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NOVEL CYTOGENETIC AND MOLECULAR TECHNIQUES IN THE DIAGNOSIS OF CONGENITAL ANOMALIES IN NEWBORNS

DIAGNOSTYKA GENETYCZNIE UWARUNKOWANYCH WRODZONYCH ZABURZEŃ ROZWOJOWYCH U NOWORODKÓW W ŚWIETLE NOWOCZESNYCH TECHNIK CYTOGENETYCZNO-MOLEKULARNYCH

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Abstract
*Knowledge of what causes developmental disorders, including congenital structural defects/anomalies, in the newborn population, facilitates the choice of further investigations, therapy and rehabilitation, allows the use of appropriate prophylaxis against comorbidities, makes it possible to specify prognosis, as well as provide reliable family counselling (both pre- and postnatal). Attempting to formulate a clinical diagnosis of a specific congenital anomaly syndrome, with or without dysmorphic features, based on history and detailed physical examination, remains crucial for the selection of the right genetic testing. Modern methods of molecular cytogenetics and molecular biology are targeted in nature (microdeletion MLPA, single gene sequencing) or are capable of analyzing the genome as a whole (array CGH, new-generation sequencing). Especially the latter techniques are now causing a rapid increase of diagnostic efficacy across different age groups, including newborns***.***

Key words: congenital defects, developmental disorders, rare disorders, newborns, genetic tests, array CGH, new generation sequencing

Streszczenie
Znajomość przyczyny wrodzonych zaburzeń rozwojowych, w tym wad/anomalii w budowie, u noworodków ułatwia prowadzenie dalszego postępowania diagnostycznego, terapeutycznego i rehabilitacyjnego, pozwala na zastosowanie odpowiedniej profilaktyki chorób współistniejących, umożliwia określenie rokowania, a także udzielenie właściwej porady rodzinnej (przedkoncepcyjnej i prenatalnej). Podjęcie próby sformułowania klinicznej diagnozy konkretnego zespołu wad/anomalii w budowie, w tym cech dysmorfii, na podstawie wywiadu i szczegółowego badania przedmiotowego pozostaje kluczowe z punktu widzenia doboru właściwego testu genetycznego. Nowoczesne metody cytogenetyki molekularnej i biologii molekularnej mają bowiem charakter celowany (MLPA mikrodelecyjne, sekwencjonowanie pojedynczego genu) lub analizują genom całościowo (CGH do mikromacierzy, sekwencjonowanie następnej generacji). Zwłaszcza ostatnie z wymienionych technik powodują obecnie lawinowy wzrost efektywności diagnostycznej w grupach chorych w różnych przedziałach wiekowych, w tym u noworodków.

Słowa kluczowe: wady rozwojowe, zaburzenie rozwoju, choroby rzadkie, noworodki, badania genetyczne, CGH do mikromacierzy, sekwencjonowanie następnej generacji

INTRODUCTION

Knowledge of what causes developmental disorders or birth defects in newborns can determine prognosis, help to choose the appropriate diagnostic, therapeutic and rehabilitation approach, and makes it possible to use the optimal prophylaxis of coexisting diseases. Establishing a specific diagnosis is also essential for the purposes of family counselling.

The key role of neonatologists or pediatricians in the diagnostic process of rare diseases relies primarily on the early identification of children from risk groups and ordering certain specialized diagnostic tests confirming the suspicion of a particular condition. Referral to a specialist genetic and/or metabolic clinic should become a standard procedure.

The application of modern methods of cytogenetic and molecular biology in the diagnostic process helps explain the etiology of a number of developmental disorders. Constant progress in molecular biology increases the chances of establishing the background of a growing number of genetic conditions.

Diagnostic, therapeutic, and social activities enable a holistic approach to a child with congenital/developmental disorder or a rare disease, improving both the patients' and their families' quality of life. In order to establish measures enabling a proper diagnosis and reduce the social consequences of disability resulting from developmental disorders, it is necessary to learn about the etiology of these defects and conditions, as well as about modern genetic diagnostic methodology [1-5].

DYSMORPHIC SYNDROMES

Dysmorphic syndromes include unique pathogenetically related symptoms, such as distinct differences in the body structure and appearance, frequent malformations of various organs, often co-existing with developmental delay and/or intellectual disability. More than 7,000 genetic syndromes are now recognized. Their diagnosis, and thus knowledge about the natural history of the disease, are essential in order to optimize medical care (including prevention of potential sequelae specific for the condition determining prognosis and appropriate therapeutic management), provide reliable genetic counselling in the families of the patients and for the purposes of uncovering the causes and mechanisms of congenital syndromes [1-4, 6-8].

CONGENITAL DEFECTS

Dysmorphic syndromes may be accompanied by malformations of various organs and systems. However, most defects of individual organs are isolated, i.e. unrelated to other health problems or anomalies of other systems. The definition of congenital malformation is very broad and includes any anatomical abnormality present at birth [1-4]. Given the complexity and nature of normal human development and the multiplicity of processes occurring during embryonic and fetal life, it should be noted that any given congenital disorder can arise due to the malfunction of any integral part of the complex process of human development [8].

The incidence of congenital defects in newborns born alive in developed countries is approximately 2-3%, and in those stillborn up to 10%. More than 50% of pregnancies with severe fetus malformations result in fetal death or early miscarriage. Due to improvements in pre- and perinatal care, the decreasing number of severe infections or injuries, and the negative impact of environmental factors on the fetus and the newborn, for several decades congenital malformations and dysmorphic syndromes have become an increasingly common reason for the hospitalization of children in neonatal or pediatric wards, as well as outpatient visits. Accordingly, they constitute serious diagnostic and therapeutic problems. These disorders often concern patients with complex histories of their condition, that is ones defined as rare diseases [1, 2, 4, 7, 8].

RARE DISEASES

A rare disease, also called an orphan disease (orphan disorder) is a condition that occurs in a population at a frequency of 1 in 2,000 or less. Sometimes the incidence of these disorders may be as low as 1 in 100,000 or 1 in 500,000 live births. Such diseases are referred to as ultrarare disorders. Close to 8,000 rare diseases have now been defined and the list is getting longer (www.orpha.net). These conditions are characterized by a variety of symptoms, a progressive course and often unfavourable prognosis. They include malformation syndromes, intellectual disability, neurological, dermatological, endocrine, cardiac, gastrological, ophthalmological, and nephrological disease in children and adults. Most of them have genetic causes, but the background can be variable (e.g. teratogenic, environmental or infectious). Every doctor, physiotherapist, therapist or other specialist in the medical field has patients with these diseases in their practice.

It is estimated that in European countries at least 28 million people, i.e. about 6% of the population, suffer from rare diseases. Rare disorders in children have a severe course in 65% of the patients, leading to death in the first year of life in 35% of the cases. Rare conditions manifest themselves in 70% of the affected children before 2 years of age, while in 50% they are accompanied by developmental delay and intellectual disability, significantly impairing the quality of life of the patients and their families (www.orpha.net).

ETIOLOGY OF DEVELOPMENTAL DISORDERS

The etiology of developmental disorders is extremely complex, because their diagnosis often requires long, labour-intensive and costly search that does not always end in success [1-3, 5]. The multitude and variety of dysmorphic syndromes, their relatively low prevalence, the difficulty in assessing dysmorphic features, as well

as phenotypes changing with the child's age, make it difficult to formulate a definitive diagnosis.

Genetic factors play an important role in the etiology of developmental disorders. Among them chromosomal aberrations, monogenic diseases and oligo/polygenic syndromes or conditions of multifactorial origin should be mentioned (tab. I) [1-4, 7]. It is important to distinguish genetic defects from those induced by environmental factors (extragenetic).

STAGES IN THE DIAGNOSIS OF DEVELOPMENTAL DISORDERS

The diagnosis of developmental disorders, birth defects or rare diseases follows the traditional model, just as in other medical specialties (tab. II). It is worth emphasizing that family history and the natural course of the disease should be collected very carefully, and physical examination would identify all, both severe and

Table I. Genetic causes of congenital defects and dysmorphic syndromes in newborns [1-4, 7].

Tabela I. Genetyczne przyczyny wad rozwojowych/zaburzeń rozwojowych i zespołów dysmorficznych u noworodków [1-4, 7].

Type of genetic defect ***Rodzaj zaburzenia genetycznego***	
Chromosomal aberrations *Aberracje chromosomowe*	Numerical aberration/*Aberracje liczbowe* Structural aberration/*Aberracje strukturalne* Microscopic aberrations (deletions, duplications) /*Aberracje mikroskopowe (delecje, duplikacje)* Mosaicism/*Mozaikowość*
Monogenic disorders *Choroby jednogenowe*	Mendelian inheritance/*Dziedziczenie mendlowskie* Autosomal dominant/*Autosomalne dominujące* Autosomal recessive/*Autosomalne recesywne* X-linked recessive /*Sprzężone z chromosomem X recesywne* X-linked dominant/*Sprzężone z chromosomem X dominujące*
Other disorder of non-Mendelian inheritance *Inne choroby (o nie-mendlowskim dziedziczeniu)*	Dynamic mutations/*Mutacje dynamiczne* Mitochondrial disorders/*Choroby mitochondrialne* Parental genomie imprinting/*Rodzicielskie piętnowanie genomowe* Somatic mutations/*Mutacje komórek somatycznych*
Multifactorial disorders *Choroby wieloczynnikowe*	Oligo-/polygenic inheritance associated with environmental factors and epigenetic changes /*Dziedziczenie oligo-/poligenowe w połączeniu* *z działaniem czynników środowiskowych* *lub wpływem zmian epigenetycznych*

Table II. Steps in genetic diagnosis of developmental defects [1-4, 6, 7].

Tabela II. Etapy diagnostyki genetycznej zaburzeń rozwojowych [1-4, 6, 7].

Clinical diagnosis/*Rozpoznanie kliniczne*
Family history/*Wywiad rodzinny*
Medical history/*Wywiad chorobowy*
Physical examination (dysmorphologic)/*Badanie przedmiotowe (dysmorfologiczne)*
Consultations and additional tests (e.g. neurological, ophthalmological, metabolic, immunological, endocrinologic, psychological, imaging of brain, skeleton and other organs, hearing tests) /*Konsultacje i inne badania dodatkowe (m.in. neurologiczne, okulistyczne,* *metaboliczne, immunologiczne, endokrynologiczne,* *gastrologiczne, psychologiczne, radiologiczne – np.: obrazowanie mózgowia,* *kośćca, audiologiczne – badanie słuchu)*
Laboratory genetic diagnosis/*Genetyczna diagnostyka laboratoryjna*

minor, abnormalities. This enables the necessary linking of anomalies/malformations into one condition, which should be followed by appropriate additional tests [7].

History taking constitutes the basis for the clinical diagnosis of any genetic condition. It concerns the family (data on possible consanguinity, ethnicity, occurrence of miscarriages or unexplained stillbirth or neonatal deaths, the health status of parents and other family members), pregnancy period, childbirth, the neonatal period, the natural history of the disorder, psychomotor development (age when sitting unsupported or walking unassisted, speaking first words). Another important part of diagnosing developmental disorders, congenital anomalies or rare diseases is the physical examination of the child.

Symptoms of genetic syndromes are extremely diverse and include malformations of internal organs, dysmorphic features of the entire body, face, head, hair, skin, neck, trunk, limbs, nails and external genitalia, as well as the impairment of psychomotor and somatic (pre- and postnatal) development [3, 4, 6, 7]. For instance, chromosomal aberrations manifest as a number of clinical signs, not just one isolated malformation. In clinical practice, the term "chromosomal phenotype" has been introduced. It refers to a phenomenon that sometimes, on the basis of characteristic/specific symptoms, makes it possible to suggest a diagnosis of a particular chromosomal aberration, or allows its strong suspicion (tab. III) [1, 2, 6, 7].

Collecting and analyzing all the data from history and physical examination allows the formulation of the initial clinical diagnosis, which should be the starting point for planning appropriate cytogenetic and molecular testing. The element that cannot be underestimated in dysmorphological diagnosis is the theoretical background, which includes studying etiology and the pathogenesis of developmental disorders, as well as the experience of the examining person combined with the recollection of specific phenotypes (*gestalt*) (4,6,7). Another factor which is crucial for establishing diagnosis is the appropriate use of additional resources (literature, databases of rare diseases, including dysmorphological databases – London Dysmorphology Database and Australian Possum database).

Orphanet's activity (www.orpha.net) cannot be overestimated. Its main objective is to provide the community with a comprehensive source of information about rare diseases and orphan drugs in order to improve the diagnosis, care and treatment of patients with a rare disease. Via the website one can access a list of more than 3,000 rare disorders described by scientists and peer-reviewed by internationally renowned experts, classified according to ICD10 (in the tab with a certain disorder, one can find information on the incidence, age of onset, as well as how specific genes are associated with a particular syndrome and how this condition is inherited). In addition, Orphanet presents a list of orphan drugs at every stage of research, the list of specialized services in 36 partner countries, provides information on specialized clinics and reference centers, medical laboratories, research projects, clinical trials, registries, networks, scientific, and technological platforms and patient organizations. Orphanet has now become the reference information portal for rare diseases and orphan drugs and is a major project that enables the establishment of a connection between the disease, text information about it (including links to other information pages) and appropriate services for the patients.

Objective naming of the observed phenotypic traits is extremely important, allowing a uniform description of the characteristics observed, and thus a comparison of the phenotypes of different patients, both for daily practice diagnostic and research purposes, as well as for etiology and pathogenesis, epidemiology, and searching for new therapies. A group of geneticists in Poland made an effort to translate into Polish the definitions of dysmorphic features of the head, neck, hands and feet (Elements of Morphology, AJMG, 2009), which had been developed by an international team of dysmorphologists (9,10), and published in Polish in the supplement of Standardy Medyczne – Pediatria (http://www.standardy.pl/index/archiwum/id_numeru/34).

Table III. Characteristic clinical features in children with chromosomal aberrations [1-4, 6, 7].
Tabela III. Cechy kliniczne występujące u dzieci z aberracjami chromosomowymi [1-4, 6, 7].

Chromosomal phenotype/*Fenotyp chromosomowy*
• dysmorphic facial features, and features of cranium, neck, limbs, nails, external genitalia, hair and skin /*cechy dysmorfii ciała, twarzy, czaszki, szyi, tułowia, kończyn, paznokci, narządów płciowych, włosów i skóry*
• inaproppriate muscle tone/*nieprawidłowe napięcie mięśniowe*
• one or more structural congenital defects/*jedna lub więcej strukturalnych wad wrodzonych*
• prenatal (IUGR) and postnatal somatic retardation/*prenatalne (IUGR) oraz postnatalne opóźnienie rozwoju somatycznego*
• delay of psychomotor and intellectual development/*opóźnienie rozwoju psychoruchowego i intelektualnego*
• behavioural phenotype, autistic symptoms/*zaburzenia zachowania, objawy autystyczne*
• positive family history (miscarriages, stillbirths, deaths after delivery, congenital defects, intellectual disability) /*obciążony wywiad rodzinny (poronienia, urodzenie martwego płodu, zgony dzieci po urodzeniu, wady rozwojowe, niepełnosprawność intelektualna)*

However, as it has been mentioned above, success may not always be achieved in the dysmorphologic diagnosis. The recognition of dysmorphic syndromes and causes of malformations amounts to no more than 60% even in the best reference centers with specialists in the field and a variety of modern diagnostic methodology, i.e. in 40% of pediatric cases. Despite advances in medicine, we are unable to find the cause of impaired development or malformation.

DIAGNOSTICS OF THE GENETIC CAUSES OF DEVELOPMENTAL DISORDERS

The methods of genetic testing used in the diagnostics of genetic malformations are constantly being improved and are subject to change with further progress of cytogenetics and molecular biology (tab. IV) [11]. The current algorithm for the genetic diagnostics of developmental disorders involves, in the first place, the exclusion of unbalanced chromosomal aberrations using classical cytogenetic techniques of GTG banding (G bands by trypsin using Giemsa), allowing the assessment of the patient's karyotype. GTG banding at a minimum resolution of 550 bands enables the detection of structural chromosomal aberrations of the size of 5-10 Mb (Mbp). The smallest change that can subsequently be detected using conventional chromosomal analysis techniques comprises approximately 2-7 x 10^6 base pairs (bp), depending on band resolution [11].

The most common material for cytogenetic testing are peripheral blood lymphocytes. In the case of certain genetic disorders it is advisable that testing of other tissue be performed, e.g. skin fibroblasts or a mouth swab are made [11]. For example, such a procedure takes place on suspicion of Pallister-Killian syndrome (mosaic form tetrasomy and/or trisomy of chromosome 12p), and when the child is observed for such clinical characteristics as body asymmetry, linear skin stains or discolorations. The above symptoms present in the syndromes are so characteristic that the suggestion of a chromosomal aberration in mosaic form can be made (e.g. trisomy 8, 16, 20). In such cases, further karyotype studies of skin fibroblasts should be ordered.

On suspicion of disorders of sex determination, cytogenetic analysis of gonadal tissue obtained during surgery is sometimes performed. Receiving the cytogenetic test result may not necessarily and usually does not mean the end of the diagnostic process. This holds true for both normal and abnormal test results [7].

One of the more important types of unbalanced structural chromosome aberrations, which are a significant cause of developmental disorders and intellectual disability, are rearrangements in the subtelomeric regions of chromosomes. The GTG banding technique makes the ends of all chromosomes look alike, which prevents their accurate analysis using classical cytogenetics. Subtelomeric aberrations with an incidence of 2.1 per 10,000 live births are identified in approximately 5-30% of patients with developmental disorders and intellectual disability [12].

Cytogenetics techniques that analyze chromosomes using the techniques of modern molecular biology, and allow the detection of structural chromosomal microaberrations invisible with classical cytogenetics, are applied in many other cases of developmental disorders. One of such recognized techniques is Fluorescent In Situ Hybridization (FISH), utilizing specific probe hybridizing to a particular chromosomal region, which allows the detection of changes of 40 kb-250 kb. This technique, however, requires formulating a clinical diagnosis of a certain syndrome, which allows for the correct choice of a fluorescent probe for the analysis. In the case of suspected subtelomeric microaberrations, it is necessary to use the entire panel of subtelomeric probes (for all the chromosomes). In addition, the FISH technique is used to identify marker chromosomes of unknown origin, additions, or complex translocations [11].

An alternative technique for testing microaberrations is MLPA (Multiplex Ligation-dependent Probe Amplification), which enables the detection of deletions and duplications of individual exons and allows the quantitative assessment of up to forty or so different loci in a single run. This translates into relatively low MLPA reagent costs. In a single MLPA reaction from 20 to 100ng of the patient's DNA is required. MLPA is used in the diagnostics of microdeletions/microduplications, including ones which are subtelomeric, interstitial autosomal or located on the X chromosome. MLPA does not allow the identification of balanced rearrangements. During the run, specific probes that hybridize to complementary segments of a patient's DNA are used, which, in the case of correct hybridization undisturbed by mutations existing in the

Table IV. Laboratory genetic diagnostic tests.

Tabela IV. Laboratoryjna diagnostyka genetyczna.

Classical cytogenetic test (karyotype)/*Badanie cytogenetyczne klasyczne – kariotyp*
Molecular cytogenetic tests for the detection of chromosomal microaberrations (e.g.: FISH, MLPA, array CGH) /*Badania metodami cytogenetyki molekularnej w kierunku mikroaberracji chromosomowych (m.in.: FISH, MLPA, CGH do mikromacierzy)*
Molecular tests in monogenic disorders (e.g.: PCR, RLFP, Sanger sequencing, New-Generation Sequencing (NGS) of coding region of genes) /*Badania metodami molekularnymi w kierunku konkretnych chorób monogenowych (m.in.: PCR, RLFP, sekwencjonowanie sangerowskie), sekwencjonowanie nowej generacji (NGS) regionów kodujących genów)*

patient, are then ligated and used as a template for DNA polymerase. The application of labeled primers in a PCR reaction makes it possible to quantify the PCR products for the individual sequences being analyzed. Such quantitative assessment enables the detection of mutations, or deletions/duplications present in the patient in heterozygous state. The comparison of the result obtained for the patient with a normal control DNA, makes it possible to identify changes in the DNA tested. Abnormal results of MLPA testing require confirmation with a different technique, usually FISH or CGH (Comparative Genomic Hybridization) [11, 12].

a. Application of MLPA in multiple congenital anomalies

According to the results of studies using the MLPA technique in patients with multiple congenital anomalies/intellectual disability in different age groups, the application of MLPA enables the detection of double the number of ascertained pathogenic rearrangements compared with the diagnostic efficacy of the karyotype (12-14). In an interesting work by Jehee et al., the authors evaluated the effects of the application of several sets of MLPA probes in patients with intellectual disability and/or congenital anomalies as an alternative to more expensive submicroscopic analysis techniques [15]. In the above publication three times as many microaberrations were identified using several sets of MLPA compared with routine cytogenetic testing. So far, however, no one has assessed the usefulness of the MLPA technique in the population of newborns with multiple congenital anomalies.

b. Application of MLPA in isolated malformations

In the group of 39 children with isolated conotruncal defects, Campos et al. detected seven *de novo* pathogenic microrearrangements, including five encompassing regions other than the critical region of the 22q11.2 deletion syndrome [16]. This indicates the potential utility of the MLPA technique for the early diagnosis of the causes of the above isolated heart defects, including the subpopulation of neonates. In another study using MLPA with subtelomeric probes in 72 fetuses and 109 liveborn infants with a diagnosis of holoprosencephaly, 11 pathogenic microaberrations in subtelomeric regions of chromosomes were identified, confirming the suggestions about the genetic heterogeneity of this malformation.[17].The most advanced method of molecular cytogenetics enabling the detection of very small quantitative changes in the genome (i.e. Copy Number Variations, CNVs, invisible with classic cytogenetics) is microarray comparative genomic hybridization (the so-called array CGH or molecular karyotyping) (tab. V) (11). Other methods, e.g. oligonucleotide (SNP array), can be used as an integral part of array analyses.

Table V. Comparison of chosen parameters of two methods for identification of chromosomal microaberrations: MLPA and aCGH.

Tabela V. Porównanie wybranych parametrów dwóch metod służących do diagnostyki mikroaberracji chromosomowych: MLPA i aCGH.

	MLPA	**aCGH**
Coverage of the genome *Pokrycie genomu*	Up to about 40 chosen genome regions */Do około 40 wybranych regionów genomu*	Whole genome/*Cały genom*
Resolution of the test *Rozdzielczość badania*	Identifies also small rearrangements (in practise thousands of bp) */Wykrywa także małe rearanżacje (w praktyce kilkaset kpz)*	Depends on the number and size of probes (in practice about 5-50kbp in regions enriched with known genes) */Zależna od liczby i rozmiaru sond (w praktyce około 20-50kpz w regionach znanych genów)*
Complexity of the method *Złożoność metody*	Technically simple/*Prosta technicznie*	More difficult technique */Trudniejsza technika*
Cost *Koszt*	Relatively low/*Względnie niski*	Quite high (about 3 times the cost of a single MLPA test) */Dość wysoki (około 3x koszt MLPA)*
Availability *Dostępność*	Available in all genetic diagnostic centers in Poland */Dostępne we wszystkich ośrodkach diagnostyki genetycznej w Polsce*	Available in chosen genetic diagnostic centers in Poland */Dostępne w niektórych ośrodkach diagnostyki genetycznej w Polsce*
Limitations *Ograniczenia metody*	Does not detect all microrearrangements (limited to the panel used) and balanced rearrangements; it is a screening method; does not detect point mutations *Nie wykrywa wszystkich mikrorearażacji (ograniczony do zastosowanego panelu) i rearanżacji zrównoważonych, jest badaniem przesiewowym, nie wykrywa mutacji punktowych*	Does not detect balanced rearrangements; limited resolution dependent on the number and location of probes; does not detect point mutations *Nie wykrywa: rearanżacji zrównoważonych, ograniczona rozdzielczość w zależności liczby i rozmieszczenia sond, nie wykrywa mutacji punktowych*

Oftentimes, the changes identified on arrays have no clinical significance, i.e. they are only polymorphic (constitute a benign variation of chromosome structure). Currently, in many countries, and in some centers in Poland, the array technique is the first-choice strategy and replaces conventional cytogenetics (karyotype) in patients with congenital anomalies, dysmorphic features or delayed psychomotor development, whose clinical picture does not allow a clinical suspicion of a specific chromosome aberration, or monogenic condition. The application of this method for the diagnosis of developmental disorders in Poland is still limited, due to its relatively high cost.

c. Application of the array CGH technique in newborns with multiple malformations

The only work evaluating the clinical utility of a CGH in the population of infants with multiple malformations was the publication by Lu et al. [18]. They identified clinically significant copy number variations (CNVs) in 32 of the 179 patients diagnosed with multiple congenital anomalies (18%). Another paper confirmed the presence of pathogenic CNVs in 20% of their patients with multiple congenital anomalies in different age groups [19]. Other research groups have focused their efforts on the search for clinically relevant microaberrations in mixed subpopulations in which the leading symptom was intellectual disability or developmental delay with or without accompanying multiple congenital anomalies. Miller et al. recommend the use of array CGH as the first-line technique on the basis of the results obtained from the study of 21,698 patients with intellectual disability/developmental delay, multiple congenital anomalies and the autism spectrum disorder. The diagnostic efficacy of this method is 12.2% in patients with a normal karyotype result [20].

According to the guidelines of the working group assessing diagnostic applications of array CGH, the size of clinically significant CNVs is defined at about 400 kb [20]. However, the British recommendations included in the Deciphering Developmental Disorders Program (DDD; www.ddduk.org) also take into account duplications as small as 250 kb and deletions of 100kb, both de novo and inherited from an affected parent, as potentially clinically relevant. Due to the specific characteristics of the newborn population, even minor changes can be classified as potentially pathogenic until the developmental potential of the affected child is verified at a later age.

d. Application of the array CGH technique in newborns with isolated congenital malformations

The use of array CGH in diagnosing the causes of isolated malformations enables the recognition of candidate genes, which, when defective, play a key role in the pathogenesis of a certain disorder, as well as makes it possible to identify pathogenic microaberration (defined as the well-known syndromic CNV). This particularly concerns the neonatal subpopulation, where the definitive diagnosis of an isolated malformation can be made only after the exclusion of developmental delay or intellectual disability later in life. The usefulness of array CGH in isolated malformations has so far been assessed mainly for cleft lip and palate, diaphragmatic hernia, congenital heart defects, and urogenital tract anomalies. The relationship between clefting and pathogenic CNVs in different age groups, including neonates, was evaluated by Maarse et al. who summed up the results of 13 studies [21]. The diagnostic efficacy of array CGH, defined as clinically significant CNVs, was 1% for the isolated cleft lip and palate. The results of a study in a group of 52 Polish newborns with the diagnosis of isolated cleft lip and palate allowed the identification of eight rearrangements, including two potentially explaining the presence of the congenital anomaly [22]. In addition, two new candidate genes *CHN2* and *CDH19* were put forward, which could play an important role in the pathogenesis of cleft lip and palate. A significant prevalence of pathogenic CNVs in isolated cardiac defects was observed by Erdogan et al. and Greenway et al. in different age groups [23, 24]. Erdogan et al., identified 18 rearrangements in 105 patients, including one deletion and two *de novo* duplications [23]. On the other hand, according to the work of Greenway et al., potentially pathogenic CNVs are present in about 1% of patients with tetralogy of Fallot [24]. For other isolated defects, such as congenital diaphragmatic hernia or urogenital tract anomalies, the frequency of potentially pathogenic CNVs may reach 10%, but the results of previous studies need to be confirmed in larger cohorts [25-27].

MOLECULAR ANALYSIS OF SINGLE GENES

A variety of molecular techniques for DNA analysis have now become routine diagnostic methods. The Polymerase Chain Reaction (PCR) is commonly used for the amplification of the specific DNA sequence. Most commonly, this method is used in combination with a number of other molecular techniques, e.g. digestion with restriction enzymes [11]. The most detailed method for testing gene structure is its sequencing, which involves reading the types and sequence of nucleotides i.e. creating a record of the gene. A very dynamic development of novel molecular techniques, such as New-Generation Sequencing (NGS), with its varieties of Whole-Genome Sequencing (WGS) and sequencing of coding regions (WES – Whole-Exome Sequencing) allows a relatively rapid and extensive analysis of thousands of genes in one run.

The future of dysmorphology relies on the correct clinical interpretation of the results of the so-called massively parallel sequencing, which generates a large amount of data from the whole genome of a single patient [7]. When assessing the clinical effectiveness of diagnostic NGS in patients with very diverse indications for the study (of which 80% are neurological symptoms), the result is approximately 25% [28]. Although this technique is mainly suited for the diagnosis of monogenic disorders, it is estimated that it can make a substantial contribution to the identification of the background of multiple or isolated malformations, where making a clinical diagnosis prior to applying the test is not possible. A model example is the study by Keren Carss et al., who performed an NGS study, enriched with the possibility of CNVs identification, in fetuses and neonates with variable structural anomalies identified in prenatal ultrasound and excluded chromosomal aneuploidy syndromes [29].

The authors found 35 single nucleotide changes, as well as small deletions and duplications. In another study, five patients have demonstrated the presence of recessive or sex-linked *de novo* variants in candidate genes. The NGS technique is, therefore, according to the authors, of similar effectiveness as diagnostic array CGH, but also allows the identification of pathogenic changes in single nucleotides and very small chromosomal rearrangements.

Array CGH and new generation sequencing technologies are considered by some authors applicable even in neonatal screening [30, 31]. The aim of such screening would be the early identification of rare genetic disorders already in the neonatal period, as well as the creation of databases of pathogenic mutations in humans in order to make the research on potential treatment for genetic conditions of later onset more efficient. As previously described, the array and NGS identify many variants of unknown significance or lack of clinical significance with respect to coded proteins and human health. Furthermore, the NGS technique identifies healthy carriers of certain mutations in the genome [30-33]. Too many and excessively difficult unsolvable questions appear during the discussion on the use of the latest genetic testing technologies in screening assays performed in healthy newborns, as a result of which no country is quick to implement projects of this type.

SUMMARY

Knowledge of what causes developmental disorders in children can determine the appropriate therapeutic regimen of rehabilitation. Proper diagnosis of genetic disease is important also for genetic counselling involving both the patient and his family. Misdiagnosis of the disease, unconfirmed by means of an objective clinical examination, may on the one hand result in an inappropriately conducted therapy, while on the other hand create no opportunities to provide proper genetic counselling for the family members of the patient with a genetic condition. Hence, a major problem is the need to refer every child who presents with dysmorphic features and/or birth defects, developmental delay and/or intellectual disability to genetics clinics. The application of modern methods of cytogenetics and molecular biology in diagnostics helps to explain the etiology of multiple phenotypic abnormalities in patients with normal karyotype. Steady progress in the development of this field of medicine gives rise to the possibility of determining the etiopathogenesis of developmental disorders. It should be noted, however, that in order to reach the proper diagnosis of the dysmorphic syndrome and verify it using the right genetic testing, it is essential to carry out a comprehensive physical and dysmorphological examination of the patient and to know the most distinctive and most common chromosomal aberration phenotypes.

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Author's contributions/Wkład Autorów
According to the order of the Authorship/Według kolejności

Conflicts of interest/Konflikt interesu
The Authors declare no conflict of interest.
Autorzy pracy nie zgłaszają konfliktu interesów.

Received/Nadesłano: 09.06.2015 r.
Accepted/Zaakceptowano: 06.10.2015 r.

Published online/Dostępne online

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