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Evaluation of potential prognostic value of Bmi-1 gene product and selected markers of proliferation (Ki-67) and apoptosis (p53) in the neuroblastoma group of tumors

Ocena potencjalnej wartości prognostycznej produktu genu Bmi-1 oraz wybranych czynników związanych z proliferacją (Ki-67) i apoptozą (p53) w guzach typu neuroblastycznego

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Introduction: Cancer in children is a very important issue in pediatrics. The least satisfactory treatment outcome occurs among patients with clinically advanced neuroblastomas. Despite much research, the biology of this tumor still remains unclear, and new prognostic factors are sought. The Bmi-1 gene product is a currently highly investigated protein which belongs to the Polycomb group (PcG) and has been identified as a regulator of primary neural crest cells. It is believed that Bmi-1 and N-myc act together and are both involved in the pathogenesis of neuroblastoma. The aim of the study was to assess the potential prognostic value of Bmi-1 protein and its relations with mechanisms of proliferation and apoptosis in the neuroblastoma group of tumors.

Material/Methods: 29 formalin-fixed and paraffin-embedded neuroblastoma tissue sections were examined using mouse monoclonal antibodies anti-Bmi-1, anti-p53 and anti-Ki-67 according to the manufacturer's instructions.

Results: There were found statistically significant correlations between Bmi-1 expression and tumor histology and age of patients.

Conclusions: Bmi-1 seems to be a promising marker in the neuroblastoma group of tumors whose expression correlates with widely accepted prognostic parameters. The pattern of BMI-1 expression may indicate that the examined protein is also involved in maturation processes in tumor tissue.

Keywords: **Bmi-1 • neuroblastoma • prognosis • apoptosis • proliferation**

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Introduction

Cancer in children is a very important issue in pediatrics. Of all cancers in childhood the group of solid tumors deserves special attention. For years, there was seen a steady increase in the incidence rate of these tumors by approx. 0.9% per year [26]. This clinical and macroscopic term 'solid tumors' defines a very large group of malignancies, extremely diverse in their pathogenesis, clinical course and prognosis [13,40,41,43]. The most representative entity of these neoplasms is neuroblastoma, commonly called in the literature 'a clinical enigma' [13,48]. This cancer covers a very wide spectrum of behavior from spontaneous or chemically induced regression to completely unexpected overall therapeutic failures [7]. The least satisfactory treatment outcome appears among children with clinically advanced neuroblastomas, and this fact leads to a search for new prognostic factors in this group of tumors.

The Bmi-1 gene product is a protein which belongs to the Polycomb group (PcG). It is a modern, highly investigated marker involved in some physiological processes and oncogenesis. Recent research showed the effect of Bmi-1 on the self-regeneration, differentiation and aging mechanisms and on regulation of stem cell activators [9,20]. Bmi-1 can also act as an oncogene [22]. The amplification and overexpression of Bmi-1 was also observed in certain human neoplasms e.g. lung, breast, ovarian, stomach and nasopharyngeal cancers, suggesting an important role of this protein in malignant transformation [17,24,28,38]. Bmi-1 has also been identified as a regulator in the self-regulation of primary neural crest cells [8,16]. It is believed that Bmi-1 and N-myc act together and both are involved in pathogenesis of neuroblastoma [30]. The Ki-67 and p53 proteins are widely evaluated markers of proliferation and apoptosis. Analysis of Ki-67 in neuroblastoma has revealed a correlation between expression of this protein and amplification of the N-myc gene [25,47]. Tp53 albumin (the p53 gene product) by affecting the cell cycle is the most important guardian of the genome. Mutations of this gene are found in many human neoplasms, e.g. colon, esophagus, nasopharyngeal or breast cancers and among patients with Li-Fraumeni syndrome. In pediatric cancers p53 mutations are also observed, e.g. in leukemias, lymphomas, soft tissue sarcomas and nephroblastoma, but they are rarely recognized in neuroblastoma [13,18,21,45,46]. The aim of this study was to assess the potential prognostic value of Bmi-1 protein and its relations with mechanisms of proliferation and apoptosis in the neuroblastoma group of tumors.

Materials and methods

Based on the agreement of the Bioethics Committee of the Medical University of Lodz (no. RNN/114/07/KE), 29 formalin-fixed and paraffin-embedded neuroblastoma tissue sections from the files of the Department of Pathology, Konopnicka Memorial Hospital, Medical University of Lodz, were selected for our study. From these tissue samples, slides with the thickness of about 3-4 μm were prepared and stained with hematoxylin and eosin (HE). All the cases were reviewed routinely by two pathologists and confirmed by immunohistochemistry and the previously diagnosed tumors became reclassified according to current criteria for this group. Examined tissue samples were obtained from 29 children (16 boys (55%) and 13 girls (45%)) aged from 14 days to 13 years (including 10 children up to 1 year old (34%)). The tumors were diagnosed as neuroblastoma (24 cases – 83%, including 13 cases of well-differentiated and 11 poorly differentiated types) and ganglioneuroblastoma (5 cases – 17%). There were 18 children (63.3%) with favorable histology, while unfavorable type appeared in 11 cases (36.7%). In 16 patients the N-myc amplification was evaluated and revealed in 4 children (31%): 3 boys and 1 girl. In the course of neoplastic disease recurrences were diagnosed in 9 cases, and those 9 children died due to cancer progression (31%).

For immunohistochemistry, mouse monoclonal antibodies were used. There were: anti-Bmi-1 (clone F6, Upstate, # 05-637), anti-p53 (clone DO-7, Novocastra, NCL-p53-D07) and anti- Ki-67 (clone MM1, Novocastra, NCL-Ki67-MM1). The Envision system (DAKO) was used and target retrieval procedures according to the manufacturer's instructions were performed.

The estimation of the expression of investigated proteins was examined with a computer image analysis system (Multi Scan Base v. 8.08 - Computer Scanning System, Ltd.). The positive reaction was found to be brown coloration of cell nuclei and positive results were showed as an index per 1000 examined cells.

Expression of investigated proteins and selected histoclinical features – age, histological type of the tumor, N-myc amplification and time of treatment – were analyzed using the statistical package STATISTICA 5.0., license no. SP125579705G51). In measurable values the mean and the median, standard deviation and the range were determined. For the estimation of differences in examined groups the Kruskal-Wallis or Mann-Whitney test was used. In subgroups of less than 5 of cases, Fisher's test and/or Yates' correction were done. Pearson's r was calculated to evaluate the correlations.

Results

Bmi-1 expression was observed in 24 of 29 examined tumor tissue samples and the mean Bmi-1 index was 80.2 (minimum 32.0, maximum 99.0, median 91.5, standard deviation ± 21.11).

In the subgroup of children who survived the mean Bmi-1 index was 85.2 (±19.71), and among children who died it was 70.3 (± 22.37). There was no statistically significant difference between the two groups.

The mean Bmi-1 index in favorable histology cases was higher than among unfavorable cases, without a statistically significant difference; 83.9 (± 18.01) and 77.9 (±23.10), respectively. In ganglioneuromas the mean Bmi-1 index was 55.3 (± 25.71), while in neuroblastomas Bmi-1 expression was higher and the mean index was 84.7 (± 17.21). The difference between Bmi-1 expression in ganglioneuromas and neuroblastomas was statistically significant ($p<0.05$). It is presented in Figure 1.

A statistically significant correlation between Bmi-1 index and age of patients was found ($p<0.05$, $r = -0.49$). It is shown in Figure 2.

A statistically significant difference between expression of Bmi-1 in tumor tissue with and without N-myc amplification was not found.

Ki-67 expression was observed in all the examined tumor tissue samples.

The mean Ki-67 index was 31.4 (minimum 2.0, maximum 95.0, median 31.5, standard deviation ± 26.76).

In the subgroup of children who survived, the mean Ki-67 index was 32.0 (±24.79), and among children who died it was 33.9 (± 33.51); there was no statistically significant difference between the two groups.

The mean Ki-67 index in favorable histology cases was lower than among unfavorable cases, without a statistically significant difference, 27.7 (± 24.86) and 38.1 (± 30.06), respectively.

P-53 expression was observed in all the examined tumor tissue samples.

The mean p-53 index was 15.1 (minimum 1.0, maximum 95.0, median 15.1, standard deviation ± 25.84).

In the subgroup of children who survived, the mean p-53 index was 12.3 (±22.61), and among children who died it was 19.7 (± 34.27). There was no statistically significant difference between the two groups.

A statistically significant correlation was found between Ki-67 and p-53 indices ($p<0.05$, $r = 0.69$).

The mean treatment time was 548.9 days (minimum 90.0, maximum 1976.0, median 468.0, standard deviation ± 452.89). A statistically significant correlation was

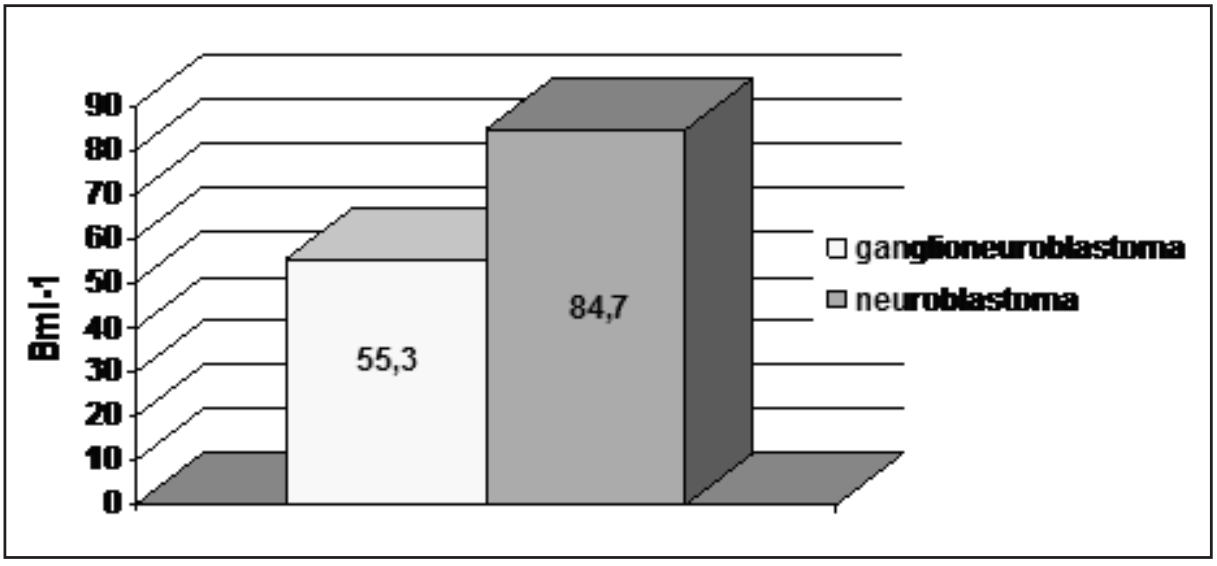

Fig. 1. The mean Bmi-1 index in ganglioneuroblastoma and neuroblastoma group of tumors

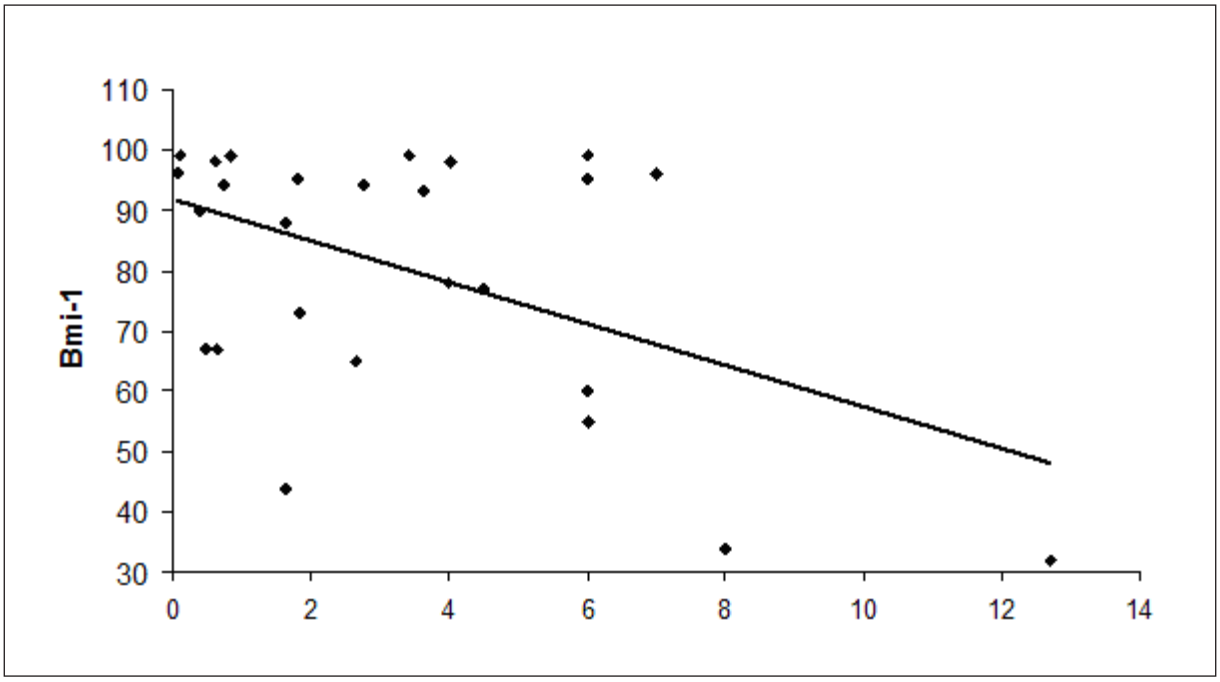

Fig. 2. Statistically important correlation between Bmi-1 index and age of the patient (axis y – Bmi-1 index, axis x – age of the patients in years)

Table 1. Results of statistical analysis of histoclinical features in nephroblastoma group of tumors

Feature	Age	Bmi-1	Ki-67	p53	Treatment days
Age	1.00	–0.49	–0.09	–0.01	0.66
Bmi–1	–0.49	1.00	0.16	0.05	–0.15
Ki–67	–0.09	0.16	1.00	0.69	–0.15
p53	–0.01	0.05	0.69	1.00	–0.06
Treatment days	0.66	–0.15	–0.15	–0.06	1.00

found between treatment time and age of the patient ($p<0.05$, $r = 0.66$).

A summary of the data investigated in the statistical analysis is presented in Table 1.

Discussion

The current list of recognized prognostic factors in neuroblastoma includes the patient's age at diagnosis, tumor location, regional lymph node involvement, microscopic picture of the tumor, response to therapy [26,32] and some biological factors, e.g. N-MYC amplification and chromosomal aberrations [2,6,12,34]. However, the results of treatment in neuroblastoma are still not satisfactory; total survival is approximately 60% and in the high risk group only 31%.

In addition to established prognostic factors, there is a very large group of highly investigated markers, whose influence on the tumor biology is not clearly explained and the potential prognostic value remains unconfirmed. Some of these factors are evaluated in various tumor types as well as in neuroblastoma, while some others show a close relation to neuroblastoma pathogenesis. Among the latter, Bmi-1 seems to be the most characteristic one.

Bmi-1 is a protein gene product from the Polycomb group which is classified as an oncogene, and it is responsible for encoding a group of transcriptional factors necessary for embryonic development and the process of self-renewal of the stem cells [30,39]. Bmi-1 also plays an important role in the differentiation of lymphocytes, and its expression was observed in immature T and B lymphocytes. What is the most important for neuroblastoma studies, it is proved that in addition to regulation of physiological processes of regeneration and self-renewal the Polycomb gene family is associated with tumorigenesis [8,10,15,19] and that Bmi-1 plays an important role in cancer cells in humans [27,37].

Recent studies have shown that the Bmi-1 gene regulates the regeneration of hematopoietic stem cells, leukemic cells, as well as primary cells of the central and sympathetic nervous system [19,23,28,31]. It is proved that the

Bmi-1 protein affects the activity and self-regeneration of the cells of the neural crest from which neuroblastoma is derived [28]. Most likely during embryogenesis the level of Bmi-1 influences on the direction of differentiation of primary neural crest cells and various levels of Bmi-1 gene expression decide the fate of cells. It was observed that Bmi-1 regulates differentiation of neuroblastoma stem cells in concentration dependent manner. Medium level of Bmi-1 is observed in neuroblastoma stem cells. Low concentration is connected with glial/Schwann-like pattern, and high causes differentiation into neuron-like cells. This kind of self-renewal and differentiation stays in compliance with widely discussed morphogen gradients in controlling cell fate in embryo [39].

The role of Bmi-1 in tumor pathogenesis and prognostic significance of its expression have been documented in many cancers. Overexpression of Bmi-1 has been found in leukemias and solid tumors of adults such as lung, colon, prostate and breast carcinomas. Bmi-1 expression in these tumors accompanied disease progression [17,23,24,27,29,49]. Recent studies have also demonstrated the prognostic significance of Bmi-1 expression in liver [49] and nasopharyngeal cancers. In the latter case, it was found that high expression of Bmi-1 correlated with poor prognosis for the patients. Bmi-1 overexpression was found in 29 of 75 patients (38.7%). The five-year survival rate was 74.2% in the group in which there was no expression of Bmi-1, whereas in those with Bmi-1 expression it was only 47.6% [38]. The results of this research are consistent with the observations of other researchers available in the literature. Bmi-1 expression has been found in the majority of examined tumor tissues, and its indices were higher in tumors with worse prognosis. What is especially interesting, in our study a statistically significant difference in Bmi-1 expression between tumors with features of maturity (ganglioneuroblastomas) and the less differentiated group (neuroblastomas) was found.

The degree of maturity of tumor cells is a basic element in histological classifications [36] and became a widely accepted prognostic factor in the neuroblastoma group of tumors. The cases are classified based on microscopic examination, which assesses the tumor stroma, mitotic--apoptotic index, and patient's age. A currently used modification of Shimada's classification is the International Neuroblastoma Pathology Classification System (INPC) [4]. The microscopic picture of the tumor determines the type and duration of the treatment and the use of adjuvant therapy. The significantly lower Bmi-1 indices in more mature tumors might argue for the role of this protein in the still unknown mechanism of the maturation process in the neuroblastoma group of tumors, which should be taken into consideration during treatment planning.

It was found that in the group with poor prognosis the average index Bmi-1 was higher, but there was no statistically significant difference between favorable and unfavorable histology cases in our studies. It is well known that age of the patient is a very important prognostic factor in neuroblastoma. The best prognosis is for children under 1 year of age; in this age group the survival reaches 75%. After two years of life the survival rate of children with neuroblastoma decreases to 30%.

A statistically significant correlation between patient's age and Bmi-1 expression was found in our research. The Bmi-1 index decreased with age. This may explain the fact that in older children mostly ganglioneuroblastomas are recognized and spontaneous or chemotherapy-induced maturation appears. Once again, this suggests an important role of Bmi-1 in neuroblastoma cell maturation processes.

The main marker of poor prognosis in the neuroblastoma group of tumors is the amplification of N-myc proto--oncogene, which is defined as presence of more than ten copies of the gene. This feature is observed in 16% to 25% of neuroblastomas [3], and it is related to: deletion of 1p observed in 25-35% of cases [2,14,32,35], the presence of additional copies of 17q, considered as an independent predictor of poor prognosis [3], and DNA ploidy [2].

It was found that Bmi-1 may be activated by the same factors as N-myc [30]. Consequently, Bmi-1 in cooperation with N-myc may contribute to disease progression and act as a marker of poor prognosis. In our study, however, there was no relationship between the expression of Bmi-1 and N-myc amplification. There was no significant correlation between the expression of Bmi-1 and Ki-67, either, despite the fact that in the literature there are reports that higher proliferation indices in neuroblastoma are associated with worse prognosis, shorter survival time and lower histological differentiation of the tumor [33].

There is little documented research on Bmi-1 in cancers in childhood. However, some of them indicate that the Bmi-1 protein plays an important role in the development and progression of neuroblastoma. Our study confirms the prognostic significance of Bmi-1 expression in the neuroblastoma group of tumors; high expression of this protein was associated with immaturity of the tumor, and hence with a poor prognosis for the patients.

The observed difference between the expression of Bmi-1 and p53 was of borderline statistical significance, which seems to be an interesting finding of our research. Mutation of the p53 gene is observed in many cancers, including children, but it is rarely observed in cases of neuroblastoma [48]. Despite the fact that genetic studies have demonstrated that the function of p53 is regulated by a variety of genes, including N-myc [18,46], the role of p53 protein in the pathogenesis of neuroblastoma is not clear. Some researchers believe that there is overexpression of mutant p53 in neuroblastoma cell nuclei [5], while others believe that mutations of the p53 gene are characteristic for cell lines of patients with recurrent neuroblastoma only [18,22,45,48]. There are also studies on compound p53 disorders and the p53-dependent phenomenon of drug resistance in treatment of neuroblastoma [42,44].

The results of the present study demonstrate the significant and complex role of Bmi-1 in pathogenesis and progression in the neuroblastoma group of tumors. The revealed statistically significant relations between Bmi-1 expression and widely accepted prognostic factors and the association with expression of the p53 gene clearly indicate the need for further research that can potentially contribute to explanation of the complex neuroblastoma biology and may have a positive impact on the results of treatment in this so problematic tumor.

Conclusion

Bmi-1 is a promising marker in the neuroblastoma group of tumors which correlates with widely accepted prognostic parameters, tumor histology and age of the patient. The pattern of BMI-1 expression may indicate that the examined protein is involved in maturation processes in tumor tissue.

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