The role of N-Myc gene amplification in neuroblastoma childhood tumour – single-centre experience

Przemysław Kaczkówka¹, Aleksandra Wieczorek¹,², Małgorzata Czogała¹,², Teofila Książek¹,², Katarzyna Szewczyk¹,², Walentyna Balwierz¹,²

¹Department of Paediatric Oncology and Haematology, Institute of Paediatrics, Jagiellonian University Medical College, Krakow, Poland
²Department of Paediatric Oncology and Haematology, University Children’s Hospital of Krakow, Poland
³Department of Medical Genetics, Faculty of Medicine, Institute of Paediatrics, Jagiellonian University Medical College, Krakow, Poland
⁴Department of Medical Genetics, University Children’s Hospital of Krakow, Poland

Introduction

Neuroblastoma (NBL) is the most commonly occurring extracranial malignant solid cancer in children and comprises 8–10% of all tumours in children. Median age at diagnosis is 17 months [1]. Neuroblastoma arises from the neural crest, which usually gives rise to parts of sympathetic nervous system and adrenal glands. The primary tumour is usually located in the abdomen (retroperitoneal space) or posterior mediastinum, but it can be found in all parts of the body where embryonic cells of sympathetic nervous system occur. In Poland there are approximately 60–70 new cases of NBL annually [2]. Among all children’s deaths due to cancer, 15% die of neuroblastoma [3]. The characteristic neuroblastoma feature is the diversity of its course: both rapid metastasis and spontaneous regression with differentiation to benign tumours, e.g. ganglioneuroma, can be observed. The percentage of spontaneous regressions is one of the highest from all human cancers [4, 5]. Five-year overall survival has increased sharply from 52% (1975–1977) to 74% (1999–2005), but it is mainly because of better curability in the low-risk group. For patients that are classified as high risk, the results of treatment are still unsatisfying [6].

Age at diagnosis (better prognosis in children younger than 18 months) and stage of disease are important prognostic factors influencing the treatment [6, 7]. Neuroblastoma affects boys more often than it does girls [8–10].

The most significant genetic marker of poor prognosis is N-Myc oncogene amplification (over four-fold increase of the N-Myc signal number in relation to the number of reference signals on chromosomes 2) [11]. Therapy should be stratified on the basis of the presence of N-Myc amplification [12, 13]. N-Myc belongs to the proto-oncogene family that products particles taking part in processes such as cell growth, differentiation, and apoptosis [14]. Disturbance in the regulation of their courses may lead to excessive and unrestricted cell proliferation, which is widely observed in cancers [15]. There are some other genetic lesions coexisting with N-Myc, which may have an influence on prognosis, such as DDX1 amplification, but their clinical meaning is still unclear [16]. Crucial predictive factors are also deletion of the long arm of chromosome 11 and deletion of the short arm of chromosome 1, which both, independently, correlate with poor prognosis [17]. Gain of the long arm of chromosome 17 is another prognostic factor [18]. Distinct factors that correlate with good prognosis are triploidy of tumour cells (as opposed to dip-
loidal and tetraploidy) and high expression of neurotrophin receptor TrkA [19, 20]. In the case of lack of N-Myc amplification, currently the presence or absence of segmental chromosomal aberrations in NBL tumour cells is the most important biological prognostic factor [21].

The aim of the study was a retrospective analysis of the impact of N-Myc amplification on disease course in neuroblastoma patients.

**Material and methods**

From the group of 160 children with NBL, who were treated from 1991 to 2015 in the Department of Paediatric Oncology and Haematology, University Children's Hospital of Krakow in Poland, the investigation focused on 140 children (87.5%) whose N-Myc amplification status had been verified at the onset of disease. Observation was finished in December 2017. Before 2002 intensity of the treatment was based mainly on disease advance and age. N-Myc gene status did not influence the therapy. There was no uniform protocol for infants, they were treated according to data available in publications. Therapy for older children was based on Japanese protocols (Tokyo). From 2002 European protocols were introduced in Poland. N-Myc gene amplification become an important risk factor. Standard-risk infants were treated according to Infant-NBL-99. From 2006 high-risk infants (stage 2–4 with N-Myc gene amplification) were treated according to the protocol for high-risk patients older than one year (HR-NBL-1/ESIOP) comprising combined chemotherapy, stem cell mobilisation and apheresis, surgery, mega-dose chemotherapy with autotransplantation, radiotherapy, and the treatment of residual disease with retinoid acid, and from 2012 also with immunotherapy. Vanillylmandelic acid (VMA) and homovanillic acid (HVA) levels were tested in 24-hour urine collection. Because the analysed group was not numerous and very diversified regarding the treatment, no analysis comprising methods of therapy was performed. Patients were between eight days and 14.4 (median 1.6 years) years old at diagnosis. Diagnosis was made on the basis of primary tumour histopathological examination or the presence of cancer cells in the bone marrow and increased catecholamines and/or their metabolites’ levels in urine according to international criteria. The following examinations were done in all patients: radiological imaging, whole-body scintigraphy with MIBG or bone scintigraphy with technetium (in MIBG non-avid patients), and biochemical tests and genetic examination of the tumour. N-Myc amplification tests were performed on touch prints with immunohistochemistry and fluorescence in situ hybridisation using two-colour molecular probe N-MYC (MYCN) Amplification (Cytocell Ltd., Cambridge). Stage of disease was established on the basis of the International Neuroblastoma Staging System. Clinical data was reported on the basis of medical documentation. The kind and intensity of therapy depended on children’s age, stage, and biological features of disease [2].

The study was carried out in two groups. The first group was represented by children with NBL with N-Myc amplification (analysed group, n = 25), the second group was represented by children with NBL (control group, n = 115) without N-Myc amplification. Comparative analyses were performed for the following parameters: age at diagnosis, sex, stage of disease, and initial levels of: ferritin, dopamine, VMA, HVA, neurospecific enolase (NSE), and lactate dehydrogenase (LDH). Treatment results were also evaluated.

Statistical calculations were carried out with Statistica® software package. One-way analyses were conducted using χ² test. Nonparametric Kolmogorov-Smirnov test was used to compare biochemical parameters in two groups of patients with and without N-Myc amplification. Overall survival (OS, time from diagnosis to death or last observation) and failure-free survival (FFS, time from diagnosis to relapse or progression of disease before obtaining complete remission, or last observation) were estimated using Kaplan-Meier method. Differences between curves were analysed with Log-Rank test. Statistical significance was set at p = 0.05.

**Results**

Among 140 NBL evaluated patients, we found 25 (17.9%) children with presence of N-Myc gene amplification. N-Myc amplification was not found in any of the nine children in stage 1. The amplification was present in: 1 (6.3%), 6 (13.3%), 2 (18.2%), and 16 (27.1%) patients, in stage 2, 3, 4S, and 4 of disease, respectively. All children with N-Myc amplification had unfavourable histopathology evaluated according to International Neuroblastoma Pathology Classification [22].

The results of comparison of biochemical parameters in groups with and without N-Myc amplification are shown in Table 1. N-Myc amplification occurred with similar frequency in boys and girls (18% in each group). Median age in both groups was comparable (1.98 years in the analysed group, 1.45 years in the control group). In the N-Myc amplification-positive group there were 18 (72%) children older than one year, and in the control group there were 67 (58.3%) children older than one year. The difference was not statistically significant (p > 0.1). Among the children with N-Myc amplification more frequent stage was 4 (64%), but compared to control group (37%) there was no statistical significance (p > 0.1).

In the analysed patients, ferritin level at diagnosis of the disease was checked in 109 cases (77.9% of patients with known N-Myc status). In the group with N-Myc amplification (n = 21) the median ferritin concentration was higher than in the control group (n = 88), at 132.6 µg/l and 288.6 µg/l, respectively. The difference was statistically significant (p < 0.01). Among the children with N-Myc amplification the most frequent NBL stage was 4 (64%), but compared to control group (37%) there was no statistical significance (p > 0.1).

In the analysed patients, ferritin level at diagnosis of the disease was checked in 109 cases (77.9% of patients with known N-Myc status). In the group with N-Myc amplification (n = 21) the median ferritin concentration was higher than in the control group (n = 88), at 132.6 µg/l and 288.6 µg/l, respectively. The difference was statistically significant (p < 0.01). Among the children with N-Myc amplification the most frequent NBL stage was 4 (64%), but compared to control group (37%) there was no statistical significance (p > 0.1).

Dopamine level was initially checked in 103 cases (73.6% of patients with known N-Myc status). In the N-Myc amplification-positive group (n = 17) and control group (n = 86) median dopamine concentration was 12.726 µg/mg creatinine and 2.045 µg/mg creatinine, respectively. The difference was statistically significant (p < 0.025). NSE level was checked at diagnosis in 80 cases (57.1% of patients with specified N-Myc status). In the analysed group (n = 12) and
control group (n = 68), the median NSE concentration was 418.2 ng/ml and 67.745 ng/ml, respectively. The difference was statistically significant (p < 0.01). LDH activity was initially checked in 125 cases (89.3% of patients with specified N-Myc status). In the group with amplification (n = 21) and without amplification (n = 104) median LDH activity was 3989 U/l and 1015.5 U/l, respectively. The difference was statistically significant (p < 0.01).

Vanillylmandelic acid level was initially checked in 115 cases (82.1% of patients with specified N-Myc status). In the N-Myc amplification-positive group (n = 19) and control group (n = 96) median VMA concentration was 22.935 µg/mg creatinine and 68.626 µg/mg creatinine, respectively. The level of VMA in urine was considerably higher in the group without N-Myc amplification. The difference was statistically significant (p < 0.005). HVA level was checked at the onset of disease in 107 cases (76.4% of patients with specified N-Myc status). In the analysed group (n = 18) and control group (n = 89) median was 61.525 µg/mg creatinine and 69.438 µg/mg creatinine, respectively. The difference was not statistically significant (p > 0.1).

Because the main aim of the study was the evaluation of the impact of N-Myc amplification on disease course in patients with NBL, we decided to exclude from the OS assessment the patients whose death was not caused by NBL. Treatment complications were the reason of death in one patient from the N-Myc amplification-positive group and five children from the control group. Deaths because of NBL occurred in 11 (44.0%) out of 25 patients with N-Myc amplification and 27 (23.5%) out of 115 patients in the control group. The difference was not statistically significant (p > 0.1).

Five-year OS for patients with and without N-Myc amplification was 58 ±10% and 79 ±4% (Fig. 1), respectively. The difference was statistically significant (p = 0.03).
Therapy failure defined as progression or relapse occurred in 12 (48.0%) out of 25 patients with amplification of N-Myc and 33 (28.7%) out of 115 patients in the control group. The difference was not statistically significant ($p > 0.1$). Five-year FFS for patients with and without N-Myc amplification was 50 ±10% and 72 ±4%, respectively (Fig. 2). The difference was statistically significant ($p = 0.03$).

In a subgroup of 81 patients in stage 1, 2, 3, and 4s of NBL there were nine patients with N-Myc amplification and 72 without amplification. Five-year OS for patients with and without N-Myc amplification was 89 ±10% and 91 ±3%, respectively. Five-year FFS for patients with and without N-Myc amplification was 89 ±10% and 89 ±4%, respectively. There was no significant difference in OS and FFS between patients with and without N-Myc amplification in this group ($p > 0.1$).

In a subgroup of 59 patients with stage 4 NBL there were 16 patients with N-Myc amplification and 43 without N-Myc amplification. Five-year OS was 38 ±13% and 55 ±8% in patients with and without N-Myc amplification, respectively (Fig. 3). Although the difference was not statistically significant ($p = 0.096$), some trend could be
observed towards OS improvement in the non-amplified group. The difference in FFS between two groups was not statistically significant ($p > 0.1$).

In a subgroup of 55 patients who were younger than one year old (≤12 months) at diagnosis there were seven patients with N-Myc amplification and 45 without N-Myc amplification. Five-year OS was 71 ±17% and 98 ±2% (Fig. 4) for patients with and without N-Myc amplification, respectively. The difference was statistically significant ($p = 0.004$), five-year FFS was 71 ±17% and 96 ±3%, respectively for patients with and without N-Myc amplification (Fig. 5). The difference was statistically significant ($p = 0.02$).

In a subgroup of 85 patients who were older than one year (18 children with and 67 children without N-Myc amplification) five-year OS was 51 ±13% and 64 ±6% for these two subgroups, respectively. The difference was not statistically significant ($p > 0.1$). No significant difference in five-year FFS was found (40 ±12% vs. 55 ±6%, respectively, $p > 0.1$).

We compared OS and FFS in children with N-Myc amplification depending on age at diagnosis. Among 25 patients with N-Myc amplification there were 18 over one year old and seven who were below one year old at diagnosis. No significant difference in five-year OS was found (51 ±13% vs. 71 ±18%, respectively, $p = 0.4$). Similarly, there was no significant difference in five-year FFS (40 ±12% vs. 71 ±17%, respectively, $p = 0.3$).

**Discussion**

Amplification of N-Myc oncogene is a known marker of poor prognosis in neuroblastoma. [9, 10]. In our study N-Myc status was known in 87.5% of all children diagnosed with NBL in our centre from 1992 to 2015. In 12.5% of patients there were no data concerning N-Myc, or examination of the marker was not done. N-Myc amplification occurred slightly less often (17.9%) than was reported by other authors (25%) [23]. It was also found out that children with N-Myc amplification were more often (64%) classified to stage 4 than the patients without amplification (37%), which relates to other studies (Table 1) [24]. Moreover, no N-Myc amplification was found in children with stage 1 NBL. It is worth noting that tumours in all children with N-Myc amplification had unfavourable histopathology, which is one of the crucial aspects when it comes to prognostic analysis [25].

High serum ferritin level without a corresponding increase in tissue iron storage is observed in patients with neuroblastoma. Moreover, when the serum ferritin level normalises, the probability of remission increases [26]. It is interesting that in the study the differences between groups were statistically significant (the group with N-Myc amplification had significantly higher levels of ferritin). Elevated dopamine level in urine is associated with poor prognosis in neuroblastoma. It occurs especially in stages 3 and 4 [27]. This biochemical parameter was also statistically significantly increased in the group with N-Myc amplification. The levels of both NSE and LDH in serum are positively associated with worse prognosis in neuroblastoma and are useful in the monitoring of treatment [28]. In our study both parameters were statistically significantly higher in the group of patients with N-Myc amplification in comparison with the group without amplification (Table 1). Elevated levels of VMA and HVA in urine are observed in 68–95% patients diagnosed with neuroblastoma [29]. In our study both levels were lower in the group of children with N-Myc amplification, which can lead to the conclusion that it is the HVA/VMA ratio, not their absolute levels, that could be a predictive factor, as was discovered in previous examinations [30].

In analysis concerning treatment outcome we found that children without N-Myc amplification had significantly higher probability of overall and failure-free survival. We analysed also subgroups of patients. The difference in the probability of OS and FFS was statistically significant only in patients younger than one year of age (Figs. 4 and 5). In older children N-Myc amplification did not influence significantly the treatment outcome. Probably this is due to the high number of stage 4 tumours in this group. No statistically significant differences in OS and FFS depending on N-Myc status were found in children with NBL stage 1, 2, 3, and 4s, but the number of patients with N-Myc amplification in this group was low (9/81, 11%). In patients with NBL stage 4 there was a trend for worse survival when N-Myc amplification was confirmed. However, the difference was not statistically significant (Fig. 3).

To sum up, we can state that N-Myc amplification is associated with elevated levels of biochemical markers corresponding to the disease activity and tumour mass. It also has a negative impact on life expectancy and is rightly perceived as one of the crucial factors that indicate bad prognosis in this cancer (Figs. 1 and 2). N-Myc status is obligatory checked in all patients with diagnosis of NBL. There is a need to continue the studies concerning N-Myc amplification. Further studies on larger groups of patients are needed. It is very important to confirm if it still is an
important prognostic factor in patients treated with more intensive protocols and if they should receive the same therapy as patients without N-Myc amplification. It is also evaluated whether it could be used for targeted therapy [31].

The authors declare no conflict of interest.

References

1. London WB, Castleberry RB, Matthay KK, et al. Evidence for an age cut-off greater than 365 days for neuroblastoma risk group stratification in the Children’s Oncology Group. J Clin Oncol 2005; 23: 6459-6465.

2. Balwierz W, Szurgot M. Nerwiak zarodkowy współczulny. In: Za-lecenia postępowania diagnostyczno-terapeutycznego w nowotworach złośliwych. Vol. III. Krzakowski M, Warzocha K (eds.). Via Medica 2013; 1082-1098.

3. Tang XX, Shimada H. Clinical Implications of Neuroblastoma Stem Cells. In: Neuroblastoma. Shimada H (eds.). InTech 2013; 291-302.

4. Schwab M, Westermann F, Hero B, Berthold F. Neuroblastoma: biology and molecular and chromosomal pathology. Lancet Oncol 2003; 4: 472-480.

5. Maris JM. Recent advances in neuroblastoma. New Engl J Med 2010; 362: 2202-2211.

6. Maris JM, Hogarty MD, Bagatell R, Cohn SL. Neuroblastoma. Lancet 2007; 369: 2106-2120.

7. Vo KT, Matthyay KK, Neuhaus J et al. Clinical, biologic, and prognostic differences on the basis of primary tumor site in neuroblastoma: a report from the international neuroblastoma risk group project. J Clin Oncol 2014; 32: 3169-3176.

8. Ward E, DeSantis C, Robbins A, Kohler B, Jemal A. Childhood and adolescent cancer statistics. CA Cancer J Clin 2014; 64: 83-103.

9. Balwierz W, Wieczorek A. New international staging system and classification of risk groups in neuroblastoma. Przegl Lek 2010; 67: 345-349.

10. Morgenstern DA, Pötschger U, Moreno L, et al. Risk stratification of high-risk metastatic neuroblastoma: A report from the HR-NBL-1/SIOPEN study. Pediatr Blood Cancer 2018; 65: e27363.

11. Ambros PF, Ambros IM, Brodeur GM, et al. International consensus for neuroblastoma molecular diagnostics: report from the International Neuroblastoma Risk Group (INRG) Biology Committee. Br J Cancer 2009; 100: 1471-1482.

12. Seeger RC, Brodeur GM, Sather H, Dalton A, Siegel SE, Wong KY, Hammond D. Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas. New Engl J Med 1985; 313: 1111-1116.

13. Rubie H, Hartmann O, Michon J, et al. N-Myc gene amplification is a major prognostic factor in localized neuroblastoma: results of the French NBL 90 study. Neuroblastoma Study Group of the Société Francaise d’Oncologie Pédiatrique. J Clin Oncol 1997; 15: 1171-1182.

14. Okubo T, Knoepfli PS, Eisenman RN, Hogan BL. Nmyc plays an essential role during lung development as a dosage-sensitive regulator of progenitor cell proliferation and differentiation. Development 2005; 132: 1363-1374.

15. Wilde BR, Ayer DE. Interactions between Myc and MondoA transcription factors in metabolism and tumourigenesis. Br J Cancer 2015; 113: 1529-1533.

16. Weber A, Imisch P, Bergmann E, Christiansen H. Coamplification of DDX1 correlates with an improved survival probability in children with MYCN-amplified human neuroblastoma. J Clin Oncol 2004; 22: 2681-2690.

17. Attiyeh EF, London WB, Mossé YP et al. Chromosome 1p and 11q deletions and outcome in neuroblastoma. N Engl J Med 2005; 353: 2243-2253.

18. Bown N, Cotterill S, Lastowska M, et al. Gain of chromosome arm 17q and adverse outcome in patients with neuroblastoma. N Engl J Med 1999; 340: 1954-1961.

19. Spitz R, Betts DR, Simon T, et al. Favorable outcome of triploid neuroblastomas: a contribution to the special oncogenesis of neuroblastoma. Cancer Genet Cytogenet 2006; 167: 51-56.

20. Brodeur GM, Bagatell R. Mechanisms of neuroblastoma regress- sion. Nat Rev Clin Oncol 2014; 11: 704-713.

21. Schleiermacher G, Mosseri V, London WB, et al. Segmental chromosomal alterations have prognostic impact in neuroblastoma: a report from the INRG project. Br J Cancer 2012; 107: 1418-1422.

22. Shimada H, Umehara S, Monobe Y, et al. International neuro- blastoma pathology classification for prognostic evaluation of patients with peripheral neuroblastic tumors: a report from the Children’s Cancer Group. Cancer 2001; 92: 2451-2461.

23. Huang M, Weiss WA. Neuroblastoma and MYCN. Cold Spring Harb Perspect Med 2013; 3: a014415.

24. Brodeur GM, Seeger RC, Schwab M, Varmus HE, Bishop JM. Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. Science 1984; 224: 1121-1124.

25. Goto S, Umehara S, Gerbing RB, et al. Histopathology (International Neuroblastoma Pathology Classification) and MYCN status in patients with peripheral neuroblastic tumors: a report from the Children’s Cancer Group. Cancer 2001; 92: 2699-2708.

26. Hann HW, Levy HM, Evans AE. Serum ferritin as a guide to therapy in neuroblastoma. Cancer Res 1980; 40: 1411-1413.

27. Nakagawara A, Ikeda K, Tasaka H. Dopaminergic neuroblastoma as a poor prognostic subgroup. J Pediatr Surg 1988; 23: 346-349.

28. Pang QM, Li K, Ma Ll, Sun RP. Clinical research on neuroblastoma based on serum lactate dehydrogenase. J Biol Regul Homeost Agents 2015; 29: 131-134.

29. Smith SJ, Diehl NN, Smith BD, Mohney BG. Urine catecholamine levels as diagnostic markers for neuroblastoma in a defined pop- ulation: implications for ophthalmic practice. Eye (Lond) 2010; 24: 1792-1796.

30. Nishi M, Miyake H, Takeda T, et al. The relationship between ho- movanillic/vanillylmandelic acid ratios and prognosis in neuro- blastoma. Oncol Rep 1998; 5: 631-633.

31. Schnepp RW, Maris JM. Targeting MYCN: a good BET for improving neuroblastoma therapy? Cancer Discov 2013; 3: 255-257.