Detection of chromosomal aberrations in neuroblastoma pediatric tumor

Identyfikacja aberracji chromosomowych w nowotworze dziecięcym neuroblastoma

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Abstract

The broad spectrum of neuroblastoma clinical behavior depends on a genomic landscape of tumor cells. Our review focuses on recent advances in detecting chromosomal aberrations in neuroblastoma cells.

Streszczenie

Obraz kliniczny neuroblastoma jest silnie zależny od zmian nagromadzonych w genomie komórek guza. W naszej pracy koncentrujemy się na najnowszych osiągnięciach w wykrywaniu aberracji chromosomowych w komórkach neuroblastoma.

Key words:

advanced neuroblastoma, chromosomal aberrations, tumor genome screening

Słowa kluczowe:

aberracje chromosomowe, genom nowotworu, niekorzystne rokowanie neuroblastoma

Introduction

Neuroblastoma (NB) is a one of the most common malignancy in childhood (6-10%). This tumor occurs in very young children. The incidence of NB is 10.5 cases per 1 million children under 15 years [1]. Approximately 90% of patients are diagnosed before the age of 5 years old [2,3]. The primary form of NB is an extracranial solid tumor with a tendency to form distant metastases. The malignant NB is an embryonic tumor that arises from neural crest progenitor cells. Therefore, primary neoplastic lesions are presented on the route of migration of precursor cells of the sympathetic nervous system: abdomen (60-80%), chest (15%), neck (2-5%), pelvis (2-5%) [4,5].

The genetic constituent is a key component of the multistage carcinogenesis process. Changes in a DNA sequence, both point mutations and chromosomal rearrangements can lead to activation of protooncogenes and inactivation of tumor suppressors thereby promoting the excessive cell proliferation. In many cancers indication of a "causative" mutation allows better understanding of its biology and identification of genetic prognostic factors. Pointing out of these factors may lead to develop the most effective treatment that is often highly personalized [6-9]. The comprehensive characterization of tumor genetic and biological features is obligatory for NB patients at the time of diagnosis. It is associated with established risk factors for a treatment failure and its confirmed clinical significance in NB. The most unfavorable prognostic factors in NB are: age over 18 months at diagnosis, advanced International Neuroblastoma Risk Group (INRG) tumor stage, low grade of tumor differentiation, *MYCN* oncogene amplification and presence of segmental or structural chromosomal aberrations (SCA) in tumor cells [10-13].

The *MYCN* oncogene amplification is the most powerful genetic factor associated with the poor outcome in NB. This alteration is correlated with the high progression risk regardless of the disease stage [14-20]. The *MYCN* amplification occurs in approximately 20-25% of all primary NB tumors. Beside the *MYCN* amplification unfavorable prognostic factors are also SCA, in contrast to numerical chromosomal aberrations (NCA) [11,21-23]. The presence of SCA correlates both with the high progression and recurrence risk, even in case of localized tumors [22-25]. Recent studies have shown that older patient accumulate much more changes in tumor chromosomes structures than younger ones [21, 26]. Schleiermacher et al. have proved that the NB progression, especially to the bone marrow is associated with accumulation of SCA in primary tumor cells. The incidence of SCA in the following stages of NB tumors increases from 11% in stage 3, by 20% in 4S, to 59% at the highest 4 stage. It should be noted, that SCA can be combined with NCA in one tumor sample and prognosis for the patient is more unfavorable than in presence of SCA only [22].

Over decades, chromosomal aberrations detecting methods have been considerably modified, allowing for the increasingly higher resolution and holistic nature of performing tests. The objective of the study was to summarize research techniques used to detect and analyze changes as SCA and NCA in NB cells.

Methods for detection chromosomal aberrations in NB cells

Classical Cytogenetic Karyotype

This test is based on analyzing numbers and a structure of chromosomes in a single cell nucleus. The karyotype test requires the presence of dividing cells arrested at the metaphase or prometaphase stage of the cell cycle for proper performance. The maximum resolution of the classical cytogenetic karyotype is 850 bands. The identification of chromosomal aberrations with a light microscope and with the maximum accuracy of 4-5 Mbp (millions of nucleic base pairs) is possible only at the mentioned resolution. The greatest advantage of the classical karyotype test is the ability to assess balanced chromosomal aberrations and accurate estimation of the cytogenetic mosaicism, including the tumor intercellular heterogeneity [8,27-29].

Large (several tens of Mbp) unbalanced chromosomal aberrations, covering whole bands and even arms, with loss or extra copy of genetic material are typical for NB [9,30,31]. The group of specific for NB SCA with unfavorable prognosis includes:

amplification of *MYCN* oncogene located in the 2p24 region, 11q23 deletion, 17q gain [14-20]. The karyotype test is not routinely performed in NB patients despite the fact that the size of these SCA makes it possible to identify them. The character of biological material makes it difficult the classical cytogenetic analysis of NB cells. The *in vitro* culture of the primary solid tumor tissue is more difficult than breeding bone marrow, what is a routine procedure in diagnosing of proliferative diseases of bone marrow. Additionally, the long-term *in vitro* culture of the tumor tissue is involved with the high risk of *de novo* acquisition of chromosomal mutations [32]. Currently, the conventional cytogenetic karyotype is relevant only in research studies with using NB cell lines.

Fluorescence In Situ Hybridization (FISH)

The FISH is a technique used in the cytogenetic diagnostics for detecting DNA sequences by molecular probes labeled with fluorescent dyes [33,34]. The resolution of FISH that appointed the detection limit depends on the probe length, and ranges approximately several thousand of nucleic base pairs. One of the most popular types of FISH probes is cytogenetic band-specific probes hybridizing to a specific chromosomal region (Fig. 1.a.). This specific region is usually associated with a locus of a genetic marker, for example: *MYCN* oncogene [33]. The great advantage of FISH is a possibility of hybridizing molecular probes, both to a material from *in vitro* cultures, as well as interphase cell nuclei from a biological material taken directly from the patient. This last technique is known as interphase-fluorescence in situ hybridization (I-FISH). Moreover, FISH technique is based on analyzing numbers of fluorescence signals in a single nucleus that allows very accurate estimation of the intercellular genetic heterogeneity typical for the clonally growth of tumors [8,28,32].

Currently, I-FISH analysis for NB is performed on touch print samples from the tumor tissue and it is the routine "gold standard" test for evaluating the *MYCN* amplification. According to the international guidelines, this test is the obligatory part of the NB patient genetic diagnostics [14,35]. The I-FISH technique can also assess the presence of other characteristic for NB SCA with unfavorable prognosis. Furthermore, combination of fluorescent dyes allows simultaneous staining of all chromosomes, known as a spectral karyotyping-fluorescence in situ hybridization (SKY-FISH) or multicolor-fluorescence in situ hybridization (M-FISH) [34]. These types of fluorescence studies are considered to be a kind of molecular karyotype, but due to the required presence of metaphase chromosomes are not used in NB similar to the classical cytogenetic karyotype.

Multiplex Ligation-Dependent Probe Amplification (MLPA)

The MLPA technique is based on hybridization very short molecular probes (50-70 bp) to the examined DNA fragment, and subsequent ligation and PCR amplification of these probes. The PCR products undergo the capillary electrophoresis separation and evaluation. The identification of quantitative changes in even single genes that are too small for detection by FISH is possible through MLPA technique. The additional advantage of MLPA is simultaneous using from few to dozen probes to mark the interested region of chromosome (Fig. 1.b.). In this way the detection efficiency of selected aberration is increased [32,36]. Furthermore, the analysis of MLPA results is fully automated and is based on statistical calculations, thus increasing its objectivity.

International groups, which conducting study in NB recommend MLPA and microarray techniques as diagnostic tools for detection of SCA and NCA in the genome of tumor tissue cells [37-39]. Moreover, international guidelines have been developed for analysis of MLPA results in NB [40]. It should also be noted, that each technique designed to evaluate NB genome changes known as CNVs (duplications or deletions of genetic material larger than 1 kbp), based on PCR reaction requires the presence of at least 60% of tumor cells in an investigated biological sample [32,40-42]. This also applies to the fast developing next generation sequencing (NGS) technology. The material necessary to perform MLPA assay in NB is DNA isolated directly from the tumor tissue. The DNA isolated from available NB cells is treated in the analysis as a "whole" that

makes it difficult and in some cases prevents recognizing of the genetic heterogeneity.

Array Comparative Genomic Hybridization (aCGH)

The aCGH technique, also known as cytogenetic microarrays belongs to molecular methods of CNVs detection [33]. The basis of aCGH is hybridization of labeled DNA that complementary attaches to the human genome reference sequence presented as oligonucleotides immobilized on a solid carrier. The most commonly used in NB are two-color CGH arrays. This type of arrays is characterized by a competitive hybridization between the DNA isolated from biological material of the patient and the control DNA. The patient and control DNA are labeled with different fluorescent dyes that distinguish it during final signals reading. Then, qualitative data are represented as numerical values, informing about the copy number status for the loci in genome [32]. The substantial advantage of aCGH technique is its resolution from a few hundred to a few nucleotides. Moreover, DNA is isolated directly from the patient's biological sample without a need for the *in vitro* culture and results reading is fully automated. The disadvantage of aCGH is the lack of identification of balanced chromosomal aberrations, but such changes are not typical for NB. The limitation of this technique is also difficulty in detecting the genetic heterogeneity (Fig. 1.c.).

According to current treatment protocols, in each patient diagnosed with NB should be obligatory checked not only the presence of the *MYCN* oncogene amplification, but also SCA and NCA in the primary tumor tissue. The results determine the risk of treatment failure as well as a type and intensity of treatment. Therefore, popularity of aCGH techniques as a tool for screening the whole genome of NB tumor cells still increases [32,37]. A special type of aCGH is the single nucleotide polymorphism (SNP) array. Microarrays like SNP allow detecting of genomic disturbances taking place without copy number changes, including uniparental disomy (UPD) and loss of

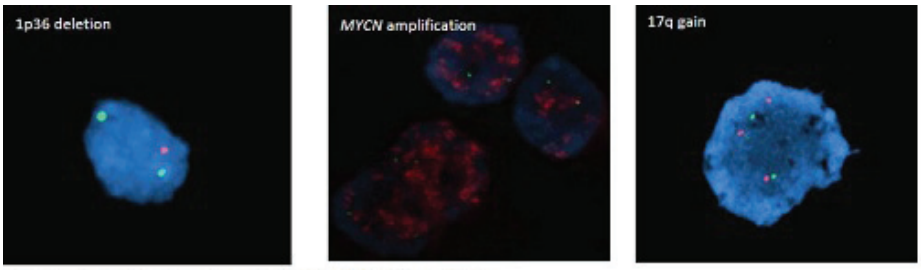

Fig.1.a. Neuroblastoma tumor I-FISH profile (Cytocell Ltd).

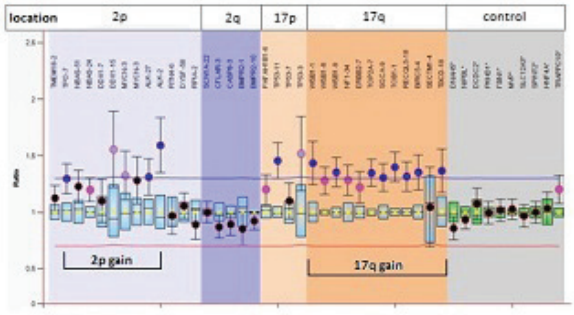

Fig.1.b. Neuroblastoma tumor MLPA profile (MRC Holland).

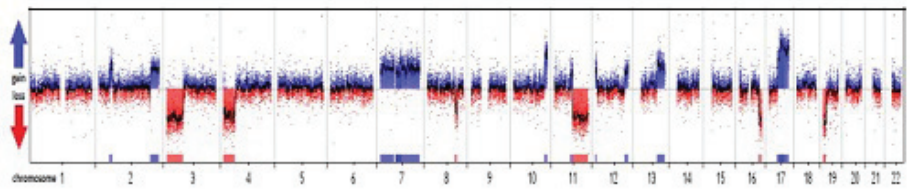

Fig.1.c. Neuroblastoma arrayCGH profile (Agilent).

Fig. 1.
Methods for identifying chromosomal aberrations in neuroblastoma

heterozygosity (LOH), as well as disclosure of low degree of mosaicism [28,43,44].

The aCGH studies performed on NB primary tumor tissues revealed new, often occurring unfavorable SCA. This group of SCA including deletions in regions: 3p, 4p, 6q, 10q, 14q, 18q, and additional copies of material: 3p, 12, 18p [9,30,32]. Moreover, detecting of first cases of UPD and LOH in NB patients was enabled by using aCGH arrays enriched with SNP [43-45]. Additionally, in the molecular karyotype, how often are called aCGH it was reported cases of the chromothripsis in NB cells [43]. A group of genes with a possible prognostic importance that were in detail analyzed in NGS was also indicated base on microarrays results especially with SNP [9]. Moreover, aCGH technique was also used for studying the genomic copy number profile of cell free DNA (cfDNA) isolated from plasma of NB patients and compared this to primary tumors as well as formalin-fixed paraffin-embedded (FFPE) specimens [37,39,46].

Next-Generation Sequencing (NGS)

Nowadays, rapidly developing NGS technologies provide the sensitive and accurate approach for the comprehensive characterization of CNVs. Platforms for NGS analysis are based on various approach of cyclic sequencing [47,48]. These platforms allow for the simultaneously sequencing millions of short (50--250 bp) DNA fragments (reads) and may process a whole human genome in short time [49,50]. The DNA strands in the sample are fragmented into very short segments, tagged and amplified. Then, each segment is aligned to the reference genome to determine its origin in genome [33].

The advantages of NGS approach include: higher coverage and resolution, more accurate estimation of the copy number status, more precise detection of breakpoints, and higher capability to identify novel CNVs simultaneously with mutational analysis than other molecular techniques. In aCGH probes are predefined for limited genomic regions. Short reads from NGS platforms, compared to aCGH are randomly sampled from the entire genome and detects small or novel CNVs that arrays often miss. Moreover, what is very important, NGS as a molecular technique permits for detection of balanced rearrangements [32]. Therefore, currently microarrays are replaced by NGS as the leading platform for investigation of genomic rearrangements. However, computational processing, analyzing, and interpreting massive amounts of data and genetic variants produced by NGS still remains complicated and very time consuming.

The NGS as the most effective tool is primarily used for genotyping in NB study. Results from different research teams indicate the most frequent mutation locus in NB. The mutation hot-spots in NB are localized in *ALK, ATRX*, *TERT* and *LMO1, ARID1A*, *ARID1B* genes. Additionally, in relapsed NB it was shown that often occurs mutation in *CHD9* gene and in RAS-MAPK pathway genes [9,51-55]. Moreover, the first effort was taken to compare two the most efficient methods for CNVs detection in NB – aCGH and NGS. Results obtained from these two methods were in accordance with SCA and NCA. However, it should be noted, that NGS allowed detecting of additional minor changes in tumor genome that were not apparent in aCGH [56].

Summary

During the past several years, considerable advances in methods used to detect chromosomal aberrations have been made. Nowadays, genetic variants from large structural rearrangements to CNVs can be detected with the reasonable sensitivity from a whole genome. Currently, I-FISH technique still remains the "gold standard" in the routine analysis of the *MYCN* oncogene amplification in NB. However, aCGH is the most common chosen technique for the NB genome screening in search of SCA and NCA in this tumor, mainly by price of analysis and equipment. It is worth noting, that results of all above techniques should be recorded in accordance with international guidelines the International System for Human Cytogenomic Nomenclature (ISCN) 2016.

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