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European Journal of Cancer 40 (2004) 2753–2759

European Journal of Cancer

www.ejconline.com

MYCN-status in neuroblastoma: characteristics of tumours showing amplification, gain, and non-amplification

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Received 27 February 2004; received in revised form 5 May 2004; accepted 6 May 2004
Available online 24 June 2004

Abstract

While the role of *MYCN*-amplification (MNA) for risk assessment in neuroblastoma is undisputed, the phenomenon of gene copy excess below the amplification threshold is rarely described. To discuss biological characteristics and the clinical impact of the so- called *MYCN*-gain *versus* amplified or non-amplified cases, we investigated the *MYCN* status of 659 patients uniformly analysed by fluorescence *in situ* hybridisation. The number of *MYCN*-amplified tumours in our cohort was 18% (116/659); an additional 38 tumours (6%) displayed *MYCN*-gain. Both alterations were associated with an advanced stage disease, an increased patient age and further chromosomal alterations. Most of the amplified neuroblastomas displayed 1p aberrations, whereas *MYCN*-gain tumours correlated with 11q alterations. In contrast to the amplified cases, tumours with gain displayed no increased *MYCN* RNA levels. MNA *versus* non-amplification discriminated between good and poor outcomes, independent of stage, age and the degree of amplification. However, patients with amplified tumours showed a significantly better outcome when this was combined with non-stage 4 disease and age <1 year *versus* stage 4 and age >1 year. Although *MYCN*-gain was associated with poor event-free-survival (EFS) in stages 1–3, 4S ($P = 0.005$), this might be related to associated genetic aberrations and not to the *MYCN*-gain itself. A survival difference between neuroblastomas with gain and single copy *MYCN* could not be delineated. In conclusion, MNA predicts a poor outcome for neuroblastoma patients of all stages and age. *MYCN*-gain is also a characteristic feature of advanced stage tumours and older patients, but is not associated with higher *MYCN* expression and appears not to be discriminative in predicting patient outcome.

Keywords: Neuroblastoma; *MYCN*-gain; FISH; Q-RT–PCR

1. Introduction

Since its first description in 1983 [1], the prognostic role for the *MYCN* oncogene in neuroblastoma is undisuted. Amplification of the gene, although the term 'amplification' is not uniformly defined, discriminates between favourable and unfavourable outcomes in neuroblastoma, even in 'high-risk' stage 4 patients over 1 year of age [2]. The tumorigenic potential is most probably the consequence of an increased gene dose as mutations within the gene were not detected [3] and all copies were shown to be transcriptionally active [4]. Consequently, the adverse effect was shown to be stronger in tumours with high level amplifications compared with low level amplifications [5].

The multiple functions and interactions of this transcription factor range from cell cycle progression [6] to an increased sensitivity to apoptosis [7,8]. Gene expression analyses recently identified more than 200 genes strongly associated with *MYCN* expression in neuroblastomas [9].

Although Southern blotting is the 'gold standard' for determining the *MYCN*-status, reliable identification of low-level amplification by comparison of band intensities is sometimes difficult. In recent years, fluorescence *in situ* hybridisation (FISH) has evolved as an alternative

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doi:10.1016/j.ejca.2004.05.002

technique. It has a higher sensitivity because the *MYCN*-copy number can be counted in each single cell. Using this technique, it was recently shown that a few additional *MYCN* copies resulting from unbalanced translocations or duplications were detected in approximately 8% of all neuroblastomas [10]. Due to the limited number of patients, an association between such a *MYCN*-gain and patients' outcome could not be assessed.

The aim of our study was to evaluate the biological characteristics and prognostic impact of *MYCN*-gain in a comparison of amplified and non-amplified tumours from a large neuroblastoma cohort using FISH.

1.1. Patients and methods

For prognostic reasons, we determined the *MYCN*-status of 659 neuroblastoma patients. Tissue samples were collected in 91 hospitals participating in the German cooperative trials between December 1989 and November 2003. Patients were treated according to the trials NB90/NB95 ($n = 105$) [2] and NB97 ($n = 554$) (for details see [11]). The cohort included 87 patients diagnosed in the German mass-screening programme. Most of the specimens were investigated at diagnosis (549, 83%), 17% during therapy or at relapse.

The FISH technique was applied using a DNA probe *D2Z* (centromere of chromosome 2) as a reference and the n-myc probe in 2p24 (both ONCOR, Gaithersburg, MD) in order to count the number of *MYCN* copies in relation to the number of chromosomes 2. Additionally, the status of 1p, 3p and 11q was determined with DNA probes from Oncor (Gaithersburg, MD) as described elsewhere in [12]. The utilisation of a wide range of tissue types is a great advantage of the FISH technique. In our cohort, tumour samples were available as touch preparations (303, 46%), frozen-cut sections (216, 33%), bone marrow aspirates (82, 12%) and paraffin- embedded-cut sections (58, 9%). According to the International Neuroblastoma Staging System (INSS) classification, tumours were judged as: stage 1 (26%), 2 (15%), 3 (17%), 4 (34%) and 4S (8%).

As recommended by the European Neuroblastoma Quality Assessment (ENQUA) group, amplification of *MYCN* was defined as a more than the 4-fold increase of *MYCN* signals in relation to the number of chromosomes 2. Additional copies up to the 4-fold were defined as *MYCN*-gain [13]. Before analysis, tumour cell content was determined in each preparation by a pathologist to ensure that only tumour and no normal tissue was investigated.

1.2. Quantitative reverse-transcription PCR

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using the SYBR-Green I reagent in 96-well optical reaction plates with optical caps on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The relative standard curve method was used for evaluation. Cycling conditions: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Serial dilutions (1:3, 1:9, 1:27, 1:81) of the cell line IMR 32 were used to generate a standard curve. *MYCN* Primer sequence: Forward 5′AAA TCG ACG TGG TCA CTG TGG-3′, reverse 5′ATC GTT TGA GGA TCA GCT CGC-3′.

Raw data were equalised to the standard curve and the resulting values were normalised to the geometrical mean of three housekeeping genes (PPIA, PBGD and PGK1) to minimise patient-specific fluctuations. Finally, data were quoted as relative expressions referring to the lowest value measured.

1.3. Statistical analyses

To compare variables of interest, Fisher's exact test, χ^2 test, or Mann–Whitney *U*-test were used, where appropriate. Kaplan–Meier estimates for event-free survival (EFS) were calculated and compared by the log-rank test.

Recurrence, progression of disease, and death from disease were counted as events. Death resulting from therapy complications was not counted as an event, but censored for the EFS analysis.

2. Results

In 116 of 659 patients (18%), we found an amplification of *MYCN* (MNA), whereas 505 (77%) displayed a regular number of gene copies. Additional *MYCN* copies classified as *MYCN*-gain could be detected in the remaining 38 specimens (6%).

Although MNA was associated with stage 4 disease ($P < 0.001$), more than one third (42/116, 36%) occurred in non-stage 4 tumours (stages 1–3, 4S), predominantly in stage 3 (25/42, 60%). Five cases with amplifications were found in stage 1 tumours, 6 in stage 2 and 4S neuroblastomas each. Approximately half of all amplified tumours (59/116, 51%) showed at least a 30-fold excess of *MYCN*, 39% (45/116) a 15–25-fold and 10% (12/116) a 5–10-fold amplification (ranges; amplified: 1.4–276 months; gain: 6–212 months; non-amplified: 1 day–441 months).

Thirty-three of 38 cases with *MYCN*-gain displayed one or two additional gene copies in relation to the number of chromosomes 2, only five cases had 3-fold the number. Two thirds of patients with *MYCN*- gain were associated with metastatic stage 4 (26/38, 68%, $P < 0.001$). Patients with *MYCN*-gain showed a significantly higher age at diagnosis compared with the

Table 1
Biological and clinical data according to the *MYCN* status

	MYCN-Status		
	Non-amplified	Amplified	Gain
Age (median)	15 Months	24 Months	34 Months
Stage	St. 1: 32%, 2: 17%, 3: 17%, 4: 26%, 4S: 9%	St. 1: 4%, 2: 5%, 3: 22%, 4: 63%, 4S: 5%	St. 1: 11%, 2: 11%, 3: 3%, 4: 68%, 4S: 8%
+1p-Aberration	42/379 (11%)	75/88 (85%)	11/33 (33%)
+3p-Aberration	27/214 (13%)	6/52 (12%)	11/25 (44%)
+11q-Aberration	85/365 (23%)	12/76 (16%)	22/33 (67%)
At least 1 aberration	114/421 (27%)	80/95 (84%)	28/38 (74%)

St., Stage.

MYCN-normal (median age: 34 *versus* 15 months, $P = 0.001$). Compared with children with MNA, a statistical significant difference was not demonstrated (34 *versus* 24 months, $P = 0.12$). Clinical and biological data of patients with *MYCN*-gain are given in Table 1. Tumours with *MYCN*-gain more frequently had other chromosomal alterations like 1p, 3p and 11q aberrations than tumours with normal *MYCN*. At least one abnormal marker was detected in 74% cases with *MYCN*-gain (28/38). Seven cases could not be analysed for all of the markers due to the lack of sufficient specimens.

Samples of 26 patients from the whole cohort were investigated at different time points during the course of their disease (i.e., initially, after therapy or at relapse). Two tumours displayed a *MYCN*-gain initially, but not at relapse, and a third case showed the situation *vice versa*. The remaining 23 showed consistent results. Another 17 cases were analysed at different localisations within the tumour or different manifestations (primary tumour and metastasis or bone marrow) with consistent results in each case.

Additionally, four neuroblastomas showed a heterogeneous aberration pattern. One initial stroma-poor neuroblastoma (Hughes grade 2) was amplified focally and diffusely at relapse. In another case, only a few scattered amplified cells were found in the bone marrow initially, in the relapsed tumour (Hughes grade 3) again a universal amplification was found. The bone marrow of the third patient with a low infiltration grade showed 3 amplified cells, whereas the tumour-cut section (Hughes grade 3) lacked any amplification at all. Finally, a tumour (Hughes grade 3) with a few amplified cells and nests could be analysed only after therapy.

2.1. Quantitative real-time RT-PCR

To investigate whether the higher copy number results in a higher gene expression level, we performed a quantitative RT-PCR for 27 patients (*MYCN*-normal: $n = 10$, Amplified: $n = 7$, *MYCN*-gain: $n = 10$). Analysis was limited because of the lack of available tissue for RNA isolation in the *MYCN*-gain group. While relative expression for *MYCN*-amplified patients ranged between the 2- and 113-fold compared with the lowest value measured in our cohort, both *MYCN*-gain and non-amplified tumours showed comparably low expression levels (range: 1.4–12-fold and 1.2– 6.6-fold, respectively, Fig. 1).

2.2. Patient outcome

The 3-year EFS of the whole cohort ($n = 659$) was $62 \pm 2\%$, the 3-year-overall survival (OS) was $81 \pm 2\%$. Outcome was poor for patients with amplified tumours *versus* non-amplified (3-year EFS: $33 \pm 5\%$ *versus* $71 \pm 2\%$, respectively, $P < 0.0001$; 3-year OS: $44 \pm 6\%$ *versus* $90 \pm 2\%$, respectively, $P < 0.0001$). Neuroblastomas with *MYCN*-gain were associated with a worse 3-year EFS compared with the non-amplified cases ($39 \pm 10\%$ *versus* $71 \pm 2\%$, respectively, $P = 0.005$), but OS was not statistically different compared with the non-amplified tumours (3-year OS: $76 \pm 9\%$ *versus* $90 \pm 2\%$, $P =$ Non-significant (NS)).

MNA was associated with a significantly poorer outcome in patients with non-stage 4 disease (EFS of amplified *versus* non-amplified tumours: $P < 0.001$) and in stage 4 patients ($P = 0.025$, Fig. 2). Within the stage 4

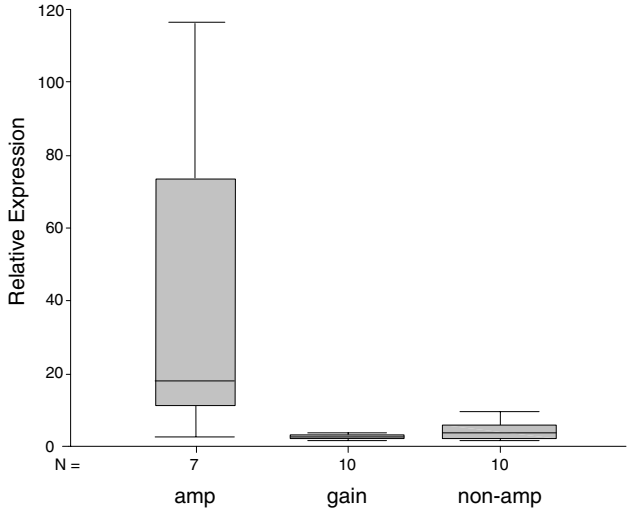


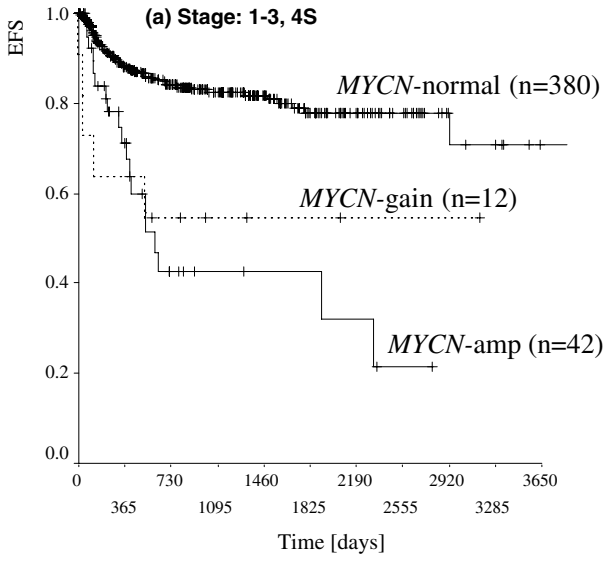
Fig. 1. Relative *MYCN* expression for each *MYCN*-status based on the lowest value measured (amp: amplified; non-amp: non-amplified).

group, patients with amplified neuroblastoma *versus* *MYCN*-normal showed a significantly poorer prognosis either for children below 1 year of age (3-years EFS: $24 \pm 6\%$ *versus* $53 \pm 17\%$, respectively, $P = 0.05$) or over 1 year ($24 \pm 6\%$ *versus* $37 \pm 5\%$, respectively, $P = 0.006$). Patients with non-stage 4 and *MYCN*-gain had worse EFS compared with the non-amplified patients ($P = 0.005$). It was non-significant for OS ($P = 0.14$). However, in stage 4 the outcome of patients with *MYCN*-gain was identical to those patients with *MYCN*-single copy.

To investigate whether the *MYCN* copy number in amplified tumours influences outcome, we calculated Kaplan–Meier analyses using different cut-off points (5–10-fold *versus* more than 10-fold; 5–20-fold *versus* more than 20-fold; 5–50-fold *versus* more than 50-fold). We could not find any differences either in the whole group ($n = 116$) or for patients with stage 4 or for patients with stages 1–3 and 4S.

Within the group of amplified tumours, those patients with non-stage 4 disease displayed no difference in EFS compared with stage 4 patients (3-year EFS: $43 \pm 10\%$ *versus* $29 \pm 6\%$, respectively, $P = 0.36$), but a significantly better OS (3-year OS: $58 \pm 11\%$ *versus* $39 \pm 7\%$, respectively, $P = 0.04$). Amplified tumours of stages 1 and 2 *versus* stage 3 displayed a 3-year EFS of $40 \pm 21\%$ and $33 \pm 11\%$, a non-significant difference.

The analysis of MNA tumours by age demonstrated that patients below 1 year of age showed a better 3-years EFS of $62 \pm 13\%$ (Fig. 3) compared with $25 \pm 5\%$ ($P = 0.01$) in older children (3-years OS: $74 \pm 12\%$, *versus* $38 \pm 6\%$, respectively, $P = 0.009$). This phenomenon cannot be explained by a different stage distribution because within the amplified cohort, stage 4 patients < 1year showed a significantly better outcome than stage 4 children >1year (3-years-EFS: $53 \pm 17\%$ *versus* $24 \pm 6\%$, respectively, $P = 0.05$; 3-year OS: $64 \pm 17\%$ *versus* $33 \pm 7\%$, respectively, $P = 0.04$).



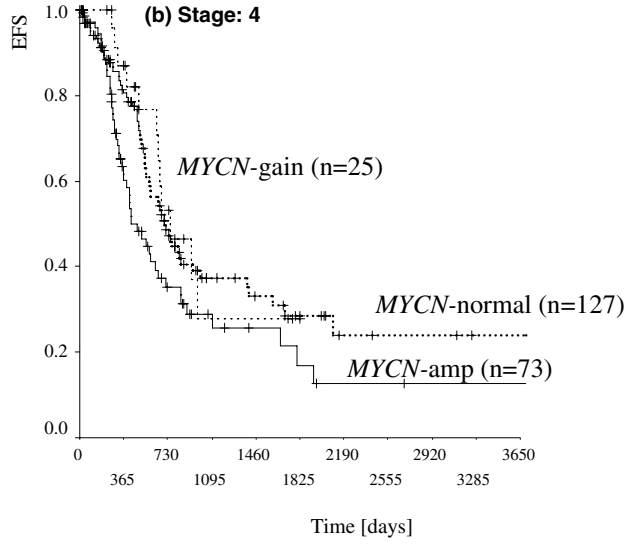


Fig. 2. Event-free-survival (EFS) according to *MYCN*-status and stage: (a) Stages 1–3, 4S: 3-year EFS *MYCN*-normal: $82 \pm 2\%$, -gain: $55 \pm 15\%$, -amplified: $43 \pm 9\%$ (normal *versus* amplified: $P < 0.001$, normal *versus* gain: $P = 0.005$); (b) Stage 4: 3-year EFS normal: $38 \pm 5\%$, gain: $31 \pm 11\%$, amplified: $29 \pm 6\%$ (normal *versus* amplified: $P = 0.025$, normal *versus* gain: non-significant (NS).

3. Discussion

Southern blotting is frequently used to determine the *MYCN*-status. Although providing reliable results, the evaluation of low-level amplification may be ambiguous.

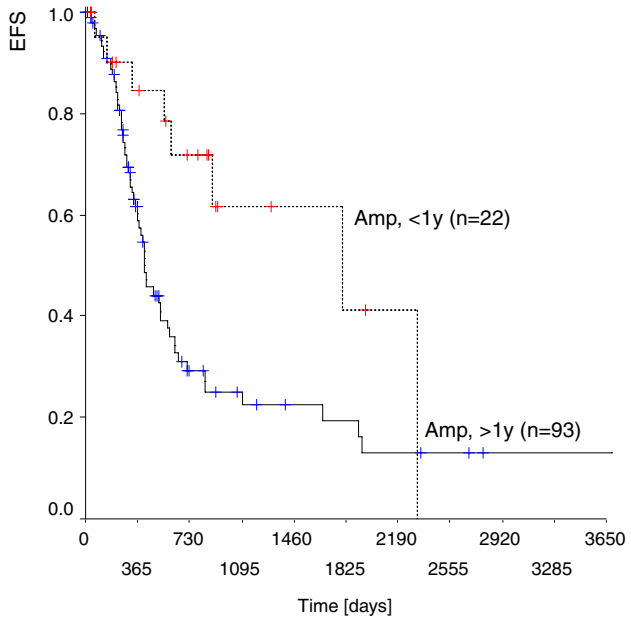


Fig. 3. EFS of patients with amplified tumours according to age at diagnosis: 3-year EFS < 1 year of age: $62 \pm 13\%$, >1 year: $25 \pm 5\%$ ($P = 0.01$).

Like many other groups, we therefore decided to use FISH as this technique allows the determination of *MYCN*-copies in single cells and is more sensitive in cases of low-level amplification. Thus, 6% of our investigated tumours showed only one or a few additional *MYCN* copies, which is defined as *MYCN*-gain. This phenomenon has been suggested to represent rearrangements, such as duplications or unbalanced translocations, including the distal short arm of chromosome 2 [14,15]. Similar to amplifications, *MYCN*-gain is more frequently detected in stage 4 disease, but is also present in a small portion of localised (= stages 1–3) and 4S tumours. In contrast to amplifications, the simultaneous occurrence of 11q aberrations is very frequent in samples with *MYCN*-gain, a phenomenon that was also observed by comparative genomic hybridisation (CGH) analyses [16]. Since 11q aberrations and MNA are rarely associated [17,18], *MYCN*-gain appears not to be a prelude to amplification, but an independent and late event in the complex aberration pattern of advanced neuroblastomas. The percentage of amplifications (18%) in our large cohort of 659 tumours was somewhat lower compared with that reported in other large studies (22%) [19], probably as a result of a mass-screening bias towards localised neuroblastomas. Excluding the screening-positive tumours, the portion of amplified cases (20%) is representative for the German neuroblastoma cohort and is in good agreement with other patient samples tested.

While *MYCN* gene amplification is a well-established factor for an unfavourable prognosis, the clinical relevance of the *MYCN* expression level remains controversial. Multiple gene copies usually lead to higher RNA levels, but the latter is not necessarily the consequence of an increased gene dosage [20]. Therefore, the expression of the *MYCN* oncogene might not be of prognostic significance, particularly in non-amplified tumours [20,21] and in infants under 1 year of age [22].

Using quantitative RT-PCR, the expression analysis in our series displayed an increase of *MYCN* expression in a wide range of amplified tumours. However, compared with *MYCN*-single copy cases, *MYCN*-gain was not associated with a higher RNA level in most cases. Valent and colleagues [10] described a slightly higher expression using quantitative RT-PCR in three investigated tumours. Due to different normalisation factors and the limited number of patients the results are not completely comparable. We would assume that a few additional copies are obviously insufficient to noticebly elevate the expression.

In contrast to MNA, *MYCN*-gain was associated only with a poor EFS and not OS, in localised or 4S neuroblastomas and was of no prognostic relevance in stage 4 tumours in our cohort. Due to the limited number of patients with localised stages and 4S that showed *MYCN* gain, the prognostic influence is questionable and might rather be the consequence of several associated alterations in 11q, 1p or 3p in this group that were recently shown to be prognostic [18,23].

While the 5–10-fold copy number is sufficient to increase neuroblastoma growth, 1, 2 or 3 additional copies are probably not. In 1985, Seeger and colleagues [5] reported that patients with a low-level amplification (3–10-fold) as assessed by Southern analysis showed a better prognosis than those with a higher degree of amplification. To test the hypothesis that an increase of *MYCN* copies is associated with a poorer outcome, we calculated EFS and OS using different cut-off points. We found no prognostic differences between cases with high and low-level amplifications. Most amplified tumours displayed at least 10-fold copy number at diagnosis and it seems that beyond this threshold of *MYCN* copies there is an unfavourable effect on patient outcome. Unfortunately, we cannot draw any conclusions with regard to outcome for patients with tumours with a 5–10-fold copy number due to their rare occurrence ($n = 12$). The discrepancy between the results of Seeger and colleagues and our study is most likely due to their use of a less strict definition of amplification where the *MYCN*-gain might have been attributed to cases with $<$10-fold copy number. According to ENQUA guidelines, neuroblastomas with a 3 or 4-fold copy number would not today be defined as amplified. Technical reasons might also explain some of the discrepancies as FISH is suitable for exactly quantifying low-level amplification, whereas for cases with higher amplification the technique provides an estimation alone. Another aspect might be differences in the treatment protocols between the various neuroblastoma trials. The patient's age and tumour stage were important determinants of outcome for the amplified cases. Children below 1 year showed a significantly better prognosis than older children and, patients with non-stage 4 amplified disease had a better outcome than stage 4 amplified cases. Consistent with our study, Cohn and colleagues [24] and Alvarado and colleagues [25] reported patients with localised neuroblastoma had a better outcome, despite *MYCN* amplification. By contrast, we could not confirm data reporting a high mortality for infants younger than 1 year with amplified neuroblastomas [26]. Instead, we observed a significantly better prognosis for infants compared with older children with amplified tumours (Fig. 3). In Germany, infants with amplified neuroblastomas were treated in the 'high-risk' arm of treatment protocols, which may have improved outcomes in this subgroup compared with other patient subsets. The interaction between the upregulated transcription factor *MYCN* and several other genes is obviously influenced by the genetics of the tumour which differ in localised *versus* stage 4 disease, as well as with age.

Sequential analyses of samples from different tumour sites or from different times were highly concordant

indicating that changes in the cytogenetic aberration pattern, as well as a heterogeneous distribution of chromosomal alterations occur only rarely [27]. However, interestingly, heterogeneity was observed in some tumours that showed either focal amplification or only some scattered amplified cells at diagnosis, but a universal amplification in relapsed tumours. This accords well with the biological role of *MYCN* in enhancing tumour progression because amplified cells display a growth advantage. On the other hand, two specimens demonstrated a *MYCN*-gain initially, but not in the relapsed tumour. Corresponding to our prognosis data, a few additional gene copies are unlikely to exert a large growth advantage. Neuroblastoma heterogeneity, although rare, is an important observation as it might be clinically and therapeutically relevant [28].

In conclusion, our data from a large patient cohort clearly shows that MNA is the pivotal predictor defining a 'high-risk' tumour, independent of the degree of amplification and other clinical parameters. However, the impact of amplification on tumour progression seems attenuated when it occurs in the context of localised disease and at a younger age. Finally, an increase in the *MYCN* gene dosage through chromosome 2 short-arm rearrangements, leading to a few additional copies represents an independent cytogenetic event and *per se* presumably does not have substantial prognostic influence due to its close association with other genetic events like aberrations in 11q.

4. Conflicts of interest

The authors have no potential conflicts of interest.

Acknowledgements

The authors thank Joern Oestreich for his excellent technical assistance.

This study was supported by the "Deutsche Kinderkrebs-Stiftung" and "Tumour bank neuroblastoma" of the competence network "Paediatric Oncology and Haematology (KPOH)"

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