Minimal residual disease monitoring in neuroblastoma

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Chapter 6

Neuroblastoma messenger RNA is frequently detected in bone marrow at diagnosis of localized neuroblastoma patients

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Abstract

Introduction
The clinical importance of the detection of neuroblastoma mRNA in bone marrow (BM) of localized neuroblastoma patients at diagnosis remains unclear. In this prospective multicenter study, BM samples of a large cohort, were studied using Real-time quantitative PCR (qPCR).

Methods
BM samples at diagnosis from 160 patients with localized neuroblastoma were prospectively collected at Dutch and German centers between 2009 and 2013. qPCR was performed using five neuroblastoma specific markers. The association with other biological factors and the prognostic impact of BM positivity and clinical response was assessed.

Results
In 58 out of 160 patients neuroblastoma mRNA was detected in BM. In 47 of the 58 positive samples only one marker was found positive. BM positivity was significantly associated with MYCN amplification (p=0.02) and deletion of chromosome 1p (p=0.04). In total 31 patients had an event, of which only 5 patients had progression to stage 4. BM positivity was not associated with an unfavorable outcome. However, the detection of more than one marker was associated with an unfavorable outcome (systemic or local relapse) (EFS 48% vs 85%; p=0.03) in the whole cohort and in the observation group.

Conclusions
BM positivity was associated with unfavorable biological factors and might represent more aggressive tumors. Patients with qPCR positive BM should not be upstaged, because of very few systemic events in the cohort. However, for patients with more than one marker positive a more careful follow-up is advisable. These results need to be verified in a very large cohort of localized patients.
Neuroblastoma mRNA in bone marrow of localized patients

Introduction

Neuroblastoma is an extracranial solid tumor of childhood (1, 2). The presence of metastatic spread at diagnosis is the most important factor in determining outcome (3, 4). Localized patients (stage 1, 2 and 3) have a good prognosis, but a small percentage of these patients can relapse.

According to the International Neuroblastoma Risk Group, BM disease is determined by morphology on smears and biopsies (5). However, Real-time quantitative PCR (qPCR) and immunocytology are more sensitive techniques for the detection of minimal neuroblastoma cells than morphology (6-9).

Detection of GD2 positive tumor cells in BM samples of localized patients is suggested to be correlated with an unfavorable outcome (10). Neuroblastoma mRNA detection by qPCR in localized neuroblastoma has been described in a very small number of patients (4 out of 14 patients) by Shono et al. (11). Corrias et al. (12) have studied BM of 126 localized patients in a retrospective study by using qPCR. No significant association between Tyrosine Hydroxylase (TH) expression and outcome was observed, however detection of GD2 Synthase (GD2S) was surprisingly associated with a favorable outcome. Yanez et al. studied 94 BM samples and 81 PB samples of localized patients by using TH and DCX as markers. Detection of these markers in BM was not associated with a poor survival. However, detection of TH and DCX in PB was predictive of relapse (13). These results suggest that the clinical importance of neuroblastoma mRNA detection in BM at diagnosis of localized patients remains unclear. Therefore, the goal of our study was to investigate the frequency and prognostic value of the detection of a panel of 5 sensitive and specific mRNA markers (PHOX2B, DDC, TH, CHRNA3, GAP43) (9, 14-19) in BM from localized neuroblastoma patients. Our group has recently demonstrated the superiority of this panel compared to the use of single markers (9).

Methods

Patients and treatment

In this study BM samples at diagnosis were taken from children diagnosed with localized neuroblastoma between 2009 and 2013. Histologic diagnosis of neuroblastoma was established and centrally confirmed for all patients. Staging was done according to the International Neuroblastoma Staging System (INSS) (4, 20). Patients were treated according to the similar protocols of the German NB2004 trial and the Dutch DCOG09 trial. Based on the risk stratification (Supplemental Figure 1) patients were assigned to the observation, medium or high risk group. In the observation group patients underwent initial surgery and were then observed for 12 months or until the end of the second year
of life. Local progression or threatening symptoms were treated with N4 chemotherapy.
In the medium risk protocol treatment consisted of alternating N5 and N6 courses, 4 N7
courses and retinoic acid for 12 months. In the high risk protocol treatment consisted of
two N8 courses (if randomized to, German patients), alternating N5 and N6 courses (3
each), MIBG therapy and high dose chemotherapy with autologous stem cell transplan-
tation followed by retinoic acid for 12 months.

For therapy stratification, the status of the MYCN oncogene and the status of distal
chromosome 1p (1p36) were investigated using two different molecular techniques (for
MYCN: FISH, Southern Blot or array-based comparative genomic hybridization and for 1p
deletion: FISH, PCR or array-based CGH) in the DCOG and GPOH reference Laboratories.
The test result for each parameter was given according to the criteria of the European
Neuroblastoma Pathology, Biology, and Bone Marrow Group. (21)
The GD2 immunocytology of BM samples was done according to the internationally
standardized protocol described by Swertz et al. 2005. For all Dutch and German pa-
tients this was performed in Cologne, Germany (22).
Written informed consent from parents or guardians was obtained for all patients.
The study was approved by the Medical Research Ethics Committee of the Academic
Medical Center (Amsterdam, the Netherlands) and the University of Cologne (Cologne,
Germany). Also ethical approval was acquired from all participating hospitals locally.

RNA extraction, reverse transcription and real-time quantitative PCR
Whole BM RNA was isolated from PAX blood RNA tubes according to the instructions
of the manufacturer (Qiagen, Venlo, Netherlands) with the PAX blood RNA Kit. cDNA
was synthesized from 2-3 μg of RNA, using 25 μmol/L random hexamers (Invitrogen,
Carlsbad, CA, USA), 1 mmol/L dNTPs (Promega, Madison, WI, USA) and 100U of MMLV
transcriptase (Invitrogen), in a total reaction volume of 20 μl and incubated at 42°C for
45 minutes. Finally, the reverse transcriptase was inactivated by heating and the volume
was diluted to 100 μl. qPCR for paired-likehomeobox2B (PHOX2B), tyrosine hydroxylase
(TH), dopa decarboxylase (DDC), cholinergic receptor alpha 3 (CHRNA3) and growth-
associated protein 43 (GAP43) was performed using beta-glucoronidase (GUS) on the
Step-One-Plus (Applied Biosystems, Carlsbad, CA, USA). Primer and probe sequences
have been described previously (9). Reactions were carried out in 20 μl (10 μl Taqman
Fast Universal PCR Mastermix (Applied Biosystems), 0.8 μl of 7.5 μM forward and reverse
primer and 0.8 μl of 5μM probe and 5μl cDNA. Initial heating was done for 20 s at 95°C,
followed by 50 cycles of 1s at 95°C and 20 s at 60°C. All qPCR experiments were carried
out in triplicate and mean values were used. If more than one sample of a patient was
available, samples were tested separately. In case of discrepant results, the positive
sample was used in the analysis.
Data analysis
A sample was scored positive if one out of five markers was above the threshold for positivity as has been described previously (10). In short, PHOX2B was scored positive if the Ct value of the sample was <50 and the other markers were scored positive if the ΔCt of the sample was >3 Ct than the ΔCt found in control BM samples. The quantitative range was defined as described by van Velden et al. 2007 (23). To calculate the level of infiltration, the expression level of the RNA PCR targets were related to the expression level in neuroblastoma cell line IMR32, according to the following formula: $2^{\Delta \Delta Ct} \times 100\%$. To study the association between qPCR positivity and other biological factors Fisher’s exact test was used. Survival rates were estimated by employing Kaplan-Meier’s methodology. To assess the significant differences between the estimated survival curves the log-rank test has been used. The median follow-up was assessed by the reverse Kaplan-Meier method. All statistical analyses were performed by using SPSS version 21.

Results

Patient Characteristics
One hundred sixty localized patients were included in the study (39 stage 1, 61 stage 2 and 57 stage 3) (**Table 1**). Patient age ranged from 0 to 6215 days, with a median of 423 days. An estimated 5-years OS and EFS of 94% (+/- 2) and 80% (+/- 3.7), respectively, was observed. The median follow up after diagnosis was 32 months (range 2.7 - 79.5). Most patients (123/160) were treated according to the observation protocol of the GPOH NB04/DCOG NB09 protocol.

Detection of neuroblastoma mRNA in BM samples
In 58/160 patients (36%) neuroblastoma mRNA was detected in BM at diagnosis. In 47 of the 58 positive samples (83%) only one marker was positive (**Figure 1A**). This indicates a very low level of infiltration. PHOX2B was most often positive (46/58; 79%), whereas TH and DDC were only positive in 5 samples (9%). In 11 patients (27%) more than one marker was detected. In only 2 patients all 5 markers showed positive results. Most patients with positive qPCR results had very low levels of infiltration (**Figure 1B**). In 10 patients a level of infiltration of more than 0.01 percent was observed. In most of these patients (8/10) more than one marker was positive.

Association between qPCR positivity and biological factors
Detection of neuroblastoma mRNA was not associated with stage and age (**Figure 2A and B**). However, there was a significant association between unfavorable biological
### Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>Patient characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (days)</strong></td>
<td>423 (0 – 6215)</td>
</tr>
<tr>
<td>Median (range)</td>
<td>423 (0 – 6215)</td>
</tr>
<tr>
<td>&lt; 12 months</td>
<td>68/160 (43%)</td>
</tr>
<tr>
<td><strong>Country</strong></td>
<td></td>
</tr>
<tr>
<td>Netherlands</td>
<td>24/160 (15%)</td>
</tr>
<tr>
<td>Germany</td>
<td>136/160 (85%)</td>
</tr>
<tr>
<td><strong>INSS stage</strong></td>
<td></td>
</tr>
<tr>
<td>Stage 1</td>
<td>39/160 (24%)</td>
</tr>
<tr>
<td>Stage 2</td>
<td>61/160 (38%)</td>
</tr>
<tr>
<td>Stage 3</td>
<td>57/160 (36%)</td>
</tr>
<tr>
<td>Multilocular</td>
<td>2/160 (1%)</td>
</tr>
<tr>
<td>Localized but stage unknown</td>
<td>1/160 (1%)</td>
</tr>
<tr>
<td><strong>Risk group</strong></td>
<td></td>
</tr>
<tr>
<td>Observation</td>
<td>123/160 (77%)</td>
</tr>
<tr>
<td>Medium Risk</td>
<td>22/160 (14%)</td>
</tr>
<tr>
<td>High Risk</td>
<td>15/160 (9%)</td>
</tr>
<tr>
<td><strong>MYCN</strong></td>
<td></td>
</tr>
<tr>
<td>Amplification</td>
<td>15/160 (9%)</td>
</tr>
<tr>
<td>No amplification</td>
<td>144/160 (90%)</td>
</tr>
<tr>
<td>Not analyzed/ unknown</td>
<td>1/160 (1%)</td>
</tr>
<tr>
<td><strong>LOH 1p</strong></td>
<td></td>
</tr>
<tr>
<td>1p normal</td>
<td>112/160 (70%)</td>
</tr>
<tr>
<td>1p aberration</td>
<td>34/160 (21%)</td>
</tr>
<tr>
<td>Not analyzed/ unknown</td>
<td>14/160 (9%)</td>
</tr>
<tr>
<td><strong>Treatment observation group</strong></td>
<td></td>
</tr>
<tr>
<td>Observation</td>
<td>29/123 (24%)</td>
</tr>
<tr>
<td>Complete resection</td>
<td>52/123 (42%)</td>
</tr>
<tr>
<td>Partial resection</td>
<td>10/123 (8%)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>32/123 (26%)</td>
</tr>
<tr>
<td><strong>Survival (years)</strong></td>
<td></td>
</tr>
<tr>
<td>Mean EFS (± SE)</td>
<td>5.2 (±0.2)</td>
</tr>
<tr>
<td>5-years EFS (± SE)</td>
<td>77% (± 3.7)</td>
</tr>
<tr>
<td>Mean OS (± SE)</td>
<td>6.2 (±0.1)</td>
</tr>
<tr>
<td>5-years OS (± SE)</td>
<td>94% (± 2.0)</td>
</tr>
<tr>
<td><strong>Observation time (months)</strong></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>32 (2.7 – 79.5)</td>
</tr>
</tbody>
</table>
Neuroblastoma mRNA in bone marrow of localized patients

Factors and qPCR positivity (Figure 2C and D). In 10/15 patients (67%) with a MYCN amplification qPCR results of the BM were positive, whereas in only 47/144 patients (33%) with a MYCN single copy tumor positive results were observed (p=0.02). Furthermore, in 18/34 patients (53%) with a 1p aberration positive qPCR results were observed, whereas in only 36/112 patients (32%) with a normal 1p status minimal disease was detected.

**Events and qPCR positivity**

In total, 31/160 patients (19%) had an event (23 local progression, 5 progression to stage 4 and 3 patients progression to stage 4s) (Figure 3A). Treatment after disease progression was limited in 12 children (further observation only, surgical resection, N4 chemotherapy). Eight patients received more intensive chemotherapy. Eleven patient who were initially treated in the HRG or MRG, were treated according to different relapse protocols (Supplemental table 1).

In patients with an event, neuroblastoma mRNA in BM at diagnosis was more often detected than in patients without an event, respectively 52% (16/31) versus 32% (42/129) (Figure 3B and C). However, this difference was not statistically significant (p=0.1). For the 31 patients with an event, qPCR results at time of event were also available for 16 patients. In 10/16 patients (63%) qPCR results were positive at time of event (Supplemental table 2). We also analyzed the large observation group separately (n=123), because in this group qPCR positivity might change clinical intervention, in contrast to the patients who were already treated according to the MRG (n=22) and the HRG (n=15).
In this group 21/123 patients (17%) had an event (15 local progression, 3 progression to stage 4 and 3 patients progression to stage 4s) (Figure 3D). There was no difference in qPCR positivity between patients with and without an event, respectively 38% (8/21) versus 31% (31/102) (Figure 3E and 3F). So overall, there was no significant association between qPCR positivity and the number of patients with progressive disease. Furthermore, only very few systemic events occurred in this cohort.

**Systemic events and BM involvement**

Eight patients developed a systemic event (5 progression to stage 4, 3 progression to stage 4s). Six of these 8 patients had qPCR positive BM at diagnosis (3 patients with progression to stage 4 and all 3 patients progressing to stage 4s) (Supplemental table 3). Of the 5 patients progressing to stage 4, 4 patients developed BM metastases and 1 patient developed isolated liver metastases without BM involvement. For 3 of these patients qPCR results were available at time of event and in 2 patients qPCR results were positive at time of event. Two of the patients progressing to stage 4s developed liver metastases, BM was not assessed at that time. The third patient with progression to stage 4s...
Figure 3. Events and qPCR positivity. 

**a.** Number of events in the whole cohort.

**b.** qPCR results in patients with an event in the whole cohort.

**c.** qPCR results in patients without an event in the whole cohort.

**d.** Number of events in the observation group.

**e.** qPCR results in patients with an event in the observation group.

**f.** qPCR results in patients without an event in the observation group.
further progressed to stage 4. This patient developed liver metastases when progressing to stage 4s. BM morphology results were inconclusive at that time point, whereas qPCR results were positive. Progression to stage 4 was diagnosed by a distant lymph node metastasis without morphological BM involvement. In summary, of the 8 patients with systemic events 4 patients had BM disease according to the INSS guidelines at time of event (3 of these 4 patients had qPCR positive BM at diagnosis).

**Association between qPCR positivity and event free survival**

Positive qPCR results were not associated with significant impaired EFS in the whole cohort (n=160; 5-years EFS 70% vs 81% p=0.09) (**Figure 4A**) and in the observation group (n=123; 5-years EFS 78% vs 81% p=0.5) (**Figure 4B**). Also positive qPCR results for one of the single markers was not associated with significant impaired EFS in the

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**Figure 4.** Event free survival for qPCR positive and negative patients. **a.** EFS and qPCR positivity in the whole cohort (grey curve = qPCR pos; black curve = qPCR neg). **b.** EFS and qPCR positivity in the observation group (grey curve = qPCR pos; black curve = qPCR neg). **c.** EFS and qPCR positivity with >1 marker in the whole cohort (grey curve = qPCR neg; black curve = qPCR positive). **d.** EFS and qPCR positivity with >1 marker in the observation group (grey curve = qPCR pos; black curve = qPCR neg).
whole cohort and in the observation group (supplemental figures 2 and 3). However, detection of more than one marker was associated with an unfavorable outcome in the whole cohort (5-years EFS 55% vs 79% p=0.03) (Figure 4C) and the observation group (5-years EFS 56% vs 82% p=0.03) (Figure 4D). Five out of 11 patients with more than one marker positive had an event (2 local progression, 2 progression to stage 4 and 1 patients progression to stage 4s and later to stage 4) (Table 2). In 4 of these 5 patients GD2 immunocytology results were also positive. One of the patients showing progression to stage 4 was already treated according to the high risk protocol. The other patient with progression to stage 4 was initially treated according to the observation protocol and underwent partial resection. The patient with progression to stage 4s and subsequently to stage 4 was only observed until the progression to stage 4. So overall, detection of more than one marker might identify a small group of patients with an increased risk of tumor progression.

Table 2. Characteristics of the patients with >1 marker positive

<table>
<thead>
<tr>
<th>Ptn ID</th>
<th>Stage</th>
<th>Risk group</th>
<th>Gender</th>
<th>Age</th>
<th>MYCN</th>
<th>1p aberration</th>
<th>IC</th>
<th>Treatment</th>
<th>Event</th>
<th>Death</th>
<th>Last control state</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>OG</td>
<td>M</td>
<td>2368</td>
<td>0</td>
<td>0</td>
<td>9</td>
<td>complete resection</td>
<td>no</td>
<td>no</td>
<td>CR</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>OG</td>
<td>M</td>
<td>451</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>complete resection</td>
<td>no</td>
<td>no</td>
<td>CR</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>OG</td>
<td>M</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>observation</td>
<td>progression to stage 4s and further stage 4</td>
<td>no</td>
<td>CR</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>OG</td>
<td>F</td>
<td>21</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>chemotherapy</td>
<td>local progression</td>
<td>no</td>
<td>CR</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>OG</td>
<td>F</td>
<td>274</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>chemotherapy</td>
<td>local progression</td>
<td>no</td>
<td>CR</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>OG</td>
<td>M</td>
<td>373</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>partial resection</td>
<td>progression to stage 4</td>
<td>no</td>
<td>CR</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>OG</td>
<td>M</td>
<td>42</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>complete resection</td>
<td>no</td>
<td>no</td>
<td>CR</td>
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<tr>
<td>8</td>
<td>2</td>
<td>HRG</td>
<td>M</td>
<td>478</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>high risk protocol</td>
<td>progression to stage 4</td>
<td>yes</td>
<td>died</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>OG</td>
<td>F</td>
<td>258</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>observation</td>
<td>no</td>
<td>no</td>
<td>CR</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>OG</td>
<td>M</td>
<td>477</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>chemotherapy</td>
<td>no</td>
<td>no</td>
<td>CR</td>
</tr>
<tr>
<td>11</td>
<td>2</td>
<td>OG</td>
<td>M</td>
<td>455</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>chemotherapy</td>
<td>no</td>
<td>no</td>
<td>CR</td>
</tr>
</tbody>
</table>

Stage: according to the International Neuroblastoma Staging System; Risk group: OG = observation group, HRG = High risk group; Gender: M = male, F= female; Age: in days at diagnosis; MYCN amp: 1= MYCN amplification, 0= MYCN single copy; 1p aberration: 1= 1p aberration, 0= 1p normal; IC (GD2 immunocytology): 1= positive, 0= negative, 3= inconclusive, 9= not available; Last control state (according to the International Neurobastoma Response Criteria): CR = complete remission
Discussion

In this large prospective study we have investigated the clinical significance of neuroblastoma mRNA detection by qPCR in patients with localized disease. This is, to our knowledge, the first prospective study addressing this question in a large cohort of neuroblastoma patients with localized disease.

Several studies have shown that localized patients have a good prognosis (24-29) and that local relapse or progression is the most common event (30), especially in patients observed without cytotoxic treatment (31). In our study only 5 patients (3%) showed progression to stage 4 (3 of these patients had neuroblastoma mRNA detected in BM at diagnosis), and 2 of these patients were already treated according to the high risk treatment protocol. Furthermore, two patients with a systemic event did not have minimal BM disease at diagnosis. Since very few systemic events occurred and qPCR positivity was much more frequent, localized patients with qPCR positive BM should not be upstaged to stage 4.

Deletion of 1p36 and MYCN amplification are the most common chromosomal changes observed in neuroblastoma and are associated with a poor prognosis (32, 33). In our study we show that there is a significant association between detection of neuroblastoma mRNA and deletion of 1p and/or MYCN amplification. These results can be an indication that patients with qPCR positivity may have more aggressive tumors, also in cases without 1p aberration or MYCN amplification. Several other factors that may also affect prognosis in neuroblastoma (gene expression signatures (34), the presence or absence of other structural abnormalities (35) and DNA ploidy (36, 37)) have been described. Unfortunately these type of data were not available for the patients in our study. In a future study it will be interesting to study the association between qPCR positivity in BM and these other prognostic factors.

Although we found an association between the detection of neuroblastoma mRNA and unfavorable biological factors qPCR positivity was not associated with an unfavorable outcome. The high frequency of qPCR positivity in this group of patients and the fact that we did not find an association with outcome may be explained by the very low level of infiltration detected in many patients. It is possible that the detected mRNA originated from circulating and not infiltrating cells. Since it has been demonstrated that also extracellular vesicles can contain tumor specific mRNA (38, 39), this may be another possible source of low levels of mRNA in BM originating from the primary tumor. More aggressive tumors may have more proliferating cells undergoing apoptosis with shedding of tumor RNA. However, this is unknown for neuroblastoma. In this regard it is relevant to mention that in our study RNA was isolated from BM lysed in PAX tubes and not from intact cells. Using this approach also RNA present in microvesicles is isolated.
Indeed Hamaoui et al have used the PAX isolation method for the detection of circulating rhodospin RNA in diabetes patients (40).

When analyzing the patients with more than one marker positive (n=11), we found an association with a worse outcome in the whole cohort and in the observation group. This is in line with the results from Corrias et al. 2008 (10). They detected minimal BM disease in 19/145 patients (13%), by using GD2 immunocytology, and demonstrated that detection of minimal BM disease was associated with an unfavorable outcome. In our study 4 out of 5 relapse patients with more than one marker positive also had minimal disease detected by GD2 immunocytology. This suggests that patients with higher levels of BM infiltration may be at increased risk for disease progression and require more careful attention on their clinical course. However, further studies on even larger cohorts of patients are needed to verify this.

Because localized patients have a good prognosis, a large number of patients need to be studied to identify a group of patients with an increased risk of relapse. Although we have studied a large cohort of 160 patients, survival analyses may be different when an even larger group of patients is studied.

In conclusion, in this group of patients very few systemic events occur, whereas qPCR positivity in BM is frequent. Therefore, patients with localized disease and qPCR positivity should not be upstaged. qPCR positivity was associated with unfavorable biological factors, suggesting that patients with qPCR positivity in BM at diagnosis might have more aggressive tumors. The detection of more than one marker was associated with an unfavorable outcome and therefore for these patients a more careful follow-up is advisable. However, much larger studies will be necessary to identify a group of patients with an increased risk of relapse in this cohort with good prognosis.

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Clinic for Pediatrics, Helios Klinikum Berlin-Buch (L. Schweigerer),
Gilead Children’s Hospital, Bielefeld (N. Jorch),
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Neuroblastoma mRNA in bone marrow of localized patients

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Conflicts of interest statement: none declared
Reference List


Supplemental data

**Supplemental table 1.** Treatment after event

<table>
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<th>Treatment after event</th>
<th>Treatment after event (n=31)</th>
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<td>Observation only</td>
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<tr>
<td>Surgery</td>
<td>3</td>
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<tr>
<td>N4 chemotherapy</td>
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<tr>
<td>N4 chemotherapy and surgery</td>
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<tr>
<td>Intensive chemotherapy (MR or HR protocols)</td>
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<tr>
<td>Other relapse protocol (Initially treated in MRG/HRG)</td>
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**Supplemental table 2.** qPCR results at time of event

<table>
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<tr>
<th>Diagnosis</th>
<th>RQ-PCR pos</th>
<th>RQ-PCR neg</th>
<th>total</th>
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<tr>
<td>At event</td>
<td>RQ-PCR pos</td>
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<td>6</td>
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<tr>
<td></td>
<td>RQ-PCR neg</td>
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<td>5</td>
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| BM available | 16 |

**Supplemental table 3.** qPCR and morphology results for patients with systemic events

<table>
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<th>Patients with systemic events (n=8)</th>
<th>qPCR pos diagnosis</th>
<th>Morphology event</th>
<th>qPCR event</th>
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</thead>
<tbody>
<tr>
<td>Progression to stage 4 (n=5)</td>
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<td>4 pos, 1 neg</td>
<td>2 pos, 1 neg, 2 NA</td>
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<tr>
<td>Progression to stage 4s (n=3)</td>
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<td>1 inconclusive and 2 NA</td>
<td>1 pos, 2 NA</td>
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</table>
Supplemental figure 1. Risk stratification for localized patients in the NB04/DCOG09 protocols
Supplemental figure 2. Event free survival for qPCR positive and negative patients for the single markers in the whole group. a. PHOX2B (grey curve = qPCR pos; black curve = qPCR neg). b. TH (grey curve = qPCR pos; black curve = qPCR neg). c. DDC (grey curve = qPCR pos; black curve = qPCR neg). d. CHRNA3 (grey curve = qPCR pos; black curve = qPCR neg). e. GAP43 (grey curve = qPCR pos; black curve = qPCR neg).
Supplemental figure 3. Event free survival for qPCR positive and negative patients for the single markers in the observation group group. **a.** Phox2B (grey curve = qPCR pos; black curve = qPCR neg). **b.** TH (grey curve = qPCR pos; black curve = qPCR neg). **c.** DDC (grey curve = qPCR pos; black curve = qPCR neg). **d.** CHRNA3 (grey curve = qPCR pos; black curve = qPCR neg). **e.** GAP43 (grey curve = qPCR pos; black curve = qPCR neg).