Neuroblastoma (NB) is an embryonal tumor arising in tissues of the sympathetic nervous system and the most common cancer diagnosed during the first year of life. NB accounts for 15% of all deaths due to childhood malignancies. Despite intense multimodality therapy, at least half of high-risk patients relapse that is almost always fatal [1]. Much of the difficulty in devising effective therapies for this latter group of patients lies in the heterogeneity of disease, which can be attributed largely to the interaction of multiple genetic and epigenetic factors [2].

Unlike many adult malignancies most NB do initially respond to chemotherapy and it is likely that the presence of functionally active p53 is at least partly involved in this initial chemosensitivity. However, over half of previously responsive NB eventually relapses with chemoresistant disease and there is evidence for p53 inactivation at this stage in some cases [3]. p53 is a potent transcription factor that modulates a large number of genes involved in apoptosis, metabolism, epigenetics, and cell cycle regulation [4]. p53 is usually expressed at low levels in the cell where it has a short half-life of only 30 min [5]. NB is a p53 wild type malignancy at diagnosis and repression of p53 signaling plays an important role in its pathogenesis. Chemotherapy induces apoptosis and tumor regression primarily through activation of p53-mediated transcription. A number of previous observations confirm that wild-type p53 alleles are present in the vast majority of cases of newly diagnosed NB, but that p53/MDM2/adenosine diphosphate-ribosylation factor (ARF) responses to chemotherapy are repressed, in part due to unscheduled inhibition of p53 by MDM2. This suggests that downregulation of the p53 axis may underlie the treatment resistance typically seen in high-risk NB [4]. The expression, function and stabilization of p53 are governed by a complex network, which includes its regulators p14ARF and MDM2 [5].

Originally identified as an amplified gene located on a double minute chromosome in a transformed mouse cell line, MDM2 was later shown to have a critical role in the process of cellular transformation. Thus, deregulation of MDM2 gene expression likely contributes to the pathogenesis of a wide range of human tumors. Amplification and/or overexpression of MDM2 is found in about 10% of all human tumors. In gliomas, for example, MDM2 amplification identifies a subset of high-risk patients that do not have p53 mutations [6]. In many soft tissue sarcomas, MDM2 amplification and overexpression correlates with poor prognosis.

Additionally, p53 activates the MDM2 gene that encodes p53 antagonist forming an autoregulatory feedback loop that tightly controls expression of both p53 and MDM2 [7]. MDM2 helps to target p53 for degradation through its E3 ubiquitin ligase activity.

Recent literature demonstrates that p53 is generally functional, accumulates in the nucleus in response to DNA damage and is efficiently degraded by MDM2 in NB cells. There are reports that p53 function may be compromised as a consequence of aberrant MDM2 expression levels in NB. For instance, a study examining etoposide-induced p53 activity in a NB cell lines suggested an important role for elevated MDM2 expression levels in the regulation of p53 nuclear-cytoplasm translocation [8].

Also, elevated MDM2 expression and accompanying loss of p53 function is associated with multidrug
resistance in some NB cell lines. MDM2 ubiquitin ligase activity is rate-limiting for p53 degradation and is critical in NB [9]. Taken together, these data demonstrate that the MDM2/p53 interaction is intact in NB and suggest that deficiencies in p53 functions may be a consequence of aberrant MDM2 expression.

Amplification of the MYCN gene (MNA) has emerged as one of the most reliable indicators of aggressive and treatment-resistant NB, yet 30% to 40% of high-risk tumors lack this feature [1]. MNA is associated with an exceptionally poor prognosis in NB. Furthermore, the crucial effectors of MYCN responsible for this aggressive subset of NB await characterization [8]. MYC oncogenes activate both proliferative and apoptotic cellular pathways and, accordingly, inhibition of p53-mediated apoptosis is a prerequisite for MYC-driven tumorigenesis [10]. A critical negative regulator of the p53 tumor suppressor, MDM2, has been recently characterized in NB cell lines as a transcriptional target of MYCN. Targeted inhibition of MYCN results in reduced MDM2 expression levels, with concomitant stabilization of p53 and stimulation of apoptosis in MNA NB cell lines [8]. Moreover, MDM2 has a p53-independent role in the regulation of both MYCN mRNA stabilization and its translation, suggesting that MDM2-mediated MYCN expression is one mechanism associated with growth of MYCN-associated NB and disease progression [11]. MYCN also transcriptionally upregulates p53 expression in NB and may be an important mechanism by which MYCN induces apoptosis [12]. Scheme of MDM2 and p53 regulation involving MYCN is shown in Fig. 1.

Fig. 1. Scheme of MDM2 and p53 regulation involving MYCN oncogene

In this article, we have investigated the association of MDM2 mRNA expression in NB with clinical characteristics and unfavorable disease factors (occurrence age, disease stage, MYCN gene status, metastases and relapses) to determine the possibility of it usage as a prognostic marker of NB.

MATERIALS AND METHODS

Patients. 91 patients with verified diagnosis of NB have been enrolled into this study. The age of patients ranged from 1 month to 17 years. Mean age was 39.45 ± 4.81 months. Age of 28.6% patients did not exceed 18 months. Stages distribution: I–II stage — 21.25% of patients, III stage — 22.5% of patients, IV stage — 56.25% of patients. All patients were treated in accordance with international standards of treatment for NB. Informed consent was obtained according to the requirements of the Ethics Commission of the National Cancer Institute.

Material. Tumor tissue material was obtained by biopsy or tumor resection. 87.9% of samples were obtained from primary NB tumors, 4.4% — from recurrent tumors, 7.7% — from metastatic focus. All biomaterial was taken before treatment. Fresh tumor samples for Q-PCR analysis were stored in “RNA-later” (“Ambion”, USA) to stabilize the RNA and DNA; for fluorescent in situ hybridization (FISH) formalin-fixed, paraffin embedded tissue (FFPE) samples were used.

Analysis of TP53 gene deletion. Deletion of TP53 gene was investigated in NB tumors using FISH analysis. Preparation of FFPE tissue sample slides was performed using Paraffin Pretreatment Reagent Kit I (Abbott, USA). FISH analysis was performed using dual-color Vysis LSI p53 (17p13.1)/CEP17 Probe (Abbott, USA). Analysis of the hybridization was carried out using a fluorescence microscope Axio Imager M1 (Zeiss, Germany).

Analysis of MNA. Determination of MYCN gene status was assessed in NB fresh tumors by Q-PCR analysis and FISH for FFPE samples. Genomic DNA from tumor samples was isolated using “NucleoSpin Tissue” (Macherey-Nagel, Germany). Sequences of primers were experimentally selected with Primer Express® Software v3.0 (Applied Biosystems, USA) and synthesized by “Applied Biosystems”, they are shown in the Table 1. Primers and TaqMan probes were used at 0.6 μM and 0.4 μM concentrations, respectively. TaqMan Universal PCR Master Mix (Applied Biosystems, USA) and 5 ng of DNA were used to prepare the reaction mixture in a total volume of 25 μl. 45 cycles real-time PCR (94 °C — 3 sec, 60 °C — 30 sec and 72 °C — 30 sec) were run on 7300/7500 Real-Time PCR Systems, “Applied Biosystems”, USA. MYCN gene copy number was calculated by the formula:

\[ x = 2^{-ΔCt} \]

where \( x \) — MYCN copy number, \( ΔCt = Ct (β\text{-actin}) − Ct (MYCN) \).

FISH analysis was performed using dual-color Vysis LSI®N-MYC Spectrum Green/CEP 2 Spectrum Orange™ (Abbott, USA). According to the European Network for Quality Assurance in Higher Education guidelines [13], MNA was defined as >4-fold increase of MYCN signal number in relation to the number of chromosome 2.

Table 1. Sequences of primers (F — forward, R — reverse) for PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>F: 5′CCCCGTGCTGCTGGCGCGTT3′</th>
<th>R: 3′GGCGAAGTAGAAGTCATCTT5′</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>F: 5′CACCCACACTGTCATCACTCA3′</td>
<td>R: 3′CAGGGAAACCGCTATTCGAGA5′</td>
</tr>
<tr>
<td>MDM2</td>
<td>F: 5′CAGCCCAAGCAGCGCGAGA3′</td>
<td>R: 3′GGTTACAGCATCCAGTAGTCA5′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: 5′CACCCACACTGTCATCACTCA3′</td>
<td>R: 3′CAGCGGAACCGCTATTCGAGA5′</td>
</tr>
</tbody>
</table>
**Detection of MDM2 mRNA expression level.**

RNA was isolated from tumor tissue using “Total Nucleic Acid Isolation Kit” (Ambion, USA). Reverse transcription was conducted using PCR test set “High Capacity cDNA Reverse Transcription Kit” (Applied Biosystems, USA) in a total volume of 20 μl with 1 μl of total RNA. The reverse transcription reaction was run under the following conditions: 25 °C — 10 min; 37 °C — 10 min and 85 °C — 5 sec. GAPDH was used to normalize levels of mRNA for the relative quantification method of analysis. Sequences of primers (Applied Biosystems, USA) are shown in the Table 1. Primers and TaqMan probes were used at 0.3 μM and 0.2 μM concentrations, respectively. PCR reaction was performed in accordance with such as in the detection of MNA. Calculations were performed using the ΔΔCt relative quantification method. The thresholds and baselines were set manually in SDS and Ct values were extracted.

**Statistical analysis.** All experiments were repeated independently at least three times. Gaussian distribution of the group was checked with Kolmogorov — Smirnov tests. Analysis of the association between the studied markers and clinical characteristics of patients was performed using U-Mann — Whitney test. Prognostic significance of markers was verified with the ROC-curve (Receiver Operating Characteristic curve). Event free survival was evaluated by Kaplan — Meier estimator, statistical significance of parameters differences was determined using F-Cox criterion. The impact of gene expression in NB patients’ survival was evaluated using the Cox proportional hazards regression analysis [14]. The data were processed with the software package Statistica 6.1 using parametric and nonparametric methods of statistical analysis and MedCalc 12.1.4.0. The difference was considered statistically significant at p < 0.05.

**RESULTS AND DISCUSSION**

Mutations in the TP53 gene rarely are observed in childhood cancer of central and peripheral nervous system. In primary NB tumors frequency of its mutation does not exceed 1–2% and 15% in recurrent and/or progressive tumors. Loss of 17p chromosome region, mapping TP53 gene, is more usual for NB [4]. In our study no tumor sample with 17p chromosome region deletion was found in patients with NB. But we suggest that deficiencies in p53 functions may be a consequence of aberrant MDM2 expression showed further.

Patients were dichotomized by age into patients younger than 18 months and patients 18 months of age and older. Stage was dichotomized for the entire NB cohort with respect to metastatic progression (i.e., stage I, II, and III vs stage IV). MNA was found in 27.5% NB patients. These patients were classified as high risk group and treated with the relevant protocols. The average age of patients with MNA was 29.91 ± 3.59 months. Among all patients with MNA 36% of patients were with localized disease stages, 64% — IV stage. The frequency of MNA detected in our study (27.5%) did not differ from the data obtained by other research groups [2].

We have investigated whether MDM2 overexpression was associated with various clinical features (Table 2).

**Table 2. Clinical and biological characteristics and MDM2 mRNA expression in NB tumor samples**

<table>
<thead>
<tr>
<th>Nº</th>
<th>Clinical and biological features</th>
<th>MDM2 mRNA expression level, a.u.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Biological material</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Primary tumor</td>
<td>n = 80 (87.9%)</td>
</tr>
<tr>
<td></td>
<td>Recurrent tumor</td>
<td>n = 4 (4.4%)</td>
</tr>
<tr>
<td></td>
<td>Metastatic foci</td>
<td>n = 7 (7.7%)</td>
</tr>
<tr>
<td></td>
<td>Primary tumors:</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Age (n = 79)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Under 18 months</td>
<td>n = 29 (36.7%)</td>
</tr>
<tr>
<td></td>
<td>Over 18 months</td>
<td>n = 50 (63.3%)</td>
</tr>
<tr>
<td>3</td>
<td>Stage (n = 69)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>I–III</td>
<td>n = 32 (46.4%)</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>n = 37 (53.6%)</td>
</tr>
<tr>
<td>4</td>
<td>MYCN gene status (n = 78)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Non-amplified</td>
<td>n = 56 (71.8%)</td>
</tr>
<tr>
<td></td>
<td>Amplified</td>
<td>n = 22 (28.2%)</td>
</tr>
</tbody>
</table>

Note: a.u. — arbitrary units; “p < 0.001” — significant regarding primary tumors; “p < 0.001” — significant regarding I–III stages; “p < 0.03” — significant regarding MYCN non-amplified.

**MDM2 mRNA expression level** was significantly higher in recurrent tumors compared to primary tumors and metastatic sites (p < 0.001). Primary tumors were characterized by the lowest level of MDM2 expression. These data do not contradict the findings of other research groups [15], and can testify in favor of the theory that the inactivation of p53 by MDM2 in NB often develops during treatment and may be involved in the progression and metastasis of the disease and the occurrence of relapses.

In primary tumors the value of MDM2 mRNA expression varied quite widely, especially depending on the disease stage and MYCN gene status. MDM2 expression level was significantly higher in tumor samples obtained from patients with disseminated disease stage compared with early stages and in patients with MNA compared with MYCN-negative tumors. Thus, higher MDM2 expression was associated with unfavorable clinical NB features (MNA, disease stage), indicating a link between MDM2 overexpression and the high-risk phenotype.

We conducted ROC-analysis to assess the prognostic significance of MDM2 mRNA expression in NB progression. ROC-analysis is one of the most accurate statistical methods to assess the diagnostic and prognostic significance of the marker. For patients distribution on groups according to MDM2 mRNA expression level we applied event free survival rate as the criterion of clinical efficacy. As a result, optimal criterion for definition of high and low MDM2 mRNA expression level was established, it was > 0.0822 a.u. It was found that the area under the curve (AUC) was 0.75 (p = 0.001). According to the ROC-analysis (Fig. 2) high MDM2 expression was shown as a marker of unfavorable NB and risk of disease recurrence. This marker had sufficient sensitivity and specificity and may serve as an independent marker of prognosis and stratification of NB patients on risk groups.

To confirm obtained results we have analyzed the event free survival of NB patients depending on MDM2 mRNA expression level. With this aim all patients were divided into 2 groups according to MDM2 expression level (high and low expression) under ROC-analysis optimal criterion (Fig. 3).
As the result, overexpression of the MDM2 was found in 52.5% (42/80) of patients. We have found that the high level of MDM2 expression was associated with a significant decrease in event free survival of NB patients, regardless MYCN gene status and disease stage (p < 0.001). Accordingly, the rate of 3-year event free survival of patients with MDM2 mRNA high expression was only 12%, whereas patients with low expression — 66%. Such a significant difference indicates that this marker has substantial role in the pathogenesis and progression of NB and MDM2/p53 imbalance leads to poor prognosis in patients. As follows, along with other clinical features it can be used as prognostic and possibly therapeutic approach for treatment optimization.

In Cox proportional hazards model of the risk factors, high MDM2 expression was an independent factor for event free survival of NB patients (p = 0.002, CI 95% 1.06–1.30). Therefore, raised MDM2 expression level is associated with the risk of disease recurrence.

Since MYCN oncogene transactivates MDM2 [8, 11], in NB with MNA, increased expression/activity of MDM2 could significantly decrease the activity of p53, resulting in failure to undergo appropriate cell cycle arrest and/or apoptosis. We have analyzed changes of MDM2 expression and event free survival in tumors with different MYCN gene status.

In determining of optimal criterion for MDM2 mRNA expression level by ROC-analysis in MNA group no precise parameter for distribution on risk groups was established. With Cox regression analysis we have found that changes in MDM2 expression level in this group does not affect event free survival (p = 0.14, CI 95% 0.97–1.26). Changes of MDM2 mRNA expression in this group can be explained by MDM2/p53 interaction malfunction caused by different reasons. Also they may be caused by MNA activating effect on MDM2 and MDM2-mediated MYCN expression promotion. In any case, this leads to poor prognosis; disease progression that often occurs even in patients with MNA and low MDM2 gene expression, indicating the complexity of NB biology.

Particular attention of oncologists and scientists attracts MYCN non-amplified NB, as it has yet to be known factors that lead to rapid progression and aggressive course of the disease in some patients or spontaneous regression and favorable prognosis in others. For this NB group (n = 56) with ROC-analysis we have established the possibility of MDM2 mRNA expression using as prognostic marker and have identified the optimal criterion for distribution on favorable/unfavorable prognostic groups (Fig. 4).

Finally, we investigated the prognostic value of MDM2 expression in this group. Kaplan — Meier survival curves revealed that the clinical outcome (event free survival) was on 64% higher in low MDM2 expression group compared to high MDM2 expression group, p < 0.001 (Fig. 5). Higher than optimal criterion expression level was detected in 46.4% tumor samples. With Cox regression model we have found that changes in MDM2 mRNA expression level in MYCN non-amplified NB predetermines event free survival (p < 0.05, CI 95% 1.004–1.59).

![ROC curve for MDM2 mRNA expression ratio in primary NB tumors (p < 0.001)](image)

![Kaplan — Meier event free survival curves according to optimal criterion of MDM2 mRNA expression level in primary NB tumors (p < 0.001)](image)

![ROC curve for MDM2 mRNA expression ratio in MYCN non-amplified NB](image)

![Kaplan — Meier event free survival curves according to optimal criterion of MDM2 mRNA expression level in MYCN non-amplified NB (p < 0.001)](image)
These data suggest that MDM2 can be useful in prediction of NB prognosis and outcome. In MYCN non-amplified NB regulatory contribution of MYCN is offset. In this group, deterioration in disease course of the patient with high MDM2 mRNA expression is directly connected with dysregulation in MDM2/p53 interaction.

CONCLUSIONS

Our results have shown that MDM2 mRNA expression is an important factor in NB prognosis. For patients that do not show chromosomal rearrangements of TP53 gene locus, elevated expression of MDM2 mRNA can serve as an effective and useful prognostic marker under certain circumstances. Thus, when MDM2 is expressed at the high level in a group with MNA, this has a reinforcing effect on the progression of the disease. Elevated levels of MDM2 in NB without MNA might be used as the significant prognostic marker for both, patient stratification on risk groups and for treatment optimization.

REFERENCES