Original Article

RT-PCR demonstrates superior sensitivity and specificity in detecting the five neuroblastoma genes compared to the flow cytometry method for measurable residual disease

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Background: Exploring sensitive prognostic methods for patients with relapsed or refractory neuroblastoma (NB) is critical. The five NB genes (NB5) share a common trait: they are highly expressed in NB. Previous studies have identified their expression levels as markers for guiding micrometastasis. This study aimed to explore whether an improved NB5 detection method is superior to flow cytometry for predicting NB metastasis, measurable residual disease (MRD), and prognosis, and whether this result could serve as an independent factor to influence progression-free survival (PFS).

Methods: We utilized reverse transcriptase polymerase chain reaction (RT-PCR) to assess the expression of NB5 (*CHGA*, *DCX*, *DDC*, *PHOX2B*, and *TH*) in bone marrow (BM), peripheral blood (PB), or cerebrospinal fluid (CSF) samples collected from 71 patients. The correlation between gene expression changes and clinical characteristics, as well as survival rates, based on 113 detections were analyzed. The NB5 detection results' sensitivity and specificity in all 71 patients collected from six research centers with a median follow-up of 14 months were assessed.

Results: PB specimens showed 100% concordance with the BM specimens in terms of positive results. Furthermore, the BM specimens exhibited an additional 45.455% (5/11) positive results compared to the 34.091% (30/88) of PB specimens. The BM specimens were positive for NB5 assay, which was significantly higher than the positive results of flow cytometric MRD (15/88, 17.045%). NB5 was mainly expressed in newly diagnosed patients (P=0.043) and positive patients with flow cytometric MRD (P<0.001) or BM morphology (P<0.001). Positive rates of droplet digital PCR (ddPCR) were consistent with those of quantitative RT-PCR (qRT-PCR) in BM (13/18, 72.222%). However, in PB, the positive rate of ddPCR (2/5, 40.000%) was higher than that of qRT-PCR. A total of 38 specimens (BM, PB, CSF) were detected as positive under qRT-PCR. Among the positive results, the analysis revealed a significant difference between the *CHGA* and *TH* in pairwise comparisons (P=0.005). PFS analysis showed that among MRD-negative patients, the survival time of the NB5-positive group was significantly lower than that of NB5-negative group (27.408±10.791 *vs.* 35.961±3.084 months; P=0.034), and in the Cox regression model, risk stratification based on NB5 expression level was an independent prognostic factor for relapsed or refractory

disease [95% confidence interval (CI):1.020 to 9.099, hazard ratio (HR) =3.046, P=0.046]. Combining the follow-up results, we found that the sensitivity and specificity of NB5 detection were both 100%.

Conclusions: In our study, the improved NB5 detection method showed significantly higher sensitivity in assessing tumor relapse or residual disease compared to flow cytometric MRD. Moreover, it provided a more accurate assessment of treatment efficacy and prognosis. These findings support NB5 detection as an effective method for further stratification and monitoring of patients with relapsed or refractory NB.

Keywords: Neuroblastoma (NB); NB5 assay; survival

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Introduction

Neuroblastoma (NB) is highly heterogeneous and has a highly variable prognosis, ranging from spontaneous regression or untreated maturation to high aggressiveness (1). Approximately 50% of pediatric patients diagnosed with NB have metastatic disease (2). The International Neuroblastoma Risk Group (INRG) reports multiple factors as independent prognostic factors for recurrence (3-5).

A variety of different therapeutic strategies are being used to improve the prognosis of patients with relapsed or refractory NB. Therapeutic targets identified using next-generation sequencing or immunology, radiation therapy, autologous stem cell transplant, targeting of the tumor microenvironment associated with promoting survival or drug resistance, and the use of the differentiating agent isotretinoin have largely improved the survival of patients with high-risk NB (6-9). However, despite intensive multimodal treatment and bone marrow (BM) transplant, the prognosis for these patients is still unsatisfactory (10). Therefore, finding more sensitive monitoring methods may be of great significance in predicting the prognosis of patients with NB.

The most common metastatic sites of NB are the BM, cortical bone, and lymph nodes. In cases of relapsed or refractory NB, most patients have BM infiltration. Although the current gold standard for BM metastasis is BM cell morphology, residual tumor cells may not be detected or clearly reflect tumor viability and metabolism as compared to progression (11,12). ^{123}I-meta-iodobenzylguanidine (MIBG) technology can be used to assess the degree of disease progression. Unfortunately, MIBG cannot accurately quantify changes in disease sites, identify new sites of minimal disease, or distinguish parts of disease sites (13,14). Using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and droplet digital PCR (ddPCR) to evaluate the mRNA expression level of specific genes in peripheral blood (PB) and BM can provide early and rapid assessment of the response and prognosis of patients with NB at the molecular level and help to characterize areas that are not accessible by other detection methods. Related research indicates that the use of qRT-PCR or ddPCR in BM and PB at diagnosis or BM after induction treatment is capable of detecting the high expression of specific genes and of providing more comprehensive prognostic information for patients with NB (15-18).

Highlight box

Key findings

- The detection of five neuroblastoma genes (NB5) can have a more accurate assessment of prognosis than flow cytometry for measurable residual disease (MRD). It represents a potential new method for better assessing disease status and guiding treatment.

What is known and what is new?

- The five NB5 genes are characterized by rare or no expression in bone marrow and peripheral blood but strong expression in neuroblastoma (NB) in vivo or in cell lines.
- Our study demonstrated that NB5 detection shows significantly higher sensitivity in assessing tumor relapse or residual disease compared to flow cytometric MRD detection. Improved NB5 assay serves as new standard to redefine disease status and is independently associated with progression-free survival in relapsed/refractory NB.

What is the implication, and what should change now?

- NB5 assay is a potential method to better guide treatment and assess prognosis. Although the number of false positives and false negatives in NB5 detection is small, larger multicenter studies that combine more clinical features and use a diversity of detection methods are still needed to comprehensively evaluate and explore the application value of these five genes in NB.

Several studies have identified multiple individual messenger RNA (mRNA) with stable expression in NB (19-21). Due to the limited sensitivity of a single marker, a set of multiple markers was identified for the qRT-PCR testing of patients with NB (22-26). A recent study identified five genes, chromogranin A (*CHGA*), doublecortin (*DCX*), dopa decarboxylase (*DDC*), paired-like homeobox 2b (*PHOX2B*), and tyrosine hydroxylase (*TH*), that were rarely or never expressed in BM or PB but that were strongly expressed in NB *in vivo* or in cell lines (14,17). However, in previous studies, the calculation of multiple markers was primarily based on signature approach. This method of calculation can easily lead to false-negative outcomes because the expression levels of some genes do not increase. In consideration of the stability of gene expression across various risk groups of NB, their correlation with progression-free survival (PFS) (the time from patient enrollment to the onset of disease progression) or overall survival (OS) (time from enrollment to death from any cause), and their expression levels in other hematopoietic cells, the aforementioned five genes were chosen. They will be combined with appropriate calculation methods for further analysis. We detected the expression levels of five genes in different specimens [BM, PB, and cerebrospinal fluid (CSF)] and found the results were associated with clinical evaluation.

This study aimed to evaluate the sensitivity and specificity of five genes in disease progression, clarify their role in disease burden, and determine whether an NB5 assay is superior to flow cytometry of measurable residual disease (MRD) in predicting metastasis or residual disease and the prognosis of NB. Our results showed that in relapsed or refractory NB, NB5 provided earlier and more accurate detection than did flow cytometric MRD and that NB5 can be used as an independent factor for assessing NB clinical disease and prognosis. Our results can provide value in further risk stratification for more accurate assessment of NB metastasis and residual disease, better guidance of treatment, and superior assessment of prognosis. We present this article in accordance with the REMARK reporting checklist (available at https://tp.amegroups.com/article/view/10.21037/tp-23-545/rc).

Methods

Patient information

From October 2018 to June 2023, a total of 71 cases aged 1 to 13 years old (median, 5 years old) with NB were enrolled across six research centers (Hunan Provincial People's Hospital, Children's Hospital Affiliated to Medical College of Zhejiang University, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Anhui Provincial Children's Hospital, Children's Hospital of Chongqing Medical University, Children's Hospital of Fudan University). All cases were classified according to the 2009 International Neuroblastoma Risk Group Staging System (INRGSS), the Chinese expert consensus on diagnosis and treatment of pediatric neuroblastoma (2015 or 2021 edition), or the 2007 Children's Oncology Group (COG) Risk Staging System. They were categorized into low-risk, intermediate-risk, and high-risk groups. Among them, three patients were classified as low risk, two as intermediate risk, and the rest as high risk. All cases were diagnosed and treated according to the guidelines of the Expert Consensus on Diagnosis and Treatment of Pediatric Neuroblastoma of 2015 and the Expert Consensus on Diagnosis and Treatment of Pediatric Neuroblastoma 2021 (CCCG-NB-2021) protocol. Regular treatments were administered based on these guidelines. Three patients underwent prospective collection of BM, PB, or CSF specimens for NB5 assay. Additionally, all patients underwent concurrent BM morphology flow cytometric MRD, and qRT-PCR detection. We collected all patients with the above detect items and meeting the above criteria during the above time period for this study. A total of 182 standard disease assessments were performed and made available for analysis. The follow-up duration was 5 years, the final follow-up time was June 2023, and the median follow-up time was 14 months. During the treatment process, tumor markers were reassessed for each course of treatment, and imaging was reviewed for every two courses of treatment. Following the completion of treatment, tumor markers and imaging examinations will be reviewed every 3 months in the first year, every 4 months in the second year, and every 6 months in the third and fourth years. Follow-up was conducted by telephone interviews and outpatient review. Loss to follow-up is defined as no follow-up for more than 6 months after the end of treatment, and the last follow-up time is as of July 9, 2023. If new lesions are detected during imaging reexamination during treatment and are accompanied by an increase in neuron-specific enolase (NSE) or vanillylmandelic acid (VMA), recurrence is diagnosed. A comprehensive disease evaluation was performed on BM aspirate/biopsy prior to treatment. During the course of treatment, tumor biomarkers and

BM MRD (BM-MRD) levels were examined to assess tumor burden. Additionally, the expression levels of NB5 mRNA in BM and/or PB were also tested at the same time point. The gold standard for assessing whether there is BM infiltration is BM cell morphology. This study obtained approval from the research ethics committees of each participating institution and was conducted in accordance with the approved guidelines. Investigators obtained written informed consent from the legal guardians of all the patients involved. All the aforementioned patients' various detection parameters are compared with the qRT-PCR results to assess the correlation between qRT-PCR and other testing methods at the same time point. This analysis aims to evaluate the sensitivity, specificity, and prognostic guidance of detecting the five genes. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and approved by the Medical Ethics Committee of Hunan Provincial People's Hospital (approval No. 2023-106).

Sample processing and NB5 assay

BM, PB, or CSF total RNA extraction was performed using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and stored at −80 ℃ until use. The RNA integrity value (RIN) was determined using a bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), with RIN >5.5 being considered acceptable. The mean RIN for BM was 8.5 (range, 6.3–9.7), and that for PB was 8.2 (range, 7.38–9.09). Reverse transcription of 2,000 ng total RNA was performed using 20 µL of reaction volume and Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase. The expression levels of *CHGA*, *DCX*, *DDC*, *PHOX2B*, and *TH* were quantitatively analyzed with beta-2-microglobulin (*B2M*) serving as the housekeeping gene for normalization. A qRT-PCR analysis was performed using predesigned and preoptimized probe and primer sets on an ABI 7500 Fast Real-time PCR system (Applied Biosystems, Thermo Fisher Scientific). Relative mRNA expression was evaluated using the Δ threshold cycle (ΔCt) method as described previously (14). The Ct value for each gene was defined as the cycle number where the amplification signal exceeded the baseline threshold by at least 0.5 cycles. If the threshold was not reached by the 45th cycle, it was designated as 45. A lower Ct value indicated a higher expression level of the NB5 genes. ddPCR was performed using a QX200 ddPCR system (BioRad Laboratories, Hercules, CA, USA) with a total volume of 20 µL. To correct for differences in total RNA quantity and complement DNA synthesis efficiency, *B2M* was used as a housekeeping gene to normalize the target copy numbers. Relative copy numbers of each NB5 mRNA were calculated as the copy numbers of each NB5 mRNA divided by the copy numbers of *B2M* mRNA multiplied by 100%. ddPCR analysis was performed following the "Digital MIQE Guidelines: Minimum Information for Publication of Quantitative Digital PCR Experiments" (27). The primer and probe sets are listed in Table S1.

Disease evaluation

BM aspirate or trephine biopsy was evaluated for the presence of NB cells during diagnosis by at least two experts. Flow cytometry was used to detect MRD. At each disease evaluation time point during BM assessment, the responses assigned included complete remission (CR) (regardless of BM infiltration at baseline, no BM infiltration was seen when BM was re-evaluated), stable disease (SD) [BM infiltration, the extent of BM infiltration is >5% on re-evaluation but does not meet the criteria for CR, very good partial remission (VGPR) and PD], partial remission (PR) (there is no infiltration in the BM, and the extent of BM infiltration is >5% during re-evaluation, or there is infiltration in the BM, and the extent of BM infiltration is >2 times and the extent of infiltration is >20% during re-evaluation), and VGPR (if any of the following conditions occurs, it will be evaluated as VGPR. The range of BM infiltration is <5%, when re-evaluated, the BM infiltrates, but it is between 0 to 5%; There is no infiltration in the BM, when re-evaluated, there is infiltration in the BM, but the range of infiltration is between 0 to 5%; BM infiltration range >20%, BM infiltration was re-evaluated but between 0 to 5%).

Statistical analysis

Statistical computations were performed using SPSS 26.0 software (IBM Corp., Armonk, NY, USA). Standard descriptive and analytic statistical methods were employed at appropriate points, including analysis of variance (ANOVA), χ^2 test, and contingency table analysis (28). The significance in PFS was assessed using Kaplan-Meier survival analysis, and the two-sided log-rank test was used to compare the survival curves. Univariate analysis of predicted values of NB5 ΔCt and other variables was completed via the Cox proportional hazards regression model. Evaluate

Table 1 Patients, clinical disease assessment, and NB5 expression detection

Characteristics	Category description	Values
Sex	Male	44 (61.972)
	Female	27 (38.028)
Age (months)	–	5 [1–13]
Risk group	Low-risk group	3 (4.225)
	Medium-risk group	2 (2.817)
	High-risk group	66 (92.958)
Bone marrow morphology	Positive/total†	8/91 (8.791)
MRD (flow cytometric)	Positive/total†	15/91 (16.484)
Bone marrow NB5 qRT-PCR	Positive/total†	30/89 (33.708)
Blood NB5 qRT-PCR	Positive/total†	6/22 (27.273)
Cerebrospinal fluid NB5 qRT-PCR	Positive/total†	2/2 (100.000)
Bone marrow NB5 ddPCR	Positive/total†	13/18 (72.222)
Blood NB5 ddPCR	Positive/total†	2/5 (40.000)
Cerebrospinal fluid NB5 ddPCR	Positive/total†	1/1 (100.000)

Data are shown as number (%) or median [range]. †, number of assessments. NB5, five neuroblastoma genes (*CHGA, DCX, DDC, PHOX2B*, and *TH*); *CHGA*, chromogranin A; *DCX*, doublecortin; *DDC*, dopa decarboxylase; *PHOX2B*, paired-like homeobox 2b; *TH*, tyrosine hydroxylase; MRD, measurable residual disease; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; ddPCR, droplet digital PCR.

the sensitivity and specificity of NB5 detection based on the follow-up results. P values <0.05 were considered statistically significant for all tests.

Results

Patient and sample characteristics

A total of 71 newly diagnosed patients with NB who had submitted at least one specimen for NB5 assay were included in this study (*Table 1*). A total of 182 standard disease assessments were performed, with 113 NB5 assays performed via qRT-PCR and 24 via ddPCR. The median time between disease assessment and qRT-PCR or ddPCR detection was 2 days (*Table 1*, Figure S1). Among the 71 patients, there were slightly more male patients than female patients (1.63:1), the median age was 5 years old (range, 1–13 years), and 92.958% (66/71) of patients were in the high-risk group (*Table 1*).

Comparison of clinical biological characteristics based on the expression levels of NB5

Before conducting this study, a baseline was established using 30 negative BM specimens from the Chinese population and 100 clinical BM specimens from patients with NB. The Ct value-positive threshold was set at 39, while the ΔCt-positive threshold was set below 18. Among the five genes, a positive result was determined if there was at least one gene with a ΔCt value below 18. Patients were divided into two groups based on the expression results of NB5. A total of 38 specimens were in the NB5-positive group, and the remaining 75 specimens were in the negative group. Furthermore, we conducted a comparison of their clinical symptoms. There were no significant differences between the NB5 qRT-PCR positive group and the negative group in gender, risk group, or treatment stage. However, significant associations were found between NB5 genes and diagnosis in both the positive and negative groups (P=0.043), and there were significant differences in MRD (flow cytometry), BM morphology, and the current states of the patient (P<0.001) (*Table 2*).

NB5 qRT-PCR assay of both BM and PB specimens

A total of 21 paired BM and PB specimens were included in this study. It was observed that PB specimens showed 100% concordance with the BM specimens in terms of positive results. Furthermore, the BM specimens exhibited an additional five positive results compared to the PB specimens (*Figure 1* and Table S2). The analysis of ΔCt values for the five genes revealed a strong correlation between BM and PB specimens. Notably, the ΔCt values in the BM were consistently smaller than those in the PB for all the genes. Each gene was positive in BM but negative in PB. These results suggest a lower concentration of NB cells in the PB than in BM. In the specimens with strong expression of the five genes by qRT-PCR (ΔCt <10), the

Table 2 Clinical characteristic comparison based on the NB5 assay

Characteristics	NB5 qRT-PCR, n (%)		P value*
	Positive	Negative	
Sex			0.588
Male	11 (25.000)	33 (75.000)	
Female	9 (33.333)	18 (66.667)	
Risk group			0.468
Low-risk group	0	3 (100.000)	
Medium-risk group	0	2 (100.000)	
High-risk group	38 (35.185)	70 (64.815)	
Treatment stage			0.110
Induction therapy	30 (42.857)	40 (57.143)	
Consolidation therapy	1 (10.000)	9 (90.000)	
Maintenance therapy	7 (31.818)	15 (68.182)	
End of treatment	0	11 (100.000)	
Diagnosis			0.043
Primary	22 (30.556)	50 (69.444)	
Relapse	16 (53.333)	14 (46.667)	
End of treatment	0	11 (100.000)	
MRD (flow cytometry)			<0.001
Positive	15 (100.000)	0	
Negative	18 (23.684)	58 (76.316)	
No disease assessments	22		
Bone marrow morphology			<0.001
Positive	8 (100.000)	0	
Negative	24 (28.916)	59 (71.084)	
No disease assessments	22		
State			<0.001
CR	8 (13.333)	52 (86.667)	
SD	9 (64.286)	5 (35.714)	
PR	14 (70.000)	6 (30.000)	
VGPR	7 (36.842)	12 (63.158)	

*, P value <0.05 indicates a statistically significant difference. MRD and bone marrow morphology assessments were not performed in 22 times each. NB5, five neuroblastoma genes (*CHGA, DCX, DDC, PHOX2B*, and *TH*); *CHGA*, chromogranin A; *DCX*, doublecortin; *DDC*, dopa decarboxylase; *PHOX2B*, paired-like homeobox 2b; *TH*, tyrosine hydroxylase; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; MRD, measurable residual disease; CR, complete remission; SD, stable disease; PR, partial remission; VGPR, very good partial remission.

expressions of *TH*, *DCX*, *DDC*, *PHOX2B*, and *CHGA* in the BM were 7.008±0.745, 5.211±1.183, 5.552±1.050, 4.309±1.256, and 4.736±1.076, respectively, which were much stronger than those in PB (*Figure 1*).

NB5 qRT-PCR assay of the clinical disease state in BM, PB, and CSF

To investigate the correlation between NB5 ΔCt values in different specimens and different clinical disease statuses, we performed a simple linear regression analysis. The results demonstrated a negative correlation between ΔCt values of all five genes and the tumor cell content of flow cytometric MRD (*Figure 2* and *Table 3*). Among 88 BM specimens, 30 (34.091%) of them were positive in the NB5 assay, which was a significantly higher proportion than that of flow cytometric MRD (15/88, 17.045%). Moreover, 15 of the 30 positive NB5 assay results were consistent with the positive flow cytometric MRD results (Table S3). Additionally, there were 15 positive results detected in NB5 assay that were not detected in the flow cytometric MRD. NB5 positive results were 17.046% higher than flow cytometric MRD, and these patients were found to have different degrees of disease progression or poor treatment effect in the later stage, which was consistent with the NB5 assay.

To analyze the correlation between disease burden and NB5 expression levels, the disease status of all patients was evaluated and classified as remission (CR, PR, VGPR) or SD. The expression level of NB5 in BM specimens changed with disease status. In the comparison between the remission stable groups in BM specimens, all five genes showed significant differences, with the stable group exhibiting lower ΔCt values (higher expression) compared to the remission group (*Figure 3*). In the comparison between the high-risk and low/medium-risk groups, only *PHOX2B* exhibited a significant difference (Figure S2). In BM, there was a significant difference in the ΔCt values of the five genes between positive and negative specimens (BM morphology or flow cytometric MRD) according to the *t*-test (Figures S3,S4). ANOVA was performed to evaluate whether tumor burden made an independent contribution to NB5 ΔCt values. The relationship between NB5 ΔCt and flow cytometric MRD and BM morphology was analyzed. The results indicated that flow cytometric MRD was independently associated with a stronger NB5 genes expression level in both BM and PB (*Table 4*). Moreover, in the interaction terms between BM morphology and flow cytometric MRD, no significant differences in NB5 genes

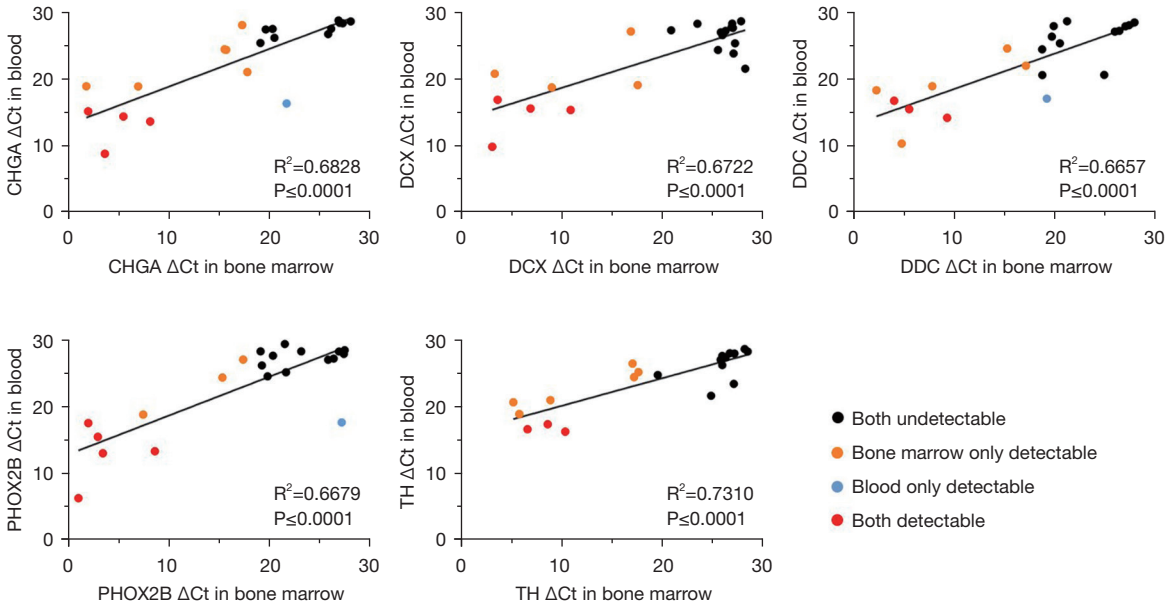

Figure 1 NB5 assay correlations between ΔCt bone marrow and blood. Undetectable: both bone marrow and blood ΔCt were below 18. NB5, five neuroblastoma genes (*CHGA*, *DCX*, *DDC*, *PHOX2B*, and *TH*); ΔCt, Δ threshold cycle; *CHGA*, chromogranin A; *DCX*, doublecortin; *DDC*, dopa decarboxylase; *PHOX2B*, paired-like homeobox 2b; *TH*, tyrosine hydroxylase.

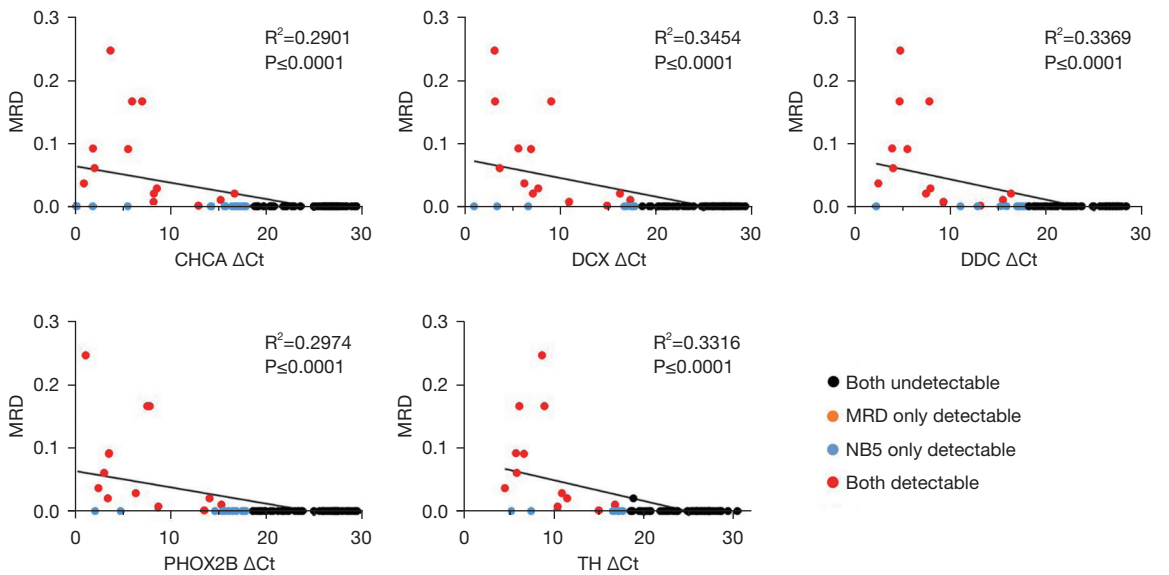

Figure 2 Correlation analysis between NB5 ΔCt level and MRD results. Undetectable: both bone marrow and blood ΔCt were below 18. MRD, measurable residual disease; ΔCt, Δ threshold cycle; NB5, five neuroblastoma genes (*CHGA*, *DCX*, *DDC*, *PHOX2B*, and *TH*); *CHGA*, chromogranin A; *DCX*, doublecortin; *DDC*, dopa decarboxylase; *PHOX2B*, paired-like homeobox 2b; *TH*, tyrosine hydroxylase.

Table 3 NB5 ΔCt and clinical status

NB5 assay ΔCt and clinical disease	BM NB5 assay ΔCt			PB NB5 assay ΔCt		
	Positive/total, n (%)	Mean ΔCt for total (SE)	Mean ΔCt for positive (SE)	Positive/total, n (%)	Mean ΔCt for total (SE)	Mean ΔCt for positive (SE)
All NB5 assays						
CHGA	27/88 (30.682)	18.297 (3.073)	11.572 (1.354)	5/21 (23.810)	19.239 (3.065)	12.783 (2.084)
DCX	21/88 (23.864)	18.387 (3.195)	11.282 (0.602)	4/21 (19.048)	19.413 (2.743)	13.747 (1.995)
DDC	25/88 (28.409)	17.700 (2.749)	11.673 (1.058)	5/21 (23.810)	19.384 (2.623)	13.947 (1.939)
PHOX2B	23/88 (26.136)	17.575 (3.254)	10.366 (0.950)	6/21 (28.571)	20.187 (2.900)	13.870 (1.384)
TH	19/88 (21.591)	18.218 (3.020)	11.591 (1.231)	3/21 (14.286)	20.967 (1.913)	16.724 (0.324)
Bone marrow morphology						
CHGA	8/88 (9.091)	15.048 (3.235)	7.865 (0.298)	No specimen	No specimen	No specimen
DCX	8/88 (9.091)	16.007 (3.355)	8.557 (0.594)	No specimen	No specimen	No specimen
DDC	8/88 (9.091)	14.701 (3.111)	7.855 (0.286)	No specimen	No specimen	No specimen
PHOX2B	8/88 (9.091)	14.672 (3.525)	6.913 (1.079)	No specimen	No specimen	No specimen
TH	8/88 (9.091)	16.578 (2.998)	9.971 (0.605)	No specimen	No specimen	No specimen
MRD (flow cytometry)						
CHGA	15/88 (17.045)	16.053 (3.326)	8.682 (0.847)	0/1	–	–
DCX	15/88 (17.045)	17.380 (3.268)	10.200 (1.348)	0/1	–	–
DDC	15/88 (17.045)	15.812 (3.103)	9.010 (1.068)	0/1	–	–
PHOX2B	15/88 (17.045)	16.099 (3.418)	8.600 (1.436)	1/1 (100.000)	–	–
TH	15/88 (17.045)	17.559 (3.038)	10.868 (1.029)	0/1	–	–

NB5, five neuroblastoma genes (*CHGA, DCX, DDC, PHOX2B*, and *TH*); ΔCt, Δ threshold cycle; BM, bone marrow; PB, peripheral blood; SE, standard error; *CHGA*, chromogranin A; *DCX*, doublecortin; *DDC*, dopa decarboxylase; *PHOX2B*, paired-like homeobox 2b; *TH*, tyrosine hydroxylase; MRD, measurable residual disease.

expression were observed.

Detection and analysis of NB5 ddPCR and qRT-PCR in BM, PB, and CSF

For different specimens (BM, PB, and CSF), we performed ddPCR on 24 specimens (20 patients). Based on previous testing of positive specimens, a threshold line of 0.010% (relative copy number) was established for ddPCR. The results, as shown in *Table 5*, revealed that the positive rates of ddPCR were consistent with qRT-PCR in BM (13/18, 72.222%) and CSF (1/1, 100.000%). However, in PB (2/5, 40.000%), the positive rate of ddPCR was higher than that of qRT-PCR (*Table 5*).

The contribution of the NB5 genes to disease

A total of 38 specimens (BM, PB, and CSF) were detected positive as with qRT-PCR. Among the positive results, we assessed the frequency of the five genes in the positive specimens and used the χ^2 test to determine if there were significant differences. The analysis revealed a significant difference (P=0.005) between the *CHGA* and *TH* genes in pairwise comparisons. No significant differences were observed in the pairwise comparisons of the other genes. Thus, based on qRT-PCR results, the contribution of the five genes was ranked as follows: *CHGA* ≈ *DCX* ≈ *DDC* ≈ *PHOX2B* and *CHGA* > *TH* (*Table 6*). In addition, we conducted the same analysis using ddPCR data but did

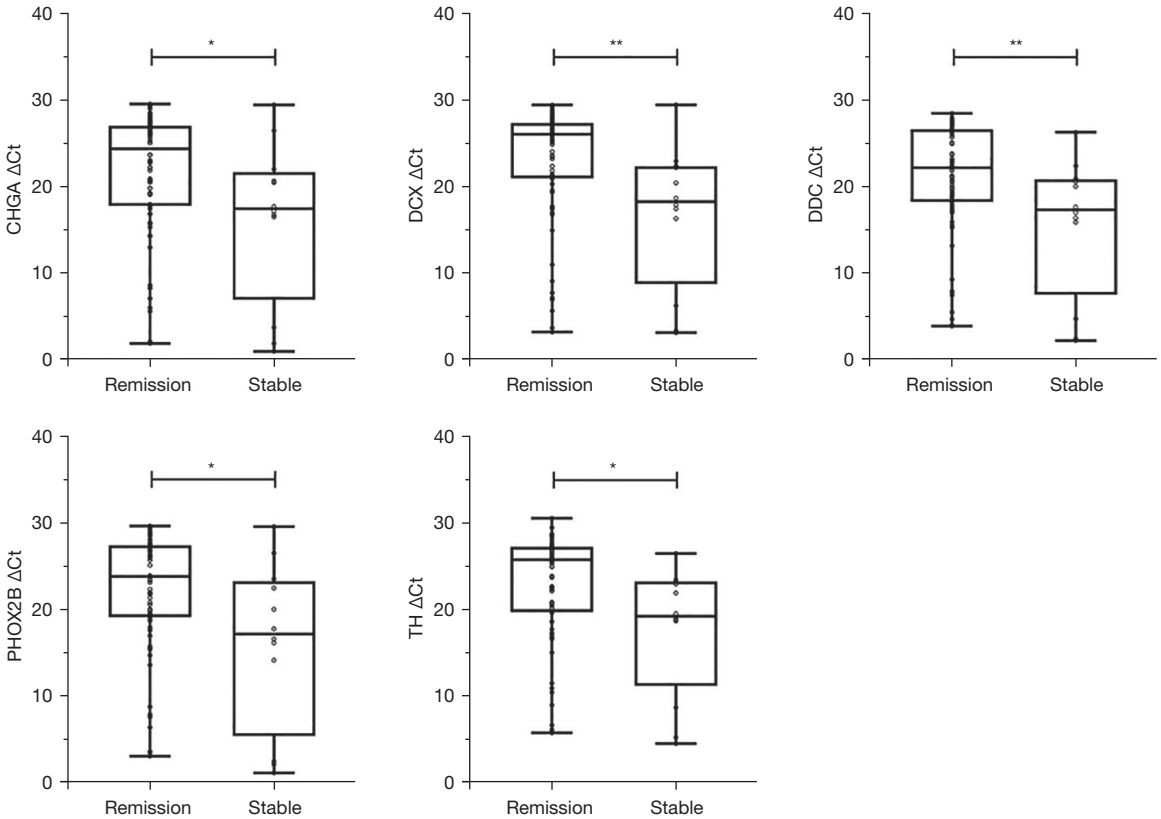

Figure 3 Level of NB5 ΔCt in bone marrow for each disease status. n=76 and n=12 in the remission group and stable group, respectively. *, P<0.05; **, P<0.01. ΔCt, Δ threshold cycle; NB5, five neuroblastoma genes (*CHGA*, *DCX*, *DDC*, *PHOX2B*, and *TH*); *CHGA*, chromogranin A; *DCX*, doublecortin; *DDC*, dopa decarboxylase; *PHOX2B*, paired-like homeobox 2b; *TH*, tyrosine hydroxylase.

not obtain results similar to those from qRT-PCR. This discrepancy may be related to the limited number of specimens used in the analysis (Table S4).

NB5 assay ΔCt and PFS

The analysis included time-dependent covariate analysis on ΔCt and PFS, with a Cox proportional hazards regression model was used to assess the hazard ratios (HRs) of different influencing factors. Our results showed that although there were no significant differences in flow cytometric MRD, BM morphology, or NB5 ΔCt for PFS, the grouping based on NB5 ΔCt demonstrated a tendency toward better prognostic stratification (*Figure 4* and Table S5). In order to further evaluate the significance of NB5 ΔCt in prognostic stratification, additional analyses were conducted. We analyzed the PFS of patients without flow cytometric MRD detectable in correlation with NB5 ΔCt at two groups (ΔCt ≤18 and ΔCt >18). The average survival time in the positive group (NB5 ΔCt ≤18) of patients at initial diagnosis was 27.408±10.791 months (median, 21.000±3.914 months) compared with 35.961±3.084 months (median, 38.000±6.244 months) (P=0.034) in the negative group (NB5 ΔCt >18). Furthermore, the Cox analysis revealed that in flow cytometric MRD negative patients, the HR of the NB5 ΔCt positive group (ΔCt ≤18) was 3.046 times higher than that of the NB5 ΔCt negative group (ΔCt >18) (*Figure 4D* and Table S5). This result suggests that NB5 provides a more accurate assessment of disease progression and prognosis than does flow cytometric MRD and can be used as an independent factor to assess the role of clinical disease and prognosis.

Receiver operating characteristic (ROC) analysis was carried out for the ΔCt of the five genes. Additionally, we analyzed the geometric mean (GM) (GM Ct value of the five genes minus the GM of the housekeeping gene Ct, NB5 signature ΔCt) of the five genes and the other signatures (NB5 minus *CHGA*, NB5 minus *DDC*, NB5

Table 4 Main effects via ANOVA of different clinical disease comparison[†]

Clinical disease comparison	BM NB5 assay ΔCt		PB NB5 assay ΔCt	
	ΔCt difference (SE)	P	ΔCt difference (SE)	P
Bone marrow morphology (negative *vs.* positive patients)				
CHGA	14.365 (1.110)	0.437	7.767 (2.188)	0.856
DCX	14.899 (0.071)	0.164	7.142 (1.727)	0.998
DDC	13.691 (1.242)	0.279	6.229 (1.889)	0.546
PHOX2B	15.518 (0.334)	0.222	8.327 (2.168)	0.773
TH	13.215 (1.564)	0.303	6.061 (1.617)	0.817
MRD (flow cytometry) (negative *vs.* positive patients)				
CHGA	14.743 (1.321)	<0.001	8.754 (2.546)	0.040
DCX	14.361 (1.392)	<0.001	7.665 (1.708)	0.047
DDC	13.605 (1.898)	<0.001	8.659 (1.982)	0.006
PHOX2B	14.997 (1.773)	<0.001	9.990 (3.073)	0.014
TH	13.381 (1.603)	<0.001	6.648 (1.574)	0.014

[†], for main effects via ANOVA, ΔCt difference represents the difference between negative patients and positive patients of NB5 ΔCt. P<0.05 is a statistically significant difference. ANOVA, analysis of variance; NB5, five neuroblastoma genes (*CHGA, DCX, DDC, PHOX2B*, and *TH*); *CHGA*, chromogranin A; *DCX*, doublecortin; *DDC*, dopa decarboxylase; *PHOX2B*, paired-like homeobox 2b; *TH*, tyrosine hydroxylase; BM, bone marrow; PB, peripheral blood; SE, standard error; ΔCt, Δ threshold cycle; MRD, measurable residual disease.

Table 5 Comparison of ddPCR and qRT-PCR results across the different samples

Method	Sample	Category description	N (%)
ddPCR	Bone marrow	Positive/total[†]	13/18 (72.222)
	Peripheral blood	Positive/total[†]	2/5 (40.000)
	Cerebrospinal fluid	Positive/total[†]	1/1 (100.000)
qRT-PCR	Bone marrow	Positive/total[†]	13/18 (72.222)
	Peripheral blood	Positive/total[†]	1/5 (20.000)
	Cerebrospinal fluid	Positive/total[†]	1/1 (100.000)

[†], number of assessments. ddPCR, droplet digital polymerase chain reaction; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction.

Table 6 qRT-PCR positive samples for NB5 detection results

Gene	Positive[†]	Negative[†]	Total
CHGA	33[a] (86.84)	5[a] (13.16)	38 (100.00)
DCX	27[a,b] (71.05)	11[a,b] (28.95)	38 (100.00)
DDC	31[a,b] (81.58)	7[a,b] (18.42)	38 (100.00)
PHOX2B	30[a,b] (78.95)	8[a,b] (21.05)	38 (100.00)
TH	21[b] (55.26)	17[b] (44.74)	38 (100.00)
Total	142 (74.74)	48 (25.26)	190 (100.00)

Data are shown as n (%). [†], the letters "[a]" and "[b]" indicate whether there is a statistical difference; containing the same letters means there is no significant difference, otherwise there is a significant difference. qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; NB5, five neuroblastoma genes (*CHGA, DCX, DDC, PHOX2B*, and *TH*); *CHGA*, chromogranin A; *DCX*, doublecortin; *DDC*, dopa decarboxylase; *PHOX2B*, paired-like homeobox 2b; *TH*, tyrosine hydroxylase.

minus *DCX*, NB5 minus *PHOX2B*, *TH*/*DCX*, *PHOX2B*/*TH*/*DDC*, *PHOX2B*/*TH*/*DCX*, and *PHOX2B*/*TH*), and a similar analysis was performed. Based on these results, the most influential single gene was found to be *DCX*, with an area under the curve (AUC) of 0.733. Its removal resulted in a 6.589% drop in the AUC (NB5 minus *DCX*). The results showed that the prediction of PFS by the *DCX* gene had the highest AUC (*DCX* > *DDC* > *PHOX2B* > *CHGA* > *TH*), and the AUC prediction result of the NB5 signature ΔCt was between *DCX* and *DDC* (Figure S5 and Table S6).

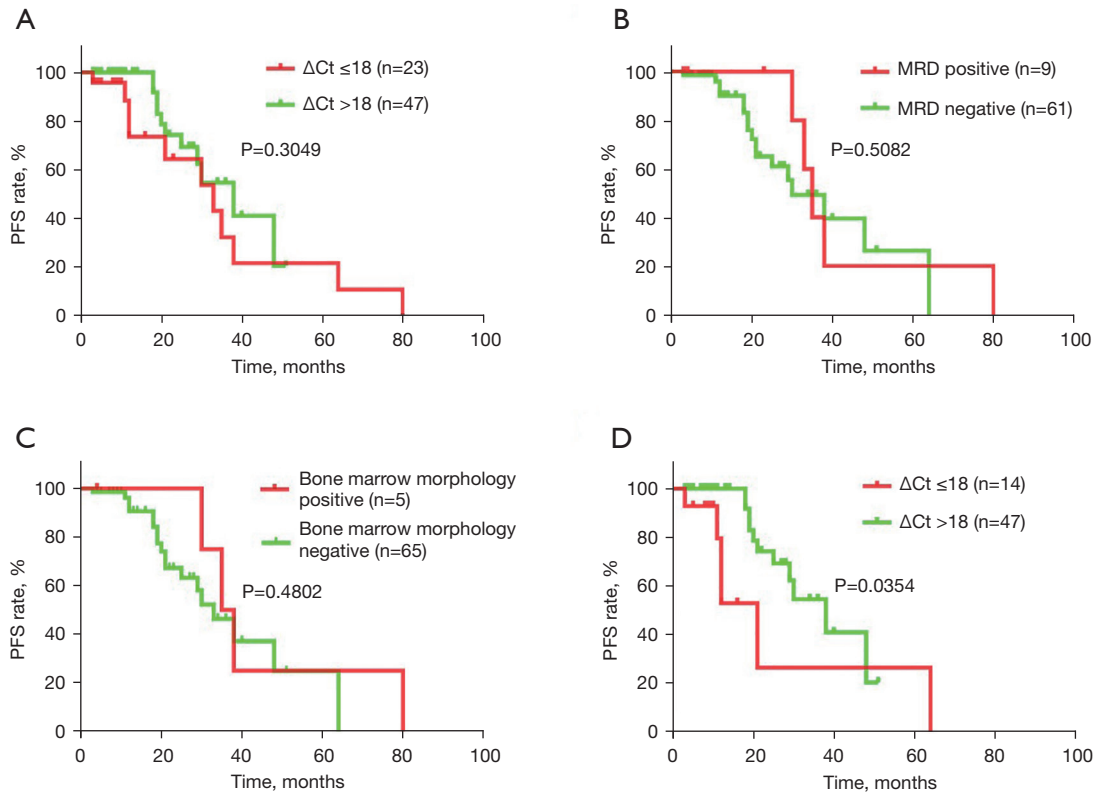

Figure 4 Correlation of NB5 assay ΔCt in bone marrow with PFS. (A) PFS of patients with positive and negative detection of NB5; (B) PFS of patients with positive and negative detection of MRD; (C) PFS of patients with positive and negative detection in bone marrow morphology; (D) PFS of all patients with positive and negative detection for NB5 and negative detection for MRD. ΔCt ≤18: positive; ΔCt >18: negative. PFS, progression-free survival; ΔCt, Δ threshold cycle; MRD, measurable residual disease; NB5, five neuroblastoma genes (*CHGA*, *DCX*, *DDC*, *PHOX2B*, and *TH*); *CHGA*, chromogranin A; *DCX*, doublecortin; *DDC*, dopa decarboxylase; *PHOX2B*, paired-like homeobox 2b; *TH*, tyrosine hydroxylase.

Discussion

Disease assessment is critical for therapeutic decisions and prognostic assessment. Several potential factors are associated with the risk stratification of high-risk NB, including clinical factors [e.g., age, serum lactate dehydrogenase (LDH), serum ferritin], host factors, tumor biology (e.g., *MYCN* amplification, genomic instability), disease response (e.g., MIBG scoring), and MRD monitoring (29). Among these numerous factors, rapid and accurate monitoring of MRD plays a crucial role in evaluating NB metastasis and residual disease, guiding treatment, and assessing prognosis. Previous studies have shown that the expression levels of *CHGA*, *DCX*, *DDC*, *PHOX2B*, and *TH* can be used alone or in combination as markers to guide micrometastasis (14,30).

CHGA is a member of the chromogranin/secretogranin family and is involved in encoding neurosecretory granules that promoting NB cell differentiation (31). The *DCX* gene encodes a protein belonging to the doublecortin family, which is specifically expressed in migrating neurons of the central and peripheral nervous systems. The expressed protein guides neuron migration by regulating microtubule organization and stability (32). *DDC* gene encodes a protein that catalyzes the formation of dopamine and is considered a sensitive marker for NB cells (33). The *PHOX2B* gene encodes a protein belonging to the paired family of homeodomain transcription factor, which is involved in neural maturation and differentiation. It is highly expressed in NB and has been identified as a specific marker for MRD in NB (34). The *TH* gene encodes a protein that functions as the rate-limiting enzyme in the synthesis of catecholamines and plays a key role in the physiology of adrenergic neurons. Given that catecholamines are

predominantly produced by NB cells, *TH* plays a significant role in micrometastasis detection (35). Therefore, assessing the expression of these five genes is of great clinical value in NB detection.

qRT-PCR was utilized to detect three different specimens (BM, PB, CSF). The results indicated that all three specimens were capable of yielding positive results. In the CSF specimens, one patient in the consolidation therapy stage experienced seizure symptoms after radiotherapy, raising strong suspicion of central nervous system relapse. In the qRT-PCR analysis, four genes (*DCX*, *DDC*, *PHOX2B*, and *CHGA*) were found to be positive. However, after a few days of treatment, only three genes (*DCX*, *PHOX2B*, and *CHGA*) remained positive. Although the number of specimens was limited, the results strongly indicate the potential of applying qRT-PCR detection methods in different specimens (*Table 5*). In both ddPCR and qRT-PCR analyses, the positive results obtained from ddPCR were found to be 100% concordant with the results from qRT-PCR (*Table 5*). Interestingly, in PB specimens, ddPCR exhibited a higher positivity rate compared to qRT-PCR (with an additional positive detection). Concurrent assessment of BM MRD (flow cytometry) indicated a positive result (16.500%) for the same period in the patients. Based on this data, we speculate that for patients who are unable to provide a BM specimen, using ddPCR for PB diagnostic evaluation could be a viable strategy worth exploring. Nino *et al.* used ddPCR to detect MRD in PB stem cell grafts of patients with high-risk NB and found that MRD had an impact on the prognosis (36). Through the analysis of seven genes, they observed that patients with high gene expression had significantly lower event-free survival (EFS) (36). Our results, consistent with Nino's findings, suggest that ddPCR holds greater potential for the application of MRD monitoring in NB.

Using individual genes for evaluating the results can enhance the sensitivity of the detection. However, to avoid false-positive results, we adopted a strategy inspired by previous studies (17,37,38) and compared the performance of the five individual genes with that of the NB5-signature approach (Figure S5 and Table S6). We also compared the results with other previously reported signatures (18,30,39,40). The results demonstrated that among all the different gene signature approaches, *DCX* alone consistently had the highest AUC (Figure S5 and Table S6). Furthermore, we observed that some patients showed positivity in only one gene (one case for *TH*, two cases for *PHOX2B*, and two cases for *CHGA*). Among these patients, the individual with *TH* positivity (flow cytometric MRD was negative) showed evidence of increased bone metabolism in the left scapula on imaging, suggestive of tumor bone metastasis. Remarkably, these patients demonstrated significant improvement in their condition after treatment. These findings further demonstrate that even when individual genes are positive, they can accurately assess the degree of disease progression.

In our study, a high correlation was observed between ΔCt values of the five genes in paired BM and PB specimens. Furthermore, NB5 ΔCt was detectable when BM morphology or MRD (flow cytometry) standard evaluations were negative, and these patients were found to have different degrees of disease progression or poor treatment effect in the later stage, which was consistent with the NB5 assay (*Figure 2*, *Table 4*).

The most notable results of this study are the correlation between NB5 expression levels in BM and PFS in patients with refractory or relapsed NB. This was especially evident in patients with negative for MRD according to flow cytometry. The data with undetected NB5 expression in BM was consistently associated with negative MRD results on flow cytometry and indicated a higher proportion of PFS. In contrast, among patients with MRD negative status on flow cytometry, those with NB5 positivity demonstrated poorer PFS according to the survival analysis and also had a higher HR compared to NB5-negative patients (*Figure 4* and Table S5). For example, 1 patient was initially detected as positive for NB5 (*DCX*, *DDC*, *PHOX2B*, *CHGA*) but was negative for MRD on flow cytometry. After 6 months, a follow-up BM examination showed that NB5 (*TH*, *DCX*, *DDC*, *PHOX2B*, *CHGA*) remained positive. At this time, both flow cytometric MRD and BM morphology showed positive results. Another patient was negative on both the first and second NB5 assay, and was in the maintenance treatment phase in a CR state. In the third NB5 assay, all five genes were found to be positive, but BM morphology and flow cytometric MRD monitoring were all negative. Additionally, imaging examinations did not reveal any signs of recurrence. Close follow-up monitoring was conducted, and a re-examination half a month later showed MIBG uptake near the left renal hilum, the bilateral mandibular branches, bilateral the iliac bones, and a focally in a lesion on the right ischium, suggestive of bone metastasis. The Curie score was 5 points, indicating widespread relapse. These two cases clearly illustrate that BM NB5 detection is more sensitive and more accurate than is BM flow cytometric MRD in reflecting the BM conditions of

patients with NB. In addition, when the NB5 detection result change from negative to positive, this could predict NB recurrence earlier, and early intervention and treatment can greatly improve the final prognosis of patients.

We further found that BM NB5 detection has advantages over BM flow cytometric MRD detection in assessing treatment effect. In a 3-year-old girl, the first BM NB5 detection was positive for *TH* and *DDC*, and flow cytometric MRD (0.010%) was also positive. After induction therapy was continued, the second BM examination showed that flow cytometric MRD was negative. However, *TH* and *DDC* genes were still detected, and the patient still had multiple lesions in the bone. At the time of writing, the patient is in a PR state. Our data suggest that for patients with relapsed/refractory NB, even in cases where MRD is clearly negative, NB5 results can still accurately predict PFS. The expression of NB5 in BM enables early risk stratification for patients with relapsed or refractory NB before and during treatment. Several studies have explored the use of different genes to the assess survival rate in NB. Viprey *et al.* investigated the expression levels of *PHOX2B*, *TH*, and *DCX* in BM and found that these expression levels could be used to predict EFS and OS, as well as monitor treatment response (30). Additionally, Marachelian *et al.* used *CHGA*, *DCX*, *DDC*, *PHOX2B*, and *TH* to assess expression levels and their significant correlation with PFS (14). Despite the small number of false positives and false negatives, multiple larger multicenter studies, the inclusion of additional clinical features, and use of diverse detection methods are required to comprehensively evaluate and explore the application value of these five genes in NB.

Conclusions

Our findings demonstrate that NB5 detection in the BM of patients with NB is more sensitive and provides a more accurate reflection of the BM status of patients with NB as compared to BM flow cytometric MRD. It can precisely assess prognosis and prevent tumor recurrence in patients with NB. Furthermore, BM NB5 detection exhibited higher sensitivity and accuracy than did PB NB5 detection. BM NB5 detection may also serve as an independent prognostic factor for patients with relapsed or refractory NB.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and approved by the Medical Ethics Committee of Hunan Provincial People's Hospital (approval No. 2023-106). Informed consent was obtained from all the patients' legal guardians.

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