Minimal residual disease detection and monitoring in children with neuroblastoma
Stutterheim, J.

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Minimal residual disease detection and monitoring in children with neuroblastoma
Chapter 7

Methylated RASSF1a is the first specific DNA marker for minimal residual disease testing in neuroblastoma
(submitted)

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Abstract

Purpose
PCR-based detection of minimal residual disease (MRD) in neuroblastoma is presently based on neuroblastoma-specific transcripts. However, the expression of these targets varies between patients and upon treatment, and only PHOX2B is truly specific. RASSF1a is methylated (RASSF1aM) in neuroblastoma and we investigated whether it can serve as a specific and stable DNA MRD marker.

Patients and methods
The RASSF1aM specific real-time quantitative (RQ)-PCR was tested on control bone marrow (BM) (n=50), on 71 neuroblastoma tumors and on 159 clinical BM samples at diagnosis and at follow up of 77 patients. Results were compared to a panel of RNA markers and correlated with prognosis.

Results
RASSF1aM was present in all stage 4 and 4s tumors (n=50) and in 86% stage 1-3 tumors (n=21). The level of methylation in stage 4 neuroblastoma was correlated with overall survival (p=0.02). RASSF1aM-PCR was highly specific (only one amplification in 50 control samples tested in triplicate) and had a similar sensitivity as the RNA-based PCRs as shown on clinical samples. Moreover, RASSF1aM enabled accurate quantification without need for the original tumor.

Conclusion
RASSF1aM is a novel, highly specific DNA marker for MRD detection in neuroblastoma, equal to PHOX2B in specificity and sensitivity, and better suitable for MRD quantification. We propose to include RASSF1aM in further prospective MRD studies in neuroblastoma alongside RNA MRD markers. In addition, this assay can be used for sensitive detection and quantitation of circulating cells in all cancers with hypermethylated RASSF1a.
Introduction

In the search for specific methods to assess bone marrow (BM) in patients with neuroblastoma (NB), real-time quantitative PCR (RQ-PCR) using RNA markers\textsuperscript{1-3} and GD2-immunocytology\textsuperscript{4} are now being standardized for monitoring minimal residual disease (MRD).\textsuperscript{5} So far, PHOX2B is the only neuroblastoma specific marker for MRD detection in NB using RQ-PCR.\textsuperscript{6} To overcome heterogeneity in expression between tumors, detection of tumor cells by RQ-PCR is preferably done using a panel of neuroblastoma specific RNA markers which detects tumor cells with a sensitivity up to 1 in 10\textsuperscript{6} normal nucleated bone marrow cells.\textsuperscript{7}

The expression of the RNA markers in primary tumors varies with a factor 1000.\textsuperscript{7} So for accurate quantification of MRD levels the primary tumor is needed and it is furthermore not known whether these markers are stably expressed during treatment. DNA is more stable than RNA and is not dependant on level of gene expression. Thus a DNA marker will enable more accurate quantification of MRD in BM of NB patients. However, a general neuroblastoma specific DNA aberration has not been found yet. Common epigenetic changes, such as aberrant DNA methylation, have been described in neuroblastoma.\textsuperscript{8-10} A gene, which is often silenced by promoter region methylation\textsuperscript{11} in neuroblastoma and not in normal tissues, is RASSF1a.\textsuperscript{8-10, 12, 13}

The purpose of our study was to determine whether methylated RASSF1a (RASSF1a\textsubscript{M}) can serve as a DNA RQ-PCR marker for MRD detection in patients with NB with similar sensitivity and specificity as RNA markers and to determine whether MRD levels in the BM can be more accurately quantified using this marker.
Materials and Methods

Patients and samples
BM samples of patients with NB, treated at the Emma Children’s Hospital/AMC, Amsterdam, the Netherlands, have been collected between 1995 and 2010. We selected samples with already available RQ-PCR results: 77 BM samples obtained at diagnosis from 45 patients with INNS14 stage 4, 26 stage 1-3 and 6 stage 4s, and 50 BM samples during treatment from 28 patients with stage 4. From 64 of these patients primary tumor was available. From an additional 7 patients the primary tumor was tested without corresponding BM samples. Patients’ characteristics are shown in supplementary table 1. Written informed consent was obtained from parents/guardians. The Medical Research Ethics Committee of the AMC approved the study.

Control samples
To determine background of the RASSF1aM RQ-PCR in normal BM, BM samples obtained from 50 children with acute lymphoblastic leukemia in molecular remission, as determined by antigen-receptor RQ-PCR, were tested.15

RNA extraction, reverse transcription and real-time quantitative PCR
Total cellular RNA from tumor and BM samples was extracted with Trizol according to the manufacturer’s instructions (Invitrogen, Carslbad, USA). cDNA was synthesized as described.6 RQ-PCR for PHOX2B, tyrosine hydroxylase (TH), dopamine decarbohydroxylase (DDC), cholinergic receptor3 (CHRNA3) and growth associated protein 43 (GAP43) was performed using β-glucuronidase (GUS) for normalization.7

DNA extraction and methylation specific real-time quantitative PCR
DNA was extracted using the Qiamp DNA blood mini kit. Bisulfite conversion was with an Epitect Bisulfite kit (both, Qiagen, Germantown, USA). Input for bisulfite treatment was 100ng for tumor samples and 2μg for BM samples and eluted in 40μl. To control for DNA input, β-actin RQ-PCR was performed. Primers and probes for β-actin and RASSF1aM were modified from Lehmann et al.16 Primers and probe for unmethylated RASSF1a (RASSF1aU) were designed in the same region (spanning exon 1) as RASSF1aM, primer-probe combinations listed supplementary table 2. Oligonucleotides were synthesized by Eurogentec (Liège, Belgium). RQ-PCR was performed in a Step-one-Plus (PE Biosystems, Darmstadt, Germany). Reactions were carried out in 20μl (10μl Taqman Fast Universal PCR
Mastermix (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands), 2.6μl H₂O, 300nM forward and reverse primer, 200nM Taqman probe, 5μl (c)DNA starting with 20 sec 95°C followed by 50 cycles (1 sec 95°C, 20 sec 60°C). RQ-PCR assays for RASSF1aₘ were carried out in triplicate, RASSF1aₜ and β-actin in duplicate.

**Assay sensitivity by in vitro serial dilutions**

Sensitivity of the RQ-PCR assay was assessed by 10-fold diluting DNA isolated from IMR32 neuroblastoma cells into mononuclear cells (MNC) DNA. Sensitivity was defined as the lowest dilution with at least one positive replicate ≥ 1.0 Ct lower than the lowest Ct in MNC. The quantitative range was defined as the lowest dilution giving a reproducible amplification (dCt of all replicates ≤ 1.5) and with a mean Ct value within 2.6-4.0 Cts from the previous dilution point, with all Ct values ≥ 3.0 lower than the lowest Ct of MNC.¹⁷

**Data and statistical analysis**

Clinical samples were scored positive if at least one of three replicates was ≥ 1.0 Ct lower than the lowest Ct in control BM.

The percentage of RASSF1a methylation was defined as; [RASSF1aₘ] / ([RASSF1aₘ] + [RASSF1aₜ]). In tumors we corrected for the percentage of tumor cells quantified by morphology.

For quantification with RNA markers, relative values were calculated using the formula: 2^ddCt (dCt primary tumor– dCt BM diagnosis) * 100%.

The median relative expression was determined by taking the median of the relative values of all PCR targets.

To compare levels of methylation the Mann-Whitney test was used, correlation analyses were done using Spearman's test. Survival analyses were done using Kaplan-Meier and log rank statistics, all in SPSS 15.0.
**Results**

**DNA methylation of RASSF1a in neuroblastoma tumors**
We first tested RASSF1a methylation status in 71 NB tumors. DNA was bisulfite converted and tested with an RQ-PCR specific for methylated RASSF1a and one specific for unmethylated RASSF1a.

In the neuroblastoma cell line IMR32 only methylated RASSF1a sequence and in normal white blood cells only unmethylated RASSF1a sequence was detected (figure 1). In all stage 4 (n=45) and 4s (n=5) tumors RASSF1a was methylated, with a median percentage of 88% (range 11-100) and 65% (range 21-100), respectively. In non-metastatic NB (stage 1-3) RASSF1a\textsubscript{M} was detected in 18 of 21 (86%) of tumors with a median percentage of 30% (range 0-100).

**Background of methylated RASSF1a in control bone marrow samples**
To define threshold for positivity in BM, we determined the background of RASSF1a\textsubscript{M} in control BM (supplementary table 3). In only 1 out of 50 BM samples amplification in one of the triplicates was observed (Ct 40.9). The threshold for positivity for RASSF1a\textsubscript{M} in BM was arbitrarily set on 39.9.

**Sensitivity of the RASSF1a\textsubscript{M} RQ-PCR assay**
The sensitivity of RASSF1a\textsubscript{M} was tested by assessing dilutions of IMR32 DNA into normal MNC DNA. The percentage tumor cells measured correlated with the percentage tumor cells expected (quantitative range of 10\textsuperscript{-4}) (figure 2), indicating that the percentage of tumor cell infiltration can be accurately quantified assuming that all tumor cells are methylated. The assay reached a sensitivity of detection of 1 tumor cell in 10\textsuperscript{5} MNC (10\textsuperscript{-5}), equivalent to detection of 2.5pg (~ 1 allele).
RASSF1aM, RASSF1aU, and β-actin were tested on white blood cells (WBC) from a healthy control, on IMR32 cell line cells, and on neuroblastoma tumors of different disease stages: stage 1-3 (n=21), stage 4s (n=5), stage 4 (n=45).

Sensitivity of the RQ-PCR assay was assessed by diluting DNA isolated from IMR32 neuroblastoma cells into MNC DNA to obtain a dilution range from $10^{-1}$ to $10^{-6}$. Each dilution was tested in triplicate for RASSF1aM. In the table the average Ct values of RASSF1aM are depicted with its standard deviation (sd). The percentage of tumor cells was quantified using the formula: $\frac{[\text{RASSF1a}_M]}{([\text{RASSF1a}_M] + [\text{RASSF1a}_U])}$. The table shows the number of NB cells in 1 million normal WBCs, mean Ct value, theoretical percentage tumor cells, and quantified percentage tumor cells.
Comparison of RASSF1aM RQ-PCR results with BM cytology

The RQ-PCR results for RASSF1aM of all available BM samples (n=127; 77 at diagnosis (13 stage 1 or 2, 13 stage 3, 6 stage 4s and 45 stage 4) and 50 during treatment from 28 stage 4 patients) were compared to BM cytology; As expected all BM samples from stage 1 and 2 patients were negative for RASSF1aM, showing again the specificity of the assay (table 1a). In contrast, in 7 out of 13 BM samples from stage 3 patients RASSF1aM was detected, but in none of these samples all triplicates were positive (table 1a), indicating a low level of infiltration (supplementary table 3). In stage 4 and 4s patients, RASSF1aM was positive in all cytology positive samples (n=39). Furthermore, in 9 cytology negative samples, RASSF1aM was positive. In samples taken at follow-up from stage 4 patients many cytology-negative samples were positive for RASSF1aM (n=18), (table 1b).

Comparison of RASSF1aM RQ-PCR results with RQ-PCR results of a panel of RNA markers in BM samples

To investigate the performance of RASSF1aM for MRD-testing, the assay was tested on clinical samples (n=127), which were also tested with a panel of RNA markers. As shown in table 1, in 79% of the samples both assays gave similar results (71 positive and 37 negative samples). In 10 samples the RASSF1aM was negative, while the RNA panel gave a positive result, and vice versa in 9 cases the RASSF1aM was positive and the RNA panel negative. Most discrepant samples were seen in patients with low tumor load (BM obtained from patients at diagnosis with stage 1 – 3 and BM obtained during or after treatment from stage 4 patients). RASSF1aM detected one extra positive sample compared to the RNA panel in a stage 4s patient at diagnosis, and one panel positive sample of a stage 4 patient at diagnosis was negative for RASSF1aM (table 1a). The MRD level of this latter sample was low, since PHOX2B was the only positive marker of the panel. The tumor of the only stage 4 patient in which both DNA and RNA markers did not detect MRD in the BM at diagnosis, showed 90% RASSF1a methylation and had high expression of all RNA markers.

In conclusion, the performance of the RASSF1aM–PCR on clinical BM samples of NB patients is comparable to that of the PCR panel of RNA markers.
Results of PCR based quantification by either RASSF1a<sub>m</sub> or a panel of PCR targets in BM samples at diagnosis (n=21) and BM samples at follow up (n=9). Quantification by PCR targets was performed relative to the primary tumor and the median relative expression of 5 RNA PCR targets is depicted. Spearman’s tests were performed to assess the significance of the relationship between the two variables.
**Figure 4.** Correlation between tumor cell infiltration in the bone marrow at diagnosis and survival in patients with stage 4 disease older than 1 year of age.

Survival curve according to percentage of RASSF1a methylation in BM at diagnosis: black line = low infiltration (<1%) (n=15), grey line = medium infiltration (1-20%) (n=19), dotted line = high infiltration (>20%) (n=7); log rank test, 0.04.

**Figure 5.** High levels of RASSF1a methylation in neuroblastoma tumors is correlated with poor outcome in patients with stage 4 disease older than 1 year of age.

Survival curve according to percentage of RASSF1a methylation in neuroblastoma tumors of patients with stage 4 disease older than 1 year of age, dotted line = high methylation level (>70%) (n=25), black line = low methylation level (<70%) (n=16); log rank test, 0.02.
Comparison of quantification of MRD levels in BM applying RASSF1a\textsubscript{M} and a panel of RNA markers

For the quantification of MRD levels based on RNA RQ-PCRs, the expression level of the PCR targets in the BM has to be related to their expression in the primary tumor. From 21 patients with stage 4, primary tumor RNA was available. Since the estimated MRD level in the BM varied for the different RNA targets, the median MRD level of the panel of 5 RNA targets was determined for each BM sample as the best estimate for MRD level. In figure 3 the correlation between MRD levels (all within the quantitative range) in BM samples determined by RASSF1a\textsubscript{M} and the median MRD level determined by the panel of RNA markers is shown. In 23 out of 30 BM samples, both methods quantified more or less (within 1 log) the same MRD level (R\textsuperscript{2} = 0.76; Spearman's test, p<0.001). In 3 of 30 samples, RASSF1a\textsubscript{M} showed MRD levels higher than 1 log than the panel of RNA markers. Since we assumed that all NB cells were methylated, an overestimation of RASSF1a\textsubscript{M} is excluded. Moreover, two of these samples showed >1% BM infiltration with morphology, confirming that the MRD level is underestimated by the RNA panel. In conclusion, MRD quantification in BM is possible using RASSF1a\textsubscript{M} with the advantage that the primary tumor is not needed for MRD quantification.

Now we have shown that accurate quantification is possible using RASSF1a\textsubscript{M}, we investigated whether the level of BM infiltration at diagnosis is prognostic for survival. As shown in figure 4, stage 4 patients with >20% of NB cells in the BM had a worse outcome compared to patients with lower levels of BM infiltration (p=0.04). Remarkably, there was no difference between patients with low (<1%) or intermediate (1-20%) MRD levels.

Extent of RASSF1a methylation in neuroblastoma tumors and survival

We also investigated if differences in methylation levels in the primary tumor were prognostic for overall survival (OS) in patients with stage 4 neuroblastoma older than 1 year. Patients with tumors with a high percentage RASSF1a methylation (>70%) had a significantly worse outcome than patients with a low percentage of RASSF1a methylation (5 years-OS 19% ± 9 versus 56% ± 12, respectively; p=0.02), figure 5. The extent of RASSF1a methylation was higher in MYCN amplified tumors compared to MYCN non-amplified tumors (supplementary figure 1a and 1b), in both patients without metastasis (stage 1-3) and with metastasis (stage 4). Thus the extent of RASSF1a methylation seems to correspond to more aggressive neuroblastoma.
Discussion

In this study we describe methylated RASSF1a as the first DNA marker, which can be used for MRD detection in patients with NB. In contrast to most RNA markers, RASSF1aM was shown to be almost totally specific for neuroblastoma tumor cells. Furthermore, the DNA-PCR specific for RASSF1aM was found to reach similar sensitivity as the panel of RNA-PCRs when tested on a large panel of clinical samples of NB patients. In samples with tumor load around the detection limits of both assays, the two PCR approaches were complementary, thereby increasing the sensitivity of PCR-based MRD detection. Another advantage is that using RASSF1aM as target, MRD levels in BM can be more accurately quantified than by using an RNA panel, since the DNA-PCR is unlike the RNA targets not dependent on variable expression levels, and the primary tumor is not needed anymore.

We chose RASSF1aM as a potential DNA MRD marker, since RASSF1a is one of the most frequently methylated genes in neuroblastoma and is not methylated in hematological cells. In previous studies it was shown that RASSF1a was methylated in only a subset of NB, which varied from 70 to 94% of all neuroblastoma. However, we now showed that RASSF1a was methylated in all metastatic (stage 4 and 4s) tumors and only in some stage 1-3 tumors (3 out of 21 tested) no methylation of RASSF1a was observed. None of the three patients with an unmethylated RASSF1aM tumor had BM involvement at diagnosis, determined by RQ-PCR. Yang et al. also found that the frequency of RASSF1a methylation was higher in stage 4. In conclusion, although RASSF1a is not methylated in all NB tumors, RASSF1a methylation appeared to be present in all tumors that are able to metastasize to the BM. Furthermore it remains methylated in tumor cells metastasized to the BM, so it is applicable in most if not all patients for MRD detection in the BM.

The applicability of a PCR target for MRD detection is also determined by its background expression in hematological cells. All RNA targets except PHOX2B, used for MRD detection in NB, are hampered by this background. In contrast, RASSF1aM was only amplified in one of a triplicate (> Ct 40) in 50 control BM samples. Furthermore, the RASSF1aM RQ-PCR reached the maximal theoretical sensitivity of detection of 1 tumor cell in 100,000 MNC cells, which equals to detection of 2.5pg DNA (~ 1 allele of DNA). This also shows that the bisulfite conversion of the DNA is robust and complete, and that conversion does not lead to degradation of DNA. Hence RASSF1aM is much more specific than most RNA PCR markers, with a maximal sensitivity.
Next, we tested the RASSF1a\textsubscript{M} PCR on a panel of clinical samples and compared it to a panel of RNA markers. Qualitative PCR results for both assays were almost similar in diagnostic BM samples; tumor cells were detected by RASSF1a\textsubscript{M} in all but one RNA-panel positive samples. In BM samples during treatment there were more discrepancies between the DNA and RNA markers; 4 of 25 RASSF1a\textsubscript{M} positive samples were negative for the panel and 6 of 27 panel positive samples were negative for RASSF1a\textsubscript{M}. All these ten samples had MRD levels around the detection limit for the positive PCR assay. Thus, RASSF1a\textsubscript{M} appears to be of additional value for MRD detection especially around the detection limit of the assays.

In addition, quantitative results for both assays were compared. For the CpG-rich region of RASSF1a analyzed in this study a close correlation between methylation and gene silencing has been shown.\textsuperscript{11,19} In addition, RASSF1a gene inactivation by allelic loss of chromosome 3p21.3 is a rare event in neuroblastoma.\textsuperscript{8,20,21} Therefore the percentage methylation corresponds to the percentage of tumor cell infiltration. Theoretically, a DNA marker is more suitable for MRD detection than an RNA marker. The expression levels of RNA markers vary greatly (up to 3 log) between tumors and in many cases RNA of the primary tumor is not available, especially not at diagnosis. Moreover, we observed that the expression level of RNA markers could change during treatment (manuscript in preparation). Although the percentage of methylation varies between tumors and might be changed during therapy, this has less impact (within 1-log) on the estimated MRD level compared to the 3-log variation in expression level seen for RNA markers. Indeed we show that in some cases RNA markers were down-regulated in BM metastasis, while RASSF1a\textsubscript{M} showed comparable quantification as BM morphology. Using RASSF1a\textsubscript{M} for quantification of BM infiltration, we now observed that in patients with BM involvement, there was no difference in survival between patients with low (<1\%) or intermediate (1-20\%) BM infiltration. Only the few patients with >20\% NB cells had a significant worse prognosis. Although it has been suggested by Trager and colleagues\textsuperscript{22} that high expression of TH at diagnosis corresponds to poor outcome, our finding indicates that the mere presence of BM infiltration is the major prognostic factor rather than the estimated level of infiltration. This can be explained by the patchy growth pattern of neuroblastoma, where the place of the biopsy might determine the level of infiltration.

Now we have shown that RASSF1A\textsubscript{M} can be used as an MRD marker for high-risk neuroblastoma, we also investigated whether the level of
methylation in neuroblastoma tumors was related to prognosis. Indeed, even
in this heterogeneously treated group of patients, a high level of RASSF1a
methylation (>70%) was found to be correlated with poor survival in stage
4 patients (p=0.02). In both non-metastatic and metastatic NB the extent
of RASSF1a methylation was higher in MYCN amplified tumors. This can
be explained by the model proposed by Palakurthy et al. in which MYC
facilitates recruitment of the DNA methyltransferase DNMT3B to the
RASSF1a promoter, which causes methylation of RASSF1a.23

RASSF1a methylation is not specific for neuroblastoma, as it occurs in a
broad spectrum of tumors.24 The PCR on bisulfite converted genomic DNA
described in this paper could therefore also be used to detect circulating
tumor cells in for example breast or lung cancer in which now tumor-
associated transcripts, such as CK1925, and CEA26, are used.

In conclusion, in this paper we present a novel sensitive and specific
DNA RQ-PCR marker for MRD detection in BM cells of patients with
neuroblastoma; methylated RASSF1a. The data presented, suggest that
methylated RASSF1a should be included, as a marker in prospective MRD
studies of neuroblastoma, not only for detection but also for accurate
quantification of MRD levels.
Chapter 7 RASSF1a: a novel DNA neuroblastoma MRD marker

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22 Trager C, Vernby A, Kullman A et al. mRNAs of tyrosine hydroxylase and dopa decarboxylase but not of GD2 synthase are specific for neuroblastoma minimal disease and predicts outcome for children with high-risk disease when measured at diagnosis. Int J Cancer 2008; 123:2849-2855.


24 Hesson LB, Cooper WN, Latif F. The role of RASSF1A methylation in cancer. Dis Markers 2007; 23:73-87


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Appendix Chapter 7

Supplementary data
**supplementary table 1. Patients’ characteristics**

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<th>Patient Characteristics (n=84)</th>
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**supplementary table 2. Primer- probe combinations**

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Minimal residual disease detection and monitoring in children with neuroblastoma
supplementary table 3. RASSF1a methylation in control bone marrow samples (n=50) and clinical bone marrow samples (n=127)

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<tr>
<td>stage 4 BM diagnosis</td>
<td>2</td>
<td>1**</td>
<td>3</td>
<td>39</td>
<td>45</td>
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<tr>
<td>BM follow up</td>
<td>25</td>
<td>4**</td>
<td>4</td>
<td>17</td>
<td>50</td>
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</tr>
</tbody>
</table>

+ none of triplicate amplified;
† one of triplicate amplified;
§ two of triplicate amplified;
‡ three of triplicate amplified;
* Ct value 40.9;
** Ct values below arbitrary threshold of 39.9 Ct

supplementary figure 1. Percentage of RASSF1a methylation in MYCNA tumors compared to non-MYCNA tumors in patients with different disease stages determined by real time quantitative PCR.

A Comparison of the percentage of RASSF1a methylation in MYCNA (n=3) versus non-MYCNA tumors (n=18) in non-metastatic patients (Mann-Whitney test, p=0.006).

B Comparison of the percentage of RASSF1a methylation in MYCNA (n=19) versus non-MYCNA tumors (n=26) in stage 4 patients (Mann-Whitney test, p=0.68).

Abbreviations:
MYCNA, MYCN amplified tumor