VOLUME 32 · NUMBER 10 · APRIL 1 2014

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

Neuroblastoma mRNAs Predict Outcome in Children With Stage 4 Neuroblastoma: A European HR-NBL1/SIOPEN Study

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Published online ahead of print at www.jco.org on March 3, 2014.

Support information appears at the end of this article.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

Clinical trial information: NCT01704716.

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0732-183X/14/3210w-1074w/$20.00

DOI: 10.1200/JCO.2013.53.3604

ABSTRACT

Purpose
To evaluate the hypothesis that detection of neuroblastoma mRNAs by reverse transcriptase quantitative polymerase chain reaction (RTqPCR) in peripheral blood (PB) and bone marrow aspirates (BM) from children with stage 4 neuroblastoma are clinically useful biomarkers of risk.

Methods
RTqPCR for paired-like homeobox 2b (PHOX2B), tyrosine hydroxylase (TH), and doublecortin (DCX) mRNA in PB and BM of children enrolled onto the High-Risk Neuroblastoma Trial-1 of the European Society of Pediatric Oncology Neuroblastoma Group (HR-NBL1/SIOPEN) was performed at diagnosis and after induction therapy.

Results
High levels of TH, PHOX2B, or DCX mRNA in PB or BM at diagnosis strongly predicted for worse event-free survival (EFS) and overall survival (OS) in a cohort of 290 children. After induction therapy, high levels of these mRNAs predicted worse EFS and OS in BM but not in PB. Combinations of mRNAs in BM did not add to the predictive power of any single mRNA. However, in the original (n = 182) and validation (n = 137) PB cohorts, high TH ($\log_{10}$TH > 0.8) or high PHOX2B ($\log_{10}$PHOX2B > 0.28) identify 19% of children as ultrahigh risk, with 5-year EFS and OS rates of 0%; OS rate was 25% (95% CI, 16% to 36%) and EFS rate was 38% (95% CI, 28% to 49%) in the remaining children. The magnitude of reduction in mRNA level between diagnosis and postinduction therapy in BM or PB was not of additional predictive value.

Conclusion
High levels of TH and PHOX2B mRNA in PB at diagnosis objectively identify children with ultrahigh-risk disease who may benefit from novel treatment approaches. The level of TH, PHOX2B, and DCX mRNA in BM and/or PB at diagnosis might contribute to an algorithm to improve stratification of children for treatment.

J Clin Oncol 32:1074-1083. © 2014 by American Society of Clinical Oncology

INTRODUCTION

Neuroblastoma is the most frequent cancer diagnosed in children younger than 5 years old and is responsible for 15% of cancer deaths in this age group. Neuroblastoma has a heterogeneous clinical course, ranging from spontaneous regression to incurable high-risk disease. The majority of children present older than age 18 months with poor-prognosis metastatic disease in bone marrow (BM) and bone.[1,2,3] Although consensus approaches to pretreatment risk stratification based on clinical[4] and genomic tumor[5] characteristics have been described, these do not reliably predict outcome differences for the large group of children diagnosed with high-risk disseminated disease.

We and others have previously demonstrated that reverse transcriptase polymerase chain reaction for neuroblastoma mRNAs in BM or peripheral blood (PB) can be predictive of outcome.[6-15] However, the clinical utility of this approach remains controversial, most likely reflecting the small number of children investigated and methodologic differences between studies.[16-18] To date, there are no reports of a prospective study investigating the predictive power of mRNAs in BM and PB embedded in a multicenter trial of high-risk neuroblastoma. Therefore, we have prospectively investigated the independent clinical significance of detecting a selected panel of three neuroblastoma mRNAs[19] by reverse transcriptase quantitative polymerase chain reaction (RTqPCR) in BM and PB from children with stage 4 neuroblastoma treated in the High-Risk

Table 1. Event-Free Survival Predicted by the Level of TH, PHOX2B, and DCX mRNA in BM and PB at Diagnosis and After Induction Therapy

Variable	Cox Survival Model Using log(RTqPCR) As a Continuous Variable, χ^2	Cut Point (Log RQ mRNA)	95% CI on Cut Point	Cut-Point Simulation *P*	Patients (No.)			Cox Survival Model Using RTqPCR Cut Point Log_{10} Value ($> v <$ cut point)					
								Univariable			Multivariable (adjusted for age, *MYCN*, BM cytology, trephine histology)		
					Total	Below Cut Point	Above Cut Point	χ^2	HR	95% CI	χ^2	HR	95% CI
Diagnosis													
BM													
TH	6.5*	2.36	−1.21 to 3.99	.74	198	113	85	6.7†	1.59	1.12 to 2.27	3.20	1.43	0.96 to 2.13
PHOX2B	6.2*	−2.63	−3.13 to −1.37	.19	174	18	156	11.3‡	3.94	1.45 to 10.71	8.50†	3.61	1.29 to 10.12
DCX	6.3*	−0.45	−4.00 to 2.26	.35	166	38	128	10.1†	2.25	1.30 to 3.89	6.46*	2.34	1.18 to 4.64
PB													
TH	6.4*	0.80	0.16 to 0.94	.07	190	172	18	13.3‡	3.01	1.78 to 5.07	15.38‡	3.38	1.98 to 5.79
PHOX2B	5.0*	0.28	0.25 to 0.36	.007	182	158	24	15.9‡	2.91	1.82 to 4.66	16.24‡	3.01	1.86 to 4.89
DCX	4.1*	0.41	0.23 to 0.97	.03	175	131	43	12.0‡	2.12	1.42 to 3.16	12.20‡	2.26	1.46 to 3.51
After induction therapy													
BM													
TH	4.7*	−1.88	−2.52 to −0.51	.41	151	50	102	8.1†	1.88	1.19 to 2.97	6.79†	1.88	1.15 to 3.10
PHOX2B	4.5*	−1.69	−1.82 to −1.15	.19	141	88	53	9.3†	1.89	1.26 to 2.83	6.89†	1.81	1.16 to 2.83
DCX	1.1	−0.65	−0.99 to −0.16	.054	118	81	37	6.7†	1.84	1.17 to 2.89	4.47*	1.78	1.04 to 3.05
PB													
TH	0.12	−0.18	—§	.20	136	134	2	2.9	NA		3.11	NA	
PHOX2B	0.92	−3.27	—§	.98	129	118	11	1.7	1.69	0.81 to 3.50	1.78	1.77	0.80 to 3.88
DCX	< 0.01	−1.19	—§	.04	107	105	2	4.3*	NA		4.27*	NA	

NOTE. χ^2 values, *P* values, and 95% CIs of HRs for univariable and multivariable Cox model analyses are indicated, using levels of mRNA as continuous variable or using a single cut point. The cut-point simulation *P* value evaluates whether the observed cut-point χ^2 is likely to be a chance finding given the χ^2 from analyzing the RTqPCR as a continuous variable (see Methods and Data Supplement).

Abbreviations: BM, bone marrow; DCX, doublecortin; HR, hazard ratio; Log RQ, log_{10} relative quantification normalized value; NA, not applicable because of low numbers of children with levels greater than the cut point; PB, peripheral blood; PHOX2B, paired-like homeobox 2b; RTqPCR, reverse transcriptase quantitative polymerase chain reaction; TH, tyrosine hydroxylase.

*$P < .05$.

†$P < .01$.

‡$P < .001$.

§Not possible to determine a reliable cut point.

Table 2. Overall Survival Predicted by TH, PHOX2B, and DCX mRNA Levels in BM and PB at Diagnosis and After Induction Therapy

Variable	Cox Survival Model Using log(RTqPCR) As a Continuous Variable, χ^2	Cut Point (Log RQ mRNA)	95% CI on Cut Point	Cut-Point Simulation *P*	Patients (No.)			Cox Survival Model Using RTqPCR Cut Point Log_{10} Value ($> v <$ cut point)					
								Univariable			Multivariable (adjusted for age, *MYCN*, BM cytology, trephine histology)		
					Total	Below Cut Point	Above Cut Point	χ^2	HR	95% CI	χ^2	HR	95% CI
Diagnosis													
BM													
TH	3.4	2.00	1.91 to 2.55	.025	298	100	98	11.4*	2.01	1.33 to 3.05	10.93*	2.38	1.38 to 4.09
PHOX2B	4.1†	2.12	1.78 to 2.29	.16	174	97	77	10.3‡	2.05	1.32 to 3.19	10.29‡	2.47	1.39 to 4.37
DCX	5.6†	2.47	2.06 to 3.16	.38	166	93	73	9.2‡	1.99	1.28 to 3.12	10.07‡	2.47	1.38 to 4.41
PB													
TH	1.9	0.27	0.01 to 0.58	.017	190	147	43	9.6‡	2.06	1.33 to 3.18	8.74‡	2.03	1.29 to 3.18
PHOX2B	6.8‡	−0.23	−0.37 to −0.21	.34	182	123	59	16.1*	2.41	1.59 to 3.65	15.35*	2.55	1.61 to 4.06
DCX	7.4‡	0.41	0.26 to 0.43	.22	175	131	43	17.9*	2.74	1.76 to 4.26	15.80*	2.77	1.70 to 4.50
After induction therapy													
BM													
TH	1.9	−1.88	−2.49 to −0.54	.98	152	50	102	6.5†	1.93	1.13 to 3.29	5.97†	1.98	1.11 to 3.51
PHOX2B	2.9	−1.31	−1.82 to −0.36	.08	141	96	45	8.9‡	2.03	1.29 to 3.21	7.86‡	2.07	1.25 to 3.44
DCX	0.13	−0.49	−0.98 to −0.46	.007	118	86	32	6.8‡	2.07	1.22 to 3.52	9.23‡	2.67	1.43 to 5.01
PB													
TH	0.08	−0.53	—§	.11	136	133	3	3.4	NA		3.30	NA	
PHOX2B	0.12	−2.75	—§	.85	129	124	5	1.0	NA		1.39	NA	
DCX	0.26	−1.60	—§	.02	107	102	5	5.7†	NA		6.13†	NA	

NOTE. χ^2 values, *P* values, and 95% CIs of HRs for univariable and multivariable Cox model analyses are indicated, using levels of mRNA as continuous variable or using a single cut point. The cut-point simulation *P* value evaluates whether the observed cut-point χ^2 is likely to be a chance finding given the χ^2 from analyzing the RTqPCR as a continuous variable (see Methods and Data Supplement).

Abbreviations: BM, bone marrow; DCX, doublecortin; HR, hazard ratio; Log RQ, log_{10} relative quantification normalized value; NA, not applicable because of low numbers of children with levels greater than the cut point; PB, peripheral blood; PHOX2B, paired-like homeobox 2b; RTqPCR, reverse transcriptase quantitative polymerase chain reaction; TH, tyrosine hydroxylase.

*$P < .001$.

†$P < .05$.

‡$P < .01$.

§Not possible to determine a reliable cut point.

Neuroblastoma Trial-1 of the European Society of Pediatric Oncology Neuroblastoma Group (HR-NBL1/SIOPEN).

METHODS

Clinical Samples, Children, and Trial

BM (2×0.5 mL from the right and left site, not pooled) and PB (1×2 mL) were taken from children diagnosed with stage 4 neuroblastoma enrolled onto HR-NBL1/SIOPEN between January 2003 and June 2009 (www.SIOPEN-R-NET.org).[20] Samples were collected at the time of entry onto the study (diagnosis) and after dose-intensive induction chemotherapy (rapid cisplatin, vincristine, carboplatin, etoposide, and cyclophosphamide)[20,21] a median of 89 days later (interquartile range, 85 to 98 days), but before myeloablative therapy. All children were evaluated according to the study protocol.[22] Written informed consent was obtained from the guardian of each child, and the trial was approved ethically in each participating country by the competent authorities. Data and safety monitoring was undertaken by an independent Data Monitoring and Safety Committee. This observational, blind, biologic study did not impact on clinical management of children in HR-NBL1/SIOPEN.

Sample Processing and Analysis

All samples were collected into PAXgene Blood RNA Tubes (PreAnalytiX, Hombrechtikon, Switzerland) and transferred to one of five SIOPEN Molecular Monitoring Group reference laboratories for analysis using optimized standard operating procedures.[17] The $\log_{10}$ relative quantification normalized values were obtained after normalization to a fixed RNA standard (800 pg of IMR-32 RNA in 400 ng of RNA from PB of a healthy donor) prepared by the central laboratory in Leeds, United Kingdom, and distributed to participating centers throughout the study.[17] Quality assurance was maintained across the reference laboratories by analysis of quality control samples biannually.[17] Details of primers and probes are provided in the Data Supplement. The triplicate result for the three target mRNAs, tyrosine hydroxylase (TH), paired-like homeobox 2b (PHOX2B), and doublecortin (DCX), and the housekeeping gene mRNA β_2-microglobulin (β2M) were recorded blind to clinical information in the SIOPEN-R-NET database.

Statistical Analysis

Event-free survival (EFS) was calculated from the time of diagnosis to an event (which could be recurrence, progression, or death caused by disease) or the date of the last assessment without event. Overall survival (OS) was defined as the time from diagnosis to death caused by disease or the date the child was last reported alive. A simple mean imputation approach[23] was used for the relatively small number of missing values, and the results were not sensitive to this assumption.

Fractional polynomials, which use a series of predefined transformations of predictor variables,[24,25] were initially calculated in an attempt to find a continuous relationship between $\log_{10}$ mRNA levels and EFS and OS. None of the transformations were significantly better than the simple linear model, which in turn proved to be substantially inferior to using a single cut point for many of the variables, particularly in PB at diagnosis.

To verify this, data were simulated assuming that the mRNA values followed a continuous linear model and were prognostic of EFS and OS. Data were simulated assuming negative exponential survival distributions; the number of patients in the simulation, distribution of follow-up times, and magnitude of the continuous linear model effect were all as found in the actual data. One million simulations were performed, resulting in a distribution of χ^2 statistics. Using the observed data, the optimal cut point and its associated χ^2 value were calculated. An exact P value was derived based on the location of the observed χ^2 value relative to the distribution of χ^2 values from the simulations. This P value addressed the null hypothesis that the observed linear model result could likely have occurred by chance. $P < .05$ suggested that the cut point model was preferred. This approach was repeated for each mRNA. Importantly, separate Cox model analyses selecting only patients above, and then below, the single optimized cut point for each mRNA could not demonstrate additional prognostic discrimination in these children.

The simulations were repeated, this time assuming the mRNA values were not prognostic of EFS and OS. This enabled us to calculate an adjusted P value for each variable by quantifying the proportion of these simulations where the χ^2 from the optimum (most significant) cut point in the simulation was greater than the analogous optimum χ^2 derived from the actual data. Therefore, these adjusted P values provide a true significance level for the analysis at that (optimum) cut point; the unadjusted P value is overstated because the chosen cut point was the optimal such cut point. These adjusted P values were used to generate the Kaplan-Meier EFS and OS curves, applying a proportional hazards adjustment to all the deaths so that the two curves came closer together, such that the log-rank tests produced P values that matched the adjusted P values. Therefore, the EFS and OS curves allow for the calculation of optimum cut points and represent best estimates for the true magnitudes of the effect on EFS and OS for these variables. Where given, the percentage

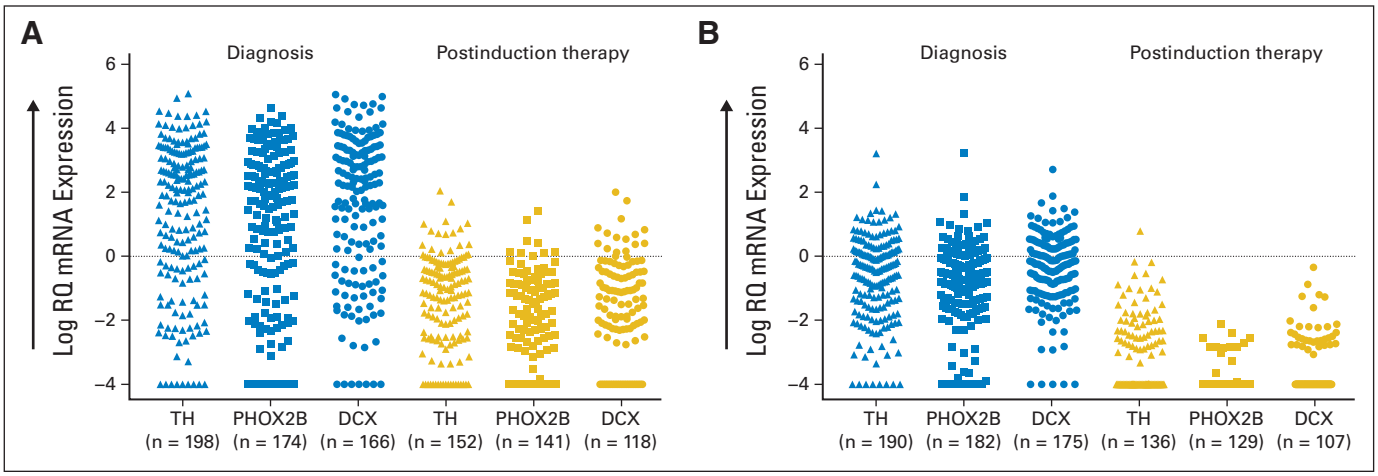

Fig 1. Levels of paired-like homeobox 2b (PHOX2B), tyrosine hydroxylase (TH), and doublecortin (DCX) mRNA at diagnosis and after induction therapy in (A) bone marrow aspirates (BM) and (B) peripheral blood (PB) of children with stage 4 neuroblastoma enrolled onto the High-Risk Neuroblastoma Trial-1 of the European Society of Pediatric Oncology Neuroblastoma Group (HR-NBL1/SIOPEN). TH, PHOX2B, and DCX mRNA levels were measured using reverse transcriptase quantitative polymerase chain reaction ($\log_{10}$ relative quantification normalized values [Log RQ]). Expression for any single mRNA in BM at diagnosis varies by 8 $\log_{10}$ (10^8-fold) and in PB by 7 $\log_{10}$ (10^7-fold). Median expression for all markers in BM and PB after induction therapy was lower than that at diagnosis by a value of at least 3 $\log_{10}$ (10^3-fold) and a maximum of 7 $\log_{10}$ (10^7-fold).

of children alive at 5 years is taken from these adjusted curves. Finally, 95% CIs were derived for the cut points for each variable using Cox analyses at additional cut points above and below the optimum cut point and using the likelihood ratio statistic to evaluate the significance of the difference between the likelihood for these cut points and that for the optimum cut point. Such CIs facilitate comparison with future results from other studies that also examine cut points for these variables (Tables 1 and 2 and Data Supplement).

These analyses and observations led to the conclusion that for the mRNAs where there was a substantial effect on EFS or OS, there is a threshold beyond which the mRNAs most reliably predict worse EFS or OS, and the analyses are therefore presented in this manner. We have also presented the *P* values obtained by considering the variable as continuous for comparison.

RESULTS

Patient Characteristics

Two hundred ninety children with stage 4 disease were studied in the original cohort, with a median age of 35 months (range, 5 to 205

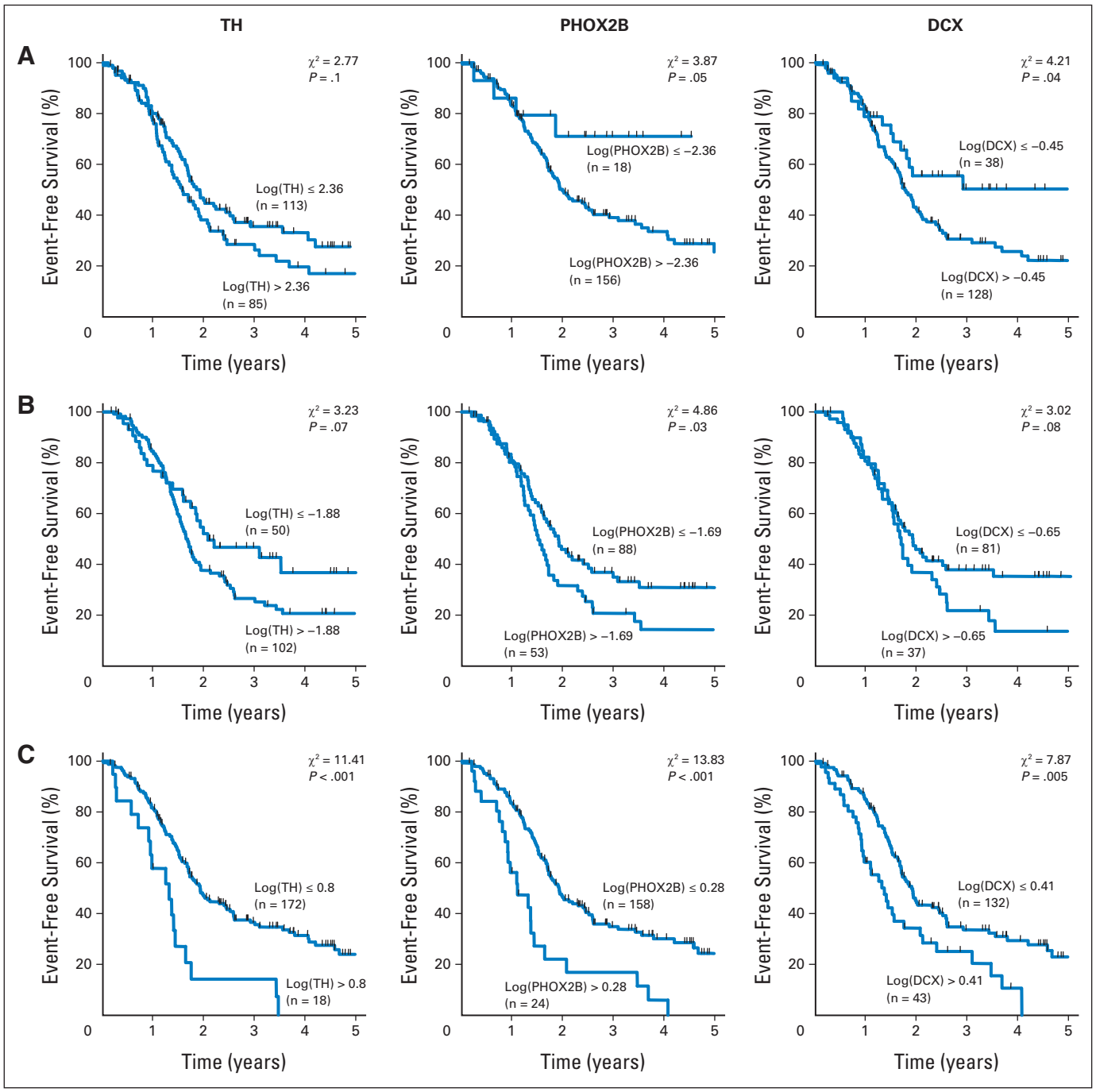

Fig 2. Prognostic and predictive power of mRNAs detected by reverse transcriptase quantitative polymerase chain reaction in bone marrow (BM) and peripheral blood (PB) from children with stage 4 neuroblastoma. Kaplan-Meier event-free survival curves, according to the level of mRNA detected in (A) BM at diagnosis and (B) after induction therapy and in (C) PB at diagnosis. (Continued on next page.)

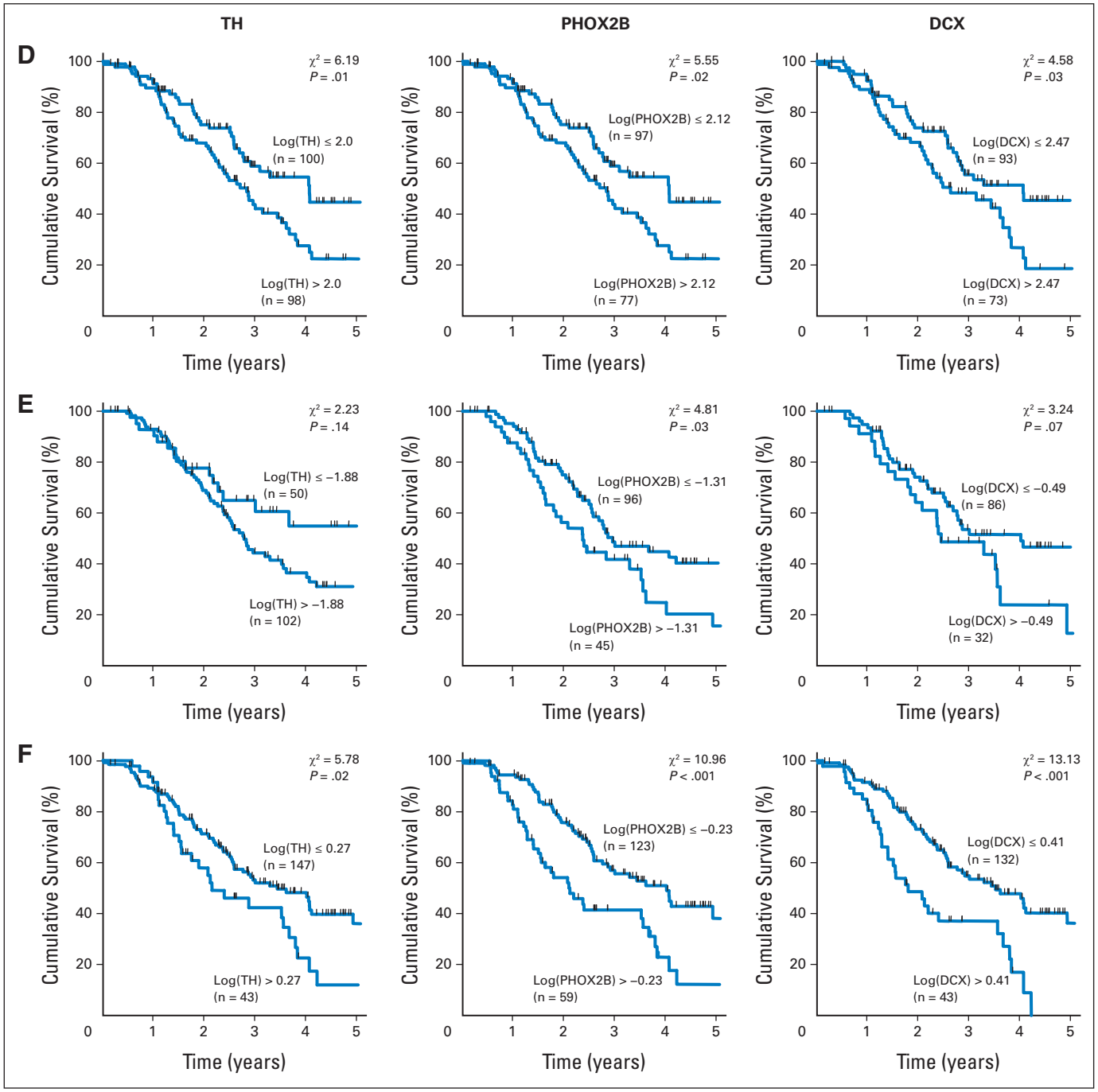

Fig 2. (Continued). Kaplan-Meier overall survival curves, according to the level of mRNA detected in (D) BM at diagnosis and (E) after induction therapy and in (F) PB at diagnosis. Log-rank χ^2 values, *P* values, and number of children with levels greater than and less than the indicated optimal mRNA cut points are shown. The number of children at risk with time for each marker is provided in the Data Supplement. DCX, doublecortin; PHOX2B, paired-like homeobox 2b; TH, tyrosine hydroxylase.

months) at diagnosis and a median follow-up time of 27 months (range, 1 to 83 months). PB from a second cohort of 175 children with stage 4 disease recruited later into HR-NBL1/SIOPEN was also analyzed (PB validation cohort); these children had a median age of 38 months (range, 2 to 238 months) at diagnosis and a median follow-up time of 23 months (range, 3 to 44 months). OS and EFS were similar in the original and validation cohorts, with $P = .79$ for OS and $P = .51$ for EFS when comparing the two. Primary tumor *MYCN* status was available in 70% of children; of these, 40% had *MYCN*-amplified tumors. BM aspirate cytology/trephine histology at diagnosis and after induction therapy was available in 91% and 92% of patients, respectively, and metaiodobenzylguanidine (MIBG) status was available in 84% of patients at diagnosis.

Frequency and Levels of TH, PHOX2B, and DCX mRNA in BM and PB

The quantitative data show that the levels of TH, PHOX2B, and DCX mRNA in BM and PB vary greatly between children and time of sample collection (Fig 1). In BM, the median levels of expression for all three mRNAs were higher at diagnosis or after induction therapy than in PB taken at the same time. There was a high concordance between levels of PHOX2B, TH, and DCX mRNA in BM; the correlation was $r = 0.92$ between TH and PHOX2B mRNA, $r = 0.89$ between TH and DCX, and $r = 0.95$ between DCX and PHOX2B. After induction therapy in BM and at diagnosis in PB, the median level of mRNAs was lower than that at diagnosis in BM, and expression of the three mRNAs was slightly less highly correlated. For example, in PB at diagnosis, the correlation was $r = 0.79$ between TH and PHOX2B, $r = 0.68$ between TH and DCX, and $r = 0.84$ between DCX and PHOX2B (Data Supplement). After induction therapy (when the levels of mRNAs in PB were frequently low or undetectable), there was no correlation between the level of mRNAs in PB. The number of samples it was possible to analyze for all three mRNAs varied depending on the RNA yield from each sample (Data Supplement). Correlations between the mRNAs and skeletal MIBG uptake (which was positive in 85% of patients with available data) at diagnosis were low, with r values ranging between 0.22 and 0.25 for mRNAs in BM and between 0.07 and 0.15 in PB. Not surprisingly, there was a higher correlation between the level of mRNAs in BM and the presence of neuroblastoma cells detected by BM cytology and histology at diagnosis, with r values ranging between 0.58 and 0.60.

Prognostic and Predictive Significance of TH, PHOX2B, and DCX mRNA in BM

High levels of PHOX2B, TH, or DCX mRNA strongly predicted for EFS when detected in BM at diagnosis in both univariable and multivariable analyses (Table 1). No single mRNA had substantially greater predictive capacity than any other mRNA in BM at diagnosis (Tables 1 and 2, Fig 2). There was considerable overlap between the patients identified by the three mRNAs. For example, all children with good prognosis identified by low expression of PHOX2B (n = 18) also had low TH and low DCX. High expression of PHOX2B, TH, or DCX mRNA was also predictive of EFS and OS when detected in BM after induction therapy (Tables 1 and 2, Fig 2). Age, *MYCN* status of primary tumor, cytology of BM aspirate, or trephine histology at diagnosis did not add substantially to the predictive value of the

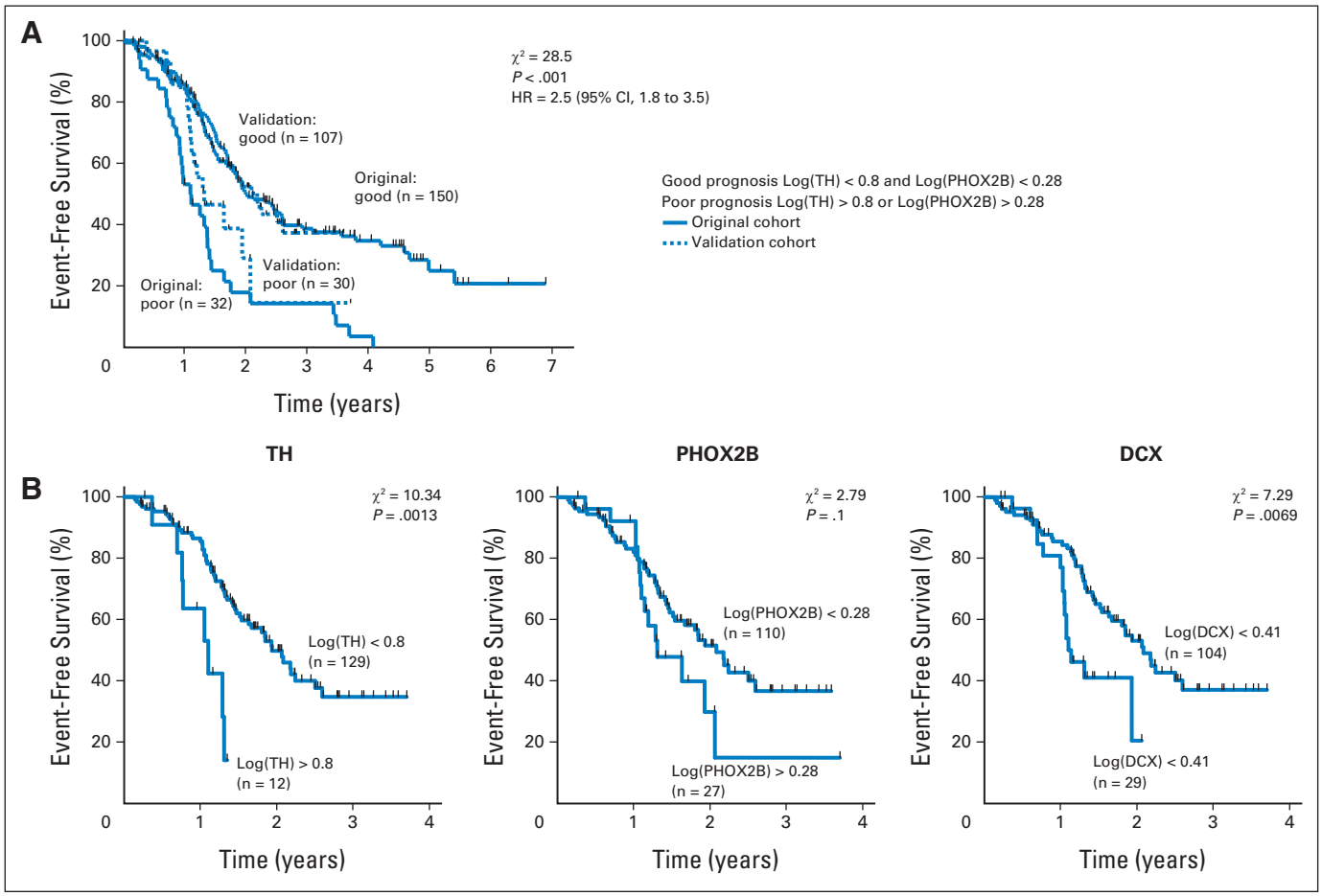

Fig 3. Prognostic and predictive power of tyrosine hydroxylase (TH) and paired-like homeobox 2b (PHOX2B) mRNAs detected by reverse transcriptase quantitative polymerase chain reaction in peripheral blood (PB) from children with stage 4 neuroblastoma at diagnosis. Kaplan-Meier event-free survival curves according to combined levels of $\log_{10}$(TH) and $\log_{10}$(PHOX2B) mRNA detected in PB at diagnosis (A) in the original study cohort and a validation cohort using the same cut points and (B) according to the level of mRNAs detected in PB at diagnosis in the validation set using the cut points identified in the original study cohort. Log-rank χ^2 values, P values, and number of children with levels greater than and less than the indicated optimal mRNA cut points are shown. The number of children at risk with time for TH and PHOX2B is provided in the Data Supplement. DCX, doublecortin; HR, hazard ratio.

mRNAs. For example, $\log_{10}$TH showed a Cox model χ^2 of 11.4 ($P < .001$) for OS (using a cut point of 2; Table 2), whereas age, the most significant clinical prognostic factor in this data set, had a χ^2 of only 3.2 ($P = .07$). Further comprehensive multivariable analyses demonstrated that postinduction therapy mRNA levels did not add to the predictive capacity of mRNA values at diagnosis, suggesting that the mRNA levels at diagnosis are the key determinants of outcome.

Prognostic and Predictive Significance of TH, PHOX2B, and DCX mRNA in PB

High levels of any of the three mRNAs in PB at diagnosis strongly predict for inferior EFS (Table 1, Fig 2) and OS (Table 2, Fig 2). Levels of TH, PHOX2B, or DCX mRNA in PB taken at diagnosis robustly predict for worse EFS, identifying 9% (18 of 190 children), 13% (24 of 182 children), and 25% (43 of 175 children) of children, respectively, with a two- to three-fold increased risk of relapse (Table 1, Fig 2). The predictive power of these mRNAs in PB at diagnosis was confirmed in the PB validation cohort (n = 175). Strikingly, children with levels of TH or PHOX2B mRNA greater than the respective derived cut points in both the original and validation cohorts had an exceptionally poor EFS (Fig 3, Table 3) and OS (Data Supplement); high TH ($\log_{10}$TH > 0.8) or high PHOX2B ($\log_{10}$PHOX2B > 0.28) levels identified 19% of children as ultrahigh risk, with 5-year EFS and OS rates of 0%. This compares to 5-year EFS and OS rates of 25% (95% CI, 16% to 36%) and 38% (95% CI, 28% to 49%), respectively, for the remaining children. Similarly, clear discriminations for EFS and OS, with overall log-rank χ^2 values of 21.5 and 22.0, respectively (HR, 2.0 and 2.1, respectively), were also evident using the lower PB OS cut points for TH and PHOX2B of 0.27 and −0.23, respectively; these identified a slightly larger group of children (39%) with medium risk (data not shown). DCX did not add to the discriminatory effect of TH and PHOX2B combined, reflecting the overlap of PHOX2B and DCX mRNA distribution (results not shown). These observations suggest that TH and PHOX2B mRNAs are attractive propositions for disease prognostication in PB at diagnosis. The same was not the case in PB taken after induction therapy, where none of the mRNAs were predictive of EFS or OS (Tables 1 and 2, respectively).

Table 3. Comparison of Event-Free Survival HRs and 95% CIs for TH, PHOX2B, and DCX mRNAs Detected by RTqPCR in PB From the Children With Stage 4 Neuroblastoma at Diagnosis in the Original Cohort and Validation Set

	Original Cohort (n = 182)		Validation Set (n = 137)	
Time and Target	HR*	95% CI	HR*	95% CI
Diagnosis				
TH	1.22	1.04 to 1.44	1.25	1.06 to 1.47
DCX	1.20	1.00 to 1.45	1.32	1.12 to 1.56
PHOX2B	1.20	1.02 to 1.42	1.28	1.08 to 1.51
After induction therapy				
TH	1.03	0.85 to 1.25	0.98	0.76 to 1.26
DCX	1.00	0.76 to 1.31	0.96	0.72 to 1.29
PHOX2B	1.32	0.77 to 2.24	1.04	0.74 to 1.47

Abbreviations: DCX, doublecortin; HR, hazard ratio; PB, peripheral blood; PHOX2B, paired-like homeobox 2b; RTqPCR, reverse transcriptase quantitative polymerase chain reaction; TH, tyrosine hydroxylase.

*HRs are given treating the log (base 10) RTqPCR as a continuous variable, so that they measure the effect of a unit difference in these variables. Hazards considered in this way are multiplicative; thus, for example, the HR for a 5-$\log_{10}$ difference between diagnosis $\log_{10}$(TH) values, where the values were for example −1 and 4, would be 1.22^{-5}, which equals 2.7.

Magnitude of TH, PHOX2B, and DCX mRNA Clearance Does Not Predict Outcome

There is no indication that the magnitude of difference in the level of any mRNA or combination of mRNAs measured at diagnosis and after induction therapy (so-called clearance) in PB or BM predicts EFS or OS (Data Supplement). Although the number of children in these analyses is small (< 100 children) compared with those examining the predictive power of individual mRNAs, in a Cox model, none of the multivariable *P* values are close to significant and the level of mRNA clearance ($\log_{10}$ reduction) from diagnosis to after induction therapy did not add any additional predictive power over that of individual mRNA levels. Any contribution that clearance might have is negated by the predictive power of the initial mRNA level in this group of children, and it is the level of mRNA at diagnosis that dominates in the predictive analysis.

DISCUSSION

The current study demonstrates for the first time, to our knowledge, in a prospective trial that levels of PHOX2B, TH, or DCX mRNA in BM and PB at diagnosis and in BM after induction therapy are independent predictors of EFS and OS. The predictive power of these mRNAs at diagnosis reveals how their quantification could be used to identify upfront children with stage 4 disease for whom current treatment is failing and who may be candidates for alternative novel experimental therapeutics, with the anticipated logistical, financial, and outcome advantages mandating a change in current clinical practice.

In two independent data sets, high levels of TH or PHOX2B mRNA in PB at diagnosis identify 19% of children as having ultrahigh-risk disease, who may be candidates for alternative treatment strategies. The detection of these mRNAs in PB at diagnosis is particularly attractive because this compartment is easy to sample, making it suitable for development of a minimally invasive and cost-effective RTqPCR biomarker assay for patient classification. In BM at diagnosis, high levels of these mRNAs also predict for worse EFS and OS, although based on current data, we expect analysis of PB at this time to be more informative and therefore potentially of greater value for guiding treatment choice. After induction therapy, the levels of PHOX2B, TH, and DCX mRNA were predictive of EFS and OS in BM, suggesting these mRNAs could be used to monitor patient status throughout the disease and treatment course in real time, with expression of these mRNAs being consistent with failure of current induction therapy to effectively clear disease in a group of high-risk children. However, this did not add to the predictive power of a single mRNA detected at diagnosis, indicating that the level of mRNAs at diagnosis is the most powerful predictor of outcome in this data set, and therefore selecting children for treatment based on mRNA level at diagnosis might be more clinically informative for some children than modifying treatment based on response criteria measured after induction therapy. The predictive value of these mRNAs should be compared with response assessed after induction therapy measured using an MIBG score, which has recently been shown to be prognostic in children with stage 4 disease.[26] It will be important in future studies to determine whether RTqPCR and MIBG are identifying the same

ultrahigh-risk children. The predictive value of mRNAs in this large series of stage 4 children with neuroblastoma is consistent with reports from smaller studies in PB[7,12,14,27] and BM[8-14,26,28] and demonstrates the suitability of this quantitative objective approach across multiple laboratories.

Several groups have reasoned that given the heterogeneity of the neuroblastoma cell, multiple RNAs would be more informative than a single mRNA.[14,19,27,29,30] Consistent with this hypothesis, TH and PHOX2B mRNA levels are of greatest prognostic value in PB at diagnosis. However, no single mRNA or mRNA combination proved superior in BM at diagnosis or after induction therapy. These observations are consistent with the high degree of correlation between all three mRNAs in BM and the difference in TH mRNA distribution compared with that of PHOX2B and DCX in PB. This distribution will impact on the optimal predictive cut points for each mRNA and may be influenced by treatment efficacy, which requires further investigation. International studies are now under way to compare the independent predictive value of RTqPCR for these neuroblastoma mRNAs at diagnosis with that of genomic aberrations,[31] multigene expression signatures,[32,33] and BM histology and cytology[34] to develop a risk classification algorithm for children with high-risk disease that most reliably identifies those with ultrahigh-risk disease so that they may be offered alternative experimental treatment.

The results from this RTqPCR study warrant a biomarker qualification study, stratifying children with high-risk neuroblastoma for therapy based on the level of TH and PHOX2B mRNAs in PB at diagnosis. The dynamic range and prognostic value of these neuroblastoma mRNAs may contribute to the development of a prognostic score for integration into clinical practice, informing treatment choice for children with high-risk neuroblastoma.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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REFERENCES

1. Matthay KK, Villablanca JG, Seeger RC, et al: Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis-retinoic acid: Children's Cancer Group. N Engl J Med 341:1165-1173, 1999
2. London WB, Castleberry RP, Matthay KK, et al: Evidence for an age cutoff greater than 365 days for neuroblastoma risk group stratification in the Children's Oncology Group. J Clin Oncol 23:6459-6465, 2005
3. Monclair T, Brodeur GM, Ambros PF, et al: The International Neuroblastoma Risk Group (INRG) staging system: An INRG Task Force report. J Clin Oncol 27:298-303, 2009
4. Cohn SL, Pearson AD, London WB, et al: The International Neuroblastoma Risk Group (INRG) classification system: An INRG Task Force report. J Clin Oncol 27:289-297, 2009
5. Ambros PF, Ambros IM, Brodeur GM, et al: International consensus for neuroblastoma molecular diagnostics: Report from the International Neuroblastoma Risk Group (INRG) Biology Committee. Br J Cancer 100:1471-1482, 2009
6. Burchill SA, Selby PJ: Molecular detection of low-level disease in patients with cancer. J Pathol 190:6-14, 2000
7. Burchill SA, Lewis IJ, Abrams KR, et al: Circulating neuroblastoma cells detected by reverse transcriptase polymerase chain reaction for tyrosine hydroxylase mRNA are an independent poor prognostic indicator in stage 4 neuroblastoma in children over 1 year. J Clin Oncol 19:1795-1801, 2001
8. Horibe K, Fukuda M, Miyajima Y, et al: Outcome prediction by molecular detection of minimal residual disease in bone marrow for advanced neuroblastoma. Med Pediatr Oncol 36:203-204, 2001
9. Tchirkov A, Paillard C, Halle P, et al: Significance of molecular quantification of minimal residual disease in metastatic neuroblastoma. J Hematother Stem Cell Res 12:435-442, 2003
10. Cheung IY, Lo Piccolo MS, Kushner BH, et al: Early molecular response of marrow disease to biologic therapy is highly prognostic in neuroblastoma. J Clin Oncol 21:3853-3858, 2003
11. Pession A, Libri V, Sartini R, et al: Real-time RT-PCR of tyrosine hydroxylase to detect bone marrow involvement in advanced neuroblastoma. Oncol Rep 10:357-362, 2003
12. Corrias MV, Faulkner LB, Pistorio A, et al: Detection of neuroblastoma cells in bone marrow and peripheral blood by different techniques: Accuracy and relationship with clinical features of patients. Clin Cancer Res 10:7978-7985, 2004
13. Parareda A, Gallego S, Roma J, et al: Prognostic impact of the detection of microcirculating tumor cells by a real-time RT-PCR assay of tyrosine hydroxylase in patients with advanced neuroblastoma. Oncol Rep 14:1021-1027, 2005
14. Träger 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 123:2849-2855, 2008
15. Stutterheim J, Zappeij-Kannegieter L, Versteeg R, et al: The prognostic value of fast molecular response of marrow disease in patients aged over 1 year with stage 4 neuroblastoma. Eur J Cancer 47:1193-1202, 2011
16. Burchill SA: Micrometastases in neuroblastoma: Are they clinically important? J Clin Pathol 57:14-20, 2004
17. Viprey VF, Corrias MV, Kagedal B, et al: Standardisation of operating procedures for the detection of minimal disease by QRT-PCR in children with neuroblastoma: Quality assurance on behalf of SIOPEN-R-NET. Eur J Cancer 43:341-350, 2007
18. Kågedal B: Detecting minimal residual disease in neuroblastoma: Still a ways to go. Clin Chem 55:1268-1270, 2009
19. Viprey VF, Lastowska MA, Corrias MV, et al: Minimal disease monitoring by QRT-PCR: Guidelines for identification and systematic validation of molecular markers prior to evaluation in prospective clinical trials. J Pathol 216:245-252, 2008
20. Ladenstein R, Valteau-Couanet D, Brock P, et al: Randomized trial of prophylactic granulocyte colony-stimulating factor during rapid COJEC induction in pediatric patients with high-risk neuroblastoma: The European HR-NBL1/SIOPEN study. J Clin Oncol 28:3516-3524, 2010
21. Pearson AD, Pinkerton CR, Lewis IJ, et al: High-dose rapid and standard induction chemotherapy for patients aged over 1 year with stage 4 neuroblastoma: A randomised trial. Lancet Oncol 9:247-256, 2008
22. Brodeur GM, Pritchard J, Berthold F, et al: Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. J Clin Oncol 11:1466-1477, 1993
23. Gelman A, Hill J: Missing-data imputation, in Data Analysis Using Regression and Multilevel/Hierarchical Models. Cambridge, United Kingdom, Cambridge University Press, 2007, pp 529-543
24. Royston P, Altman DG: Regression using fractional polynomials of continuous covariates: Parsimonious parametric modelling (with discussion). Appl Stat 43:429-467, 1994
25. Altman DG, Royston P: The cost of dichotomising continuous variables. BMJ 332:1080, 2006
26. Yanik GA, Parisi MT, Shulkin BL, et al: Semiquantitative mIBG scoring as a prognostic indicator

in patients with stage 4 neuroblastoma: A report from the Children's Oncology Group. J Nucl Med 54:541-548, 2013

27. Corrias MV, Haupt R, Carlini B, et al: Multiple target molecular monitoring in bone marrow and peripheral blood samples from patients with localized neuroblastoma and healthy donors. Pediatr Blood Cancer 58:43-49, 2012

28. Stutterheim J, Gerritsen A, Zappeij-Kannegieter L, et al: PHOX2B is a novel and specific marker for minimal residual disease testing in neuroblastoma. J Clin Oncol 26:5443-5449, 2008

29. Cheung IY, Feng Y, Gerald W, et al: Exploiting gene expression profiling to identify novel minimal residual disease markers of neuroblastoma. Clin Cancer Res 14:7020-7027, 2008

30. Stutterheim J, Gerritsen A, Zappeij-Kannegieter L, et al: Detecting minimal residual disease in neuroblastoma: The superiority of a panel of real-time quantitative PCR markers. Clin Chem 55:1316-1326, 2009

31. Maris JM: Recent advances in neuroblastoma. N Engl J Med 362:2202-2211, 2010

32. Vermeulen J, De Preter K, Naranjo A, et al: Predicting outcomes for children with neuroblastoma using a multigene-expression signature: A retrospective SIOPEN/COG/GPOH study. Lancet Oncol 10:663-771, 2009

33. Oberthuer A, Hero B, Berthold F, et al: Prognostic impact of gene expression-based classification for neuroblastoma. J Clin Oncol 28:3506-3511, 2010

34. Beiske K, Burchill SA, Cheung IY, et al: Consensus criteria for sensitive detection of minimal neuroblastoma cells in bone marrow, blood and stem cell preparations by immunocytology and QRT-PCR: Recommendations by the International Neuroblastoma Risk Group Task Force. Br J Cancer 100:1627-1637, 2009

Support

Supported by Cancer Research UK (Grant No. C5651/A9663; S.A.B.), the Neuroblastoma Society (S.A.B.), the Italian Neuroblastoma Foundation (M.V.C.), the French National Hospital Research Project (A.T.), Ministry of Health–Czech Republic Conceptual Development of Research Organization, University Hospital Motol (A.V.), and the University of Leeds.

Acknowledgment

We thank Ulrike Pötschger for the collection and assembly of the clinical outcome data and Günter Schreier for maintenance of SIOPEN-R-NET. We thank the parents, guardians, and children who participated in this study and the hospital staff at the contributing centers.

This work was presented in part at the 44th Annual Congress of the International Society of Pediatric Oncology, October 5-8, 2012, London, United Kingdom.

V.F.V., M.V.C., A.T., K.S., A.V., S.D., and S.A.B. are members of the International Society of Pediatric Oncology European Neuroblastoma Research Network Molecular Monitoring Group.