Methods for detection of minimal residual disease in neuroblastoma pediatric tumor

Metody detekcji minimalnej choroby resztkowej w nowotworze dziecięcym neuroblastoma

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Abstract

The bone marrow examination is obligatory during the neuroblastoma diagnosis and a treatment. We focus on significant improvements in currently available examinations methods allow perceive a single tumor cell.

Streszczenie

Badanie szpiku kostnego jest obowiązkowe podczas diagnozy i leczenia neuroblastoma. Praca jest komentarzem obecnie dostępnych metod badawczych umożliwiających detekcję pojedynczej komórki nowotworowej.

Key words:

neuroblastoma metastasis, neuroblastoma molecular markers, neuroblastoma residual disease

Słowa kluczowe:

choroba resztkowa w neuroblastoma, markery molekularne neuroblastoma, przerzuty neuroblastoma

Introduction

Neuroblastoma (NB) is the most common extracranial solid tumor of childhood (6-10%). This tumor has a very heterogeneous clinical course ranging from spontaneous regression to a high-risk disease with a tendency to form distant metastases [1,2]. The disseminated disease is presented in approximately 50% of newly diagnosed NB [1-3]. The high-advanced NB tumor stage with the presence of the metastatic spread, especially to bone marrow (BM) at diagnosis is the most important unfavorable prognostic factor in determining outcome.

The curability of NB patients increased from 43% (5 year event free survival (EFS) in 1974-1989) to 70% (5 year EFS in 1997-2002) [2,4]. Despite this, the survival rate for children with the high-risk NB remains low. Approximately 50% of those who will attain a complete or partial remission will relapse and die within 5 years of the diagnosis [1,5]. One of the most important reasons of treatment failure is the presence of persistent neoplastic cells following chemotherapy, known as the minimal residual disease (MRD). Disease as MRD can persist locally in the primary NB tumor or distantly in blood, bone marrow or distant metastatic organs. Moreover, it is thought that among MRD is presented cancer stem cells (CSC) subpopulation. The subpopulation of CSCs embed particular in bone marrow cavities which may explain the high incidence of metastases to BM into NB patients (60% in children, 80% in neonates) [5-9]. Therefore, the BM examination is the obligatory part of the diagnostic pathway. It is necessary to confirm or exclude the presence of NB neoplastic cells in BM, both during the initial diagnosis and determining the disease stage and also at certain checkpoints of a therapy. The currently available research techniques allow detection of even a single NB cell and very accurate monitoring of MRD and thus evaluate the effectiveness of a treatment [7,9-12,14].

Methods for detection MRD in NB

Light Microscopy

According to the International Neuroblastoma Risk Group (INRG) a basic investigation of BM should be performed by the cytomorphology of smears collected by the fine needle aspiration biopsy and the histopatology of the trephine biopsy. Samples are collected bilateral, stained according a manufacturer's protocol and evaluated using a light microscope. The presence of NB metastatic cells may be excluded only if all evaluated samples are negative. If only one of evaluated samples is found positive, this is the basis for accepting the diagnosis of dissemination of tumor NB cells in BM [13,14].

Neoplastic NB cells have several morphological features characteristic for small-round-cell tumors. First of all, the NB cell has a circular shape, a very large hyperchromatic nucleus and a negligible amount of cytoplasm. NB tumor cells are slightly or much greater than erythrocytes. However, these characteristics are not sufficiently specific, based on which it is possible to recognize the NB cell especially when only one occurring between normal hematopoietic cells in the BM sample. Moreover, the detection of NB cells in BM is hampered by an uneven distribution of tumor cells and a tendency to form aggregates called *pseudorosettes* [15, 16]. Such clusters of tumor cells may randomly appear or not in the analyzed smear what increases the risk of false negative results. The classic cytomorphology assessment is a low sensitive method. The reliable estimation of NB cell is possible when tumor cells represent more than 0.1%, and in some cases even 10% of BM cells [7,8,10,11]. Therefore, it is recommended to supplement the NB BM diagnostics including MRD monitoring with methods having higher estimation accuracy.

Flow Cytometry

The flow cytometry is a fast and fully automated diagnostic method using fluorescently labeled cells' components passed through a laser beam. The great advantage of the flow cytometry is a simultaneous multiparameter quantitative and qualitative analysis [17-19].

Neoplastic NB cells are marked by intensive production of N-CAM protein (neural cell adhesion molecule). The glycoprotein N-CAM is responsible for a neural cell adhesion and is recognized by CD56 antibody. In addition, NB cells do not present any antigens specific for leukocytes for which

CD45 antibody is complementary. Therefore, the disclosure of NB tumor cells micrometastases to BM is usually based on the immunophenotype CD45-/CD56+ or GD2+/CD9+/CD57+/CD81+ [20-21]. Antigens CD9 and CD81 are transmembrane proteins belonging to the tetraspanin family involved in cells adhesion and migration [22-24]. The antigen CD57 is a fragment of protein known as an epitope that is directly recognized by antibodies. It is produced in a number of neuronal cells and its tissue-specific expression changes during the nervous system development [25].

It is worth noting that, the CD45-/CD56+ immunophenotype profile is not specific only to NB cells. It is also used for assaying Ewing's sarcoma cells, rhabdomyosarcoma and other small-cell cancers or neuroendocrine tumours as well as abnormal leukocytes [26]. The sensitivity of the flow cytometry method is estimated to be from 10^{-4} to 10^{-5} [14].

Enzymatic And Fluorescent Immunocytochemistry

The immunocytochemistry technique is commonly used to detect studied cells and visualize particular protein targets by specific binding of a primary antibody to an antigen. The antibody-antigen formed complex can be pictured by a fluorescent dye directly attached to the antibody. The alternative is an indirect method using an enzymatic reaction with a chromogen in which a substrate is converted into a colored product. The advantage of fluorescent staining is an ability to a simultaneous indication of several antigens in a cell, whereas the disadvantage is a rapid degradation of a fluorochrome by light. The more permanent image for examination is obtained using the chromogen but then the number of testing antigens is limited to two that additionally must be located in different cellular compartments. Another advantage of antibodies enzymatic labeling is a possibility for insight into a cell morphology. This is an important aspect of NB cells verification, especially in the non-specific immunocytochemical staining that usually concerns macrophages [14,16,20].

The disialoganglioside GD2 is the antigen used for detection of NB cells in BM. This antigen is presented by neuroectodermal cells and occurring only on a cell membrane of neurons as well as peripheral nerve fibers. The disialoganglioside GD2 is also exposed on the NB cell surface [14,27,28].

The immunocytochemistry technique is currently one of the most sensitive methods for detection of tumor cells in BM (10^{-5} do 10^{-6}) that has been considered as the diagnostic standard for NB patients [10]. However, it requires a lot of experience from a person who evaluates a sample under a microscope. In addition, it has been reported that during an anticancer therapy the GD2 antigen biosynthesis can be inhibited in NB cells what may reflected in false negative MRD results [29,30].

Polymerase Chain Reaction With Reverse Transcription

The assumption of RT-PCR (reverse transcription-PCR) and qRT-PCR (quantitative real time-PCR) techniques used to detection and monitoring MRD in NB patients is to determine a level of molecular markers expression in BM samples. Higher expression of the marker gene in comparison to a reference gene should be interpreted as higher level of the BM infiltration. The ideal molecular marker should be specific to a tumor and does not show expression in normal cells. Moreover, it's expression should be stable in time and the level should not change within a single cell under a treatment. The possible loss of expression should only arises from elimination of tumor cells and not be driven by inhibitory effects of a therapy on the neoplastic cell metabolism [10,11,31].

So far, there have not been unambiguously indicated specific molecular markers for NB cells [11,32]. In the past, the most commonly used mRNAs as NB tags were: disialoganglioside synthase (*GD2S*) and tyrosine hydroksylase (*TH*) [12,32-35]. Unfortunately, *GD2S* was expressed in almost all normal BM cells at a level similar to NB cells. However, this does not exclude the GD2 antigen as the reliable marker for immunocytochemistry assays because this examination takes into account the additional verification of NB cells based on its morphological characteristics. The protein TH has been evidenced as the first enzyme on the metabolic trail of catecholamines that excessive secretion is typical for NB cells. Initially, *TH* gene has

been evaluated as the reliable single molecular marker for detection of MRD in NB patients, but its expression was observed in 30% of peripheral blood and BM normal samples [8,10,11,31,32,34,36-38]. Therefore, other genes involved in the central nervous system development and in the biosynthesis and metabolism of neurotransmitters are intensively validated as possible molecular markers of MRD in NB: paired-like homeobox 2b (*PHOX2B*) [11,12,14,32,34,35], dopa decarboxylase (*DDC*) [18,10,37-39], doublecortin (*DCX*) [32,37,40,41], growth associated protein 43 (*GAP43*), cholinergic receptor nicotinic alpha 3 (*CHRNA3* [8,31,38,39], cyclin D1 (*CCND1*), collapsin response mediator protein 1 (*CRMP1*), GABA A receptor β3 (*GABRB3*), ISL LIM homeobox 1 (*ISL1*), kinesin family member 1A (*KIF1A*), transforming acidic coiled-coil containing protein 2 (*TACC2*), dopamine β-hydroxylase (*DBH*) [36,42,43]. It has been showed that *PHOX2B* is now the most specific marker gene for NB [8,10,11,31,32,34,36-38]. Moreover, it has been confirmed the stability of *PHOX2B* and *TH* genes expression in NB cells during an applied anticancer therapy [31]. In the future, these observations allow avoiding false negative MRD results. These pretended results would be associated with a decrease of molecular markers expression levels in a single cancer cell after a treatment only but not with decreasing number of these cells in BM. Additionally, it is recommend using a panel of marker genes for estimating the presence of NB cells in BM. This can improve the specificity and sensitivity of an assay, particularly during and after a treatment [8,32,36-40]. Nowadays, qRT-PCR beside anti-GD2 immunocytochemistry is the most sensitive detection methods of NB tumor cells in BM (10^{-6}) [10].

Summary

According to the latest research programs coordinated by the INRG group and the Society of Pediatric Oncology European Neuroblastoma Network (SIOPEN) committee the evaluation of BM samples in NB patients should be performed using: the conventional cytomorphological and histopathological analysis, and at the same time the immunocytochemistry with anti-GD2, and further the molecular technique qRT-PCR with *TH* gene as the marker [12,14,43]. International guidelines for a preparation and evaluation of BM samples by the anti-GD2 immunocytochemical assay have been established [10,14,44]. Whereas, there is a need for further studies concerning the expression of molecular marker genes in NB cells in order to verify their specificity in qualitative and quantitative determinations of surviving cancer cells after a chemotherapy and immunotherapy. There is also a need to develop coherent guidelines for presentating molecular results. Especially, qRT-PCR results published by various researchers are difficult to compare. The main reason is another reference gene but also a way of data analyzing. NB investigators have adopted different criteria for qualitative and quantitative evaluation, including both a relative and absolute estimation. In addition, to the absolute evaluation have been used different NB cell lines that may vary among themselves in the expression level of selected marker genes [45]. Therefore, it should aim to standardize of qRT-PCR method, taking into account selected marker genes so as results obtained in various centers will be consistent.

The intensive development of molecular techniques and their popularization, not only in research, but also in a clinical approach to patients is continuously observed. Next generation sequencing (NGS) study contributed to the detailed knowledge of genome, transcriptome and epigenome in many cancers. The high throughput and the comprehensive analytical potential of NGS have been also used in the NB MRD investigation. Van Wezel et al. identified 42 chromosomal breakpoints in NB primary tumor samples by whole-genome sequencing. Afterwards, tumor-specific chromosomal rearrangements have been used as DNA targets in quantitative real-time PCR BM analysis. It is worth nothing, that markers at DNA level like abnormalities in chromosomes structures are more stable than RNA because do not depend on gene expression level [46]. There is still need to search and evaluate new NB markers for indicating tumor cells progression and clinical prognosis. These markers should be specific and stable both during treatment and also between primary and metastatic sites as well as patients' biodiversity what currently is a challenge in NB.

References

1. Hallett A, Traunecker H. A review and update on neuroblastoma. Paediatr Child Health 2012; 22: 103-107.
2. Gains J, Mandeville H, Cork N, [et al.]. Ten challenges in the management of neuroblastoma. Future Oncol 2012; 8: 839-858.
3. Øra I, Eggert A. Progress in treatment and risk stratification of neuroblastoma: Impact on future clinical and basic research. Semin Cancer Biol 2011; 21: 217-228.
4. Moroz V, Machin D, Faldum A, [et al.]. Changes over three decades in outcome and the prognostic influence of age-at-diagnosis in young patients with neuroblastoma: a report from the International Neuroblastoma Risk Group Project. Eur J Cancer 2011; 47: 561-71.
5. Maris JM. Recent advances in neuroblastoma. N Engl J Med 2010; 362(23): 2202-2211.
6. Lagasse E. Cancer stem cells with genetic instability: The best vehicle with the best engine for cancer. Gene Ther 2008; 15: 136-142.
7. Okcu MF, Wang RY, Bueso-Ramos C, et al. Flow cytometry and fluorescence in situ hybridization to detect residual neuroblastoma cells in bone marrow. Pediatr Blood Cancer 2005; 45: 787-95.
8. Stutterheim J, Gerritsen A, Zappeij-Kannegieter L, et al. Detecting minimal residual disease in neuroblastoma: the superiority of a panel of real-time quantitative PCR markers. Clin Chem 2009; 55: 1316-26.
9. Tsang KS, Li CK, Tsoi WC, et al. Detection of micrometastasis of neuroblastoma to bone marrow and tumor dissemination to hematopoietic autografts using flow cytometry and reverse transcriptase-polymerase chain reaction. Cancer 2003; 97: 2887-97.
10. Beiske K, Ambros PF, Burchill SA, et al. Detecting minimal residual disease in neuroblastoma patients-the present state of the art. Cancer Lett 2005; 228: 229-40.
11. Stutterheim J, Gerritsen A, Zappeij-Kannegieter L, Kleijn I, Dee R, Hooft L, et al. PHOX2B is a novel and specific marker for minimal residual disease testing in neuroblastoma. J Clin Oncol 2008; 26: 5443-9.
12. Viprey VF, Corrias MV, Kagedal B, et al. Standardisation of operating procedures for the detection of minimal disease by QRT-PCR in children with neuroblastoma: quality assurance on behalf of SIOPEN-R-NET. Eur J Cancer 2007; 43: 341-50.
13. Ambros PF, Ambros IM, SIOP Europe Neuroblastoma Pathology, Biology, and Bone Marrow Group. Pathology and biology guidelines for resectable and unresectable neuroblastic tumors and bone marrow examination guidelines. Med Pediatr Oncol 2001; 37: 492-504.
14. Beiske K, Burchill SA, Cheung IY, et al. Consensus criteria for sensitive detection of minimal neuroblastoma cells in bone marrow, blood and stem cell preparations by immunocytology and QRT-PCR: recommendations by the International Neuroblastoma Risk Group Task Force. Br J Cancer 2009; 100: 1627-37.
15. Rajwanshi A, Srinivas R, Upasana G. Malignant small round cell tumors. J Cytol 2009; 26: 1-10.
16. Ambros PF, Mehes G, Ambros IM, [et al.]. Disseminated tumor cells in the bone marrow - chances and consequences of microscopical detection methods. Cancer Lett. 2003;197:29-34.
17. Autissier P, Soulas C, Burdo TH, et al. Evaluation of a 12-color flow cytometry panel to study lymphocyte, monocyte, and dendritic cell subsets in humans. Cytometry A 2010; 77: 410-9.
18. Davidson B, Dong HP, Berner A, et al. The diagnostic and research applications of flow cytometry in cytopathology. Diagn Cytopathol 2012; 40: 525-35.
19. De Tute RM. Flow cytometry and its use in the diagnosis and management of mature lymphoid malignancies. Histopathology 2011; 58: 90-105.
20. Okcu MF, Wang RY, Bueso-Ramos C, et al. Flow cytometry and fluorescence in situ hybridization to detect residual neuroblastoma cells in bone marrow. Pediatr Blood Cancer 2005; 45: 787-95.
21. Tsang KS, Li CK, Tsoi WC, et al. Detection of micrometastasis of neuroblastoma to bone marrow and tumor dissemination to hematopoietic autografts using flow cytometry and reverse transcriptase-polymerase chain reaction. Cancer 2003; 97: 2887-97.
22. Spriel AB, Figdor CG. The role of tetraspanins in the pathogenesis of infectious diseases. Microbes Infect 2010; 12: 106-12.
23. Leccia F, Nardone A, Corvigno S, et al. Cytometric and biochemical characterization of human breast cancer cells reveals heterogeneous myoepithelial phenotypes. Cytometry A 2012; 81: 960-72.
24. Powner D, Kopp PM, Monkley SJ, et al. Tetraspanin CD9 in cell migration. Biochem Soc Trans. 2011; 39: 563-7.

25. Focosi D, Bestagno M, Burrone O, et al. CD57+ T lymphocytes and functional immune deficiency. J Leukoc Biol 2010; 87: 107-16.
26. Bozzi F, Gambirasio F, Luksch R, et al. Detecting CD56+/NB84+/CD45- immunophenotype in the bone marrow of patients with metastatic neuroblastoma using flow cytometry. Anticancer Res 2006; 26: 3281-7.
27. Berthold F, Schneider A, Schumacher R, et al. Detection of minimal disease in bone marrow of neuroblastoma patients by immunofluorescnce. Pediatr Hematol Oncol 1989; 6: 73-83.
28. Czaplicki D, Horwacik I, Kowalczyk A, et al. New method for quantitative analysis of GD2 ganglioside in plasma of neuroblastoma patients. Acta Biochim Pol 2009; 56: 423-31.
29. Schumacher-Kuckelkorn R, Hero B, Ernestus K, Berthold F. Lacking immunocytological GD2 expression in neuroblastoma: report of 3 cases. Pediatr Blood Cancer 2005; 45: 195-201.
30. Träger C, Vernby A, Kullman A, et al. mRNAs of tyrosine hydroxylase and dopa decarboxylase but not of GD2 synthase are specific for neuroblastoma minimal disease and predicts outcome for children with high-risk disease when measured at diagnosis. Int J Cancer 2008; 123: 2849-55.
31. Stutterheim J, Zappeij-Kannegieter L, Ora I, et al. Stability of PCR targets for monitoring minimal residual disease in neuroblastoma. J Mol Diagn 2012; 14: 168-75.
32. Viprey VF, Lastowska MA, Corrias MV, et al. Minimal disease monitoring by QRT-PCR: guidelines for identification and systematic validation of molecular markers prior to evaluation in prospective clinical trials. J Pathol 2008; 216: 245-52.
33. Cheung IY, Lo Piccolo MS, Kushner BH, et al. Quantitation of GD2 synthase mRNA by real-time reverse transcriptase polymerase chain reaction: clinical utility in evaluating adjuvant therapy in neuroblastoma. J Clin Oncol 2003; 21: 1087-93.
34. Lambooy LH, Gidding CE, van den Heuvel LP, et al. Real-time analysis of tyrosine hydroxylase gene expression: a sensitive and semiquantitative marker for minimal residual disease detection of neuroblastoma. Clin Cancer Res 2003; 9: 812-9.
35. Viprey VF, Burchill SA. Gene expression profiling for discovery of novel markers of minimal disease. Clin Cancer Res 2009; 15: 6742.
36. Cheung IY, Feng Y, Gerald W, et al. Exploiting gene expression profiling to identify novel minimal residual disease markers of neuroblastoma. Clin Cancer Res 2008; 14: 7020-7.
37. Corrias MV, Haupt R, Carlini B, et al. Multiple target molecular monitoring of bone marrow and peripheral blood samples from patients with localized neuroblastoma and healthy donors. Pediatr Blood Cancer 2012; 58: 43-9.
38. Stutterheim J, Zappeij-Kannegieter L, Versteeg R, et al. The prognostic value of fast molecular response of marrow disease in patients aged over 1 year with stage 4 neuroblastoma. Eur J Cancer 2011; 47: 1193-202.
39. Kågedal B. Detecting minimal residual disease in neuroblastoma: still a ways to go. Clin Chem 2009; 55: 1268-70.
40. Yáñez Y, Grau E, Oltra S, et al. Minimal disease detection in peripheral blood and bone marrow from patients with non-metastatic neuroblastoma. J Cancer Res Clin Oncol 2011; 137: 1263-72.
41. Oltra S, Martinez F, Orellana C, et al. The doublecortin gene, a new molecular marker to detect minimal residual disease in neuroblastoma. Diagn Mol Pathol 2005; 14: 53-7.
42. Hirase S, Saitoh A, Hartomo TB, et al. Early detection of tumor relapse/regrowth by consecutive minimal residual disease monitoring in high-risk neuroblastoma patients. Oncology Letters 2016; 12: 1119-1123.
43. Villamón E, Berbegall AP, Piqueras M, et al. Genetic instability and intratumoral heterogeneity in neuroblastoma with MYCN amplification plus 11q deletion. PLoS One 2013; 8: e53740.
44. Swerts K, Ambros PF, Brouzes C, et al. Standardization of the immunocytochemical detection of neuroblastoma cells in bone marrow. J Histochem Cytochem 2005; 53: 1433-40.
45. Hartomo TB, Kozaki A, Hasegawa D, et al. Minimal residual disease monitoring in neuroblastoma patients based on the expression of a set of real-time RT-PCR markers in tumor-initiating cells. Oncology Report 2013; 29: 1629-36.
46. Van Wezel EM, Zwijnenburg D, Zappeij-Kannegieter L, Bus E, van Noesel MM, Molenaar JJ, et al. Whole-genome sequencing identifies patient-specific DNA minimal residual disease markers in neuroblastoma. *Journal* of *Molecular Diagnostics* 2015; 17: 43-52.