Minimal residual disease monitoring in neuroblastoma

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Chapter 4

Circulating tumor DNA for disease monitoring in neuroblastoma

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Abstract

Introduction
Circulating tumor DNA (ctDNA) has been used for disease monitoring in several types of cancer. Therefore, the aim of our study was to investigate whether ctDNA can be used for treatment response monitoring in neuroblastoma.

Method
121 plasma samples from 50 patients were analyzed for the detection of hypermethylated RASSF1A DNA. DNA was isolated and total cell free DNA (cfDNA) was determined by qPCR for albumin and the amount of ctDNA was determined by qPCR for methylated RASSF1A after bisulfite conversion. Detection of ctDNA was compared with clinic-biological patient characteristics, such as BM and PB MRD (qPCR), MIBG scans and urinary catecholamines.

Results
In 13/20 diagnostic samples ctDNA was detected. In all 7 diagnostic samples from patients with localized disease no ctDNA was detected. During induction chemotherapy (stage 4 patients only) in 13/42 samples ctDNA was detected (30%). At relapse in 8 out of 14 samples ctDNA was detected (57%). The amount of cfDNA was significantly higher in neuroblastoma patient at time of diagnosis and relapse than in healthy controls and stage 4 patients had the highest amount of cfDNA. There was a significant correlation between ctDNA and PB or BM MRD, when tumor levels were high or no tumor was detected. Discrepancies were observed in 20 samples and were studied in detail. The discrepancies were mainly observed in samples during treatment when tumor burden was lower.

Conclusion
Hypermethylated RASSF1A can be used as marker for monitoring of ctDNA in neuroblastoma patients and highly correlates with the disease status at diagnosis. Our data indicate that there is no correlation when tumor load is lower because during treatment in several samples discrepancies were observed. It is likely that ctDNA can originate from both primary tumor as metastases and may be of special interest for disease monitoring in patients relapsing in other organs than the BM.
Introduction

Neuroblastoma is an extracranial solid tumor of childhood, with a broad spectrum of clinical behavior (1, 2). The presence of metastatic spread at diagnosis is the most important factor in determining outcome in patients with neuroblastoma (3-5). About 50% of patients present with bone marrow (BM) metastases at diagnosis. Patients at the highest risk for disease progression and mortality are those who are older than 18 months of age and have disseminated disease, or localized disease with unfavorable markers such as MYCN amplification (5). High risk patients are treated by a multimodality approach, including chemotherapy, surgical resection and high dose chemotherapy with autologous stem cell rescue (6-8).

To assess the response to treatment, evaluation is based on the International Neuroblastoma Response Criteria (INRC). Results of BM morphological examinations, measurement of urinary catecholamine levels, MIBG scintigraphy and imaging (MRI/PET scans) are combined to assess the extent of disease (9). Immunocytology and qPCR are more sensitive techniques to detect and monitor BM metastases in neuroblastoma and several prospective studies are ongoing or have been published investigating the clinical significance of these techniques for BM response evaluation in high risk neuroblastoma (10-12). However, many patients with negative MRD results during treatment can suffer from recurrent disease.

Circulating DNA fragments that carry tumor specific alterations (circulating tumor DNA; ctDNA) can be found in plasma of cancer patients (13, 14). Several studies have shown the feasibility of using ctDNA to monitor disease in various types of cancer (15-20). In neuroblastoma detection of ctDNA at diagnosis has been described with the use of methylated RASSF1A, MYCN, MPO/Survivin (located on 17q) and ALK as markers (21-25). However, in all these studies ctDNA has only been used at diagnosis and was not used for disease and treatment monitoring. Therefore, the aim of our study was to investigate whether ctDNA, by using hypermethylated RASSF1A as marker (26), can be used to monitor treatment response in neuroblastoma patients and is of additive value compared with other techniques like MIBG scans, urinary catecholamines and qPCR based MRD detection in BM and PB.

Methods

Patients and samples

Peripheral blood samples (n=121) from 50 neuroblastoma patients were collected between 2013 and 2015. Patients were treated at the Emma Children’s Hospital/AMC, Amsterdam, the Sophia Children’s Hospital/EMC, Rotterdam or the Princess Maxima Center for
Pediatric Oncology, Utrecht in the Netherlands. 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). Peripheral blood samples from healthy donors (n=75) (healthy male volunteers or regular blood donors) at Sanquin Blood Supply Amsterdam were collected as controls. Blood samples were collected in EDTA tube and centrifuged within 24 hours to separate plasma from the peripheral blood cells (2420 rpm for 10 minutes, without brake). Subsequently, the plasma was stored at - 20 °C.

**DNA isolation and bisulfite conversion**

Depending on the available amount of plasma, DNA was extracted by using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) (for 200µl plasma) or using the MagNA Pure 96 isolation robot (Roche, Basel, Switzerland) (for 500- 1000 µl plasma).

After isolation, bisulfite conversion was performed using the EpiTect Bisulfite Kit (Qiagen) according to the instructions of the manufacturer. The sample was eluted in 40 µl. Converted DNA samples were used directly or stored at - 20 °C.

**Real-time quantitative PCR**

Real-time quantitative PCR (qPCR) was performed on the StepOnePlus™ thermal cycler (Applied Biosystems, Foster city, CA, USA). Reactions were carried out in 20 µl, consisting of 2.6 µl distilled water, 0.8 µl of 7.5 µM forward primer, 0.8 µl of 7.5 µM reverse primer, 0.8 µl of 5.0 µM TaqMan® probe, 10 µl TaqMan® Fast Universal PCR Master Mix (Applied Biosystems®) and 5.0 µl DNA sample.

Primers and probes were obtained from Eurogentec (Liege, Belgium). Primer and probe sequences have been described previously (26). To control for DNA input albumin (before bisulfite conversion) and β-actin (after bisulfite conversion) PCRs were carried out. Subsequently, PCRs for unmethylated and methylated RASSF1A were performed. PCRs for albumin, β-actin and unmethylated RASSF1A were performed in duplicate, methylated RASSF1A was tested in triplicate. Calibration (standard) curves were made using DNA from IMR-32 cell line for methylated RASSF1A and pooled mono-nuclear cells (MNC) from healthy individuals for unmethylated RASSF1a.

**PB and BM MRD assays**

Total RNA from whole blood and BM samples was extracted using the PAX blood RNA tubes and kit (PreAnalytix, Qiagen, Germany) according to the instructions of the manufacturer. cDNA was synthesized and qPCR for paired-like homeobox 2B (PHOX2B), tyrosine hydroxylase (TH), dopamine decarboxylase (DDC), cholinergic receptor 3 (CHRNA3), dopamine beta-hydroxylase (DBH) and growth associated protein 43 (GAP43) was performed using β-glucorindase (GUS) for normalization as has been described previously (27).
Data analysis

Clinical data (including urinary catecholamines and imaging data (MIBG score (28))) were collected from electronic patient files at the different hospitals involved in the study. Circulating tumor DNA was scored positive not quantifiable if not all wells of the triplicate were positive or the variations between replicates was > 1.5 dCt (29). Quantification of methylated RASSF1A was performed relative to cell line IMR32. For quantification with RNA markers, relative values were calculated using the formula: \(2^{\Delta \Delta \text{Ct}}\) (dCt sample – dCt IMR32) * 100%. The median relative expression of 5 markers was used for the analysis. Correlation analysis between ctDNA and PB and BM MRD levels were done using Spearman’s test. Unpaired t-tests were performed to compare significant differences between groups. All statistical analyses were performed in SPSS version 22 or Graphpad prism version 6.

Table 1. Patient characteristics.

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of patients</td>
<td>50</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
</tr>
<tr>
<td>male</td>
<td>30</td>
</tr>
<tr>
<td>female</td>
<td>21</td>
</tr>
<tr>
<td><strong>Age at diagnosis (months)</strong></td>
<td></td>
</tr>
<tr>
<td>minimum</td>
<td>0.6</td>
</tr>
<tr>
<td>maximum</td>
<td>395</td>
</tr>
<tr>
<td>mean</td>
<td>51.5</td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
</tr>
<tr>
<td>stage 4</td>
<td>37</td>
</tr>
<tr>
<td>stage 4s</td>
<td>3</td>
</tr>
<tr>
<td>stage 1,2 and 3</td>
<td>9</td>
</tr>
<tr>
<td>unknown</td>
<td>1</td>
</tr>
<tr>
<td><strong>MYCN</strong></td>
<td></td>
</tr>
<tr>
<td>amplified</td>
<td>12</td>
</tr>
<tr>
<td>not amplified</td>
<td>34</td>
</tr>
<tr>
<td>unknown</td>
<td>4</td>
</tr>
<tr>
<td><strong>Chr 1p status</strong></td>
<td></td>
</tr>
<tr>
<td>1p del</td>
<td>11</td>
</tr>
<tr>
<td>1p normal</td>
<td>27</td>
</tr>
<tr>
<td>unknown</td>
<td>12</td>
</tr>
</tbody>
</table>

*Stage according to INSS
Results

Patients and samples
From 50 children 121 samples were tested in this study. Patient characteristics are shown in table 1. The mean age at diagnosis was 51.5 months. Most patients had stage 4 disease (n=36). Primary tumor MYCN status was available for more than 90% of children; of these, 27% had MYCN amplification.

Methylated RASSF1A PCR was performed for 75 control samples. Occasionally (in 3/75 samples) amplification was observed, however this never occurred in all of the three replicates.

From 20 patients a sample at diagnosis was available and in 13 of these samples ctDNA was detected (12 of these patients had stage 4 disease and 1 patient stage 4s)). In all 7 diagnostic samples from patients with localized disease no ctDNA was detected. During induction chemotherapy (all samples were from stage 4 patients) in 13/42 samples ctDNA was detected (30%). At relapse in 8 out of 14 samples ctDNA was detected (57%). Results are summarized in table 2.

Amount of cell free DNA and circulating tumor DNA
The amount of cell free DNA (cfDNA) per ml of plasma was determined by using albumin PCR in 75 control samples and compared with the samples from neuroblastoma patients at diagnosis or relapse (n=34). Neuroblastoma patients at diagnosis or relapse had significantly more cell free DNA than healthy controls. Stage 4 patients had the highest amount of cfDNA (figure 1A). The amount of cfDNA was highest at diagnosis, decreased during and after induction therapy and was again higher at time of relapse (figure 1B).
the samples where ctDNA was detected (n= 42), the amount of cfDNA was significantly higher than in the healthy controls. In the 7 patients, where no ctDNA was detected at diagnosis, cfDNA levels were comparable with control samples (grey triangles) (figure 1C). At diagnosis levels of ctDNA were highest, decreased during induction therapy and were not detected after autologous stem cell transplantation (ASCT). At relapse the amount of ctDNA was comparable to levels at diagnosis (figure 1D).

Figure 1. Amount of cell free and circulating tumor DNA. a. Comparison of amount cell free DNA between neuroblastoma patients and healthy donors. b. Amount of cell free DNA at different time points during treatment. c. Amount of cell free DNA in samples with circulating tumor DNA compared with samples from healthy donors and samples without circulating tumor DNA detected. d. Amount of circulating tumor DNA at different time points during treatment.
Comparison of ctDNA and the detection of neuroblastoma mRNA in blood and BM

Data comparing ctDNA and the detection of neuroblastoma mRNA in blood samples were available for 69 serial time points (14 samples were derived at diagnosis or relapse) (figure 2). There was a significant correlation of qualitative (positive or negative) results between PB MRD and ctDNA, with 84% concordant results. qPCR based MRD was detected in 26 samples and ctDNA was detected in 25 samples, with 38 double negative, 20 double positive and 11 samples positive for either ctDNA (n=5) or PB MRD (n=6). In 8 samples high levels of ctDNA were observed whereas PB MRD was not detectable or very low levels were detected (figure 2, blue circle). In 4 samples PB MRD levels were relatively high whereas ctDNA was not detected (figure 2, orange circle). These samples will be discussed in detail below. These discrepancies were mainly observed in samples obtained during treatment (10/12).

Correlation Pb MRD and ctDNA

Figure 2. Correlation between PB MRD and ctDNA. PB MRD level relative to IMR32. Circulating tumor DNA level relative to IMR32. In blue the samples with relatively high ctDNA levels compared with PB MRD. In red the samples with relatively high PB MRD compared with ctDNA. The open circles indicated relapse/diagnosis samples.
Data comparing ctDNA and the detection of neuroblastoma mRNA in BM samples were available for 40 serial time points (13 samples were derived at diagnosis or relapse) (figure 3). BM MRD was detected in 20 samples and ctDNA was detected in 19 samples. There was a significant correlation of qualitative (positive or negative) results between BM MRD and ctDNA, with 92% concordant results. 18 out of 40 samples were positive for both qPCR based BM MRD and ctDNA, 19 samples were double negative and only 3 samples were positive for either ctDNA (n=2) or BM MRD (n=1). However, in 7 samples high levels of ctDNA were found whereas BM MRD was negative or very low levels were detected (figure 3, blue circle). In 6 samples relatively high levels of BM MRD were found whereas ctDNA levels were negative or very low (figure 3, orange circle). These samples will be discussed in detail in the next paragraph. These discrepancies were mainly observed in samples obtained during treatment (10/14).

Figure 3. Correlation between BM MRD and ctDNA. BM MRD level relative to IMR32. Circulating tumor DNA level relative to IMR32. In blue the samples with relatively high ctDNA levels compared with PB MRD. In red the samples with relatively high PB MRD compared with ctDNA. The open circles indicated relapse/diagnosis samples.
Table 3. Assessment of discrepant samples

<table>
<thead>
<tr>
<th>ptn ID</th>
<th>Age</th>
<th>Stage</th>
<th>Time point</th>
<th>ctDNA vs PB or BM MRD</th>
<th>Imaging PT</th>
<th>Comments</th>
<th>Urinary catecholamines</th>
</tr>
</thead>
<tbody>
<tr>
<td>2001</td>
<td>6.2</td>
<td>4</td>
<td>Diagnosis</td>
<td>RASSF1A_M 0.61875, PB_MRD 0.0003, BM_MRD 0.00009</td>
<td>1</td>
<td>Large PT, diaphragm metastases</td>
<td>Y</td>
</tr>
<tr>
<td>N732</td>
<td>1.56</td>
<td>4</td>
<td>Local relapse</td>
<td>48.54122, PB_MRD 0.0002, BM_MRD 0.00005</td>
<td>0</td>
<td>Local relapse</td>
<td>N</td>
</tr>
<tr>
<td>N841</td>
<td>0.7</td>
<td>4</td>
<td>Diagnosis</td>
<td>29.83121, PB_MRD 0.03, BM_MRD 0.6</td>
<td>7</td>
<td>Cervical mass</td>
<td>Y</td>
</tr>
<tr>
<td>N841</td>
<td>0.7</td>
<td>4</td>
<td>Induction chemotherapy</td>
<td>Pos NQR, PB_MRD 0.00002, BM_MRD Neg</td>
<td>8</td>
<td>Stable</td>
<td>N</td>
</tr>
<tr>
<td>N850</td>
<td>4.15</td>
<td>4</td>
<td>Diagnosis</td>
<td>27, PB_MRD Neg, BM_MRD 0.00001</td>
<td>857.8ml</td>
<td>MIBG negative</td>
<td>Y</td>
</tr>
<tr>
<td>N850</td>
<td>4.15</td>
<td>4</td>
<td>Induction chemotherapy</td>
<td>0.15475, PB_MRD Neg, BM_MRD 0.00002</td>
<td>384.5ml</td>
<td></td>
<td>N</td>
</tr>
<tr>
<td>N777</td>
<td>9.42</td>
<td>4</td>
<td>9 months after ASCT</td>
<td>PB_MRD Neg 0.0003, BM_MRD Neg</td>
<td>0</td>
<td>CR</td>
<td>N</td>
</tr>
<tr>
<td>N798</td>
<td>6.59</td>
<td>4</td>
<td>15 months after ASCT</td>
<td>PB_MRD Neg 0.0008</td>
<td>0</td>
<td>CR</td>
<td>N</td>
</tr>
<tr>
<td>N802</td>
<td>4.7</td>
<td>4</td>
<td>Relapse therapy</td>
<td>PB_MRD Neg 0.00004, BM_MRD Neg</td>
<td>missing Relapse CNS</td>
<td></td>
<td>N</td>
</tr>
<tr>
<td>N834</td>
<td>1.78</td>
<td>4</td>
<td>Induction chemotherapy</td>
<td>PB_MRD Neg neg 0.002</td>
<td>0</td>
<td>CR</td>
<td>N</td>
</tr>
<tr>
<td>N848</td>
<td>0.24</td>
<td>4s</td>
<td>Diagnosis</td>
<td>PB_MRD Pos NQR 0.003, BM_MRD 0.05</td>
<td>2</td>
<td></td>
<td>Y</td>
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<tr>
<td>N621</td>
<td>4.35</td>
<td>4</td>
<td>Relapse</td>
<td>PB_MRD Pos NQR 0.02, BM_MRD 0.14</td>
<td>20</td>
<td>No PT</td>
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<tr>
<td>N621</td>
<td>4.35</td>
<td>4</td>
<td>Relapse therapy</td>
<td>PB_MRD 6.01635, BM_MRD Neg 0.05</td>
<td>14</td>
<td>No PT</td>
<td>Y</td>
</tr>
<tr>
<td>N621</td>
<td>4.35</td>
<td>4</td>
<td>Relapse therapy</td>
<td>PB_MRD Pos NQR 0.00001, BM_MRD 1</td>
<td>9</td>
<td>No PT</td>
<td>N</td>
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<tr>
<td>N649</td>
<td>1.96</td>
<td>4</td>
<td>Relapse therapy</td>
<td>PB_MRD 1.65886, BM_MRD 0.0003</td>
<td>20</td>
<td>New lesion</td>
<td>Y</td>
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<tr>
<td>N649</td>
<td>1.96</td>
<td>4</td>
<td>Relapse therapy</td>
<td>PB_MRD 6.71248, BM_MRD 0.00002</td>
<td>20</td>
<td>stable</td>
<td>Y</td>
</tr>
<tr>
<td>N649</td>
<td>1.96</td>
<td>4</td>
<td>Relapse therapy</td>
<td>PB_MRD 0.001, BM_MRD 0.007</td>
<td>0.2</td>
<td>stable</td>
<td>Y</td>
</tr>
</tbody>
</table>

Age in years; RASSF1A_M level relative to IMR32 = ctDNA; PB and BM MRD level relative to IMR32; red: relatively high level of PB or BM MRD compared with ctDNA; blue: relatively high level of ctDNA compared with PB or BM MRD; MIBG = curie score (28); VMA and HVA in millimol/mol creatinine, Y= elevated, N= not elevated
Assessing discrepant findings between ctDNA and PB or BM MRD

Quantitative discrepant results when comparing PB MRD or BM MRD and ctDNA were found for 13 samples matched with BM MRD and 12 samples matched with PB MRD. In 5 samples discrepancies for both PB and BM MRD as compared with ctDNA were observed. Therefore, for these 20 discrepant samples from 12 patients we summarized the results in table 3. If available, imaging and urinary catecholamine results of the same time point were retrieved.

In the samples from 5 patients (2001, N732, N841, N850 and N770) relatively high levels of ctDNA were detected compared with PB or BM MRD. In all these patients no or very little BM infiltration was detected, however these patients had large primary or relapse tumors and elevated urinary catecholamines. Patient 2001, N850 and N841 had very low levels of BM infiltration at diagnosis and BM became negative during treatment. Patients N732 and N770 suffered from local recurrences, without BM involvement. Therefore, in these patients with high tumor load without or with minimal BM involvement it is likely that the ctDNA originated from the primary tumor.

In the samples from patients N777, N798, N834 and N802 no ctDNA was detected, whereas very low levels of PB or BM MRD were detected. N777, N798 and N834 were all in complete remission at that time point (MIBS score 0 and no elevated urinary catecholamines) and subsequent samples (if available) showed negative MRD results. Therefore, it is likely that the tumor load in these patients is very low. N802 was treated for a central nervous system (CNS) relapse. Therefore, also the tumor load is very low in this patient.

In the diagnosis sample from stage 4s patient N848 relatively high levels of BM MRD were detected compared with ctDNA. It should be mentioned that RASSF1A hypermethylation can be variable in stage 4s and localized patients and therefore we may underestimate the level of ctDNA in this sample.

In relapse patients N649 and N621 fluctuating levels of ctDNA were detected during relapse treatment. Both patients suffered from mainly metastatic relapses and it is therefore likely that the ctDNA originated from these metastases and that the fluctuating levels of ctDNA were caused by therapy effects.

In summary, ctDNA can originate from both primary tumor as metastases. Furthermore, in 2 patients levels of ctDNA can fluctuate during treatment.

Discussion

In this study we show that the detection of ctDNA in plasma is a suitable non-invasive tool for disease monitoring in neuroblastoma. Hypermethylated RASSF1A was selected as marker because this gene has been shown to be frequently hypermethylated in stage 4 neuroblastoma tumors and can be used
as DNA marker for MRD detection in neuroblastoma (26). Furthermore, detection of RASSF1A in pretreatment serum of 64 neuroblastoma patients from all stages was described as a prognostic marker (21).

In breast cancer and other solid cancers circulating tumor DNA has been demonstrated as sensitive tool for treatment response assessment (18-20). To our knowledge we are the first to report on ctDNA in neuroblastoma for disease monitoring.

We first investigated whether levels of cfDNA are higher in neuroblastoma patients than in healthy controls. Indeed at diagnosis and relapse neuroblastoma patients had significantly higher levels of cfDNA. Stage 4 patients had the highest amounts of cfDNA. During induction chemotherapy these levels decreased and were high again at relapse. Suggesting that with increase of tumor burden cfDNA levels also increase. This was supported by analyzing the amount of cfDNA in the samples where we detected ctDNA. Indeed samples where we detected ctDNA had higher levels of cfDNA than healthy individuals. Several studies have indeed shown that higher concentrations of cfDNA are present in cancer patients (30-32).

Hypermethylation of RASSF1A has been described in several types of cancer and in physiological circumstances in placental cells (33). Furthermore, RASSF1A is not methylated in normal hematological cells (26, 33, 34). In our study we tested 75 plasma samples from healthy individuals. In 3 out of these 75 samples we detected very low levels of hypermethylated RASSF1A. Also in other studies infrequent detection of hypermethylated RASSF1A has been observed in plasma samples from healthy controls (35, 36). When detecting very low levels of hypermethylated RASSF1A in neuroblastoma patients (indicated as positive not in quantitative range), results should be analyzed with caution.

We tested 121 samples from 50 neuroblastoma patients and detected ctDNA in 42 samples. At diagnosis in 13/20 diagnosis samples (62%) ctDNA was detected (12/12 positive samples from stage 4 patients and 1/8 positive samples from localized or 4s patients). Misawa et al. described detection of hypermethylated RASSF1A in 17/68 patients (25%), however most of these patients had localized disease. In 11/18 stage 4 patients hypermethylated RASSF1A was observed in serum (61%). Our group has previously described that hypermethylation of RASSF1A is variable in stage 4s and localized patients (26), therefore the level of ctDNA can be underestimated in these patients when using hypermethylated RASSF1A as marker.

We compared the performance of ctDNA with PB and BM MRD in 69 and 40 samples respectively. There was a strong correlation between ctDNA and BM MRD when tumor burden was high or no tumor was detected. However, in some samples discrepancies were observed. These discrepant samples were studied in detail and additional clinical data concerning tumor response status (imaging and urinary catecholamines) were retrieved. In the samples where we detected relatively high levels of ctDNA compared with PB or BM MRD it is very likely that the ctDNA originated from the primary tumor,
since at time of sampling for these patients still considerable tumor amounts were present. In samples from four patients with very low levels of PB or BM MRD no ctDNA was detected. Three of these patients were in complete remission. In two patients with metastatic relapses fluctuating levels of ctDNA were observed in response to treatment, possibly because dying tumor cells can shed more cell free DNA.

It would be of interest to investigate whether detection of hypermethylated RASSF1A at diagnosis and during and after induction chemotherapy in stage 4 patients is associated with an unfavorable outcome. We need to increase our sample size and follow up time to be able to perform these survival analyses.

Since we show that it is likely that ctDNA can originate from both primary tumor and metastases we investigated whether ctDNA can have additive predictive value in relapsing patients without detectable BM MRD after 2 courses of GD2 immunocytology (11) in 45 serum samples (data not shown). By using ctDNA as biomarker we were able to detect an additional 5 patients, however only shortly before the relapse became clinically evident. So overall, at this time point detection of ctDNA was not of predictive value. However, interestingly 3 of these 5 patients had a relapse in the central nervous system. Indeed Kramer et al. described that the CNS may be a sanctuary site for neuroblastoma and that with the improvements in therapy there may be an increase in CNS relapses. Frequently in patients with relapses in the CNS this is the sole site of recurrence (37, 38). Therefore, it will be interesting to look into specific subgroups of patients relapsing in other organs than the BM and this work is currently ongoing.

In concluding, hypermethylated RASSF1A can be used as marker for the detection of ctDNA in neuroblastoma patients. Detection and monitoring of ctDNA is a non-invasive tool for response assessment and is likely to originate from both primary tumor and metastases. Detection of ctDNA highly correlates with disease status as evaluated by PB and BM MRD, urinary catecholamines and imaging (MIBG scans and MRI), mainly when high tumor levels are present or no tumor is detected. During treatment discrepancies were observed in level between ctDNA and PB or BM MRD. Survival analyses need to be performed to assess the prognostic value of detection of ctDNA at diagnosis and during or after induction chemotherapy. Furthermore, detection of ctDNA may be of additive value to monitor disease or detect relapse in patients without BM involvement.
Reference List

ctDNA for disease monitoring in neuroblastoma


