Heterogeneity of the MYCN Oncogene in Neuroblastoma

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Abstract

Purpose: MYCN amplification is an important therapy-stratifying marker in neuroblastoma. Fluorescence in situ hybridization with signal detection on the single-cell level allows a critical judgement of MYCN intratumoral heterogeneity.

Experimental Design: The MYCN status was investigated by fluorescence in situ hybridization at diagnosis and relapse. Heterogeneity was defined as the simultaneous presence of amplified cells (≥5 cells per slide) and nonamplified cells within one tumor or sequential change of the amplification status during the course of the disease. Likewise, heterogeneity can be detected between primary tumor and metastasis.

Results: From 1,341 patients analyzed, 1,071 showed no amplification, 250 showed homogeneous amplification, and 20 patients showed MYCN heterogeneity. Of the patients with heterogeneity, 12 of 20 had clusters of MYCN amplifications, 3 of 20 had amplified single cells, 3 of 20 showed MYCN amplifications in the bone marrow but not in the primary tumor, and 2 of 20 acquired MYCN amplification during the course of the disease. All stage 4 patients were treated according to high-risk protocols; 7 of 8 later progressed. Four patients with localized disease were treated according to high-risk protocol because of MYCN-amplified clusters; 1 of 4 later progressed. One patient treated with mild chemotherapy experienced progression. Seven patients with localized/4S disease underwent no chemotherapy: 4 of 5 patients with MYCN heterogeneity at diagnosis remained disease-free, and 1 of 5 experienced local progression. Two patients had normal MYCN status at diagnosis but acquired MYCN amplification during the course of the disease.

Conclusion: MYCN heterogeneity is rare. Our results suggest that small amounts of MYCN-amplified cells are not correlated to adverse outcomes. More patients with heterogeneity are warranted to clarify the role of MYCN heterogeneity for risk classification.

Since its first description in 1983 (1), the outstanding role of the MYCN oncogene particularly as a prognostic factor in neuroblastoma is undisputed. Amplification of the gene discriminates between favorable and unfavorable outcome in neuroblastoma, even in high-risk stage 4 patients (2–5). Gene expression analyses recently identified more than 200 genes (6). Although Southern blotting has been the standard for determining the MYCN status, reliable identification of low-level amplification by comparison of band intensities is sometimes difficult. Fluorescence in situ hybridization (FISH) has a higher sensitivity because it detects the MYCN copy number on the single-cell level and allows correlation of morphologic details. Neuroblastomas are considered to be stable with respect to MYCN amplification (7). Intratumoral heterogeneity of MYCN in neuroblastomas has rarely been described thus far (7–11).

The aim of our study was the evaluation of biological characteristics and the prognostic effect of the heterogeneity of MYCN amplification in comparison with homogeneous amplified and nonamplified tumors done on the large German neuroblastoma cohort. We add evidence that heterogeneity of MYCN amplification may exist in localized and disseminated neuroblastoma, report on frequency of MYCN heterogeneity, and discuss the influence on therapeutic decisions on microscopic foci harboring this aberration.

Materials and Methods

Patients and therapy. We determined the MYCN status in 1,731 specimen of 1,341 neuroblastoma patients diagnosed between December 1989 and December 2007 and registered in the German cooperative

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MYCN amplification were checked for tumor cell content and cytotoxic treatment, quality of the FISH slide (cross-hybridization or insufficient hybridization), and exclusion of tumor cell contamination (floating cells, contaminated tools); furthermore, each slide was reviewed by at least one second investigator experienced in the FISH technique. The nature of nonamplified cells was evaluated by numerical aberrations or even NB84 immunostaining. All tumors with heterogeneity were classified according to the International Neuroblastoma Pathology Committee classification. Except for one patient with ganglioneuroblastoma (patient no. 8), all other 19 patients showed Schwannian stroma-poor and poorly differentiated neuroblastic tumor at the time of MYCN heterogeneity or amplification presentation.

Southern blot, PCR, and FISH in reference laboratories. In addition to FISH analysis, MYCN status was assessed in a second reference lab by Southern blot, PCR, or FISH as reported elsewhere (1, 13). As recommended by the European Neuroblastoma Quality Assessment group (12), amplification was defined as a >4-fold increase of band intensity in relation to the band from the internal standard on chromosome 2.

Results

Of 1,341 patients analyzed, 1,071 (79.9%) showed no amplification and 250 (18.6%) had homogeneous amplification, but 20 (1.5%) patients revealed heterogeneity according to the MYCN status. Gain of 2p24 (MYCN gene locus) was included in the group with no amplification and was present in 79 (5.9%) patients. The frequency and type of alteration of 20 patients with any heterogeneity are summarized in Table 1.

Intratumoral heterogeneity occurred in 15 patients either as clusters of MYCN amplification (n = 12) or as single amplified cells (5-30 amplified cells; n = 3). Clusters occur in one or several foci of MYCN-amplified cells (here at least 20 cells) surrounded by nonamplified tumor cells. A FISH example with amplified clusters is given in Fig. 1. Intratumoral heterogeneity of clusters was more frequently observed at higher-stage or metastatic tumors (1 × stage 1, 4 × stage 3, 5 × stage 4, 2 × stage 4S). Patient no. 6 with stage 4S disease showed sidebar to MYCN-amplified cell clusters, simultaneous 2p24 gain in about one third of cells, and normal MYCN signals in the remaining cells (touch preparation and frozen-cut sections).

A different MYCN status of primary tumor and metastasis was seen in three patients. All showed MYCN amplification in bone marrow–infiltrating neuroblastoma cells but not in the primary tumor. Homogeneous amplification of MYCN in tumor cells of bone marrow was found in stage 4 (n = 2), and amplified single cells in one stage 4S patient. Additionally, in one patient (no. 17), the bone marrow tumor cells were heterogeneous: Clusters of MYCN-amplified tumor cells were surrounded by nonamplified neuroblastoma cells at diagnosis (heterogeneity by site and heterogeneity by cells).

As third group, two patients (1 × stage 3, 1 × stage 4) acquired MYCN amplification during the course of the disease, both at relapse (Fig. 2).

Comparison with Southern blot and PCR methods was possible in 11 of 15 patients with intratumoral heterogeneity. The MYCN amplification found by FISH analysis was rarely confirmed by the second method. Southern blot showed MYCN amplification in the initial tumor of a patient (patient 9) who developed MYCN heterogeneity at relapse. Only in one patient with amplified cell clusters (of eight patients investigated) was amplification confirmed by Southern blot. In the
patients with single amplified cells, Southern blot and PCR were unable to detect MYCN amplification. In contrast, in both patients with amplification at relapse, this was confirmed by Southern blot or PCR.

The chromosome 1(p36) status was investigated in 15 of 15 patients with intratumoral MYCN heterogeneity. In 5 of these 15 patients, aberrations of 1p were found. Deletion of 1(p36) was found in 3 of 12 patients with MYCN-amplified cell clusters. Two of them (patients 4 and 8) showed 1(p36) deletion in the MYCN-amplified subclone only. In the third case (patient 11), deletion of 1(p36) was not clearly restricted to the MYCN-amplified cluster. In addition, two patients showed 1p imbalances not restricted to the amplified cells: one with MYCN-amplified single cells (patient 13) and one

**Table 1. Characteristics of all 20 neuroblastoma patients with genetic heterogeneity of MYCN amplification**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Stage</th>
<th>Age at dx (d)</th>
<th>MYCN status (FISH)</th>
<th>MYCN status by SB/PCR</th>
<th>1p status (FISH)</th>
<th>Therapy</th>
<th>Outcome (d)</th>
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<tr>
<td></td>
<td></td>
<td></td>
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<td>Tumor at relapse</td>
<td>Tumor at dx</td>
<td>Tumor at relapse</td>
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<td>1</td>
<td>1</td>
<td>734</td>
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<td>—</td>
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<td>Adf 1,798</td>
</tr>
<tr>
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<td>3</td>
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<td>—</td>
<td>—</td>
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<td>3</td>
<td>448</td>
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<td>—</td>
<td>—</td>
<td>Adf 1,417</td>
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<td>4</td>
<td>3</td>
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<td>—</td>
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<td>1,536</td>
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<td>—</td>
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<td>—</td>
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<td>—</td>
<td>—</td>
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<td>9</td>
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<td>2,162</td>
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<td>—</td>
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<tr>
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<td>1</td>
<td>98</td>
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<td>—</td>
<td>—</td>
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<td>87</td>
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<td>—</td>
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<td>—</td>
<td>—</td>
<td>Adf 542</td>
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<tr>
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<td>4</td>
<td>1,733</td>
<td>hMNA</td>
<td>Normal</td>
<td>Normal</td>
<td>—</td>
<td>Adf 301, DOD 856</td>
</tr>
</tbody>
</table>

NOTE: Patients are listed by numbers and lined out by intratumoral heterogeneity (cell cluster or single cell), bone marrow amplification versus nonamplified tumors, or heterogeneity during the course of disease. Clinical features and therapy application are indicated. Abbreviations: BM, bone marrow; dx, diagnosis; n.d., not done; n.i., not infiltrated; SB, Southern blot; MNA, MYCN amplification, hMNA, homogeneous MNA; Adf, alive disease-free; progr., progression of tumor; Met., metastatic; Rel., relapse of tumor; DOD, died of disease.

*Complete resection of tumor.

Indicate therapy due to MYCN amplification.

Incomplete resection.

Surgical biopsy.
Fig. 1. Heterogeneity of MYCN gene copies demonstrated by FISH showing MYCN-amplified foci surrounded by nonamplified tumor cells. Dual-color FISH analysis of touch preparation of one neuroblastoma patient (patient 7 in Table 1) to show MYCN gene copies (red) in comparison with reference probe for chromosome 2 (green) counterstained with 4,6-diamidino-2-phenylindole (blue). Tumor is composed of two subclones showing either MYCN amplification with >20 signals in nuclei (arrows) or normal status of MYCN with equal signal number to centromere. This case was scored as nonamplified by Southern blotting. Bar, 20 μm.

with MYCN-amplified cluster at relapse (patient 9). Although we have found only 5 of 15 patients with aberrant 1(p36) status, we cannot exclude more aberrations in the other patients due to methodical limitations.

**Therapy and outcome.** Eight of the 20 patients with MYCN heterogeneity had stage 4 and were treated according to high-risk protocols. One patient received intermediate-risk chemotherapy for clinical reasons (age and stage 3). Among nine patients with localized disease and MYCN heterogeneity at diagnosis, four patients were classified and treated as a high-risk neuroblastoma because of MYCN-amplified clusters (1× stage 1, patient 1; 3× stage 3, patients 2, 4, and 5). Five patients with localized/4S disease and MYCN heterogeneity at diagnosis received no chemotherapy, including two children with single amplified cells (1× stage 1, patient 13; 1× stage 3, patient 15) and three with amplified clusters (1× stage 3, patient 3; 2× stage 4S, patients 6 and 7). Additionally, two patients with normal MYCN status at diagnosis received no chemotherapy but showed either intratumoral heterogeneity (patient 14, stage 2) or single amplified cells in the bone marrow (stage 4S) at relapse (patient 16). Particularly, the first of these patients (patient 14) experienced marginal progression during observation that was cured by surgery alone. In the primary tumor resected after the observation period, few amplified cells were found.

The median observation time was 3.3 years (range, 1-11.5 years). Seven of eight patients with stage 4 showed progression on high-risk regimens. Tumor progression was also observed in the patient with intermediate therapy, wherein the first tumor at diagnosis showed normal MYCN, but relapsed material had homogeneous amplification (patient 19). One of four patients with localized disease and intensive chemotherapy due to MYCN-amplified cluster showed progression (patient 5); the others lived disease-free 1,205 to 1,798 days after diagnosis. Of note is that among the five children without chemotherapy showing intratumoral heterogeneity at diagnosis, only one suffered progression (patient 3). This patient with amplified cluster at relapse showed local tumor progression, but interestingly, in the samples of the resection after progression only tumor cells with normal MYCN status were found. Four patients were disease-free 378 to 4,225 days after diagnosis (stage 1, patient 13, and stage 3, patient 15, patient 15, patient with amplified single cells; 2× stage 4S patients with clusters, patients 6 and 7). One of these children (patient 6) with MYCN-amplified clusters had a short follow-up (378 days). Other two patients (patients 14 and 16) experienced progression without chemotherapy and acquired heterogeneous amplification only at relapse.

**Discussion**

In many trials, high-risk patients are identified by the presence or absence of MYCN amplification, particularly those with localized and 4S tumors (14). Southern blot has frequently been used to determine the MYCN status based on pooled DNA from tumor cells. The implementation of FISH
analysis allows the determination in situ of single cells and allows a more critical judgment of intratumoral heterogeneity. Over the threshold of four copies, the growth advantage of MYCN-amplified tumor cells is independent of the amount of MYCN amplification and of other clinical parameters (15).

Reports indicate genetic heterogeneity in a great variety of human tumors showing variation in loss of heterozygosity, copy number of genes, or mutation behavior (16–19). It was shown that gene amplification even in single foci can lead to metastatic growth in prostate carcinoma (20). Only few publications describe heterogeneity of MYCN status in neuroblastoma (8). In three case reports, heterogeneous MYCN amplification was documented in children with neuroblastoma (9, 10, 21). One further study identified 11 neuroblastic tumors where MYCN amplification was documented in children with neuroblastoma (9, 10, 21). One further study identified 11 neuroblastic tumors showing intratumoral heterogeneity for MYCN amplification or chromosome 1p deletion, or aberrations of these genetic markers that occurred during tumor progression (7). In our cohort, MYCN heterogeneity was found in 20 patients and supports the concept of genetic heterogeneity in neuroblastoma patients.

The clinical effect of MYCN heterogeneity has not been clearly elucidated. One might argue that even one MYCN-amplified cell remaining in the body can be the seed for relapse or progression. Our results are conflicting: On one hand, relapse, progression, or dissemination seems to be in correlation with a selection or formation of MYCN-amplified cells. One of our patients having only some scattered amplified cells in the tumor material at diagnosis showed universal amplification in the relapse tumors. The same observation was made in the bone marrow of a child without any amplification in tumor material. A further two patients showed homogeneous MYCN amplification only in the relapsed tumor. In one patient, MYCN status was normal at diagnosis and single amplified cells occurred at relapse. Most probably, amplification was not acquired during disease progression, but few amplified cells at diagnosis were missed due to incomplete resection. On the other hand, MYCN amplification seems not to be essential for relapse, progression, or dissemination. Two patients in our cohort had MYCN-amplified clusters in the primary tumor but normal MYCN status at progression. However, only one of five patients with heterogeneous MYCN amplification at diagnosis without chemotherapy showed progression. Accordingly, four patients with localized disease showing up to 10 amplified cells or small cell clusters remained free of progression without chemotherapy. Additionally, four patients exist in our cohort displaying only two to three amplified cells, but in line with methodical limitation, they were not classified and treated as amplified patients. These patients live disease-free without chemotherapy. Therefore, it seems unlikely that only a few amplified cells exert a large growth advantage. It might be important to define a threshold of counted amplified cells in patients with influence on tumor outcome. In our cohort, we arbitrarily defined heterogeneity as at least five amplified cells, but from our data we assumed that a clinical influence might demand a higher amount of aberrant cells.

Another kind of heterogeneity is obvious in three patients who have MYCN amplification in the bone marrow but not in the primary tumor. In all children, the tumor was incompletely resected, and thus some amplified cells probably may have been left in situ. Imbalance of chromosome 1 could be detected in the tumor of one patient. Why these neuroblastoma cells do not show amplification is unclear. Again, an explanation could be heterogeneity not visible in investigated sections due to incomplete resection. In addition, one patient had intratumoral heterogeneity in the bone marrow at diagnosis, with MYCN-amplified cluster surrounded by nonamplified cells.

Besides MYCN amplification heterogeneity, 2p24 gain (MYCN gene locus) can be observed heterogeneously as well in neuroblastoma. This could reflect gain of short arm 2p or gain of MYCN gene. FISH analysis could clearly detect gain of 2p24 as a 1.5- to 4-fold excess of MYCN copies in relation to reference probe on chromosome 2q. In contrast to amplification, unbalanced gain seems to be an independent cytogenetic event in neuroblastoma and is frequently detected in stage 4 diseases. Its prognostic influence is limited because of the close association with other genetic events like aberrations in 11q (22). Here we describe one patient with heterogeneous MYCN-amplified cluster showing also a gain of 2p24. In literature, such heterogeneity was also observed in three tumors exhibiting simultaneous gain and amplification detected in neighboring cells (11).

Thorner et al. (23) reported on a different kind of MYCN heterogeneity independent of heterogeneity between gain or nonamplification and amplification. Investigators defined cases as heterogeneous when there was a ≥50% difference in copy number from cell to cell. By this definition, they found 4 of 14 patients showing heterogeneity of MYCN copy number. We observed this phenomenon in our cohort in a few cases (data not shown) but did not include these patients in our analysis.

MYCN amplification is highly correlated with partial deletion of chromosome 1 (15, 24). Therefore, one would expect 1p36 deletion or imbalance of the MYCN-amplified cells in the majority of the 20 patients with MYCN heterogeneity. Technically, the occurrence of 1p aberrations in subclones is more difficult to identify by FISH compared with MYCN amplification. A low amount of aberrations is often not methodically distinguishable from hybridization artifacts. Indeed, only three patients showed clear aberrant deletion of 1p36 with intratumoral MYCN heterogeneity. At least in two children (third most probably) occurrence of deletion was restricted to the same subclone as MYCN amplification. Other patients had normal 1p status. We hypothesize that, most likely, focal aberration of 1p status exists in some cells, maybe not visible due to less aberrant cells in sample. Important new insights were given by two patients with proven imbalance in chromosome 1 and MYCN-amplified cluster or single cells. In both cases, the imbalance was not restricted to cells with MYCN amplification; it was even more existing in another subclone. The limited number of amplified single cells hindered the analysis of whether MYCN-amplified cells themselves harbor imbalance or not. However, the examples clearly indicate at least two subclones in tumor material showing either (a) amplification of the MYCN gene (and 1p imbalance?) or (b) 1p imbalance. Kerbl et al. (21) showed that different clones of neuroblastoma cells may exist within one patient and even within a single tumor. Alterations of MYCN and 1p36 status were complementary in their case. Cells not amplified for MYCN displayed 1p imbalance, whereas MYCN-amplified cells had a normal 1p36 status. Altogether, the presence of different genetic subpopulations in tumor cells can be postulated.
MYCN heterogeneity infrequently occurs in neuroblastoma. The identification by FISH on the single-cell level and careful workup of tumor material is important. We observed three different types of MYCN heterogeneity in our cohort: intratumoral heterogeneity within amplified clusters or single cells (heterogeneity by cells), MYCN heterogeneity between primary tumors and metastasis (heterogeneity by site), and changing MYCN status during the course of the disease (heterogeneity by time). In stage 4 patients, the clinical value of this observation is limited because they receive intensive chemotherapy anyway. These patients seem to do as poorly as those with homogeneous amplification. However, particularly young children with low percentages of amplified cell cluster or single cells and otherwise low-risk neuroblastoma may not necessarily require high-dose chemotherapy regimens. More patients will provide more information about the establishment of risk stratifications of heterogeneity. From the data presented here, an up-staging of otherwise low-risk patients to the high-risk category cannot be recommended.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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