The Coamplification Pattern of the MYCN Amplicon Is an Invariable Attribute of Most MYCN-Amplified Human Neuroblastomas

Axel Weber, Sven Starke, Eckhard Bergmann, and Holger Christiansen

Abstract

Purpose: Fifteen percent to 20% of human neuroblastomas show amplification of the MYCN oncogene physiologically located at chromosome 2p24-25, indicating an aggressive subtype of human neuroblastoma with a poor clinical outcome. Recent findings revealed that the structure of the amplicon differs interindividually and that coamplification of genes in telomeric proximity to MYCN might play a relevant role in neuroblastoma development and response to treatment, respectively. We now asked if the amplicon structure is an invariable attribute of an individual tumor or if the coamplification pattern could change during progress or in case of recurrent disease.

Experimental Design: We used a previously described multiplex PCR approach to analyze the coamplification status of MYCN-amplified human neuroblastomas (n = 33) in tumor tissue at the time of initial diagnosis and in consecutive tissue specimens at later time points after initial treatment or from relapsing disease. The MYCN copy number per haploid genome (Mcn/hg) in these specimens was determined in a separate duplex PCR.

Results: In 32 of the 33 investigated tumors, the amplicon structure showed no changes after initial chemotherapy and in recurrent disease. Mcn/hg showed a decrease after initial treatment (n = 23), whereas we found a significant increase in recurrent disease (n = 10).

Conclusion: Our data indicate that the initial determined structure of the 2p24-25 amplicon is a consistent attribute in the great majority of the individual MYCN-amplified neuroblastomas and shows no plasticity during or after chemotherapy. Observed changes in the Mcn/hg over the course of disease are in line with preexisting cell culture findings.

Amplification of the MYCN oncogene is the most extensively studied biological marker for an unfavorable prognostic outcome in patients with neuroblastoma (1–3). The overall survival probability for patients with MYCN amplification is <30% in the first 6 years after initial diagnosis (own data of 216 patients with MYCN-amplified neuroblastoma). Within this population, patients that show no metastatic disease at initial diagnosis (stages I,II, and III) respond better to current therapy strategies, including high-dose chemotherapy, autologous stem cell transplantation, and treatment with 13-cis-retinoic acid compared with patients with initial stage IV disease. Examining primary tumor samples of a large cohort of patients for coamplification pattern of different genes telomeric and centromeric to MYCN, we recently showed that the coamplification of the DDX1 gene, in proximal telomeric vicinity to MYCN, identifies a subgroup of patients with an improved survival probability compared with patients without this coamplification, independently of stage and age (4).

However, not all of the long-time-surviving patients harbored DDX1 coamplification and not every patient that underwent rapid progression of disease lacked DDX1 coamplification. Other investigators found only a trend toward a better prognosis (5) or even described a trend toward a worse prognosis for patients with DDX1-coamplified tumors (6–9). Because of the relative homogeneity regarding prognosis of the patients in these studies and the small numbers of investigated patients, statistical significance was not obtained. We hypothesized that DDX1 might influence the response of neuroblastoma cells to chemotherapy under certain conditions but does not serve as a warrant for survival per se.

In cell culture studies on methotrexate-treated Chinese hamster and mouse cells, Hahn et al. (10, 11) showed that an elevation in the amplification number of the amplified dihydrofolate reductase gene occurs under increasing selective pressure. Furthermore, the authors described a trend to a reduction in the amplicon size under these strong selective conditions. In general, the size of the amplified DNA fragments in those cell models is described to be a stable attribute independent of the manner of amplification as double-minute chromosomes or homogenously staining regions (11, 12).

These findings and our previously determined data of coamplification pattern in primary neuroblastoma prompted us to ask if the structure and the copy number of the chromosome 2p24-25 amplicon, once determined at initial neuroblastoma development, are invariable attributes for each
individual MYCN-amplified neuroblastoma. To answer that question, we analyzed the coamplification pattern of subsequent tissue specimens taken at initial diagnosis and at different time intervals after treatment initiation of 33 patients with MYCN-amplified neuroblastoma using the two multiplex PCRs previously described (4). Furthermore, we determined the number of amplified copies of MYCN in relation to inhibin-β-b in a separate, semiquantitative duplex PCR (Supplementary Fig. S1).

Patients and Methods

Patients. We studied primary tumor specimens from 33 children with MYCN-amplified neuroblastomas diagnosed in Germany from 1986 to 2003. The 33 neuroblastomas were selected out of a total number of 174 tumor specimens with MYCN amplification within this period of time. The selection criterion was the availability of tumor tissue of comparable quality to different time points during therapy of each individual patient for DNA isolation.

All neuroblastoma diagnoses were confirmed by histologic assessment of a tumor specimen obtained at surgery. The tumors were classified according to the International Neuroblastoma Staging System criteria (13). All patients were treated according to previously described protocols with confirmed consent for therapy and study procedures (14, 15). Therapy included surgery, polychemotherapy, and, dependent on the randomization procedure, high-dose chemotherapy with autologous stem cell transplantation, anti-GD2 antibody, and retinoic acid treatment.

Our study group consisted of 1 stage I, 0 stage II, 10 stage III, 17 stage IV, and 5 stage IV-S tumors. The median patient age at diagnosis was 30.8 months (range 0.6-163.3 months). The median follow-up time for all 33 patients was 25.2 months (range 7.5-150.1 months). The median follow-up time of the patients that died of the disease was 18.4 months (n = 21) compared with 82.6 months for patients alive (n = 12).

The mean time interval between first and second biopsy of all investigated patients (n = 33) was 4.6 months (range 1.0-76.9 months). The mean time between first and third biopsy (n = 3) was 22.5 months. Most of the second biopsies were taken from the site of the initial tumor during operation after initial chemotherapy treatment. The mean time between first and second biopsy of these patients (n = 23) was 4.1 months (range 1.0-20.3 months). For 10 patients, recurrent disease was diagnosed based on the clinical criteria that before relapse, a full relapse of tumor was diagnosed that again was defined as stage I. To the best of our knowledge, this was the first time that a recurrent disease was diagnosed after initial treatment (Fig. 2).

Table 1. Some 2p24-25 and the Mcn/hg for each investigated tumor are given in Table 1.

The coamplification pattern of the investigated genes on chromosome 2p24-25 and the Mcn/hg for each investigated tumor are given in Table 1.

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Statistics. Fisher’s exact test was used to compare the amplification status for each gene determined at initial diagnosis and at later time points. The paired t test was used to compare the Mcn/hg determined at initial diagnosis and at later time points. Statistical analyses were done with the SPSS version 10.0 software (SPSS, Inc., Chicago, IL). P values of ≤0.05 were regarded as significant.

Results

The coamplification pattern within the MYCN amplicon is an invariable attribute of most neuroblastoma tumors. In the present cohort besides MYCN amplification, 15 (46%) of the investigated tumors showed coamplification for DDX1, 11 (33%) for NAG, 5 (15%) for NSE1, 2 (6%) for LPIN1, 31 (94%) for EST-AA581763, 2 (6%) for SMC6, and 1 (3%) for SDC1 (Fig. 1; Table 1).

The coamplification of genes located telomeric to MYCN (DDX1, NAG, and NSE1) was less frequent compared with our previously published data that were determined on a larger cohort (n = 98 patients; ref. 4). However, the differences were not statistically significant and could be explained by the smaller patient numbers in the present study and the relatively high number of patients with recurrent disease selected for investigation within this study.

In the cohort of 33 patients investigated in this study, all patients except one did not show any change in the coamplification pattern during the course of disease, which means that the amplicon structure was invariable at the different time points of subsequent biopsies.

Patient 18 showed a loss of primarily coamplified genes (NSE1, NAG, and DDX1) within the amplicon at the second biopsy date, 76.9 months after the initial diagnosis. This patient was initially diagnosed as a stage I tumor at the age of 6.6 months. At the age of 6.4 years (83.5 months), a recurrent tumor was diagnosed that again was defined as stage I. To the date of our investigation, the patient was still alive (114 months after initial diagnosis; Fig. 2).

In three patients, three consecutive tumor biopsies were investigated. In DNA isolated from third biopsy tissue (n = 3), no change in the amplicon structure was found compared with the first or second biopsy in all three patients. Coamplification of DDX1 was found in only one of these three specimens. No tumor showed amplification of genes located further telomeric to MYCN (Table 1).

The number of amplified MYCN copies decreases after initial chemotherapy and increases in recurrent disease. Unlike the...
coamplification pattern, a significant change in amplification number was observed in the investigated cohort (Fig. 3).

Thus, we found the Mcn/hg to be decreased after initial chemotherapy (n = 23; Fig. 3A). The median Mcn/hg at initial diagnosis was 25 Mcn/hg compared with 20 Mcn/hg after initial chemotherapy (25-75% percentile: 25-40 Mcn/hg compared with 20-30 Mcn/hg, respectively).

In contrast, we found an increase of Mcn/hg in tumor samples of recurrent disease compared with their primary counterparts (n = 10; Fig. 3B). The median MYCN copy number at primary diagnosis was 27 Mnc/hg compared with 46 Mnc/hg in recurrent disease (25-75% percentile: 10-36 Mnc/hg compared with 30-64 Mnc/hg, respectively). Both alterations are statistically significant (P < 0.05, paired t test).

Discussion

Not much is known about the initiation and genesis of the MYCN amplicon in neuroblastoma development. Cheng et al. (16) revealed that, preferentially, the paternal allele undergoes the amplification process. Referring to patient age, recent data indicate that amplification of MYCN occurs to a defined point of time in neuroblastoma development as 65% of patients with MYCN-amplified neuroblastomas are between 12 and 40 months of age (17). Our data from about 247 patients are in consent with this age distribution, as, in our cohort, 55% MYCN-amplified tumors are initially diagnosed at an age between 12 and 40 months.

The ampiclon size varies between 350 and >1,000 kb in length, including different coamplified genes telomeric and centromeric of MYCN (4, 18). The ampiclon number varies from 2 to 256, and, in some cases, more copies per haploid genome in neuroblastoma cells at that time of diagnosis. In the great majority of MYCN-amplified neuroblastomas, the amplified genomic regions are initially present in the form of extrachromosomal double minutes rather than in homogeneously staining regions, which can be found mainly in recurrent disease (19, 20).

Table 1. Clinical data and coamplification pattern of the 33 neuroblastoma patients investigated

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NOTE: Only patient 18 showed a change in amplification pattern. The amplification pattern for all other patients is given only once and showed no changes in subsequent specimens.

Abbreviations: Tu, primary tumor; Rec, recurrent tumor; % Viab. t.c., % viable tumor cells; NA, not available; Ampl., amplified gene; s.c., single copy per haploid genome.

*Age at different time points in months.

†Stage of disease at diagnosis (according to International Neuroblastoma Staging System criteria).

‡Tissue for coamplification investigation from primary or recurrent tumor.
Extensive DNA rearrangements can be found within the amplified region, leading in part to the phenomenon that coamplified genes might be presented not equally in copy number compared with MYCN in either homogenously staining regions or double-minute chromosomes (21, 22).

Yoshimoto et al. (20) described the possibility of coexisting double-minute chromosomes and homogenously staining regions in single tumor cells besides cells with either double-minute chromosomes or homogenously staining regions in one neuroblastoma tumor. They claim a possible transition from double-minute chromosomes to homogenously staining regions during tumor development based on the finding that cells that reintegrate amplified oncogenes into the genome show a growth advantage over cells harboring the amplified gene copies in extrachromosomal double-minute chromosomes (23).

A change or evolution of the amplicon structure has not yet been reported for neuroblastoma. Our findings show that a change of the amplicon structure is uncommon during the course of disease but is possible in neuroblastoma recurrent disease. In the great majority of cases, we found the amplicon structure to be an invariable attribute of the individual tumor that does not undergo rearrangement during therapy. The observed change in the coamplification pattern in one patient could be explained by subsequent selection of more therapy-resistant neuroblastoma cell clones harboring the MYCN amplicon of smaller extent (Fig. 2B). As a prerequisite for this explanation, a heterogeneous ancestor cell population, which contains cells harboring MYCN amplicons of different sizes at determination of the individual neuroblastoma, has to be presumed. Alternatively, a successive change of the amplicon structure, caused coincidentally in highly proliferating cells or by DNA interfering agents and favoring cells with amplicons of smaller size over a given period of time, could be a possible mechanism. The latter explanation is more consistent with observations in common cell culture models (reviewed in ref. 23).

We previously showed coamplification and overexpression of DDX1 to occur preferentially in long-time-surviving patients and discussed a possible influence on the response to chemotherapeutics (4). Hypothetically, cells that lose this coamplification telomeric to MYCN should become more resistant to chemotherapeutic treatment. Our finding in the neuroblastoma of patient 18 that lost coamplification of NSE1, NAG, and DDX1 in the relapsing tumor is consistent with this hypothesis.

Interestingly, for amplified copies of the c-myc and MYCN genes occurring in double-minute chromosomes, a reduction in copy number after treatment with hydroxyurea can be observed, but not for copies in homogenously staining regions. After hydroxyurea treatment, the amplicon number can recur to the initial count (24). These findings show a possibility to influence gene amplification by medical treatment but also

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indicate that these exogenously induced genomic changes are not irreversible. In 1987, Brodeur et al. (25) investigated consecutive tumor samples of 12 patients with MYCN-amplified neuroblastomas and describe no change in the amplicon number for their investigated cohort. However, considering the given data of each individual patient, the findings of Brodeur et al. are strongly consistent with our findings. In their study, the Mcn/hg was found to be increased in tumor tissue of four of the seven patients with recurrent disease when compared with the primary tumor tissue. In consecutive specimens of patients during treatment, the Mcn/hg decreased in three of four reported patients.

These findings and our own observation of a reduction of the Mcn/hg after initial chemotherapeutic treatment indicate that the reported observations in cell culture regarding elimination of extrachromosomal amplified MYCN in response to treatment is also a possible mechanism of adaptation in primary MYCN-amplified neuroblastomas. In a cellular context, this regulation is reasonable as cells that eliminate extrachromosomal copies of MYCN might decelerate MYCN-induced proliferation. This is an observed mechanism to avoid massive apoptosis triggered by chemotherapy and enforced by myc oncogenes (26). As we lack the information if MYCN amplification is present in double-minute chromosomes or homogenously staining regions in neuroblastomas of our investigated patients, a correlation of the molecular phenotype of amplification to a decline of the amplification number is not possible. On the other hand, we observed a significant increase in the Mcn/hg in recurrent disease compared with the initially diagnosed primary neuroblastoma. These cells might represent highly proliferating clones that could bypass myc-enforced apoptosis and, thus, have benefit from harboring a higher Mcn/hg. We have to annotate that the observed changes of the Mcn/hg could also result from a different content of viable tumor cells within the subsequent tissue specimens, based on the used PCR method. However, the tumor cell content of the investigated specimens in this study is interindividually and intra-individually comparable (Table 1).

![Figure 1](image1.png)

**Fig. 1.** Frequency of coamplification of the investigated genes on chromosome 2p24-25 in primary tumor tissue at the time of initial diagnosis (white columns, n = 33) and at the time of the second biopsy (gray columns, primary tumor site (p.t.; n = 23) and black columns, recurrent disease (rec.; n = 10)).

![Figure 2](image2.png)

**Fig. 2.** A, different coamplification pattern of patient 18 as indicated by the two described multiplex PCR (bp, molecular weight marker; R, healthy human kidney DNA was used as single copy reference). B, different amplicon structures of patient 18 derived from the two multiplex PCR. Distance (kbp) of the seven investigated genes in relation to MYCN (BLAT assignment information).

![Figure 3](image3.png)

**Fig. 3.** Copy number of MYCN per haploid genome at the time of initial diagnosis (1st) and time of second biopsy (2nd; box-whisker plot). A, patients (n = 23) with second biopsy at the primary tumor site after initial chemotherapy (P = 0.017, paired t test). B, patients (n = 10) with recurrent disease (P = 0.02, paired t test).
The observed changes in the amplicon copy number over time might reflect abided regulatory mechanisms in neuroblasto-
toma cells comparable with cell culture models of acquired gene amplification. In contrast to an acquired gene amplifica-
tion under selective pressure, a reason for an initialization of the MYCN amplification in developing neuroblastic cells, with
the possibility to lead to MYCN-amplified neuroblastoma, remains unclear. Once initiated, the MYCN amplification is
found to be irreversible in neuroblastoma tumors, and, once determined at time of initial amplification, the structure of the
MYCN amplicon is invariable during the course of disease, including chemotherapy with DNA-damaging agents, in the
majority of neuroblastoma tumors.

A fundamental question arises from our findings. Is there a cellular program superior to MYCN amplification that advises a
cell, deriving from the neural crest, to amplify MYCN in a
certain developmental condition, which then could lead to
MYCN-amplified neuroblastoma if this genomic condition becomes an irreversible attribute?

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The Coamplification Pattern of the MYCN Amplicon Is an Invariable Attribute of Most MYCN-Amplified Human Neuroblastomas

Axel Weber, Sven Starke, Eckhard Bergmann, et al.


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