Changes in MYCN expression in human neuroblastoma cell lines following cisplatin treatment may not be related to MYCN copy numbers

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Abstract. Neuroblastoma is a tumor accounting for approximately 10% of all childhood malignancies and 50% of all childhood cancer-related deaths. MYCN gene copy number variation represents the most important prognostic factor in neuroblastoma. Prognostic significance of MYCN gene expression is more complicated and may depend on other factors such as MYCN gene copy number status. In the present study, we assessed MYCN gene expression using real-time RT-PCR following cisplatin treatment in three human neuroblastoma cell lines (UKF-NB-3, UKF-NB-4 and SK-N-AS) and their cisplatin-resistant counterparts. We also examined MYCN gene status and copy number (gain and amplification) variations using interphase and metaphase fluorescent in situ hybridization (FISH) and multiplex ligation-dependent probe amplification (MLPA). Only cisplatin-sensitive UKF-NB-4 cells exhibited decreased MYCN expression following treatment with cisplatin. Other sensitive neuroblastoma cells did not exhibit a change in MYCN expression. In contrast, cisplatin-resistant UKF-NB-4 and SK-N-AS cells exhibited increased MYCN expression irrespective of the number of MYCN copies or concentration of cisplatin in the medium. In MYCN-amplified neuroblastoma cells we did not observe any significant change in the number of MYCN copies after cisplatin treatment, whereas MYCN-non-amplified SK-N-AS cells revealed during cisplatin treatment an increased number of MYCN gene copies caused by 2p gain in the majority of cells by FISH. We postulated that cisplatin treatment does not result directly in altered transcription of MYCN. A functional change in MYCN mRNA levels and increased MYCN expression in cisplatin-resistant neuroblastoma cells do not have a clear relationship to MYCN copy numbers. These findings may further contribute to the understanding of cisplatin chemotherapy in connection with MYCN expression, and the possible copy number variations in MYCN neuroblastoma cells may be of importance since targeting of MYCN is being tested as neuroblastoma therapy.

Introduction

Neuroblastoma (NBL), a tumor of the sympathetic nervous system, is the most common solid extracranial childhood tumor, and is responsible for 50% of all childhood cancer-related deaths. V-myc myelocytomatosis viral-related oncogene, neuroblastoma-derived (MYCN) was the first amplified oncogene that was found to be of high clinical significance due to its association with the most aggressive form of NBL (1,2). MYCN belongs to the family of MYC genes (MYC, MYCN and MYCL) which encode transcription regulators that participate in the control of both cell growth and apoptosis depending on the cellular context (3). In healthy humans, MYCN is expressed only in certain embryonal tissues, and in adult cells its expression is very low or missing (4). The human MYCN gene is normally located on the short arm of chromosome 2 (2p24). The term 2p24 gain is suggested as a descriptive generic term for MYCN signal numbers exceeding up to 4-fold the number of reference signals on the chromosomal arm 2q. This pattern could reflect either a gain of short arms of chromosome 2 (2p gain) or a gain of the MYCN gene (MYCN gain) which is not an independent prognostic factor in NBL (5) and could indicate an ‘incipient’ MYCN amplification (6). MYCN amplification is defined as a >4-fold increase in the MYCN signal number compared to the reference probe, located on chromosome 2q (6). Amplified MYCN can take the form of either extra-chromosomal double minutes or as homogeneously staining chromosomal regions (HSCRs), which are usually located on chromosomes other than 2p (normal MYCN location) (7).
Three morphologically distinct cell types termed NBL (neuroblastic), S (substrate adherent) and I (intermediate) occur in NBL. The biological differences between S and N subtypes may be of clinical relevance (8); S-type cells are often prone to develop drug resistance (9). An effective therapy for children with high-risk neuroblastoma (HR NBL) remains one of the greatest challenges for pediatric oncologists. Over the past two decades, numerous attempts have been made to improve the outcomes of these patients by delivering intensive platinum cytostatics based on induction therapy (10). Unfortunately, intensive chemotherapy, which is usually used for treatment of HR NBL, can cause genetic and expression changes in NBL cells. These cellular changes can cause development of drug resistance to the drugs used, thus, ultimately rendering the chemotherapy ineffective. Drug resistance has been described as common in children suffering from NBL, particularly in those whose tumors display amplification and high-level expression of the MYCN oncogene (11). High-level MYCN expression is associated with unfavorable outcomes and a poor prognosis in tumors with amplified MYCN (12). However, high-level mRNA MYCN expression in NBL lacking MYCN amplification appears to be associated with favorable outcomes (13). High levels of MYCN mRNA in NBL with MYCN amplification may be explained by the fact that copies of the amplified gene are actively transcribed (14). Overexpression of the MYC gene family generally induces unrestricted cell proliferation, inhibition of differentiation, cell growth, angiogenesis, reduced cell adhesion, metastasis and genomic instability (15). MYCN protein has been observed to induce apoptosis in different cells, including NBL and cells with apoptosis induced by cytostatics. NBL cells with MYCN amplification (and increased gene expression) can resist treatment only when there is additional dysfunction in the apoptotic pathways, such as caspase deficiency (16). MYCN protein can also contribute to the acquired drug-resistance phenotype of cancer cells by modulating the regulation of a specific set of ATP-binding cassette transporter genes to mediate the efflux of chemotherapeutic agents from cancer cells (17).

The aim of our study was to examine MYCN gene expression in different NBL cell lines, including cisplatin (CDDP)-sensitive or -resistant N and S types, following a wide range (100-3,000 ng/ml) of different CDDP doses. The novel aspect of the study includes a comparison of MYCN gene expression and copies of MYCN gene in NBL cells, following long-term CDDP treatment. MYCN gene copy numbers and its corresponding expression attracted our particular attention since this gene has at the present become a frequent therapeutic target of NBL (18-20).

Materials and methods

Characteristics of the investigated cell lines. UKF-NB-3 and UKF-NB-4 cell lines with MYCN amplification were established from bone marrow metastases of two patients with HR NBL. The UKF-NB-4 cell line that was established from recurrent disease already possessed the intrinsic multidrug resistance phenotype including 7q21 gain (21). The cell lines were kindly provided by Professor J. Cinatl Jr (Institute for Medical Virology, Hospital of the Johann Wolfgang Goethe University, Frankfurt am Main, Germany). Cells were grown in Iscove's modified Dulbecco's medium (IMDM) with 10% fetal calf serum (PAA Laboratories, Pasching, Austria). The SK-N-AS cell line was derived from bone marrow metastasis of a female patient with HR NBL. This cell line, which has normal MYCN status, was purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and was cultivated according to the manufacturer's instructions.

The CDDP-resistant counterparts, designated as UKF-NB-3\textsuperscript{CDDP} and UKF-NB-4\textsuperscript{CDDP}, were also kindly provided by Professor J. Cinatl Jr. SK-N-AS\textsuperscript{CDDP} was prepared in our laboratory by incubation of parental SK-N-AS with increasing concentrations of CDDP either for 14 or 24 months. Solutions of CDDP (Ebewe Pharma Ges.m.b.H. Nfg. KG, Unterach, Austria) were prepared according to the manufacturer's instructions. CDDP-resistant cell lines were cultivated in a medium containing 1 µg/ml of CDDP.

Subsequent CDDP treatment. The above characterized cells were exposed to 100, 1,000, 2,000 and 3,000 ng/ml CDDP 24 h after seeding. After 24 h, cells were harvested by trypsinization, washed in sterile phosphate-buffered saline and frozen with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) at -80°C until RNA isolation.

MTT test. The sensitivity of cell lines to CDDP was determined by using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test (22). The IC\textsubscript{50} was assessed using an MTT dye reduction assay as described in a previous report (23). The IC\textsubscript{50} values were calculated from at least 3 independent experiments using linear regression of the dose-log response curves using SoftMax Pro microplate data software (Molecular Devices, Sunnyvale, CA, USA).

RNA isolation and real-time reverse transcriptase PCR. Total RNA was extracted from all cell lines using TRIzol reagent (Invitrogen). The quality of isolated RNA was verified by horizontal agarose gel electrophoresis, and RNA quantity was measured using a Biomate 3 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Isolated RNA (1 µg) was reverse transcribed to cDNA using random hexamers and a reverse transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Real-time RT-PCR was assessed using the 7500 Real-Time PCR System (Applied Biosystems) using MYCN and β-2-microglobulin (B2M) primers and probe kits from Generi Biotech (Hradec Kralove, Czech Republic) and TaqMan® Gene Expression Master Mix (Applied Biosystems). Results were expressed using the relative expression software tool (REST) (24) employing B2M as the reference gene. B2M was chosen for its stable expression at consistent levels in NBL, regardless of clinical stage, and have been used as reference genes in a number of studies of gene expression in NBLs or NBL cell lines (25-27).

Fluorescent in situ hybridization and karyotype. Interphase fluorescent in situ hybridization (FISH) analyses were performed using an ON MYCN (2p24)/LAF (Kreatech Biotechnology B.V., Amsterdam, The Netherlands) and by using Vysis LSI N-MYC (2p24) SpectrumOrange Probe (Vysis, Downers Grove, IL, USA) simultaneously with Alpha
Satellite 2 Green Probe (Cytocell Technologies, Cambridge, UK), in accordance with the manufacturer's instructions. The use of a 2p-specific probe simultaneously with 2q signals is recommended by the International Neuroblastoma Risk Group Biology Committee (6). Slides were scored with an Olympus AX70 (Olympus Optical Co., Ltd., Japan) fluorescence microscope with a CCD camera ( Jenoptik, Jena, Germany). Images of the interphase nuclei were digitally captured using an appropriate software program (MetaSystems, Altlussheim, Germany). Two hundred nuclei were investigated from each cell line as recommended for evaluation by FISH analyses (28).

For karyotype analysis, exponentially growing cells cultivated in medium without CDDP were harvested with trypsin, treated with hypotonic solution, and fixed with 3:1 methanol/acetic acid. Slides were banded using standard trypsin-Giemsa banding (GTG-banding) or were used for metaphase FISH analysis performed by Vysis LSI N-MYC (2p24) SpectrumOrange Probe simultaneously with Alpha Satellite 2 Green Probe in accordance with the manufacturer’s instructions.

**Multiplex ligation-dependent probe amplification.** Multiplex ligation-dependent probe amplification (MLPA) analysis was performed to identify whether the region gained or amplified included adjacent genes in addition to the MYCN gene. We used the neuroblastoma-specific SALSA MLPA P252B Probe Kit (MRC-Holland, Amsterdam, The Netherlands) which analyzes chromosomes 2 and 17. Detailed information on probe sequences, gene loci and chromosome locations can be found at www.mlpa.com. Genomic DNA (50-200 ng) was denatured and the probes were allowed to hybridize (16 h at 60°C). PCR was performed on the samples in a volume of 50 µl containing 10 µl of the ligation reaction mixture and using a thermal cycler Mastercycler ep gradient (Eppendorf, Hamburg, Germany). Aliquots of 1 µl of the PCR reaction were combined with 0.5 µl ROX-labeled internal size standard (Applied Biosystems) and 12 µl deionized formamide. Fragments were separated by electrophoresis on an Applied 3130xl capillary sequencer and quantified using the intermediate version Coffalyser (MRC Holland). For copy-number detection, four normal control DNA samples were included in each set of MLPA experiments. Interpretation was based on the comparison of peak heights between the control DNA and the tumor sample.

**Statistical analysis.** All numerical data are presented as means ± standard deviation and analyzed statistically using the Student's t-test; mRNA expression results were analyzed using REST software (24).

**Results**

The sensitivity of cell lines to CDDP. The sensitivity of the cell lines to CDDP was verified using the MTT test. The resistant cell lines showed at least a 2-fold increase in resistance to CDDP measured as IC₅₀. The data presented in Table I indicated the UKF-NB-3 cell line as the most sensitive and SK-N-AS CDDP and UKF-NB-4 CDDP as the most resistant towards CDDP.

### Table I. Levels of the drug-resistance of the tested NBL cell lines expressed as IC₅₀.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>NBL type</th>
<th>IC₅₀ (µM) CDDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-N-AS</td>
<td>S</td>
<td>3.71±0.67</td>
</tr>
<tr>
<td>SK-N-ASC CDDP</td>
<td>S</td>
<td>11.68±2.84</td>
</tr>
<tr>
<td>UKF-NB-3</td>
<td>N</td>
<td>0.99±0.01</td>
</tr>
<tr>
<td>UKF-NB-3 CDDP</td>
<td>N</td>
<td>7.68±1.37</td>
</tr>
<tr>
<td>UKF-NB-4</td>
<td>S</td>
<td>4.60±0.2</td>
</tr>
<tr>
<td>UKF-NB-4 CDDP</td>
<td>S</td>
<td>11.33±1.12</td>
</tr>
</tbody>
</table>

IC₅₀ values were calculated from the linear regression of the dose-log response curves after a 96-h exposure to CDDP, as determined by the MTT assay. Values are the means ± SD of at least 3 independent experiments. S, S-type of NBL; N, N-type of NBL.

**MYCN expression levels in CDDP-sensitive and -resistant NBL cell lines.** Both MYCN-amplified UKF-NB-3 and UKF-NB-4 cell lines had significantly higher MYCN mRNA expression as compared to the MYCN non-amplified line SK-N-AS (14.8, respectively 13.4-fold higher in 2-log scale, P<0.001) (Fig. 1). SK-N-AS CDDP had 3.8-fold higher expression of MYCN (P<0.01) than SK-N-AS. We did not find any distinguishable change in MYCN expression between CDDP-sensitive UKF-NB-3 and its CDDP-resistant counterpart (Fig. 1), while UKF-NB-4 CDDP had a 1.6-fold higher expression of MYCN (P<0.05) in comparison to that in the sensitive UKF-NB-4 cell line. There were no significant differences in MYCN expression between UKF-NB-3 and UKF-NB-4 cells nor between UKF-NB-3 CDDP and UKF-NB-4 CDDP cell lines. Regarding the CDDP-resistant cell lines, the highest MYCN expression in comparison with SK-N-AS CDDP was found in the UKF-NB-4 CDDP cell line (11.3-fold higher; P<0.01), followed by UKF-NB-3 CDDP (10.8-fold higher; P<0.01).

**MYCN expression levels in CDDP-sensitive and -resistant NBL cells following short-term CDDP treatment.** CDDP-
sensitive UKF-NB-4 cells showed a significant decrease in MYCN expression after cultivation with CDDP concentrations of 1000 ng/ml and higher (P=0.001) (Fig. 2A). CDDP-resistant UKF-NB-4 cells showed significant upregulation of MYCN expression (P=0.001) in comparison with the sensitive cells, however, a single CDDP dose did not significantly modulate MYCN expression levels in the resistant cell lines (Fig. 2). These expression changes were not associated with senescence, as determined by staining of investigated cell cultures for the senescence-associated β-galactosidase marker (data not shown).

In the sensitive SK-N-AS cells, we did not observe any significant changes in MYCN expression after cultivation with any CDDP dose (P>0.3). The SK-N-AS<sup>CDDP</sup> cell line showed a significant increase in MYCN expression relative to the control maternal line independent of the presence or absence of CDDP in the medium (P<0.05) (Fig. 2B).

**MYCN status in CDDP-treated NBL cells.** Using FISH, we observed MYCN amplification in all 200 investigated nuclei of UKF-NB-3 and UKF-NB-4 cells and their CDDP-resistant counterparts (Fig. 3A and B). UKF-NB-3 and UKF-NB-4 amplified MYCN in the form of HSRs. Regarding the UKF-NB-3 cells, multiplex ligation-dependent probe amplification (MLPA) revealed disomy of chromosome 2 without any other genetic aberration in 2p except for MYCN amplification. In UKF-NB-3<sup>CDDP</sup> cells we found a loss of the 2p11.2-2p25.3 region except for 2p23.2 (ALK) and MYCN-amplified copies. Both CDDP-sensitive and -resistant UKF-NB-4 cells exhibited an amplified 2p24.3 region (NAG, DDX1, and MYCN genes), but without any other significant change in 2p.

Using interphase FISH analysis with two different sets of probes, the MYCN-non-amplified SK-N-AS cell line showed...
normal MYCN status in the majority of cells (Fig. 3C) with a sporadically (3.5%) occurring clone having an extra 2p (Fig. 4A). After short-term (14 months) and long-term (24 months) cultivation with CDDP, SK-N-AS<sup>CDDP</sup> cells were examined again for the MYCN gene status and for the number of chromosome 2. The CDDP cultivation resulted in a substantial clonal expansion of cells with 2p gain, since the SK-N-AS<sup>CDDP</sup> cells had 60 and 77% of cells with three 2p (Fig. 3D) after short-term and long-term cultivation, respectively (Fig. 4A). In contrast, using MLPA we did not find any significant 2p change in SK-N-AS<sup>CDDP</sup> cells, but MLPA analyses whole genomic material regardless of heterogeneity in the cell population as in FISH.

Metaphase FISH analyses of SK-N-AS cells also revealed a predominant occurrence of cells with two chromosomes 2 in mitotic cell and a subsequent increase in cells with 2p gain after CDDP treatment was noted (Fig. 3E). We also observed cells with tetrasomy or monosomy, but their proportion was rather minor (Fig. 4B).

**Discussion**

In the present study, 6 NBL cell lines (4 with MYCN amplification and 2 cell lines without MYCN amplification) were analyzed. The cell line SK-N-AS<sup>CDDP</sup> (without MYCN amplification), which may serve as a model for the development of drug-resistant NBL, was established and characterized in our laboratory. In the context of CDDP-based NBL therapy, we investigated mRNA MYCN expression and MYCN gene status. In the SK-N-AS and SK-N-AS<sup>CDDP</sup> cells we found a clear link between the number of MYCN gene copies and their expression. This finding was in agreement with the study of Valent et al (29) who found higher MYCN expression in three of four NBLs with 2p/MYCN gain in comparison with the SK-N-SH cell line that has 1 MYCN copy in the haploid genome. The cells with high-level MYCN amplification (UKF-NB-3, UKF-NB-4 and their CDDP-resistant counterparts) exhibit several-fold higher mRNA MYCN expression than those without MYCN amplification. In the case of NBL cell lines with normal number of MYCN copies and in NBL cell lines with one extra MYCN copy, the difference in MYCN expression was significant. The biological and clinical characteristics of 2p/MYCN gain have not been to date as clearly defined as MYCN amplification (30). Approximately 10% of diagnosed NBLs could contain an MYCN gain (30,31). Patients with MYCN gain demonstrated a significantly higher age at diagnosis and according to several studies are associated with an advanced stage as compared to MYCN-normal cases (30,31). In contrast to the study of Spitz et al (31) who reported that NBLs with MYCN gain displayed no increase in MYCN mRNA expression, and in agreement with Valent et al (29) who found in the majority of NBLs cases a 2p/MYCN gain increased expression, we found a tendency of MYCN gain and higher MYCN expression in the SK-N-AS<sup>CDDP</sup> cells.

Numeric changes of 2p in SK-N-AS cells may suggest the selection advantage of cells with 2p gain in the presence of CDDP. The simultaneous coexistence of both cell types, cells with disomy of chromosome 2 and cells with 2p gain, is documented in Fig. 4B. The subsequent clonal expansion of cells with 2p gain in the presence of CDDP may be a realistic interpretation of this phenomenon as described by Cohen et al (32) in the case of the SK-N-SH cell line. Apparently, senescence has no effect on 2p gain or increased MYCN expression in SK-N-AS<sup>CDDP</sup> cells. Notably, observations based on metaphase FISH analyses of SK-N-AS<sup>CDDP</sup> cells (revealing cells with 2p gain, sporadic tetrasomy or monosomy) have not been confirmed by MLPA analysis, which did not confirm any significant 2p change in both SK-N-AS or SK-N-AS<sup>CDDP</sup> cell lines. This discrepancy may be due to the nature of the two techniques: FISH may be more subjective due to the visual evaluation, while capable of taking into account the heterogeneity in the cell population, whereas MLPA, an accurate and sensitive technique, analyzes whole genomic material irrespective of the situation in particular cells (33). Our data suggest the need to analyze amplification of the genetic material by two independent methods in order to avoid false interpretations.
In addition to clinical (clinical stage, age), biochemical (LDH levels) and genetic (MYCN amplification, 1p36 deletion) prognostic parameters, the expression of several genes have also been reported to predict the outcome of NBL. Overexpressed genes encoding tyrosine kinase receptors (TrkA and EPHB6) and cell surface molecules (CD44, EFNB2, and EFNB3) have been described as favorable markers (13), but the significance of MYCN expression remains controversial since the survival of children suffering from NBL does not correlate with its expression (13,34,35). A high expression of MYCN in NBL cells may be associated with both a favorable (cells without MYCN amplification) and adverse prognosis (cells with amplified MYCN) (12). Similarly, MYC overexpression without amplification has also been linked to a more favorable prognosis in patients with breast (36) and colorectal cancers (37). Better outcomes of patients with tumors that overexpress MYC or MYCN are due to either increased apoptosis or enhanced drug-sensitivity evoked by higher proliferation (35). Clinical observation has also been confirmed in in vitro experiments. Transfection of cells with a vector containing MYCN significantly decreased the viability of NBL cells without MYCN amplification. MYCN protein induced apoptosis and enhanced expression of genes that were prognostically favorable (13). In vitro studies using an NBL cell line with a controlled MYCN expression system showed that NBL cells with induced MYCN expression have higher cytostatically induced apoptosis than NBL cells without induction of MYCN expression. Cytostatic drugs with various mechanisms of action including CDDP have been tested. All tested compounds were more effective in cells overexpressing MYCN, except for betulinic acid, which induces apoptosis by direct effects on mitochondria (38). The above-mentioned results reporting equal or increased MYCN expression in CDDP-resistant cell seem to differ from our findings. An explanation may be that the induction of multiple changes in the genome of resistant cells indicates that cytostatics induce drug-resistance through multiple mechanisms (21,39). It is possible that NBL cells without amplification of the MYCN used in the above mentioned study (38) have normally functioning apoptotic pathways, but in relation to drug-resistance, there is a defect in one of these pathways, possibly due to increased MYCN expression. Van Noesel et al (40) analyzed a panel of NBL cell lines with MYCN amplification and showed that MYCN induces both caspase-8- and caspase-9-mediated apoptosis. The authors concluded that an epigenetic downregulation of caspase-8 in NBLs is unlikely to be induced by overexpression of MYCN. Additional defects in apoptosis may be involved in the caspase-9 route to apoptosis. It appears that defects in apoptotic routes in NBL tumors do have a neutralizing effect on MYCN (40).

As an in vitro model of drug-resistance (22), we chose CDDP-resistant sublines prepared by incubation of parental NBL cells with increasing concentrations of CDDP. We found that the NBL cell line, with predominantly normal status of the MYCN gene, had increased numbers of cells with 2p gain and increased MYCN expression after CDDP treatment. Wasenius et al (41) described both deletion and gain of 2p in CDDP-resistant ovarian cancer cell lines but our finding of 2p gain in NBL in connection with drug-resistance was described for the first time here. We also studied MYCN expression in CDDP-sensitive and -resistant NBL cells after exposure to CDDP as an in vitro model of chemotherapy. MYCN amplified cell lines, cultivated with CDDP, did not change MYCN expression. Therefore, cisplatin resistance does not result in a functional change to MYCN mRNA levels. Our findings contribute to the understanding of the influence of chemotherapy on MYCN expression in NBL cell lines. This is of particular importance since targeting the MYCN gene may be a therapeutic alternative for NBL (42).

Acknowledgements

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