Expression of Five Neuroblastoma Genes in Bone Marrow or Blood of Patients with Relapsed/Refractory Neuroblastoma Provides a New Biomarker for Disease and Prognosis

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Abstract

Purpose: We determined whether quantifying neuroblastoma-associated mRNAs (NB-mRNAs) in bone marrow and blood improves assessment of disease and prediction of disease progression in patients with relapsed/refractory neuroblastoma.

Experimental Design: mRNA for CHGA, DCK, DDC, PHOX2B, and TH was quantified in bone marrow and blood from 101 patients concurrently with clinical disease evaluations. Correlation between NB-mRNA (delta cycle threshold, ΔCt, for the geometric mean of genes from the TaqMan Low Density Array NB5 assay) and morphologically defined tumor cell percentage in bone marrow, 123I-meta-iodobenzylguanidine (MIBG) Curie score, and CT/MRI-defined tumor longest diameter was determined. Time-dependent covariate Cox regression was used to analyze the relationship between ΔCt and progression-free survival (PFS).

Results: NB-mRNA was detectable in 83% of bone marrow (185/223) and 63% (89/142) of blood specimens, and their ΔCt values were correlated (Spearman r = 0.67, P < 0.0001), although bone marrow Ct was 7.9 ± 0.5 C, stronger than blood C. When bone marrow morphology, MIBG, or CT/MRI were positive, NB-mRNA was detected in 99% (99/100), 88% (100/113), and 81% (82/101) of bone marrow samples. When all three were negative, NB-mRNA was detected in 55% (11/20) of bone marrow samples. Bone marrow NB-mRNA correlated with bone marrow morphology or MIBG positivity (P < 0.0001 and P = 0.007). Bone marrow and blood ΔCt values correlated with PFS (P < 0.001; P = 0.001) even when bone marrow was morphologically negative (P = 0.001; P = 0.014). Multivariate analysis showed that bone marrow and blood ΔCt values were associated with PFS independently of clinical disease and MYCN gene status (P < 0.001; P = 0.055).

Conclusions: This five-gene NB5 assay for NB-mRNA improves definition of disease status and correlates independently with PFS in relapsed/refractory neuroblastoma. Clin Cancer Res; 23(18): 5374–83. ©2017 AACR.
Quantitation of neuroblastoma-associated mRNA in blood, bone marrow, and peripheral blood stem cells with reverse-transcriptase polymerase chain reaction is sensitive and can provide prognostic information for patients with high-risk neuroblastoma prior to relapse. We provide a systematic comparison of disease burden quantification in relapsed patients by standard evaluations versus a new TaqMan Low Density Array assay for mRNA of five neuroblastoma genes (NB5 assay) and show that the NB5 assay enhances standard evaluations and provides independent prognostic information in this population. This biomarker assay has been incorporated into all NANT consortium therapeutic studies in order to validate the assay and prospectively analyze disease burden assessment and to provide standard clinical disease response testing in the context of uniform therapy in large numbers of patients.

**Translational Relevance**

Quantitation of neuroblastoma-associated mRNA in blood, bone marrow, and peripheral blood stem cells with reverse-transcriptase polymerase chain reaction is sensitive and can provide prognostic information for patients with high-risk neuroblastoma prior to relapse. We provide a systematic comparison of disease burden quantification in relapsed patients by standard evaluations versus a new TaqMan Low Density Array assay for mRNA of five neuroblastoma genes (NB5 assay) and show that the NB5 assay enhances standard evaluations and provides independent prognostic information in this population. This biomarker assay has been incorporated into all NANT consortium therapeutic studies in order to validate the assay and prospectively analyze disease burden assessment and to provide standard clinical disease response testing in the context of uniform therapy in large numbers of patients.

Testing in the context of uniform therapy in large numbers of patients.

Relapsed/refractory neuroblastoma patients were studied because they generally have detectable disease and because evaluations of changes in disease burden could be enhanced by a sensitive and quantitative assay. This is the first prospective study to quantify expression of five neuroblastoma-associated genes in bone marrow and blood of patients with relapsed/refractory neuroblastoma and to correlate expression with concurrent CT/MRI and MIBG imaging and morphologic bone marrow evaluations. The NB5 assay improves definition of disease burden and provides prognostic information that is independent from that derived from clinical disease and MYCN gene assessments.

**Patients and Methods**

**Patients**

The study was designed and conducted in accordance with the U.S. Common Rule, the Declaration of Helsinki, and local regulations including the US Code of Federal Regulations Title 21. The study was performed after approval of the protocol and any informed consent documents by an institutional review board at each site. Investigators obtained written consent from all subjects prior to study enrollment.

Patients were treated at a New Approaches to Neuroblastoma Therapy (NANT) Consortium institution and were enrolled in the NANT Biology Study (NANT 2004-05). All patients had high-risk neuroblastoma as defined by the INRC (ref. 1) and for the purposes of this study, only patients with relapsed/progressive disease (at any time point prior to enrollment) or no response (refractory disease) per International neuroblastoma response criteria (INRC; ref. 24) were included. Therapy at the time of clinical and NB5 assay assessments varied, and included NANT therapeutic protocols and other therapies for relapsed/refractory disease (Supplementary Table S1). Once enrolled in NANT 2004-05, bone marrow and blood samples were obtained prospectively for the NB5 assay. All patients submitted at least one bone marrow and/or blood sample between October 11, 2011, and April 24, 2014, for quantification of neuroblastoma mRNA with the NB5 assay at the time of standard disease evaluation that included bone marrow morphology (bilateral aspirates/biopsies) and MIBG and CT/MRI imaging. All imaging, bone marrow aspirates/biopsies, and samples for NB5 assay were obtained within one month of each other (median, 3 days) with exception of those for two patients that spanned 38 and 53 days.

**Sample processing and NB5 assay**

NB5 assays were performed on mononuclear cells from heparinized blood and bone marrow (pooled bilateral aspirates) isolated by density separation with Ficoll–Hypaque (25). The bilateral aspirates were pooled in order to have a sample more reflective of overall bone marrow disease burden detectable by this assay. The NB5 assay quantified expression of neuroblastoma-associated genes CHGA, DCX, DDC, PHOX2B, and TH and of housekeeping genes B2M, GAPDH, HPRT1, and SDHA with predesigned and preoptimized probe and primer sets (Supplementary Table S2), 2,500 ng of cDNA, and standard cycling conditions using the 7900HT fast real-time PCR system (Applied
biosystems]. The cycle threshold (Ct) value for each gene was the cycle number where the amplification signal reached a threshold of 0.5 over baseline, and Ct 40 was assigned when this threshold was not reached by the 40th cycle. A summary ΔCt for the five detection genes was computed as the geometric mean (GM) of the Ct values for the five detection genes minus the GM of the Ct values of the four housekeeping genes (NB5 assay ΔCt). Lower ΔCt values indicate higher NB-mRNA. NB-mRNA was “undetectable” when none of the five neuroblastoma genes had a Ct < 40. Experiments in which neuroblastoma cell line RNA was seeded into peripheral blood mononuclear cell RNA showed that the NB5 assay can detect one neuroblastoma cell among 108 PBMCs (5). ΔCt was chosen instead of Ct alone as it accounts for RNA quality of the samples obtained. Note that ΔCt values may be equivalent when one specimen may have no detectable neuroblastoma mRNA and other may be mildly positive. This is due to variability of the geometric mean of housekeeping genes. Undetectable versus detectable samples are distinguished in all analyses. Details are provided in the Supplementary Patients and Methods section.

Disease evaluation

For all patients included in this analysis, disease evaluations and central review were performed as follows (including patients treated on NANT therapeutic trials or non-NANT therapies). CT/MRI images were reviewed by one radiologist (F. Goodarzian) using RECIST 1.0 criteria for presence and size of target and nontarget lesions (26), and the sum of the longest diameter (LD) of all target lesions was determined. MIBG scans were reviewed by one radiologist (H.A. Lai), who performed Curie scoring (27, 28). Histopathology of bilateral bone marrow biopsies was reviewed by one pathologist (H. Shimada), and the maximum percentage of tumor cells from either side was assigned as the percent of neuroblastoma cells (29).

Response was graded at each disease evaluation time point for CT/MRI, MIBG, and bone marrow assessments, and these were combined into an overall response per the NANT Response Criteria (v1.0; ref. 30). The overall response assigned was CR, PR, mixed response (MR), SD, or PD. For patients with multiple disease evaluation time points, each disease progression was considered a new baseline for subsequent response evaluation. Time to progression was calculated from the first baseline and after each subsequent progression. Details are provided in the Supplementary Patients and Methods section.

Statistical analysis

Where appropriate, standard descriptive and analytic statistical methods such as t test, ANOVA, ordinary least squares regression, and contingency table analyses were used (31). Reported P values were two sided, with P < 0.05 considered significant. Statistical computations were performed with Stata 11 (Stata Statistical Software: Release 11). Individual NB5 assay or disease evaluations were the analytic units and therefore a single patient may contribute more than one time point with paired NB5 and disease assessment. As the majority of patients had only one or two of each assessment type, formally accounting for repeated measures had negligible effect on the results in most analysis or was technically not feasible in selected analyses. Therefore, for consistency we report the analyses that do not account for repeated measures. For the ANOVA analysis of differences in average ΔCt between groups and for regression of ΔCt on clinical evaluations, assays classified as undetectable represented right censored data, and they were analyzed using normal theory maximum likelihood interval regression analysis as implemented in Stata module “intreg.”

Because individual patients could have multiple disease evaluations performed during follow-up, and the results of these evaluations could change, implying that the patient is now potentially at higher or lower risk of relapse or progression, time-dependent covariate (TDC) Cox regression analysis was used to examine the influence of ΔCt and other variables of interest (e.g., CT/MRI, bone marrow morphology) on time to progression (32, 33). Individual patients could contribute multiple follow-up periods in this analysis, with time 0 reset at study entry or at occurrence of disease progression, and where values of ΔCt, CT/MRI, Curie score, maximum percent bone marrow involvement by morphology were all time varying, that is could change at each evaluation during the follow-up period thus shifting individuals to different risk groups at the times of these changes. P values were based on the likelihood ratio test. Product limit (Kaplan–Meier) curves using these intervals were constructed to visualize the magnitude of the NB5 effect represented in a univariate TDC analysis of NB5. As above, time 0 is reset at study entry or at progression, and patients whose NB5 status changes during follow-up are shifted to the curve representing that new status at the time of the change. Although any one curve may not reflect the outcome of a particular well-defined group of patients with a known NB5 assessment history, the difference between curves effectively conveys the magnitude of the difference in outcome resulting from different NB5 assay results.

Results

Patients and disease status evaluations

One hundred one patients who submitted at least one specimen for TLDA analysis with relapsed (n = 81) or refractory (n = 20) neuroblastoma are included in this study (Table 1). A total of 305 standard disease evaluations were performed, and concurrent NB5 assay data were obtained for 259 with a median of 3 days between the disease evaluation and NB5 assay (Table 1 and Supplementary Table A3). RNA integrity numbers (RINs) of the specimens for NB5 assays were the following: mean RINs for bone marrow 9.4 (range, 6.1–10) and blood 9.3 (range, 8.1–10). Patients were allowed to submit samples at the time of each disease evaluation, generally every 2 and 3 months. The median time from the first to last assessment, which included CT/MRI and MIBG scans, bone marrow morphology, or NB5 analysis, was 24 weeks (range, 5–127 weeks). The number of evaluation time points was 1 (n = 33 patients), 2 (n = 28), 3 (n = 11), and 4+ (n = 29). Review of disease evaluations performed demonstrates the following: MIBG scans were most frequently positive (77%) whereas CT/MRI and bone marrow evaluations were positive in 53% and 45% of assessments. The percentage of morphologically identified neuroblastoma cells in bone marrow correlated with the MIBG Curie score (Supplementary Fig. S1; Spearman r = 0.4; P < 0.0001) but not with CT/MRI-defined tumor LD. All bone marrow samples with >30% neuroblastoma cells on routine morphology were associated with positive MIBG scans, whereas morphologically negative bone marrow samples were associated with a range of Curie scores.
Neuroblastoma mRNA for at least one of the five genes was detected in 185/223 (83%) of bone marrow and 89/142 (63%) of blood specimens using the NB5 assay (Table 1). Eighty-three of 101 (82%) bone marrow and blood specimens were obtained on the same day while the remaining 18 pairs were obtained a median of 2 days apart (range, 1–6 days). Analysis of $\Delta C_\text{T}$ at these 106 time points showed high correlation between bone marrow and blood (Spearman $r = 0.67$, $P < 0.0001$), but also showed that the level of neuroblastoma mRNA is less in blood than in bone marrow (Fig. 1). In 19 strongly positive bone marrow specimens ($\Delta C_\text{T} \leq 8$), bone marrow gave a signal that

![Figure 1](image-url)

**Figure 1.**
Correlation of NB5 assay $\Delta C_\text{T}$ between bone marrow (BM) and blood. NB5 analysis was performed on bone marrow and blood specimens that were obtained concurrently from 106 time points where both assays were performed. Spearman $r = 0.67$, $P < 0.0001$. Values are denoted “undetectable” when mRNA for none of the five neuroblastoma (NB) genes had a $C_\text{T} < 40$ (see Patients and Methods).

<table>
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<th>Variable</th>
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<th>Summary/count</th>
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<tbody>
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</tr>
<tr>
<td>Sex</td>
<td>Male</td>
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</tr>
<tr>
<td></td>
<td>Female</td>
<td>35</td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td>Median (range) in months</td>
<td>50 (3–236)*</td>
</tr>
<tr>
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</tr>
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<td></td>
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<tr>
<td></td>
<td>Non-amplified</td>
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</tr>
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<td></td>
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</tr>
<tr>
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<td>6</td>
</tr>
<tr>
<td></td>
<td>Unfavorable</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>Unknown/not done</td>
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<tr>
<td>Response to frontline therapy</td>
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</tr>
<tr>
<td></td>
<td>Relapsed, progression</td>
<td>81</td>
</tr>
<tr>
<td>CT/MRI</td>
<td># Positive/# total* (%)</td>
<td>147/279 (52.7%)</td>
</tr>
<tr>
<td></td>
<td>Median (range) LD in cm for positives</td>
<td>3.7 (1–36)</td>
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<tr>
<td>MIBG</td>
<td># Positive/# total* (%)</td>
<td>204/264 (77.3%)</td>
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<tr>
<td></td>
<td>Median (range) Curie score for positives</td>
<td>7.0 (2–7)*</td>
</tr>
<tr>
<td>Bone marrow morphology</td>
<td># Positive/# total* (%)</td>
<td>110/243 (45.3%)</td>
</tr>
<tr>
<td></td>
<td>Median (range) % neuroblastoma cells for positives</td>
<td>5 (0.5–95)</td>
</tr>
<tr>
<td>Bone marrow NB5 TLDA</td>
<td># Detectable/# total* (%)</td>
<td>185/223 (83%)</td>
</tr>
<tr>
<td></td>
<td>Median (range) $\Delta C_\text{T}$ for detectable</td>
<td>15.3 (0.02–21.2)</td>
</tr>
<tr>
<td>Blood NB5 TLDA</td>
<td># Detectable/# total* (%)</td>
<td>89/142 (63%)</td>
</tr>
<tr>
<td></td>
<td>Median (range) $\Delta C_\text{T}$ for detectable</td>
<td>18.2 (6.8–21.8)</td>
</tr>
</tbody>
</table>

* $N = 99$ patients.

* $n = 145$ positive evaluations with Curie score; 59 assessments were positive but Curie score unknown.
was 7.9 ± 0.46 C₅₇ stronger than blood. Bone marrow was negative in only 8% of instances (5/62) when blood was positive, but blood was negative in 35% of instances (31/88) when bone marrow was positive (Fig. 1).

**NB5 assay ΔC₅₇ and clinical disease status**

In univariate analysis, bone marrow and blood NB5 ΔC₅₇ correlated with both the percentage of neuroblastoma cells in bone marrow by morphology and with the MIBG Curie score (Fig. 2A–D; P < 0.0001). 81/177 (69%) of bone marrow with negative morphology had detectable neuroblastoma mRNA with the NB5 assay. Nondetectable neuroblastoma mRNA in bone marrow in all but one case was associated with morphologically nondetectable neuroblastoma cells. The bone marrow from this patient had large areas of pink neuropil on one side but no neuroblastoma cells. Two patients with a high Curie score of 10 and 20 and nondetectable neuroblastoma mRNA in bone marrow had large soft tissue tumors 7 years from diagnosis (12 and 35 cm LD on anatomical imaging), extensive bone disease and no morphologically detectable

![Diagram](https://example.com/diagram.png)

**Figure 2.**

Correlation of NB5 ΔC₅₇ in bone marrow (BM) and blood with concurrently performed standard disease evaluations. **A** and **B**, Correlation of bone marrow and blood NB5 ΔC₅₇ and the percent of neuroblastoma (NB) cells in bone marrow defined morphologically. **C** and **D**, Correlation of bone marrow and blood NB5 ΔC₅₇ and MIBG Curie score. **E** and **F**, Correlation of bone marrow and blood NB5 ΔC₅₇ and the CT/MRI defined tumor LD. Specimens in which NB mRNA was detectable or nondetectable with the NB5 assay are coded blue or red, respectively. Inset tables provide summary data for each comparison.
Table 2. Clinical disease status and NB5 assay $\Delta C_t$

<table>
<thead>
<tr>
<th>NBS assay and clinical disease assessments</th>
<th>Bone marrow NB5 assay $\Delta C_t$</th>
<th>Blood NB5 assay $\Delta C_t$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N positive/total (%)</td>
<td>Mean $\Delta C_t$ (SE)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>for positive (SE)</td>
</tr>
<tr>
<td>All NBS assays</td>
<td>185/223 (83)</td>
<td>15.7 (0.46)</td>
</tr>
<tr>
<td>All NBS assays with concurrent clinical disease assessments</td>
<td>158/190 (83)</td>
<td>15.6 (0.50)</td>
</tr>
<tr>
<td>No disease found</td>
<td>11/20 (55)</td>
<td>20.0 (1.40)</td>
</tr>
<tr>
<td>Soft tissue only</td>
<td>2/7 (29)</td>
<td>24.5 (2.79)</td>
</tr>
<tr>
<td>MIBG only</td>
<td>28/34 (82)</td>
<td>17.6 (1.00)</td>
</tr>
<tr>
<td>Bone marrow only</td>
<td>9/9 (100)</td>
<td>15.3 (1.91)</td>
</tr>
<tr>
<td>MIBG and bone marrow</td>
<td>13/33 (97)</td>
<td>11.2 (2.00)</td>
</tr>
<tr>
<td>MIBG and soft tissue</td>
<td>27/38 (71)</td>
<td>18.7 (0.97)</td>
</tr>
<tr>
<td>Soft tissue and BM</td>
<td>8/8 (100)</td>
<td>12.8 (2.02)</td>
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<tr>
<td>Soft tissue, bone marrow, and MIBG</td>
<td>4/11 (00)</td>
<td>11.7 (0.89)</td>
</tr>
</tbody>
</table>

Main effects ANOVA$^b$

<table>
<thead>
<tr>
<th>BM NB5 $\Delta C_t$, 190 time points$^d$</th>
<th>Blood NB5 $\Delta C_t$, 104 time points$^e$</th>
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<tbody>
<tr>
<td>Clinical disease comparison</td>
<td></td>
</tr>
<tr>
<td>$\Delta C_t$ difference (SE) $^a$</td>
<td>$\Delta C_t$ difference (SE) $^a$</td>
</tr>
<tr>
<td>$P$</td>
<td>$P$</td>
</tr>
<tr>
<td>Bone marrow (negative vs. positive patients)</td>
<td>6.8 (0.78)</td>
</tr>
<tr>
<td>MIBG (negative vs. positive patients)</td>
<td>2.9 (1.07)</td>
</tr>
<tr>
<td>CT/MRI (negative vs. positive patients)</td>
<td>-0.77 (0.87)</td>
</tr>
</tbody>
</table>

$^a$Means and SEs of NB5 $\Delta C_t$ were obtained from an analysis that accounts for right censoring due to mRNA levels below the level of detectability of the NB5 assay (see Patients and Methods). These represent the best estimate of average NB5 $\Delta C_t$ in the different disease status groups regardless of their detection status. This analysis was performed for patients who had CT/MIBG/bone marrow clinical evaluations and NB5 assay testing.

$^b$For the main effects analysis of variance, values represent the difference in the mean NB5 $\Delta C_t$ in patients negative for a clinical assessment compared to positive patients, controlling for the status of the remaining clinical assessments. Interaction terms were not significant and were excluded from this model.

$^c$All NB5 assays with concurrent clinical disease assessments.

Neuroblastomas in bone marrow. NB5 $\Delta C_t$ in blood, but not in bone marrow, correlated with the CT/MRI defined LD of soft tissue tumors (Figs. 2E and F).

Bone marrow and blood NB5 $\Delta C_t$ values associated with bone marrow, MIBG, and CT/MRI findings on clinical evaluations as shown in Table 2. Note, only evaluations with no missing evaluations of either bone marrow, MIBG, and CT/MRI were included in this analysis. Notably, 11/20 (55%) of patients with no clinically detectable disease had neuroblastoma mRNA detectable in bone marrow and/or blood. Analysis of variance assessed whether tumor burden (nondetectable or mRNA detectable in bone marrow and/or blood. Analysis of variance assessed whether tumor burden (nondetectable or detectable disease) contributed independently to the average NB5 $\Delta C_t$. (Table 2). Analysis of variance of the association between NB5 $\Delta C_t$ and tumor burden assessed by bone marrow morphology, MIBG, or CT/MRI (nondetectable vs. detectable disease) showed that the presence of disease by bone marrow morphology and MIBG imaging were independently associated with a stronger NB5 $\Delta C_t$ signal in bone marrow and in blood (Table 2, bottom). In contrast, presence of soft tissue disease by CT/MRI was not associated with strengthening of the NB5 assay signal in either bone marrow or blood independently of bone marrow morphology and MIBG imaging. Change in NB5 $\Delta C_t$ was assessed between pairs of time points where both NB5 $\Delta C_t$ and clinical disease assessments were performed (Supplementary Fig. S2). Change in NB5 $\Delta C_t$ in these sequential bone marrow samples was correlated with change in the percentage of bone marrow neuroblastoma cells (Spearman $r = -0.34$, $P < 0.0001$), the MIBG Curie score ($r = -0.48$, $P < 0.0001$), and the CT/MRI LD ($r = -0.21$, $P < 0.01$). For bone marrow, the change in NB5 $\Delta C_t$ that was associated with progressive disease ($\Delta C_t$ for PD specimen $-\Delta C_t$ for previous specimen) was $-2.7 \pm 0.83$ and with nonprogressive disease was $1.9 \pm 0.27$ ($P < 0.0001$, two-sample t test). For blood, these values were $-2.0 \pm 0.66$ and $0.52 \pm 0.25$, respectively ($P < 0.0001$).

NB5 assay $\Delta C_t$ and progression-free survival

Time-dependent covariate analysis of $\Delta C_t$ and progression-free survival (PFS) was performed. NB5 assay results were classified as undetectable, $\Delta C_t > 15$ and $\Delta C_t \leq 15$, where the cut-off point of 15 is approximately the median of $\Delta C_t$ detectable values. All NB5 assays were included in this analysis if they met the criterion of having one subsequent clinical disease assessment. On univariate analysis, both bone marrow and blood NB5 $\Delta C_t$ were significant predictors of subsequent progression for all patients including those without morphologically detectable neuroblastoma cells in bone marrow (Figs. 3A–D; Table 3). When comparing the baseline characteristics of the undetectable versus detectable patients by NB5 assay, there were no statistically significant differences in MYCN status, age at diagnosis, or history of prior relapse.

The NB5 assay using all five genes was compared in this dataset with other analyses using other signatures (PHOX2B, TH; PHOX2B, TH; and TH) based on prior publications (refs. 17, 19, 34, 35; Supplementary Fig. S3). The correlation between these signatures and the NB5 assay was very high, but there was a larger number of undetectable results in the signatures with fewer than five genes. Dividing the sample in Supplementary Fig. S3 into two groups at various cut-off points and comparing 1-year PFS, the NB5 signature resulted in a wider separation between groups than the other signatures. ROC analysis confirmed that NB5 signature resulted in a higher AUC than the other signatures for predicting PFS. In addition, individual genes were removed from the NB5 assay and change in AUC was calculated (Supplementary Table S4). Based on this analysis, no one gene dominates all the others but the singly most in the different disease status groups regardless of their detection status. This analysis was performed for previous specimen) was $-2.7 \pm 0.83$ and with nonprogressive disease was $1.9 \pm 0.27$ ($P < 0.0001$, two-sample t test). For blood, these values were $-2.0 \pm 0.66$ and $0.52 \pm 0.25$, respectively ($P < 0.0001$).

To determine whether NB5 $\Delta C_t$ was associated with disease progression independently of clinically-defined disease and MYCN gene status, multivariate time-dependent covariate analysis was performed using time points where all three clinical
assessments (CT/MRI, MIBG, and bone marrow morphology) and MYCN data were available and classified as either positive or negative. These analyses confirmed that NB5 ΔCt is significantly associated with PFS independently of clinical disease and MYCN gene status (Table 3). Analyses using ΔCt as a continuous rather than a categorical variable yielded similar results.

**Figure 3.**
Correlation of NB5 assay ΔCt in bone marrow (BM) and blood with PFS. The probability of progression for all patients (A and B), for patients with negative bone marrow by morphology (C and D), and for patients with positive bone marrow by morphology (E and F) in relationship to NB5 ΔCt in bone marrow (A, C, E) and blood (B, D, F) is shown. P values in the figures correspond to the categorical univariate likelihood ratio test from the time-dependent covariate analysis shown in Table 3 (see Patients and Methods). ΔCt of 15 chosen as the median of positive ΔCt values. N is the number of unique patients/number of samples.

**Discussion**
Disease evaluation is important for guiding therapeutic decisions and assessing prognosis. This is the first study of patients with high-risk neuroblastoma to quantify expression of five neuroblastoma-associated genes in bone marrow and blood.

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Table 3. Univariate and multivariate time-dependent covariate analysis of association of NB5 assay ΔCt with PFS

<table>
<thead>
<tr>
<th>Type of analysis</th>
<th>Patient group</th>
<th>Bone marrow NB5 assay</th>
<th>Blood NB5 assay</th>
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<td></td>
<td></td>
<td>N</td>
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<tr>
<td>Univariate</td>
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<td>Univariate</td>
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<td>Multivariateb</td>
<td>All</td>
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</tr>
<tr>
<td>Multivariateb</td>
<td>Bone marrow morph. positive</td>
<td>86</td>
<td>0.098</td>
</tr>
</tbody>
</table>

*For the categorical analysis, P values are based on two degree of freedom Cox likelihood ratio test using three NB5 ΔCt categories as shown in Fig. 3, and for the continuous analysis they are based on a two degree of freedom likelihood ratio test for linear and quadratic terms for NB5 ΔCt.

b Multivariate analysis includes only time points where the NB5 assay and CT/MRI, MIBG, and bone marrow morphology assessments of disease status were performed. The multivariate analysis simultaneously adjusts for positive/negative status by CT/MRI, MIBG, and bone marrow morphology and includes NB MYCN gene status (amplified or nonamplified) for the 94 patients with available data.

concurrently with standard disease evaluations at several sequential times. We show a high correlation between the quantity of neuroblastoma-associated mRNA in bone marrow and blood and disease status defined by percent of neuroblastoma cells in bone marrow biopsy and MIBG Curie score but also show that neuroblastoma mRNA is detectable when these standard evaluations are negative. Importantly, quantifying neuroblastoma mRNA in both bone marrow and blood provides an independent predictor of PFS. Thus, the NB5 assay provides a useful new molecular biomarker that improves both assessment of disease and prognostication.

Neuroblastoma mRNA in bone marrow and blood, as quantified by the NB5 assay, were strongly correlated, but the amount in bone marrow was significantly greater. Although it is less invasive to perform this assay on blood samples, our data suggest that bone marrow samples provide more sensitive detection. Indeed, when bone marrow was positive, 35% of blood specimens did not have detectable neuroblastoma mRNA, which indicates that testing blood cannot be a sensitive surrogate for bone marrow disease. NB5 assay ΔCt values of both bone marrow and blood correlated with the percentage of neuroblastoma cells in bone marrow and with the MIBG Curie score. Furthermore, even when controlling for the status of the other clinical disease assessments, stronger bone marrow and blood NB5 ΔCt values correlated with neuroblastoma cells in bone marrow (present or absent) and with MIBG uptake (present or absent). Correlation of blood ΔCt with both bone marrow and MIBG-defined disease implies that both can contribute to circulating neuroblastoma cells. Blood ΔCt (not bone marrow) also correlated with soft tissue tumor size, which suggests that soft tissue disease also can contribute to circulating tumor cells. The absence of correlation between bone marrow NB5 ΔCt and tumor size suggests that neuroblastoma cell growth/survival in the two sites may be independent.

In many instances, quantification of neuroblastoma-associated mRNA in bone marrow and blood using the NB5 assay detected disease when CT/MRI and MIBG scans and morphologic assessment of bone marrow were negative. For example, the NB5 assay detected neuroblastoma mRNA in bone marrow of 11 of 20 patients and in blood of four of nine patients who were in complete clinical remission. Although not significant by logrank test, 5 of 11 patients with NB5 detectable versus 1 of 9 with NB5 nondetectable disease in bone marrow subsequently developed progressive disease. Improved sensitivity of disease quantification in bone marrow and blood will likely improve assessment of response to treatment and provide an early surrogate for outcome. Although others have reported that quantitative RT-PCR is more sensitive than morphology for detecting bone marrow disease (14, 16, 36, 37), our study is the first to comprehensively compare quantification of mRNA (for five neuroblastoma-associated genes) in bone marrow and blood to current standard imaging and bone marrow evaluations. Our data shows that the NB5 assay for mRNA in bone marrow and blood improves assessment of neuroblastoma disease status.

The most striking result of this study is the association of neuroblastoma mRNA quantity in bone marrow and blood with PFS of patients with refractory/relapsed neuroblastoma. This was highly significant for all patients and even for those whose bone marrow samples were negative morphologically. In fact, the absence of NB5 detectable mRNA in bone marrow, which is almost always associated with a negative bone marrow morphology, predicts a high probability of PFS, whereas, any level of neuroblastoma mRNA in bone marrow, even when neuroblastoma cells were not detected morphologically, predicted a poor PFS. Importantly, multivariate analysis demonstrated that the quantity of neuroblastoma mRNA in bone marrow and blood defined with our NB5 assay predicts PFS for all patients and for those with morphologically negative bone marrow samples independently from disease status defined with standard clinical evaluations and from MYCN gene status. Although time to first relapse, age, stage of disease, and MYCN gene copy number have been reported to be independently predictive of postrelapse survival (6), our study is the first to demonstrate that subgroups of patients with relapsed/refractory neuroblastoma can be identified who have different likelihoods of PFS when treated in early phase clinical trials. Our data suggest that quantifying neuroblastoma mRNA for multiple neuroblastoma-associated genes in bone marrow and blood will enable risk stratification of patients with relapsed/refractory neuroblastoma before and during their treatment in early phase clinical trials.

A criticism of an assay using five genes would be that there could be increased sensitivity but with addition of false positivity. To answer this, a comparison of the 5-gene signature to other published gene combination signatures was performed (Supplementary Fig. S3). This showed that the NB5 assay is detectible more often and also better able to predict PFS at different cut-off points. This argues that the positive predictive value is enhanced by quantifying expression of all five genes.

Predicting PFS by quantifying neuroblastoma mRNA with a multigene assay could be affected by clinical variables. First, aspirating a single site may not provide bone marrow that is representative of multiple sites with sufficient RNA quality (36, 38), although one study that utilized immunocytoLOGY to identify neuroblastoma cells indicated that a sensitive assay can
overcome this potential problem (39). To circumvent this problem, we obtained specimens from both iliac crests and pooled them for testing, and the RNA quality for this multi-institution study was excellent. Second, some neuroblastoma cells may be tightly adherent in the bone marrow microenvironment and not readily aspirated. However, we used biopsy specimens to define neuroblastoma cells morphologically, and there was only one specimen that had neuropil in the biopsy but that did not have detectable neuroblastoma mRNA, which suggests that the sensitivity of the NB5 assay renders this an unlikely limitation. Although testing blood could potentially overcome sampling concerns, there is less neuroblastoma mRNA in blood than bone marrow, and some blood specimens did not have detectable neuroblastoma mRNA even though disease was present by standard evaluations. The type of therapy could affect test results, but our finding that NB5 ΔCt predicts PFS for patients enrolled in a variety of early phase clinical trials suggests independence from treatment. However, this needs to be further studied in trials in which patients are uniformly treated and have complete disease assessments along with NB5 assay testing of bone marrow and blood.

Other studies have shown that quantifying mRNA for three and five neuroblastoma-associated genes [TH, PHOX2B, and DCX; (17) PHOX2B, TH, DDC, GAP43, and CHRNB3; (21)] in bone marrow provides prognostic information for patients with stage 4, high-risk neuroblastoma at diagnosis and during and at the end of induction therapy (17, 21). We previously reported that subgroups of high-risk neuroblastoma patients with different outcomes can be identified by quantifying neuroblastoma mRNA in peripheral blood stem cells obtained during induction therapy with the NB5 assay used in this study (5). Quantification of mRNA for four neuroblastoma-associated genes (BAGALNT1, PHOX2B, CCND1, and ISL1) has been reported to predict PFS and overall survival for patients in first or second remission or with refractory disease and treated with the anti-GD2 antibody 3F8 (19). Our study demonstrates for the first time an improvement in the definition of disease status by quantifying mRNA for five neuroblastoma-associated genes (CHGA, DCX, DDC, PHOX2B, and TH) in bone marrow and blood and confirms the correlation with PFS in relapsed or refractory neuroblastoma. Analysis of bone marrow and blood with this new molecular biomarker assay predicts PFS independently of standard clinical evaluations of disease and of MYCN gene status. Further studies that utilize this assay in this and other contexts will aim to confirm its utility in managing patients with neuroblastoma. To this end, the NB5 assay has been incorporated into all NANT consortium therapeutic studies in order to validate the assay and prospectively analyze disease burden assessment and to provide standard clinical disease response testing in the context of uniform therapy in large numbers of patients.

Disclosure of Potential Conflicts of Interest

H.A. Lai reports receiving commercial research grants from Siemens Medical. No potential conflicts of interest were disclosed by the other authors.

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Other (acquisition of data via laboratory assays, provision of data for statistical analysis, reporting and organizing data, database entry, etc.): J.A. Parra
Leader of NANT: oversee all studies: K.K. Matthay

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References

Expression of Five Neuroblastoma Genes in Bone Marrow or Blood of Patients with Relapsed/Refractory Neuroblastoma Provides a New Biomarker for Disease and Prognosis

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