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NEUROFIBROMIN IN NEUROFIBROMATOSIS TYPE 1 – MUTATIONS IN *NF1* GENE AS A CAUSE OF DISEASE*

NEUROFIBROMINA W NERWIAKOWŁÓKNIAKOWATOŚCI TYPU I – MUTACJE W GENIE *NF1* JAKO PRZYCZYNA CHOROBY

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Abstract

Neurofibromatosis type I (NF1) is a disease associated with the presence of benign neurofibromas and malignant tumours of the central and peripheral nervous system, that are accompanied by characteristic changes in the skin, such as café-au-lait spots or axillary freckling. In 50% of NF1 patients, the clinical symptoms become apparent below 1st year and in 97%, before the age of 8 years. The disease is mainly caused by the presence of mutation in the NF1 gene that encodes neurofibromin – a protein involved in the regulation of several cellular signaling pathways responsible for cell proliferation and differentiation. Neurofibromin is necessary for embryonic development and involved mainly in the differentiation of neural crest derived cells, mesenchymal cells, neural cells, melanocytes and bone cells. Type I neurofibromatosis is inherited in autosomal dominant manner, nevertheless about 50% of detected mutations are de novo ones. The mutations have full penetrance, although they also have significant pleiotropic effect. Over 1485 different mutations have been identified in the NF1 gene so far, most of which lead to a synthesis of truncated, non-functional protein. It is estimated that the point mutations are responsible for approximately 90% of cases of NF1. The remaining 5-7% of NF1 cases are associated with the presence of a single exon or whole NF1 gene deletion (17q11.2 microdeletion syndrome). The article discusses the role of neurofibromin in cell signaling with the special attention to RAS/MAPK pathway regulation as well as in organism development. Also the basic methods of molecular analysis of NF1 gene are presented in the context of their application in the diagnosis and clinical differentiation of the disease.

Key words: neurofibromin, neurofibromatosis type I, RAS/MAPK signaling pathway, NF1

Streszczenie

Nerwiakowłókniakowatość typu I (NF1) jest chorobą związaną z występowaniem łagodnych i złośliwych guzów centralnego i obwodowego układu nerwowego – nerwiakowłókniaków, którym towarzyszą charakterystyczne zmiany skórne takie jak plamy w kolorze kawy z mlekiem czy piegowate nakrapianie pach i pachwin. U 50% pacjentów objawy kliniczne choroby ujawniają się poniżej 1 roku, zaś u 97% przed ukończeniem 8 roku życia. Przyczyną choroby są mutacje w genie NF1 kodującym neurofibrominę – białko biorące udział w regulacji wielu szlaków sygnałowych w komórce odpowiedzialnych za proliferację i różnicowanie komórek. Neurofibromina jest niezbędna w rozwoju embrionalnym organizmu, bierze udział w rozwoju i różnicowaniu przede wszystkim komórek wywodzących się z grzebienia mezenchymalnego, komórek nerwowych, melanocytów i komórek tkanki kostnej. Nerwiakowłókniakowatość typu I dziedziczona jest autosomalnie dominująco, jednak około 50% wykrywanych mutacji ma charakter de novo. Mutacje mają pełną penetrację, ale obserwuje się dla nich znaczny efekt plejotropowy. Do tej pory zidentyfikowano ponad 1485 różnych zmian w genie NF1, z których większość prowadzi do powstania skróconej, niefunkcjonalnej formy białka. Szacuje się że zmiany na poziomie sekwencji DNA odpowiedzialne są za około 90% przypadków NF1. Pozostałe 5-7% przypadków choroby związanych

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jest z obecnością delecji pojedynczych eksonów genu NF1 lub dużych delecji w obszarze 17q11.2 obejmujących cały gen NF1. W artykule omówiona została rola neurofibrominy w funkcjonowaniu komórki oraz rozwoju całego organizmu, z uwzględnieniem regulacji szlaku RAS/MAPK. Przedstawione zostały podstawowe metody analizy molekularnej genu NF1 w kontekście ich zastosowania w diagnostyce i różnicowaniu klinicznym choroby.

Słowa kluczowe: neurofibromina, nerwiakowłókniakowatość typu 1, szlak RAS/MAPK, NF1

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INTRODUCTION

Neurofibromatosis (NF) is a heterogeneous group of diseases associated with occurrence of tumours of central and peripheral nervous system. Most frequent NF types are: neurofibromatosis type I (96-97%) and neurofibromatosis type II (2-3%). Both clinical entities are characterized by the presence of neoplasms – benign or malignant tumours, that are accompanied by characteristic changes in the skin such as cafe-au-lait spots (CAL) or axillary freckling (1).

Neurofibromatosis type I appears in the early childhood. It is estimated that approximately 50% of sporadic cases of NF1 develop characteristic clinical features by the age of 1, and in 97% of cases - before the age of 8 years (2).

Neurofibromatosis type I belongs to RASopathies – a group of diseases associated with improper functioning of RAS/MAPK signal transduction pathway – a kinase cascade activated by mitogenic factors. The RAS/MAPK pathway is one of the major cellular signaling pathways that mediates cell growth and proliferation stimulated by growth factor. Proper activity of this pathway is also essential for cell differentiation during organism development (3). Abnormal activation of the RAS/MAPK pathway not only contributes to neoplastic transformation, but also can cause a wide spectrum of clinical symptoms, like pigmentation disturbances, cardiovascular defects and changes in skeletal and nervous system. Apart from neurofibromatosis type I there are other inherited diseases caused by a defect in the genes encoding RAS/MAPK pathway proteins, for instance Noonan syndrome and its allelic form Leopard syndrome, cardiofaciocutaneous syndrome and Costello syndrome (4).

THE ROLE OF NEUROFIBROMIN IN CELL DIFFERENTIATION AND ORGANISM DEVELOPMENT

Neurofibromatosis type I is a disease caused by the presence of mutation in *NF1* gene located on chromosome 17 (17q11.2). The protein product of *NF1* gene is neurofibromin, a cytoplasmic protein consisting of 2818 amino acids and molecular weight of 280kDa (5, 6). Additional studies have shown that neurofibromin may be also located in the nucleus in certain cell types (7). The protein is expressed in all cells, but its expression level is the highest in neurons, Schwann cells, glial cells and leukocytes (8). Neurofibromin is highly conserved throughout evolution – shares up to 98% homology with its murine analogue (9) and 60% with the *Drosophila* protein (10).

Neurofibromin is a multifunctional protein involved in the regulation of many cellular signaling pathways. The protein regulates the activity of the RAS/MAPK and mTOR pathways, adenylate cyclase activity and interacts with proteins such as microtubulin, kinesin-1, protein kinase A and C, caveolin and amyloid precursor protein (11). Probably many functions of neurofibromin remain to be elucidated, but these that have been described so far can partly explain the broad spectrum of clinical symptoms observed in patients with NF1.

Neurofibromin is involved in the regulation of a number of signaling pathways related to the cell growth and proliferation. Neurofibromin mainly acts by modulating the activity of the RAS-family proteins. The NF1 protein belongs to a group of Ras GTPase-activating proteins (GAP), which accelerate the hydrolysis of the GTP bound to active RAS protein**, what leads to RAS inactivation and subsequent inhibition of RAS/MAPK pathway. Improper function or decreased level of neurofibromin lead to increased activity of the RAS/MAPK pathway and thereby promotes uncontrolled cell proliferation (3, 12). For this reason, the proteins from the GAP family are referred as tumor suppressors. Their activity is essential for appropriate regulation of cell growth and proliferation.

The *NF1* gene is also mentioned in the context of Knudson hypothesis (multiple-hit hypothesis) concerning the mechanisms of malignant transformation (13). The somatic mutations in *NF1* gene have been observed in specimens of benign and malignant tumours in patients with confirmed molecularly neurofibromatosis type I. Moreover, the activity of the RAS/MAPK pathway is significantly increased in tumour cells derived from the NF1 patients (14).

**RAS family of proteins, also referred to as p21, are encoded by *HRAS*, *KRAS* and *NRAS* genes. The names of the genes are derived from the original descriptions of RAS proteins in the context of their oncogenic role: Harvey rat sarcoma viral oncogene – *HRAS*, Kirsten rat sarcoma viral oncogene homolog - *KRAS* and neuroblastoma RAS viral oncogene homolog - *NRAS*. RAS proteins have a molecular weight of 21kDa and play a key role in the signal transduction from the membrane to the nucleus. The RAS protein activates the cascade of the mitogen activated kinases (MAPKs). RAS protein binds GTP or GDP in its active and inactive form, respectively.

In addition to the RAS/MAPK pathway, neurofibromin plays an important role in the regulation of the activity of mTOR signal transduction pathway. Dysregulation of this signaling pathway is characteristic of a group of diseases called phakomatoses. The *NF1* deficient cells show continuous activation of the mTOR pathway resulting from the presence of an activated RAS protein form (15). Furthermore, the cell lines derived from malignant tumours from patients with NF1 are sensitive to rapamycin – mTOR inhibitor. This suggests a potential utility of rapamycin in the NF1 treatment (15).

Neurofibromin is essential for normal embryonic development in mammals. Studies in murine models have shown that the lack of neurofibromin is lethal. Embryos that did not have a functional *NF1* gene died between 12.5 and 13.5 day of embryonic development, mainly because of the altered development of blood vessels. In turn, selective inactivation of *NF1* in endothelial cells or nervous cells led to abnormalities in the development of cardiovascular or nervous system (problems with the formation of cerebral cortex) (11).

The *in vitro* studies have shown that NF1-deficient cells exhibited reduced adhesion, increased motility and changed actin cytoskeleton structure during cell movement. The important role of neurofibromin in embryonic development, particularly in the differentiation and functioning of mesenchymal cells, explains typical signs of neurofibromatosis type I, especially those which are not related to enhanced cell proliferation (16).

Due to the very high prevalence of pigmentation disturbances (CAL spots, freckling) in patients with NF1, it was suggested that neurofibromin may be necessary for proper development, differentiation and function of melanocytes (17). *In vitro* studies showed that the expression of the genes encoding markers characteristic of the melanocytes is controlled by the RAS/MAPK signal transduction pathway. The loss of one copy of the *NF1* gene, leading to an increase in activity of the RAS protein, is associated with increased levels of transcription of genes specific to melanocytes, which can lead to disturbances in the distribution of pigment in the skin (17).

Another characteristic sign of NF1 are bone and skeletal defects, observed in about 30% of the patients. As suggested by the functional *in vitro* studies, this may be related to the specific role of NF1 protein in the control of differentiation and function of bone cells, especially osteoclasts and osteoblasts (18). It was found that the decrease of neurofibromin expression is associated with decreased expression of osteoblast-specific bone markers and reduced level of bone mineralization. In the case of osteoclasts, decreased *NF1* gene expression led to the activation of the RAS/MAPK pathway, that stimulated cell survival and promoted bone matrix degradation (19). Any disturbances in the functioning of the cells involved in bone metabolism may explain the skeletal defects observed in patients with NF1.

MOLECULAR BASIS OF NEUROFIBROMATOSIS TYPE I

Type I neurofibromatosis is inherited in autosomal dominant manner. Approximately 50% of mutations found in patients with a clinical diagnosis of NF1 are *de novo* ones. Identified mutations not only have a full penetration, but also have pleiotropic effect. Patients with the same mutation, even in the same family, may have extremely distinct clinical signs of the disease. This suggests the significant role of modifier genes or epigenetic factors that are responsible for the variability of NF1 phenotypic expression (20).

The *NF1* gene was identified with positional cloning*** in 1990 (21). It is one of the biggest genes in the human genome that encompasses approximately 280kbp of genomic DNA. The basic transcript consists of 57 exons, although the gene has four alternatively spliced exons 9bR, 10A2, 23a and 48a (fig. 1). The *NF1* gene contains two large introns, 1 and 35, (1 and 27b according to the previous numbering), comprising more than 60 kb each. Within the intron 35, there are three small genes *EVI2A*, *EVI2B* (ecotropic viral integration site) and *OMG* (oligodendrocyte myelin glycoprotein), whose expression occurs in the opposite direction to the *NF1* gene. The *EVI2B* and *EVI2A* are human homologs of the *Evi-2A* and *Evi-2* mouse genes that encode proteins related to the development of leukemia in mice. The *OMG* gene product is a membrane glycoprotein expressed in the human central nervous system during the myelination process of nerve cells (22).

The mRNA of *NF1* gene has over 11-13kbp in length with 8457bp open reading frame and 3.5 kb of 3' untranslated region. It has been observed that *NF1* transcripts vary in size, depending on the tissue, as a result of alternative splicing of pre-mRNA. The most common form of *NF1* mRNA is NM_000267 transcript that has about 8.5kb, contains 57 exons and encodes protein consisting of 2818 amino acids (23).

Besides the alternative mRNA splicing, the transcript of the *NF1* gene may undergo editing by several proteins like P66, P44, APOBEC-1, that form so-called editosome. This protein complex converts the cytosine located at the position c.3916 to uracil, thus generating a premature STOP codon. The protein encoded by this transcript would not contain the crucial GRD domain. In addition, this type of transcript, like all containing premature STOP codon, is unstable and undergoes rapid degradation called nonsense mediated decay. In such a situation, a nonequivalent

***Positional cloning is a method for the identification of genes responsible for the occurrence of a specific phenotype without a precise knowledge about its pathogenesis. Formerly described as reverse genetics. The method is based on the analysis of the polymorphic markers throughout the genome in all family members in which a specific disease occurred. Such analysis allows to identify of a specific haplotype (set of genetic markers) which is inherited by the affected individuals. This enables the determination of the approximate chromosomal region associated with a particular phenotype. Matching chromosomal region allows to select potential gene/genes associated with the pathogenesis of the disease. Subsequently, sequencing of the predicted gene and further functional studies enable the identification of gene associated with the occurrence of a specific phenotype.

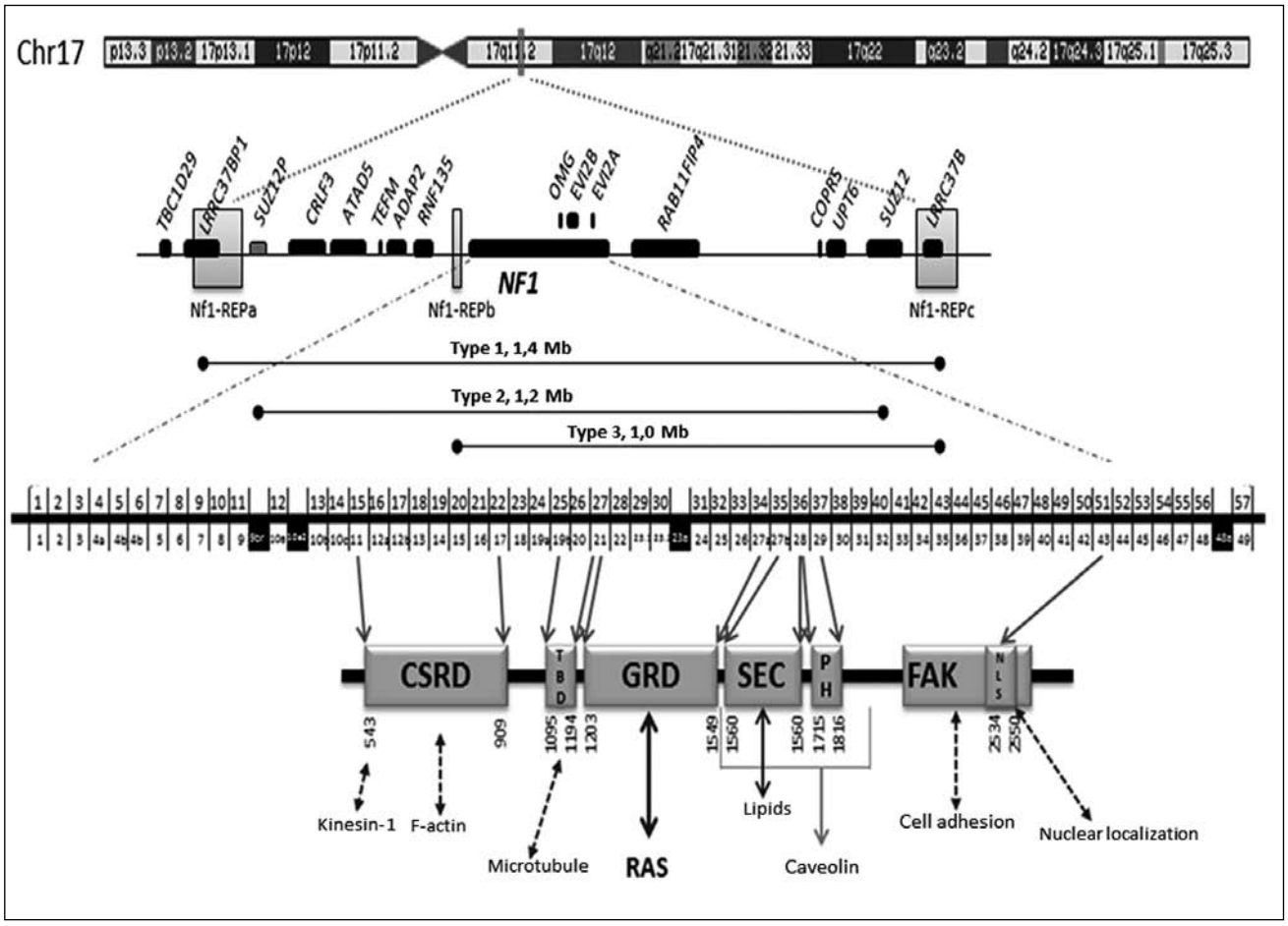


Fig. 1. The scheme of NF1 gene and neurofibromin protein (figure prepared by author).
From the upper part: the localization of NF1 gene in chromosome 17 and 17q11.2 locus; the scheme of NF1 gene with all coding exons of NM_000267 NF1 transcript described according to current and previous nomenclature, the exons undergoing alternative splicing are shown in black (the scheme is not in scale); the scheme of functional domains of neurofibromin, the proteins interacting with neurofibromin are shown.
Neurofibromin domains: the CSRD domain – cysteine and serine rich domain, the TBD domain – tubulin binding domain, the GRD domain – domain responsible for interaction with RAS and GTP hydrolysis; the Sec14-PH domain – bipartite lipid binding domain; the FAK domain – focal adhesion kinase-interacting domain; NLS sequence – nuclear localization sequence.

Ryc. 1. Schematyczne przedstawienie struktury genu NF1 oraz białka NF1 (opracowanie własne).
Od góry: lokalizacja genu NF1 w obrębie chromosomu 17 oraz locus 17q11.2; schemat struktury genu NF1 przedstawiający wszystkie eksony kodujące podstawowy transkrypt NF1 (NM_000267) wraz z podaniem obowiązującej i starszej numeracji eksonów, eksony biorące udział w alternatywnym składaniu przedstawione są w kolorze czarnym (rycina nie jest przedstawiona w skali); schemat domen funkcjonalnych neurofibrominy oraz nazwy białek, z którymi oddziaływują poszczególne domeny.
Domeny białka NF1: domena CSRD – domena bogata w cysteinę i serynę, domena TBD – domena wiążąca tubulinę, domena GRD – domena odpowiedzialna za oddziaływanie z białkiem RAS i hydrolizę związanego z nim GTP, domena Sec14-PH – dwuczęściowa domena wiążąca kwasy tłuszczowe, domena FAK – odpowiedzialna za oddziaływanie NF1 z kinazą adhezji miejscowej, sekwencja NLS – sygnał lokalizacji jądrowej.

expression of both *NF1* alleles is observed and this results from different transcript stability or altered transcript transport from the nucleus to the cytoplasm (24).

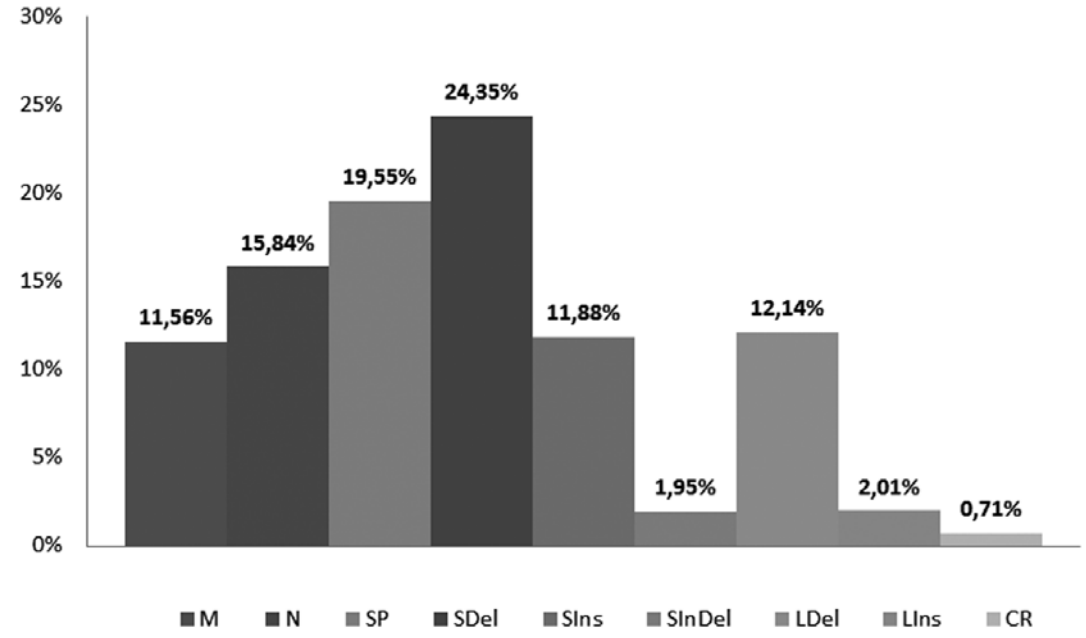
Neurofibromin expression is regulated at the level of transcription and translation, that can result in rapid change of mRNA and protein level. Proteins and non-coding RNA molecules interacting with the *NF1* transcript may cause its degradation and therefore affect the availability of the protein. Such factors might be potential modifiers of the clinical expression of neurofibromatosis type I (25).

MUTATIONS IN THE GENE *NF1* AS A CAUSE OF NEUROFIBROMATOSIS TYPE I

As mentioned earlier, neurofibromatosis type I is associated with the presence of mutations in *NF1* gene. About 50% of the mutations are *de novo* ones and are not inherited from the parent of a patient. The incidence of *de novo* mutations in *NF1* gene *locus* is very high compared to other genes involved in the pathogenesis of genetically determined diseases and the mutation

rate is approximately 1 x 10^{-4} per gamete per generation (26). The large size of *NF1* gene does not fully explain such a high rate of new mutation formation. Mutations identified so far are randomly distributed within the gene – they are not localized in "hot spot" regions, which are more prone to the mutation occurrence. Mutations are quite common in exons 3, 5 and 27 (according to previous numbering 3, 4b and 21) which contain structural elements susceptible to mutations (e.g., in exon 5 tandem repeat sequence), although they cannot be referred as mutational "hot spots" (27 and based on the Human Gene Mutation Database).

To date, more than 1485 various changes were detected in the *NF1* gene. Most of these changes (≈ 80%) result in truncated form of the protein (fig. 2). Mutations of this type are often observed in the genes encoding tumour suppressors, such as *APC*, *TSC1* (≈100% mutation leads to truncated protein), *RB1*, *BRCA1*, *ATM* (≈80% of detected mutations) (based on the Human Gene Mutation Database). In addition to nonsense mutations and small deletions or insertions causing a frame shift and a premature termination of the protein, point mutations disturbing the process of *NF1* mRNA splicing are commonly observed. These mutations include not only the changes in the consensus sequences GT-AG at the intron-exon boundaries, but also substitutions in the regulatory sequences (like exonic splicing enhancers /ESE/ sequences). Among them we can distinguish: (1) mutations in the conserved 3' or 5' end of the intron resulting in the entire exon skipping, (2) intronic mutations causing insertion of a cryptic exon, (3) exonic mutations leading to the creation of a new splicing site, resulting in deletion of exon fragment, (4) point mutations in conserved splicing sites, favouring insertion of a cryptic exon and intron fragment and causing partial deletion of an exon or insertion of intron fragment and (5) exonic mutations causing deletion of an entire exon. Mutations of this type detected at genomic DNA level may be improperly classified either as "silent"



Mutation type / *Typ mutacji*	Number of mutation / *Liczba mutacji*
Missense mutations / *Mutacje typu missens* (M)	178
Mutacje typu nonsens / *Nonsense mutations* (N)	244
Splicing mutations */Mutacje powodujące zaburzenia składania mRNA* (SP)	301
Small deletions / *Małe delecje* (SDel)	375
Small insertions / *Małe insercje* (SIns)	183
Small indels / *Małe insercje/delecje* (SInDel)	30
Large deletions / *Duże delecje* (LDel)	187
Large insertions / *Duże insercje* (LIns)	31
Complex rearrangements / *Złożone rearanżacje* (CR)	11

Fig. 2. The frequency of different mutation types in NF1 gene.
Fig. 2. In table, the number of identified mutation of each type is listed, at the chart – their frequency is shown.

Ryc. 2. Częstość występowania różnych typów mutacji w genie NF1.
Ryc. 2. W tabeli podano liczbę zidentyfikowanych mutacji, a na wykresie zaznaczono częstość ich występowania.

mutations or mutations resulting in amino acid change. This inaccurate classification may lead to underestimation of their role as a potential pathogenic mutation (28, 29, 30). The remaining 20% of point mutations are missense mutations leading to amino acid changes that alter the activity of the neurofibromin protein (based on the Human Gene Mutation Database).

It is estimated that changes in DNA sequence are responsible for approximately 90% of NF1 cases. The remaining 5-7% of cases are associated with the presence of a *NF1* single exon deletion or large deletions on chromosome 17q11.2 encompassing entire *NF1* gene (fig. 1). There are three types of large deletions: type I of about 1.4 Mb, comprising 14 different genes located between the regions of NF1-LCR (*Nf1-RepA* and *Nf1-RepC*), type II of about 1.2 Mb, spanning 13 different genes located between *JJAZ1* gene and its pseudogene and type III of about 1 Mb, that includes the genes located between the sequences and *Nf1-RepB* and *Nf1-RepC* (31).

The analysis of mutation inheritance has revealed that the point mutations often occur on the chromosome of the paternal origin (>80%), while microdeletions are more common on the maternal chromosome (32, 33).

MOLECULAR ANALYSIS OF THE *NF1* GENE

Despite growing knowledge about the role of *NF1* gene mutations in the pathogenesis of neurofibromatosis type I, the molecular diagnostics of this disease is still difficult and molecular basis of the diseases requires further studies. This is mainly due to the size of the gene (large number of exons) as well as the presence of *NF1* pseudogenes on the other chromosomes. Both these factors mean that the molecular analysis of *NF1* gene based on the genomic DNA sequence is a huge challenge. The analysis of RNA may facilitate molecular studies, but there is a problem with RNA stability and possible premature degradation of transcripts containing STOP codon so called nonsense-mediated mRNA decay (34).

In the past, molecular studies of *NF1* gene were performed primarily with the protein truncation test (PTT) that was based on the *in vitro* synthesis of peptide fragments on the *NF1* fragments template. This method enabled the identification of mutations that resulted in the synthesis of shortened protein. However, it was very laborious and required adequate laboratory facilities. In accordance with the current standards of molecular diagnostics, the PTT test is not sufficiently precise – it does not detect missense mutations or small insertions/deletions, which do not alter the open reading frame (35, 36).

The PTT method has been gradually displaced by the sequencing of *NF1* gene on the mRNA template. This procedure requires one additional reverse transcription step – a synthesis of cDNA from RNA. At the same time, this method allows for faster *NF1* sequence analysis because of the smaller number of amplicons to analyze as compared to the sequencing of genomic DNA. The identification of a mutation at the transcript level is an indication for the sequencing of a proper region of genomic DNA in order to identify a nucleotide variation causing improper mRNA splicing. Moreover, the identification of mutation at the genomic DNA level facilitates carrier testing in proband's family. It was estimated that the sequencing analysis at the mRNA level allows to detect approximately 90% of mutations in patients with a clinical diagnosis of NF1 (37).

Obviously the molecular analysis of *NF1* gene can be limited to genomic DNA and sequencing with classical Sanger sequencing method or next generation sequencing (fig. 3). In the first case, the analysis process itself will last much longer than sequencing of RNA or cDNA, which is a significant disadvantage of this method. On the other hand, use of the next generation sequencing allows for concurrent analysis of all exons of the *NF1* gene. However, such approach involves much higher cost of performance, especially when it is done for a single patient. A significant limitation of both methods is unfeasibility of direct identification of mutations responsible for splicing abnormalities, which represent 21% of mutations in *NF1* gene identified so far. Application of those tests can lead to incorrect classification of changes that actually cause abnormal *NF1* splicing. Therefore, the analysis of genomic DNA must always be followed by careful *in silico* analysis using computer packages, which allow to identify potential splicing sites and regulatory elements controlling this process (38) .

The detection of deletion of a fragment or entire *NF1* gene is based on techniques enabling the investigation of copy number of specific DNA fragment. Originally, the deletion identification was possible only with cytogenetic methods, particularly fluorescence in situ hybridization (FISH) or high resolution karyotype analysis, although only large deletions involving whole *NF1* gene could be found with these methods. Also the analysis of polymorphic microsatellite sequences in NF1 families was used to detect large deletions (39).

Currently, a common method used to analyze *NF1* gene and 17q11.2 region is a multiplex ligation dependent probe amplification (MLPA) introduced in 2004. This method allows quantitative assessment of specific DNA fragments and detection of deletions or duplications in the tested *locus* (fig. 3). The main advantage of this method is the possibility to detect a single exon deletion, which was not achievable in the case of the FISH or karyotype analysis (40, 41).

It is estimated that the combination of several methods (e.g., sequencing analysis of *NF1* transcript and MLPA) in the analysis of molecular pathogenesis of neurofibromatosis type I, allows to detect mutations in up to 95% of patients with a clinical diagnosis of the disease. In the remaining 5% of cases, more complex changes involving *NF1* gene may occur, such as chromosomal rearrangements e.g translocation or regulatory mutations in the promoter or regulatory regions of the *NF1* gene (38).

GENOTYPE-PHENOTYPE CORRELATION

The spectrum of mutations in the *NF1* gene is very broad, and clinical studies have shown no significant relationship between the type of mutation and the clinical status of the disease. The deletion of the entire gene,

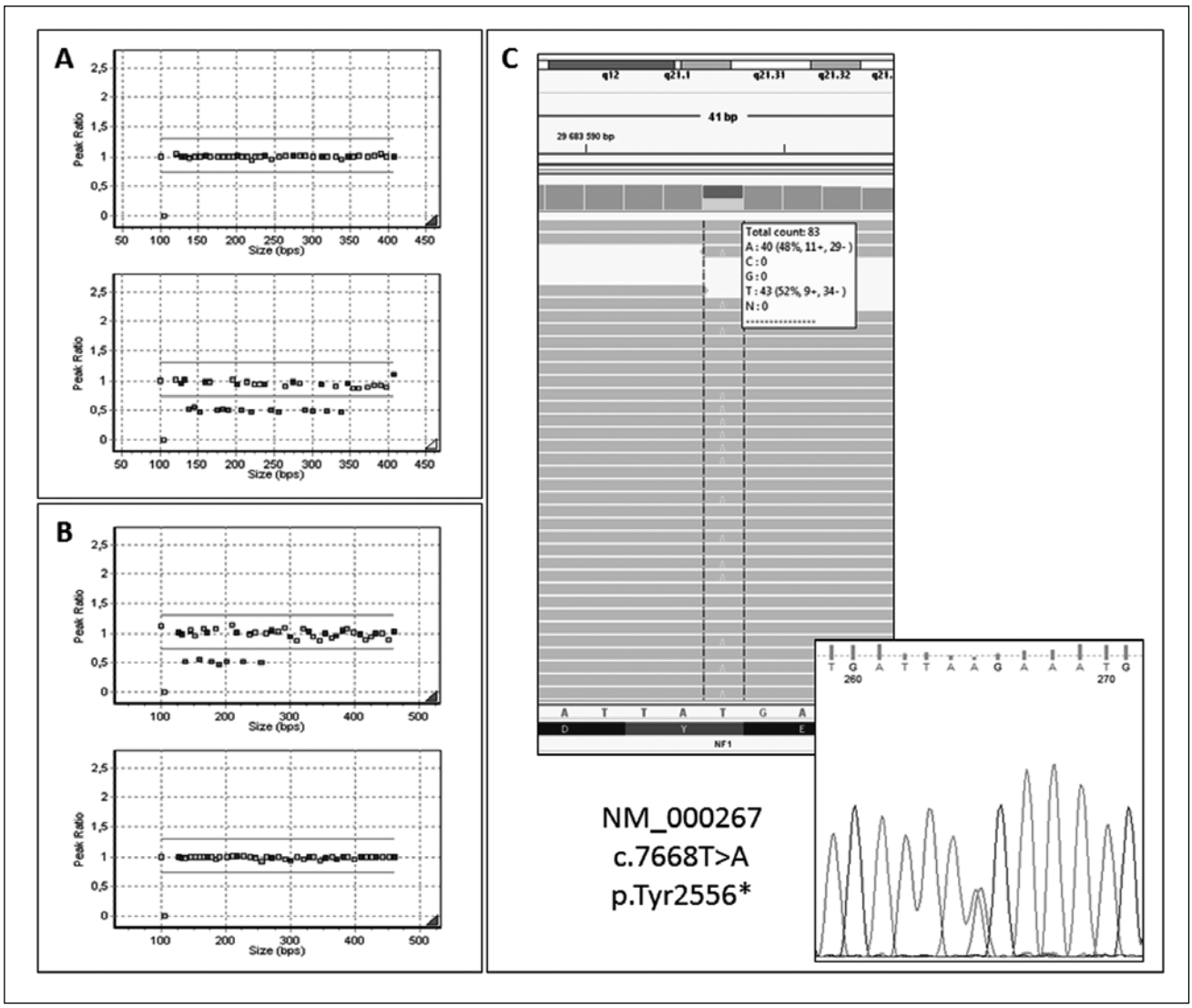


Fig. 3. The results of molecular analysis for patients with neurofibromatosis type I clinical suspicion.
a) The MLPA analysis with P122 MLPA kit containing probes for NF1 and other genes from 17q11.2 region; upper panel – normal results for a control person; lower panel – deletion in 17q11.2 region that encompass NF1 and neighboring genes: SUZ12P, CRLF3, ATAD5, ADAP2, RNF135, UTP6, SUZ12, LRRC37B;
b) The MLPA analysis with P082 MLPA kit containing probes for selected exons of NF1 gene; upper panel – normal results for a control person; lower panel – deletion in NF1 gene encompassing exons 1-12 (lower signal for probes corresponding to exons: 1, 3, 5, 8, 10 and 12);
c) The NF1 gene sequencing with next generation sequencing (left side) the confirmation of mutation presence with classic Sanger sequencing (right). The results of NGS and classic sequencing were prepared with Integrative Genomics Viewer and FinchTV software, respectively.

Ryc. 3. Wyniki analizy DNA pacjentów z podejrzeniem klinicznym nerwiakowłókniakowatości typu I.
a) Analiza MLPA z użyciem zestawu P122 zawierającego sondy dla genu NF1 oraz innych z regionu 17q11.2; górny panel - wynik prawidłowy dla osoby dla osoby zdrowej, dolny panel – delecja w regionie 17q11.2 obejmująca gen NF1 oraz sąsiadujące z nim geny: SUZ12P, CRLF3, ATAD5, ADAP2, RNF135, UTP6, SUZ12, LRRC37B;
b) Analiza MLPA z użyciem zestawu P082 zawierającego sondy dla wybranych eksonów genu NF1. górny panel – wynik prawidłowy dla osoby dla osoby zdrowej, dolny panel – delecja w genie NF1 obejmująca eksony 1-12 (niższy sygnał dla sond odpowiadających eksonom: 1, 3, 5, 8, 10 i 12);
c) Sekwencjonowanie genu NF1 z wykorzystaniem sekwencjonowania następnej generacji (z lewej) wraz z potwierdzeniem mutacji sekwencjonowaniem klasyczną metodą Sangera (z prawej). Wyniki sekwencjonowania opracowane w programie Integrative Genomics Viewer (NGS) i Finch TV (Sanger).

referred to as 17q11.2 microdeletion, affects about 5% of NF1 patients (31, 39). It is suggested that 17q11.2 microdeletion is associated with a more severe form of the disease, in the course of which neurofibromas, dysmorphic features and learning difficulties are observed (42, 43).

It has also been observed that people with a specific mutation – deletion of three nucleotides in exon 17 of *NF1* gene (c.2970-2972 delAAT), which does not change reading frame, develop much moderate symptoms of the disease. Those patients have pigmentation defects and Lisch nodules typical for NF1, whereas cutaneous neurofibromas

or clinically detectable plexiform neurofibromas are not present (44).

Furthermore, the less prominent forms of NF1 can be caused by mosaicism – the coexistence of genetically different cell lines in one organism. In the case of neurofibromatosis type I, mosaicism is a result of the *NF1* mutation appearance during postzygotic period and depending on the time of mutation occurrence may lead to generalized, segmental or gonadal mosaicism (45). The somatic mutations that occurred at an early stage of embryonic development result in generalized mosaicism, which results in a mild form of the disease, resembling classical NF1. Mutations that arise at a later stage of prenatal development result in segmental NF1 whose clinical symptoms are limited to the one part of the body. Gonadal mosaicism is relatively rare. The pathogenic mutation is present only in germline cells and there is no evidence of clinical symptoms. It is recognized in families where healthy parents have two or more children affected with NF1 (46).

Also the neurofibromatosis-Noonan syndrome (NFNS, OMIM 601321) may be classified as a subtype of NF1. Patients with this diagnosis have characteristic clinical features of Noonan syndrome such as hypertelorism, downslanting palpebral fissures, low-set ears, webbed neck or pulmonary valve stenosis and characteristic of NF1-CAL spots and other skin changes. These patients do not have the mutation in *PTPN11* gene that is frequently mutated (up to 50% of cases) in patients with classical Noonan syndrome. However, they do have mutations in the *NF1* gene. It was suggested that the specific phenotype of NFNS patients may result from specific location of mutations in *NF1* gene that result in the disturbed interaction of neurofibromin with RAS protein (4, 47).

SUMMARY

Neurofibromatosis type I is a multisystem disease with a complex phenotype. It is characterized by the number of characteristic clinical features that are not unique to this disease and may occur in other clinical entities. One example is the Legius syndrome (OMIM 611431) which is also inherited in an autosomal dominant manner and shares many phenotypic features with NF1, such as CAL spots, axillary freckling, mild intellectual disability, macrocephaly and facial dysmorphic features. However, patients with this disorder, do not develop neurofibromas, Lisch nodules or tumours of central nervous system that are characteristic for neurofibromatosis type I (48). Legius syndrome is caused by the presence of pathogenic mutation in *SPRED1* gene (15q13.2), which encodes a protein that is a negative regulator of RAS/MAPK pathway. Originally, the mutations in this gene were detected in patients with NF1 clinical diagnosis, who did not have mutation in *NF1* gene. The overlap of clinical symptoms observed in NF1 and Legius syndrome patients can be explained by the fact that proteins encoded by *NF1* and *SPRED1* genes play a similar role in the cell signaling and both are negative regulators of the RAS/MAPK pathway. Moreover, if specific dysmorphic features are present in a patient with pigmentation disturbances, other clinical entities from RASopathy group should be considered in addition to the NF1 and Legius syndrome (49).

Clinical features, related to NF1, especially in young children, can also be observed in the Turcot syndrome – autosomal recessive disorder which is caused by mutations in *MLH1* and *MSH2* genes that encode proteins involved in DNA mismatch repair system. The clinical picture of Turcot syndrome resembles NF1, especially in the early stages of postnatal development, but the inheritance of the disease is completely different as the disease is inherited in autosomal recessive manner (50, 51).

The examples of Legius and Turcot syndromes prove the real need for careful clinical examination of the patient before beginning of molecular analysis to confirm the clinical diagnosis. Moreover, the fact that NF1 is inherited in an autosomal dominant manner and half of the identified mutations are familial ones, each molecular analysis should be followed by the genetic counselling for the patient and its family. The identification of the causative pathogenic mutation is also an indication for the molecular testing of the patient's parents. This ensures the appropriate genetic and/or other expertise counseling in the NF1 families.

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