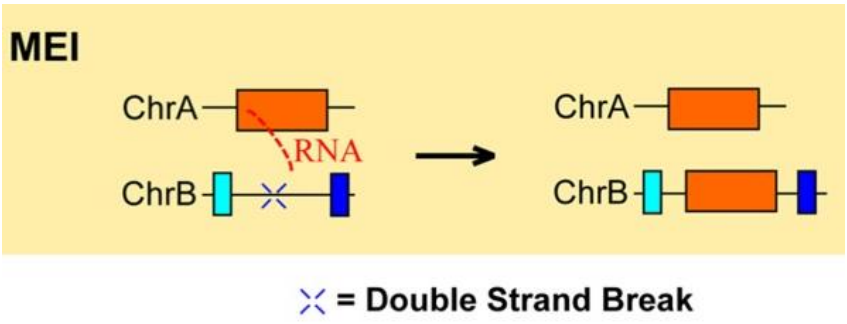


Figure 9. *Mobile element insertion (84)*

Knowing and understanding the mechanism underlying CNV formation is a significant part of identifying and predicting factors and events contributing to a variety of structural variants and their manifestation in an individual.

1.1.4.3. Detection of copy number variations

Accurate screening and characterization of CNVs are challenging because of their wide range of length. From the first methods used for the analysis of CNV at a microscopic scale to the newest generation of sequencing techniques, numerous molecular diagnostic methods have been developed and applied (Figure 10). As detection methods have evolved, the resolution of detection improved over time, giving the possibility of the identification of CNVs with various sizes. After initial analysis of entire chromosomes

with karyotyping, hybridization-based methods have led to major advances in the identification of CNVs and a more detailed analysis of the genome. Fluorescence *in situ* hybridization (FISH) remained a commonly used technique for confirmation of chromosomal abnormalities from metaphase or interphase using fluorescent probes (23, 112).

Nowadays, primarily microarray-based platforms (array-based comparative genomic hybridization), Multiplex Ligation-dependent Probe Amplification (MLPA) and Next Generation Sequencing (NGS) are used for CNV detection.

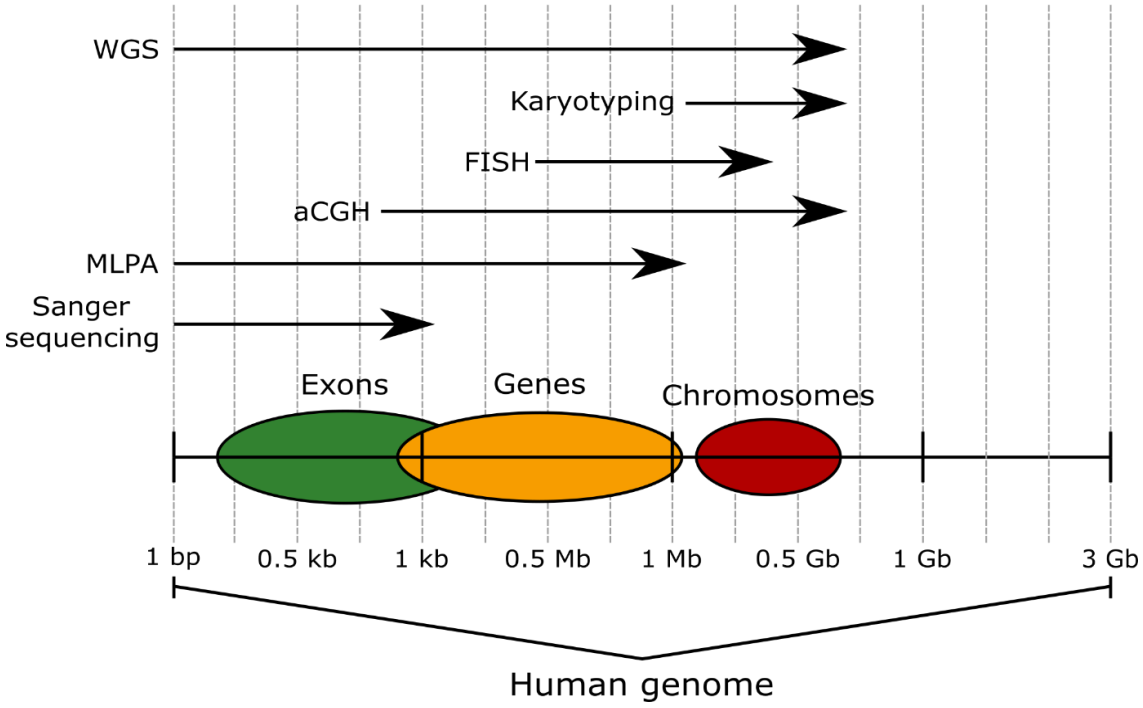


Figure 10. *Schematic representation of the resolution of different methods capable to detect CNVs*

Comparative Genomic Hybridization (CGH) is a technique developed in the early nineties to identify chromosomal gains and losses along the whole genome (113). A methodological advancement of classical cytogenetics has resulted in the development of a high-resolution, genome-wide screening technique known as array comparative genomic hybridization (aCGH) (114).

aCGH can be applied to search for imbalances relatively efficiently and quickly throughout the genome. It uses differently labelled fluorescent genomic DNA samples. The signal intensity ratio seen between two samples is a representation of the copy number balance of certain chromosomal targets. The first important observations came from approaches using BAC clones (112). However, poor resolution resulted in

overestimation of CNV sizes. The usage of long oligonucleotide arrays facilitated a more accurate determination of CNVs, provided a more complete genome coverage, better signal-to-noise ratio and helped the process of chip development. aCGH or DNA microarrays are mostly used to search for clinically important variants (23, 114). SNP microarrays correlate signal intensities from a sample with clustered intensities and from a set of reference samples, and analyze single sample per microarray (112).

Although aCGH is capable of detecting large CNVs simultaneously throughout the whole genome, it is usually not applicable to detect smaller CNVs, especially at the exon level. In addition, it is not capable to precisely characterize the breakpoints.

MLPA is a molecular genetic diagnostic method capable of investigate copy number differences at multiple loci. Relatively low amounts of genomic DNA can be amplified by PCR reaction after the steps of denaturation, hybridization and ligation (115). The technique involves the amplification of oligonucleotides that have been previously hybridized to the genomic DNA template, thus only the ligated oligonucleotides will be amplified. The efficiency of the method to analyze highly homologous sequences is based on the sensitivity of the ligation step. MLPA can easily and successfully identify multiple exons simultaneously. The advantages of MLPA are that it is fast, cost-effective, and multiple loci can be analyzed. A significant limitation is that polymorphisms at or near the ligation sites might affect the ligation step and influence the results. Like aCGH, MLPA is also ineffective for precise breakpoint characterization (115).

High-throughput sequencing provides numerous data as a result of sequencing millions of short reads with high productivity, reproducibility and accuracy. However, early usage of NGS was based on the detection of SNPs and small indels, and the improvements in the quality of NGS made it available to identify CNVs, as well. Consequently, with the abundant numbers of short overlapping fragments the detection resolution has been greatly improved, therefore providing a better viewpoint of structural variations of various sizes. The proper detection of structural variations is still a difficult task and many detection algorithms and methodologies are being developed including *de novo* assembly, split read and read depth methods (RD) and paired end mapping (116). For instance, determining of absolute copy number variations, read depth method is a useful technique as seen in previous research (117). In addition, RD is capable to detect exact breakpoints with high accuracy (118). Although mixed approaches are also being used. A significant limitation of short-read sequencing is connected to unique mapping of short reads to

homologous regions, for example SDs. The most accurate approach to discover SVs is complete genome assembly compared to a high quality reference genome (112). Using new algorithms and longer read technologies will potentially improve *de novo* assembly. Overall, NGS is capable to identify genetic variations in a wide range from SNPs to large CNVs. Although most of the advancements coming from whole genome or whole exome sequencing, more and more computational pipelines are being developed to detect smaller CNVs (118). The improvement of algorithms and bioinformatics tools detecting structural variations significantly helped the definition and determination of SVs (23, 119).

In order to detect CNVs several laboratory techniques have been developed, either for genome-wide or locus specific analysis. However, exact determination at base-pair resolution of breakpoint junctions stands as a challenging task. Sequencing of CNV breakpoints after amplification of the junction by long-range polymerase chain reaction (PCR) is a conventional method for the determination of the breakpoints (67, 120). DNA targets over 5kb can be amplified by long range PCR. Recently new methods are being introduced for proper determination of the breakpoint junctions, such as asymmetry linker-mediated nested PCR walking, and capture and single-molecule real-time sequencing. They have been demonstrated to improve CNV detection, in cases where structural complexity is present (for instance CNVs in Pelizaeus-Merzbacher disease) (121).

It is clear that no single techniques are capable to accurately identify the various forms of CNVs. Each methodology from cytogenetics to massively parallel sequencing has its own advantages and limitations. In general, aCGH is applied for genome-wide analysis of large CNVs without exact characterization of the breakpoints. However, MLPA is a more targeted approach, it is usually applied to examine CNVs affecting one or a few exons to several genes. However, in combination they supplement each other in resolution and accuracy. Furthermore, usage of different methods is essential for the verification and confirmation of detected CNVs. With the latest advancement in next generation sequencing, primarily whole genome sequencing, and bioinformatics, conceivably SNVs and various sizes of CNVs can be properly analyzed simultaneously in the future, thus simplifying and accelerating the diagnostic process.

1.2. Rare Diseases

There are several ways to define rare diseases, thus there is no universal definition for this term. In some cases the definition is based on the number of affected people, in other cases it relies on the severity of the disease and/or the existing/non-existing treatments. By the definitions of the European Commission on Public health, rare diseases are "life-threatening or chronically debilitating diseases which are of such low prevalence that special combined efforts are needed to address them". The prevalence number is specified as less than 1/2000 people. Currently around 7000 rare diseases are acknowledged. Most of them have a genetic cause, only a small portion are generated by infections (bacterial, viral), allergies, and other environmental factors. The disorders can be inherited from parents or generated *de novo*. To our current knowledge ca. 50% of rare diseases affect children. The Global Genes Project estimates the number of affected people as 300 million. The manifestations of the disorders are vastly variable, even among families affected by the same diseases. Both disease progression and the manifestation of the disorder are different amongst patients. In conclusion, the determination and diagnosis of each disorder remain a challenging issue (122, 123).

1.2.1. Marfan syndrome

Marfan syndrome (MFS; OMIM #154700) is a multi-systemic disease with high clinical heterogeneity. It is an autosomal dominant disorder affecting mainly the skeletal, ocular and cardiovascular systems. The expression of the disease can vary in a spectrum from mild isolated features to severe and progressive multiorgan disease (124).

The most life-threatening complication in MFS is connected to the cardiovascular system, including dilatation of the aortic root and ascending aorta, which can result in aortic dissection and sudden death (125, 126). MFS shows complete penetrance (127). The estimated prevalence of MFS is about 1/5000-1/10000 (124). Characteristic inter- and intrafamilial variability have been found in the disease progression and clinical presentation. The disease is caused by mutations in the fibrillin-1 (*FBN1*) gene, which consists of 65 coding exons and is located on the long arm of chromosome 15 (15q21.1). It encodes a major component of microfibrils in the extracellular matrix, called fibrillin-1.

Pathogenic variations in the genes encoding transforming growth factor β receptors 1 and 2 (*TGFBR1* and *TGFBR2*, respectively) have also been reported in patients with MFS (128, 129). Interestingly, most of the disease-causing mutations in *TGFBR1* and *TGFBR2* are responsible for another inherited connective tissue disorder, called Loeys-Dietz syndrome (130). Moreover, there are other MFS-related disorders known as Loeys-Dietz syndrome, homocystinuria, Ehlers-Danlos syndrome vascular types, stiff skin syndrome, MASS syndrome (mitral valve prolapse, aortic enlargement, skin and skeletal findings) and congenital contractural arachnodactyly. These have overlapping phenotypic features with MFS, therefore diagnostics and/or genetic testing is essential in the establishment of the precise diagnosis (131, 132).

According to ClinVar (https://www.ncbi.nlm.nih.gov/clinvar) database, ca. 2500 disease-causing variations (pathogenic, likely pathogenic) and ~1700 alterations with uncertain significance are known in the *FBN1* gene. Although missense mutations are the most prevalent forms represented (133) several frameshift, splice-site or nonsense mutations and in-frame deletions and insertions have also been identified. In addition, the number of large structural variations are increasing as well. Both single, multiple exons and whole *FBN1* deletions have been reported so far (130, 134-159). In the case of *FBN1*, 2-7% of MFS patients have been reported to carry a copy number variation (CNV) (130, 142). Until now, no genomic rearrangements were detected in either *TGFBR1* or *TGFBR2*. Copy number changes of entire *TGFBR1* or *TGFBR2* were identified as part of microdeletion or microduplication involving several other genes (157, 158).

1.2.2. Neurofibromatosis

Neurofibromatosis has multiple distinct types, although the three most frequent are neurofibromatosis type 1 and 2 (NF1 and NF2) and schwannomatosis. Neurofibromatosis has no gender or racial predilection. The most common type, the neurofibromatosis 1 (NF1; MIM#162200), also known as von Recklinghausen disease, is an autosomal dominant disorder caused by genetic alterations in the gene called *NF1*. The disease was described first in 1881 by a German pathologist, Friedrich Von Recklinghausen. Its incidence at birth is 1 in 2500-3000, while the incidence of segmental NF1 is estimated at 1 in 36,000 to 40,000 (160, 161). The main clinical features of NF1 are the hyperpigmented skin macules, called café-au-lait spots (CALS), and the pathognomonic

neurofibromas. The neurofibromas are mostly noncancerous (benign) tumours, localized on or just under the skin. It's a composition of Schwann cells, blood vessels, mast cells and fibroblasts. Neurofibromatosis has a tremendous spectrum of clinical variability, including Lisch nodules, skeletal abnormalities, vascular disease, central nervous system tumours and cognitive dysfunction (attention deficit, learning disabilities), as well. Skeletal abnormalities such as dysplasia of the long bones are characteristic for NF1 patients. Many features increase in frequency with aging and show age-dependent penetrance (162). Characterization differs not just amongst unrelated individuals, but also between relatives in a single family. Socialization is heavily impacted by cognitive and developmental delay.

The tumour suppressor gene encodes a Ras-specific GTPase-activating protein, called neurofibromin (NM_000267) (163). Its major role is to negatively regulate the Ras/MAPK signaling cascade and to regulate the mTOR pathway activity, therefore participating in the regulation of cell growth and differentiation. Its expression affects a vast number of cells in the nervous system including neurons, astrocytes, oligodendrocytes, microglia and Schwann cells (162, 163).

NF1 is a ca. 290 kilobases long segment localized on the long arm of chromosome 17 (17q11.2), consisting of 57 exons. The loss-of-function mutations in the *NF1* gene cause neurofibromatosis type 1. The penetrance and the mutation rate is quite high with 80% of paternal origin (164). Mutations can be inherited from the parents or arise *de novo*, without a family history. Novel mutations occur primarily in paternally derived chromosomes, and the probability of these mutations increases with the paternal age (164). These *de novo* mutations occur approximately in 50% of the cases. Most of the time the molecular basis for NF1 is the haploinsufficiency for neurofibromin. Worth mentioning that, epigenetic modifications could cause variability in the phenotypic expression. NF1 is caused by mutations in the *NF1* gene and rarely by 17q11 microdeletion (162). A great number of germline mutations are intragenic and their effect results in truncated neurofibromin (165). According to the ClinVar database, currently approximately 3300 disease-causing mutations and more than 3500 variations of uncertain significance are dispersed through the gene. The molecular interpretation is made challenging by the facts, that the gene's introns contain coding sequence, the gene has three alternatively spliced exons (9a, 23a, 48a) with different tissue specificities and

there are pseudogenes on different chromosomes (for instance: 2q21.1, 14q11.1, 18p11.21, 22q11.1 etc.).

Present day nearby 5-11% of NF1 patients have copy number variations (CNVs), specifically deletions encompassing the *NF1* and contiguous genes (166, 167). A recent work of Hildegard Kehrer-Sawatzki et al. indicates that a difference is observable in the clinical phenotypic features between the general NF1 population and those patients who have type-1 *NF1* microdeletions (168, 169). According to previous studies, more severe clinical manifestations of NF1 are associated to *NF1* microdeletions compared to intragenic point mutations (162, 169). For instance, a previous study showed that patients who have *NF1* microdeletions have a higher incidence of learning disabilities and facial dysmorphism (162). Currently, there are 4 types of microdeletions, called types 1, 2, 3 and atypical. (Figure 11)

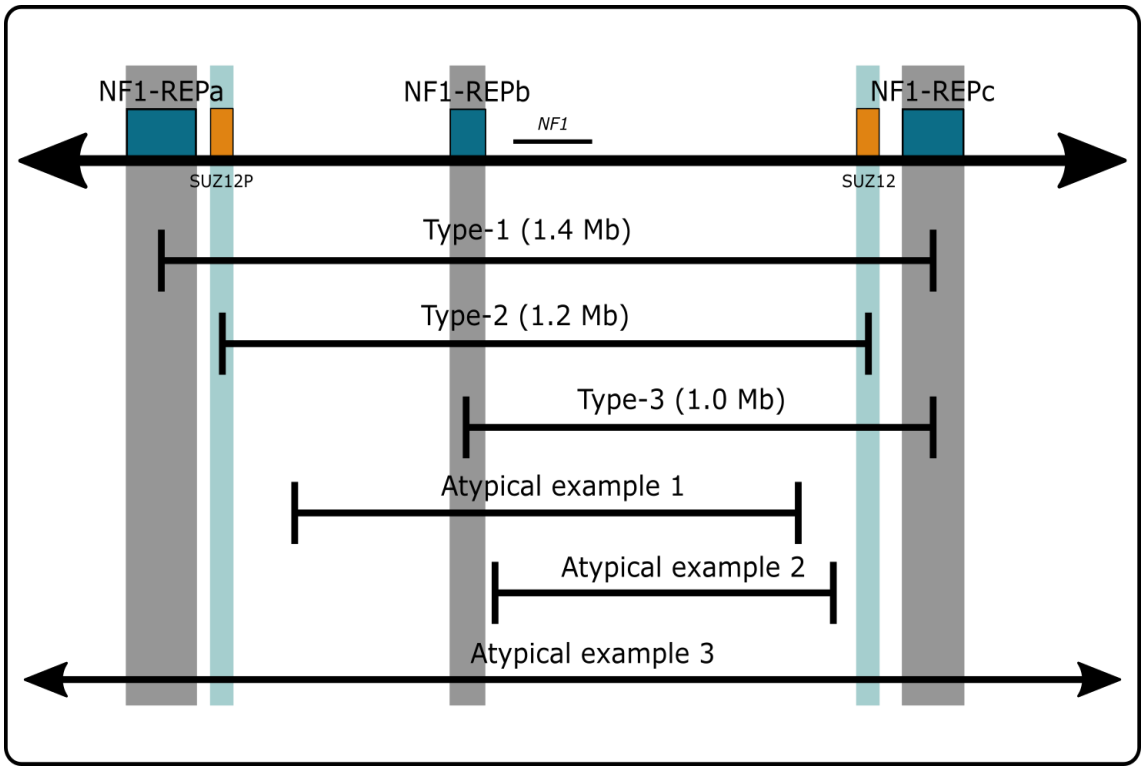


Figure 11. *Schematic representation of the different NF1 deletion types*

The main difference between them is the breakpoint location, the size involved, and the affected region, specifically the affected genes inside the deletions range. The most frequent form is the type-1 *NF1* microdeletion (76-80%), which is 1.4 Mb long and

include 14 protein coding genes, and four microRNA genes, as well (170). This high frequency is a result of the architecture of this chromosomal segment, specifically the flanking regions of the *NF1* gene. The neighbouring region contains low-copy repeat segments, thus giving susceptibility to the reoccurrence of mutations. These low copy repeats are paralogous sequences called *NF1-REP-a* and *NF1-REP-c*. The main characteristics of the different types are described briefly in *Table 1* (162).

Table 1. *Different types of NF1 microdeletions and their features*

	Type 1	**Type 2**	**Type 3**	**Atypical**
Size	1.4 Mb	1.2 Mb	1.0 Mb	heterogeneous
Affected genes	14 protein coding + 4 microRNA	13 protein coding + 2 microRNA	9 protein coding + 2 microRNA	heterogeneous
Frequency	76-80%	10%	1-4%	8-10%
Breakpoints	*NF1-REP-a* and *NF1-REP-c*	*SUZ12P* and *SUZ12*	*NF1-REP-b* and *NF1-REP-c*	heterogeneous

Type 1, 2 and 3 are caused by interchromosomal recombination, known as non-allelic homologous recombination during either meiosis (type 1, type 3), or mitosis (type 2). This is the most well-known mechanism, and majority of CNVs are related to it.

In the case of atypical microdeletions, the causes are heterogeneous, several mechanisms have been related to their formation, including aberrant DNA double-strand break repair and/or replication, and retrotransposon-mediated mechanisms. An increasing number of cases highlight the importance of transposable elements in the formation of genomic rearrangements (77, 108-111). Atypical *NF1* microdeletions do not present recurrent breakpoints, the affected genes and size also vary. Both postzygotic and germline origins can occur.

The first 17q11.2 microdeletion patient was reported in 1992. Since then, more than 150 subjects have been described (171).

2. Aims

Our aim was to

1. determine the frequency and the type of copy number variations among patients with type 1 neurofibromatosis;
2. explore the genotype-phenotype correlation between different types of copy number variations in the NF1 microdeletion patient cohort;
3. compare the differences in the clinical course of the intragenic and microdeletion patient cohort suffering from type 1 neurofibromatosis ;
4. reveal an association between the detected large *FBN1* deletions so far and the severity of the cardiovascular manifestations;
5. investigate the contribution of the deletion of regulatory elements in the clinical course of Marfan syndrome;
6. explore the mechanism underlying the large deletion of *FBN1*.

3. Materials and methods

3.1. Patients and sample preparation

Patients with suspected syndromes for Marfan syndrome or neurofibromatosis were referred for genetic testing at our institute (Department of Medical Genetics). The patients and their families included in the study underwent clinical examination and sampling in the context of genetic counselling. Written informed consent was obtained from all patients or their legal guardians and peripheral blood samples were collected. All experiments were performed in accordance with the Helsinki Declaration of 1975 and with the Hungarian legal requirements of genetic examination, research and biobanking. The research was approved by the ethics committee of the University of Pecs.

Our research included 41 patients with suspected Marfan syndrome or a related connective tissue disorder. These patients originated from 38 unrelated families [13 females, 28 males; mean age: 23 years (age range: 1-47 years)]. Preliminary analysis of the *FBN1*, *TGFBR1* and *TGFBR2* genes were performed by Sanger sequencing with negative results. As a control, 15 patients [7 females, 8 males, mean age at the time of examination: 28 years, (age range: 0.5-59 years)] with intragenic *FBN1* mutations were enrolled into the study, as well. All of the patients fulfilled the revised Ghent criteria. The diagnostic criteria of Ghent nosology for Marfan syndrome without family/genetic history requires a major criterion in two systems (ocular and skeletal systems) and the involvement of one additional organ system (cardiovascular and/or skin).

Our research included 640 unrelated patients with suspected neurofibromatosis. After Sanger sequencing of the *NF1* gene or NGS analyses of *NF1*, *NF2*, *KIT*, *PTPN11*, *RAF1*, *SMARCB1*, and *SPRED1* genes no disease-causing mutations have been identified in 252 patients. Of these, 17 patients (10 males, 7 females; mean age at time of examination: 12.9 years, age range: 2-36 years) with large *NF1* deletion were identified by MLPA. The patient cohort consisted of mainly children (14 out of 17) with the ages between 2 and 17 years. As a control, 33 patients age and sex matched (14 females, 19 males; mean age at the time of examination: 15.2 years, age range: 6 months - 47 years) with intragenic *NF1* mutations were enrolled into the study, as well.

3.2. Clinical investigation of NF1 patients

Phenotypic features of the 17 microdeletion and the 33 control patients were collected using the same standardized questionnaire collection protocol in four HCPs (health care provider). Most symptoms were confirmed by physical examination. In all cases, the same clinician examined and followed up the same patient. An ophthalmologist diagnosed the Lisch nodules and other ocular manifestations. Dysmorphic features were assessed by an expert clinical syndromologist based on international guidelines (http://elementsofmorphology.nih.gov/) (172, 173). All the patients were investigated by cranial MRI. Age and race-related percentile curves were applied to evaluate childhood overgrowth.

Evaluation of the intellectual functions, developmental delay and learning disabilities were determined by various psychological tests appropriate to their age: ages between 0 and 5 years (Walter Strassmeier's developmental scale) (174), ages between 1 and 42 months (Bayley Scales test) (175) and ages between 3 and 14 years (Budapest Binet test) (176). When IQ was not measured, it was estimated to be >70 based on the fact that the patient attended a regular kindergarten or school (with special educational needs). In order to determine attention deficit hyperactivity disorder (ADHD), international guidelines were applied (https://www.nhs.uk/conditions/attention-deficit-hyperactivity-disorder-adhd/symptoms/). In case a patient was not able to speak or had a problem with the language content, structure and expressive vocabulary and grammar, the term "speech difficulties" were applied. In our cases, the speech difficulties were connected to delayed language development and not the neurological symptoms.

3.3. Methods

3.3.1. DNA isolation

DNA was isolated from peripheral blood leukocytes with E.Z.N.A.® Blood DNA Maxi kit (Omega BIO-TEK, Norcross, USA). The concentration and purity of extracted DNA were measured with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

3.3.2. MLPA analysis

MLPA analysis was applied to search for copy number variations in the genes *FBN1, TGFBR1, TGFBR2, NF1* and its neighboring region. Commercially available SALSA MLPA probemix P065-C1 and P066-C1 (MRC-Holland, Amsterdam, The Netherlands) were used for *FBN1, TGFBR1, TGFBR2* for investigating Marfan syndrome patients. In case of type 1 NF patients, SALSA MLPA kits P081-D1 and P082-C2 were used for *NF1*. Moreover, SALSA MLPA kit P122-D1 NF1 area mix was used for the examination of the contiguous genes in the flanking regions. The probemix contained 20 probes for 16 genes (*MYO1D, PSMD11, ZNF207, LRRC37B, SUZ12, UTP6, RNF135, ADAP2, ATAD5, CRLF3, SUZ12P, CPD, BLMH, TRAF4, PMP22, ASPA*), which were localized upstream and downstream, as well. Besides, it also contained probes for five distinct *NF1* exons (1, 17, 30, 49, 57). Information about the localization and exact sequences of the probes are available on the manufacturer's website. According to the manufacturer's instructions, a total of 100–200 ng of genomic DNA of each patient was used. Capillary electrophoresis was applied on an ABI3130 Genetic analyzer (Life Technologies, USA). The results were analyzed using Coffalyser software (MRC-Holland, Amsterdam, The Netherlands). Each MLPA signal was normalized and compared to the corresponding peak area obtained from the three control samples. Deletions and duplications of the targeted regions were suspected when the signal ratio exceeded 30 % deviation. Positive results were confirmed by repeated MLPA experiments.

3.3.3. Whole genome array comparative genomic hybridization analysis

Whole Genome Array Comparative Genomic Hybridization Analysis was performed using the Affymetrix CytoScan 750 K Array. Genomic DNA samples were digested, ligated, amplified, fragmented, labeled, and hybridized to the CytoScan 750 K Array platform according to the manufacturer's instructions. The raw data were analyzed by ChAS v2.0 Software (Affymetrix, Thermo Fisher Scientific, Waltham, MA)

3.3.4. CNV interpretation detected by aCGH

The interpretation of CNVs was performed with the help of several public databases and websites including Ensembl and ECARUCA (European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations) (177), UCSC database, DGV (Database of Genomic Variants), and DECIPHER (Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources) (178).

3.3.5. Somatic mosaicism determination in *NF1* microdeletion

In order to determine the presence and extent or absence of somatic mosaicism in patients examined by aCGH assay in the NF1 patient cohort, allele difference plot and B allele frequency (BAF) plot were evaluated together with Log2 ratios and weighted Log2 ratios with the help of ChAS software. In the samples investigated by MLPA, the ratio values for each MLPA probe were used to assess mosaicism. Values between 0.4-0.6 were considered as non-mosaic deletion, values around 0.7 or up to 0.8 were considered as mosaic deletion.

3.3.6. Characterization of breakpoints in *FBN1* deletion

Long-range PCR and subsequent Sanger sequencing were applied to confirm the *FBN1* deletion and determine the breakpoints. We designed primers targeting the flanking region of the predicted deletion (45F: 5'-TCTTGGTTGCTTCCAAATTC-3' 47R: 5'-GCTGGAACACTAGAGATGATG-3') and QIAGEN Long Range PCR kit (Qiagen, Hilden, Germany) was applied. The following cycling process according to the manufacturer's instructions was applied: 3-min initial denaturation at 93 °C, 35 cycles of 15 s at 93 °C, 30 s at 55 °C and 8 min at 68 °C. The PCR analysis displayed a ca. 1.5kb and a 6kb (wild type) product. The smaller fragment was excised from agarose gel and cleaned with the help of Nucleospin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany). The purified PCR product was sequenced with the help of BigDye Terminator Cycle Sequencing Ready Reaction kit v1.1 in an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Forster City, USA) and the aforementioned 45F, 47R and newly designed internal primers. The designed internal primers are shown below:

- FBN1delF: 5’-CAGGAAGAATGTGTTATTTTGCTC-3’
- FBN1delR: 5’-GTCTCAGAATGTATCCCTCAC-3’

3.3.7. In silico analysis of the *FBN1* gene in patients suffering from Marfan syndrome

In silico analysis of the neighboring region of the breakpoints were applied with the help of Tandem Repeats Finder (179), REPFINDER (180), RepeatMasker (181), REPEATAROUND (67) and QGRS MAPPER (182). Tandem Repeats Finder was applied to display and locate tandem repeats in the DNA sequence. In case of identifying inverted repeat and/or identical direct repeat sequences REPFINDER was applied. RepeatMasker with HMMER and Cross_match search engine with low sensitivity was applied for screening interspersed repeats and low complexity DNA sequences. REPEATAROUND was used to identify mirror repeats, direct and inverted repeats. The examined size range was determined at the range of 30 bases both downstream and upstream from the breakpoints. QGRS MAPPER was applied for the examination of G-rich sequences.

3.3.8. Analyses of regulatory elements in *FBN1*

Regulatory elements were analysed within the *FBN1* gene with the help of the UCSC genome browser. *In silico* preliminary analysis included the usage of the TFBS Conserved, ENCODE Transcription Binding Factors, Vista Enhancers, UCSC genes, base positions and ORegAnno tracks. Throughout the whole *FBN1* gene, the ORegAnno identifiers were collected with the associated transcription factor names and their genomic positions. ORegAnno (Open Regulatory Annotation) shows literature-curated, experimentally proven regulatory regions and polymorphisms, and transcription factor binding sites (TFBS) as well. The positions were correlated to the *FBN1* deletions with known genomic positions. Conversion of the breakpoints were applied into GRCh37/hg19 genome build where it was essential.

The presented regulatory elements are from PAZAR and JASPAR datasets via UCSC. PAZAR is a public database of regulatory sequence and transcription factor annotations.

JASPAR database includes curated and non-redundant experimentally determined TFBS in different eukaryote organisms. Preliminary association analysis was applied with the data exported from previous Chip-seq analyses which provided several various TFBS mapped to the *FBN1* gene.

In order to make the data comparable, genomic localization of the regulatory elements and all published *FBN1* deletions affecting a single or a few exons have been harmonized with the GRCh37/hg19 genome build.

3.3.9. Statistical analysis

SPSS version 27 (SPSS inc, Chicago, IL) was applied for the statistical analysis. Two-tailed Fisher's exact test was used to measure differences in the frequencies of clinical features between patients with copy number variations (*NF1* microdeletion and *FBN1* large deletion) and patients with intragenic mutations. A difference with $p<0.05$ was considered as significant.

4. Results

4.1. Marfan syndrome

4.1.1. CNV analysis

Large *FBN1* rearrangements were screened by MLPA in 41 patients. Among these, one novel large deletion was identified in a 22-year old female and her 1-year-old son. The mother's clinical examination started when she was 12 months old. Initial symptoms included long arms, arachnodactyly, myopia, lens subluxation and pectus excavatum. Clinical evaluation did not fulfill the Ghent criteria at the age of four. Her initial symptoms and the observed elevated homocysteine level in her urine suggested homocystinuria as a clinical diagnosis. Mutation analysis of the *CBS* gene (cystathionine beta synthase) displayed negative results. As a result of the appearing new symptoms (mitral valve prolapse, skin striae, pectus carinatum, scoliosis, joint hypermobility) later in her life, finally fulfilled the diagnostic criteria for Ghent nosology. The 1-year-old male patient also showed several symptoms, including arachnodactyly, positive wrist and thumb sign, pectus excavatum, scoliosis and a tendency toward tall stature. His cardiovascular system was intact and had mild myopia.

During MLPA analysis a novel large deletion encompassing exons 46-47 was identified (Figure 12). As a consequence, the 31st and 32nd calcium binding EGF-like domains of the fibrillin-1 protein are deleted which contributes to the development of the Marfan syndrome. The molecular testing of the female patient's parents confirmed the *de novo* origin of the deletion.

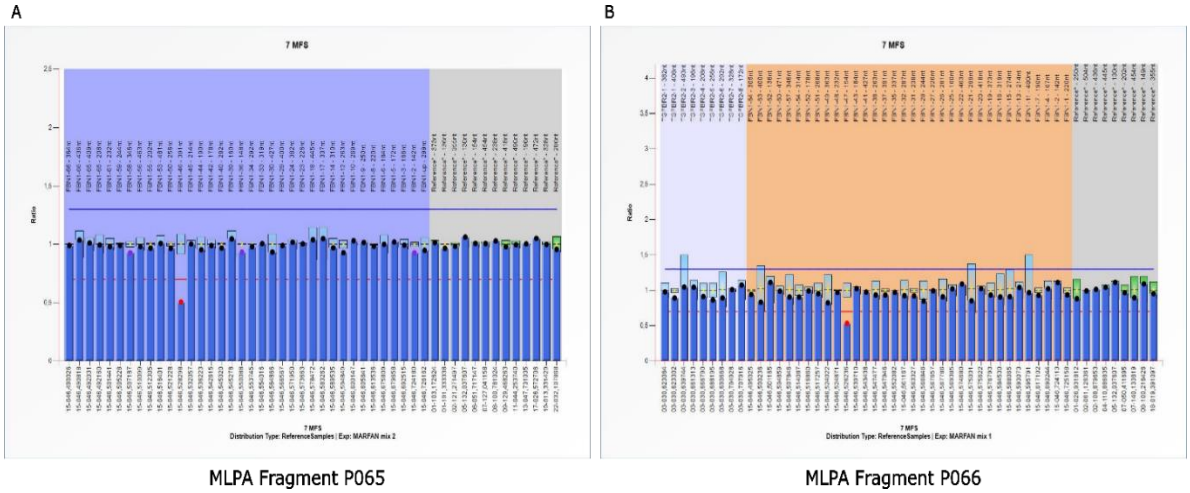


Figure 12 *Heterozygous deletion of exon 46-47 indicated by the reduced peak areas after MLPA analysis*

4.1.2. Breakpoint analyses and possible molecular mechanism exploration

Further analysis by long range PCR confirmed the deletion and determined the exact breakpoints. The examination revealed a 6kb (wild type) and a 1.5 kb long fragment (deleted allele). The excision and purification of the smaller fragment followed up by direct sequencing presented a 4916 bp long deletion. Interpretation and identification of the sequences showed that the breakpoints localize into intronic regions resulting in an in frame mutation. A TG insertion was found near the breakpoints, which was not observed in the parents' sequence. (Figure 13)

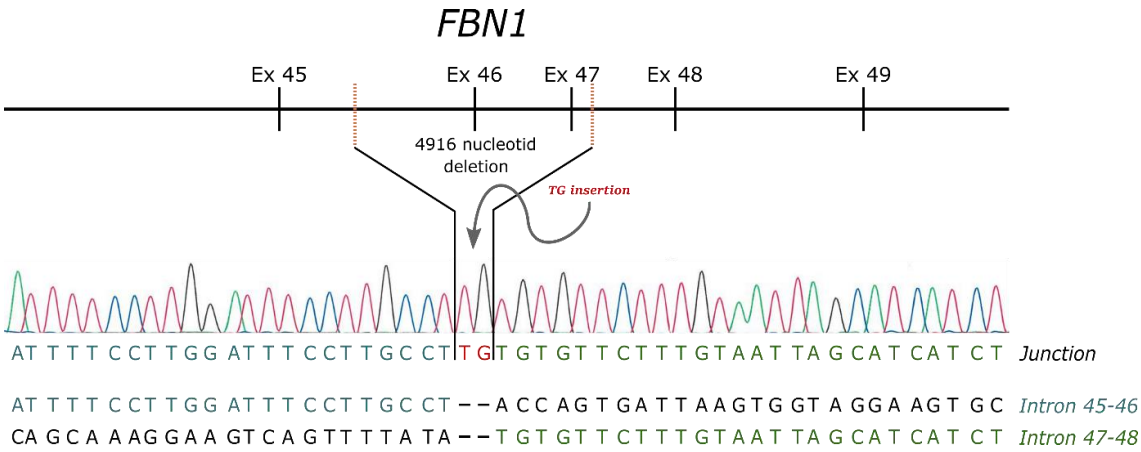


Figure 13. *Breakpoint characterization. Sequences of intron 45 (blue) and intron 47 (green) illustrated by different colors. Exons are represented by bars and marked with the corresponding number. Orange dotted lines mark the position of the breakpoints. Black letters denote the sequences of the deleted regions. All nucleotide positions are represented in relation to the human genome reference sequence (NCBI build hg19).*

Figure 13 presents the sequences of PCR products spanning the breakpoints junction of the deletion with the TG dinucleotide insertion (indicated by curved arrow).

In order to explore the formation of the deletion, preliminary in silico analysis was performed. REPFINDER and Tandem Repeats Finder Program did not reveal remarkable sequence homology in close proximity of the deletion region. The aforementioned programs were applied for the analysis of the genomic sequence between exon 44 and 50. No significant repeat sequences were revealed. RepeatMasker did not expose any L1, Alu, LTR, MIR DNA elements between exon 45 and 48. Tetraplex formation of a single strand of DNA with another unpaired single strand could be generated by G-rich sequences. No significant G-rich sequences were detected by QGRS MAPPER. REPEATAROUND showed slight differences in the repeat variations (Table 2) with or without 'TG' insertion.

Table 2 *Representation of the detected repeats (direct, indirect, mirror, complementary) by REPEATAROUND*

	5' breakpoint with TG insertion	5' breakpoint without TG insertion
Direct repeat	4 base (2)	4 base (2)
	5 base (4)	5 base (3)
	8 base (1)	8 base (1)
Indirect repeat	4 base (6)	4 base (6)
	5 base (2)	5 base (1)
Mirror repeat	4 base (4)	4 base (2)
Complementary repeat	4 base (5)	4 base (5)
	5 base (2)	5 base (2)

4.1.3. Investigation of the association between the severity of cardiovascular manifestations and CNVs

Cardiovascular (CV) symptoms were classified into two distinct groups, called minor and major CV. The former includes annulus mitralis calcification (age of onset, <40y), pulmonary artery dilatation, mitral valve prolapse, aorta descendens or aorta abdominalis dilatation or dissection (age of onset, <50y). Major symptoms include aorta ascendens, aortic ascendens dilatation with or without aortic regurgitation and involvement of the sinuses of Valsalva.

The CV symptoms of patients with a large deletion of the *FBN1* gene observed in the literature so far are summarized in the tables below (Tables 3, 4, 5). A great portion of the patients carrying single-exon deletion showed major CV symptoms (10 out of 16;

63%), in addition, one patient had minor symptoms, and two patients had no manifestations in the cardiovascular system. Data was not available for three patients and in one case the clinical information was not clear. Patients with multiple exon deletions showed much higher frequency of major CV symptoms (16 out of 19; 84%). Furthermore, 11 patients had minor symptoms besides their major CV symptoms. The remaining five patients did not show minor CV symptoms, one patient (our case) had minor CV symptoms only and no clear clinical information was available in two cases. In case of whole gene deletion, 11 out of 16 patients (69%) displayed major CV symptoms, where eight patients belong to two families.

Among our control patients (intragenic mutations) six patients showed major (40%), four patients displayed minor CV manifestations (mitral valve prolapse only, 27%) and five patients did not have any CV symptoms. The observed frequencies of the major CV manifestations demonstrated a significant difference (73 vs 40%, respectively; p=0.031) between patients with large deletion and the control patient cohort. In case of patients with multiple exon deletions the results were quite similar (84% vs 40%; p=0.012). Finally, no significant difference was presented between the patients carrying single exon deletion and the patients with an intragenic *FBN1* mutation.

Table 3 Summary of MFS cases with single-exon deletion in FBN1 gene

Original exon numbering	IF / OF	Affected domains	Technique	Confirmation/breakpoints determination	Phenotype in paper	Cardiovascular symptoms		Ref
						Major	Minor	
Ex1 and promoter region	OF	–	MLPA	Affymetrix Human Mapping 500 K Array Set	Severe MFS	X	X	(148)
Ex1 and promoter region *	OF	–	MLPA	Sequencing	Classic MFS	N/A	N/A	(149)
Ex1*	OF	–	MLPA	N/A	Classic MFS	N/A	N/A	(130)
Ex2	IF	–	PCR then SSCP and CSGE	N/A	Classic MFS	X	-	(147)
Ex3	OF	1st EGF-like	High-Throughput Microarray and MLPA	N/A	MFS	N/A	N/A	(150)
Ex6	IF	3rd EGF-like	MLPA	gap PCR and sequencing	Potential MFS	-	-	(145)
Ex18	IF	11th cbEGF-like	N/A	N/A	Potential MFS	-	-	(159)
Ex29*	IF	18th cbEGF-like	SSCP	N/A	Neonatal MFS	X	-	(155)
Ex30	IF	19–20th cbEGF-like	aCGH	MLPA	Neonatal MFS	X	-	(156)
Ex33 (no probes for exon 32)	IF	21–22th cbEGF-like	DHPLC/MLPA	N/A	Neonatal MFS	X	-	(136)
Ex36	IF	25–26th cbEGF-like	PCR-DHPLC/MLPA	N/A	Classic MFS	#	#	(143)
Ex43	IF	7th TB, 29th cbEGF-like	MLPA	qPCR/long range PCR then Sanger sequencing	Classic MFS	X	-	(157)
Ex50	IF	35th cbEGF-like	MLPA	qPCR/long range PCR then Sanger sequencing	Suspected MFS	X	-	(157)
Ex52*	IF	8th TB, 36th cbEGF-like	PCR then SSCP and CSGE	N/A	Classic MFS	X	-	(147)
Ex54	IF	37–38th cbEGF-like	MLPA	qPCR/long range PCR then Sanger sequencing	Suspected MFS	X	-	(157)
Ex56	IF	39–40th cbEGF-like	MLPA	qPCR/long range PCR then Sanger sequencing	Classic MFS	X	-	(157)

*N/A: not available, #: no explicit clinical information, IF: in frame, OF: out of frame *:it is not clear whether the referred paper uses the 65 exon or 66 exon numbering, **the referred paper probably uses the 65 exon numbering convention*

Table 4 *Summary of MFS cases with multiple exon deletions in FBN1 gene*

Original exon numbering	IF / OF	Affected domains	Technique	Confirmation/breakpoints determination	Phenotype in paper	Cardiovascular symptoms		Ref
						Major	Minor	
Ex1-5	OF	1–3rd EGF-like	MLPA/Array-CGH	N/A	Classic MFS	X	X	(141)
Ex1-16	OF	1–3rd EGF-like, 1st TB, 4–10th cbEGF-like	MLPA	Affymetrix Array	Classic MFS	X	X	(148)
Ex1-36	OF	1–3rd EGF-like, 4–26th cbEGF-like, 1–5th TB	MLPA	gap PCR and sequencing	Classic MFS	X	X	(145)
Ex2-4	OF	1–2nd EGF-like	NGS (panel then WGS)	MLPA and PCR with sanger sequencing	Classic MFS	X	X	(135)
Ex6–65**	OF	3rd EGF-like, 4–47th cbEGF-like, 1–9th TB	MLPA	N/A	Classic MFS	#	#	(130)
Ex13–49	IF	7–34th cbEGF-like, 3–7th TB	DHPLC/MLPA	N/A	MFS	X	X	(136)
Ex24–26*	IF	14–16th cbEGF-like	N/A	N/A	Neonatal MFS	X	X	(134)
Ex33–38	IF	21–26th cbEGF-like, 6th TB	MLPA	N/A	Neonatal MFS	X	X	(151)
Ex34–43	IF	23–29th cbEGF-like, 6–7th TB	MLPA	N/A	Classic MFS	X	X	(158)
Ex37–65**	OF	26–47th cbEGF-like, 3–9th TB	MLPA	N/A	Classic MFS	#	#	(130)
Ex42–43	IF	7th TB, 29th cbEGF-like	sequencing and RT-PCR	N/A	Classic MFS	X	-	(146)
Ex44–46	IF	29–31th cbEGF-like	sequencing and RT-PCR	N/A	Neonatal MFS	X	X	(146)
Ex44–66	OF	29–47th cbEGF-like, 8–9th TB	MLPA	qPCR/long range PCR then sequencing	Classic MFS	X	-	(157)
Ex46-47	**IF**	**31-32th cbEGF-like**	**MLPA**	**long range PCR then Sanger sequencing**	**Juvenile onset MFS**	**-**	**X**	**Our case**
Ex48–53	IF	33–37th cbEGF-like, 8th TB	MLPA	gap PCR and sequencing	Neonatal MFS	X	X	(145)
Ex49–50	IF	34–35th cbEGF-like	MLPA	gap PCR and sequencing	Neonatal MFS	X	X	(145)
Ex50–63*	OF	35–46th cbEGF-like, 8–9th TB	DHPLC	N/A	MFS	X	-	(154)
Ex58–63	OF	41–46th cbEGF-like	FISH, Southern blot, sequencing, Western blot	N/A	Juvenile onset classic MFS	X	-	(153)
Ex60-62*	IF	43-45th cbEGF-like	SSCP/Southern-blot	N/A	Classic MFS	X	-	(144, 152)

*N/A: not available, #: no explicit clinical information, IF: in frame, OF: out of frame *:it is not clear whether the referred paper uses the 65 exon or 66 exon numbering convention, **the referred paper probably uses the 65 exon numbering convention*

Table 5 *Summary of MFS cases with whole FBN1 gene deletion*

Original exon numbering	IF / OF	Affected domains	Technique	Confirmation/breakpoints determination	Phenotype in paper	Cardiovascular symptoms		Ref
						Major	Minor	
FBN1:Ex1–66	-	Full gene	MLPA	FISH/aCGH	Incomplete MFS	-	X	(183)
FBN1:Ex1–66	-	Full gene	MLPA	Array-CGH 385 K Chromosome 15 Specific Array	Classic MFS	X*	-	(141)
FBN1:Ex1–66	-	Full gene	MLPA	SNP array	(Potential) MFS	#	#	(142)
FBN1:Ex1–66	-	Full gene	Conventional chromosome analysis / Affymetrix Cytogenetics Array 2.7	FISH	Classic MFS	X	X	(139)
FBN1:Ex1–66	-	Full gene	MLPA	aCGH	MFS	X	X	(184)

*N/A: not available #: Referred paper discussed 10 patients, where 6 of them had major cardiovascular symptoms, three of this six patients had minor symptoms also. In addition, one patient had only minor symptoms and one patient had no cardiovascular symptoms. IF: in frame, OF: out of frame * referred study reported 3 patients with whole FBN1 gene deletion, all of them had major CV symptoms.*

The deletions can generate either in-frame (IF) or out-of-frame (OF) variations, however the severity of the observed CV symptoms are not affected by whether the deletion is IF or OF.

4.1.4. Analyses of regulatory elements within *FBN1* gene focusing on transcription factor binding sites

Several tissue-specific enhancer regions were revealed in the intronic regions of the *FBN1* gene by preliminary in silico analysis. The possible associations between the affected TFBS and CV manifestation were examined. Those cases were selected for the analysis who carried a single exon deletion with known genomic positions or a few exons were affected by the deletion. Results of in silico analyses are shown below in Table 6.

Table 6 *Summary of transcription factor binding site analyses of FBN1 gene*

Genomic position (GRCh37)	Affected region	Transcription factors	Active regulatory elements in aorta	Patient Age (y)	Cardiovascular symptoms	
					Major	Minor
Single exon deletion						
g.48,941,579 - 48,914,693	Ex1 and promoter	MAFK (x2), RBL2 (x2), ZNF263, ZBTB33, E2F4, EBF1 (x2), E2F1, CTCF, PRDM1, SMARCA4, GATA3, CEBPB, USF1, BATF::JUN, FOXA1, **STAT3**, STAT1	+	25	X	X
g.48,890,441 - 48,873,891	Exon 6	SMARCA4 (x4), CEBPB (x2), EBF1 (x3), DUX4, TP63, ZNF263	+	49	-	-
~g.48,778,050 - 48,777,400	Exon 30	-	-	<1	X	-
g.48,753,819 - 48,749,026	Exon 43	FOXA1, JUN (var.2), JUNB, FOS, STAT1, GATA3, RBL2 (x2), MAX	-	24	XX	-
g.48,734,801 - 48,730,690	Exon 50	PRDM1	+	14	X	-
g.48,727,672 - 48,726,338	Exon 54	-	+	5	X	-
g.48,724,560 - 48,722,281	Exon 56	-	-	38	X	-
Few exon deletion						
N/A	Exon 1-5	E2F4, EBF1 (x2 + x1), E2F1, CTCF, PRDM1, SMARCA4 (x2), GATA3, CEBPB (x2 + x1), USF1, BATF::JUN (x2), FOXA1 (x2), MAFK, **STAT3**, STAT1, DUX4 (x2), MAX, JUND (var.2)	+	27	X	X
g.48,922,918 - 48,890,962	Exon 2-4	EBF1, FOXA1 (x2), MAFK, **STAT3**, STAT1, BATF::JUN, DUX4 (x2), MAX, JUND (var.2), CEBPB	+	32	X	X
N/A	Exon 24-26	[MAFK, MAFF, JUNB], AR	-	After birth	XX	X
N/A	Exon 33-38	AR, DUX4, FOXA1, CEBPB, IGF1R	-	1	X	X
g.48,754,954 - 48,748,519	Exon 42-43	JUN, JUND (var.2), FOXA1, JUN (var.2), JUNB, FOS, STAT1, GATA3, RBL2 (x2), MAX, **STAT3**	-	>46	X	-
g.48,745,163 - 48,738,026	Exon 44-46	GATA2, FOS (x2), JUN (var.2)	-	>6	X	X
g.48,743,774 - 48,738,856	***Exon 46-47***	*GATA2, FOS (x2), JUN (var.2)*	-	***22***	-	**X**
g.48,738,131 - 48,727,786	Exon 48-53	PRDM1	+	15	X	X
g.48,737,397 - 48,732,835	Exon 49-50	-	+	3	X	X
g.48,718,149 - 48,706,671	Exon 58-63	FOS, PRDM1, TFAP2A, JUN, JUND (var.2), FOXA1 (x5), MAFF, MAFK, ELF1 (x2), GATA2, **STAT3**, JUND	+	17	XX	-
N/A	Exon 60-62	FOS, PRDM1, TFAP2A	+	48	X	-

Active regulatory elements also include promoter and/or enhancer elements. X: aortic ascendens dilatation, XX: aortic ascendens dilatation along with dissection

Numerous TFBS have been found in the region of *FBN1* gene affected by different CNVs. Although, the deletion seen in our patient involves only a few TFBS (Figure 14). According to the preliminary in silico analysis among the presented TFBS, STAT3 shows a potential correlation with CV symptoms. Five cases presented a deletion involving STAT3 binding sites. Out of them, four patients developed aortic dilatations and one

patient suffered from an acute dissection of the ascending aorta and right coronary artery as well. The comprehensive evaluation of the connection between the deleted TFBS and the CV symptoms of the patient, suggests that STAT3 binding site is supposed to play a role in the development of cardiovascular manifestations. Furthermore, several regulatory elements (promoters and/or enhancers) known to be active in the aorta have been found in the region of *FBN1* gene affected by different CNVs.

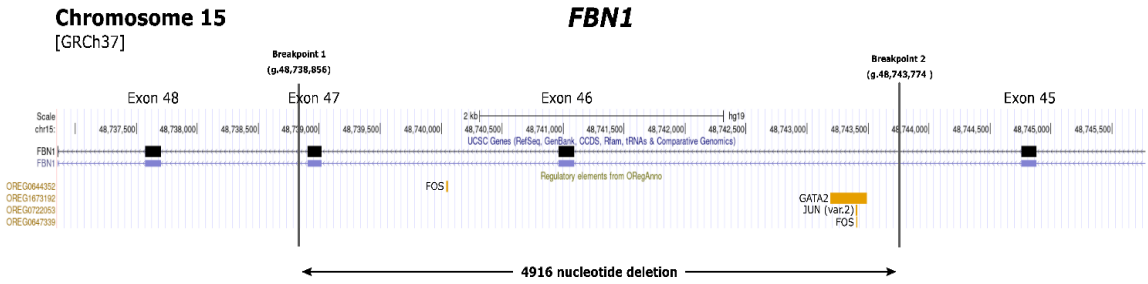


Figure 14 *Localizations of TF binding sites within the deleted region of FBN1 gene as found in our patient. Regulatory elements are indicated by amber markings, the exons displayed by black rectangles. Data were based on GRCh37.*

Figure 14 represents the affected regulatory elements (GATA2, JUN, FOS) in our patient's deletion.

4.2. Neurofibromatosis

4.2.1. Characterization of the *NF1* microdeletions

A total of 252 patients in whom mutation analysis did not find any pathogenic *NF1* point mutations or intragenic insertions/deletions were screened for large *NF1* rearrangements by MLPA. Of these, 17 patients showed heterozygous deletions of the entire *NF1* gene and several contiguous genes in its flanking regions. The MLPA analysis revealed twelve type-1 and five atypical deletions. An aCGH analysis was applied in ten patients (eight patients with type-1 and two patients with atypical deletions) for the confirmation of the MLPA results. The aCGH tests were not applicable in the remaining seven cases (four patients with type-1 and three with atypical deletions) due to the quality of the available samples.

Similar results were found by MLPA and aCGH in eight cases (seven type-1 deletion and one atypical). According to the aCGH test, one of the patients (85/NF) showed type-2 deletion (MLPA displayed as an atypical deletion) and another patient (4672016)

revealed atypical deletion (MLPA demonstrated it as a type-1 deletion). The evaluation of patient 4672016 resulted in the conclusion that this patient has type-1 deletion.

The differences in the results generated by the methods originate from the different localization of the probes. The breakpoints of the deletion detected in our patient (85/NF) were localized within the region covered by *SUZ12* and *SUZ12P* probes of P-122 set. According to the manufacturer's description Cytoscan 750K chip contains more probes for *SUZ12* (at least 50) and its pseudogene *SUZ12P* (at least 7 probes), compared to the MLPA P122 probe set (one probe for *SUZ12* gene exon 10 and two probes for *SUZ12P* exon 3 and 1), thus in this case aCGH was capable to identify the type-2 deletion.

Altogether twelve type-1 (eight determined and four potential type-1 deletions), one type-2 and four atypical deletions were identified in our patient cohort (Figure 15). Besides, no type-3 microdeletion was detected. The aCGH analyses demonstrated four type-1 deletions with identical estimated breakpoints (ca. 1.37 Mb deletion size). Moreover, three distinct novel atypical deletions were detected. Patient 134/NF and 260/NF are close relatives (mother and child), so they possess the same deletion. The results of MLPA and aCGH analyses are seen in the figure below (Figure 15).

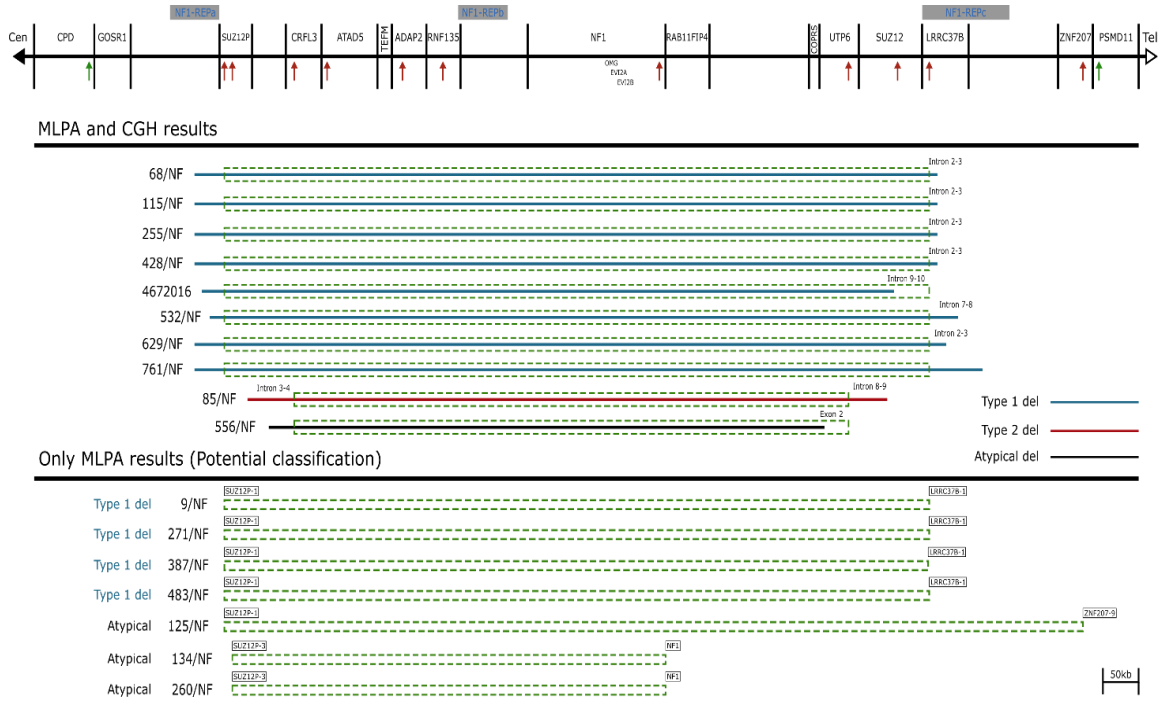


Figure 15. *Schematic representation of the NF1 gene and flanking regions. Red (affected) and green (not not affected) arrows demonstrate the localization of MLPA probes, solid lines indicate the deletion range with known breakpoints determined by aCGH probes. Dotted rectangles correspond to the deleted range determined by MLPA probes. Colored solid lines represent the deletion types (blue: type-1, red: type-2, black: atypical).*

The top of the figure schematically displays the affected genes and the localization of NF1-REP regions in both figures (Figure 15 and 16).

The known atypical NF1 cases together with our atypical deletions are summarized in Figure 16. Two out of three novel atypical deletions were identified by MLPA. However, remarkable overlap is observed with the published cases, the deletions seen in our patients are typically smaller.

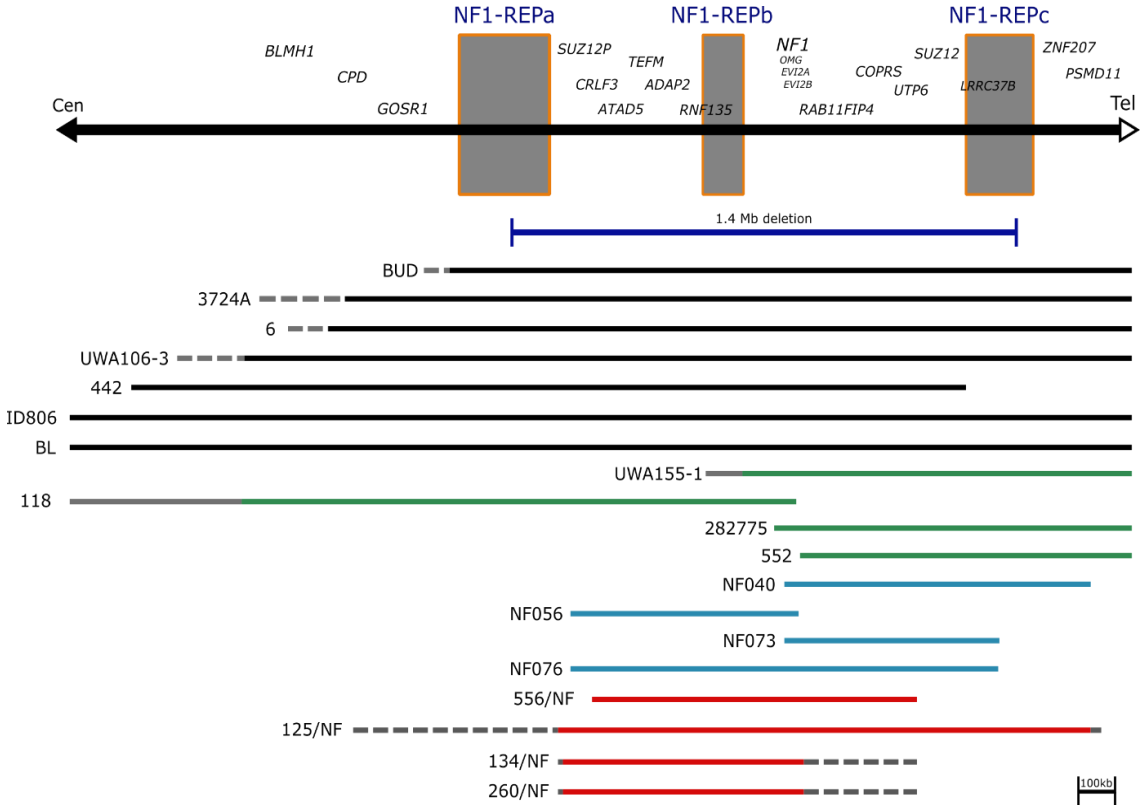


Figure 16 *Schematic representation of atypical NF1 deletions. The known deleted regions are indicated by solid lines, while dotted lines display the potential deletion range. Horizontal black, green and blue lines demonstrate the known atypical NF1 cases. Horizontal red lines refer to our cases.*

Based on the probe localization of the SALSA kit, MLPA is only capable to estimate the location of the breakpoints. SALSA P122 probe set contains 23 probes within the 17q region and the distance between the adjacent probes are quite variable from 11kb up to 1500 kb, therefore exact breakpoints are potentially localized far (somewhere in the dotted line) from the breakpoint boundaries determined by only MLPA probes. The exact localization can be defined precisely by breakpoint-spanning PCR (185).

4.2.2. Assessment of somatic mosaicism

Out of the ten patients examined by aCGH, one patient (556/NF) with atypical deletion showed somatic mosaicism with an extent of ca. 30%. In the remaining cases investigated by MLPA, the ratio values do not imply the presence of somatic mosaicism. Even though, neither MLPA nor aCGH is able to detect low-grade mosaicism (below 20%) due to the limitations of the techniques.

In order to completely rule out mosaicism, the examination of additional tissues, for instance buccal, urine or fibroblast cells is necessary. Based on previous research, the occurrence of somatic mosaicism seems to be very rare in patients with type-1 *NF1* microdeletion (186), therefore our patients with type-1 deletion are considered to be non-mosaic cases. The only patient with type-2 deletion inherited the deletion from her mother, consequently she does not possess somatic mosaicism. Patient 260/NF inherited the deletion from his mother, therefore this patient is considered as non-mosaic, too. His mother (134/NF) is supposed to be a non-mosaic case as well, since she has a positive family history (her mother and her grandmother were also affected, however, without laboratory diagnosis) and the MLPA results (peak ratios were between 0.49-0.55) also supported this assumption. MLPA peak ratios were between 0.49 and 0.55 also for patient 125/NF, therefore we supposed this patient to be a non-mosaic, as well.

4.2.3. Clinical characterization of our patients with different types of *NF1* microdeletion

The numerous clinical features and neuropsychological manifestations are presented in the *NF1* microdeletion patient cohort. Seven major categories were determined and selected for genotype-phenotype association analysis (Table 7).

Table 7 *Clinical features of our patients with different type of NF1 microdeletions*

	Deletion type	Type 1								Type 1				Type 2	Atypical			
	Applied method	CGH								MLPA				CGH	CGH	MLPA		
	Patients	68/ NF	115/ NF	255 NF	428 NF	467/ 2016	532/ NF	629/ NF	761/ NF	9/ NF	271/ NF	387/ NF	483/ NF	85/ NF	556/ NF	125/ NF	134/ NF	260/ NF
	Gender	M	F	M	M	F	M	F	M	M	M	F	M	F	M	F	F	M
	Age of onset	26 y	5 mo	at birth	at birth	N/A	12 y	at birth	bh	at birth	at birth	at birth	5 y	1 mo	6.5 y	at birth	3 y	at birth
	Age at examination	36 y	9 y	14 y	5 y	9 y	14 y	4.5y	9 y	21 y	4 y	17 y	7.5 y	13 y	10 y	2 y	40 y	8 y
Dysmorphic features	Facial dysmorphism	X	X	X	X	-	X	X	-	X	-	-	X	-	-	-	-	X
	Hypertelorism	X	X	X	X	-	X	X	-	-	-	-	X	-	-	-	X	X
	Facial asymmetry	-	-	-	-	-	X	-	X	X	-	-	-	-	-	-	-	-
	Coarse face	X	-	X	X	-	X	X	X	X	-	-	X	X	-	-	-	-
	Broad neck	-	-	X	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Large hands, feet	-	X	X	X	-	X	X	X	X	-	-	X	X	-	-	-	-
Skin manifestations	CALs	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	Freckling	-	X	X	X	X	-	X	X	X	X	X	X	-	X	X	-	X
	Excess soft tissue	-	-	X	X	-	-	X	-	X	-	-	-	X	-	-	-	-
	SBC neurofibromas	X	X	X	X	-	-	-	X	-	X	X	-	-	-	-	X	-
	CT neurofibromas	-	-	-	-	-	-	-	-	X	-	-	-	-	-	-	-	-
	PL neurofibromas*	-	-	-	-	-	-	-	-	X	-	X	-	-	-	-	-	-
Education and behavior problems	SDiCD	X	-	X	X	X	X	X	X	X	-	-	X	-	-	-	-	X
	Learning difficulties	X	-	X	-	X	X	X	X	X	-	X	X	X	-	-	-	-
	Speech difficulties	-	-	X	X	X	X	X	X	-	-	X	X	-	-	-	-	-
	IQ < 70	-	-	-	--	-	-	-	-	X	-	-	-	-	-	-	-	-
	ADHD	-	-	-	X	-	-	-	-	X	-	-	-	-	-	-	-	-
Skeletal manifestations	Skeletal anomalies	X	X	X	X	X	X	X	X	X	X	X	-	X	X	X	X	X
	Scoliosis	X	-	X	-	-	X	-	-	X	X	-	-	X	-	-	X	-
	Pectus excavatum	-	X	-	X	-	X	-	-	X	X	-	-	-	-	X	-	X
	Bone cysts	X	n.d.	-	n.d.	n.d.	-	-	-	-	-	-	-	-	-	-	-	-
	Joint hyperflexibility	-	-	-	X	-	-	-	-	-	-	-	-	-	-	-	-	-
	Macrocephaly	-	X	X	X	X	-	X	X	-	-	X	-	X	-	-	-	X
Neurological manifestations	Muscular hypotonia	X	-	X	-	-	-	-	X	-	-	-	-	-	-	-	-	-
	Headache	-	-	-	X	-	-	-	-	-	-	-	-	-	-	-	-	-
	Coordination problem	-	-	X	X	X	-	-	X	-	-	-	-	-	-	-	-	-
	MPNST	X	-	-	-	-	-	-	-	-	-	X	-	-	-	-	-	-
	Spinal neurofibromas	-	n.d.	n.d.	n.d.	-	-	n.d.	X	-	-	X	-	n.d.	-	-	n.d.	n.d.
	T2 hyperintensities	X	X	X	X	-	-	X	X	X	X	X	X	X	-	X	n.d.	X
Ocular manifestations	Visual disturbance	-	-	-	-	-	X	-	-	-	-	X	-	X	-	-	-	-
	Lisch nodules	-	-	X	-	-	-	-	-	X	-	X	-	X	-	-	-	-
	Strabismus	-	-	-	-	-	X	-	-	-	-	-	X	-	-	-	-	-
	OPG	-	-	-	-	-	-	X	-	-	-	X	-	-	X	-	-	X
Development. problems	Tall stature	-	X	X	-	-	X	X	X	X	-	X	-	-	-	-	-	-

CALs, café-au-lait spots; CT/SBC/PL, cutaneous/subcutaneous/plexiform neurofibroma; SDiCD, significant delay in cognitive development; ADHD, Attention deficit hyperactivity disorder; MPNST, malignant peripheral nerve sheath tumours; OPG, Optic Pathway Glioma

Dysmorphic features

According to our observation, 9 out of 17 patients (53%) presented facial dysmorphism. 8 out of 12 type-1 *NF1* and 1 out of 4 atypical deletion cases presented it. A similar prevalence was recognized in case of hypertelorism, although the distribution among the deletion types was different. This clinical feature was found to roughly the same extent in type-1 deletion and atypical deletion cases (58% vs 50%, respectively). Facial asymmetry was noted only in 3 out of 12 patients with type-1 deletion. Coarse facial appearance and large hands and feet seem to be characteristic dysmorphic features of *NF1* microdeletion patients, because it was frequent in our type-1 deletion patients (8 out of 12, 67%), and both symptoms were also noted in the type-2 deletion patient. Coarse facial appearance was absent in our atypical cases. On contrary, dysmorphic traits were rare event in our intragenic *NF1* patient population. Among the examined dysmorphic features only hypertelorism (6 out of 33 controls; 18%) and facial asymmetry (2 out of 33 controls; 6%) were observed.

Skin manifestations

Regardless of the type of deletion, café-au-lait spots (CALs) were observed in all patients. However axillary and inguinal freckling was absent in the type-2 deletion patient, they showed high frequency in type-1 (10 out of 12; 83%) and atypical (3 out of 4; 75%) deletion groups. In addition to skin manifestations, excess soft tissue in hands and feet was presented among our patients, though at a lower frequency. In type-1 deletion group it was observed in 4 out of 12 patients (33%), it developed in a patient with type-2 deletion also, in contrast, it was absent in the atypical deletion patients. Skin manifestations, including CALs (30 out of 33; 91%) and axillary and inguinal freckling (17 out of 33; 52%) are characteristic of intragenic NF1 patients as well, as their high frequency indicates.

Neurofibromas and other tumours

Four different neurofibromas can be distinguished, including cutaneous, subcutaneous, plexiform and spinal neurofibromas. According to our results, subcutaneous neurofibromas were the most common among the four, although it is worth mentioning

that whole-body and spinal MRI is not part of the routine procedure in our patient management and 14 out of 17 patients were children, furthermore 10 out of 14 were under 10 years old at the age of examination. Subcutaneous neurofibromas were found more common in type-1 deletion patient cohort compared to type-2 and atypical groups. They were observed in 7 out of 12 patients (58%) with type-1 deletion, and in 1 out of 4 patients (25%) with atypical microdeletion, though none occurred in the patient with type-2 deletion. The prevalence of cutaneous neurofibromas appears to be less frequent in our patient cohort, it was observed in only one patient with type-1 deletion. Externally observable plexiform neurofibromas were seen in only two patients with type-1 deletion. None of the patients with type-2 or atypical microdeletions presented this type of neurofibromas. Spinal neurofibromas were found in the type-1 microdeletion group with low frequency (2 out of 12 patients; 17%).

Optic pathway glioma (OPG) was detected by MRI in four patients and it was not symptomatic in any of these cases. Out of the four patients, OPG was seen in two type-1 (17%) and two atypical cases (50%), hence it was absent in the patient with type-2 deletion. Among the control patients two symptomatic and two asymptomatic OPG were observed.

Malignant peripheral nerve sheath tumours (MPNST) were observed in two of our patients, one was adult and one was nearly adult (36 years and 17 years old, respectively), and both belonged to type-1 deletion group. None of the patients with type-2 or atypical microdeletions displayed this type of tumour. It is noteworthy to mention that MPNST show age-related penetrance, therefore the low frequency might be the consequence of our patient cohort consisting of mainly children under 17 years. The frequency of this type of tumour was high (50%, 2 out of 4) among adult patients.

Among our intragenic NF1 patients, subcutaneous fibromas were found with 30% (10 out of 33) frequency, the occurrence of cutaneous and plexiform neurofibromas were 18% (6 out of 33) and 6 % (2 out of 33), respectively. Spinal neurofibromas were observed in 3% (1 out of 33) of our patients. Furthermore, 4 out of 33 (12%) of the control patient cohort developed OPG, and no MPNST was observed.

Skeletal anomalies

Majority of our patients demonstrated some form of anomalies of the skeletal system (16 out of 17; 94%). Macrocephaly was the most frequent (9 out of 17; 53%), it was common in type-1 microdeletion cohort with 58% prevalence and only one patient presented in the atypical cohort.

Scoliosis was noted in 7 out of 17 patients studied here (41%). Pectus excavatum was observed in 42% of our patient cohort. In contrast to scoliosis, pectus excavatum was more frequently observed in patients with atypical microdeletion (50%) as compared to type-1 deletion group (33%) Interestingly, there were only two patients who presented scoliosis together with macrocephaly. Only one patient (type-1 microdeletion) presented bone cysts. Pes planus was observed in three patients, pes cavus was absent in our patient cohort. Interestingly, skeletal anomalies were the leading manifestations in our patient with type-2 deletion. She had macrocephaly, scoliosis, bilateral dislocation of the elbow and wrist joint. Moreover, absorption of the tibial malleolus was observed and she developed osseous malignancy as well. The intragenic NF1 patient group demonstrated skeletal anomalies less frequently (33%). Of these, scoliosis occurred most frequently with 21% prevalence. Macrocephaly and pectus excavatum were noted in 9% of the patients and 3% of them presented pes cavus.

Ocular manifestations

Ocular manifestations were observed in 7 of 17 of our patients (41%). Even though Lisch nodule is a characteristic feature for type 1 neurofibromatosis, it was observed in 3 out of 12 patients with type-1 deletion and in the patient with type-2 deletion. It was absent in the atypical patient cohort. In addition, two patients with type-1 deletion and, the type-2 deletion patient presented other ocular manifestations, including visual disturbance, proptosis and strabismus. One of the patients had hypermetropia, while the others had myopia. Somewhat similar frequencies were observed in the intragenic NF1 patient cohort. Lisch nodule was presented in 7 out of 33 (21%), and visual disturbances were seen in 5 out of 33 patients (15%). Strabismus was absent, one patient had myopia, two patients presented hypermetropia, and anisometropia was observed in two other patients.

Neuropsychological symptoms (manifestations)

Significant delay in cognitive development and general learning difficulties (9 out of 12; 75%), and speech difficulties (8 out of 12; 67%) were observed with relatively high frequency in type-1 patients. Attention deficit hyperactivity disorder was observed in two patients, and only one patient had an IQ below 70. IQ measurement was performed in only four among our patients, however, all of our pediatric patients attended regular kindergarten or school, except the one with IQ=45, and five of them had special educational needs. Besides significant delay in cognitive development (1 out of 4, 25%) and general learning difficulties seen in the type-2 patient, majority of these neuropsychological manifestations were not found in the atypical patient cohort and in the type-2 patient.

The majority of our patients presented T2 hyperintensities (13 out of 17, 76%). Of the 13 individuals, ten were type-1, one type-2 and two atypical. Nevertheless, we did not find any correlation between the age of our patients and the T2 signal intensities. Structural brain abnormalities were not observed.

Coordination problems and muscular hypotonia (33% and 25%, respectively) were documented in patients with type-1 deletion. None of these neurological symptoms were found in our type-2 and atypical deletion groups. Epilepsy and nerve pain were absent from our patient cohort. One patient with type-1 deletion complained of a headache.

Neuropsychological manifestations were rare in the NF1 intragenic patient cohort. A significant delay in cognitive development, speech difficulties and epilepsy were observed in 1 out of 33 patients (3%). Overall, muscular hypotonia (4 out of 33; 12%) and general learning difficulties (5 out of 33; 15%) were observed with slightly higher frequencies. T2 hyperintensities had the highest prevalence with 39% (13 out of 33 patients).

Connective tissue anomalies and cardiac abnormalities

Heart abnormalities and connective tissue anomalies occurred very rarely in our patient cohort. No congenital heart defect, pulmonary stenosis, ventricular septal defect, aortic stenosis, aortic dissection, mitral valve prolapses, mitral valve insufficiency or aortic valve insufficiency was found in any of the deletion groups. Patent ductus arteriousus was

detected in one patient with type-1 deletion, in addition, hypertrophic cardiomyopathy was observed in another patient with type-1 microdeletion. Atrial septal defect was seen in one patient with atypical microdeletion. It is noteworthy to mention that two of our patients were not investigated by cardiac ultrasound. Although hyperflexibility of joints was absent in our patients with type-2 or atypical deletion, 2 out of 12 type-1 deletion patients (17%) presented it.

These manifestations were rare in our patients with NF1 intragenic mutation, as well. Among the cardiac abnormalities only ventricular septal defect was observed at birth in one patient and only 2 out of 33 (6%) of our patients developed joint laxity.

Other features

Some rare clinical manifestations, such as obesity, hearing impairment, immune deficiency and milk protein allergy were observed in our patient group. However, it is hard to tell whether these symptoms are associated with the observed large deletion or are the results of an independent event.

5. Discussion

The human genome remained relatively well conserved throughout evolution, however, modern genomic tools have revealed that it is more diverse, complex, and dynamic than previously thought. Genetic variations have various forms and they are suspected to represent the 0.1% (187, 188) and 0.4% (189) of the human genome (13). One major source of genetic diversity in humans comes from structural variants. Their identification and interpretation remains the most challenging task. Majority of the observations and conclusions are coming from the most known, best detailed forms, known as copy number variations (68). CNVs are widespread in the human genome and an increasing number of studies prove their important role in phenotypic variation and evolution. Furthermore, there is growing evidence demonstrating that besides genomic disorders, CNVs may also be responsible for the development of Mendelian diseases or sporadic traits (190).

5.1. Marfan syndrome

5.1.1. Investigating the association between the detected large *FBN1* deletions and the severity of the cardiovascular manifestations

In Marfan syndrome ca 2-7 % of the disease-causing mutations belong to CNVs (130, 157). In our patient cohort (2 out 41 patients; 4.8%) a novel large deletion, affecting exons 46 and 47, was identified in the *FBN1* gene by MLPA. The *de novo* origin was revealed and confirmed by molecular genetic testing of our primary case and her parents. According to previously published data, the detection rate of CNVs in our patient cohort is quite similar, therefore MLPA is capable to detect large CNVs in a cost-effective manner in MFS patients.

FBN1 encodes a multi-domain glycoprotein called fibrillin-1, which is a major component of microfibrils in the extracellular matrix of elastic and non-elastic tissues (191). The protein consists of 47 epidermal growth factor (EGF)-like and 9 TGF-ß1 binding protein (TB) domains. Majority of the EGF-like domains are known as calcium-binding EGF (cb-EGF) domains (192) because they contain a calcium binding sequence, which plays an essential role in the structure and function of the protein by providing protection against proteolysis (193), stabilizing the microfibril architecture (194-196) and controlling the interactions between various extracellular matrix components (197). Mutations in the *FBN1* gene generally disrupt microfibril formation, therefore resulting

in the degeneration of microfibril architecture, loss of extracellular matrix integrity and weakening of the connective tissue thus leading to the final instability of the aortic wall.

In Marfan syndrome, the most serious clinical manifestations are thoracic aortic aneurysm (TAA) and dissection (TAAD). Dilatation, dissection and potential rupture of the aorta are the result of the dysregulation and/or destruction of the cellular and extracellular components of the aortic wall (198). The highly dynamic aortic wall is subject to strong hemodynamic changes and is able to properly respond to these stimuli with the help of its refined biomechanical functions. Fibrillin-1 containing microfibrils in association with essential elastin contribute significantly to the stability and elasticity of the aorta (191). Adult MFS patients can develop various cardiovascular manifestations, such as calcification of mitral and aortic valves, dilatation of aortic root, dilated cardiomyopathy, proximal ascending aorta and pulmonary artery, and arrhythmia with dissection or rupture of thoracic aortic aneurysm, which is the leading cause of sudden death in the natural history of MFS. Although severe mitral valve prolapse (MVP), valvular regurgitation and aortic root dilation with congestive heart failure are less frequent in children.

Genotype-phenotype correlations are crucial to reveal associations between mutations and disease severity. Several studies (183, 195, 199) have been published so far to connect various *FBN1* mutations to certain clinical features. For instance, it has been found that premature termination codon (PTC) mutations (frameshifts, stop codons, out-of-frame splice mutations), leading to no or a truncated form of fibrillin-1, are related to more severe skin and skeletal phenotype as compared to in-frame mutations (183). Another example was reported in previous studies, that MFS patients with mutations leading to in-frame exon skipping tend to have a severe phenotype (195, 199). Furthermore, a number of studies represented an association between the severity of the aortic phenotype and the type of *FBN1* mutations (haploinsufficient vs dominant negative). A more severe aortic phenotype was observed in patients with haploinsufficient-type *FBN1* variants (nonsense and out-of-frame), which presumably results in nonsense-mediated mRNA decay, than those with dominant-negative-type mutations (missense and in-frame), that are expected to exert loss-of-function effects (200-202). However, it is noteworthy to mention that contrarily to previous studies (201, 202) in the CNV patient cohort, the type of the mutation (IF or OF) had no effect on the severity of the cardiovascular manifestations.

Besides full *FBN1* gene deletions, there are 34 various CNVs, affecting single (Figure 17) or multiple exons (Figure 18) (Table 3, 4). Detailed clinical evaluation of the presented cases revealed severe cardiovascular manifestations (dilatation and/or dissection of the thoracic aorta) in the majority (26 of 34) of the patients. In six cases no clinical data or no clear clinical information was available. In our primary case only mitral valve prolapse was seen and her 1-year old infant's cardiovascular system was intact. Thereby, apart from CNVs, other factors supposedly play a role in the development of severe cardiovascular manifestations. In two cases, cardiovascular manifestations were absent, although they differed from our case, since they carried single exon deletion (exon 6 and 18). Interpretation of the results revealed that cardiovascular manifestations are more severe and frequent in the patients affected by CNVs compared to the patients suffering from intragenic *FBN1* gene mutations.

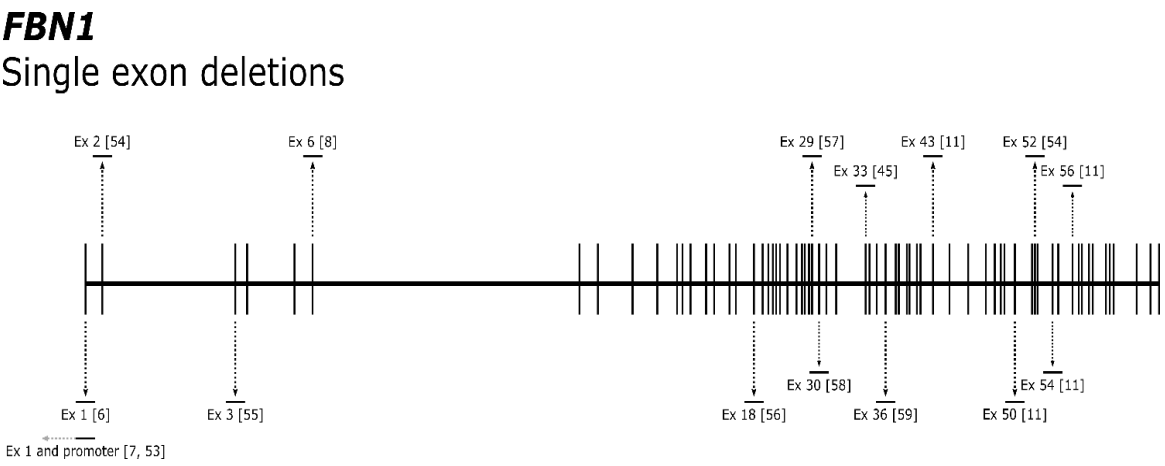


Figure 17 *Known single exon deletions in the FBN1 gene*

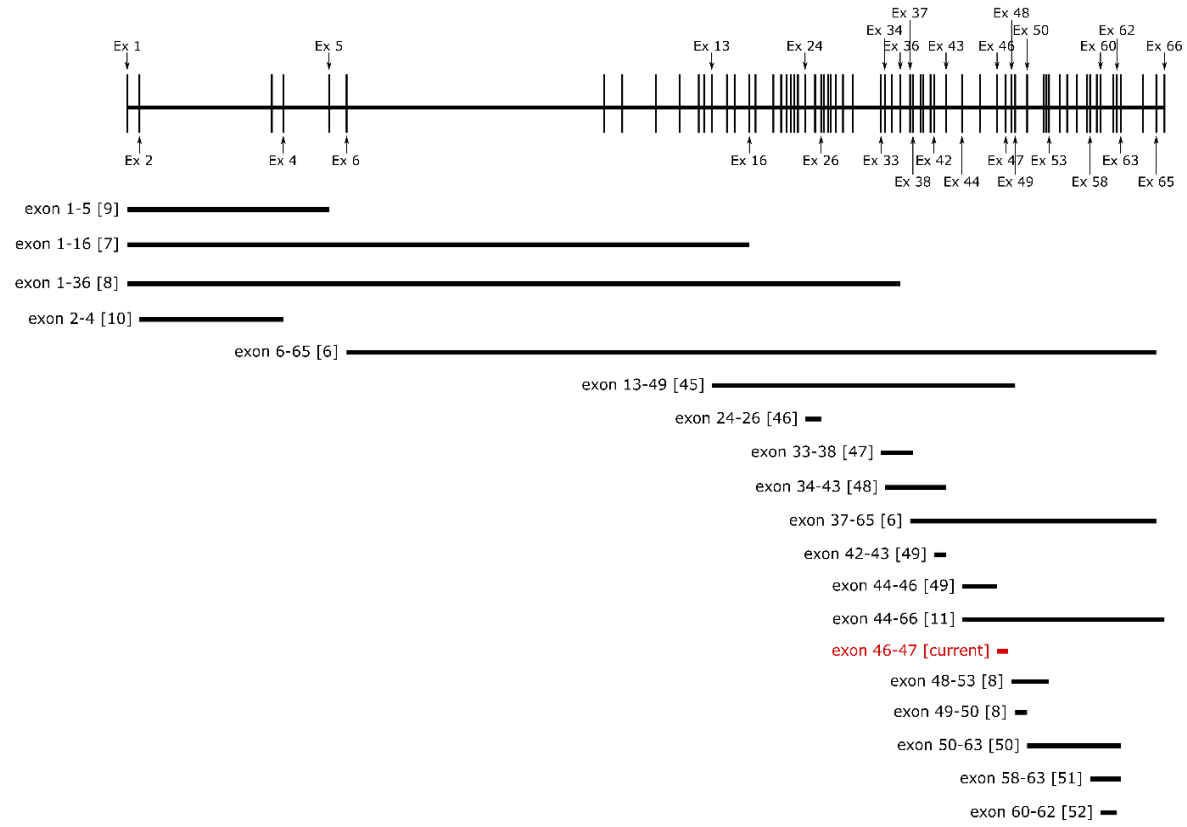


Figure 18 *Known multiple exon deletions in the FBN1 gene*

Microfibrils play a crucial role in regulating the bioavailability of the transforming growth factor beta (TGF-β), since fibrillin-1 binds to latent TGF-β–binding protein (LTBP) and sequesters TGF-β in the ECM (extracellular matrix), thus inhibiting TGF-β signaling (203, 204). As a result of mutations occurring in *FBN1*, the matrix sequestration of the latent TGF-β complex is affected, thus as a consequence the uncontrolled release of TGF-β leads to the overactivation of TGF-β signaling (205, 206). The contribution of TGF-β signaling to the aortic disease progression is suggested by experiments in MFS mice (207).

A deletion affecting TB binding domains has been observed in four cases (exon 43, exon 33-38, exon 42-43, exon 48-53, respectively), where the removal generates an in-frame mutation in all cases. The deletion results in a defective fibrillin-1 protein which potentially leads to the degeneration of microfibril architecture and indirectly to the loss of extracellular matrix integrity. We hypothesize that the deletion of TB domains (namely TB6, TB7 and TB8) in these patients causes the release of active TGF-β into ECM in the aortic wall which in turn overactivates the canonical TGF-β signaling pathway. This

effect then may superimpose to the microfibril degeneration and finally together lead to severe cardiovascular manifestations (i.e. aortic dilatation and aortic dissection) in these cases.

We observed aortic dissection in one patient (24 years old) and dilated aorta in three other patients. Since cardiovascular manifestations are age-dependent, it is possible that two of the three patients did not develop aortic dissection due to their young age.

Recently it was suggested that non-coding genetic variations might have an effect (often exerted in a tissue-specific manner) on gene regulation, which can also lead to the development of Mendelian diseases. Certain structural variations might uncouple regulatory elements from their target genes (208, 209).

Beside *FBN1* mutations, cell type-specific epigenetic predisposition may also be involved in the development of TAA, as previously demonstrated by Gomez et al through the investigation of the epigenetic control of vascular smooth muscle cells in Marfan and non-Marfan TAA (210).

After comprehensive evaluation, our *in silico* analysis of *FBN1* gene demonstrated the presence of potential transcription binding sites for STAT3 in a number of cases. According to Chandesris et al, aneurysm formation was one of the most frequent vascular abnormalities in STAT3 deficient adult patients. They supposed that the observed vascular abnormalities are the consequence of a systemic connective tissue disorder that includes arterial fragility (211). Inhibition of STAT3-dependent signaling in mouse models demonstrated a greater susceptibility to vascular aneurysm. We suppose that in CNV patients carrying a deletion involving STAT3 binding sites, the deletion itself has an effect on STAT3 signaling pathways which may superimpose on the *FBN1* gene defect and together they lead to a severe cardiovascular manifestation in these patients.

5.1.2. Discussion of the mechanism underlying the large *FBN1* deletion

Identification of the exact breakpoints of the *FBN1* deletion revealed the loss of a 4916 nucleotide long sequence with the insertion of ‘TG’ nucleotides (Figure 13). Our hypothesis is based on several previous studies. The mechanism behind the deletion might be the result of the MMBIR mechanism described earlier in detail by Hastings et al (100) and later by Ottaviani and colleagues (212). A few cases have been presented that certain

disorders, such as Cornelia de Lange syndrome and haemophilia A, are caused by non-recurrent CNVs generated by the MMBIR mechanism (181).

According to our hypotheses (Figure 19) a potential dinucleotide insertion ('TG') created a 'CCTTGCCTTG' direct repeat sequence (I.), which might interrupt the replication machinery. The insertion itself or the generated repeat potentially caused the replication fork to slow down, stall and eventually collapse. Presumably, this event resulted in a single DSB, where a 5' to 3' resection generated a sequence with a short 3' overhang (II.). As a result of the resection, a DNA segment was exposed to another DNA segment with possible microhomology in close proximity. As a consequence, a D-loop was formed with the 3' overhang part of the dsDNA invading the microhomologous region, where annealing and restarting of the synthesis occurred (III.). On the other hand, we suggest a simultaneous adenine-to-guanine substitution (IV.) due to an erroneous DNA repair, which at that position creates a microhomology on the other DNA segment, therefore eventually creating the final sequence with the ~5 kilobase long deletion supplemented by a 'TG' dinucleotide insertion at the breakpoints (V.). Since the *FBN1* gene is localized on the reverse strand, the sequence of *FBN1* was represented in the reverse orientation during exploring the mechanism behind the CNV formation. However, for easier explanation and understanding, we indicated the final sequence in regular orientation. Hereby we suggest that MMBIR were responsible for the formation of the CNV in our case, which is supported by the fact that MMBIR is often associated with small stretches (1-4bp) of microhomology (85, 213, 214).

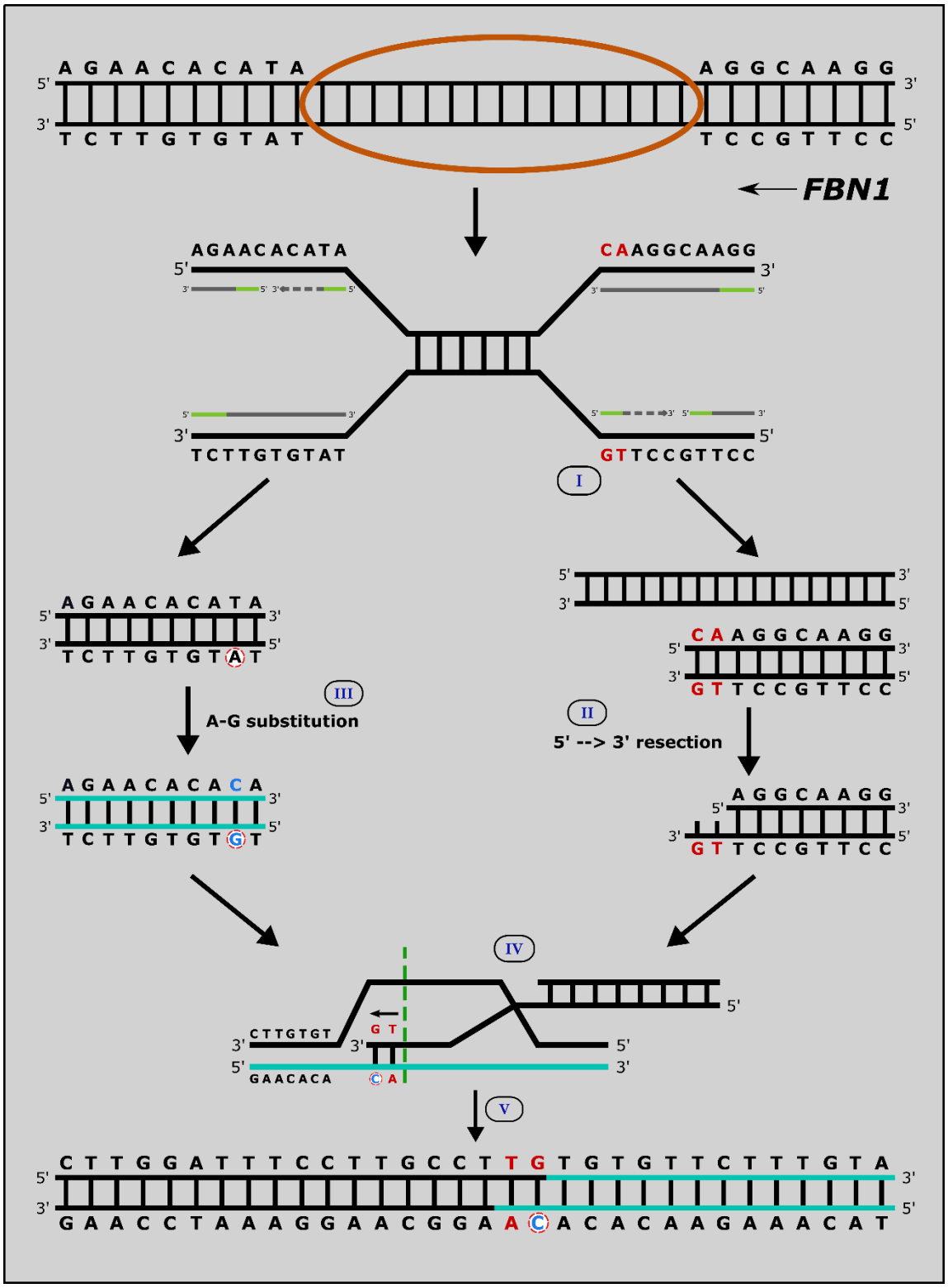


Figure 19 *Potential mechanism (MMBIR) behind the formation of the 4916 nucleotide long deletion*

5.2. Neurofibromatosis

The first case of large *NF1* microdeletion was published by Kayes et al in 1992 (171). Since then several genotype-phenotype correlations have been established, which suggested a more severe clinical phenotype among patients with *NF1* microdeletion compared to the intragenic *NF1* patient cohort. However, certain variability of clinical symptoms has been observed among individuals with *NF1* microdeletions. Most of the presented clinical information about *NF1* microdeletion patients is primarily coming from adult patient population so far. Only a few studies demonstrated pediatric clinical information (169).

In order to reveal genotype-phenotype correlations, we compared the clinical characterization of our patients with the published data on microdeletion and intragenic *NF1* patients. During our research 17 patients with large *NF1* microdeletion were identified. Among them 12 patients were demonstrated to be type-1 (eight detected by aCGH and four based on MLPA results), one patient had type-2 deletion and four patients possessed atypical deletions. The distribution of type-1 deletion in our patient cohort is somewhat similar (70%) to the prevalence determined from previous studies (70-80%) (215, 216). Somatic mosaicism with an extent of ca. 30% was detected in one patient with atypical *NF1* microdeletion.

Comparing the clinical features of previously published cases with our patients suffering from either type-1 *NF1* microdeletion or intragenic mutation, a similar difference was observed (Table 8). Remarkable difference was observed in several manifestations, such as dysmorphic features, subcutaneous neurofibromas, skeletal anomalies and neurobehavior problems. Although significant differences were recognized in certain clinical features between cases with large *NF1* microdeletion published previously and in our microdeletion patient cohort, it is noteworthy to mention that particular manifestations are age dependent. The majority of our patients (13 out of 17) were less than 15 years old at the time of the examination.

Table 8 *Clinical features of patients with type-1 NF1 microdeletion*

		Frequency in patients with type-1 *NF1* microdeletions (%)					Frequency in *NF1* non-deleted patients (%)		p
System involvement/ manifestations	Clinical features	this study (n=12)	(168) (n=29)	(215) (n=44)	(167) (n=7)	(217) (n=11)	this study (n=33)	(168) (n=29)	This study
Dysmorphic features	Facial dysmorphism	67	90	54.8	43	n.d.	0	n.d.	**<0,001**
	Hypertelorism	58	86	n.d.	n.d.	n.d.	18	n.d.	**0,022**
	Facial asymmetry	25	28	n.d.	n.d.	n.d.	6	8	0,109
	Coarse face	67	59	n.d.	n.d.	n.d.	0	n.d.	**<0,001**
	Broad neck	8	31	n.d.	n.d.	n.d.	0	n.d.	0,267
	Large hands and feet	67	46	n.d.	n.d.	n.d.	0	n.d.	**<0,001**
Skin manifestations	Café-au-lait spots	100	93	20.8	100	100	91	86-99	0,553
	Axillary and inguinal freckling	83	86	86.4	57	72.7	52	86-89	0,086
	Excess soft tissue in hands and feet	33	50	n.d.	n.d.	n.d.	0	n.d.	**0,003**
	Subcutaneous neurofibromas	58	76	37.2-41.8	29	45.5[#]	30	48	0,163
	Cutaneous neurofibromas	8	86	15.4-48.7	57	45.5[#]	18	38-84	0,655
	Plexiform neurofibromas	17	76	0.6	29	27.3	6	15-54	0,286
Education and behavior problems	SDiCD	75	48	n.d.	14	36.4	3	17	**<0,001**
	General learning difficulties	75	45	85.7	n.d.	18.2	15	31-47	**<0,001**
	Speech difficulties	67	48	n.d.	29	0	3	20-55	**<0,001**
	IQ < 70	8	38	n.d.	14	36.4	0	7-8	0,267
	ADHD	17	33	n.d.	n.d.	0	6	38-49	0,286
Skeletal manifestations	Skeletal anomalies	92	76	31+	14	45.5+	33	31	**<0,001**
	Scoliosis	42	43	31	0	9.1	21	10-28	0,254
	Pectus excavatum	42	31	n.d.	n.d.	n.d.	9	12-50	**0,022**
	Bone cysts	8	50	n.d.	n.d.	0	0	1	0,267
	Hyperflexibility of joints	8	72	n.d.	n.d.	n.d.	6	n.d.	1,0
	Pes cavus	n.d.	17	n.d.	n.d.	n.d.	3	n.d.	1,0
	Macrocephaly	58	39	11.5	14	45.5	9	24-45	**0,01**
Neurological manifestations	Muscular hypotonia	25	45	n.d.	n.d.	n.d.	12	27	0,362
	Epilepsy	0	7	n.d.	n.d.	0	3	4-13	1,0
	MPNST	17	21	7.1	0	*	0	2-7	0,067
	Spinal neurofibromas	17	64	n.d.	n.d.	n.d.	3	24-30	0,169
	T2 hyperintensities	83	45	n.d.	29	n.d.	39	34-79	**0,017**
Ocular manifestations	Visual disturbance	17	n.d.	n.d.	14	n.d.	15	n.d.	1,000
	Lisch nodules	25	93	40	14	45.5	21	63-93	1,000
	Strabismus	17	NA	n.d.	14	n.d.	0	NA	0,067
	Optic pathway gliomas	17	19	15	n.d.	0	12	11-19	0,650
Develop. problem	Tall-for-age stature	58	46	22.2	n.d.	n.d.	0	n.d.	**<0,001**
Heart problems	Congenital heart defects	0	29	n.d.	n.d.	n.d.	0	2	0 (or constant)

*n.d., not determined; NA, not assessed or no data available; #no straightforward information (only referenced as neurofibroma); *it is not clear from the manuscript (it was mentioned that 18.2% of patient had tumours); + it may be higher (there were data for scoliosis and macrocephaly only); SDiCD, significant delay in cognitive development; MPNST, malignant peripheral nerve sheath tumours; ADHD, attention deficit hyperactivity disorder*

Based on various studies *NF1* microdeletions show a certain degree of variability in the frequency of some clinical features (Table 8) (162, 167, 215, 217, 218). Dysmorphic features are characteristics for patients with *NF1* microdeletion, especially in individuals with type-1 deletions as Mautner et al. presented in a large study, where the majority of the cases (ca. 90%) had this manifestation (218). However, a lower frequency was observed in other studies (167, 215). In our type-1 patient cohort, 67% of the affected individuals possess this feature. The represented data imply that it is a very frequent symptom in patients with type-1 deletions. Although large hands and feet were not stated in microdeletion patients by most studies, our patient cohort showed a slightly higher frequency of it (67%) compared to the observed percentage (46%) demonstrated earlier by Mautner (218).

Previous studies established an early-onset of neurofibromas among *NF1* microdeletion patients. Besides the close frequency observed in our patients and others (58% and 76%, respectively) of the detected subcutaneous neurofibromas, the occurrence of cutaneous or plexiform neurofibromas was greatly lower in our patients compared to other patient groups (8% vs. 86%, 17% vs 76%, respectively). However, it is worth highlighting that cutaneous neurofibromas show age-related penetrance, therefore the difference in the observed frequency might originate from the fact that our patient cohort mainly consisted of children and adolescents. Nevertheless, the recent observation of the high frequency of cutaneous neurofibromas detected among children was found by Kehrer-Sawatzki (169). Subcutaneous neurofibromas in type-1 *NF1* patients are associated with mortality in *NF1* disease (219). In addition, patients with subcutaneous neurofibromas possess a higher risk for the development of MPNSTs, and the presence of plexiform neurofibromas involves risk for the development of malignant tumour (220).

A significant delay in cognitive development was found more frequently in our type-1 patients, but the prevalence of intellectual disability was less pronounced. The presence of connective tissue anomalies in our patient group showed significant differences compared to Mautner's patients (8% and 72%, respectively). The observed frequency of overgrowth was similar to the results of other studies.

Type-1 deletion harbors fourteen protein coding genes and four microRNA genes. Haploinsufficiency of certain co-deleted genes with *NF1* might influence some clinical manifestations, and it may contribute to the severity of the disease (168). Deletion of

RNF135 has been associated with dysmorphic facial features, reduced cognitive capability and overgrowth (221) and the removal of *ADAP2* is connected to heart defects (222). Furthermore, the loss of the tumour suppressive function of *SUZ12* and *ATAD5* promote tumour development (223, 224). Despite type-1 microdeletion patients share broadly the same gene content, remarkable clinical variability is demonstrated, which suggests that other unique genomic architecture may contribute to the observed variability.

Type-2 deletions account for 10-20 % of *NF1* large deletion cases according to previous studies. In our patient cohort one patient and her asymptomatic mother carry this type of large *NF1* deletion. No asymptomatic patient with large *NF1* microdeletion was identified so far, therefore we suppose that the mother should be a mosaic patient. Somatic mosaicism in type-2 deletion is a frequently observed phenomenon and it is associated with a milder clinical phenotype. The research group of Vogt et al. determined and discussed mosaicism in type-2 deletions through multiple studies (225, 226). Only a few non-mosaic type-2 cases with detailed phenotype have been published (167, 226) so far (Table 9).

Table 9 *Clinical features of patients with type-2 NF1 microdeletions*

Clinical features of patients with type-1 *NF1* microdeletions (frequency observed, %)			**Presence or absence of the features in patients with type-2 *NF1* deletions**			
Patients	n=29	n=12	078	P. 2429	P. 2358	85/NF
Reference	(168)	this study	(167)	(225, 227)	(225, 227)	this study
CALs	93%	100%	+	+	+	+
Freckling	86%	83%	-	+	+	-
Lisch nodule	93%	25%	?	+	+	+
Cutaneous neurofibromas	86%	8%	+	+ (multiple)	-	-
Subcutaneous neurofibromas	76%	58%	+	+ (multiple)	+	-
Plexiform neurofibromas	76%	17%	-	+ (multiple)	+	-
Facial dysmorphism	90%	67%	-	+	+	-
Large hands and feet	46%	67%	N/A	+	+	+
Macrocephaly	39%	58%	-	+	+	+
Tall stature	46%	58%	N/A	-	-	-
Learning disabilities	48%	75%	?	+	+ (mild)	+
Attention deficits	33%	17%	?	+	+	-
Scoliosis	43%	42%	+	-	N/A	+
Hyperflexibility of the joints	72%	8%	N/A	+	+	-
MPNST	21%	17%	-	+	-	-
T2 hyperintensities	45%	83%	N/A	-	+	+
Muscular hypotonia	45%	25%	N/A	N/A	+	-
Congenital heart defects	21%	0%	N/A	+	+	-

−, absent; +, present; N/A, not assessed or no data available; ? unclear result from the original article. CALs, café-au-lait spots; MPNST, malignant peripheral nerve sheath tumours.

In contrast to previous cases, neurobehavioral problems, cardiac manifestations, freckling, hyperflexibility of the joints and externally observable neurofibromas were absent in our type-2 patient's phenotype. Interestingly, the whole clinical picture is dominated by skeletal anomalies. Although type-2 deletions are typically 1.2 Mb in size, the exact localization of the breakpoints are presumably different in our patients and in the published cases. This may result in the removal of certain regulatory factors, which may finally lead to the observed variability in the phenotype.

Atypical deletions are observed in around 8-10% among patients with *NF1* microdeletions. They form a heterogeneous group with their various localization and affected size and the presented diverse clinical picture. Furthermore, somatic mosaicism can be frequently observed, which may lead to a milder phenotype. In our patient cohort we observed a higher frequency (23%) and only one patient showed mosaicism. Around 20 patients with atypical deletion were published so far without recurrent breakpoints (162, 167, 170, 171, 228-236). During our research three distinct, novel deletions were found. Apart from the major diagnostic criteria for NF1, hardly any overlapping symptoms were observed with the clinical pictures of the known cases (Table 10). Significant differences can be seen in dysmorphic features, neuropsychological manifestations and the presence of various neurofibromas.

Characteristic hallmarks of NF1 microdeletions, such as facial dysmorphia, facial asymmetry, large hands and feet and coarse face, were observed in the majority of patients with type-1 *NF1* microdeletion, and presented at least in half of the atypical cases identified so far. However, in our patient cohort only one patient showed facial dysmorphia and another had hypertelorism. Although various types of neurofibromas can be detected among the atypical *NF1* microdeletion patients, in our case only one individual, who was 40 years old, has developed subcutaneous neurofibromas. It is a known phenomenon that the number of neurofibromas may increase with the age of the patient, therefore our results may be related to the age of the patients.

Table 10 *Clinical features of patients with atypical NF1 microdeletions*

Patient	Age	Gender	Skin manifestations	Neurofibromas	Dysmorphic features	Skeletal manifestations	Ocular Manifestations	Neuropsychological manifestations	Other	Refs
BUD	14; 18	n.i.	CALs, F	Many CNF, SNF	Coarse face	SCS, genu valgum, joint laxity	n.i.	SDiCD, ID, T2 hyperintensities	Many ST	(232)
3724A	13	Female	CALs, F	Few CNF	Coarse face, FA, hypertelorism, ptosis, broad lips and nose	PE	LiN	Moderate ID	-	(228)
6	NI	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	(233, 234)
UWA106-3	18	Male	CALs, F	Many CNF, PNF, spinal NF	Coarse face, large hands	MA	n.i.	SDiCD, IQ 46	Many ST	(170, 171)
442	18; 26	Male	CALs, F	Multiple SCNF, and many CNF, PNF	Coarse face	SCS	LiN	IQ 76, severe LD	Many ST	(229)
BL	13,5	Male	CALs, F	-	FD, hypertelorism	Skeletal anomalies	-	Severe ID	-	(230)
ID806	3 mo; 3; 4	Male	CALs, F	-	Narrow palpebral fissures, ptosis, low set, rotated ears, prominent maxilla	-	-	Marked developmental delay, SP, seizure	-	(231)
UWA155-1	27	N/A	-	Multiple CNF, spinal NF	Coarse face, ptosis, large hands and feet	MA	-	Moderate ID	MPNST	(170)
118	5	Male	CALs, F	n.i.	-	-	OPG	Seizure, no LD	-	(234)
282775	n.d.	N/A	CALs	-	Noonan-like FD	-	-	PD, SP	-	(236)
552	20	Female	CALs, F	2 PNF, 4 SIN NF	Large hands and feet	PE, lumbar lordosis,	LiN, visual disturbance	Mild ID, severe LD, SP, hypotonia	-	(235)
NF040	1	Female	CALs	PNF	-	-	*	*	-	(167)
NF056	60	Female	CALs, F	CNF	-	-	*	*	-	
NF073	25	Female	CALs, F	CNF	-	-	*	*	-	
NF076	36	Female	CALs	CNF	-	-	*	*	-	
556/NF	10	Male	CALs, F	-	-	Bilateral PP	OPG	-	-	(162)
125/NF	2	Female	CALs, F	-	-	PE	-	-	-	
134/NF	40	Female	CALs	SCNF	Hypertelorism	SCS	-	-	-	
260/NF	8	Male	CALs, F	-	FD, hypertelorism	PE, MA	OPG	SDiCD, T2 hyperintensities	ASD	

*CALs, café-au-lait spots; F, freckling; FA, facial asymmetry; FD, facial dysmorphy; CNF, cutaneous neurofibroma; SCNF, subcutaneous neurofibroma; PNF, plexiform neurofibroma; SIN NF, small intramuscular nodular neurofibroma; ST, spinal tumours; MPNST, malignant peripheral nerve sheath tumours; SDiCD, significant delay in cognitive development; ID, intellectual disability; LD, learning difficulties; SP, speech delay; PD, psychomotor delay; SCS, scoliosis; PE, pectus excavatum; MA, macurocephaly; PP, pes planus; LiN, Lisch nodule; ASD, atrial septal defect. * unclear results in the original article. NA, no data available*

In addition, neuropsychological manifestations were absent in our patients, only one showed significant delay in the cognitive development. Table 11 summarizes the haploinsufficiency intolerant genes in all cases published so far. The gene content of the deleted region observed in the various forms of atypical deletions has an effect on the phenotypic features, especially genes with intolerance of haploinsufficiency. In three of the patients we studied, only MLPA measurements could be performed, suggesting that a haploinsufficiency intolerant gene, namely *RAB11FIP4*, might also be deleted. The function of this gene in disease pathogenesis is not yet clear.

Table 11 *Size of the deletions and haploinsufficient genes located within the atypical NF1 deletions.*

Patient	Deletion size (Mb)	Haploinsufficient genes (by ExAC pLI)	Haploinsufficient genes (by gnomAD pLI)	Refs
BUD	4.7	CRLF3, ATAD5, NF1, RAB11FIP4, SUZ12, LRRC37B, RHOT1, c17orf75, PSMD11	ATAD5, NF1, **OMG**, RAB11FIP4, SUZ12, PSMD11, **CDK5R1**	(232)
3724A	2.0-3.1	CRLF3, ATAD5, NF1, RAB11FIP4, SUZ12, LRRC37B, RHOT1, c17orf75, PSMD11	ATAD5, NF1, **OMG**, RAB11FIP4, SUZ12, PSMD11, **CDK5R1**	(228)
6	3	CRLF3, ATAD5, NF1, RAB11FIP4, SUZ12, LRRC37B, RHOT1, c17orf75, PSMD11	ATAD5, NF1, **OMG**, RAB11FIP4, SUZ12, PSMD11, **CDK5R1**	(233, 234)
UWA106-3	3.2-3.7	CRLF3, ATAD5, NF1, RAB11FIP4, SUZ12, LRRC37B, RHOT1, c17orf75, PSMD11	ATAD5, NF1, **OMG**, RAB11FIP4, SUZ12, PSMD11, **CDK5R1**	(170, 171)
442	2	CRLF3, ATAD5, NF1, RAB11FIP4, SUZ12	ATAD5, NF1, **OMG**, RAB11FIP4, SUZ12	(229)
BL	~3	CRLF3, ATAD5, NF1, RAB11FIP4, SUZ12, LRRC37B, RHOT1, c17orf75, PSMD11	ATAD5, NF1, **OMG**, RAB11FIP4, SUZ12, PSMD11, **CDK5R1**	(230)
ID806	~7	CRLF3, ATAD5, NF1, RAB11FIP4, SUZ12, LRRC37B, RHOT1, c17orf75, PSMD11	ATAD5, NF1, **OMG**, RAB11FIP4, SUZ12, PSMD11, **CDK5R1**	(231)
UWA155-1	2.1-2.7	NF1, RAB11FIP4, SUZ12, LRRC37B, RHOT1, c17orf75, PSMD11	NF1, **OMG**, RAB11FIP4, SUZ12, PSMD11, **CDK5R1**	(231)
118	N/A	CRLF3, ATAD5, NF1	ATAD5, NF1	(234)
282775	>1.33	NF1, RAB11FIP4, SUZ12, LRRC37B, RHOT1, c17orf75	NF1, **OMG**, RAB11FIP4, SUZ12	(236)
552	2.7	NF1, RAB11FIP4, SUZ12, LRRC37B, RHOT1, c17orf75, PSMD11	NF1, **OMG**, RAB11FIP4, SUZ12, PSMD11, **CDK5R1**	(235)
40	1.27-1.46	NF1, RAB11FIP4, SUZ12, LRRC37B, RHOT1, c17orf75	NF1, **OMG**, RAB11FIP4, SUZ12,	(167)
56	0.60-1.14	CRLF3, ATAD5, NF1	ATAD5, NF1, **OMG**	
73	0.93-1.28	NF1, RAB11FIP4, SUZ12, LRRC37B	NF1, **OMG**, RAB11FIP4, SUZ12	
76	1.26-1.63	CRLF3, ATAD5, NF1, RAB11FIP4, SUZ12, LRRC37B	ATAD5, NF1, **OMG**, RAB11FIP4, SUZ12	
556/NF	1.122	CRLF3, ATAD5, NF1, RAB11FIP4, SUZ12	ATAD5, NF1, **OMG**, RAB11FIP4, SUZ12	(162)
125/NF	1.635*	CRLF3, ATAD5, NF1, RAB11FIP4, SUZ12, LRRC37B, RHOT1, c17orf75	ATAD5, NF1, **OMG**, RAB11FIP4, SUZ12	
134/NF	0.618*	CRLF3, ATAD5, NF1	ATAD5, NF1, **OMG**	
260/NF	0.618*	CRLF3, ATAD5, NF1	ATAD5, NF1, **OMG**	

**Results originated from MLPA probes location. The probability of loss of function (pLI) metric were provided by the gnomAD browser (https://gnomad.broadinstitute.org/). According to official description, a transcript's intolerance to variation is measured by predicting the number of variants expected to be seen in the gnomAD dataset and comparing those expectations to the observed amount of variation. The scale ranges from 0 to 1, where the closer the pLI value is to 1, the more intolerant the gene appears to be to loss of function (LoF) variants. We determined as haploinsufficient a gene if the pLI value was above 0.9, which indicates extreme intolerance to LoF variants (237).*

According to previous studies (238, 239), a lower cognitive ability was revealed in patients with *NF1* microdeletion compared to the patients with intragenic mutations. Co-deletion of genes *RNF135* and *OMG* (oligodendrocyte myelin) is assumed to play a role in the development of decreased cognitive ability (168). *OMG*, which plays an important role in early brain development, was connected to certain neuropsychiatric disorders, and to the progression of intellectual disability (240, 241). Moreover, patients with autism carried a rare allele of *RNF135* gene with higher frequency (222). However, our patients encompassing *RNF135* and *OMG* hardly displayed neuropsychiatric symptoms, so further factors might be also necessary for the development of these manifestations in patients with *NF1* microdeletions.

Several genes (*ATAD5*, *COPRS*, *UTP6* and *SUZ12*) in the 17q11.2 region were supposed to be involved in tumorigenesis (168), therefore they may be accounted for an increased risk for high tumour load. In our cases co-deletion of *ATAD5*, *COPRS* and *UTP6* genes with *NF1* was observed in one patient, and two other patients' deletion included *ATAD5*. Although none of these patients developed internal tumours, a high load of internal tumours was observed in a number of patients with larger atypical deletion. In one of our patients the atypical deletion harbours all of these four genes, however, perhaps due to her young age (i.e. 2 y) no tumours were found at the age of her examination.

Genotype-phenotype analyses among our patients revealed that specific clinical manifestations, including dysmorphic facial features, macrocephaly, large hands and feet, delayed cognitive development and/or learning difficulties, speech difficulties, subcutaneous neurofibromas and overgrowth were observed more frequently in the *NF1* microdeletion patient cohort compared to the intragenic *NF1* mutation patient group. Furthermore, it seems that these symptoms are characteristic of the patient group with type-1 *NF1* microdeletion. In case of the non-mosaic type-2 *NF1* large deletion patient, only a few of the remarkable symptoms were observed, such as large hands and feet, learning difficulties and macrocephaly, as well. Skeletal manifestations were present. In our atypical *NF1* microdeletion patient cohort only facial dysmorphism, presence of the subcutaneous neurofibromas, delayed cognitive development and macrocephaly were observed. In contrast to previous reports (168, 218), joint laxity, heart defects, muscular hypotonia and bone cysts were absent in type-1 *NF1* microdeletion patients. However, it is noteworthy to mention that manifestations of several symptoms are age dependent.

6. Conclusions

1. Three types of *NF1* microdeletion (type-1, type-2 and atypical) were identified in our NF1 patient cohort. Among the detected 17 microdeletion, altogether twelve type-1 (~70%), one type-2 (~6%) and four atypical deletions (~24%) were identified. Three distinct novel atypical deletions and no type-3 microdeletion were detected.

2. Genotype-phenotype analyses among our patients revealed that specific clinical manifestations, such as dysmorphic facial features, macrocephaly, large hands and feet, delayed cognitive development and/or learning difficulties, speech difficulties, subcutaneous neurofibromas and overgrowth are characteristic for the patient group with type-1 NF1 microdeletion. Our patient with non-mosaic type-2 NF1 large deletion had only a few of the typical clinical symptoms observed in NF1 microdeletion: macrocephaly, large hands and feet, as well as learning difficulties, moreover our patient with atypical NF1 microdeletion demonstrated facial dysmorphism, presence of the subcutaneous neurofibromas, delayed cognitive development and macrocephaly.

3. We observed that patients with *NF1* large deletion presented more severe clinical phenotype compared to individuals with intragenic *NF1* mutations, possibly due to the affected gene contents and/or the loss of other regulatory DNA elements.

4. We demonstrated, with the help of the literature data and our results, that large various CNVs are often associated with severe cardiovascular manifestations in Marfan syndrome.

5. An association between severe cardiovascular symptoms and the large deletions of the *FBN1* gene was supposed, and we found that involvements of regulatory elements (lack of transcrioption binding site for STAT3) may play a role in the development of cardiovascular symptoms.

6. Breakpoint characterization of the large deletion detected in *FBN1* gene presented a 4916 nucleotide long deletion, with a TG dinucleotide insertion. With the help of previous models and bioinformatic analysis, we proposed that a rare mechanism, termed microhomology-mediated break induced replication, might be responsible for the large deletion.

7. References

1. Lappalainen T, Scott AJ, Brandt M, Hall IM. Genomic Analysis in the Age of Human Genome Sequencing. Cell. 2019;177(1):70-84.
2. International HapMap C. A haplotype map of the human genome. Nature. 2005;437(7063):1299-320.
3. Weber JL, David D, Heil J, Fan Y, Zhao C, Marth G. Human diallelic insertion/deletion polymorphisms. Am J Hum Genet. 2002;71(4):854-62.
4. McCarroll SA, Hadnott TN, Perry GH, Sabeti PC, Zody MC, Barrett JC, et al. Common deletion polymorphisms in the human genome. Nat Genet. 2006;38(1):86-92.
5. Iafrate AJ, Feuk L, Rivera MN, Listewnik ML, Donahoe PK, Qi Y, et al. Detection of large-scale variation in the human genome. Nat Genet. 2004;36(9):949-51.
6. Jacobs PA. Human chromosome heteromorphisms (variants). Prog Med Genet. 1977;2:251-74.
7. Rogozin IB, Makarova KS, Natale DA, Spiridonov AN, Tatusov RL, Wolf YI, et al. Congruent evolution of different classes of non-coding DNA in prokaryotic genomes. Nucleic Acids Res. 2002;30(19):4264-71.
8. Niu DK, Jiang L. Can ENCODE tell us how much junk DNA we carry in our genome? Biochem Biophys Res Commun. 2013;430(4):1340-3.
9. Zhen Y, Andolfatto P. Methods to detect selection on noncoding DNA. Methods Mol Biol. 2012;856:141-59.
10. Cavalli-Sforza LL, Piazza A. Human genomic diversity in Europe: a summary of recent research and prospects for the future. Eur J Hum Genet. 1993;1(1):3-18.
11. Sharp AJ, Cheng Z, Eichler EE. Structural variation of the human genome. Annu Rev Genomics Hum Genet. 2006;7:407-42.
12. Brookes AJ. The essence of SNPs. Gene. 1999;234(2):177-86.
13. Karki R, Pandya D, Elston RC, Ferlini C. Defining "mutation" and "polymorphism" in the era of personal genomics. BMC Med Genomics. 2015;8:37.
14. Davidson EH, Britten RJ. Regulation of gene expression: possible role of repetitive sequences. Science. 1979;204(4397):1052-9.
15. Kumar RP, Senthilkumar R, Singh V, Mishra RK. Repeat performance: how do genome packaging and regulation depend on simple sequence repeats? Bioessays. 2010;32(2):165-74.
16. Criscione SW, Zhang Y, Thompson W, Sedivy JM, Neretti N. Transcriptional landscape of repetitive elements in normal and cancer human cells. BMC Genomics. 2014;15:583.
17. Gokcumen O, Babb PL, Iskow RC, Zhu Q, Shi X, Mills RE, et al. Refinement of primate copy number variation hotspots identifies candidate genomic regions evolving under positive selection. Genome Biol. 2011;12(5):R52.
18. Warburton PE, Hasson D, Guillem F, Lescale C, Jin X, Abrusan G. Analysis of the largest tandemly repeated DNA families in the human genome. BMC Genomics. 2008;9:533.
19. Bose P, Hermetz KE, Conneely KN, Rudd MK. Tandem repeats and G-rich sequences are enriched at human CNV breakpoints. PLoS One. 2014;9(7):e101607.
20. Pos O, Radvanszky J, Buglyo G, Pos Z, Rusnakova D, Nagy B, et al. DNA copy number variation: Main characteristics, evolutionary significance, and pathological aspects. Biomed J. 2021;44(5):548-59.
21. Sudmant PH, Rausch T, Gardner EJ, Handsaker RE, Abyzov A, Huddleston J, et al. An integrated map of structural variation in 2,504 human genomes. Nature. 2015;526(7571):75-81.
22. Collins RL, Brand H, Karczewski KJ, Zhao X, Alfoldi J, Francioli LC, et al. A structural variation reference for medical and population genetics. Nature. 2020;581(7809):444-51.
23. Gordeeva V, Sharova E, Arapidi G. Progress in Methods for Copy Number Variation Profiling. Int J Mol Sci. 2022;23(4).

24. Pang AW, MacDonald JR, Pinto D, Wei J, Rafiq MA, Conrad DF, et al. Towards a comprehensive structural variation map of an individual human genome. Genome Biol. 2010;11(5):R52.
25. Vialle RA, de Paiva Lopes K, Bennett DA, Crary JF, Raj T. Integrating whole-genome sequencing with multi-omic data reveals the impact of structural variants on gene regulation in the human brain. Nat Neurosci. 2022;25(4):504-14.
26. Ebert P, Audano PA, Zhu Q, Rodriguez-Martin B, Porubsky D, Bonder MJ, et al. Haplotype-resolved diverse human genomes and integrated analysis of structural variation. Science. 2021;372(6537).
27. Chaisson MJP, Sanders AD, Zhao X, Malhotra A, Porubsky D, Rausch T, et al. Multi-platform discovery of haplotype-resolved structural variation in human genomes. Nat Commun. 2019;10(1):1784.
28. Perry GH, Tchinda J, McGrath SD, Zhang J, Picker SR, Caceres AM, et al. Hotspots for copy number variation in chimpanzees and humans. Proc Natl Acad Sci U S A. 2006;103(21):8006-11.
29. Mahmoud M, Gobet N, Cruz-Davalos DI, Mounier N, Dessimoz C, Sedlazeck FJ. Structural variant calling: the long and the short of it. Genome Biol. 2019;20(1):246.
30. Tuzun E, Sharp AJ, Bailey JA, Kaul R, Morrison VA, Pertz LM, et al. Fine-scale structural variation of the human genome. Nat Genet. 2005;37(7):727-32.
31. Levy-Sakin M, Pastor S, Mostovoy Y, Li L, Leung AKY, McCaffrey J, et al. Genome maps across 26 human populations reveal population-specific patterns of structural variation. Nat Commun. 2019;10(1):1025.
32. Lin YL, Gokcumen O. Fine-Scale Characterization of Genomic Structural Variation in the Human Genome Reveals Adaptive and Biomedically Relevant Hotspots. Genome Biol Evol. 2019;11(4):1136-51.
33. Liu Y, Wilson SH. DNA base excision repair: a mechanism of trinucleotide repeat expansion. Trends Biochem Sci. 2012;37(4):162-72.
34. McMurray CT. Mechanisms of trinucleotide repeat instability during human development. Nat Rev Genet. 2010;11(11):786-99.
35. Jacobs PA, Strong JA. A case of human intersexuality having a possible XXY sex-determining mechanism. Nature. 1959;183(4657):302-3.
36. Wyllie JP, Wright MJ, Burn J, Hunter S. Natural history of trisomy 13. Arch Dis Child. 1994;71(4):343-5.
37. Cereda A, Carey JC. The trisomy 18 syndrome. Orphanet J Rare Dis. 2012;7:81.
38. Jacobs PA, Baikie AG, Court Brown WM, Strong JA. The somatic chromosomes in mongolism. Lancet. 1959;1(7075):710.
39. Morin SJ, Eccles J, Iturriaga A, Zimmerman RS. Translocations, inversions and other chromosome rearrangements. Fertil Steril. 2017;107(1):19-26.
40. Jacobs PA, Browne C, Gregson N, Joyce C, White H. Estimates of the frequency of chromosome abnormalities detectable in unselected newborns using moderate levels of banding. J Med Genet. 1992;29(2):103-8.
41. Pellestor F, Anahory T, Lefort G, Puechberty J, Liehr T, Hedon B, et al. Complex chromosomal rearrangements: origin and meiotic behavior. Hum Reprod Update. 2011;17(4):476-94.
42. Batista DA, Pai GS, Stetten G. Molecular analysis of a complex chromosomal rearrangement and a review of familial cases. Am J Med Genet. 1994;53(3):255-63.
43. Sebat J, Lakshmi B, Troge J, Alexander J, Young J, Lundin P, et al. Large-scale copy number polymorphism in the human genome. Science. 2004;305(5683):525-8.
44. Escaramis G, Docampo E, Rabionet R. A decade of structural variants: description, history and methods to detect structural variation. Brief Funct Genomics. 2015;14(5):305-14.
45. Feuk L, Carson AR, Scherer SW. Structural variation in the human genome. Nat Rev Genet. 2006;7(2):85-97.
46. Conrad DF, Pinto D, Redon R, Feuk L, Gokcumen O, Zhang Y, et al. Origins and functional impact of copy number variation in the human genome. Nature. 2010;464(7289):704-12.

47. Redon R, Ishikawa S, Fitch KR, Feuk L, Perry GH, Andrews TD, et al. Global variation in copy number in the human genome. Nature. 2006;444(7118):444-54.
48. Shaikh TH, Gai X, Perin JC, Glessner JT, Xie H, Murphy K, et al. High-resolution mapping and analysis of copy number variations in the human genome: a data resource for clinical and research applications. Genome Res. 2009;19(9):1682-90.
49. Lupski JR, de Oca-Luna RM, Slaugenhaupt S, Pentao L, Guzzetta V, Trask BJ, et al. DNA duplication associated with Charcot-Marie-Tooth disease type 1A. Cell. 1991;66(2):219-32.
50. Sebat J, Lakshmi B, Malhotra D, Troge J, Lese-Martin C, Walsh T, et al. Strong association of de novo copy number mutations with autism. Science. 2007;316(5823):445-9.
51. Weiss LA, Shen Y, Korn JM, Arking DE, Miller DT, Fossdal R, et al. Association between microdeletion and microduplication at 16p11.2 and autism. N Engl J Med. 2008;358(7):667-75.
52. Bochukova EG, Huang N, Keogh J, Henning E, Purmann C, Blaszczyk K, et al. Large, rare chromosomal deletions associated with severe early-onset obesity. Nature. 2010;463(7281):666-70.
53. Vacic V, McCarthy S, Malhotra D, Murray F, Chou HH, Peoples A, et al. Duplications of the neuropeptide receptor gene VIPR2 confer significant risk for schizophrenia. Nature. 2011;471(7339):499-503.
54. Chance PF, Alderson MK, Leppig KA, Lensch MW, Matsunami N, Smith B, et al. DNA deletion associated with hereditary neuropathy with liability to pressure palsies. Cell. 1993;72(1):143-51.
55. Lupski JR, Wise CA, Kuwano A, Pentao L, Parke JT, Glaze DG, et al. Gene dosage is a mechanism for Charcot-Marie-Tooth disease type 1A. Nat Genet. 1992;1(1):29-33.
56. Vogels A, Fryns JP. The Prader-Willi syndrome and the Angelman syndrome. Genet Couns. 2002;13(4):385-96.
57. McDonald-McGinn DM, Sullivan KE, Marino B, Philip N, Swillen A, Vorstman JA, et al. 22q11.2 deletion syndrome. Nat Rev Dis Primers. 2015;1:15071.
58. Potocki L, Bi W, Treadwell-Deering D, Carvalho CM, Eifert A, Friedman EM, et al. Characterization of Potocki-Lupski syndrome (dup(17)(p11.2p11.2)) and delineation of a dosage-sensitive critical interval that can convey an autism phenotype. Am J Hum Genet. 2007;80(4):633-49.
59. Bayes M, Magano LF, Rivera N, Flores R, Perez Jurado LA. Mutational mechanisms of Williams-Beuren syndrome deletions. Am J Hum Genet. 2003;73(1):131-51.
60. Walsh T, McClellan JM, McCarthy SE, Addington AM, Pierce SB, Cooper GM, et al. Rare structural variants disrupt multiple genes in neurodevelopmental pathways in schizophrenia. Science. 2008;320(5875):539-43.
61. Xu B, Roos JL, Levy S, van Rensburg EJ, Gogos JA, Karayiorgou M. Strong association of de novo copy number mutations with sporadic schizophrenia. Nat Genet. 2008;40(7):880-5.
62. Gonzalez E, Kulkarni H, Bolivar H, Mangano A, Sanchez R, Catano G, et al. The influence of CCL3L1 gene-containing segmental duplications on HIV-1/AIDS susceptibility. Science. 2005;307(5714):1434-40.
63. Kuhn L, Schramm DB, Donninger S, Meddows-Taylor S, Coovadia AH, Sherman GG, et al. African infants' CCL3 gene copies influence perinatal HIV transmission in the absence of maternal nevirapine. AIDS. 2007;21(13):1753-61.
64. Dumas L, Kim YH, Karimpour-Fard A, Cox M, Hopkins J, Pollack JR, et al. Gene copy number variation spanning 60 million years of human and primate evolution. Genome Res. 2007;17(9):1266-77.
65. Lupski JR. An evolution revolution provides further revelation. Bioessays. 2007;29(12):1182-4.
66. van Ommen GJ. Frequency of new copy number variation in humans. Nat Genet. 2005;37(4):333-4.
67. Lee JA, Carvalho CM, Lupski JR. A DNA replication mechanism for generating nonrecurrent rearrangements associated with genomic disorders. Cell. 2007;131(7):1235-47.
68. Zhang F, Gu W, Hurles ME, Lupski JR. Copy number variation in human health, disease, and evolution. Annu Rev Genomics Hum Genet. 2009;10:451-81.

69. Shaw CJ, Lupski JR. Implications of human genome architecture for rearrangement-based disorders: the genomic basis of disease. Hum Mol Genet. 2004;13 Spec No 1:R57-64.
70. Stankiewicz P, Lupski JR. Genome architecture, rearrangements and genomic disorders. Trends Genet. 2002;18(2):74-82.
71. Zhou W, Zhang F, Chen X, Shen Y, Lupski JR, Jin L. Increased genome instability in human DNA segments with self-chains: homology-induced structural variations via replicative mechanisms. Hum Mol Genet. 2013;22(13):2642-51.
72. Bailey JA, Eichler EE. Primate segmental duplications: crucibles of evolution, diversity and disease. Nat Rev Genet. 2006;7(7):552-64.
73. Gu W, Zhang F, Lupski JR. Mechanisms for human genomic rearrangements. Pathogenetics. 2008;1(1):4.
74. Mun S, Kim S, Lee W, Kang K, Meyer TJ, Han BG, et al. A study of transposable element-associated structural variations (TASVs) using a de novo-assembled Korean genome. Exp Mol Med. 2021;53(4):615-30.
75. Arlt MF, Mulle JG, Schaibley VM, Ragland RL, Durkin SG, Warren ST, et al. Replication stress induces genome-wide copy number changes in human cells that resemble polymorphic and pathogenic variants. Am J Hum Genet. 2009;84(3):339-50.
76. Arlt MF, Wilson TE, Glover TW. Replication stress and mechanisms of CNV formation. Curr Opin Genet Dev. 2012;22(3):204-10.
77. Kim PM, Lam HY, Urban AE, Korbel JO, Affourtit J, Grubert F, et al. Analysis of copy number variants and segmental duplications in the human genome: Evidence for a change in the process of formation in recent evolutionary history. Genome Res. 2008;18(12):1865-74.
78. Girirajan S, Dennis MY, Baker C, Malig M, Coe BP, Campbell CD, et al. Refinement and discovery of new hotspots of copy-number variation associated with autism spectrum disorder. Am J Hum Genet. 2013;92(2):221-37.
79. Peng Z, Zhou W, Fu W, Du R, Jin L, Zhang F. Correlation between frequency of non-allelic homologous recombination and homology properties: evidence from homology-mediated CNV mutations in the human genome. Hum Mol Genet. 2015;24(5):1225-33.
80. Chen L, Zhou W, Zhang L, Zhang F. Genome architecture and its roles in human copy number variation. Genomics Inform. 2014;12(4):136-44.
81. Inoue K, Lupski JR. Molecular mechanisms for genomic disorders. Annu Rev Genomics Hum Genet. 2002;3:199-242.
82. Lupski JR, Stankiewicz P. Genomic disorders: molecular mechanisms for rearrangements and conveyed phenotypes. PLoS Genet. 2005;1(6):e49.
83. Jiang Y, Chu WK. Potential Roles of the Retinoblastoma Protein in Regulating Genome Editing. Front Cell Dev Biol. 2018;6:81.
84. Bickhart DM, Liu GE. The challenges and importance of structural variation detection in livestock. Front Genet. 2014;5:37.
85. Shaw CJ, Lupski JR. Non-recurrent 17p11.2 deletions are generated by homologous and non-homologous mechanisms. Hum Genet. 2005;116(1-2):1-7.
86. Lee JA, Inoue K, Cheung SW, Shaw CA, Stankiewicz P, Lupski JR. Role of genomic architecture in PLP1 duplication causing Pelizaeus-Merzbacher disease. Hum Mol Genet. 2006;15(14):2250-65.
87. Chen C, Umezu K, Kolodner RD. Chromosomal rearrangements occur in S. cerevisiae rfa1 mutator mutants due to mutagenic lesions processed by double-strand-break repair. Mol Cell. 1998;2(1):9-22.
88. Weinstock DM, Brunet E, Jasin M. Formation of NHEJ-derived reciprocal chromosomal translocations does not require Ku70. Nat Cell Biol. 2007;9(8):978-81.
89. Welcker AJ, de Montigny J, Potier S, Souciet JL. Involvement of very short DNA tandem repeats and the influence of the RAD52 gene on the occurrence of deletions in Saccharomyces cerevisiae. Genetics. 2000;156(2):549-57.
90. McVey M, Lee SE. MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings. Trends Genet. 2008;24(11):529-38.
91. Roerink SF, van Schendel R, Tijsterman M. Polymerase theta-mediated end joining of replication-associated DNA breaks in C. elegans. Genome Res. 2014;24(6):954-62.

92. Schimmel J, van Schendel R, den Dunnen JT, Tijsterman M. Templated Insertions: A Smoking Gun for Polymerase Theta-Mediated End Joining. Trends Genet. 2019;35(9):632-44.
93. Slack A, Thornton PC, Magner DB, Rosenberg SM, Hastings PJ. On the mechanism of gene amplification induced under stress in Escherichia coli. PLoS Genet. 2006;2(4):e48.
94. Langston LD, Indiani C, O'Donnell M. Whither the replisome: emerging perspectives on the dynamic nature of the DNA replication machinery. Cell Cycle. 2009;8(17):2686-91.
95. Indiani C, Langston LD, Yurieva O, Goodman MF, O'Donnell M. Translesion DNA polymerases remodel the replisome and alter the speed of the replicative helicase. Proc Natl Acad Sci U S A. 2009;106(15):6031-8.
96. Viguera E, Canceill D, Ehrlich SD. Replication slippage involves DNA polymerase pausing and dissociation. EMBO J. 2001;20(10):2587-95.
97. Liskay RM, Letsou A, Stachelek JL. Homology requirement for efficient gene conversion between duplicated chromosomal sequences in mammalian cells. Genetics. 1987;115(1):161-7.
98. Reiter LT, Hastings PJ, Nelis E, De Jonghe P, Van Broeckhoven C, Lupski JR. Human meiotic recombination products revealed by sequencing a hotspot for homologous strand exchange in multiple HNPP deletion patients. Am J Hum Genet. 1998;62(5):1023-33.
99. Lovett ST, Hurley RL, Sutera VA, Jr., Aubuchon RH, Lebedeva MA. Crossing over between regions of limited homology in Escherichia coli. RecA-dependent and RecA-independent pathways. Genetics. 2002;160(3):851-9.
100. Hastings PJ, Ira G, Lupski JR. A microhomology-mediated break-induced replication model for the origin of human copy number variation. PLoS Genet. 2009;5(1):e1000327.
101. McIlwraith MJ, Vaisman A, Liu Y, Fanning E, Woodgate R, West SC. Human DNA polymerase eta promotes DNA synthesis from strand invasion intermediates of homologous recombination. Mol Cell. 2005;20(5):783-92.
102. Cannistraro VJ, Taylor JS. Ability of polymerase eta and T7 DNA polymerase to bypass bulge structures. J Biol Chem. 2007;282(15):11188-96.
103. Verma P, Greenberg RA. Noncanonical views of homology-directed DNA repair. Genes Dev. 2016;30(10):1138-54.
104. Chen JM, Chuzhanova N, Stenson PD, Ferec C, Cooper DN. Complex gene rearrangements caused by serial replication slippage. Hum Mutat. 2005;26(2):125-34.
105. Tancredi M, Sensi E, Cipollini G, Aretini P, Lombardi G, Di Cristofano C, et al. Haplotype analysis of BRCA1 gene reveals a new gene rearrangement: characterization of a 19.9 KBP deletion. Eur J Hum Genet. 2004;12(9):775-7.
106. Chen JM, Chuzhanova N, Stenson PD, Ferec C, Cooper DN. Meta-analysis of gross insertions causing human genetic disease: novel mutational mechanisms and the role of replication slippage. Hum Mutat. 2005;25(2):207-21.
107. Prak ET, Kazazian HH, Jr. Mobile elements and the human genome. Nat Rev Genet. 2000;1(2):134-44.
108. Callinan PA, Batzer MA. Retrotransposable elements and human disease. Genome Dyn. 2006;1:104-15.
109. Bailey JA, Liu G, Eichler EE. An Alu transposition model for the origin and expansion of human segmental duplications. Am J Hum Genet. 2003;73(4):823-34.
110. Xing J, Zhang Y, Han K, Salem AH, Sen SK, Huff CD, et al. Mobile elements create structural variation: analysis of a complete human genome. Genome Res. 2009;19(9):1516-26.
111. Deininger PL, Batzer MA. Alu repeats and human disease. Mol Genet Metab. 1999;67(3):183-93.
112. Cantsilieris S, Baird PN, White SJ. Molecular methods for genotyping complex copy number polymorphisms. Genomics. 2013;101(2):86-93.
113. Weiss MM, Hermsen MA, Meijer GA, van Grieken NC, Baak JP, Kuipers EJ, et al. Comparative genomic hybridisation. Mol Pathol. 1999;52(5):243-51.

114. McCarroll SA, Kuruvilla FG, Korn JM, Cawley S, Nemesh J, Wysoker A, et al. Integrated detection and population-genetic analysis of SNPs and copy number variation. Nat Genet. 2008;40(10):1166-74.
115. Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G. Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res. 2002;30(12):e57.
116. Medvedev P, Stanciu M, Brudno M. Computational methods for discovering structural variation with next-generation sequencing. Nat Methods. 2009;6(11 Suppl):S13-20.
117. Sudmant PH, Kitzman JO, Antonacci F, Alkan C, Malig M, Tsalenko A, et al. Diversity of human copy number variation and multicopy genes. Science. 2010;330(6004):641-6.
118. Singh AK, Olsen MF, Lavik LAS, Vold T, Drablos F, Sjursen W. Detecting copy number variation in next generation sequencing data from diagnostic gene panels. BMC Med Genomics. 2021;14(1):214.
119. Moreno-Cabrera JM, Del Valle J, Castellanos E, Feliubadalo L, Pineda M, Brunet J, et al. Evaluation of CNV detection tools for NGS panel data in genetic diagnostics. Eur J Hum Genet. 2020;28(12):1645-55.
120. Zhang F, Khajavi M, Connolly AM, Towne CF, Batish SD, Lupski JR. The DNA replication FoSTeS/MMBIR mechanism can generate genomic, genic and exonic complex rearrangements in humans. Nat Genet. 2009;41(7):849-53.
121. Zhang L, Wang J, Zhang C, Li D, Carvalho CMB, Ji H, et al. Efficient CNV breakpoint analysis reveals unexpected structural complexity and correlation of dosage-sensitive genes with clinical severity in genomic disorders. Hum Mol Genet. 2017;26(10):1927-41.
122. Wright CF, FitzPatrick DR, Firth HV. Paediatric genomics: diagnosing rare disease in children. Nat Rev Genet. 2018;19(5):325.
123. Amberger JS, Bocchini CA, Schiettecatte F, Scott AF, Hamosh A. OMIM.org: Online Mendelian Inheritance in Man (OMIM(R)), an online catalog of human genes and genetic disorders. Nucleic Acids Res. 2015;43(Database issue):D789-98.
124. Judge DP, Dietz HC. Marfan's syndrome. Lancet. 2005;366(9501):1965-76.
125. Canadas V, Vilacosta I, Bruna I, Fuster V. Marfan syndrome. Part 1: pathophysiology and diagnosis. Nat Rev Cardiol. 2010;7(5):256-65.
126. Adams JN, Trent RJ. Aortic complications of Marfan's syndrome. Lancet. 1998;352(9142):1722-3.
127. Scriver CR. The Metabolic & Molecular Bases of Inherited Disease: McGraw-Hill; 2001.
128. Mizuguchi T, Collod-Beroud G, Akiyama T, Abifadel M, Harada N, Morisaki T, et al. Heterozygous TGFBR2 mutations in Marfan syndrome. Nat Genet. 2004;36(8):855-60.
129. Chung BH, Lam ST, Tong TM, Li SY, Lun KS, Chan DH, et al. Identification of novel FBN1 and TGFBR2 mutations in 65 probands with Marfan syndrome or Marfan-like phenotypes. Am J Med Genet A. 2009;149A(7):1452-9.
130. Lerner-Ellis JP, Aldubayan SH, Hernandez AL, Kelly MA, Stuenkel AJ, Walsh J, et al. The spectrum of FBN1, TGFbetaR1, TGFbetaR2 and ACTA2 variants in 594 individuals with suspected Marfan Syndrome, Loeys-Dietz Syndrome or Thoracic Aortic Aneurysms and Dissections (TAAD). Mol Genet Metab. 2014;112(2):171-6.
131. Ramachandra CJ, Mehta A, Guo KW, Wong P, Tan JL, Shim W. Molecular pathogenesis of Marfan syndrome. Int J Cardiol. 2015;187:585-91.
132. Hoffjan S. Genetic dissection of marfan syndrome and related connective tissue disorders: an update 2012. Mol Syndromol. 2012;3(2):47-58.
133. Collod-Beroud G, Le Bourdelles S, Ades L, Ala-Kokko L, Booms P, Boxer M, et al. Update of the UMD-FBN1 mutation database and creation of an FBN1 polymorphism database. Hum Mutat. 2003;22(3):199-208.
134. Apitz C, Mackensen-Haen S, Girisch M, Kerst G, Wiegand G, Stuhrmann M, et al. Neonatal Marfan syndrome: unusually large deletion of exons 24-26 of FBN1 associated with poor prognosis. Klin Padiatr. 2010;222(4):261-3.
135. Benke K, Agg B, Meienberg J, Kopps AM, Fattorini N, Stengl R, et al. Hungarian Marfan family with large FBN1 deletion calls attention to copy number variation detection in the current NGS era. J Thorac Dis. 2018;10(4):2456-60.

136. Blyth M, Foulds N, Turner C, Bunyan D. Severe Marfan syndrome due to FBN1 exon deletions. Am J Med Genet A. 2008;146A(10):1320-4.
137. Breckpot J, Budts W, De Zegher F, Vermeesch JR, Devriendt K. Duplication of the TGFBR1 gene causes features of Loeys-Dietz syndrome. Eur J Med Genet. 2010;53(6):408-10.
138. Campbell IM, Kolodziejska KE, Quach MM, Wolf VL, Cheung SW, Lalani SR, et al. TGFBR2 deletion in a 20-month-old female with developmental delay and microcephaly. Am J Med Genet A. 2011;155A(6):1442-7.
139. Colovati ME, da Silva LR, Takeno SS, Mancini TI, AR ND, Guilherme RS, et al. Marfan syndrome with a complex chromosomal rearrangement including deletion of the FBN1 gene. Mol Cytogenet. 2012;5:5.
140. Faivre L, Khau Van Kien P, Callier P, Ruiz-Pallares N, Baudoin C, Plancke A, et al. De novo 15q21.1q21.2 deletion identified through FBN1 MLPA and refined by 244K array-CGH in a female teenager with incomplete Marfan syndrome. Eur J Med Genet. 2010;53(4):208-12.
141. Furtado LV, Wooderchak-Donahue W, Rope AF, Yetman AT, Lewis T, Plant P, et al. Characterization of large genomic deletions in the FBN1 gene using multiplex ligation-dependent probe amplification. BMC Med Genet. 2011;12:119.
142. Hilhorst-Hofstee Y, Hamel BC, Verheij JB, Rijlaarsdam ME, Mancini GM, Cobben JM, et al. The clinical spectrum of complete FBN1 allele deletions. Eur J Hum Genet. 2011;19(3):247-52.
143. Hung CC, Lin SY, Lee CN, Cheng HY, Lin SP, Chen MR, et al. Mutation spectrum of the fibrillin-1 (FBN1) gene in Taiwanese patients with Marfan syndrome. Ann Hum Genet. 2009;73(Pt 6):559-67.
144. Kainulainen K, Sakai LY, Child A, Pope FM, Puhakka L, Ryhanen L, et al. Two mutations in Marfan syndrome resulting in truncated fibrillin polypeptides. Proc Natl Acad Sci U S A. 1992;89(13):5917-21.
145. Li J, Wu W, Lu C, Liu Y, Wang R, Si N, et al. Gross deletions in FBN1 results in variable phenotypes of Marfan syndrome. Clin Chim Acta. 2017;474:54-9.
146. Liu W, Schrijver I, Brenn T, Furthmayr H, Francke U. Multi-exon deletions of the FBN1 gene in Marfan syndrome. BMC Med Genet. 2001;2:11.
147. Loeys B, Nuytinck L, Delvaux I, De Bie S, De Paepe A. Genotype and phenotype analysis of 171 patients referred for molecular study of the fibrillin-1 gene FBN1 because of suspected Marfan syndrome. Arch Intern Med. 2001;161(20):2447-54.
148. Matyas G, Alonso S, Patrignani A, Marti M, Arnold E, Magyar I, et al. Large genomic fibrillin-1 (FBN1) gene deletions provide evidence for true haploinsufficiency in Marfan syndrome. Hum Genet. 2007;122(1):23-32.
149. McInerney-Leo AM, Marshall MS, Gardiner B, Coucke PJ, Van Laer L, Loeys BL, et al. Whole exome sequencing is an efficient, sensitive and specific method of mutation detection in osteogenesis imperfecta and Marfan syndrome. Bonekey Rep. 2013;2:456.
150. Ogawa N, Imai Y, Takahashi Y, Nawata K, Hara K, Nishimura H, et al. Evaluating Japanese patients with the Marfan syndrome using high-throughput microarray-based mutational analysis of fibrillin-1 gene. Am J Cardiol. 2011;108(12):1801-7.
151. Pees C, Michel-Behnke I, Hagl M, Laccone F. Detection of 15 novel mutations in 52 children from 40 families with the Marfan or Loeys-Dietz syndrome and phenotype-genotype correlations. Clin Genet. 2014;86(6):552-7.
152. Raghunath M, Kielty CM, Kainulainen K, Child A, Peltonen L, Steinmann B. Analyses of truncated fibrillin caused by a 366 bp deletion in the FBN1 gene resulting in Marfan syndrome. Biochem J. 1994;302 (Pt 3):889-96.
153. Singh KK, Elligsen D, Liersch R, Schubert S, Pabst B, Arslan-Kirchner M, et al. Multi-exon out of frame deletion of the FBN1 gene leading to a severe juvenile onset cardiovascular phenotype in Marfan syndrome. J Mol Cell Cardiol. 2007;42(2):352-6.
154. Voermans N, Timmermans J, van Alfen N, Pillen S, op den Akker J, Lammens M, et al. Neuromuscular features in Marfan syndrome. Clin Genet. 2009;76(1):25-37.
155. Weidenbach M, Brenner R, Rantamaki T, Redel DA. Acute mitral regurgitation due to chordal rupture in a patient with neonatal Marfan syndrome caused by a deletion in exon 29 of the FBN1 gene. Pediatr Cardiol. 1999;20(5):382-5.

156. Wooderchak-Donahue W, VanSant-Webb C, Tvrdik T, Plant P, Lewis T, Stocks J, et al. Clinical utility of a next generation sequencing panel assay for Marfan and Marfan-like syndromes featuring aortopathy. Am J Med Genet A. 2015;167A(8):1747-57.
157. Yang H, Ma Y, Luo M, Zhao K, Zhang Y, Zhu G, et al. Identification of gross deletions in FBN1 gene by MLPA. Hum Genomics. 2018;12(1):46.
158. Yoo EH, Woo H, Ki CS, Lee HJ, Kim DK, Kang IS, et al. Clinical and genetic analysis of Korean patients with Marfan syndrome: possible ethnic differences in clinical manifestation. Clin Genet. 2010;77(2):177-82.
159. LU Xin-xin HX-l, WANG Ren, CHEN Xi-jun, RAO Hui-ying, WU Wen-bing, QIU Li-ping, HUANG Yi, WU Yan-an Detection of deletion mutations of FBN1 in two patients with Marfan syndrome using next generation sequencing (NGS) and multiplex ligation-dependent probe amplification(MLPA) technique. Chinese Journal of Clinical Laboratory Science. 2015(10):744-7.
160. Lammert M, Friedman JM, Kluwe L, Mautner VF. Prevalence of neurofibromatosis 1 in German children at elementary school enrollment. Arch Dermatol. 2005;141(1):71-4.
161. Uusitalo E, Leppavirta J, Koffert A, Suominen S, Vahtera J, Vahlberg T, et al. Incidence and mortality of neurofibromatosis: a total population study in Finland. J Invest Dermatol. 2015;135(3):904-6.
162. Buki G, Zsigmond A, Czako M, Szalai R, Antal G, Farkas V, et al. Genotype-Phenotype Associations in Patients With Type-1, Type-2, and Atypical NF1 Microdeletions. Front Genet. 2021;12:673025.
163. Jett K, Friedman JM. Clinical and genetic aspects of neurofibromatosis 1. Genet Med. 2010;12(1):1-11.
164. Stephens K, Kayes L, Riccardi VM, Rising M, Sybert VP, Pagon RA. Preferential mutation of the neurofibromatosis type 1 gene in paternally derived chromosomes. Hum Genet. 1992;88(3):279-82.
165. Park VM, Pivnick EK. Neurofibromatosis type 1 (NF1): a protein truncation assay yielding identification of mutations in 73% of patients. J Med Genet. 1998;35(10):813-20.
166. Cnossen MH, van der Est MN, Breuning MH, van Asperen CJ, Breslau-Siderius EJ, van der Ploeg AT, et al. Deletions spanning the neurofibromatosis type 1 gene: implications for genotype-phenotype correlations in neurofibromatosis type 1? Hum Mutat. 1997;9(5):458-64.
167. Zhang J, Tong H, Fu X, Zhang Y, Liu J, Cheng R, et al. Molecular Characterization of NF1 and Neurofibromatosis Type 1 Genotype-Phenotype Correlations in a Chinese Population. Sci Rep. 2015;5:11291.
168. Kehrer-Sawatzki H, Mautner VF, Cooper DN. Emerging genotype-phenotype relationships in patients with large NF1 deletions. Hum Genet. 2017;136(4):349-76.
169. Kehrer-Sawatzki H, Kluwe L, Salamon J, Well L, Farschtschi S, Rosenbaum T, et al. Clinical characterization of children and adolescents with NF1 microdeletions. Childs Nerv Syst. 2020;36(10):2297-310.
170. Dorschner MO, Sybert VP, Weaver M, Pletcher BA, Stephens K. NF1 microdeletion breakpoints are clustered at flanking repetitive sequences. Hum Mol Genet. 2000;9(1):35-46.
171. Kayes LM, Riccardi VM, Burke W, Bennett RL, Stephens K. Large de novo DNA deletion in a patient with sporadic neurofibromatosis 1, mental retardation, and dysmorphism. J Med Genet. 1992;29(10):686-90.
172. Allanson JE, Cunniff C, Hoyme HE, McGaughran J, Muenke M, Neri G. Elements of morphology: standard terminology for the head and face. Am J Med Genet A. 2009;149A(1):6-28.
173. Hall BD, Graham JM, Jr., Cassidy SB, Opitz JM. Elements of morphology: standard terminology for the periorbital region. Am J Med Genet A. 2009;149A(1):29-39.
174. Strassmeier W. Early intervention programs for handicapped and retarded children from age 0 to 5. Int J Rehabil Res. 1980;3(4):533-5.
175. Bayley N RG. Bayley scales of infant and toddler development: Bayley-III. San Antonio: Harcourt Assessment, Psych Corporation. 2006;7.

176. Bass L, Borbély S, Jászberényi M, Lányiné EÁ, Sarkady K, Gerebenné VK, et al. A differenciált beiskolázás néhány mérőeszköze. A Budapesti Binet-teszttel végzett vizsgálatokról: Akadémiai Kiadó; 1989. p. 29-49 (in hungarian).
177. Vulto-van Silfhout AT, van Ravenswaaij CM, Hehir-Kwa JY, Verwiel ET, Dirks R, van Vooren S, et al. An update on ECARUCA, the European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations. Eur J Med Genet. 2013;56(9):471-4.
178. Firth HV, Richards SM, Bevan AP, Clayton S, Corpas M, Rajan D, et al. DECIPHER: Database of Chromosomal Imbalance and Phenotype in Humans Using Ensembl Resources. Am J Hum Genet. 2009;84(4):524-33.
179. Benson G. Tandem repeats finder: a program to analyze DNA sequences. Nucleic Acids Res. 1999;27(2):573-80.
180. Xie X, Liu W, Dong G, Zhu Q, Liu YG. MMEJ-KO: a web tool for designing paired CRISPR guide RNAs for microhomology-mediated end joining fragment deletion. Sci China Life Sci. 2021;64(6):1021-4.
181. Garcia-Muse T, Aguilera A. Transcription-replication conflicts: how they occur and how they are resolved. Nat Rev Mol Cell Biol. 2016;17(9):553-63.
182. Hastings PJ, Lupski JR, Rosenberg SM, Ira G. Mechanisms of change in gene copy number. Nat Rev Genet. 2009;10(8):551-64.
183. Faivre L, Collod-Beroud G, Loeys BL, Child A, Binquet C, Gautier E, et al. Effect of mutation type and location on clinical outcome in 1,013 probands with Marfan syndrome or related phenotypes and FBN1 mutations: an international study. Am J Hum Genet. 2007;81(3):454-66.
184. Dordoni C, Ciaccio C, Santoro G, Venturini M, Cavallari U, Ritelli M, et al. Marfan syndrome: Report of a complex phenotype due to a 15q21.1 contiguos gene deletion encompassing FBN1, and literature review. Am J Med Genet A. 2017;173(1):200-6.
185. Summerer A, Mautner VF, Upadhyaya M, Claes KBM, Hogel J, Cooper DN, et al. Extreme clustering of type-1 NF1 deletion breakpoints co-locating with G-quadruplex forming sequences. Hum Genet. 2018;137(6-7):511-20.
186. Summerer A, Schafer E, Mautner VF, Messiaen L, Cooper DN, Kehrer-Sawatzki H. Ultra-deep amplicon sequencing indicates absence of low-grade mosaicism with normal cells in patients with type-1 NF1 deletions. Hum Genet. 2019;138(1):73-81.
187. Sachidanandam R, Weissman D, Schmidt SC, Kakol JM, Stein LD, Marth G, et al. A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. Nature. 2001;409(6822):928-33.
188. Jorde LB, Wooding SP. Genetic variation, classification and 'race'. Nat Genet. 2004;36(11 Suppl):S28-33.
189. Tishkoff SA, Kidd KK. Implications of biogeography of human populations for 'race' and medicine. Nat Genet. 2004;36(11 Suppl):S21-7.
190. Harel T, Lupski JR. Genomic disorders 20 years on-mechanisms for clinical manifestations. Clin Genet. 2018;93(3):439-49.
191. Sakai LY, Keene DR, Engvall E. Fibrillin, a new 350-kD glycoprotein, is a component of extracellular microfibrils. J Cell Biol. 1986;103(6 Pt 1):2499-509.
192. Corson GM, Chalberg SC, Dietz HC, Charbonneau NL, Sakai LY. Fibrillin binds calcium and is coded by cDNAs that reveal a multidomain structure and alternatively spliced exons at the 5' end. Genomics. 1993;17(2):476-84.
193. Reinhardt DP, Ono RN, Sakai LY. Calcium stabilizes fibrillin-1 against proteolytic degradation. J Biol Chem. 1997;272(2):1231-6.
194. Handford PA. Fibrillin-1, a calcium binding protein of extracellular matrix. Biochim Biophys Acta. 2000;1498(2-3):84-90.
195. Jensen SA, Handford PA. New insights into the structure, assembly and biological roles of 10-12 nm connective tissue microfibrils from fibrillin-1 studies. Biochem J. 2016;473(7):827-38.
196. Werner JM, Knott V, Handford PA, Campbell ID, Downing AK. Backbone dynamics of a cbEGF domain pair in the presence of calcium. J Mol Biol. 2000;296(4):1065-78.

197. Rock MJ, Cain SA, Freeman LJ, Morgan A, Mellody K, Marson A, et al. Molecular basis of elastic fiber formation. Critical interactions and a tropoelastin-fibrillin-1 cross-link. J Biol Chem. 2004;279(22):23748-58.
198. Shen YH, LeMaire SA. Molecular pathogenesis of genetic and sporadic aortic aneurysms and dissections. Curr Probl Surg. 2017;54(3):95-155.
199. Liu W, Qian C, Comeau K, Brenn T, Furthmayr H, Francke U. Mutant fibrillin-1 monomers lacking EGF-like domains disrupt microfibril assembly and cause severe marfan syndrome. Hum Mol Genet. 1996;5(10):1581-7.
200. Baudhuin LM, Kotzer KE, Lagerstedt SA. Increased frequency of FBN1 truncating and splicing variants in Marfan syndrome patients with aortic events. Genet Med. 2015;17(3):177-87.
201. Franken R, Teixido-Tura G, Brion M, Forteza A, Rodriguez-Palomares J, Gutierrez L, et al. Relationship between fibrillin-1 genotype and severity of cardiovascular involvement in Marfan syndrome. Heart. 2017;103(22):1795-9.
202. Takeda N, Inuzuka R, Maemura S, Morita H, Nawata K, Fujita D, et al. Impact of Pathogenic FBN1 Variant Types on the Progression of Aortic Disease in Patients With Marfan Syndrome. Circ Genom Precis Med. 2018;11(6):e002058.
203. Dallas SL, Miyazono K, Skerry TM, Mundy GR, Bonewald LF. Dual role for the latent transforming growth factor-beta binding protein in storage of latent TGF-beta in the extracellular matrix and as a structural matrix protein. J Cell Biol. 1995;131(2):539-49.
204. Isogai Z, Ono RN, Ushiro S, Keene DR, Chen Y, Mazzieri R, et al. Latent transforming growth factor beta-binding protein 1 interacts with fibrillin and is a microfibril-associated protein. J Biol Chem. 2003;278(4):2750-7.
205. Habashi JP, Judge DP, Holm TM, Cohn RD, Loeys BL, Cooper TK, et al. Losartan, an AT1 antagonist, prevents aortic aneurysm in a mouse model of Marfan syndrome. Science. 2006;312(5770):117-21.
206. Neptune ER, Frischmeyer PA, Arking DE, Myers L, Bunton TE, Gayraud B, et al. Dysregulation of TGF-beta activation contributes to pathogenesis in Marfan syndrome. Nat Genet. 2003;33(3):407-11.
207. Zilberberg L, Phoon CK, Robertson I, Dabovic B, Ramirez F, Rifkin DB. Genetic analysis of the contribution of LTBP-3 to thoracic aneurysm in Marfan syndrome. Proc Natl Acad Sci U S A. 2015;112(45):14012-7.
208. Maurano MT, Humbert R, Rynes E, Thurman RE, Haugen E, Wang H, et al. Systematic localization of common disease-associated variation in regulatory DNA. Science. 2012;337(6099):1190-5.
209. Vockley CM, Barrera A, Reddy TE. Decoding the role of regulatory element polymorphisms in complex disease. Curr Opin Genet Dev. 2017;43:38-45.
210. Gomez D, Coyet A, Ollivier V, Jeunemaitre X, Jondeau G, Michel JB, et al. Epigenetic control of vascular smooth muscle cells in Marfan and non-Marfan thoracic aortic aneurysms. Cardiovasc Res. 2011;89(2):446-56.
211. Chandesris MO, Azarine A, Ong KT, Taleb S, Boutouyrie P, Mousseaux E, et al. Frequent and widespread vascular abnormalities in human signal transducer and activator of transcription 3 deficiency. Circ Cardiovasc Genet. 2012;5(1):25-34.
212. Ottaviani D, LeCain M, Sheer D. The role of microhomology in genomic structural variation. Trends Genet. 2014;30(3):85-94.
213. Toffolatti L, Cardazzo B, Nobile C, Danieli GA, Gualandi F, Muntoni F, et al. Investigating the mechanism of chromosomal deletion: characterization of 39 deletion breakpoints in introns 47 and 48 of the human dystrophin gene. Genomics. 2002;80(5):523-30.
214. Nobile C, Toffolatti L, Rizzi F, Simionati B, Nigro V, Cardazzo B, et al. Analysis of 22 deletion breakpoints in dystrophin intron 49. Hum Genet. 2002;110(5):418-21.
215. Pasmant E, Sabbagh A, Spurlock G, Laurendeau I, Grillo E, Hamel MJ, et al. NF1 microdeletions in neurofibromatosis type 1: from genotype to phenotype. Hum Mutat. 2010;31(6):E1506-18.

216. Messiaen L, Vogt J, Bengesser K, Fu C, Mikhail F, Serra E, et al. Mosaic type-1 NF1 microdeletions as a cause of both generalized and segmental neurofibromatosis type-1 (NF1). Hum Mutat. 2011;32(2):213-9.
217. Bianchessi D, Morosini S, Saletti V, Ibba MC, Natacci F, Esposito S, et al. 126 novel mutations in Italian patients with neurofibromatosis type 1. Mol Genet Genomic Med. 2015;3(6):513-25.
218. Mautner VF, Kluwe L, Friedrich RE, Roehl AC, Bammert S, Hogel J, et al. Clinical characterisation of 29 neurofibromatosis type-1 patients with molecularly ascertained 1.4 Mb type-1 NF1 deletions. J Med Genet. 2010;47(9):623-30.
219. Tucker T, Wolkenstein P, Revuz J, Zeller J, Friedman JM. Association between benign and malignant peripheral nerve sheath tumors in NF1. Neurology. 2005;65(2):205-11.
220. Waggoner DJ, Towbin J, Gottesman G, Gutmann DH. Clinic-based study of plexiform neurofibromas in neurofibromatosis 1. Am J Med Genet. 2000;92(2):132-5.
221. Tastet J, Decalonne L, Marouillat S, Malvy J, Thepault RA, Toutain A, et al. Mutation screening of the ubiquitin ligase gene RNF135 in French patients with autism. Psychiatr Genet. 2015;25(6):263-7.
222. Venturin M, Carra S, Gaudenzi G, Brunelli S, Gallo GR, Moncini S, et al. ADAP2 in heart development: a candidate gene for the occurrence of cardiovascular malformations in NF1 microdeletion syndrome. J Med Genet. 2014;51(7):436-43.
223. Zhang M, Wang Y, Jones S, Sausen M, McMahon K, Sharma R, et al. Somatic mutations of SUZ12 in malignant peripheral nerve sheath tumors. Nat Genet. 2014;46(11):1170-2.
224. Bell DW, Sikdar N, Lee KY, Price JC, Chatterjee R, Park HD, et al. Predisposition to cancer caused by genetic and functional defects of mammalian Atad5. PLoS Genet. 2011;7(8):e1002245.
225. Vogt J, Mussotter T, Bengesser K, Claes K, Hogel J, Chuzhanova N, et al. Identification of recurrent type-2 NF1 microdeletions reveals a mitotic nonallelic homologous recombination hotspot underlying a human genomic disorder. Hum Mutat. 2012;33(11):1599-609.
226. Vogt J, Nguyen R, Kluwe L, Schuhmann M, Roehl AC, Mussotter T, et al. Delineation of the clinical phenotype associated with non-mosaic type-2 NF1 deletions: two case reports. J Med Case Rep. 2011;5:577.
227. Roehl AC, Vogt J, Mussotter T, Zickler AN, Spoti H, Hogel J, et al. Intrachromosomal mitotic nonallelic homologous recombination is the major molecular mechanism underlying type-2 NF1 deletions. Hum Mutat. 2010;31(10):1163-73.
228. Cnossen MH, de Goede-Bolder A, van den Broek KM, Waasdorp CM, Oranje AP, Stroink H, et al. A prospective 10 year follow up study of patients with neurofibromatosis type 1. Arch Dis Child. 1998;78(5):408-12.
229. Kehrer-Sawatzki H, Kluwe L, Funsterer C, Mautner VF. Extensively high load of internal tumors determined by whole body MRI scanning in a patient with neurofibromatosis type 1 and a non-LCR-mediated 2-Mb deletion in 17q11.2. Hum Genet. 2005;116(6):466-75.
230. Riva P, Corrado L, Natacci F, Castorina P, Wu BL, Schneider GH, et al. NF1 microdeletion syndrome: refined FISH characterization of sporadic and familial deletions with locus-specific probes. Am J Hum Genet. 2000;66(1):100-9.
231. Upadhyaya M, Roberts SH, Maynard J, Sorour E, Thompson PW, Vaughan M, et al. A cytogenetic deletion, del(17)(q11.22q21.1), in a patient with sporadic neurofibromatosis type 1 (NF1) associated with dysmorphism and developmental delay. J Med Genet. 1996;33(2):148-52.
232. Kehrer-Sawatzki H, Tinschert S, Jenne DE. Heterogeneity of breakpoints in non-LCR-mediated large constitutional deletions of the 17q11.2 NF1 tumour suppressor region. J Med Genet. 2003;40(10):e116.
233. Venturin M, Gervasini C, Orzan F, Bentivegna A, Corrado L, Colapietro P, et al. Evidence for non-homologous end joining and non-allelic homologous recombination in atypical NF1 microdeletions. Hum Genet. 2004;115(1):69-80.
234. Venturin M, Guarnieri P, Natacci F, Stabile M, Tenconi R, Clementi M, et al. Mental retardation and cardiovascular malformations in NF1 microdeleted patients point to candidate genes in 17q11.2. J Med Genet. 2004;41(1):35-41.

235. Kehrer-Sawatzki H, Schmid E, Funsterer C, Kluwe L, Mautner VF. Absence of cutaneous neurofibromas in an NF1 patient with an atypical deletion partially overlapping the common 1.4 Mb microdeleted region. Am J Med Genet A. 2008;146A(6):691-9.
236. Mantripragada KK, Thuresson AC, Piotrowski A, Diaz de Stahl T, Menzel U, Grigelionis G, et al. Identification of novel deletion breakpoints bordered by segmental duplications in the NF1 locus using high resolution array-CGH. J Med Genet. 2006;43(1):28-38.
237. Karczewski KJ, Francioli LC, Tiao G, Cummings BB, Alfoldi J, Wang Q, et al. The mutational constraint spectrum quantified from variation in 141,456 humans. Nature. 2020;581(7809):434-43.
238. Descheemaeker MJ, Roelandts K, De Raedt T, Brems H, Fryns JP, Legius E. Intelligence in individuals with a neurofibromatosis type 1 microdeletion. Am J Med Genet A. 2004;131(3):325-6.
239. Ottenhoff MJ, Rietman AB, Mous SE, Plasschaert E, Gawehns D, Brems H, et al. Examination of the genetic factors underlying the cognitive variability associated with neurofibromatosis type 1. Genet Med. 2020;22(5):889-97.
240. Bernardinelli Y, Nikonenko I, Muller D. Structural plasticity: mechanisms and contribution to developmental psychiatric disorders. Front Neuroanat. 2014;8:123.
241. Martin I, Andres CR, Vedrine S, Tabagh R, Michelle C, Jourdan ML, et al. Effect of the oligodendrocyte myelin glycoprotein (OMgp) on the expansion and neuronal differentiation of rat neural stem cells. Brain Res. 2009;1284:22-30.

8. List Of Publications

8.1. Papers on which the thesis is based

1. Genotype-Phenotype Associations in Patients With Type-1, Type-2, and Atypical NF1 Microdeletions

G Büki, A Zsigmond, M Czakó, R Szalai, G Antal, V Farkas, G Fekete, D Nagy, M Széll, Tihanyi, B Melegh, K Hadzsiev, Judit Bene

Frontiers in Genetics 2021 Jun 8;12:673025. doi: 10.3389/fgene.2021.673025. eCollection 2021.

Impact factor: 4.772

2. Microhomology-Mediated Break-Induced Replication: A Possible Molecular Mechanism of the Formation of a Large CNV in FBN1 Gene in a Patient with Marfan Syndrome

G Buki, K Hadzsiev, J Bene

Current Molecular Medicine April 2022 DOI: 10.2174/1566524022666220428111943

Impact factor: 2.616

3. Neurofibromatosis-1 microdeletiós szindróma: Molekuláris genetika és klinikai heterogenitás

G Buki, A Till, A Zsigmond, J Bene, K Hadzsiev

Orvosi Hetilap 2022; 163(51): 2041–2051. DOI:10.1556/650.2022.32673

Impact factor: 0.707

Summed impact factor: 8.095

4. Potential association between large FBN1 deletions and severe cardiovascular phenotype in Marfan syndrome due to probable tissue specific enhancers abolishment

G Buki, R Szalai, A Pinter, K Hadzsiev, B Melegh, T Rauch, J Bene

Under publication

Impact factor: -

8.2. Other Publications

1. **Identification, presence, and possible multifunctional regulatory role of invertebrate gonadotropin-releasing hormone/corazonin molecule in the great pond snail (Lymnaea stagnalis)**

I Fodor, Z Zrinyi, R Horváth, P Urbán, R Herczeg, G Büki, J M Koene, P-S Tsai, Z Pirger

Gen Comp Endocrinol. 2020 Dec 1;299:113621. doi: 10.1016/j.ygcen.2020.113621. Epub 2020 Sep 20.

Impact factor: 2.63

2. Revealing the impact of the Caucasus region on the genetic legacy of Romani people from genome-wide data

Z Bánfai, V Ádám, E Pöstyéni, G Büki, M Czakó, A Miseta, B Melegh

PLoS One. 2018 Sep 10;13(9):e0202890. doi: 10.1371/journal.pone.0202890. eCollection 2018.

Impact factor: 3.24

3. A rare form of ion channel gene mutation identified as underlying cause of generalized epilepsy

Á Till, R Szalai, M Hegyi, E Kövesdi, G Büki, K Hadzsiev, B Melegh

Orv Hetil. 2019 May;160(21):835-838. doi: 10.1556/650.2019.31404.

Impact factor: 0.41

4. Genome-Wide Marker Data-Based Comparative Population Analysis of Szeklers From Korond, Transylvania, and From Transylvania Living Non-Szekler Hungarians

V Ádám, Z Bánfai, K Sümegi, G Büki, A Szabó, L Magyari, A Miseta, M Kásler, B Melegh

Front Genet. 2022 Mar 28;13:841769. doi: 10.3389/fgene.2022.841769. eCollection 2022.

Impact factor: 4.599

8.3. Citable abstracts

1. Examining the molecular mechanism behind a non recurrent CNV in the *FBN1* gene

G Büki, J Bene

XVIII. János Szentágothai Multidisciplinary Conference and Student Competition Book of Abstracts

8.4. Other abstracts

1. **Survey on Medical Education for Undergraduates and Postgraduates** 2018

G Büki

Magyar Humángenetika Társaság XII. kongresszus JARC (Joint Action on Rare Cancers) roundtable

2. A **kaukázus roma genetikai örökségre való hatásának feltárása teljes genom adatokon alapuló vizsgálatokkal** 2018

Zs Bánfai, V Ádám, G Büki, M Czakó, B Melegh

Magyar Humángenetika Társaság XII. kongresszus

3. **Identification of a novel two-exon deletion of FBN1 gene in a patient with Marfan syndrome and homocystinuria** 2019

G Büki, K Hadzsiev, L Pintér, B Melegh, J Bene

European Society of Human Genetics

4. **Detection of a novel large deletion in *FBN1* gene by MLPA** 2019

J. Bene, G. Büki, L. Pintér, B. Melegh, K. Hadzsiev

American Society of Human Genetics

5. **Investigation of copy number variations in the development of rare diseases.** 2019

G Büki, B Melegh, J Bene

5th International Cholnoky Symposium

6. **A recurrent *ZC4H2* gene mutation associated with diverse neurological symptoms in a patient with Wieacker-Wolff syndrome.** 2020

G. Buki, J. Zima, B. Galik, A. Gyenesei, K. Hadzsiev, B. Melegh, J. Bene

European Society of Human Genetics

7. **Glycosylphosphatidylinositol biosynthesis defect due to digenic heterozygous mutations in *PIGT* and *PIGV* genes in a patient with psychomotor and cognitive delay** 2021

G. Buki, A. Zsigmond, A. Till, A. Gyenesei, B. Melegh, K. Hadzsiev, J. Bene

European Society of Human Genetics

8. **FBN1 génben előforduló nem rekurrens CNV hátterében álló molekuláris mechanizmus felderítése** 2021

G Büki, R Szalai, Á Till, A Zsigmond, K Hadzsiev, J Bene

Magyar Humángenetika Társaság XIII. kongresszus

9. **Egy ritka kötőszöveti rendellenességet eredményező kópiaszámbeli eltérés molekuláris mechanizmusának feltárása** 2022

G Büki, J Bene

Tavaszi Szél konferencia

9. Acknowledgements

I would like to take this opportunity to express my gratitude to all those, whose assistance contributed to my PhD work.

I am primarily grateful to my supervisor Dr. Judit Bene who encouraged and helped me to join the Doctoral School of Interdisciplinary Medicine, Medical School, University of Pécs. She monitored and guided my professional activity throughout. She supported from the start and provided with outstanding professional knowledge and experience.

I would like to thank Professor Dr. Béla Melegh for providing me the opportunity to start my PhD work in the Department of Medical Genetics and supported me throughout. Also I am grateful to Dr. Kinga Hadzsiev for the continuous support and providing ongoing assistance.

Special thanks to the colleagues of the Department of Medical Genetics, including Zsolt Bánfai, András, Szabó, Márta Czakó, Anita Maász, Lili Magyari, Alexandra Mikó, Katalin Sümegi, Renáta Szalai, Ágnes Till, Anna Zsigmond who helped me with their competent, conscientious work and professional experience. I would not have been able to carry out my work without the help of assistants, whose expertise has been invaluable throughout this project.

Finally, I owe a debt of gratitude to my family who made this work possible with their endless support. Finally yet importantly, I would like to thank Henrietta Horváth for the enormous patience and encouragement coming from her, as well as her helpful advices.

.

Publications directly related to the topic of the thesis

frontiers in Genetics

ORIGINAL RESEARCH
published: 08 June 2021
doi: 10.3389/fgene.2021.673025

Genotype-Phenotype Associations in Patients With Type-1, Type-2, and Atypical *NF1* Microdeletions

*Gergely Büki[1,2], Anna Zsigmond[1], Márta Czakó[1,2], Renáta Szalai[1,2], Gréta Antal[1], Viktor Farkas[3], György Fekete[4], Dóra Nagy[5], Márta Széll[5], Marianna Tihanyi[6], Béla Melegh[1,2,7], Kinga Hadzsiev[1,2] and Judit Bene[1,2,7]**

[1] Department of Medical Genetics, Clinical Center, Medical School, University of Pécs, Pécs, Hungary, [2] Szentágothai Research Centre, University of Pécs, Pécs, Hungary, [3] 1st Department of Pediatrics, Semmelweis University, Budapest, Hungary, [4] 2nd Department of Pediatrics, Semmelweis University, Budapest, Hungary, [5] Department of Medical Genetics, Faculty of Medicine, University of Szeged, Szeged, Hungary, [6] Genetic Laboratory, Szent Rafael Hospital of Zala County, Zalaegerszeg, Hungary, [7] Full member of the European Reference Network on Genetic Tumour Risk Syndromes (ERN GENTURIS) – Project ID No. 739547, Pécs, Hungary

OPEN ACCESS

Edited by:
Ramcés Falfán-Valencia,
Instituto Nacional de Enfermedades Respiratorias-México, Mexico

Reviewed by:
Feng Zhang,
Fudan University, China
Hildegard Kehrer-Sawatzki,
Ulm University Medical Center, Germany
Eric Pasmant,
Université de Paris, France

***Correspondence:**
Judit Bene
bene.judit@pte.hu

Specialty section:
This article was submitted to Human and Medical Genomics, a section of the journal Frontiers in Genetics

Received: 26 February 2021
Accepted: 12 May 2021
Published: 08 June 2021

Citation:
Büki G, Zsigmond A, Czakó M, Szalai R, Antal G, Farkas V, Fekete G, Nagy D, Széll M, Tihanyi M, Melegh B, Hadzsiev K and Bene J (2021) Genotype-Phenotype Associations in Patients With Type-1, Type-2, and Atypical NF1 Microdeletions. Front. Genet. 12:673025. doi: 10.3389/fgene.2021.673025

Neurofibromatosis type 1 is a tumor predisposition syndrome inherited in autosomal dominant manner. Besides the intragenic loss-of-function mutations in *NF1* gene, large deletions encompassing the *NF1* gene and its flanking regions are responsible for the development of the variable clinical phenotype. These large deletions titled as *NF1* microdeletions lead to a more severe clinical phenotype than those observed in patients with intragenic *NF1* mutations. Around 5-10% of the cases harbor large deletion and four major types of *NF1* microdeletions (type 1, 2, 3 and atypical) have been identified so far. They are distinguishable in term of their size and the location of the breakpoints, by the frequency of somatic mosaicism with normal cells not harboring the deletion and by the number of the affected genes within the deleted region. In our study genotype-phenotype analyses have been performed in 17 mostly pediatric patients with *NF1* microdeletion syndrome identified by multiplex ligation-dependent probe amplification after systematic sequencing of the *NF1* gene. Confirmation and classification of the *NF1* large deletions were performed using array comparative genomic hybridization, where it was feasible. In our patient cohort 70% of the patients possess type-1 deletion, one patient harbors type-2 deletion and 23% of our cases have atypical *NF1* deletion. All the atypical deletions identified in this study proved to be novel. One patient with atypical deletion displayed mosaicism. In our study *NF1* microdeletion patients presented dysmorphic facial features, macrocephaly, large hands and feet, delayed cognitive development and/or learning difficulties, speech difficulties, overgrowth more often than patients with intragenic *NF1* mutations. Moreover, neurobehavior problems, macrocephaly and overgrowth were less frequent in atypical cases compared to type-1 deletion. Proper diagnosis is challenging in certain patients since several clinical manifestations show age-dependency. Large tumor load exhibited more frequently in this type of disorder, therefore better understanding of genotype-phenotype correlations

and progress of the disease is essential for individuals suffering from neurofibromatosis to improve the quality of their life. Our study presented additional clinical data related to *NF1* microdeletion patients especially for pediatric cases and it contributes to the better understanding of this type of disorder.

Keywords: copy number variation, type-1 *NF1* microdeletion, type-2 *NF1* microdeletion, atypical *NF1* microdeletion, 17q11.2 deletion syndrome, array-CGH, multiplex ligation-probe dependent amplification, *NF1* gene

INTRODUCTION

Neurofibromatosis type 1 (NF1; MIM#162200), also known as von Recklinghausen disease, is an autosomal dominant disorder caused by loss-of-function mutations in the neurofibromin 1 (*NF1)* gene. The incidence of NF1 at birth is approximately 1 in 2500-3000 and the disease frequency shows no gender or racial predilection (Lammert et al., 2005; Uusitalo et al., 2015). The typical clinical features of NF1 are the hyperpigmented skin macules, called as café-au-lait spots (CALs), freckling of the axillary and inguinal regions, the pathognomonic neurofibromas and Lisch nodules. The neurofibromas are mostly benign tumors, localized on or under the skin (Huson and Hughes, 1994). They consist of a mixed cell types including Schwann cells, perineural cells, mast cells and fibroblasts. However, neurofibromatosis has a tremendous spectrum of clinical variability, including skeletal abnormalities, vascular disease, central nervous system tumors and cognitive dysfunction (attention deficit, learning disabilities) as well. Skeletal abnormalities such as dysplasia of the long bones are also characteristic for NF1 patients. Many features increase in frequency with aging and shows age-dependent manifestations. Moreover, strong intra- and interfamilial phenotypic variability can be observed among individuals carrying the same pathogenic mutations (Jett and Friedman, 2010).

Neurofibromin 1 gene is located on the long arm of the chromosome 17 (17q11.2) and codes for neurofibromin, a tumor suppressor that functions in the RAS/MAPK and mTOR pathways and controls the cell growth and proliferation (Jett and Friedman, 2010). The penetrance is complete and the mutation rate is high. Most of the intragenic *NF1* mutations are of paternal origin. Half of the known patients inherit the mutation, and the other half have a spontaneous mutation. Novel mutations occur primarily in paternally derived chromosomes, and the probability of these mutations increases with the paternal age (Stephens et al., 1992). A great number of germline mutations are intragenic and their effect causes a truncated neurofibromin (Park and Pivnick, 1998). Currently approximately 2000 mutations (nonsense, frameshift, point mutations etc.) are dispersed through the gene (Abramowicz and Gos, 2014).

The general NF1 population is mostly affected by point mutations or small indels, although a number of cases reported large deletions encompassing the *NF1* gene and its flanking regions. These large deletions titled as *NF1* microdeletions lead to a more severe clinical phenotype than those observed in patients with intragenic *NF1* gene mutations. These severe clinical features include large numbers of early-onset neurofibromas, cognitive deficits, dysmorphic features and an increased risk for the development of malignant peripheral nerve sheath tumors (MPNSTs) (Kehrer-Sawatzki et al., 2017).

Approximately 5-10% of NF1 patients have large deletions and the numbers are continuously increasing as a result of technological innovations (Cnossen et al., 1997; Kluwe et al., 2004; Zhang et al., 2015). Four major types of *NF1* microdeletions (type 1, 2, 3 and atypical) have been identified so far. The main difference among them are the breakpoint location, the size of the deletion, and the number of the affected genes within the deleted region (Kehrer-Sawatzki et al., 2017). The most frequent form is the type-1 *NF1* microdeletion, which is 1.4 Mb long and includes 14 protein-coding genes and four microRNA genes as well (Dorschner et al., 2000; Lopez-Correa et al., 2001). Type-1 deletions account for 70-80% of all large *NF1* deletions (Pasmant et al., 2010; Messiaen et al., 2011). Type-2 *NF1* deletions are less common than type-1 and they represent ca. 10-20% of all large *NF1* deletions (Mautner et al., 2010; Pasmant et al., 2010; Messiaen et al., 2011). Type-2 deletions are 1.2 Mb in size and result in the deletion of 13 genes. In contrast to type-1 and type-2 *NF1* deletions, type-3 *NF1* deletions are very rare, their occurrence is around 1-4% of patients with *NF1* microdeletions (Bengesser et al., 2010; Pasmant et al., 2010; Messiaen et al., 2011). This type of deletion spans 1 Mb and leads to the loss of 9 protein coding genes.

Type-1, 2, and 3 NF1 microdeletions are generated by non-allelic homologous recombination (NAHR) between low-copy repeats (LCRs) during either meiosis (type-1, type-3), or mitosis (type-2) (Dorschner et al., 2000; Jenne et al., 2001; Lopez-Correa et al., 2001; Bengesser et al., 2010; Pasmant et al., 2010; Roehl et al., 2010; Zickler et al., 2012; Hillmer et al., 2016). Type-1 cases are usually maternally inherited germline deletions (Neuhausler et al., 2018), while type-2 ones are predominantly of postzygotic origin (Kehrer-Sawatzki et al., 2004; Steinmann et al., 2008; Vogt et al., 2012). Besides these three types of recurrent microdeletions, atypical *NF1* deletions have been identified in a number of patients. In atypical deletions non-recurrent breakpoints have been discovered, thereby the size of the deletion and the number of the affected genes also vary (Kehrer-Sawatzki et al., 2003, 2005, 2008; Mantripragada et al., 2006; Pasmant et al., 2010; Messiaen et al., 2011). Non-homologous end joining mechanism has been associated mostly with atypical deletions (Venturin et al., 2004a). However, either aberrant DNA double strand break repair and/or replication, and retrotransposon-mediated mechanisms have also been supposed to be involved in the background of their formation (Vogt et al., 2014). Atypical microdeletions may occur

approximately in 8-10% of all patients with NF1 microdeletions (Pasmant et al., 2010).

Somatic mosaicism with normal cells not harboring large *NF1* deletion can be observed with different frequencies in different types of *NF1* deletions. This phenomenon is rare among type-1 deletions, vast majority (more than 95%) of the patients with type-1 deletion is non-mosaic (Messiaen et al., 2011; Summerer et al., 2019). Contrast to type-1 deletion, somatic mosaicism is quite common in type-2 *NF1* deletions, it occurs in at least 63% of all type-2 deletions (Vogt et al., 2012). Atypical *NF1* deletions also display mosaicism frequently. In a study reported by Vogt et al. (2014), approximately 60% of the cases were associated with somatic mosaicism (Vogt et al., 2014). It is worth to note that somatic mosaicism with normal cells without the deletion has a considerable effect on the disease phenotype, however it is difficult to assess its presence.

In addition to the extent of somatic mosaicism, the age of the patients is also an important confounding factor in phenotypic comparisons of NF1 patient cohort, since many symptoms are progressive in onset and some of them appears later in life (Cnossen et al., 1998).

Several research groups have investigated different aspects of *NF1* microdeletions, however only a few studies presented profound clinical examinations. Here we report clinical and genotype data from 17 patients, mainly (82%) children and adolescents, carrying different types of microdeletion. One of the patients with atypical deletion showed somatic mosaicism. The aim of our study was to characterize the detected deletions in our patient cohort and elucidate genotype-phenotype correlations through clinical data collection.

MATERIALS AND METHODS

Participants

Between 2009 and 2019, our laboratory tested 640 unrelated patients with suspected neurofibromatosis. After Sanger sequencing of the *NF1* gene or NGS analyses of *NF1, NF2, KIT, PTPN11, RAF1, SMARCB1, SPRED1* genes no disease-causing mutations have been identified in 252 patients. Of these, 17 patients (7 females, 10 males; mean age at time of examination:12.9 years, age range:2-36 years) with large *NF1* deletion were identified by MLPA and were enrolled into this study. Our patient cohort mostly (14 out of 17) consisted of children between the ages of 2 and 17. Two patients inherited the deletion from their mothers (patients 85 and 260), while in the remaining 15 patients the deletions had *de novo* origin based on the negative MLPA results of the parents or the absence of a clinically affected parent. However, in the latter case low grade or tissue specific mosaicism cannot be ruled out. The mother of patient 260 (patient 134) was clinically affected as well, therefore she was also included in the analysis. The mother of patient 85 was sine morbo. As a control, age and sex matched 33 patients (14 females, 19 males; mean age at the time of examination: 15.2 years, age range:6 months-47 years) with intragenic *NF1* mutations were enrolled into the study as well.

The study was approved by the ethics committee of the University of Pecs (Protocol 8581-7/2017/EUIG). Written informed consent was obtained from all patients or their legal guardians and peripheral blood samples were collected. All experiments were performed in accordance with the Helsinki Declaration of 1975 and with the Hungarian legal requirements of genetic examination, research and biobanking.

All of the patients fulfilled the diagnostic NIH criteria for NF1. Main clinical characteristics of our patient cohort are summarized in **Table 1**. Phenotypic data was obtained from our Genetic counseling unit and from our collaborator clinicians.

Sample Preparation and MLPA Analysis

DNA was isolated from peripheral blood leukocytes with E.Z.N.A.® Blood DNA Maxi kit (Omega BIO-TEK, Norcross, United States). The concentration and purity of extracted DNAs were measured with the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States).

Multiplex ligation-dependent probe amplification (MLPA) assays were performed for screening large deletions or duplications in *NF1* gene using the commercially available SALSA MLPA kits P081-D1 and P082-C2 (MRC-Holland, Amsterdam, The Netherlands). The two probemixes contained together one probe for each exon, three probes for exon 1, one probe for intron 1, and two probes for the exons 15, 21, 23, 51, and 58 of the *NF1* gene. Additionally, one upstream and one downstream probe of *NF1* gene and two probes for the *OMG* gene (located within intron 36 of *NF1* gene) were applied. Moreover, SALSA MLPA kit P122-D1 *NF1* area mix was used for the examination of the contiguous genes in the flanking regions. The probemix contained 20 probes for 16 genes (*MYO1D*, *PSMD11*, *ZNF207*, *LRRC37B*, *SUZ12*, *UTP6*, *RNF135*, *ADAP2*, *ATAD5*, *CRLF3*, *SUZ12P*, *CPD*, *BLMH*, *TRAF4*, *PMP22*, *ASPA*), which were localized upstream and downstream as well. Besides, it also contained probes for five distinct *NF1* exons (1, 17, 30, 49, 57). According to the manufacturer's instructions, a total of 100–200 ng of genomic DNA of each patient and the same amount of three control genomic DNA was used for hybridization. Amplification products were separated by capillary electrophoresis on an ABI 3130 Genetic Analyzer (Life Technologies, United States) and the results were analyzed using Coffalyser software (MRC-Holland, Amsterdam, Netherlands). Each MLPA signal was normalized and compared to the corresponding peak area obtained from the three control samples. Deletions and duplications of the targeted regions were suspected when the signal ratio exceeded 30% deviation. Positive results were confirmed by repeated MLPA experiments and further investigated with array CGH.

Whole Genome Array Comparative Genomic Hybridization Analysis

Array comparative genomic hybridization (aCGH) was performed using the Affymetrix CytoScan 750 K Array. Genomic DNA samples were digested, ligated, amplified, fragmented, labeled, and hybridized to the CytoScan 750 K Array platform according to the manufacturer's instructions. The raw data were

TABLE 1 | Clinical features of our patients with different type of *NF1* microdeletions.

	Deletion type	**Type 1**								**Type 1**				**Type 2**	**Atypical**			
	Applied method	**aCGH**								**MLPA**				**aCGH**	**aCGH**	**MLPA**		
	Patients	**68/NF**	**115/NF**	**255NF**	**428NF**	**467/2016**	**532/NF**	**629/NF**	**761/NF**	**9/NF**	**271/NF**	**387/NF**	**483/NF**	**85/NF**	**556/NF**	**125/NF**	**134/NF**	**260/NF**
	Gender	**M**	**F**	**M**	**M**	**F**	**M**	**F**	**M**	**M**	**M**	**F**	**M**	**F**	**M**	**F**	**F**	**M**
	Age of onset	**26 y**	**5 mo**	**at birth**	**at birth**	**N/A**	**12 y**	**at birth**	**at birth**	**at birth**	**at birth**	**at birth**	**5 y**	**1 mo**	**6.5 y**	**at birth**	**3 y**	**at birth**
	Age at examination	**36 y**	**9 y**	**14 y**	**5 y**	**9 y**	**14 y**	**4.5y**	**9 y**	**21 y**	**4 y**	**17 y**	**7.5 y**	**13 y**	**10 y**	**2 y**	**40 y**	**8 y**
Dysmorphic features	Facial dysmorphism	X	X	X	X	-	X	X	-	X	-	-	X	-	-	-	-	X
	Hypertelorism	X	X	X	X	-	X	X	-	-	-	-	X	-	-	-	X	X
	Facial asymmetry	-	-	-	-	-	X	-	X	X	-	-	-	-	-	-	-	-
	Coarse face	X	-	X	X	-	X	X	X	X	-	-	X	X	-	-	-	-
	Broad neck	-	-	X	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Large hands, feet	-	X	X	X	-	X	X	X	X	-	-	X	X	-	-	-	-
Skin manifestations	CALs	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	Freckling	-	X	X	X	X	-	X	X	X	X	X	X	-	X	X	-	X
	Excess soft tissue	-	-	X	X	-	-	X	-	X	-	-	-	X	-	-	-	-
	SBC neurofibromas	X	X	X	X	-	-	-	X	-	X	X	-	-	-	-	X	-
	CT neurofibromas	-	-	-	-	-	-	-	-	X	-	-	-	-	-	-	-	-
	PL neurofibromas*	-	-	-	-	-	-	-	-	X	-	X	-	-	-	-	-	-
Education and behavior problems	SDiCD	X	-	X	X	X	X	X	X	X	-	-	X	-	-	-	-	X
	Learning difficulties	X	-	X	-	X	X	X	X	X	-	X	X	X	-	-	-	-
	Speech difficulties	-	-	X	X	X	X	X	X	-	-	X	X	-	-	-	-	-
	IQ < 70	-	-	-	–	-	-	-	-	X	-	-	-	-	-	-	-	-
	ADHD	-	-	-	X	-	-	-	-	X	-	-	-	-	-	-	-	-
Skeletal manifestations	Skeletal anomalies	X	X	X	X	X	X	X	X	X	X	X	-	X	X	X	X	X
	Scoliosis	X	-	X	-	-	X	-	-	X	X	-	-	X	-	-	X	-
	Pectus excavatum	-	X	-	X	-	X	-	-	X	X	-	-	-	-	X	-	X
	Bone cysts	X	n.d.	-	n.d.	n.d.	-	-	-	-	-	-	-	-	-	-	-	-
	Joint hyperflexibility	-	-	-	X	-	-	-	-	-	-	-	-	-	-	-	-	-
	Macrocephaly	-	X	X	X	X	-	X	X	-	-	X	-	X	-	-	-	X
Neurological manifestations	Muscular hypotonia	X	-	X	-	-	-	-	X	-	-	-	-	-	-	-	-	-
	Headache	-	-	-	X	-	-	-	-	-	-	-	-	-	-	-	-	-
	Coordination problem	-	-	X	X	X	-	-	X	-	-	-	-	-	-	-	-	-
	MPNST	X	-	-	-	-	-	-	-	-	-	X	-	-	-	-	-	-

(Continued)

TABLE 1 | Continued

	Deletion type	Type 1														Type 1				Type 2		Atypical			
	Applied method	aCGH														MLPA				aCGH	aCGH	MLPA			
	Patients	68/NF	115/NF	255NF	428NF	467/2016	532/NF	629/NF	761/NF	9/NF	271/NF	387/NF	483/NF	85/NF	556/NF	125/NF	134/NF	260/NF							
	Gender	M	F	M	M	F	M	F	M	M	M	F	M	F	M	F	M	M							
	Age of onset	26 y	5 mo	at birth	at birth	N/A	12 y	at birth	at birth	at birth	at birth	at birth	5 y	1 mo	6.5 y	at birth	3 y	at birth							
	Age at examination	36 y	9 y	14 y	5 y	9 y	14 y	4.5y	9 y	21 y	4 y	17 y	7.5 y	13 y	10 y	2 y	40 y	8 y							
	Spinal neurofibromas	-	n.d.	n.d.	n.d.	-	-	n.d.	X	-	-	X	-	n.d.	-	-	n.d.	n.d.							
	T2 hyperintensities	X	X	X	X	-	-	X	X	X	X	X	X	X	-	X	n.d.	X							
Ocular manifestations	Visual disturbance	-	-	-	-	-	X	-	-	-	-	X	-	X	-	-	-	-							
	Lisch nodules	-	-	X	-	-	-	-	-	X	-	X	-	X	-	-	-	-							
	Strabismus	-	-	-	-	-	X	-	-	-	-	-	X	-	-	-	-	-							
	OPG	-	-	-	-	-	-	X	-	-	-	X	-	-	X	-	-	X							
Development. problems	Tall stature	-	X	X	-	-	X	X	X	X	-	X	-	-	-	-	-	-							

*CALs, café-au-lait spots; CT/SBC/PL, cutaneous/subcutaneous/plexiform neurofibroma; SDiCD, significant delay in cognitive development; ADHD, Attention deficit hyperactivity disorder; MPNST, malignant peripheral nerve sheath tumors; OPG, Optic Pathway Gliomas. * means externally observable plexiform neurofibroma. -, absent; X, present, n.d., not determined.*

analyzed by ChAS v2.0 Software (Affymetrix, Thermo Fisher Scientific, Waltham, MA).

CNV Interpretation

DNA sequence information of the identified CNVs refer to the public UCSC database (GRCh37/hg19). CNV interpretation was performed with the help of the following databases and websites: DECIPHER (Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources) (Firth et al., 2009), DGV (Database of Genomic Variants), Ensembl and ECARUCA (European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations) (Vulto-van Silfhout et al., 2013). The estimated size of the deletions and the estimated breakpoints were assessed using the known locations of the last proximal and first distal deleted probes.

Somatic Mosaicism Determination

In patients examined by aCGH assay, allele difference plot and B allele frequency (BAF) plot were evaluated together with Log2 ratios and weighted Log2 ratios with the help of ChAS software to assess the presence and extent or absence of somatic mosaicism. In those samples investigated by MLPA, the ratio values for each MLPA probe were used to assess mosaicism. Values between 0.4-0.6 were considered as non-mosaic deletion, values around 0.7 or up to 0.8 were considered as mosaic deletion.

Clinical Investigation

Phenotypic features of the 17 microdeletion and the 33 control patients were collected using the same standardized questionnaire collection protocol in four HCPs (health care provider). The same patient was always examined and followed up by the same clinician. Most features were identified by physical examination. Dysmorphic features were assessed by expert clinical syndromologist following international guidelines[1] (Allanson et al., 2009; Hall et al., 2009). Lisch nodules and other ocular manifestations were diagnosed by an ophthalmologist. To evaluate childhood overgrowth age and race-related percentile curve was applied. All the patients were investigated by cranial MRI. To evaluate intellectual functions, developmental delay and learning disabilities, patients were assessed by various psychological tests appropriate to their age (Walter Strassmeier's developmental scale: ages between 0 and 5 years (Strassmeier, 1980), Bayley Scales test (BSID-III): ages between 1 and 42 months (Bayley, 2006), Budapest Binet test: ages between 3 and 14 years (Bass et al., 1989)). When IQ was not measured, it was estimated to be > 70 based on the fact that the patient attended a regular kindergarten or school (with special educational needs). ADHD was diagnosed following international guidelines[2]. The term "speech difficulties" was used in those cases when the patient did not speak or he or she had a problem with the language content, language structure and expressive vocabulary and grammar. We assigned it to delayed language development and not neurological symptoms (dysarthria or orofacial dyskinesis).

[1]http://elementsofmorphology.nih.gov/

[2]https://www.nhs.uk/conditions/attention-deficit-hyperactivity-disorder-adhd/symptoms/

Statistical Analysis

All statistical analyses were performed with SPSS version 27 (SPSS Inc,. Chicago, IL, United States). Two-tailed Fisher's exact test was used to assess whether there is a difference in the frequency of clinical features between patients with type-1 *NF1* microdeletion and patients with intragenic *NF1* mutations. A difference with $p < 0.05$ was considered as significant.

RESULTS

Characterization of the *NF1* Microdeletions

A total of 252 patients in whom mutation analysis did not find any pathogenic *NF1* point mutations or intragenic insertions/deletions were screened for large *NF1* rearrangements by MLPA. Heterozygous deletions of the entire *NF1* gene and its flanking regions were identified in 17 patients using SALSA P081/082 assay. To determine the contiguous genes involved in the deletion, the SALSA P122 assay was applied. As a result, majority of our cases (12/17) had type-1 deletion. Moreover, the MLPA analysis revealed atypical deletions in 5 patients. The estimated proximal and distal breakpoints, preceding and following marker locations and the estimated size of the deletions identified by MLPA are summarized in **Supplementary Table 1**. To confirm the MLPA results, array comparative genomic hybridization (aCGH) analyses were performed in 10 patients (8 patients with type-1 and 2 patients with atypical deletions). The estimated location of proximal and distal breakpoints, preceding and following markers and the estimated size of the deletions determined by aCGH are summarized in **Supplementary Table 2**. The classification by MLPA and by aCGH were found to be the same in eight cases (7 type-1 deletion and 1 atypical). In patient 85/NF the aCGH finally revealed the existence of type-2 deletion although the MLPA showed atypical deletion. In patient 4672016 the aCGH test showed atypical deletion whereas MLPA detected a type-1 deletion, finally we considered this patient has type-1 deletion. The discrepancy between the MLPA and aCGH results in these cases may originate from the different localization of the probes. Type-2 deletions are characterized by breakpoints located within *SUZ12* gene and its pseudogene *SUZ12P*. SALSA P122 probe set contains only one probe for *SUZ12* gene (SUZ12-10: localized within exon 10) and 2 probes for *SUZ12P* pseudogene (SUZ12P-3, SUZ12P-1: probe localization within exon 3 and exon 1, respectively). The breakpoints of the deletion detected in our patient (85/NF) were localized within the region covered by *SUZ12* and *SUZ12P* probes of P-122 set. The applied CytoScan 750K chip contains more probes, at least 50 and 7 for *SUZ12* and *SUZ12P*, respectively. Therefore, aCGH was capable to identify this type-2 deletion. Breakpoints of type-1 deletions are located within the low-copy repeats NF1-REPa and NF1-REPc. In patient 4672016 the estimated proximal breakpoint detected by aCGH can be found within NF1-REPa and the estimated distal breakpoint detected by MLPA can be found within NF1-REPc, therefore we considered 4672016 patient as having type-1 deletion. In the remaining 7 cases (4 patients with type-1 and three with atypical deletions), aCGH tests were not feasible due to the quality of the available samples. After all, 8 type-1 deletions, 4 potential type-1 deletions (altogether 12 type-1 deletions), one type-2 deletion and 3 atypical deletions in four patients were identified in our patient cohort. No type-3 microdeletion was found in our patients. Among the type-1 deletions aCGH analyses revealed identical estimated breakpoints in four cases with an approximately 1.37 Mb deletion size. Among atypical cases three distinct novel deletions were detected. Patient 134/NF and 260/NF are close relatives (mother and child), so they possess the same deletion. The results of our MLPA and aCGH analyses with the localization of the MLPA probes are visualized in **Figure 1**. Novel atypical deletions identified in this study, together with the already known atypical NF1 cases, are demonstrated in **Figure 2** and **Tables 4**, **5**. Two out of three novel atypical deletions were identified by MLPA. SALSA P122 probe set contains 23 probes within the 17q region and the distance between the adjacent probes are quite variable from 11 kb up to 1500 kb. The preceding markers of the estimated proximal breakpoint and the following markers of the estimated distal breakpoint are localized far from the breakpoint boundaries. The distance between the preceding markers and the estimated proximal breakpoints are ca. 270 kb and 27 kb in case 125/NF and 260/NF (134/NF), respectively. The distance between the following markers and the estimated distal breakpoints are ca. 80 kb and 500 kb in case 125/NF and 260/NF (134/NF), respectively. MLPA is able to identify only estimated location of breakpoints, the exact localization of the breakpoints can be determined precisely by breakpoint-spanning PCR (Summerer et al., 2018). In our cases the actual breakpoints are presumably located somewhere between two MLPA probes. Therefore, the regions in proximal direction from the first probe or in distal direction from the last probe affected by the deletion until the adjacent probe are suggested as potential deleted region and represented in **Figure 2** with dotted lines.

Assessment of Somatic Mosaicism

Among 10 patients investigated by aCGH, only one subject (556/NF) with atypical *NF1* microdeletion displayed somatic mosaicism with an extent of ca. 30%. In 7 patients examined by MLPA, the ratio values do not imply the presence of any mosaicism. However, neither aCGH, nor MLPA measurements are capable to detect low-grade mosaicism below 20% due to the nature of these techniques. In this study we investigated only blood samples, so to completely rule out mosaicism, examination of additional tissues such fibroblast, buccal or urine cells are necessary. In type-1 *NF1* microdeletion the occurrence of somatic mosaicism is known to be very rare (Summerer et al., 2019), so based on our results our type-1 patients can be considered as non-mosaic cases. The only one patient with type-2 deletion inherited the deletion from her mother, consequently she does not possess somatic mosaicism. Anyway, this is compatible with the aCGH result as well. Among our four patients with atypical *NF1* deletion, the results indicated ca. 30% mosaicism in only one case (556/NF). Patient 260/NF inherited the deletion from his mother, therefore this patient is considered as non-mosaic. His mother (134/NF) is supposed to be a non-mosaic case as

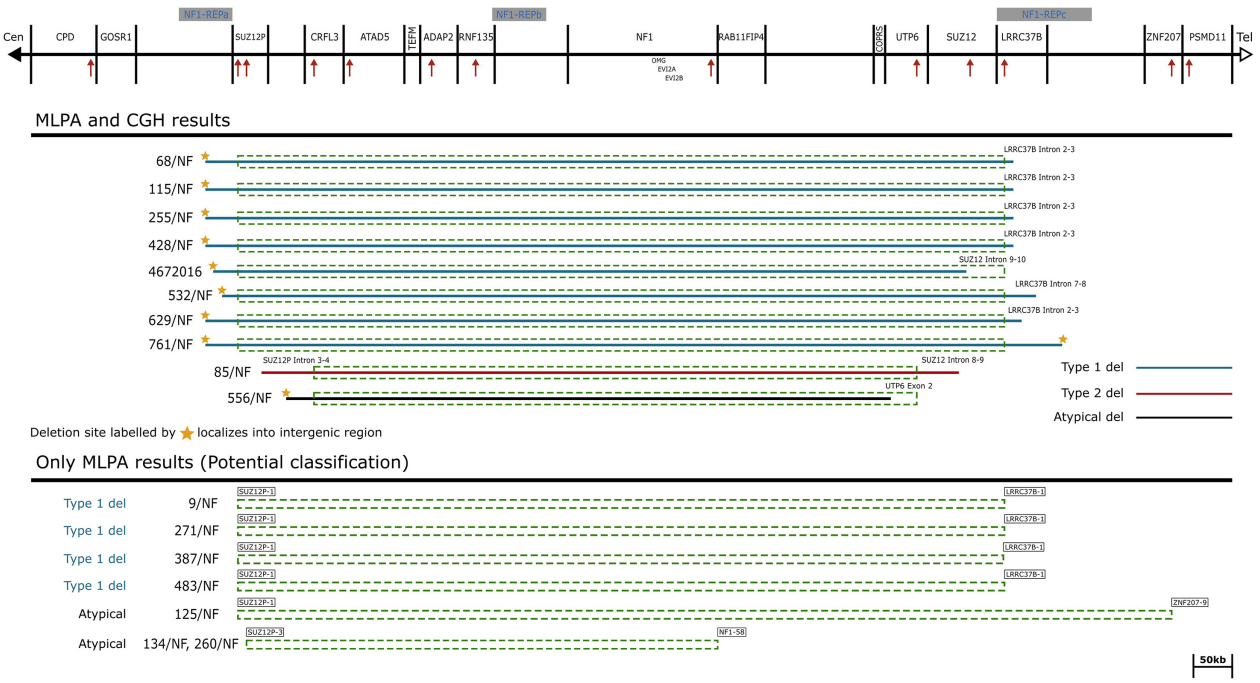


FIGURE 1 | Schematic representation of the *NF1* gene and flanking regions. The affected genes and NF1-REP regions are schematically displayed at the top of the figure. Localization of MLPA probes are demonstrated by red arrows. Solid lines symbolize the deletion range with known breakpoints determined by aCGH probes. Dotted rectangles correspond to the deleted range determined by MLPA probes. Deletion types are marked by colored solid lines, blue: type-1 deletions, red: type-2 deletion and black: the suggested atypical deletion. The last probes contained by the deletion are explicitly displayed at the ends of the deletion ranges.

well, since she has a positive family history (her mother and her grandmother were also affected, however, without laboratory diagnosis) and the MLPA results (peak ratios were between 0.49-0.55) also supported this assumption. MLPA peak ratios were between 0.49 and 0.55 also for patient 125/NF, therefore we supposed this patient to be a non-mosaic as well.

Clinical Characterization of Our Patients With Different Type of *NF1* Microdeletion

Several clinical features and neuropsychological manifestations belonging to eight major categories were selected for consideration for genotype-phenotype association analysis (**Table 1**). The frequency of each clinical feature that appeared in patients with type-1 *NF1* microdeletion is compared with frequencies observed in our control group, i.e., patients with intragenic *NF1* mutation (**Supplementary Tables 4**, **5**).

Dysmorphic Features

Facial dysmorphism was described in 9 of the 17 patients investigated (53%). It was present in 8 out of 12 patients with type-1 *NF1* deletion (67%) and in 1 out of 4 atypical *NF1* deletion (25%) patient cohort. The prevalence of hypertelorism was similar to that of facial dysmorphism, however the distribution among the deletion types was different. This clinical feature was found to roughly the same extent in type-1 deletion and atypical deletion cases (58% vs 50%, respectively). Facial asymmetry was noted only in 3 out of 12 patients with type-1 deletion. Coarse facial appearance was frequent in type-1 deletion patients (8 out of 12 patients, 67%), it was present also in the type-2 deletion patient, though it was absent in our atypical cases. Large hand and feet seem to be a characteristic dysmorphic feature of *NF1* microdeletion patients as well, since the majority of our patients with type-1 deletion (67%, 8 out of 12) showed this trait and it was also noted in the type-2 patient. Dysmorphic features were rare events in our intragenic *NF1* patient population. Of the examined dysmorphic traits only hypertelorism and facial asymmetry were found with the frequency of 18% (6 out of 33 controls) or 6% (2 out of 33 controls), respectively.

Skin Manifestations

Café-au-lait spots (CALs) were observed in each patient in our study regardless of the type of the deletion they have. Axillary and inguinal freckling occurred also in high frequency in our patient cohort. It was more common within the type-1 deletion group, 10 out of 12 patients (83%) presented this skin manifestation. In atypical deletion group 3 out of 4 patients (75%) displayed this feature, however, it was absent in the type-2 deletion patient. Moreover, another skin manifestation, i.e. excess soft tissue in hands and feet was observed among our patients, though at a lower frequency. In type-1 deletion group it was noted in 4 out of 12 patients (33%), it developed in a patient with type-2 deletion also, in contrast, it was not found in the atypical deletion patients. Skin manifestations are characteristic for intragenic *NF1* patients as well. CALs were presented in 91% (30 out of 33) of our patients

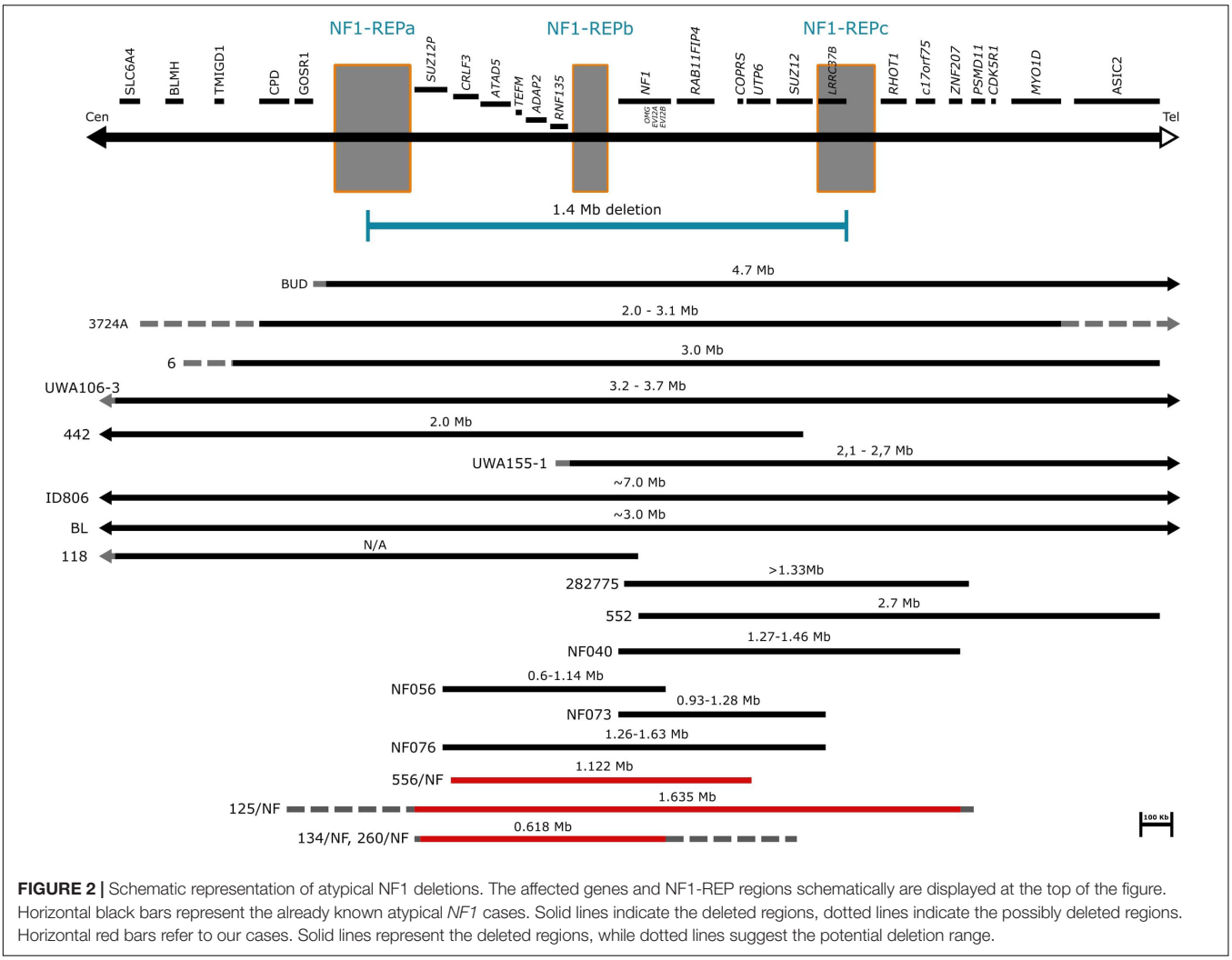


FIGURE 2 | Schematic representation of atypical NF1 deletions. The affected genes and NF1-REP regions schematically are displayed at the top of the figure. Horizontal black bars represent the already known atypical *NF1* cases. Solid lines indicate the deleted regions, dotted lines indicate the possibly deleted regions. Horizontal red bars refer to our cases. Solid lines represent the deleted regions, while dotted lines suggest the potential deletion range.

and the frequency of axillary and inguinal freckling was 52% (17 out of 33 controls).

Neurofibromas and Other Tumors

Subcutaneous neurofibromas were found more common in type-1 deletion patient cohort compared to type-2 and atypical groups. They were observed in 7 out of 12 patients (58%) with type-1 deletion, in 1 out of 4 patients (25%) with atypical microdeletion, though none occurred in the patient with type-2 deletion. The prevalence of cutaneous neurofibromas appears to be less frequent in our patient cohort, it was observed in only one patient with type-1 deletion. However, it is important to mention that 14 out of 17 patients were children and furthermore 10 out of 14 were under 10 years old at the age of examination.

Externally observable plexiform neurofibromas were seen in only 2 patients with type-1 deletion, in a 21-year-old boy and a 17-year-old girl. None of the patients with type-2 or atypical microdeletions presented this type of neurofibromas. However, this is worth to mention that whole-body MRI was not performed routinely in our patients, therefore we have no information about the internally occurring plexiform neurofibromas.

Spinal neurofibromas were found in the type-1 microdeletion group only, however, within this group, the prevalence was low, it developed in 2 out of 12 patients (17%). However, the observed low occurrence is probably the result of the fact, that spinal MRI is not part of the routine procedure in our patient management.

Optic pathway glioma (OPG) was detected by MRI in 4 patients and it was not symptomatic in any of these cases. It was more common in the atypical group with 50% prevalence. Moreover, it developed in 2 out of 12 patients (17%) with type-1 deletion but it was absent in the patient with type-2 deletion. Among the control patients 2 symptomatic and 2 asymptomatic OPG were observed.

Malignant peripheral nerve sheath tumors (MPNST) were observed in 2 of our patients, both belonging to type-1 deletion group. None of the patients with type-2 or atypical microdeletions displayed this type of tumor. MPNSTs show age-related penetrance and our patient cohort consisted of mainly children under 17 years, therefore it is not surprising to detect low occurrence among our patients. However, both patients presenting MPNSTs were adult or nearly adult (36 years and

17 years old, respectively), consequently the frequency of this type of tumor was high (50%, 2 out of 4) among adult patients.

Among our intragenic *NF1* patients, subcutaneous fibromas were found with 30% (10 out of 33) frequency, the occurrence of cutaneous and plexiform neurofibromas were 18% (6 out of 33) or 6% (2 out of 33), respectively. Spinal neurofibromas were observed in 3% (1 out of 33) of our patients. Moreover, 12% (4 out of 33) of this patient cohort developed optic pathway glioma, however, no malignant peripheral nerve sheath tumors occurred.

Skeletal Anomalies

Anomalies of the skeletal system were detected in almost all of our patients (94%, 16 out of 17). The most frequent skeletal anomaly was macrocephaly, which was observed in 9 out of 17 patients (53%). This clinical feature was common in type-1 microdeletion cohort with 58% prevalence, whereas in atypical cohort only one patient (25%) presented this symptom.

Scoliosis was noted in 7 out of 17 patients studied here (41%). It was more frequent in patients with type-1 *NF1* microdeletion than in patients with other type of *NF1* microdeletions. Interestingly, there were only 2 patients who presented scoliosis together with macrocephaly.

Pectus excavatum was observed in 35% of our patient cohort. In contrast to scoliosis, this skeletal anomaly was more frequently observed in patients with atypical microdeletion (50%) as compared to type-1 deletion group (33%).

Bone cysts were found in only one patient with type-1 microdeletion.

None of our patient displayed pes cavus, however, other foot deformities such as pes planus was observed in 3 patients.

Interestingly, skeletal anomalies were the leading manifestations in our patient with type-2 deletion. She had macrocephaly, scoliosis, bilateral dislocation of the elbow and wrist joint. Moreover, absorption of the tibial malleolus was observed and she developed osseous malignancy as well.

Skeletal anomalies were less frequently observed in the intragenic *NF1* patient group (33%). Of these, scoliosis occurred most frequently with 21% prevalence. Macrocephaly and pectus excavatum were noted in 9% of the patients and 3% of them presented pes cavus.

Ocular Manifestations

Ocular manifestations were observed in 7 of 17 our patients (41%). Lisch nodule, one of the characteristic hallmarks of type 1 neurofibromatosis, was noted only in 3 out of 12 patients with type-1 deletion and in the patient with type-2 deletion, however, it was not observed in the atypical patient cohort. Moreover, other ocular manifestations, such as visual disturbance, strabismus and proptosis were noticed in 2 patients with type-1 deletion and in the type-2 deletion patient. One of the patients had hypermetropia, while the others had myopia. The frequency of ocular manifestations was similar in the intragenic *NF1* patient cohort. Lisch nodule was noted in 21% (7 out of 33) of the patients and 15% (5 out of 33) presented visual disturbances as well. One patient had myopia, two patients had hypermetropia, and two other patients had anisometropia. However, strabismus was not observed.

Neuropsychological Manifestations

Significant delay in cognitive development and general learning difficulties were observed with high frequency (75%, 9 out of 12) in type-1 patients. Furthermore, along with the previous features, speech difficulties occurred in 67% (8 out of 12) of this patient group. One patient had an IQ below 70 and 2 patients showed attention deficit hyperactivity disorder (ADHD). IQ measurement was performed in only two among our type-1 patients (761/NF IQ:77, 9/NF IQ:47), however, all of our pediatric patients attended regular kindergarten or school, except the one with IQ = 47, and five of them have special educational needs. Therefore, we supposed these patients are not intellectually disabled, so we marked them as negative for IQ < 70 criteria in **Table 1**. Majority of these neuropsychological features were not found in atypical patient cohort (patient 556/NF IQ:89) and in the type-2 patient. Only a significant delay in cognitive development was noted in 25% (1 out of 4) of atypical patients and the type-2 patient suffered from general learning difficulties.

Structural brain abnormalities were not observed in our patients, however, T2 hyperintensities were found in the majority of our patients. It was present with 75% (9 out of 12) prevalence in type-1 deletion patient cohort, with 25% (1 out of 4) prevalence in atypical group and also in the patient with type-2 deletion. Nevertheless, we did not find any correlation between the age of our patients and the T2 signal intensities.

Muscular hypotonia and coordination problems (25% and 33%, respectively) were documented in patients with type-1 deletion. None of these neurological symptoms were found in our type-2 and atypical deletion groups.

Epilepsy and nerve pain were not noted in our patients. One patient with type-1 deletion complained of headache.

Neuropsychological manifestations were not common among the patients with *NF1* intragenic mutation. 3% (1 out of 33) of our patients presented significant delay in cognitive development, speech difficulties and epilepsy. Moreover, general learning difficulties were noted with a bit higher frequency (15%, 5 out of 33). Muscular hypotonia was observed in 12% (4 out of 33) of our patients and T2 hyperintensities were found in 39% (13 out of 33) of them.

Connective Tissue Anomalies and Cardiac Abnormalities

Connective tissue anomalies and heart abnormalities were a very rare event in our patient cohort. Hyperflexibility of joints was observed in 2 out of 12 type-1 deletion patients (17%). Such manifestation was not present in our patients with type-2 or atypical deletions. Among the cardiac abnormalities atrial septal defect was observed in one patient with atypical microdeletion. Moreover, hypertrophic cardiomyopathy was observed in one patient (8%) and patent ductus arteriosus (PDA) occurred in another patient (8%) with type-1 microdeletion. No congenital heart defect, pulmonary stenosis, ventricular septal defect, aortic stenosis, aortic dissection, mitral valve prolapses, mitral valve insufficiency, aortic valve insufficiency was found in any of the deletion groups. It should mention that two of our patients were not investigated by cardiac ultrasound.

These manifestations were rare in our patients with *NF1* intragenic mutation as well. Among the cardiac abnormalities only ventricular septal defect was observed at birth in one patient and 6% (2 out of 33) of our patients developed joint laxity.

Other Features

Some rare clinical manifestations were observed in our patient group. Obesity, hearing impairment, immune deficiency and milk protein allergy, however it is hard to tell whether these symptoms are associated with the observed large deletion or the results of an independent event.

DISCUSSION

The *NF1* gene was discovered in Viskochil et al. (1990), somewhat later the first case with large *NF1* microdeletion was published in Kayes et al. (1992). Several attempts were made to establish genotype-phenotype correlations which finally suggested a more severe clinical phenotype among patients with *NF1* microdeletion than patients with intragenic *NF1* mutations. However, certain variability of clinical symptoms has been observed among individuals with *NF1* microdeletions.

In this study, we have identified 17 patients with large *NF1* microdeletion. Among them 8 proved to be a type-1 microdeletion carrier by aCGH, 4 more patients are supposed to belong to type-1 group based on MLPA results, 1 patient has type-2 deletion and 4 patients possess atypical deletions. Somatic mosaicism with an extent of ca. 30% was detected in one patient with atypical *NF1* microdeletion. Comparison of clinical characterization of our patients with the published data on intragenic and microdeletion NF1 patients was performed to reveal distinct phenotype-genotype correlations. Moreover, the frequencies of phenotypic features in our patients with *NF1* microdeletion and with type-1 deletion were compared to frequencies observed in our patients with intragenic *NF1* mutation as well (**Supplementary Tables 3–5**).

A similar difference was found between our patients with intragenic *NF1* mutation and *NF1* microdeletion in several clinical features when comparing to those previously published by others (**Table 2**). Mainly the occurrence of dysmorphic features, subcutaneous neurofibromas, skeletal anomalies and neurobehavior problems showed significant difference. Moreover, remarkable differences in certain clinical features were observed between our patients with *NF1* microdeletion and the previously published cases with large *NF1* deletions. However, it is important to emphasize that the majority of our patients (13 out of 17) were less than 15 years old at the time of the examination. There are only few studies (Kehrer-Sawatzki et al., 2020) that demonstrated pediatric clinical data, the majority of phenotypic data published previously originated mainly from adult patient populations.

Type-1 deletion represents the largest group of *NF1* microdeletion cohort with an estimated 70-80% prevalence (Pasmant et al., 2010; Messiaen et al., 2011). The occurrence of this type of deletion among our patients was somewhat similar (70%). Significant number of articles were published on this type of deletion, however, these reports indicate that the clinical phenotype associated with *NF1* microdeletions show a certain degree of variability in the frequency of some clinical features (**Table 2**) (Mensink et al., 2006; Mautner et al., 2010; Pasmant et al., 2010; Bianchessi et al., 2015; Zhang et al., 2015). Dysmorphic features are common in individuals with large *NF1* deletions, whereas they occur rarely among intragenic NF1 patient population. Among these features facial dysmorphism is one of the most characteristic hallmarks of patients with *NF1* microdeletion. In our type-1 patient cohort 67% of the affected individuals possess this manifestation. At the same time in a large study performed by Mautner et al. involving 29 patients (Mautner et al., 2010), the majority of the cases (ca 90%) had facial dysmorphism. However, Pasmant and Zhang observed this feature with lower frequency (Pasmant et al., 2010; Zhang et al., 2015). Nevertheless, all of these data indicate that facial dysmorphic features are very frequent in type-1 deletions. Another dysmorphic feature which can be seen more often in microdeletion patients is the observed large hands and feet. It occurred with 67% prevalence in our patient cohort, it was observed in 46% of patients by Mautner (Mautner et al., 2010), however, it was not stated by others. Another observable difference can be seen in the number of the detected neurofibromas. Previous studies established an early-onset of neurofibromas among *NF1* microdeletion patients. While the frequency of the detected subcutaneous neurofibromas in our patients was close to that observed by others (58 vs 76%), the occurrence of cutaneous or plexiform neurofibromas was remarkably lower in our patients compared to other patient groups (8 vs. 86% and 17 vs. 76%, respectively). However, it is worth to highlight, that our patient cohort mainly consisted of children and adolescents, and 9 out of 17 were less than 10 years old at the time of examination. Cutaneous neurofibromas show age-related penetrance and they usually appear in adulthood, therefore this may contribute to the difference in frequency observed by us and by others. Nevertheless, a high frequency (60%) of cutaneous neurofibromas was observed among children by Kehrer-Sawatzki in a recent study (Kehrer-Sawatzki et al., 2020). The high prevalence of subcutaneous neurofibromas in type-1 *NF1* patients is important to consider, since they are associated with mortality in NF1 disease (Tucker et al., 2005). Patients with subcutaneous neurofibromas possess a higher risk for the development of MPNSTs. In addition, the presence of plexiform neurofibromas possess a risk for development of malignant tumor as well (Waggoner et al., 2000). More pronounced alteration can be seen in the cognitive ability. Although, significant delay in cognitive development was found more frequently in our type-1 patients, the prevalence of intellectual disability was less pronounced. Moreover, overgrowth, which is characteristic for type-1 *NF1* microdeletion, was observed as much as by others, however, connective tissue anomalies were fairly less frequent among our patients. It was common among Mautner's patients (72%), but it was rare (8%) in our patient cohort.

Type-1 deletion harbors 14 protein coding genes and 4 microRNA genes. Some of the genes co-deleted with *NF1* may have an influence on the clinical manifestation observed in

patients with *NF1* microdeletion, thus affecting the severity of the disease (Kehrer-Sawatzki et al., 2017). Haploinsufficiency of certain genes may contribute to dysmorphic facial features, overgrowth and reduced cognitive capability (*RNF135*) (Tastet et al., 2015) or heart defects (*ADAP2*) (Venturin et al., 2014), whereas others might have tumor suppressive function, thus their deletion promote tumor development (*SUZ12, ATAD5*) (Bell et al., 2011; Zhang et al., 2014). Although the size of the deletion and the gene content is almost the same in all patients with type-1 deletion, they demonstrate a notable clinical variability. This observation may suggest that differences in the unique genomic architecture of the patients may also contribute to the observed variability of the clinical phenotypes.

Type-2 deletions account for 10-20% of *NF1* large deletion cases according to previous studies. In our patient cohort one patient and her asymptomatic mother carries this type of large *NF1* deletion. Because of the missing phenotypic signs, we suppose that the mother should be a mosaic patient. In type-2 deletions existence of somatic mosaicism is a frequently observed phenomenon, these deletions arise during post-zygotic cell division and are associated with a milder clinical phenotype. Vogt et al. reported 18 patients with type-2 deletion, 16 of whom proved to be mosaic cases (Vogt et al., 2011). In another study the same research group identified 27 of 40 patients with mosaicism determined by FISH. That paper did not contain clinical information, because it was focused on the possible molecular mechanism behind type-2 deletion formation (Vogt et al., 2012). Only a few non-mosaic type-2 cases with detailed phenotype have been published so far (**Table 3**; Vogt et al., 2011; Zhang et al., 2015). These patients share common features, half of which can be found in our patient as well. However, some characteristic hallmarks of *NF1* microdeletion symptoms are missing from our patient's phenotype or they are presented in a mild form. This may originate from her young age (13 years). She does not have any type of externally observable neurofibromas, cardiac manifestations, those that may manifest as early as childhood, and neurobehavioral problems, whereas these features were noted in the majority of the published cases. Moreover, frequent skin manifestation such as freckling was not observed in our patient. These traits occurred in other known type-2 patients. The unique feature of our patient is that the whole clinical picture is dominated by skeletal anomalies. She underwent a number of operations affecting the skeletal system. Moreover, absorption of the tibial malleolus was observed and she developed osseous malignancy as well. After all her clinical picture possesses many features frequently observed in patients with large *NF1* deletion. Although type-2 deletions are typically 1.2 Mb in size, the exact localization of the breakpoints are presumably different in our patient and in the published cases. This may result in the removal of certain regulatory factors which may finally lead to the observed variability in the phenotype.

Atypical deletions form a heterogeneous group of *NF1* microdeletions regarding the clinical manifestations they cause as well as the size and location of the deletion. Moreover, somatic mosaicism can be frequently observed among these patients which may lead to a milder phenotype. The occurrence of atypical cases is around 8-10% among patients with *NF1* microdeletion, however, in our patient cohort we observed a higher frequency (23%) and only one patient displayed mosaicism. Around 20 patients with atypical deletion were published so far without recurrent breakpoints (Kayes et al., 1992; Upadhyaya et al., 1996; Cnossen et al., 1997; Dorschner et al., 2000; Riva et al., 2000; Kehrer-Sawatzki et al., 2003, 2005, 2008; Venturin et al., 2004a,b; Mantripragada et al., 2006; Zhang et al., 2015). In our study three distinct, novel deletions were identified. The deletions in the published cases show remarkable overlaps with those observed in our patients, though in our cases the deletions are typically smaller (**Figure 2**). However, the clinical pictures of the known cases show hardly any overlapping symptoms apart from the major diagnostic criteria for NF1 (**Table 4**). Remarkable difference can be seen in dysmorphic features, neuropsychological manifestations and the presence of various neurofibromas. Dysmorphic features such as facial dysmorphia, coarse face, facial asymmetry and large hands and feet are characteristic hallmarks of *NF1* microdeletions. They were observed in the majority of patients with type-1 *NF1* microdeletion (**Table 2**) and it was noted at least in half of the atypical cases identified so far, however, in our patient cohort only one patient displayed facial dysmorphia and another had hypertelorism. Moreover, these features were not observed in patients described by Zhang et al. (2015). In addition, notable divergence can be observed in the occurrence of various neurofibromas among the atypical *NF1* microdeletion patients. All the patients in Zhang's study manifested cutaneous or plexiform neurofibromas, 6 out of 11 other published cases had various type of neurofibromas, whereas in our study only one patient has developed subcutaneous neurofibromas. This discrepancy may be related to the age of the patients. It is a known phenomenon that the number of the neurofibromas may increase with the age of the patient. Among atypical cases the majority of the patients who presented any type of neurofibromas were teenagers or young adults. In our patient cohort, which consisted of mainly children under 10 years, the only one who had subcutaneous neurofibroma was 40 years old. In addition, observable difference can be found among the neuropsychological manifestation. These features were almost absent in our patients, only one showed significant delay in cognitive development, however, moderate to severe intellectual disability or severe learning disability were noted in almost all patients carrying larger deletion than our patients. In an atypical deletion the gene content of the deleted region has an effect on the phenotypic manifestations, particularly the genes with intolerance of haploinsufficiency are likely to have pathological consequences. **Table 5** summarized the haploinsufficiency intolerant genes in all cases published so far including this study. Although in 3 out of 4 patients of ours only MLPA measurements were feasible, the deletion of one more haploinsufficiency intolerant gene, namely *RAB11FIP4,* may be expected beyond those demonstrated in **Table 5**. The exact role of this gene in the disease pathogenesis is not clear. Previous studies (Descheemaeker et al., 2004; Ottenhoff et al., 2020) revealed that *NF1* microdeletion genotype is associated with a lower cognitive ability compared with intragenic *NF1* genotype. Co-deletion of

TABLE 2 | Clinical features of patients with type-1 *NF1* microdeletion.

System involvement/ manifestations	Clinical features	Frequency in patients with type-1 *NF1* microdeletions (%)					Frequency in *NF1* non-deleted patients (%)	
		This study (*n* = 12)	Kehrer-Sawatzki et al., 2017 (*n* = 29)	Pasmant et al., 2010 (*n* = 44)	Zhang et al., 2015 (*n* = 7)	Bianchessi et al., 2015 (*n* = 11)	This study (*n* = 33)	Kehrer-Sawatzki et al., 2017 (*n* = 29)
Dysmorphic features	Facial dysmorphism	67	90	54.8	43	n.d.	0	n.d.
	Hypertelorism	58	86	n.d.	n.d.	n.d.	18	n.d.
	Facial asymmetry	25	28	n.d.	n.d.	n.d.	6	8
	Coarse face	67	59	n.d.	n.d.	n.d.	0	n.d.
	Broad neck	8	31	n.d.	n.d.	n.d.	0	n.d.
	Large hands and feet	67	46	n.d.	n.d.	n.d.	0	n.d.
Skin manifestations	Café-au-lait spots	100	93	20.8	100	100	91	86-99
	Axillary and inguinal freckling	83	86	86.4	57	72.7	52	86-89
	Excess soft tissue in hands and feet	33	50	n.d.	n.d.	n.d.	0	n.d.
	Subcutaneous neurofibromas	58	76	37.2-41.8	29	45.5[#]	30	48
	Cutaneous neurofibromas	8	86	15.4-48.7	57	45.5[#]	18	38-84
	Plexiform neurofibromas	17	76	0.6	29	27.3	6	15-54
Education and behavior problems	SDiCD	75	48	n.d.	14	36.4	3	17
	General learning difficulties	75	45	85.7	n.d.	18.2	15	31-47
	Speech difficulties	67	48	n.d.	29	0	3	20-55
	IQ < 70	8	38	n.d.	14	36.4	0	7-8
	ADHD	17	33	n.d.	n.d.	0	6	38-49
Skeletal manifestations	Skeletal anomalies	92	76	31+	14	45.5+	33	31
	Scoliosis	42	43	31	0	9.1	21	10-28
	Pectus excavatum	33	31	n.d.	n.d.	n.d.	9	12-50
	Bone cysts	8	50	n.d.	n.d.	0	0	1
	Hyperflexibility of joints	8	72	n.d.	n.d.	n.d.	6	n.d.
	Pes cavus	n.d.	17	n.d.	n.d.	n.d.	3	n.d.
	Macrocephaly	58	39	11.5	14	45.5	9	24-45
Neurological manifestations	Muscular hypotonia	25	45	n.d.	n.d.	n.d.	12	27
	Epilepsy	0	7	n.d.	n.d.	0	3	4-13
	MPNST	17	21	7.1	0	*	0	2-7
	Spinal neurofibromas	17	64	n.d.	n.d.	n.d.	3	24-30
	T2 hyperintensities	75	45	n.d.	29	n.d.	39	34-79
Ocular manifestations	Visual disturbance	17	n.d.	n.d.	14	n.d.	15	n.d.
	Lisch nodules	25	93	40	14	45.5	21	63-93
	Strabismus	17	NA	n.d.	14	n.d.	0	NA
	Optic pathway gliomas	17	19	15	n.d.	0	12	11-19
Developmental problem	Tall-for-age stature	58	46	22.2	n.d.	n.d.	0	n.d.
Heart problems	Congenital heart defects	0	29	n.d.	n.d.	n.d.	0	2

*n.d., not determined; NA, not assessed or no data available; [#]no straightforward information (only referenced as neurofibroma); *it is not clear from the manuscript (it was mentioned that 18.2% of patient had tumors); + it may be higher (there were data for scoliosis and macrocephaly only); SDiCD, significant delay in cognitive development; MPNST, malignant peripheral nerve sheath tumors; ADHD, attention deficit hyperactivity disorder.*

TABLE 3 | Clinical features of patients with type-2 *NF1* microdeletions.

Clinical features of patients with type-1 *NF1* microdeletions (frequency observed,%)			**Presence or absence of the features in patients with "non-mosaic" type-2 *NF1* deletions**			
Patients	*n* = 29	*n* = 12	078	P. 2429	P. 2358	85/NF
Reference	Kehrer-Sawatzki et al., 2017	This study	Zhang et al., 2015	Roehl et al., 2010; Vogt et al., 2012	Roehl et al., 2010; Vogt et al., 2012	This study
CALs	93%	100%	+	+	+	+
Freckling	86%	83%	−	+	+	−
Lisch nodule	93%	25%	?	+	+	+
Cutaneous neurofibromas	86%	8%	+	+ (multiple)	−	−
Subcutaneous neurofibromas	76%	58%	+	+ (multiple)	+	−
Plexiform neurofibromas	76%	17%	−	+ (multiple)	+	−
Facial dysmorphism	90%	67%	−	+	+	−
Large hands and feet	46%	67%	N/A	+	+	+
Macrocephaly	39%	58%	−	+	+	+
Tall stature	46%	58%	N/A	−	−	−
Learning disabilities	48%	75%	?	+	+ (mild)	+
Attention deficits	33%	17%	?	+	+	−
Scoliosis	43%	42%	+	−	N/A	+
Hyperflexibility of the joints	72%	8%	N/A	+	+	−
MPNST	21%	17%	−	+	−	−
T2 hyperintensities	45%	75%	N/A	−	+	+
Muscular hypotonia	45%	25%	N/A	N/A	+	−
Congenital heart defects	21%	0%	N/A	+	+	−

−, absent; +, present; N/A, not assessed or no data available; ? unclear result from the original article. CALs, café-au-lait spots; MPNST, malignant peripheral nerve sheath tumors.

genes adjacent to *NF1*, such as *OMG* and *RNF135* are supposed to contribute to the observed decreased cognitive ability (Kehrer-Sawatzki et al., 2017). *OMG* gene encodes the oligodendrocyte myelin glycoprotein which plays an important role in early brain development (Martin et al., 2009). Moreover, *OMG* is associated with intellectual disability and neuropsychiatric disorders (Bernardinelli et al., 2014). In addition, a rare allele of *RNF135* gene has been found with higher frequency in patients with autism (Tastet et al., 2015). Although the deletion identified in our patients encompass *OMG* and *RNF135* genes as well, our patients hardly displayed neuropsychiatric symptoms. This observation implies that beyond the *OMG* and *RNF135* deletion further factors are also necessary for the development of intellectual disability or neuropsychiatric manifestations in patients with *NF1* microdeletions. Contrary to our cases, high load of internal tumors were observed in a number of patients with larger atypical deletion. Several genes (*ATAD5, COPRS, UTP6* and *SUZ12*) in the 17q11.2 region were supposed to be involved in tumorigenesis (Kehrer-Sawatzki et al., 2017). *ATAD5* was affected in our two patients, co-deletion of *ATAD5, COPRS* and *UTP6* was observed in another one. However, none of these patients of ours developed internal tumors. Co-deletion of *ATAD5, COPRS, UTP6* and *SUZ12* genes with *NF1* may possess an increased risk for high tumor load which might lead to the observed high number of tumors in patients with larger atypical deletion. In one of our patients the atypical deletion harbors all of these four genes, however, perhaps due to her young age (i.e., 2 years) no tumors were found at the age of her examination.

Genotype-phenotype analyses among our patients revealed that ones with *NF1* microdeletion more often presented dysmorphic facial features, macrocephaly, large hands and feet, delayed cognitive development and/or learning difficulties, speech difficulties, overgrowth and subcutaneous neurofibromas compared to those with intragenic *NF1* mutations. These features seemed to be characteristic for the patient group with type-1 *NF1* microdeletion, however, some of the above-mentioned traits were absent from the type-2 and atypical *NF1* microdeletion

TABLE 4 | Clinical features of patients with atypical *NF1* microdeletions.

Patient	Age (y)	Gender	Skin manifestations	Neurofibromas	Dysmorphic features	Skeletal manifestations	Ocular Manifestations	Neuropsychological manifestations	Other	References
BUD	14; 18	N/A	CALs, F	Many CNF, SNF	Coarse face	SCS, genu valgum, joint laxity	N/A	SDiCD, ID, T2 hyperintensities	Many ST	Kehrer-Sawatzki et al., 2003
3724A	13	Female	CALs, F	Few CNF	Coarse face, FA, hypertelorism, ptosis, broad lips and nose	PE	LiN	Moderate ID	-	Cnossen et al., 1997
6	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Venturin et al., 2004a,b
UWA106-3	18	Male	CALs, F	Many CNF, PNF, spinal NF	Coarse face, large hands	MA	N/A	SDiCD, IQ 46	Many ST	Dorschner et al., 2000; Kayes et al., 1992
442	18; 26	Male	CALs, F	Multiple SCNF, and many CNF, PNF	Coarse face	SCS	LiN	IQ 76, severe LD	Many ST	Kehrer-Sawatzki et al., 2005
BL	13,5	Male	CALs, F	-	FD, hypertelorism	Skeletal anomalies	-	Severe ID	-	Riva et al., 2000
ID806	3 mo; 3; 4	Male	CALs, F	-	Narrow palpebral fissures, ptosis, low set, rotated ears, prominent maxilla	-	-	Marked developmental delay, SP, seizure	-	Upadhyaya et al., 1996
UWA155-1	27	N/A	-	Multiple CNF, spinal NF	Coarse face, ptosis, large hands and feet	MA	-	Moderate ID	MPNST	Dorschner et al., 2000
118	5	Male	CALs, F	N/A	-	-	OPG	Seizure, no LD	-	Venturin et al., 2004b
282775	n.d.	N/A	CALs	-	Noonan-like FD	-	-	PD, SP	-	Mantripragada et al., 2006
552	20	Female	CALs, F	2 PNF, 4 SIN NF	Large hands and feet	PE, lumbar lordosis, pedes valgoplanus	LiN, visual disturbance	Mild ID, severe LD, SP, hypotonia	-	Kehrer-Sawatzki et al., 2008
NF040	1	Female	CALs	PNF	-	-	*	*	-	Zhang et al., 2015
NF056	60	Female	CALs, F	CNF	-	-	*	*	-	
NF073	25	Female	CALs, F	CNF	-	-	*	*	-	
NF076	36	Female	CALs	CNF	-	-	*	*	-	
556/NF	10	Male	CALs, F	-	-	Bilateral PP	OPG	-	-	this study
125/NF	2	Female	CALs, F	-	-	PE	-	-	-	
134/NF	40	Female	CALs	SCNF	Hypertelorism	SCS	-	-	-	
260/NF	8	Male	CALs, F	-	FD, hypertelorism	PE, MA	OPG	SDiCD, T2 hyperintensities	ASD	

*CALs, café-au-lait spots; F, freckling; FA, facial asymmetry; FD, facial dysmorphy; CNF, cutaneous neurofibroma; SCNF, subcutaneous neurofibroma; PNF, plexiform neurofibroma; SIN NF, small intramuscular nodular neurofibroma; ST, spinal tumors; MPNST, malignant peripheral nerve sheath tumors; SDiCD, significant delay in cognitive development; ID, intellectual disability; LD, learning difficulties; SP, speech delay; PD, psychomotor delay; SCS, scoliosis; PE, pectus excavatum; MA, macrocephaly; PP, pes planus; LiN, Lisch nodule; ASD, atrial septal defect. * unclear results in the original article. NA, no data available.*

TABLE 5 | Size of the deletions and haploinsufficient genes located within the atypical *NF1* deletions.

Patient	Deletion size (Mb)	Haploinsufficient genes (by gnomAD pLI)	References
BUD	4.7	*ATAD5, NF1, OMG, RAB11FIP4, SUZ12, PSMD11, CDK5R1, ASIC2*	Kehrer-Sawatzki et al., 2003
3724A	2.0-3.1	*ATAD5, NF1, OMG, RAB11FIP4, SUZ12, PSMD11, CDK5R1, ASIC2*	Cnossen et al., 1997
6	3	*ATAD5, NF1, OMG, RAB11FIP4, SUZ12, PSMD11, CDK5R1, ASIC2*	Venturin et al., 2004a,b
UWA106-3	3.2-3.7	*ATAD5, NF1, OMG, RAB11FIP4, SUZ12, PSMD11, CDK5R1, ASIC2*	Dorschner et al., 2000; Kayes et al., 1992; Kayes et al., 1994
442	2	*ATAD5, NF1, OMG, RAB11FIP4, SUZ12*	Kehrer-Sawatzki et al., 2005
BL	∼3	*ATAD5, NF1, OMG, RAB11FIP4, SUZ12, PSMD11, CDK5R1, ASIC2*	Riva et al., 2000
ID806	∼7	*ATAD5, NF1, OMG, RAB11FIP4, SUZ12, PSMD11, CDK5R1, ASIC2*	Upadhyaya et al., 1996
UWA155-1	2.1-2.7	*NF1, OMG, RAB11FIP4, SUZ12, PSMD11, CDK5R1, ASIC2*	Upadhyaya et al., 1996
118	N/A	*ATAD5, NF1*	Venturin et al., 2004b
282775	> 1.33	*NF1, OMG, RAB11FIP4, SUZ12*	Mantripragada et al., 2006
552	2.7	*NF1, OMG, RAB11FIP4, SUZ12, PSMD11, CDK5R1, ASIC2*	Kehrer-Sawatzki et al., 2008
40	1.27-1.46*	*NF1, OMG, RAB11FIP4, SUZ12,*	Zhang et al., 2015
56	0.60-1.14*	*ATAD5, NF1, OMG*	
73	0.93-1.28*	*NF1, OMG, RAB11FIP4, SUZ12*	
76	1.26-1.63*	*ATAD5, NF1, OMG, RAB11FIP4, SUZ12*	
556/NF	1.122	*ATAD5, NF1, OMG, RAB11FIP4, SUZ12*	This study
125/NF	1.635*	*ATAD5, NF1, OMG, RAB11FIP4, SUZ12*	
134/NF	0.618*	*ATAD5, NF1, OMG*	
260/NF	0.618*	*ATAD5, NF1, OMG*	

**Results originated from MLPA probes location. The probability of loss of function (pLI) metric were provided by the gnomAD browser (https://gnomad.broadinstitute.org/). According to official description, a transcript's intolerance to variation is measured by predicting the number of variants expected to be seen in the gnomAD dataset and comparing those expectations to the observed amount of variation. The range scales from 0 to 1, where the closer the pLI value is to 1, the more intolerant the gene appears to be to loss of function (LoF) variants. We determined as haploinsufficient a gene if the pLI value was above 0.9, which indicates extreme intolerance to LoF variants (Karczewski et al., 2020).*

patient cohort. Our patient with non-mosaic type-2 *NF1* large deletion had only a few of the typical clinical signs: macrocephaly, large hands and feet as well as learning difficulties. On the other hand, she has a strong skeletal involvement. In our atypical *NF1* microdeletion patient cohort only the facial dysmorphism, delayed cognitive development, macrocephaly and the presence of subcutaneous neurofibromas were noted. Certain clinical symptoms such as congenital heart defects, joint laxity, muscular hypotonia and bone cysts were reported by others in type-1 *NF1* microdeletion patients (Mautner et al., 2010; Kehrer-Sawatzki et al., 2017), but these were not pronounced in our patients. It is worth to mention that manifestations of several symptoms are age dependent, therefore a comprehensive study on the clinical course of patients with different type of *NF1* microdeletion could help to establish diagnostic milestones in these patients' group.

CONCLUSION

In conclusion, in our patient cohort three different types of *NF1* microdeletion have been identified. Although these deletions were associated with different clinical manifestations, possibly due to the deleted gene contents or the deletion of other regulatory DNA elements, patients with *NF1* large deletion showed more severe clinical phenotype compared to individuals with intragenic *NF1* mutations. The identification and in some cases the classification of the *NF1* microdeletions have been feasible using MLPA, a simple, cost-effective technique. This method enabled us to recognize *NF1* microdeletion patients easily among the general NF1 patients. Our study presented additional clinical data related to *NF1* microdeletion patients especially for pediatric patients and it contributes to the better understanding of this type of disorder.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the University of Pecs (Protocol 8581-7/2017/EUIG). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

JB conceived and designed the research. GB, RSz, GA, and MCz performed the genetic investigations. KH, AZs, MSz, GyF, VF,

MT, and DN performed the patient examinations. GB and JB completed data analysis and drafted the manuscript. GB prepared the figures and tables. GB, BM, KH, and JB edited and revised the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was supported by grants from the Medical School, University of Pécs (KA 2020-27), GINOP-2.3.3-15-2016-00025 and the National Scientific Research Program (NKFI) K-119540.

ACKNOWLEDGMENTS

We would like to thank all the patients and their family members who participated in this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.673025/full#supplementary-material

REFERENCES

Abramowicz, A., and Gos, M. (2014). Neurofibromin in neurofibromatosis type 1 – mutations in NF1gene as a cause of disease. *Dev. Period Med.* 18, 297–306.

Allanson, J. E., Cunniff, C., Hoyme, H. E., McGaughran, J., Muenke, M., and Neri, G. (2009). Elements of morphology: standard terminology for the head and face. *Am. J. Med. Genet. A* 149A, 6–28. doi: 10.1002/ajmg.a.32612

Bass, L., Borbély, S., Jászberényi, M., Lányiné, E. Á, Sarkady, K., Gerebenné, V. K., et al. (1989). "A budapesti binet-teszttel végzett vizsgálatokról," in *A Differenciált Beiskolázás Néhány Mérőeszköze*, eds K. G. Várbíró and T. Vidákovich (Cambridge, MA: Akadémiai Kiadó), 29–49. (in Hungarian).

Bayley, N. (2006). *Bayley Scales of Infant and Toddler Development: Bayley-III*. San Antonio, TX: Harcourt Assessment Psychological Corporation, 7.

Bell, D. W., Sikdar, N., Lee, K. Y., Price, J. C., Chatterjee, R., Park, H. D., et al. (2011). Predisposition to cancer caused by genetic and functional defects of mammalian Atad5. *PLoS Genet.* 7:e1002245. doi: 10.1371/journal.pgen.1002245

Bengesser, K., Cooper, D. N., Steinmann, K., Kluwe, L., Chuzhanova, N. A., Wimmer, K., et al. (2010). A novel third type of recurrent NF1 microdeletion mediated by nonallelic homologous recombination between LRRC37B-containing low-copy repeats in 17q11.2. *Hum. Mutat.* 31, 742–751. doi: 10.1002/humu.21254

Bernardinelli, Y., Nikonenko, I., and Muller, D. (2014). Structural plasticity: mechanisms and contribution to developmental psychiatric disorders. *Front. Neuroanat.* 8:123. doi: 10.3389/fnana.2014.00123

Bianchessi, D., Morosini, S., Saletti, V., Ibba, M. C., Natacci, F., Esposito, S., et al. (2015). 126 novel mutations in Italian patients with neurofibromatosis type 1. *Mol. Genet. Genomic Med.* 3, 513–525. doi: 10.1002/mgg3.161

Cnossen, M. H., de Goede-Bolder, A., van den Broek, K. M., Waasdorp, C. M., Oranje, A. P., Stroink, H., et al. (1998). A prospective 10 year follow up study of patients with neurofibromatosis type 1. *Arch. Dis. Child* 78, 408–412. doi: 10.1136/adc.78.5.408

Cnossen, M. H., van der Est, M. N., Breuning, M. H., van Asperen, C. J., Breslau-Siderius, E. J., van der Ploeg, A. T., et al. (1997). Deletions spanning the neurofibromatosis type 1 gene: implications for genotype-phenotype correlations in neurofibromatosis type 1? *Hum. Mutat.* 9, 458–464. doi: 10.1002/(SICI)1098-100419979:5<458::AID-HUMU13<3.0.CO;2-1

Descheemaeker, M. J., Roelandts, K., De Raedt, T., Brems, H., Fryns, J. P., and Legius, E. (2004). Intelligence in individuals with a neurofibromatosis type 1 microdeletion. *Am. J. Med. Genet. A* 131, 325–326. doi: 10.1002/ajmg.a.30346

Dorschner, M. O., Sybert, V. P., Weaver, M., Pletcher, B. A., and Stephens, K. (2000). NF1 microdeletion breakpoints are clustered at flanking repetitive sequences. *Hum. Mol. Genet.* 9, 35–46. doi: 10.1093/hmg/9.1.35

Firth, H. V., Richards, S. M., Bevan, A. P., Clayton, S., Corpas, M., Rajan, D., et al. (2009). DECIPHER: database of chromosomal imbalance and phenotype in humans using ensembl resources. *Am. J. Hum. Genet.* 84, 524–533. doi: 10.1016/j.ajhg.2009.03.010

Hall, B. D., Graham, J. M. Jr., Cassidy, S. B., and Opitz, J. M. (2009). Elements of morphology: standard terminology for the periorbital region. *Am. J. Med. Genet. A* 149A, 29–39. doi: 10.1002/ajmg.a.32597

Hillmer, M., Wagner, D., Summerer, A., Daiber, M., Mautner, V. F., Messiaen, L., et al. (2016). Fine mapping of meiotic NAHR-associated crossovers causing large NF1 deletions. *Hum. Mol. Genet.* 25, 484–496. doi: 10.1093/hmg/ddv487

Huson, S. M., and Hughes, R. A. C. (1994). *The Neurofibromatoses: A Pathogenetic and Clinical Overview*. London, NY: Chapman & Hall.

Jenne, D. E., Tinschert, S., Reimann, H., Lasinger, W., Thiel, G., Hameister, H., et al. (2001). Molecular characterization and gene content of breakpoint boundaries in patients with neurofibromatosis type 1 with 17q11.2 microdeletions. *Am. J. Hum. Genet.* 69, 516–527. doi: 10.1086/323043

Jett, K., and Friedman, J. M. (2010). Clinical and genetic aspects of neurofibromatosis 1. *Genet. Med.* 12, 1–11. doi: 10.1097/GIM.0b013e3181bf15e3

Karczewski, K. J., Francioli, L. C., Tiao, G., Cummings, B. B., Alfoldi, J., Wang, Q., et al. (2020). The mutational constraint spectrum quantified from variation in 141,456 humans. *Nature* 581, 434–443. doi: 10.1038/s41586-020-2308-7

Kayes, L. M., Riccardi, V. M., Burke, W., Bennett, R. L., and Stephens, K. (1992). Large de novo DNA deletion in a patient with sporadic neurofibromatosis 1, mental retardation, and dysmorphism. *J. Med. Genet.* 29, 686–690. doi: 10.1136/jmg.29.10.686

Kayes, L. M., Burke, W., Riccardi, V. M., Bennett, R., Ehrlich, P., Rubenstein, A., et al. (1994). Deletions spanning the neurofibromatosis 1 gene: identification and phenotype of five patients. *Am. J. Hum. Genet.* 54, 424–436.

Kehrer-Sawatzki, H., Kluwe, L., Funsterer, C., and Mautner, V. F. (2005). Extensively high load of internal tumors determined by whole body MRI scanning in a patient with neurofibromatosis type 1 and a non-LCR-mediated 2-Mb deletion in 17q11.2. *Hum. Genet.* 116, 466–475. doi: 10.1007/s00439-005-1265-4

Kehrer-Sawatzki, H., Kluwe, L., Salamon, J., Well, L., Farschtschi, S., Rosenbaum, T., et al. (2020). Clinical characterization of children and adolescents with NF1 microdeletions. *Childs Nerv. Syst.* 36, 2297–2310. doi: 10.1007/s00381-020-04717-0

Kehrer-Sawatzki, H., Kluwe, L., Sandig, C., Kohn, M., Wimmer, K., Krammer, U., et al. (2004). High frequency of mosaicism among patients with neurofibromatosis type 1 (NF1) with microdeletions caused by somatic recombination of the JJAZ1 gene. *Am. J. Hum. Genet.* 75, 410–423. doi: 10.1086/423624

Kehrer-Sawatzki, H., Mautner, V. F., and Cooper, D. N. (2017). Emerging genotype-phenotype relationships in patients with large NF1 deletions. *Hum. Genet.* 136, 349–376. doi: 10.1007/s00439-017-1766-y

Kehrer-Sawatzki, H., Schmid, E., Funsterer, C., Kluwe, L., and Mautner, V. F. (2008). Absence of cutaneous neurofibromas in an NF1 patient with an atypical deletion partially overlapping the common 1.4 Mb microdeleted region. *Am. J. Med. Genet. A* 146A, 691–699. doi: 10.1002/ajmg.a.32045

Kehrer-Sawatzki, H., Tinschert, S., and Jenne, D. E. (2003). Heterogeneity of breakpoints in non-LCR-mediated large constitutional deletions of the 17q11.2 NF1 tumour suppressor region. *J. Med. Genet.* 40:e116. doi: 10.1136/jmg.40.10.e116

Kluwe, L., Siebert, R., Gesk, S., Friedrich, R. E., Tinschert, S., Kehrer-Sawatzki, H., et al. (2004). Screening 500 unselected neurofibromatosis 1 patients for deletions of the NF1 gene. *Hum. Mutat.* 23, 111–116. doi: 10.1002/humu.10299

Lammert, M., Friedman, J. M., Kluwe, L., and Mautner, V. F. (2005). Prevalence of neurofibromatosis 1 in German children at elementary school enrollment. *Arch. Dermatol.* 141, 71–74. doi: 10.1001/archderm.141.1.71

Lopez-Correa, C., Dorschner, M., Brems, H., Lazaro, C., Clementi, M., Upadhyaya, M., et al. (2001). Recombination hotspot in NF1 microdeletion patients. *Hum. Mol. Genet.* 10, 1387–1392. doi: 10.1093/hmg/10.13.1387

Mantripragada, K. K., Thuresson, A. C., Piotrowski, A., Diaz de Stahl, T., Menzel, U., Grigelionis, G., et al. (2006). Identification of novel deletion breakpoints bordered by segmental duplications in the NF1 locus using high resolution array-CGH. *J. Med. Genet.* 43, 28–38. doi: 10.1136/jmg.2005.033795

Martin, I., Andres, C. R., Vedrine, S., Tabagh, R., Michelle, C., Jourdan, M. L., et al. (2009). Effect of the oligodendrocyte myelin glycoprotein (OMgp) on the expansion and neuronal differentiation of rat neural stem cells. *Brain Res.* 1284, 22–30. doi: 10.1016/j.brainres.2009.05.070

Mautner, V. F., Kluwe, L., Friedrich, R. E., Roehl, A. C., Bammert, S., Hogel, J., et al. (2010). Clinical characterisation of 29 neurofibromatosis type-1 patients with molecularly ascertained 1.4 Mb type-1 NF1 deletions. *J. Med. Genet.* 47, 623–630. doi: 10.1136/jmg.2009.075937

Mensink, K. A., Ketterling, R. P., Flynn, H. C., Knudson, R. A., Lindor, N. M., Heese, B. A., et al. (2006). Connective tissue dysplasia in five new patients with NF1 microdeletions: further expansion of phenotype and review of the literature. *J. Med. Genet.* 43:e8. doi: 10.1136/jmg.2005.034256

Messiaen, L., Vogt, J., Bengesser, K., Fu, C., Mikhail, F., Serra, E., et al. (2011). Mosaic type-1 NF1 microdeletions as a cause of both generalized and segmental neurofibromatosis type-1 (NF1). *Hum. Mutat.* 32, 213–219. doi: 10.1002/humu.21418

Neuhausler, L., Summerer, A., Cooper, D. N., Mautner, V. F., and Kehrer-Sawatzki, H. (2018). Pronounced maternal parent-of-origin bias for type-1 NF1 microdeletions. *Hum. Genet.* 137, 365–373. doi: 10.1007/s00439-018-1888-x

Ottenhoff, M. J., Rietman, A. B., Mous, S. E., Plasschaert, E., Gawehns, D., Brems, H., et al. (2020). Examination of the genetic factors underlying the cognitive variability associated with neurofibromatosis type 1. *Genet. Med.* 22, 889–897. doi: 10.1038/s41436-020-0752-2

Park, V. M., and Pivnick, E. K. (1998). Neurofibromatosis type 1 (NF1): a protein truncation assay yielding identification of mutations in 73% of patients. *J. Med. Genet.* 35, 813–820. doi: 10.1136/jmg.35.10.813

Pasmant, E., Sabbagh, A., Spurlock, G., Laurendeau, I., Grillo, E., Hamel, M. J., et al. (2010). NF1 microdeletions in neurofibromatosis type 1: from genotype to phenotype. *Hum. Mutat.* 31, E1506–E1518. doi: 10.1002/humu.21271

Riva, P., Corrado, L., Natacci, F., Castorina, P., Wu, B. L., Schneider, G. H., et al. (2000). NF1 microdeletion syndrome: refined FISH characterization of sporadic and familial deletions with locus-specific probes. *Am. J. Hum. Genet.* 66, 100–109. doi: 10.1086/302709

Roehl, A. C., Vogt, J., Mussotter, T., Zickler, A. N., Spoti, H., Hogel, J., et al. (2010). Intrachromosomal mitotic nonallelic homologous recombination is the major molecular mechanism underlying type-2 NF1 deletions. *Hum. Mutat.* 31, 1163–1173. doi: 10.1002/humu.21340

Steinmann, K., Kluwe, L., Cooper, D. N., Brems, H., De Raedt, T., Legius, E., et al. (2008). Copy number variations in the NF1 gene region are infrequent and do not predispose to recurrent type-1 deletions. *Eur. J. Hum. Genet.* 16, 572–580. doi: 10.1038/sj.ejhg.5202002

Stephens, K., Kayes, L., Riccardi, V. M., Rising, M., Sybert, V. P., and Pagon, R. A. (1992). Preferential mutation of the neurofibromatosis type 1 gene in paternally derived chromosomes. *Hum. Genet.* 88, 279–282. doi: 10.1007/BF00197259

Strassmeier, W. (1980). Early intervention programs for handicapped and retarded children from age 0 to 5. *Int. J. Rehabil. Res.* 3, 533–535.

Summerer, A., Mautner, V. F., Upadhyaya, M., Claes, K. B. M., Hogel, J., Cooper, D. N., et al. (2018). Extreme clustering of type-1 NF1 deletion breakpoints co-locating with G-quadruplex forming sequences. *Hum. Genet.* 137, 511–520. doi: 10.1007/s00439-018-1904-1

Summerer, A., Schafer, E., Mautner, V. F., Messiaen, L., Cooper, D. N., and Kehrer-Sawatzki, H. (2019). Ultra-deep amplicon sequencing indicates absence of low-grade mosaicism with normal cells in patients with type-1 NF1 deletions. *Hum. Genet.* 138, 73–81. doi: 10.1007/s00439-018-1961-5

Tastet, J., Decalonne, L., Marouillat, S., Malvy, J., Thepault, R. A., Toutain, A., et al. (2015). Mutation screening of the ubiquitin ligase gene RNF135 in French patients with autism. *Psychiatr. Genet.* 25, 263–267. doi: 10.1097/YPG.0000000000000100

Tucker, T., Wolkenstein, P., Revuz, J., Zeller, J., and Friedman, J. M. (2005). Association between benign and malignant peripheral nerve sheath tumors in NF1. *Neurology* 65, 205–211. doi: 10.1212/01.wnl.0000168830.79997.13

Upadhyaya, M., Roberts, S. H., Maynard, J., Sorour, E., Thompson, P. W., Vaughan, M., et al. (1996). A cytogenetic deletion, del(17)(q11.22q21.1), in a patient with sporadic neurofibromatosis type 1 (NF1) associated with dysmorphism and developmental delay. *J. Med. Genet.* 33, 148–152. doi: 10.1136/jmg.33.2.148

Uusitalo, E., Leppavirta, J., Koffert, A., Suominen, S., Vahtera, J., Vahlberg, T., et al. (2015). Incidence and mortality of neurofibromatosis: a total population study in Finland. *J. Invest. Dermatol.* 135, 904–906. doi: 10.1038/jid.2014.465

Venturin, M., Carra, S., Gaudenzi, G., Brunelli, S., Gallo, G. R., Moncini, S., et al. (2014). ADAP2 in heart development: a candidate gene for the occurrence of cardiovascular malformations in NF1 microdeletion syndrome. *J. Med. Genet.* 51, 436–443. doi: 10.1136/jmedgenet-2013-102240

Venturin, M., Gervasini, C., Orzan, F., Bentivegna, A., Corrado, L., Colapietro, P., et al. (2004a). Evidence for non-homologous end joining and non-allelic homologous recombination in atypical NF1 microdeletions. *Hum. Genet.* 115, 69–80. doi: 10.1007/s00439-004-1101-2

Venturin, M., Guarnieri, P., Natacci, F., Stabile, M., Tenconi, R., Clementi, M., et al. (2004b). Mental retardation and cardiovascular malformations in NF1 microdeleted patients point to candidate genes in 17q11.2. *J. Med. Genet.* 41, 35–41. doi: 10.1136/jmg.2003.014761

Viskochil, D., Buchberg, A. M., Xu, G., Cawthon, R. M., Stevens, J., Wolff, R. K., et al. (1990). Deletions and a translocation interrupt a cloned gene at the neurofibromatosis type 1 locus. *Cell* 62, 187–192. doi: 10.1016/0092-8674(90)90252-a

Vogt, J., Bengesser, K., Claes, K. B., Wimmer, K., Mautner, V. F., van Minkelen, R., et al. (2014). SVA retrotransposon insertion-associated deletion represents a novel mutational mechanism underlying large genomic copy number changes with non-recurrent breakpoints. *Genome Biol.* 15:R80. doi: 10.1186/gb-2014-15-6-r80

Vogt, J., Mussotter, T., Bengesser, K., Claes, K., Hogel, J., Chuzhanova, N., et al. (2012). Identification of recurrent type-2 NF1 microdeletions reveals a mitotic nonallelic homologous recombination hotspot underlying a human genomic disorder. *Hum. Mutat.* 33, 1599–1609. doi: 10.1002/humu.22171

Vogt, J., Nguyen, R., Kluwe, L., Schuhmann, M., Roehl, A. C., Mussotter, T., et al. (2011). Delineation of the clinical phenotype associated with non-mosaic type-2 NF1 deletions: two case reports. *J. Med. Case Rep.* 5:577. doi: 10.1186/1752-1947-5-577

Vulto-van Silfhout, A. T., van Ravenswaaij, C. M., Hehir-Kwa, J. Y., Verwiel, E. T., Dirks, R., van Vooren, S., et al. (2013). An update on ECARUCA, the European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations. *Eur. J. Med. Genet.* 56, 471–474. doi: 10.1016/j.ejmg.2013.06.010

Waggoner, D. J., Towbin, J., Gottesman, G., and Gutmann, D. H. (2000). Clinic-based study of plexiform neurofibromas in neurofibromatosis 1. *Am. J. Med. Genet.* 92, 132–135.

Zhang, J., Tong, H., Fu, X., Zhang, Y., Liu, J., Cheng, R., et al. (2015). Molecular characterization of NF1 and neurofibromatosis type 1 genotype-phenotype correlations in a Chinese population. *Sci. Rep.* 5:11291. doi: 10.1038/srep11291

Zhang, M., Wang, Y., Jones, S., Sausen, M., McMahon, K., Sharma, R., et al. (2014). Somatic mutations of SUZ12 in malignant peripheral nerve sheath tumors. *Nat. Genet.* 46, 1170–1172. doi: 10.1038/ng.3116

Zickler, A. M., Hampp, S., Messiaen, L., Bengesser, K., Mussotter, T., Roehl, A. C., et al. (2012). Characterization of the nonallelic homologous recombination hotspot PRS3 associated with type-3 NF1 deletions. *Hum. Mutat.* 33, 372–383. doi: 10.1002/humu.21644

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

RESEARCH ARTICLE

Microhomology-Mediated Break-Induced Replication: A Possible Molecular Mechanism of the Formation of a Large CNV in *FBN1* Gene in a Patient with Marfan Syndrome

Gergely Buki[1,2], Kinga Hadzsiev[1,2] and Judit Bene[1,2,*]

[1]*Department of Medical Genetics, Clinical Center, Medical School, University of Pécs, Pécs, Hungary;* [2]*Szentágothai Research Centre, University of Pécs, Pécs, Hungary*

Abstract: ***Background:*** Marfan syndrome (MFS) is an autosomal dominant multisystem disorder caused by mutations in the fibrillin-1 gene (*FBN1*). A small portion of them is copy number variations (CNVs), which can occur through recombination-based, replication-based mechanisms or retrotransposition. Not many have been characterized precisely in MFS.

Methods: A female patient with suspected Marfan syndrome was referred for genetic testing at our institute. After systematic sequencing of *FBN1*, *TGFBR1,* and *TGFBR2* genes, multiplex ligation-dependent probe amplification was applied. Long-range PCR, subsequent Sanger sequencing with designed primers, and preliminary *in silico* analysis were applied for the precise characterization of the breakpoints.

Results: Primary analysis displayed a *de novo* large deletion affecting exons 46 and 47 in the *FBN1* gene, which resulted in the loss of the 31st and 32nd calcium-binding EGF-like domains. Further examination of the breakpoints showed a 4916 nucleotide long deletion localized in intronic regions. Surprisingly a 'TG' dinucleotide insertion was detected at the junction. We hypothesize that the CNV formation was generated by a rare event based on the known microhomology-mediated break-induced replication (MMBIR).

Conclusion: An increasing number of CNVs are associated with Mendelian diseases and other traits. Approximately 2-7% of the cases in MFS are caused by CNVs. Up to date, hardly any model was proposed to demonstrate the formation of these genomic rearrangements in the *FBN1* gene. Hereby, with the help of previous models and breakpoint analysis, we presented a potential mechanism (based on MMBIR) in the formation of this large deletion.

ARTICLE HISTORY

Received: February 14, 2022
Revised: February 16, 2022
Accepted: March 08, 2022

DOI:
10.2174/1566524022666220428111943

Keywords: MMBIR, Marfan syndrome, *FBN1* gene, CNV, genomic rearrangement, breakpoint analyses.

1. INTRODUCTION

Marfan syndrome (MFS; OMIM #154700) is an autosomal dominant, multisystem disorder with high clinical heterogeneity. Mainly the ocular, skeletal, and cardiovascular systems are affected, where cardiovascular abnormalities can be life-threatening. The prevalence of MFS is estimated at 1/5,000 [1, 2]. The syndrome is caused by mutations in the fibrillin 1 (*FBN1*) gene [3], located on the long arm of chromosome 15 (15q21.1), comprising 65 coding exons. The gene encodes a protein called fibrillin-1, which is a major component of microfibrils in the extracellular matrix. A minority of the cases show pathogenic variations in the genes transforming growth factor β receptors 1 and 2 (*TGFBR1* and *TGFBR2*, respectively) [4, 5]. Although most of the disease-causing mutations in the aforementioned *TGFBR1* and *TGFBR2* genes are responsible for developing another inherited connective tissue disorder called Loeys-Dietz syndrome [6].

Based on the ClinVar (https://www.ncbi.nlm.nih.gov/clinvar) database, up to date, more than 5000 variations are known in the *FBN1* gene, and almost half of them are disease-causing pathogenic or likely pathogenic mutations. Among these, missense, nonsense, frameshift, splice-site, in-frame deletions, and insertions have been identified so far. Additionally, large genomic rearrangements have also been

*Address correspondence to this author at the Department of Medical Genetics, University of Pécs, H-7624 Pécs, Szigeti 12, Hungary;
Tel: (+36) 72-536427; Fax: (+36) 72-536032;
E-mail: bene.judit@pte.hu

1566-5240/XX $65.00+.00

reported. Deletion of the entire *FBN1* gene and single or multiple exons are also known [7-10]. In contrast, no duplications affecting the exons of the *FBN1* gene have been reported until now. Approximately 2-7% of MFS patients have been detected with a copy number variation (CNV) in the *FBN1* gene [11].

CNVs, encompassing losses or gains of relatively large genomic DNA segments, are represented widely in the human genome and they are one of the major sources of genetic diversity as genome-wide analysis tools and other large-scale population studies demonstrated [12, 13]. Recent research suggested that CNVs appear to have a much higher *de novo* locus-specific mutation rate than single nucleotide polymorphisms (SNPs) [14, 15]. In addition, CNVs have been associated not only with genomic disorders but also with complex traits in humans (for instance autism and schizophrenia) and may be responsible for some advantageous human-specific traits (for instance cognition and endurance running) [16-18]. Moreover, nowadays, emerging evidence shows that CNVs may cause Mendelian diseases or sporadic traits as well [18].

The formation of CNVs can occur through recombination-based [19], and replication-based mechanisms or retrotransposition [20, 21]. A couple of different mechanisms have already been suggested so far, including non-allelic homologous recombination (NAHR), non-homologous end-joining (NHEJ), or microhomology-mediated end-joining (MMEJ), along with fork stalling and template switching (FoSTeS) or microhomology-mediated break-induced replication (MMBIR). In the case of CNVs in the *FBN1* gene, accurate breakpoint analyses have been performed only in a few cases. Therefore, the precise mechanism responsible for the CNVs has not been elucidated frequently.

Hereby we present a *de novo* two exon deletion in the *FBN1* gene, which caused Marfan syndrome in a female patient. A rare rearrangement mechanism, MMBIR, is being proposed for the first time in the literature on Marfan syndrome as an underlying mechanism of this CNV formation.

2. MATERIALS AND METHODS

2.1. Participants

A female patient was referred for genetic testing to our institute (Department of Medical Genetics) because of a suspected Marfan syndrome or a related connective tissue disorder. Genetic counselling verified that the patient fulfilled the revised Ghent criteria. Systematic sequencing of *FBN1*, *TGFBR1*, and *TGFBR2* genes could not identify pathogenic point mutations. Thereby the *FBN1* and *TGFBR2* large del/dup screening was applied with MLPA. The study was approved by the ethics committee of the University of Pecs (Protocol 8770-PTE/2021). The patient gave informed consent to genetic testing according to national regulations.

2.2. Identification of the Breakpoints in our Patients

To confirm the deletion and determine the breakpoints, long-range PCR and subsequent Sanger sequencing were applied. We designed primers targeting the flanking region of the predicted deletion (45F: 5'-TCTTGGTTGCTTCCAAATTC-3' 47R: 5'-GCTGGAACACTAGAGATGATG-3'). A QIAGEN long-range PCR kit (Qiagen, Hilden, Germany) was applied according to the manufacturer's instructions with the following cycling process: 3-min initial denaturation at 93°C, 35 cycles of 15 s at 93°C, 30 s at 55°C, and 8 min at 68°C. PCR analysis displayed approximately 6 kb and 1.5 kb products. The smaller fragment was excised from agarose gel and cleaned with the help of Nucleospin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany).

After purification of the PCR product, it was sequenced using 45F,47R, and newly designed internal primers (FBN1delF:5'-CAGGAAGAATGTGTT ATTTTGCTC-3' and FBN1delR: 5'-GTCTCAGAATGTA TCCCTCAC-3') using a BigDye Terminator Cycle Sequencing Ready Reaction kit v1.1 on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Forster City, USA).

2.3. *In silico* Analysis

Tandem Repeats Finder [22] and REPFINDER [23] were applied to analyze the neighboring region of the breakpoints to reveal remarkable sequence homology. Tandem Repeats Finder was applied to locate and display tandem repeats in DNA sequences. REPFINDER was applied to identify identical direct and/or inverted repeat sequences. RepeatMasker [24] was applied to screen low complexity DNA sequences and interspersed repeats within the proximity of the breakpoints. Cross_match and HMMER search engines were used with slow sensitivity in the analysis. REPEATAROUND [25] was used to determine direct repeats, mirror repeats, and inverted repeats in the immediate vicinity of the breakpoint. The examined size range was determined at a distance of 30 bases both upstream and downstream from the breakpoints. Tetraplex formation of a single strand of DNA with another unpaired single strand could be generated by G-rich sequences. QGRS MAPPER [26] was applied to examine G-rich sequences.

3. RESULTS

A novel large deletion (exons 46-47) was identified in a 22-year-old female. As a result of this deletion, the 31st and 32nd calcium-binding EGF-like domains of the fibrillin-1 protein was affected, contributing to the development of the Marfan syndrome. The *de novo* origin of the deletion was confirmed by the molecular genetic testing of her parents.

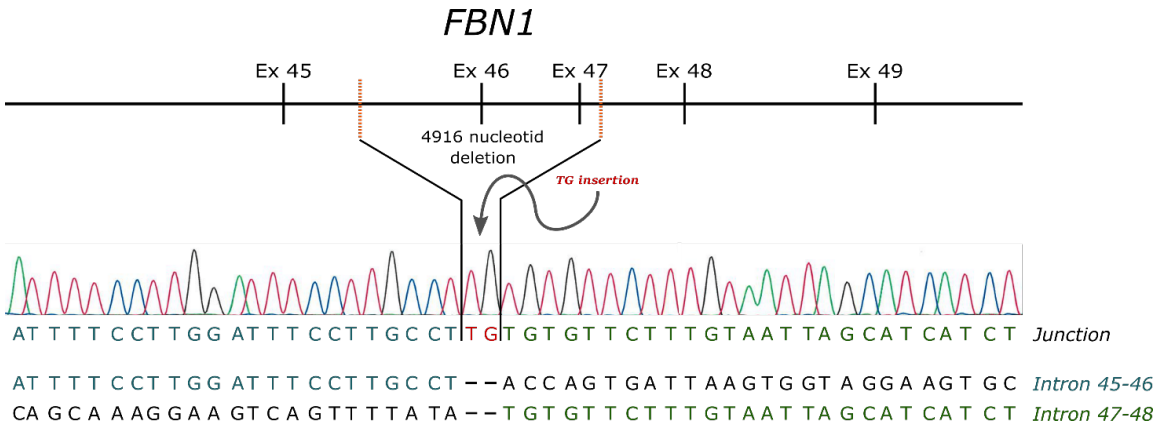


Fig. (1). Depiction of the genome in the region of the deletion and the sequences of PCR products spanning the breakpoint junctions of the deletion. Blue letters indicate intron 45 sequences and green letters indicate intron 47 sequences. The open arrow below the gene name indicates the direction of transcription. Exons are represented by bars and marked with the corresponding number. Red dotted lines mark the (most telomeric position of the possible breakpoints) position of the breakpoints. Black letters denote the sequences of the deleted regions. The curved arrow indicates TG insertion. All nucleotide positions are represented by the human genome reference sequence (NCBI build hg19). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.1. Breakpoint Analyses and Experimental Findings Supported the Proposed Mechanism Behind the Identified Deletion

The breakpoint junctions were determined by long-range PCR. A wild type (6kb fragment) and a deleted allele (1.5kb fragment) were amplified. Excision and purification of the smaller fragment and direct sequencing of the deletion junction amplicon displayed a 4916 bp long deletion. After the junction was identified, the breakpoints localized into intronic regions. Surprisingly a TG insertion was found near the breakpoints, which wasn't found in the parents' sequence (Fig. **1**).

In silico analyses of the breakpoints including the Tandem Repeats Finder Program and REPFINDER did not reveal remarkable sequence homology neighboring the deletion region. Both programs were applied for the analysis of the genomic sequence between exon 44 and exon 50. The examination did not reveal significant repeat sequences. From exon 45 to exon 48, the sequence was analyzed by RepeatMasker, which did not find any L1, Alu, LTR, or MIR DNA elements in the proximity of the deletion. REPEATAROUND was applied for a more specific analysis, showing slight differences in the repeat variations (Table **1**) with or without the 'TG' insertion. QGRS MAPPER did not detect any significant G-rich sequences.

We hypothesize that the CNV formation was generated by a rare event based on the known microhomology-mediated break-induced replication.

4. DISCUSSION

Numerous recent research findings imply that CNVs have a significant effect on human traits, diseases, and even evolution [18]. The use of high-resolution genome-wide analysis tools and platforms improved our knowledge of CNVs and their effect. Such genomic rearrangements can convey abnormal phenotypes through various molecular mechanisms including those affecting dosage sensitivity of a gene, gene fusion or gene interruption at the breakpoint junctions, or unmasking of recessive alleles. Furthermore, other noncoding cis- and trans-regulatory elements such as enhancers and promoters could be affected. The contributions of CNVs to human phenotypes, especially complex diseases and other specific traits are yet to be fully understood. Although there is growing evidence that CNVs may cause Mendelian diseases or sporadic traits as well. Approximately 10% of the Mendelian disorders are caused by CNVs. In the case of Marfan syndrome, up to date ca 2-7% of the disease-causing mutations belong to CNVs [11, 18].

According to breakpoint analysis, CNVs can be divided into two groups, namely recurrent and non-recurrent forms. The latter group is represented in MFS so far. Most of the non-recurrent CNVs are generated by NHEJ or other replication-based mechanisms (FoSTeS, MMBIR) which are increasingly accepted as mechanisms in the development of rare pathogenic CNVs [27]. Most of the recurrent CNVs are generated by NAHR.

In silico analyses of the genomic architecture surrounding the detected CNV in our patient revealed a novel non-recurrent genomic rearrangement. Three major mechanisms did not seem to explain the mechanism behind the deletion. NAHR is based on sequence homology and leads to the formation of CNVs with recurrent breakpoints. However, no extensive homology, including low copy repeats/segmental duplication, was found in the area of the breakpoints in our patient using Tandem Repeats Finder Program, REPuter, and REPFINDER software. As a consequence, the deletion is probably not mediated by the known NAHR mechanism. Some non-recurrent

Table 1. Representation of the detected repeats (direct, indirect, mirror, complementary) with and without the TG insertion by REPEATAROUND.

-	5' breakpoint with TG insertion	5' breakpoint without TG insertion
Direct repeat	4 base (2)	4 base (2)
	5 base (4)	**5 base (3)**
	8 base (1)	8 base (1)
Indirect repeat	4 base (6)	4 base (6)
	5 base (2)	5 base (1)
Mirror repeat	4 base (4)	4 base (2)
Complementary repeat	4 base (5)	4 base (5)
	5 base (2)	5 base (2)

deletions can occur *via* NHEJ, which is a major mechanism used by eukaryotic cells to repair double-strand breaks (DSBs) [28]. However, NHEJ repairs blunt ends [29], thus in the case of resected or modified ends this mechanism is blocked which is supposed to be involved in our CNV formation. An additional characteristic of NHEJ is that it often leaves informational scars at the breakpoint, including the addition or cleavage of several nucleotides [18, 30]. In our patient, a TG dinucleotide insertion was detected along with the large 4916 bp deletion. However, in the NHEJ mechanism certain repetitive elements such as MIR, LTR, LINE, Alu, and other sequence motifs (*e.g*. TTTAAA) were indicated at the breakpoints or within close proximity [31-33]. Neither of the mentioned motifs or DNA elements was present in our patient's DNA. Based on the sequential environment, NHEJ can be ruled out for the creation of deletion in our patient. The FoSTeS mechanism is frequently discussed in the complex rearrangement formation, through disengaging and invading a new replication fork in close proximity and an extension of the sequence from a microhomologous region. Moreover, in contrast to MMBIR, resection of the sequence is not involved in this model. We hypothesize that in our case the microhomologous region became available after resection, and the detected CNV was a simple rearrangement, therefore we excluded the possibility of FoSTeS.

One possible way of genomic rearrangement formation takes place through the replication machinery. Incomplete, erroneous, or untimely DNA replication events can lead to various types of mutations, including CNVs as well, which can contribute to the development of disorders [34]. Moreover, all DNA replication events are harmonized with other cellular events such as transcription and DNA repair [35, 36]. Previous studies [37, 38] outlined that replication abnormalities are often associated with various kinds of stress, including DNA binding proteins, DNA-RNA interaction, DNA damage, secondary DNA structures, and metabolic conditions [39]. This causes the replication fork to stall and collapse and a single-ended DSB will be created.

The presence of certain *de novo* DNA structures frequently contributes to forming certain genomic rearrangements with non-recurrent breakpoints. Depending on this we applied *in silico* analysis of the breakpoint and its close vicinity to search for various kinds of repeat sequences (direct, inverted, and mirror repeats) at the breakpoint. REPEATAROUND revealed, that if a 'TG' insertion occurs, a 5 base long direct repeat will appear ('CCTTGCCTTG') at the breakpoint.

We hypothesize that the deletion found in our primary case (Fig. **2**) might be the result of the MMBIR mechanism described earlier in detail by Hastings et al [38] and later by Ottaviani and colleagues [40]. According to the model the replication potentially stopped and stalled at an unexpected event. A potential dinucleotide insertion ('TG') created a 'CCTTGCCTTG' direct repeat sequence (I.) which could interrupt the replication machinery. The insertion itself or the generated repeat potentially caused the replication fork to slow down, stall and collapse. Presumably, this event resulted in a single DSB. Thereafter, a 5' to 3' resection generated a sequence with a short 3' overhang (II.). The resection exposed a DNA segment with possible microhomology to another DNA segment in close proximity. This 3' overhang part of the dsDNA invades the microhomologous region in a D-loop, (III.) then anneals to this sequence and restarts the synthesis. We suggest a simultaneous adenine-to-guanine substitution (IV.) due to an erroneous DNA repair, which at this position creates a microhomology on the other DNA segment. Eventually, the final sequence indicates a 4916 bp long deletion with inserted 'TG' nucleotides at the breakpoint (V.). In our case, a short microhomologous region is involved in the proposed molecular mechanism. Since the *FBN1* gene is localized on the reverse strand, the sequence of *FBN1* was represented in the reverse orientation during exploring the mechanism behind the CNV formation. However, for easier explanation and understanding, we indicated the final sequence in regular orientation (Fig. **2**).

In our case, we suppose MMBIR is a rare event to form the CNV. In some cases, the MMBIR model has

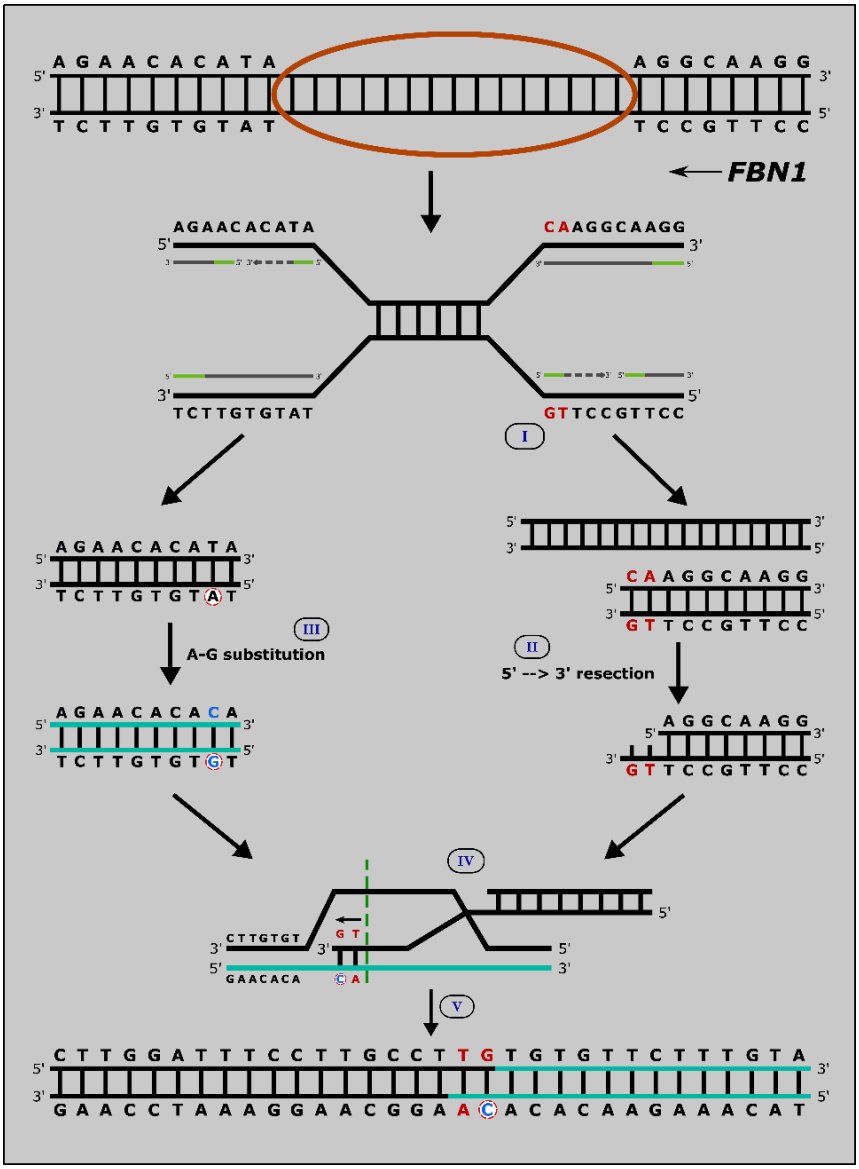


Fig. (2). A potential candidate for this mechanism is microhomology-mediated break-induced replication (MMBIR): (**I**) the replication fork stalls, collapses, and a single-ended double-strand break is created, (**II**) 5' to 3' resection generates a sequence with short 3' single-stranded overhang, (**III**) A-G substitution due to a possible error of DNA repair, (**IV**) D-loop formation by the template strand and invasion by the 3' overhang, which anneals to the microhomologous region and restarts synthesis; (**V**) synthesis is continued straight ahead. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

been associated with other non-recurrent CNVs resulting in genomic disorders such as Cornelia de Lange syndrome and haemophilia A [41]. MMBIR is often associated with small stretches (1–4 bp) of microhomology, further supporting our hypothesis [42-44].

Copy number variation analyses among Marfan patients published so far revealed single exon or multiple exon deletions in the *FBN1* gene along with the whole *FBN1* gene deletion (Fig. **3** and **4**) [6-11, 45-59]. Interestingly, accurate breakpoint analyses have been performed only in a few cases. Short stretches of identical sequences at the sites of breakpoints can be found behind the CNVs formation, however, the precise mechanism responsible for these CNVs has not been elucidated (Table **2**). In two cases, an extra nucleotide was detected similarly to our cases, however without an explanation.

CONCLUSION

CNVs are being associated more and more with certain diseases and traits, for instance, Mendelian diseases, sporadic birth defects, complex traits, and other sporadic traits, thus contributing to the genetic variation of the individuals. Various models (NHEJ, MMEJ, NAHR, FoSTeS, MMBIR, retrotransposition) have been proposed to explain the formation of the different CNVs. In the case of CNVs in the *FBN1* gene,

FBN1
Single exon deletions

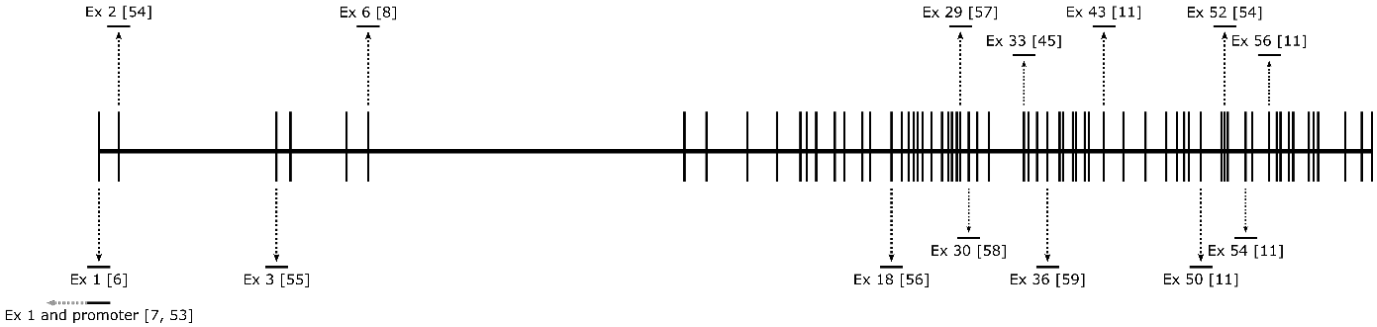


Fig. (3). Representation of the known single exon deletions throughout the *FBN1* gene. The original exon numbering was used as reported in the referred article. Solid vertical lines represent all of the exons, dotted lines indicate the known deleted exons. Numbers in the brackets indicate the reference.

FBN1
Multiple exon deletions

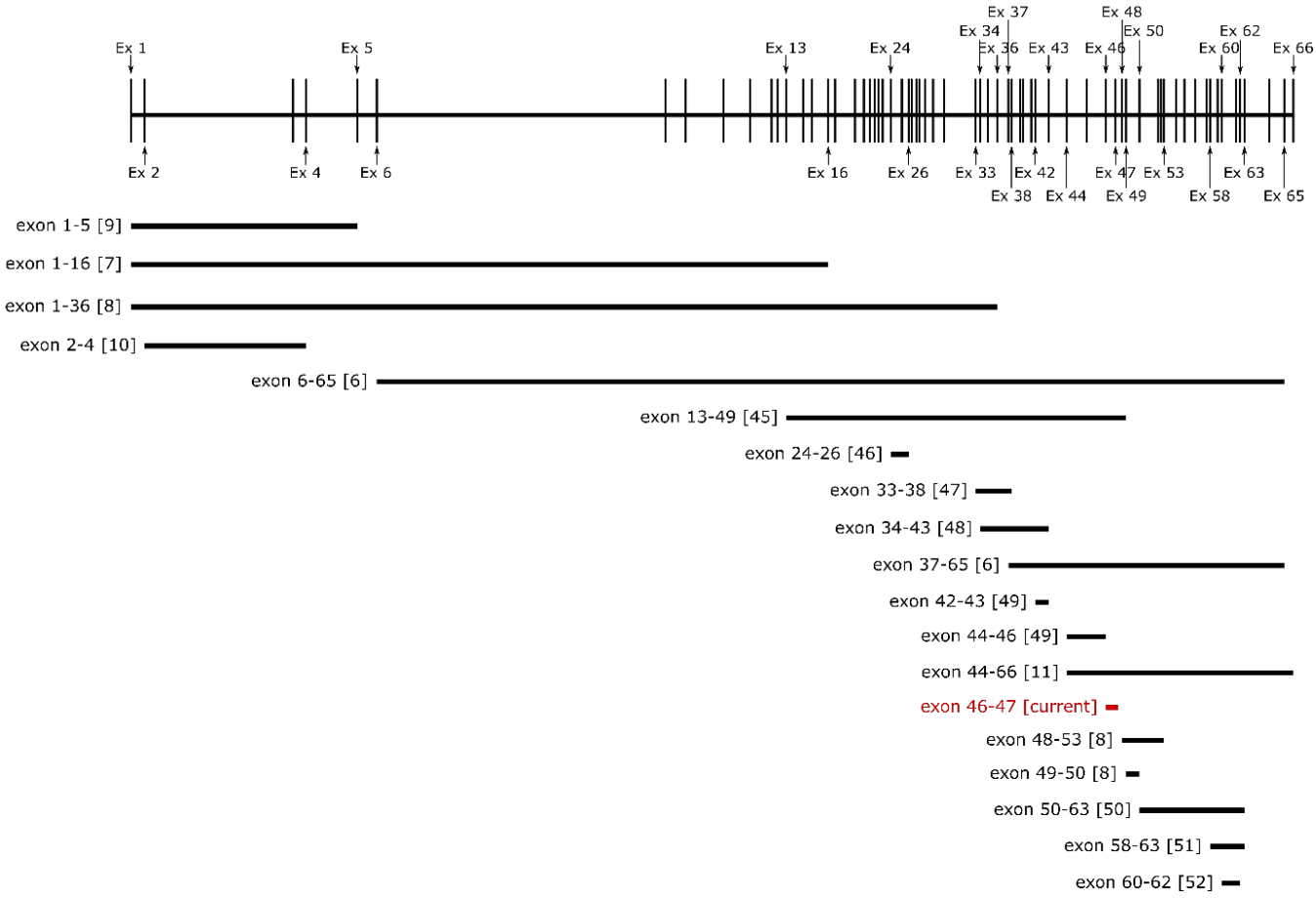


Fig. (4). Representation of the known multiple exon deletions throughout the *FBN1* gene. The original exon numbering was used as reported in the referred article. Solid vertical lines represent all of the exons. Solid horizontal lines illustrate the affected exons. Arrows display the involved exons. Numbers in the brackets indicate the reference. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 2. Characterization of the known breakpoints presented in the referring article.

Single and Multi-exon (*FBN1*)	Microhomology Present	Number of Affected Nucleotides (in Microhomology)	Insertion or Deletion of Nucleotides Near the Breakpoints	Localization of the Breakpoints	Refs.
Ex1	+	2 (GC)	-	Intron	[7]
Ex6	+	4 (CTGA)	T insertion	Intron	[8]
Ex43	N/A	N/A	-	Intron	[11]
Ex50	N/A	N/A	-	Intron	[11]
Ex54	N/A	N/A	-	Intron	[11]
Ex56	N/A	N/A	-	Intron	[11]
Ex1-16	+	2 (CC)		Intron	[7]
Ex2-4	-	-	-	Intron	[10]
Ex42–43	+	5 (CAGTA and/or GGAAA)	-	Intron	[49]
Ex44–46	+	5 (ATTTT)	-	Intron	[49]
Ex46-47	**+**	**TG**	**TG insertion**	**Intron**	**Current study**
Ex48–53	+	4 (CTGA)	-	Intron	[8]
Ex49–50	-	-	G insertion	Intron	[8]
Ex58–63	+	4 (ATTT)	-	Intron	[51]

N/A: no straightforward information available.

Exon numbering: original numbering shows the affected exons as it was reported in the referred article.

Our case (exon 46-47 deletion) corresponds to the 66 exon numbering and is represented by the human genome reference sequence (NCBI build hg19).

hardly any model was proposed to demonstrate the formation of these genomic rearrangements. More and more CNVs are demonstrated with non-recurrent breakpoints. An increasing number of these are explained by replication-based mechanisms (FoSTeS, MMBIR). Hereby we presented a potential mechanism (based on MMBIR) of the formation of a large *de novo* deletion, affecting two exons within the *FBN1* gene. A further comprehensive investigation is required to understand the precise molecular mechanism in the formation of CNVs.

LIST OF ABBREVIATIONS

MFS = Marfan syndrome

CNVs = Copy number variations

MMBIR = Microhomology-mediated break-induced replication

FBN1 = Fibrillin 1 gene

NHEJ = Non-homologous end-joining

NAHR = Non-allelic homologous recombination

MMEJ = Microhomology-mediated end-joining

AUTHORS' CONTRIBUTIONS

JB conceived and designed the research. GB performed the genetic investigations, KH performed patient examinations, GB prepared figures; GB and JB drafted the manuscript. Moreover, GB and JB edited and KH and JB revised the manuscript. Additionally, JB approved the final version of the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The Ethics Committee of the University of Pecs, Hungary (Protocol 8770-PTE 2021) reviewed and approved the studies involving human participants.

HUMAN AND ANIMAL RIGHTS

No animals were used in this study. All reported experiments on humans were performed as per the Helsinki Declaration of 1975 and with the Hungarian legal requirements of genetic examination, research and biobanking.

CONSENT FOR PUBLICATION

Informed consent was obtained from all participants included in the study.

AVAILABILITY OF DATA AND MATERIALS

The data will be available upon appropriate request.

FUNDING

This work was supported by grants from the Medical School, University of Pécs (KA 2020-27).

CONFLICT OF INTEREST

The authors declare no conflict of interests, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

REFERENCES

[1] Judge DP, Dietz HC. Marfan's syndrome. Lancet 2005; 366(9501): 1965-76. http://dx.doi.org/10.1016/S0140-6736(05)67789-6 PMID: 16325700

[2] Adams JN, Trent RJ. Aortic complications of Marfan's syndrome. Lancet 1998; 352(9142): 1722-3. http://dx.doi.org/10.1016/S0140-6736(05)79822-6 PMID: 9848345

[3] Sakai LY, Keene DR, Renard M, De Backer J. FBN1: The disease-causing gene for Marfan syndrome and other genetic disorders. Gene 2016; 591(1): 279-91. http://dx.doi.org/10.1016/j.gene.2016.07.033 PMID: 27437668

[4] Mizuguchi T, Collod-Beroud G, Akiyama T, *et al.* Heterozygous TGFBR2 mutations in Marfan syndrome. Nat Genet 2004; 36(8): 855-60. http://dx.doi.org/10.1038/ng1392 PMID: 15235604

[5] Chung BH, Lam ST, Tong TM, *et al.* Identification of novel FBN1 and TGFBR2 mutations in 65 probands with Marfan syndrome or Marfan-like phenotypes. Am J Med Genet A 2009; 149A(7): 1452-9. http://dx.doi.org/10.1002/ajmg.a.32918 PMID: 19533785

[6] Lerner-Ellis JP, Aldubayan SH, Hernandez AL, *et al.* The spectrum of FBN1, TGFβR1, TGFβR2 and ACTA2 variants in 594 individuals with suspected Marfan syndrome, Loeys-Dietz syndrome or Thoracic Aortic Aneurysms and Dissections (TAAD). Mol Genet Metab 2014; 112(2): 171-6. http://dx.doi.org/10.1016/j.ymgme.2014.03.011 PMID: 24793577

[7] Mátyás G, Alonso S, Patrignani A, *et al.* Large genomic fibrillin-1 (FBN1) gene deletions provide evidence for true haploinsufficiency in Marfan syndrome. Hum Genet 2007; 122(1): 23-32. http://dx.doi.org/10.1007/s00439-007-0371-x PMID: 17492313

[8] Li J, Wu W, Lu C, *et al.* Gross deletions in FBN1 results in variable phenotypes of Marfan syndrome. Clin Chim Acta 2017; 474: 54-9. http://dx.doi.org/10.1016/j.cca.2017.08.023 PMID: 28842177

[9] Furtado LV, Wooderchak-Donahue W, Rope AF, *et al.* Characterization of large genomic deletions in the FBN1 gene using multiplex ligation-dependent probe amplification. BMC Med Genet 2011; 12: 119. http://dx.doi.org/10.1186/1471-2350-12-119 PMID: 21936929

[10] Benke K, Ágg B, Meienberg J, *et al.* Hungarian Marfan family with large *FBN1* deletion calls attention to copy number variation detection in the current NGS era. J Thorac Dis 2018; 10(4): 2456-60. http://dx.doi.org/10.21037/jtd.2018.04.40 PMID: 29850152

[11] Yang H, Ma Y, Luo M, *et al.* Identification of gross deletions in FBN1 gene by MLPA. Hum Genomics 2018; 12(1): 46. http://dx.doi.org/10.1186/s40246-018-0178-y PMID: 30286810

[12] Mills RE, Walter K, Stewart C, *et al.* Mapping copy number variation by population-scale genome sequencing. Nature 2011; 470(7332): 59-65. http://dx.doi.org/10.1038/nature09708 PMID: 21293372

[13] Feuk L, Carson AR, Scherer SW. Structural variation in the human genome. Nat Rev Genet 2006; 7(2): 85-97. http://dx.doi.org/10.1038/nrg1767 PMID: 16418744

[14] Lupski JR. Genomic rearrangements and sporadic disease. Nat Genet 2007; 39(7)(Suppl.): S43-7. http://dx.doi.org/10.1038/ng2084 PMID: 17597781

[15] Turner DJ, Miretti M, Rajan D, *et al.* Germline rates of de novo meiotic deletions and duplications causing several genomic disorders. Nat Genet 2008; 40(1): 90-5. http://dx.doi.org/10.1038/ng.2007.40 PMID: 18059269

[16] Lupski JR. An evolution revolution provides further revelation. BioEssays 2007; 29(12): 1182-4. http://dx.doi.org/10.1002/bies.20686 PMID: 18008371

[17] Dumas L, Kim YH, Karimpour-Fard A, *et al.* Gene copy number variation spanning 60 million years of human and primate evolution. Genome Res 2007; 17(9): 1266-77. http://dx.doi.org/10.1101/gr.6557307 PMID: 17666543

[18] Zhang F, Gu W, Hurles ME, Lupski JR. Copy number variation in human health, disease, and evolution. Annu Rev Genomics Hum Genet 2009; 10: 451-81. http://dx.doi.org/10.1146/annurev.genom.9.081307.164217 PMID: 19715442

[19] Lupski JR, Stankiewicz P. Genomic disorders: Molecular mechanisms for rearrangements and conveyed phenotypes. PLoS Genet 2005; 1(6): e49. http://dx.doi.org/10.1371/journal.pgen.0010049 PMID: 16444292

[20] Kazazian HH Jr, Moran JV. The impact of L1 retrotransposons on the human genome. Nat Genet 1998; 19(1): 19-24. http://dx.doi.org/10.1038/ng0598-19 PMID: 9590283

[21] Korbel JO, Urban AE, Affourtit JP, *et al.* Paired-end mapping reveals extensive structural variation in the human genome. Science 2007; 318(5849): 420-6. http://dx.doi.org/10.1126/science.1149504 PMID: 17901297

[22] Benson G. Tandem repeats finder: A program to analyze DNA sequences. Nucleic Acids Res 1999; 27(2): 573-80. http://dx.doi.org/10.1093/nar/27.2.573 PMID: 9862982

[23] Xie X, Liu W, Dong G, Zhu Q, Liu YG. MMEJ-KO: A web tool for designing paired CRISPR guide RNAs for microhomology-mediated end joining fragment deletion. Sci China Life Sci 2021; 64(6): 1021-4. http://dx.doi.org/10.1007/s11427-020-1797-3 PMID: 32996045

[24] Smit A, Hubley R, Green P. RepeatMasker Open-4.0. 2013.

[25] Goios A, Meirinhos J, Rocha R, Lopes R, Amorim A, Pereira L. RepeatAround: A software tool for finding and visualizing repeats in circular genomes and its application to a human mtDNA database. Mitochondrion 2006; 6(4): 218-24. http://dx.doi.org/10.1016/j.mito.2006.06.001 PMID: 16854633

[26] Kikin O, D'Antonio L, Bagga PS. QGRS Mapper: A web-based server for predicting G-quadruplexes in nucleotide sequences. Nucleic Acids Res 2006; 34(Suppl_2): W676-82.

[27] Zhang F, Khajavi M, Connolly AM, Towne CF, Batish SD, Lupski JR. The DNA replication FoSTeS/MMBIR mechanism can generate genomic, genic and exonic complex rearrangements in humans. Nat Genet 2009; 41(7): 849-53. http://dx.doi.org/10.1038/ng.399 PMID: 19543269

[28] Gu W, Zhang F, Lupski JR. Mechanisms for human genomic rearrangements. PathoGenetics 2008; 1(1): 4. http://dx.doi.org/10.1186/1755-8417-1-4 PMID: 19014668

[29] Plesser Duvdevani M, Pettersson M, Eisfeldt J, *et al.* Whole-genome sequencing reveals complex chromosome rearrangement disrupting NIPBL in infant with Cornelia de Lange syndrome. Am J Med Genet A 2020; 182(5): 1143-51. http://dx.doi.org/10.1002/ajmg.a.61539 PMID: 32125084

[30] Lieber MR. The mechanism of human nonhomologous DNA end joining. J Biol Chem 2008; 283(1): 1-5. http://dx.doi.org/10.1074/jbc.R700039200 PMID: 17999957

[31] Shaw CJ, Lupski JR. Non-recurrent 17p11.2 deletions are generated by homologous and non-homologous mechanisms. Hum Genet 2005; 116(1-2): 1-7. http://dx.doi.org/10.1007/s00439-004-1204-9 PMID: 15526218

[32] Toffolatti L, Cardazzo B, Nobile C, *et al.* Investigating the mechanism of chromosomal deletion: Characterization of 39 deletion breakpoints in introns 47 and 48 of the human dystrophin gene. Genomics 2002; 80(5): 523-30. http://dx.doi.org/10.1006/geno.2002.6861 PMID: 12408970

[33] Nobile C, Toffolatti L, Rizzi F, *et al.* Analysis of 22 deletion breakpoints in dystrophin intron 49. Hum Genet 2002; 110(5): 418-21. http://dx.doi.org/10.1007/s00439-002-0721-7 PMID: 12073011

[34] Ekundayo B, Bleichert F. Origins of DNA replication. PLoS Genet 2019; 15(9): e1008320. http://dx.doi.org/10.1371/journal.pgen.1008320 PMID: 31513569

[35] García-Muse T, Aguilera A. Transcription-replication conflicts: How they occur and how they are resolved. Nat Rev Mol Cell Biol 2016; 17(9): 553-63. http://dx.doi.org/10.1038/nrm.2016.88 PMID: 27435505

[36] Lee JA, Carvalho CM, Lupski JR. A DNA replication mechanism for generating nonrecurrent rearrangements associated with genomic disorders. Cell 2007; 131(7): 1235-47. http://dx.doi.org/10.1016/j.cell.2007.11.037 PMID: 18160035

[37] Hastings PJ, Lupski JR, Rosenberg SM, Ira G. Mechanisms of change in gene copy number. Nat Rev Genet 2009; 10(8): 551-64. http://dx.doi.org/10.1038/nrg2593 PMID: 19597530

[38] Hastings PJ, Ira G, Lupski JR. A microhomology-mediated break-induced replication model for the origin of human copy number variation. PLoS Genet 2009; 5(1): e1000327. http://dx.doi.org/10.1371/journal.pgen.1000327 PMID: 19180184

[39] Zeman MK, Cimprich KA. Causes and consequences of replication stress. Nat Cell Biol 2014; 16(1): 2-9. http://dx.doi.org/10.1038/ncb2897 PMID: 24366029

[40] Ottaviani D, LeCain M, Sheer D. The role of microhomology in genomic structural variation. Trends Genet 2014; 30(3): 85-94. http://dx.doi.org/10.1016/j.tig.2014.01.001 PMID: 24503142

[41] Tokoro M, Tamura S, Suzuki N, *et al.* Aberrant X chromosomal rearrangement through multi-step template switching during sister chromatid formation in a patient with severe hemophilia A. Mol Genet Genomic Med 2020; 8(9): e1390. http://dx.doi.org/10.1002/mgg3.1390 PMID: 32627361

[42] Beck CR, Carvalho CMB, Akdemir ZC, *et al.* Megabase length hypermutation accompanies human structural variation at 17p11.2. Cell 2019; 176(6): 1310-1324.e10. http://dx.doi.org/10.1016/j.cell.2019.01.045 PMID: 30827684

[43] Liu P, Erez A, Nagamani SC, *et al.* Chromosome catastrophes involve replication mechanisms generating complex genomic rearrangements. Cell 2011; 146(6): 889-903. http://dx.doi.org/10.1016/j.cell.2011.07.042 PMID: 21925314

[44] Redin C, Brand H, Collins RL, *et al*. The genomic landscape of balanced cytogenetic abnormalities associated with human congenital anomalies. Nat Genet 2017; 49(1): 36-45. http://dx.doi.org/10.1038/ng.3720 PMID: 27841880

[45] Blyth M, Foulds N, Turner C, Bunyan D. Severe Marfan syndrome due to FBN1 exon deletions. Am J Med Genet A 2008; 146A(10): 1320-4. http://dx.doi.org/10.1002/ajmg.a.32229 PMID: 18412115

[46] Apitz C, Mackensen-Haen S, Girisch M, *et al.* Neonatal Marfan syndrome: Unusually large deletion of exons 24-26 of FBN1 associated with poor prognosis. Klin Padiatr 2010; 222(4): 261-3. http://dx.doi.org/10.1055/s-0030-1247510 PMID: 20455198

[47] Pees C, Michel-Behnke I, Hagl M, Laccone F. Detection of 15 novel mutations in 52 children from 40 families with the Marfan or Loeys-Dietz syndrome and phenotype-genotype correlations. Clin Genet 2014; 86(6): 552-7. http://dx.doi.org/10.1111/cge.12314 PMID: 24199744

[48] Yoo EH, Woo H, Ki CS, *et al*. Clinical and genetic analysis of Korean patients with Marfan syndrome: Possible ethnic differences in clinical manifestation. Clin Genet 2010; 77(2): 177-82. http://dx.doi.org/10.1111/j.1399-0004.2009.01287.x PMID: 19863550

[49] Liu W, Schrijver I, Brenn T, Furthmayr H, Francke U. Multi-exon deletions of the FBN1 gene in Marfan syndrome. BMC Med Genet 2001; 2: 11. http://dx.doi.org/10.1186/1471-2350-2-11 PMID: 11710961

[50] Voermans Nc, Timmermans J, van Alfen N, *et al.* Neuromuscular features in Marfan syndrome. Clin Genet 2009; 76(1): 25-37. http://dx.doi.org/10.1111/j.1399-0004.2009.01197.x PMID: 19659760

[51] Singh KK, Elligsen D, Liersch R, *et al.* Multi-exon out of frame deletion of the FBN1 gene leading to a severe juvenile onset cardiovascular phenotype in Marfan syndrome. J Mol Cell Cardiol 2007; 42(2): 352-6. http://dx.doi.org/10.1016/j.yjmcc.2006.11.006 PMID: 17189636

[52] Kainulainen K, Sakai LY, Child A, *et al*. Two mutations in Marfan syndrome resulting in truncated fibrillin polypeptides. Proc Natl Acad Sci USA 1992; 89(13): 5917-21. http://dx.doi.org/10.1073/pnas.89.13.5917 PMID: 1631074

[53] McInerney-Leo AM, Marshall MS, Gardiner B, *et al.* Whole exome sequencing is an efficient, sensitive and specific method of mutation detection in osteogenesis imperfecta and Marfan syndrome. Bonekey Rep 2013; 2: 456. http://dx.doi.org/10.1038/bonekey.2013.190 PMID: 24501682

[54] Loeys B, Nuytinck L, Delvaux I, De Bie S, De Paepe A. Genotype and phenotype analysis of 171 patients referred for molecular study of the fibrillin-1 gene FBN1 because of suspected Marfan syndrome. Arch Intern Med 2001; 161(20): 2447-54. http://dx.doi.org/10.1001/archinte.161.20.2447 PMID: 11700157

[55] Ogawa N, Imai Y, Takahashi Y, *et al.* Evaluating Japanese patients with the Marfan syndrome using high-throughput microarray-based mutational analysis of fibrillin-1 gene. Am J Cardiol 2011; 108(12): 1801-7. http://dx.doi.org/10.1016/j.amjcard.2011.07.053 PMID: 21907952

[56] LU Xin-xin HX-l, WANG Ren, CHEN Xi-jun, *et al.* Detection of deletion mutations of FBN1 in two patients with Marfan syndrome using Next Generation Sequencing (NGS) and Multiplex Ligation-Dependent Probe Amplification (MLPA) technique. Chin J Clin Lab Sci 2015; 10: 744-7.

[57] Weidenbach M, Brenner R, Rantamäki T, Redel DA. Acute mitral regurgitation due to chordal rupture in a patient with neonatal Marfan syndrome caused by a deletion in exon 29 of the FBN1 gene. Pediatr Cardiol 1999; 20(5): 382-5. http://dx.doi.org/10.1007/s002469900493 PMID: 10441700

[58] Wooderchak-Donahue W, VanSant-Webb C, Tvrdik T, *et al.* Clinical utility of a next generation sequencing panel assay for Marfan and Marfan-like syndromes featuring aortopathy. Am J Med Genet A 2015; 167A(8): 1747-57. http://dx.doi.org/10.1002/ajmg.a.37085 PMID: 25944730

[59] Hung CC, Lin SY, Lee CN, *et al.* Mutation spectrum of the fibrillin-1 (FBN1) gene in Taiwanese patients with Marfan syndrome. Ann Hum Genet 2009; 73(Pt 6): 559-67. http://dx.doi.org/10.1111/j.1469-1809.2009.00545.x PMID: 19839986

Neurofibromatosis-1 microdeletiós szindróma

Molekuláris genetika és klinikai heterogenitás

**Büki Gergely ▪ Till Ágnes dr. ▪ Zsigmond Anna dr.
Bene Judit dr. ▪ Hadzsiev Kinga dr.**

Pécsi Tudományegyetem, Általános Orvostudományi Kar, Klinikai Központ, Orvosi Genetikai Intézet, Pécs

Az 1-es típusú neurofibromatosis autoszomális domináns öröklésmenetet mutató, klinikailag rendkívül heterogén neurocutan kórkép, amelynek kialakulásában elsődlegesen az *NF1*-gén intragenikus funkcióvesztéses mutációi játszanak szerepet. Ugyanakkor a molekuláris diagnosztika fejlődésének köszönhetően egyre több esetben sikerül kimutatni az *NF1*-gént és az azzal szomszédos régiókat érintő kópiaszámbeli variánsokat. Genotípus-fenotípus elemzések alapján a pontmutációs eltérések okozta 1-es típusú neurofibromatosis, illetve a microdeletiós eltérések okozta, ún. 17q11.2 microdeletiós szindróma elkülöníthetők egymástól. Microdeletiók az esetek 5–10%-ában figyelhetők meg, melyek méretük, töréspontjaik genomi lokalizációja és érintett géntartalmuk alapján négy különböző típusba (1-es, 2-es, 3-as és atípusos) sorolhatók. A microdeletiós betegek gyakran súlyosabb kórlefolyást mutatnak, melyből kiemelendő a malignitások emelkedett kockázata. Az összefoglaló közleménnyel, mely a neurofibromatosis-1 microdeletiós szindróma főbb jellemzőit, molekuláris genetikai hátterét és vizsgálati módszereit tárgyalja, a microdeletiós szindrómás betegek korai diagnózishoz jutásának fontosságát szeretnénk hangsúlyozni és felhívni a figyelmet a szoros nyomon követés jelentőségére.
Orv Hetil. 2022; 163(51): 2041–2051.

Kulcsszavak: 17q11.2 microdeletiós szindróma, *NF1*-gén, kópiaszám-változás, MLPA

Neurofibromatosis-1 microdeletion syndrome

Molecular characterization and clinical heterogeneity

Neurofibromatosis type 1 is a clinically extremely heterogeneous neurocutaneous disorder, inherited in autosomal dominant manner. It is primarily caused by intragenic loss-of-function mutations in the *NF1* gene, however, as a result of improvements in molecular diagnostics, copy number variants affecting the *NF1* gene and its flanking regions are increasingly being detected. Based on genotype-phenotype analyses, two groups can be distinguished: neurofibromatosis type 1 caused by point mutations and the so-called 17q11.2 microdeletion syndrome caused by microdeletions. Microdeletions are observed in 5–10% of cases and can be divided into four different types (type 1, 2, 3 and atypical) according to the size of the deletion, the genomic location of the breakpoints and the affected gene content. Patients with microdeletions often have a more severe course of the disease, with an increased risk of malignancies. With this review, which summarizes the main characteristics and molecular genetic background of neurofibromatosis-1 microdeletion syndrome, we would like to emphasize the importance of early diagnosis of patients with microdeletion syndrome and draw attention to the importance of close follow-up.

Keywords: 17q11.2 microdeletion syndrome, *NF1* gene, copy number variation, MLPA

Büki G, Till Á, Zsigmond A, Bene J, Hadzsiev K. [Neurofibromatosis-1 microdeletion syndrome. Molecular characterization and clinical heterogeneity]. Orv Hetil. 2022; 163(51): 2041–2051.

(Beérkezett: 2022. szeptember 23.; elfogadva: 2022. október 25.)

Rövidítések

arrayCGH = (chromosomal microarray-comparative genome hybridization) kromoszomális microarray-komparatív genomiális hibridizáció; CNV = (copy number variation) kópiaszámbeli variáns; DNS = dezoxiribonukleinsav; GTP = (guanosine triphosphate) guanozin-trifoszfát; HGMD = (Human Gene Mutation Database) Humán Génmutációs Adatbázis; LCR = (low copy repeat) kis kópiaszámú ismétlődés; LoF = (loss-of-function) funkcióvesztéses; MAPK = (mitogen-activated protein kinase) mitogénaktivált proteinkináz; MEK = (mitogen-activated protein kinase/extracellular signal-regulated kinase) mitogénaktivált proteinkináz/extracelluláris szignál szabályozta kináz; MIM = (Mendelian inheritance in man) mendeli öröklődés emberben; MLPA = multiplex ligatiofüggő próbaamplifikáció; MPNST = (malignant peripheral nerve sheath tumour) malignus perifériás ideghüvely-daganat; MRI = (magnetic resonance imaging) mágnesesrezonancia-képalkotás; NAHR = (non-allelic homologus recombination) nem allélikus homológ rekombináció; NF1 = 1-es típusú neurofibromatosis; NHEJ = (non-homologous end joining) nem homológ végillesztés; NIH = (National Institutes of Health) az Egyesült Államok Nemzeti Egészségügyi Intézete; pLI = (probability of loss-of-function intolerance) a funkcióvesztéses intolerancia valószínűsége; RAS = (rat sarcoma viral oncogene homolog) patkánysarcoma virális onkogén homológ

A neurofibromatosis, vagy korábbi nevén von Recklinghausen-kór, autoszomális domináns öröklésmenetet mutató neurocutan kórkép. Több formája ismert, melyek közül a leggyakoribbak az 1-es és 2-es típusú neurofibromatosis, illetve a schwannomatosis. Kialakulása nemtől és rassztól független, kórlefolyása változatos. Legjellemzőbb formája az 1-es típusú neurofibromatosis (NF1; MIM# 162200), amely mind klinikailag, mind genetikailag eltér az említett másik két formától. Előfordulási gyakorisága a legújabb adatok szerint 1/2500–3000-ra tehető [1, 2]. A neurocutan betegség főleg a bőr és a perifériás idegrendszer területén okoz variábilis expresszivitású tüneteket, és teljes penetranciát mutat. A legszembetűnőbb tünetek a testszerte előforduló tejeskávéfoltok és a bőrön és/vagy bőr alatt kialakuló neurofibromák. A fő jellegzetességek közé tartoznak még a retinán megjelenő Lisch-nodulusok, az axillaris/inguinalis régióban megjelenő szeplőzöttség és a különböző idegrendszeri tünetek [3]. A neurofibromák többségükben jóindulatú komplex tumorok, melyeket főként Schwann-sejtek, endothelsejtek, fibroblastok és hízósejtek alkotnak [4]. A genetikai rendellenesség hatására a betegekben jelentősen megnő bizonyos daganatos megbetegedések kockázata, ezek közül a leggyakrabban az agydaganatok és a malignus perifériás ideghüvely-daganatok (MPNST-k) fordulnak elő [5]. A klinikai manifesztációk változatosak, az életkor függvényében új tünetek jelenhetnek meg, illetve a fennálló tünetek fokozatosan súlyosbodhatnak [6–8].

A rendellenesség kialakulásának hátterében a leggyakrabban a 17-es kromoszóma hosszú karján (17q11.2) található neurofibromin-1 (*NF1)*-génben előforduló funkcióvesztéses (loss-of-funtion, LoF-) mutációk állnak [9, 10]. Az *NF1*-génben többségében intragenikus, kis skálájú mutációk (pontmutációk, indelek) és kisebb, egy vagy több exont érintő kópiaszámbeli variánsok (CNV-k) fordulnak elő. Jelenleg a Human Gene Mutation Database (HGMD) alapján több mint 2800 csírasejtes mutáció, míg a ClinVar adatbázis alapján több mint 3300 patogén és valószínűleg patogén variáns ismert elszórva a gén egész területén, beleértve a CNV-ket is. Az utóbbi időben egyre több esetben mutatnak ki több gént érintő nagyobb deletiókat (a továbbiakban: microdeletiók) is, amelyek az egész *NF1*-gént, illetve a szomszédos régióban található géneket érintik. Az *NF1*-gén mutációs rátája rendkívül magas, az esetek közel 50%-ában a mutációk *de novo* alakulnak ki [11]. Ezek a *de novo* intragenikus mutációk főként az apai eredetű kromoszómán jönnek létre, melyek előfordulásának valószínűsége növekszik az apa életkorával [12]. Az *NF1*-gén 57 exonja a 2818 aminosavból álló, neurofibromin nevű fehérjét kódolja, amely mindenütt expresszálódik, az expresszió mértéke azonban a szövet típusától és a szervezet fejlődési szakaszától függően változik. A legnagyobb mértékben a felnőttneuronokban, Schwann-sejtekben, astrocytákban, leukocytákban és oligodendrocytákban fejeződik ki [13, 14]. A neurofibromin egy Ras-specifikus GTP-áz-aktiváló fehérje, melynek legfontosabb szerepe a RAS/MAPK jelátviteli kaszkád negatív regulációja, ezáltal részt vesz a sejtnövekedés és -differenciálódás szabályozásában [9, 15]. A neurofibromin fehérje tumorszuppresszorként funkcionál, hibás vagy csökkent működése így magyarázatot ad az 1-es típusú neurofibromatosisban szenvedő betegekben tapasztalt gyakoribb daganatképződésre [16].

A neurofibromatosis-1 NIH diagnosztikus kritériumai

A szerteágazó megjelenésű tünettan, illetve az ismert átfedő tünettannal rendelkező egyéb RASopathiák [17] (például Legius-szindróma) miatt megalkottak egy klaszszifikációs rendszert, amely a leggyakoribb tünetek jelenlétén/hiányán alapul [18]. Az *1. táblázat* mutatja be a NIH (National Institutes of Health) által 1987-ben létrehozott, majd 1997-ben és 2021-ben revideált kritériumrendszert, mely magában foglalja a betegség leggyakoribb tüneteit [19].

Bizonyos tünetek az életkor előrehaladtával manifesztálódnak, illetve számos esetben a meglévő tünetek progressziója figyelhető meg, így a diagnózis felállításakor az életkor figyelembevétele kulcsfontosságú lehet a kezelési lehetőségek és a pontos, egyénre igazított nyomon követés szempontjából. Korábbi tanulmányok [20, 21] alapján általánosságban bizonyított, hogy a gyermekek enyhébb tünetekkel rendelkeznek. A neurofibromatosis-1 klinikai manifesztációjának szignifikáns variabilitása miatt számos esetben nem egyértelműen állítható fel

1. táblázat A neurofibromatosis-1 diagnosztikus kritériumai a 2021. évi revideált NIH-konszenzus alapján [19]

A) A diagnózis felállításához az alábbi kritériumok közül két vagy több jelenléte szükséges, amennyiben a beteg egyik szülőjénél sem diagnosztizáltak neurofibromatosist
Hat vagy több tejeskávéfolt (pubertáskor előtt 5 mm-nél, pubertáskor után 15 mm-nél nagyobb átmérő).
Axillaris vagy inguinalis szeplőzöttség.
Két vagy több, bármilyen típusú neurofibroma vagy egy plexiform neurofibroma.
Opticus glioma.
Két vagy több, az íriszen látható Lisch-csomó vagy két vagy több érhártya-rendellenesség.
Jellegzetes csontos elváltozás (például sphenoid dysplasia, a tibia anterolateralis görbülete vagy egy hosszú csöves csont pseudoarthrosisa).
Heterozigóta patogén variáns az *NF1*-microdeletióban.
B) A neurofibromatosis-1 diagnosztikus kritériumainak megfelelő szülő gyermeke esetében az „A” kritériumok közül egy vagy több jelenléte szükséges a diagnózis felállításához

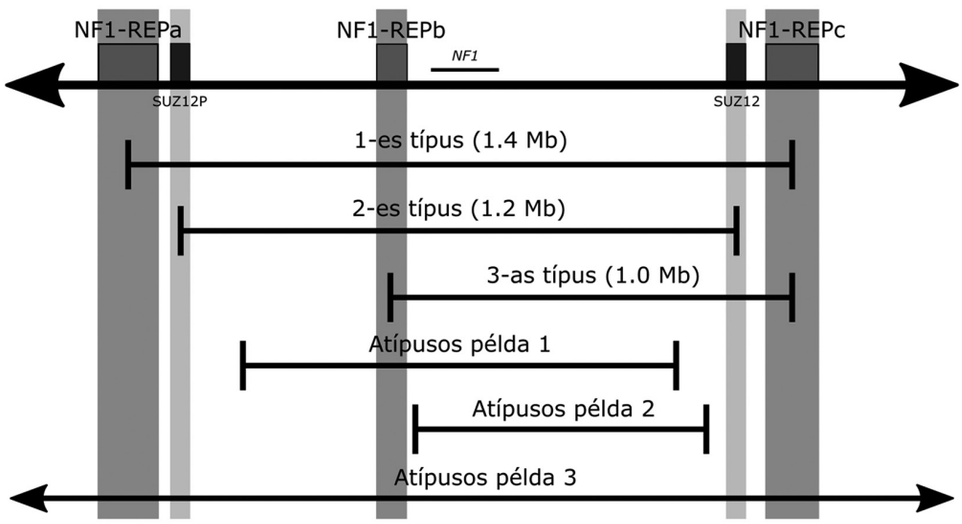
a klinikai diagnózis, így a kóroki mutációk molekuláris genetikai meghatározása fontos szerepet játszik a diagnózis megállapításában.

Microdeletiók kialakulása és csoportosításuk

Az 1-es típusú neurofibromatosist az esetek 5–10%-ában az *NF1*-gént is érintő microdeletiók okozzák [22, 23]. A rendellenesség kialakításában részt vevő microdeletiókat lokalizációjuk, méretük és érintett géntartalmuk alapján négy altípusba sorolhatjuk. Az első három típusba rekurrens deletiók tartoznak, amelyeknél az egyes típusokon belül a töréspontok jól meghatározhatóan közel azonos helyre lokalizálódnak a genomban. A 4. típust az ún. atípusos deletiók alkotják, amelyeknél mind a töréspontok, mind pedig a méret, így az érintett gének száma is heterogén *(2. táblázat)*.

A genomban szétszórtan található, nagymértékű homológiával rendelkező instabil kromoszómarégiók hajlamosítanak rekurrens microdeletiók kialakulására. A rekurrens microdeletiók gyakran alakulnak ki ún. LCR- (low copy repeat, kis kópiaszámú ismétlődés) régiók között. A szekvenciaazonosság következtében az LCR-régiók hibásan párba rendeződhetnek, emiatt nagy méretű deletiók, duplikációk és amplifikációk jöhetnek létre. Ilyen régiók találhatók a 17-es kromoszóma e szakaszán is, az *NF1*-gén környezetében. A rekurrens deletiók többségének hátterében a nem allélikus homológ rekombináció (NAHR) áll.

Az 1-es és 3-as típusú deletiók kialakításában az NF1-REP (NF1-REPa, NF-REPb, NF1-REPc) nevezetű LCR-régiók vesznek részt. Az 1-es típus kialakításában az NF1-REPa és az NF1-REPc játszik szerepet, míg a kisebb méretű 3-as típusú deletiók esetében az NF1-REPb és NF1-REPc régiók vesznek részt. A 2-es típusú deletiók kialakulása posztzigotikusan mitózis során történik a *SUZ12*-gén és az annak pszeudogénje *(SUZ12P)* közötti szekvenciaazonosság miatt. Ezzel szemben az atípusos deletiók nem rekurrens töréspontok mentén alakulnak ki. Jelenlegi ismereteink alapján a nem rekurrens deletiók többségének kialakításában a nem homológ végillesztés (NHEJ) mechanizmusa vesz részt. A deletiók keletkezésében egyéb mechanizmusokat is feltételeznek, amelyek a DNS-kettősszál törésének javításán és/vagy replikáción, illetve bizonyos retro-



1. ábra A 17q11.2 régióban előforduló, *NF1* microdeletio különböző típusainak (1-es, 2-es, 3-as és atípusos) sematikus ábrázolása. Az 1-es és 3-as típusú rekurrens deletiók töréspontjai a szürkével jelölt NF1-REPa – NF1-REPc, illetve NF1-REPb – NF1-REPc LCR-régiókban lokalizálódnak, míg a 2-es típusú deletio töréspontjai a kékkel jelölt *SUZ12*-gének és rendkívül homológ pszeudogénjén, a *SUZ12P*-n belül helyezkednek el. A nem rekurrens atípusos deletiók méretüket és töréspontjaik elhelyezkedését tekintve rendkívül heterogének.

LCR = kis kópiaszámú ismétlődés

2. táblázat | Az *NF1* microdeletiós típusok karakterisztikus jellemzői

	1-es típus	2-es típus	3-as típus	Atípusos
Méret	1.4 Mb	1.2 Mb	1.0 Mb	Heterogén
Érintett gének	14 fehérjekódoló + 4 miRNS	13 fehérjekódoló + 2 miRNS	9 fehérjekódoló + 2 miRNS	Heterogén
Gyakoriság	76–80%	10%	1–4%	8–10%
A töréspontok lokalizációja	*NF1-REPa* és *NF1-REPc* régiók	*SUZ12P* és *SUZ12* gének	*NF1-REPb* és *NF1-REPc* régiók	Heterogén

miRNS = mikro-ribonukleinsav

transzpozonok működésén alapulnak [24]. A különböző microdeletio-típusokat és a kialakításukban részt vevő LCR-régiókat az *1. ábra* szemlélteti.

Az *NF1* microdeletiók közül a leggyakoribbak az 1-es típusú deletiók, melyek anyai eredetű csíravonal-deletiók, a microdeletiós esetek 70–80%-ában felelősek a betegség kialakításáért [25, 26]. Az esetek körülbelül 10%-ában a 2-es típusú deletiók okozzák a rendellenességet. Ezekhez képest a 3-as típusú deletiókat csak ritkább esetekben (1–4%) lehetett eddig megfigyelni. Az atípusos deletiók a betegek 8–10%-ában fordulnak elő. Az egyes microdeletio-típusok összefoglaló információi a *2. táblázat*ban láthatók.

A szöveti mozaikosság kialakulása és hatása a klinikai manifesztációra

A neurofibromatosis-1 microdeletiós szindrómában bizonyos microdeletiós csoportok esetében szomatikus mozaikosság figyelhető meg, amely hatással lehet a fenotípus kialakulására. A magzati fejlődéstől kezdve a szervezet sejtjei a változó környezet folyamatos mutagén hatásainak vannak kitéve. Az osztódások során kialakuló mutációk nem minden esetben okozzák a sejt pusztulását, ezáltal két vagy akár több sejtvonal is létezhet párhuzamosan. A létrejött mutáns és normális sejtvonalak együttes jelenlétét a szervezetben a mozaikossággal jellemezzük. Attól függően, hogy a mutáció az embrionális időszak melyik időpontjában alakul ki, változhat a mozaikosság aránya, előfordulhat azonban olyan eset is, amikor a mozaikosság csak bizonyos szervre lokalizálódik, s ezáltal a megfigyelt klinikai kép is variálódhat.

Az *NF1*-gént érintő különböző típusú microdeletiós csoportokban különböző gyakorisággal figyeltek meg a mutáns sejtek mellett normálsejteket is, melyek nem hordozzák az *NF1* microdeletiót. Az *NF1* 2-es típusú és az atípusos microdeletiós esetekben gyakrabban fordul elő mozaikosság, míg az 1-es típusú microdeletiós esetekben ennél sokkal ritkábban tapasztalható, a betegek kevesebb mint 5%-ában. Az eddigi eredmények alapján az összes 2-es típusú deletio esetében közel 63%-ban, az atípusos esetekben pedig 60% körül volt tapasztalható [20]. Fontos kiemelni, hogy a szomatikus mozaikosság jelentősen megváltoztathatja a kórlefolyást, enyhébb vagy atípusos neurofibromatosis-1-es fenotípust idézhet elő [27], jelenlétét és annak mértékét azonban nehéz megállapítani.

Az *NF1* microdeletiók vizsgálati módszerei

A CNV-k vizsgálati módszerei hosszú fejlődésen mentek keresztül, kezdve a hagyományos citogenetikai módszerektől egészen az újgenerációs szekvenálási technológiákig. A vizsgálati módszerek fejlődésével javult a kimutatás felbontása, ami lehetővé tette a kisebb méretű, így a különböző microdeletiós szindrómák kialakulásában szerepet játszó variánsok azonosítását is. A molekuláris citogenetikai módszerek közé tartozó microarray-komparatív genomiális hibridizáció (arrayCGH), mely a fluoreszcensen jelölt DNS-minták teljes genomot reprezentáló oligonukleotidpróbákhoz történő hibridizációján alapul, a felbontásának (10–25 kb vagy nagy denzitású array-k esetében akár >500 bp) és pontosságának köszönhetően a CNV-k kimutatásának „gold standardjává" vált [28, 29].

A CNV-k célzottabb vizsgálatára alkalmas a multiplex ligatiofüggő próbaamplifikáció (MLPA), amely hibridizáción és multiplex polimeráz-láncreakción alapuló molekuláris genetikai diagnosztikai módszer. Az MLPA elsősorban kisebb génszakaszok deletiójának, duplikációjának, illetve amplifikációjának detektálását teszi lehetővé. Az esetek többségében intragenikus CNV-k vizsgálatára használják, bizonyos esetekben azonban nagyobb genomi régió analízisére is alkalmazható. A megfelelő próbákkal akár egy adott gén és a környező genomikus régióban található egyéb géneket érintő CNV-k is vizsgálhatóvá válnak, lehetővé téve ezáltal bizonyos microdeletiós szindrómák azonosítását is.

Az *NF1* microdeletiós szindróma laboratóriumi diagnosztizálásának egy gyors, költséghatékony módszere az MLPA. Az MRC Holland (Amszterdam, Hollandia) által kínált SALSA MLPA Probemix P081, P082 és P122 NF1 kitek alkalmasak az *NF1* microdeletió és a környező genomi régió lefedésére. Az alkalmazott próbák lehetővé teszik az 1-es, 2-es és 3-as típusú microdeletiók elkülönítését, azonban az atípusos deletiók elkülönítésére, illetve pontos meghatározására, valamint az esetlegesen előforduló szomatikus mozaikosság kimutatására a módszer csak részben alkalmas [20]. Az arrayCGH bár drágább

vizsgálati módszer, előnye, hogy képes az *NF1* microdeletiós szindróma különböző altípusait megkülönböztetni, illetve a deletiók töréspontjainak genomi lokalizációját pontosabban meghatározni. Bizonyos platformokon lehetőség van a szomatikus mozaikosság meghatározására is. Az alacsony mértékben (20% alatt) jelen lévő mozaikosság kimutatására azonban sem az MLPA, sem az arrayCGH nem alkalmas.

3. táblázat | Az *NF1* 1-es típusú microdeletiót hordozó és az *NF1* pontmutációs betegcsoportokban megfigyelt klinikai tünetek és azok előfordulási gyakorisága (%) [20, 22, 25, 27, 31]

		NF1 1-es típusú microdeletiós betegek					*NF1* pontmutációs betegek	
	A vizsgált betegek száma	n = 12	n = 29	n = 44	n = 7	n = 11	n = 33	n = 29
	Hivatkozások	Büki és mtsai [20]	Kehrer-Sawatzki és mtsai [27]	Pasmant és mtsai [25]	Zhang és mtsai [22]	Bianchessi és mtsai [31]	Büki és mtsai [20]	Kehrer-Sawatzki és mtsai [27]
Érintett szervrendszer	Klinikai tünetek							
Bőrmanifesztációk	Tejeskávéfoltok	100	93	20,8	100	100	91	86–99
	Axillaris/inguinalis szeplőzöttség	83	86	86,4	57	72,7	52	86–89
	Cutan neurofibromák	8	86	15,4–48,7	57	45,5*	18	38–84
	Subcutan neurofibromák	58	76	37,2–41,8	29	45,5*	30	48
	Plexiform neurofibromák	17	76	0,6	29	27,3	6	15–54
	Kézen és lábon túlzott lágy szövet	33	50	n/a	n/a	n/a	0	n/a
Dysmorphiás jellemzők	Arcdysmorphia	67	90	54,8	43,0	n/a	0	n/a
	Durva arc	67	59	n/a	n/a	n/a	0	n/a
	Arcaszimmetria	25	28	n/a	n/a	n/a	6	8
	Nagy kezek és lábak	67	46	n/a	n/a	n/a	0	n/a
	Hypertelorismus	58	86	n/a	n/a	n/a	18	n/a
	Széles nyak	8	31	n/a	n/a	n/a	0	n/a
Tanulási és magatartási problémák	A kognitív fejlődés szignifikáns elmaradása	75	48	n/a	14	36,4	3	17
	Általános tanulási nehézségek	75	45	85,7	n/a	18,2	15	31–47
	IQ<70	8	38	n/a	14	36,4	0	7–8
	Figyelemhiányos hiperaktivitási zavar	17	33	n/a	n/a	0	6	38–49
	Beszédkészség-problémák	67	48	n/a	29	0	3	20–55
Csontrendszeri manifesztációk	Skeletalis rendellenességek	92	76	31+	14	45,5+	33	31
	Scoliosis	42	43	31	0	9,1	21	10–28
	Macrocephalia	58	39	11,5	14	45,5	9	24–45
	Pectus excavatum	33	31	n/a	n/a	n/a	9	12–50
	Pes cavus	0	17	n/a	n/a	n/a	3	n/a
	Az ízületek hiperflexibilitása	8	72	n/a	n/a	n/a	6	n/a
	Csontcysták	8	50	n/a	n/a	0	0	1
Neurológiai manifesztációk	Izomzati hypotonia	25	45	n/a	n/a	n/a	12	27
	Malignus ideghüvelytumorok	17	21	7,1	0	*	0	2–7
	T2-hiperintenzitás	75	45	n/a	29	n/a	39	34–79
	Spinalis neurofibromák	17	64	n/a	n/a	n/a	3	24–30
	Epilepszia	0	7	n/a	n/a	0	3	4–13
Szemészeti manifesztációk	Lisch-nodulus	25	93	40	14	45,5	21	63–93
	Opticus glioma	17	19	n/a	n/a	0	12	11–19
	Látászavar	17	n/a	n/a	14	n/a	15	n/a
	Strabismus	17	n/a	15	14	n/a	0	n/a
Egyéb	Magas termet	58	46	22,2	n/a	n/a	0	n/a

n/a = nem vizsgálták, vagy nem állnak rendelkezésre adatok; * = nem egyértelmű adatok állnak rendelkezésre az eredeti közleményből

Az *NF1* microdeletiós szindróma jellegzetes tünettana

A 17q11.2 microdeletiós szindrómában (MIM# 613675), melynek becsült előfordulási gyakorisága 1/60 000 [27], az 1-es típusú neurofibromatosisban tapasztalható karakterisztikus jellegek (tejeskávéfoltok, neurofibromák, hónalji és lágyéki szeplők stb.) mellett gyakran nagy fokú klinikai variabilitás figyelhető meg a betegek körében. Korábbi tanulmányok [20, 22, 27, 30] jellegzetes különbségeket tártak fel a pontmutációs és a microdeletiós betegcsoportok klinikai manifesztációiban. A legtöbb információnk az 1-es típusú microdeletiós betegek megfigyeléséből származik. Az irodalomban eddig ismert 1-es típusú microdeletiós és pontmutációs betegcsoportokban megfigyelt tüneteket és azok gyakoriságát a *3. táblázat*ban foglaltuk össze [20, 22, 25, 27, 31]. Az 1-es típusú microdeletiós betegek körében gyakrabban tapasztalhatók bizonyos dysmorphiás vonások, a neurofibromák emelkedett száma és megjelenésük gyakorisága, az értelmi akadályozottság, illetve az ideghüvely-daganatok emelkedett kockázata és intenzívebb progressziója. A dysmorphiás jellegek közül a durva arcvonások, illetve a nagy kezek és lábak csak a microdeletiós betegeknél figyelhetők meg. A csontrendszert érintő rendellenességek is összességében nagyobb számban fordulnak elő a microdeletiós betegcsoportban. Ezek közül kiemelendő a macrocephalia, amely szignifikánsan gyakrabban jelentkezik a microdeletiós betegeknél. Jellegzetes tünet még az életkorhoz képest magas termet. Néhány tanulmányban a veleszületett szívfejlődési rendellenességek emelkedett gyakoriságát is leírták [27, 32].

A 2-es típusú deletiót hordozó betegek többségében (több mint 60%-ában) szomatikus mozaikosság fordul elő, ami enyhébb fenotípus kialakulását eredményezheti. Az irodalomban csak néhány, klinikailag jól jellemzett eset ismeretes, amelynél a betegek a deletiót nem mozaikos formában hordozzák. A betegek tüneteit a *4. táblázat*ban foglaltuk össze [20, 22, 27, 33–35]. Az alacsony esetszám által indokolt mértéktartás mellett is kiemelhető az adatokból, hogy az 1-es típusú deletiós betegekhez hasonlóan a különböző típusú neurofibromák előfordulási gyakoriságában, a csontrendszert érintő manifesztációkban, a dysmorphiás jellemzőkben, illetve a magatartási és tanulási problémák tekintetében eltérések figyelhetők meg a pontmutációs betegcsoportokhoz képest. Összehasonlítva a 2-es típusú deletiót hordozó betegek klinikai képét az 1-es típusú deletiós esetekével az is megállapítható, hogy ezen betegekben a csontrendszeri tünetek közül a macrocephalia, a dysmorphiás tünetek közül a nagy kezek és lábak, valamint a veleszületett szívfejlődési rendellenességek gyakrabban fordultak

4. táblázat | Az *NF1* nem mozaikos 2-es típusú microdeletiós betegek klinikai jellemzői [20, 22, 27, 33–35]

	A klinikai tünetek előfordulási gyakorisága (%) az 1-es típusú microdeletiós betegekben			A klinikai tünetek jelenléte vagy hiánya a nem mozaikos 2-es típusú microdeletiós betegekben				
	A vizsgált betegek száma/azonosítója	n = 29	n = 12	85/NF	078	P. 2358	P. 2429	#1
	Hivatkozások	[27]	[20]		[22]	[34, 35]		[33]
Érintett szervrendszer	Klinikai tünetek							
Bőrmanifesztációk	Axillaris/inguinalis szeplőzöttség	86	83	–	–	+	+	–
	Tejeskávéfoltok	93	100	+	+	+	+	+
	Cutan neurofibromák	86	8	–	+	–	+ (számos)	+ (számos)
	Subcutan neurofibromák	76	58	–	+	+	+ (számos)	–
	Plexiform neurofibromák	76	17	–	–	+	+ (számos)	+ (számos)
Tanulási és magatartási problémák	Tanulási nehézségek	48	75	+	?	+ (enyhe)	+	+
	Figyelemzavar	33	17	–	?	+	+	–
Neurológiai manifesztációk	T2-hiperintenzitás	45	75	+	n/a	+	–	n/a
	Izomzati hypotonia	45	25	–	n/a	+	n/a	n/a
	Malignus ideghüvelytumorok	21	17	–	–	–	+	–
Csontrendszeri manifesztációk	Az ízületek hiperflexibilitása	72	8	–	n/a	+	+	–
	Scoliosis	43	42	+	+	n/a	–	–
	Macrocephalia	39	58	+	–	+	+	–
Dysmorphiás jellemzők	Arcdysmorphia	90	67	–	–	+	+	+
	Nagy kezek és lábak	46	67	+	n/a	+	+	–
Egyéb	Magas termet	46	58	–	n/a	–	–	–
	Lisch-nodulus	93	25	+	?	+	+	+
	Veleszületett szívfejlődési rendellenességek	21	0	–	n/a	+	+	–

– = hiányzik; + = jelen van; n/a = nem vizsgálták, vagy nem állnak rendelkezésre adatok; ? = nem egyértelmű adat az eredeti cikkből

elő, mint az 1-es típusú deletiót hordozó betegekben. Megfigyelhető továbbá, hogy ezekre a betegekre az 1-es típusú betegekkel szemben nem jellemző az életkorhoz képest magas termet, és az arc dysmorphiája is ritkábban észlelhető.

A 3-as típusú microdeletio meglehetősen ritka, az irodalomban eddig összesen 11 esetet írtak le [25, 26, 36, 37]. A betegekről kevés klinikai információ áll rendelkezésre, ami azzal magyarázható, hogy a betegek többsége (81%) 10 év alatti gyermek. Az elérhető adatokat az *5. táblázat*ban foglaltuk össze [25, 36, 37]. A klinikai adatokból a kis betegszám mellett is megfigyelhető, hogy a microdeletiós betegekre jellemzően ezen betegekben is gyakrabban fordult elő az arc dysmorphiája, illetve a nagy kezek és lábak a pontmutációs esetekkel összehasonlítva. Emellett a betegek viszonylag nagyobb hányadában (36%) túlnövekedés is gyakrabban volt tapasztalható.

Az atípusos microdeletiót hordozó betegek meglehetősen heterogén tünettannal rendelkeznek. A klinikai képet tovább árnyalja a betegekben (közel 60%-ukban) jelen lévő szöveti mozaikosság. Az irodalomban eddig összesen 61 beteget azonosítottak heterogén méretű, ezáltal heterogén gén tartalmú atípusos microdeletióval, melyek közül hozzávetőlegesen 20 esetben érhető el klinikai információ. A betegek az azonosított deletio mérete és elhelyezkedése alapján két nagy csoportba sorolhatók. A betegek közel felében olyan nagy méretű deletio fordul elő, amelyek töréspontjai túlnyúlnak az 1,4 Mb méretű 1-es típusú deletión, míg a másik csoportba tartozó betegek esetében a töréspontok az 1-es típusú deletión belül helyezkednek el [38]. A betegek klinikai képe a neurofibromatosis-1 fő diagnosztikai kritériumaitól eltekintve alig mutatnak átfedéseket. Figyelemre méltó különbség tapasztalható a dysmorphiás vonásokban, a neuropszichológiai manifesztációkban és a különböző neurofibromák előfordulási gyakoriságában [20]. A betegek egy részében jellegzetes tünet a durva arcvonás és a nagy kezek, lábak. Néhány esetben súlyos, néhányban enyhe értelmi akadályozottság volt megfigyelhető. A cutan és subcutan, illetve plexiform neurofibromák kialakulása és száma is nagy fokú heterogenitást mutat.

Az 1-es típusú neurofibromatosis egyik karakterisztikus vonása a különböző jóindulatú tumorok kialakulása cutan vagy subcutan neurofibromák, illetve a potenciálisan nagy plexiform neurofibromák formájában. A plexi-

5. táblázat | Az *NF1* 3-as típusú microdeletiós betegek klinikai jellemzői [25, 36, 37]

	A klinikai tünetek jelenléte vagy hiánya a 3-as típusú *NF1* microdeletiós betegekben											
	A vizsgált betegek azonosítója	GUE	OLI	N2603	TOP	Z41/03	2176	R54307	R85918	R53520	D071	D091
	Referencia	[25]			[36]			[37]				
Érintett szervrendszer	Klinikai tünetek											
Bőrmanifesztációk	Axillaris/inguinalis szeplőzöttség	n/a	n/a	n/a	n/a	n/a	+	+	+	n/a	+	–
	Tejeskávéfoltok	+	+	+	n/a	n/a	+	+	+	+	+	+
	Cutan neurofibromák	–	–	–	+ (számos)	n/a	+ (számos)	n/a	n/a	+	–	–
	Subcutan neurofibromák	+	+	–	n/a	n/a	+ (számos)	n/a	n/a	+	n/a	–
	Plexiform neurofibromák	+	+	–	n/a	n/a	–	n/a	n/a	+	–	+
Tanulási és magatartási problémák	Tanulási nehézségek	+	n/a	n/a	n/a	n/a	+	n/a	n/a	n/a	n/a	n/a
	Értelmi akadályozottság	n/a	n/a	n/a	+	+	n/a	n/a	n/a	n/a	+	enyhe
Neurológiai manifesztációk	Malignus ideghüvely-tumorok	n/a	n/a	n/a	n/a	+	n/a	n/a	n/a	n/a	n/a	n/a
Csontrendszeri manifesztációk	Macrocephalia	n/a	n/a	n/a	n/a	n/a	–	n/a	n/a	n/a	+	n/a
	Microcephalia	n/a	n/a	n/a	n/a	n/a	+	n/a	n/a	n/a	–	n/a
	Scoliosis	n/a	n/a	n/a	n/a	n/a	+	n/a	n/a	–	n/a	n/a
Dysmorphiás jellemzők	Arcdysmorphia	+	+	+	+	+	+	n/a	n/a	n/a	n/a	+
	Nagy kezek és lábak	n/a	n/a	n/a	+	n/a	+	n/a	n/a	n/a	+	n/a
Egyéb	Magas termet	+	+	+	n/a	n/a	–	n/a	n/a	n/a	+	n/a
	Lisch-nodulus	n/a	n/a	n/a	n/a	n/a	+	n/a	n/a	n/a	–	–
	Opticus glioma	n/a	n/a	n/a	n/a	n/a	n/a	+	n/a	–	n/a	n/a

– = hiányzik; + = jelen van; n/a = nem vizsgálták, vagy nem állnak rendelkezésre adatok

form neurofibromák azonban rosszindulatú MPNST-vé alakulhatnak át, amelyek hozzájárulhatnak a neurofibromatosis-1-ben szenvedő betegek mortalitásához. A rosszindulatú transzformáció kockázati tényezői az *NF1* microdeletio jelenléte, az egész testet érintő magas daganatterhelés és a subcutan neurofibromák nagy száma [39]. A cutan neurofibromák, melyek sohasem válnak rosszindulatúvá, általában felnőttkorban jelennek meg, és a neurofibromatosis-1-ben szenvedő felnőtt populáció 80–90%-ában fordulnak elő [40]. A microdeletiós betegcsoportra azonban jellemző a cutan neurofibromák nagy száma és korai (pubertás előtti) megjelenése [27, 41]. A cutan neurofibromák mellett gyakran subcutan neurofibromák is kialakulhatnak. Az 1-es és 2-es típusú microdeletiós csoportban igen magas a subcutan neurofibromák előfordulása, ami korábbi tanulmányok szerint az MPNS kialakulásának emelkedett kockázatával társul, ezért ezekben a betegekben javasolt a szorosabb nyomon követés [42]. A külsőleg megfigyelhető neurofibromák mellett a neurofibromatosis-1-betegekben nagyszámú ún. belső neurofibroma (többségében plexiform neurofibroma) is előfordulhat, melyek sokszor csak mágnesesrezonancia-képalkotással (MRI) detektálhatók. A pontmutációs csoporthoz képest a plexiform neurofibromák nagyobb száma és gyorsabb növekedési üteme tapasztalható az *NF1* microdeletiós betegcsoportban [39]. Egyes tanulmányok szoros összefüggést figyeltek meg a belső neurofibromák jelenléte és az MPNS előfordulása között. Az MPNST-k nagyon agresszív, rossz prognózisú tumorok, melyek gyakran már meglévő plexiform neurofibromákból alakulnak ki [43]. A microdeletiós betegeknél az MPNST kialakulásának nagyobb kockázata, valamint a betegek korábbi életszakaszában való megjelenése figyelhető meg. Mindezek ismeretében a teljestest-MRI javasolt a microdeletiós betegpopulációban a nem látható, aszimptomatikus plexiform neurofibromák korai stádiumban történő kimutatása és ezáltal a korai diagnózis felállítása, valamint az időbeli kezelés elindítása céljából. Egyes megfigyelések szerint ha a gyermekekben az első MRI során nem észlelhető plexiform neurofibroma, akkor nem valószínű, hogy életük során később plexiform neurofibroma alakuljon ki [32].

A megjelenő specifikus tünetek miatt a megfelelő és alapos vizsgálatokkal jól elkülöníthetők a microdeletiós és a pontmutációs betegcsoportok. A *6. táblázat*ban összegyűjtöttük a leggyakoribb tüneteket, amelyek felhívhatják a figyelmet a microdeletio jelenlétére. Ennek ismeretében célzott vizsgálati módszer alkalmazható a rendellenesség hátterében álló genetikai eltérés feltérképezésére.

Összességében megállapítható, hogy a neurofibromatosis-1 klinikai manifesztációi a microdeletiós betegcsoportban általánosságban súlyosabbak, mint az intragenikus patogén *NF1*-mutációkkal rendelkező betegcsoportban [20, 21, 27].

A microdeletio által érintett gének funkciói és codeletióinak a fenotípusra gyakorolt lehetséges hatásai

Az *NF1* microdeletio különböző típusai változó számú gén elvesztésével járhatnak, ami a nem rekurrens, atípusos esetben a legkifejezettebb és leginkább heterogén. A rekurrens deletiók által (1-es, 2-es, 3-as típus) érintett géneket a *7. táblázat* szemlélteti [20, 27, 30, 44–54]. Az atípusos deletiókban egyéb gének is érintettek lehetnek, mint például a *CPD, GOSR1, ZNF207, PSMD11, CDK5R1.*

Az *NF1* microdeletio számos olyan, további funkcionális gént érinthet, mely feltételezhetően intoleráns a funkcióvesztésre, ezáltal a normáldózisuk megváltozása hozzájárulhat az eltérő klinikai kép kialakulásához. A legtöbb esetben a LoF-mutációk következtében kialakuló haploid elégtelenség hatására a fennmaradó egy működőképes génkópia ugyanis nem termel elegendő fehérjét. A funkcióvesztéssel szembeni intolerancia valószínűségének becslése a pLI- (probability of loss-of-function intolerance) értékkel jellemezhető. A pLI-érték alapján a gének LoF-intoleráns (pLI≥0,9) vagy LoF-toleráns (pLI≤0,1) csoportba sorolhatók. Az *NF1* microdeletiós régióban található fehérjekódoló gének közül az

6. táblázat | A microdeletiós és a pontmutációs betegcsoport leggyakoribb tünettani különbségei

Eltérést mutató jellegzetes tünetek		Pontmutációs betegcsoport	Microdeletiós betegcsoport
Dysmorphiás jellegek	Arcdysmorphia	Nem fordul elő	Gyakori
	Durva arcvonások	Nem fordul elő	Gyakori
	Nagy kezek és lábak	Nem fordul elő	Gyakori
Neurofibromák (cutan, subcutan, plexiform)		Kevesebb	Több, korábban
Macrocephalia		Ritkább	Gyakoribb
Magas termet		Nem jellemző	Gyakori
Csontrendszert érintő manifesztációk		Ritka	Gyakori
Súlyos globális fejlődési elmaradás		Ritka	Gyakori
Súlyos értelmi akadályozottság		Ritka	Gyakori

7. táblázat | A 17q11.2 *NF1* microdeletiós régióban található fehérjekódoló és miRNS-gének funkciói és a fenotípusra gyakorolt hatásuk [20, 27, 30, 44–54]

	Génelnevezés	MIM-azonosító	pLI-érték	A fenotípusra gyakorolt hatás	Génfunkció	Ref.
	LRRC37BP	–	–	–	–	–
	SUZ12P	–	–	–	–	–
	CRLF3	614853	0,00	Nem ismert.	A sejtciklus-progresszió negatív regulátora, esszenciális szerepe lehet az agyfejlődésben és az autizmus kialakulásában.	[30, 44, 45]
	ATAD5	609534	1,00	Tumorgenezis.	Tumorszuppresszor, genominstabilitás-regulátor.	[46]
	TEFM	616422	0,51	Onkogenikus szerepe lehet a hepatocellularis carcinomában.	Növeli a mitokondriális RNS-polimeráz processzivitását.	[47]
	ADAP2	608635	0,00	Cardiovascularis manifesztációkkal hozták összefüggésbe.	A szív fejlődésében játszik szerepet.	[48]
	RNF135	611358	0,00	Tumorgenezis, arcdysmorphia, túlnövekedés, csökkent kognitív képesség.	–	[49]
	MIR4733	–	–	–	Részt vesz a génexpresszió poszttranszkripciós szabályozásában.	–
	NF1	162200	1,00	Tumorgenezis, heterogén tünettan.	Tumorszuppresszor.	[20, 27]
	OMG	164345	0,97	Értelmi akadályozottság, csökkent kognitív képesség.	A korai agyfejlődésben játszik szerepet, illetve hozzájárul a központi idegrendszer myelinisatiójához.	[27, 50]
	EVI2B	158381	0,06	–	Szükséges a granulocytadifferenciálódáshoz és a haematopoeticus progenitor sejtek működéséhez.	[51]
	EVI2A	158380	0,00	Osteosarcoma kialakulásában játszhat szerepet.	–	[52]
	RAB11FIP4	611999	0,99	Hozzájárul a hasnyálmirigy-daganat progressziójához.	Részt vesz a vesicularis transzport regulációjában.	[53]
	MIR193A	614733	–	Tumorgenezis.	Részt vesz a génexpresszió poszttranszkripciós szabályozásában, tumorszuppresszori funkció.	[27]
	MIR365B	–	–	Tumorgenezis.	Részt vesz a génexpresszió poszttranszkripciós szabályozásában, tumorszuppresszori funkció.	[27]
	MIR4725	–	–	–	Részt vesz a génexpresszió poszttranszkripciós szabályozásában.	–
	COPRS	616477	0,25	A malignus ideghüvelytumorok kialakulásában játszik szerepet, tumorgenezis.	Szerepet játszik az izomsejtek differenciálódásában.	[27]
	UTP6	–	0,00	Tumorgenezis, hozzájárulhat a veleszületett szívelégtelenség gyakoribb előfordulásához.	Riboszómaszintézishez szükséges, részt vesz az apoptoszómadependens apoptózisban.	[27, 54]
	SUZ12	613675	1,00	Fokozza az ideghüvelytumorok kialakulásának kockázatát, tumorgenesis.	Tumorszuppresszori funkció.	[27]
	LRRC37B	616558	0,01	Nem ismert.	Nem ismert.	–

3-as típus | 2-es típus | 1-es típus

A pLI- (probability of loss-of-function intolerance) értékek a GnomAD böngészőből (https://gnomad.broadinstitute.org/) származnak, a GnomAD v2.1.1/GnomAD SVs v2.1 verzió alapján. A funkcióvesztés szempontjából annál intoleránsabbnak tűnik egy adott gén, minél közelebb van a pLI értéke az 1-hez. A 0,9 feletti pLI-értékkel rendelkező gének az előrejelzések szerint intoleránsak a funkcióvesztéses variánsokkal szemben.

A táblázat bal oldalán a fekete vonalak az 1-es, 2-es és 3-as típusú microdeletio méretét és az általuk érintett géneket reprezentálják.

miRNS = mikro-ribonukleinsav

ATAD5, NF1, OMG, RAB11FIP4, SUZ12 gének a LoF-intoleráns kategóriába tartoznak [30] *(7. táblázat)*, ami arra utal, hogy ezen gének haploinsufficientiája nagy valószínűséggel kóros következményekkel járhat. Ugyanakkor nem zárható ki, hogy a régióban található LoF-toleráns gének heterozigóta deletiója is valamilyen módon hozzájárulhat a klinikai kép kialakulásához. Korábbi kutatások alapján már egyre több génhez lehet funkciót, illetve fenotípusra gyakorolt hatást is társítani [20, 27, 30, 44–54]. Például az *RNF135* és *SUZ12* gének haploinsufficientiájához kapcsolható bizonyos dysmorphiás arcvonások megjelenése, túlnövekedés és csökkent kognitív képesség [30, 49], az *ADAP2* elvesztését cardiovascularis manifesztációkkal hozták összefüggésbe, míg a *COPRS* és a *SUZ12* gének az MPNST kialakulásában játszhatnak szerepet. Továbbá számos génről (például *ATAD5, RNF135, NF1, SUZ12*) megállapították, hogy részt vesznek a daganatok kialakulásában [27]. Az *NF1* microdeletiós régióban található gének eddig ismert funkcióit a *7. táblázat*ban foglaltuk össze [20, 27, 30, 44–54].

Következtetés

Az 1-es típusú neurofibromatosis neurocutan rendellenesség, amelynek hátterében a *NF1* tumorszuppresszor gén mutációi állnak. Mai ismeretünk alapján két nagyobb csoport különíthető el: a pontmutációs eltérések okozta 1-es típusú neurofibromatosis, illetve a microdeletiós eltérések okozta, ún. 17q11.2 microdeletiós szindróma. Az utóbbi esetében az 1-es típusú neurofibromatosisra jellemző karakterisztikus tünetek mellett specifikusabb és gyakran súlyosabb kórlefolyás figyelhető meg. A microdeletiós betegek fenotípusos jegyei, melyek segíthetnek a microdeletio jelenlétének korai felismerésében, a következők: dysmorphiás arcvonások, macrocephalia, nagy méretű kezek és lábak, magas termet, megkésett kognitív fejlődés és/vagy tanulási nehézség, a subcutan neurofibromák igen gyakori és nagyszámú előfordulása. A betegek körében az MPNST és egyéb malignitások kialakulásának emelkedett kockázata figyelhető meg, emiatt fontos a microdeletio jelenlétének minél korábbi azonosítása, melynek meghatározásához gyors és hatékony eszköz az MLPA, valamint a betegek szoros nyomon követése, melynek fontos része a teljestest-MRI. A rendkívül variábilis klinikum a microdeletiós betegek multidiszciplináris ellátását teszi szükségessé, magában foglalva a tünetek meghatározását, a klinikai és molekuláris vizsgálatokon alapuló diagnózis felállítását, a malignus tünetek mielőbbi felismerését és kezelését, a genetikai tanácsadást és a pszichológiai támogatást. Számos klinikai kutatás folyik hatékony terápiák kidolgozására, a betegség gyógyítása azonban a mai napig nem megoldott. Hazánkban jelenleg a plexiform neurofibroma kezelésére alkalmas MEK-inhibitor-terápia érhető el szelumetinib és trametinib néven egyedi méltányossági kérelemmel.

Anyagi támogatás: A közlemény megírása a Pécsi Tudományegyetem Általános Orvostudományi Karának támogatásával valósult meg (KA-2020-27).

Szerzői munkamegosztás: B. G., T. Á., Zs. A., B. J.: Irodalomkutatás, a kézirat megszövegezése. B. G., B. J.: Az ábrák és a táblázatok elkészítése. H. K., B. J.: A kézirat áttekintése. A közlemény végleges változatát az összes szerző elolvasta és jóváhagyta.

Érdekeltségek: A szerzőknek nincsenek érdekeltségeik.

Irodalom

[1] Lammert M, Friedman JM, Kluwe L, et al. Prevalence of neurofibromatosis 1 in German children at elementary school enrollment. Arch Dermatol. 2005; 141: 71–74.
[2] Uusitalo E, Leppävirta J, Koffert A, et al. Incidence and mortality of neurofibromatosis: a total population study in Finland. J Invest Dermatol. 2015; 135: 904–906.
[3] DeBella K, Szudek J, Friedman JM. Use of the national institutes of health criteria for diagnosis of neurofibromatosis 1 in children. Pediatrics 2000; 105: 608–614.
[4] Zheng H, Chang L, Patel N, et al. Induction of abnormal proliferation by nonmyelinating Schwann cells triggers neurofibroma formation. Cancer Cell 2008; 13: 117–128.
[5] Uusitalo E, Rantanen M, Kallionpää RA, et al. Distinctive cancer associations in patients with neurofibromatosis type 1. J Clin Oncol. 2016; 34: 1978–1986.
[6] Jett K, Friedman JM. Clinical and genetic aspects of neurofibromatosis 1. Genet Med. 2010; 12: 1–11.
[7] Gutmann DH, Ferner RE, Listernick RH, et al. Neurofibromatosis type 1. Nat Rev Dis Primers 2017; 3: 17004.
[8] Easton DF, Ponder MA, Huson SM, et al. An analysis of variation in expression of neurofibromatosis (NF) type 1 (NF1): evidence for modifying genes. Am J Hum Genet. 1993; 53: 305–313.
[9] Bergoug M, Doudeau M, Godin F, et al. Neurofibromin structure, functions and regulation. Cells 2020; 9: 2365.
[10] Marchuk DA, Saulino AM, Tavakkol R, et al. cDNA cloning of the type 1 neurofibromatosis gene: complete sequence of the *NF1* gene product. Genomics 1991; 11: 931–940.
[11] McKeever K, Shepherd CW, Crawford H, et al. An epidemiological, clinical and genetic survey of neurofibromatosis type 1 in children under sixteen years of age. Ulster Med J. 2008; 77: 160–163.
[12] Stephens K, Kayes L, Riccardi VM, et al. Preferential mutation of the neurofibromatosis type 1 gene in paternally derived chromosomes. Hum Genet. 1992; 88: 279–282.
[13] Trovó-Marqui AB, Tajara EH. Neurofibromin: a general outlook. Clin Genet. 2006; 70: 1–13.
[14] Scheffzek K, Welti S. Neurofibromin: protein domains and functional characteristics. In: Upadhyaya M, Cooper ND (eds.) Neurofibromatosis type 1. Molecular and Cellular biology. Springer, Berlin, 2012; pp. 305–326.
[15] Gutmann DH, Donahoe J, Brown T, et al. Loss of neurofibromatosis 1 *(NF1)* gene expression in NF1-associated pilocytic astrocytomas. Neuropathol Appl Neurobiol. 2000; 26: 361–367.
[16] Patrakitkomjorn S, Kobayashi D, Morikawa T, et al. Neurofibromatosis type 1 (NF1) tumor suppressor, neurofibromin, regulates the neuronal differentiation of PC12 cells via its associating protein, CRMP-2. J Biol Chem. 2008; 283: 9399–9413.
[17] Rauen KA. The RASopathies. Annu Rev Genomics Hum Genet. 2013; 14: 355–369.
[18] Riccardi VM. Neurofibromatosis: clinical heterogeneity. Curr Probl Cancer 1982; 7: 1–34.

[19] Legius E, Messiaen L, Wolkenstein P, et al. Revised diagnostic criteria for neurofibromatosis type 1 and Legius syndrome: an international consensus recommendation. Genet Med. 2021; 23: 1506–1513.
[20] Büki G, Zsigmond A, Czakó M, et al. Genotype-phenotype associations in patients with type-1, type-2, and atypical *NF1* microdeletions. Front Genet. 2021; 12: 673025.
[21] Kehrer-Sawatzki H, Kluwe L, Salamon J, et al. Clinical characterization of children and adolescents with NF1 microdeletions. Childs Nerv Syst. 2020; 36: 2297–2310.
[22] Zhang J, Tong H, Fu X, et al. Molecular characterization of *NF1* and neurofibromatosis type 1 genotype-phenotype correlations in a Chinese population. Sci Rep. 2015; 5: 11291.
[23] Cnossen MH, van der Est MN, Breuning MH, et al. Deletions spanning the neurofibromatosis type 1 gene: implications for genotype-phenotype correlations in neurofibromatosis type 1? Hum Mutat. 1997; 9: 458–464.
[24] Vogt J, Bengesser K, Claes KB, et al. SVA retrotransposon insertion-associated deletion represents a novel mutational mechanism underlying large genomic copy number changes with non-recurrent breakpoints. Genome Biol. 2014; 15: R80.
[25] Pasmant E, Sabbagh A, Spurlock G, et al. *NF1* microdeletions in neurofibromatosis type 1: from genotype to phenotype. Hum Mutat. 2010; 31: E1506–E1518.
[26] Messiaen L, Vogt J, Bengesser K, et al. Mosaic type-1 *NF1* microdeletions as a cause of both generalized and segmental neurofibromatosis type-1 (NF1). Hum Mutat. 2011; 32: 213–219.
[27] Kehrer-Sawatzki H, Mautner VF, Cooper DN. Emerging genotype-phenotype relationships in patients with large *NF1* deletions. Hum Genet. 2017; 136: 349–376.
[28] Pös O, Radvanszky J, Styk J, et al. Copy number variation: methods and clinical applications. Appl Sci. 2021; 11: 819.
[29] Carter NP. Methods and strategies for analyzing copy number variation using DNA microarrays. Nat Genet. 2007; 39(Suppl 7): S16–S21.
[30] Kehrer-Sawatzki H, Cooper DN. Classification of *NF1* microdeletions and its importance for establishing genotype/phenotype correlations in patients with *NF1* microdeletions. Hum Genet. 2021; 140: 1635–1649.
[31] Bianchessi D, Morosini S, Saletti V, et al. 126 novel mutations in Italian patients with neurofibromatosis type 1. Mol Genet Genomic Med. 2015; 3: 513–525.
[32] Nguyen R, Dombi E, Widemann BC, et al. Growth dynamics of plexiform neurofibromas: a retrospective cohort study of 201 patients with neurofibromatosis 1. Orphanet J Rare Dis. 2012; 7: 75.
[33] Yethindra V, Tagaev T, Mamytova E, et al. A rare case of patient with neurofibromatosis type 1 in a genotype-phenotype correlation revealing a submicroscopic deletion on the long arm of chromosome 17. Clin Case Rep. 2021; 9: 2397–2399.
[34] Roehl AC, Vogt J, Mussotter T, et al. Intrachromosomal mitotic nonallelic homologous recombination is the major molecular mechanism underlying type-2 *NF1* deletions. Hum Mutat. 2010; 31: 1163–1173.
[35] Vogt J, Mussotter T, Bengesser K, et al. Identification of recurrent type-2 *NF1* microdeletions reveals a mitotic nonallelic homologous recombination hotspot underlying a human genomic disorder. Hum Mutat. 2012; 33: 1599–1609.
[36] Bengesser K, Cooper DN, Steinmann K, et al. A novel third type of recurrent *NF1* microdeletion mediated by nonallelic homologous recombination between *LRRC37B*-containing low-copy repeats in 17q11.2. Hum Mutat. 2010; 31: 742–751.
[37] Zickler AM, Hampp S, Messiaen L, et al. Characterization of the nonallelic homologous recombination hotspot PRS3 associated with type-3 *NF1* deletions. Hum Mutat. 2012; 33: 372–383.
[38] Kehrer-Sawatzki H, Wahlländer U, Cooper DN, et al. Atypical *NF1* microdeletions: challenges and opportunities for genotype/phenotype correlations in patients with large *NF1* deletions. Genes (Basel) 2021; 12: 1639.
[39] Well L, Döbel K, Kluwe L, et al. Genotype-phenotype correlation in neurofibromatosis type-1: *NF1* whole gene deletions lead to high tumor-burden and increased tumor-growth. PLOS Genet. 2021; 17: e1009517.
[40] Plotkin SR, Bredella MA, Cai W, et al. Quantitative assessment of whole-body tumor burden in adult patients with neurofibromatosis. PLOS ONE 2012; 7: e35711.
[41] Kehrer-Sawatzki H, Cooper DN. Challenges in the diagnosis of neurofibromatosis type 1 (NF1) in young children facilitated by means of revised diagnostic criteria including genetic testing for pathogenic *NF1* gene variants. Hum Genet. 2022; 141: 177–191.
[42] Tucker T, Wolkenstein P, Revuz J, et al. Association between benign and malignant peripheral nerve sheath tumors in NF1. Neurology 2005; 65: 205–211.
[43] Ferner RE, Gutmann DH. International consensus statement on malignant peripheral nerve sheath tumors in neurofibromatosis. Cancer Res. 2002; 62: 1573–1577.
[44] Hahn N, Büschgens L, Schwedhelm-Domeyer N, et al. The orphan cytokine receptor CRLF3 emerged with the origin of the nervous system and is a neuroprotective erythropoietin receptor in locusts. Front Mol Neurosci. 2019; 12: 251.
[45] Wegscheid ML, Anastasaki C, Hartigan KA, et al. Patient-derived iPSC-cerebral organoid modeling of the 17q11.2 microdeletion syndrome establishes CRLF3 as a critical regulator of neurogenesis. Cell Rep. 2021; 36: 109315.
[46] Park SH, Kang N, Song E, et al. ATAD5 promotes replication restart by regulating RAD51 and PCNA in response to replication stress. Nat Commun. 2019; 10: 5718.
[47] Wan L, Wang Y, Zhang Z, et al. Elevated TEFM expression promotes growth and metastasis through activation of ROS/ERK signaling in hepatocellular carcinoma. Cell Death Dis. 2021; 12: 325.
[48] Venturin M, Carra S, Gaudenzi G, et al. *ADAP2* in heart development: a candidate gene for the occurrence of cardiovascular malformations in NF1 microdeletion syndrome. J Med Genet. 2014; 51: 436–443.
[49] Douglas J, Cilliers D, Coleman K, et al. Mutations in *RNF135*, a gene within the *NF1* microdeletion region, cause phenotypic abnormalities including overgrowth. Nat Genet. 2007; 39: 963–965.
[50] Brussa Reis L, Turchetto-Zolet AC, Fonini M, et al. The role of co-deleted genes in neurofibromatosis type 1 microdeletions: an evolutive approach. Genes (Basel) 2019; 10: 839.
[51] Zjablovskaja P, Kardosova M, Danek P, et al. Correction to: EVI2B is a C/EBPalpha target gene required for granulocytic differentiation and functionality of hematopoietic progenitors. Cell Death Differ. 2019; 26: 198. Erratum: Cell Death Differ. 2017; 24: 705–716.
[52] Li S, Yang F, Yang YK, et al. Increased expression of ecotropic viral integration site 2A indicates a poor prognosis and promotes osteosarcoma evolution through activating MEK/ERK pathway. J Recept Signal Transduct Res. 2019; 39: 368–372.
[53] Hu F, Deng X, Yang X, et al. Hypoxia upregulates Rab11-family interacting protein 4 through HIF-1alpha to promote the metastasis of hepatocellular carcinoma. Oncogene 2015; 34: 6007–6017.
[54] Venturin M, Bentivegna A, Moroni R, et al. Evidence by expression analysis of candidate genes for congenital heart defects in the NF1 microdeletion interval. Ann Hum Genet. 2005; 69: 508–516.

(Bene Judit dr.,
Pécs, Szigeti út 12., 7624
e-mail: bene.judit@pte.hu)