PHOX2B expression in bone marrow and peripheral blood is associated with metastasis and prognosis in patients with neuroblastoma

Hongjun Fan
Beijing Children's Hospital https://orcid.org/0000-0002-1034-3221
Tianyu Xing
Beijing Children's Hospital
Huimin Hong
Beijing Children's Hospital
Chao Duan
Beijing Children's Hospital
Wen Zhao
Beijing Children's Hospital
Qian Zhao
Beijing Children's Hospital
Xisi Wang
Beijing Children's Hospital
Cheng Huang
Beijing Children's Hospital
Shuai Zhu
Beijing Children's Hospital
Mei Jin
Beijing Children's Hospital
Yan Su
Beijing Children's Hospital
Chao Gao
Beijing Children's Hospital
Xiaoli Ma (mxl1123@vip.sina.com)
https://orcid.org/0000-0001-7930-4944

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Abstract

Background Paired-like homeobox 2B (PHOX2B) is specifically expressed in the nervous system including neuroblastoma cells, but little is known about the clinical significance of the expression of PHOX2B in bone marrow (BM) and peripheral blood (PB) samples of newly diagnosed neuroblastoma patients. Methods The expression of PHOX2B in 276 paired BM and PB samples of neuroblastoma patients at diagnosis was tested by quantitative reverse transcriptase polymerase chain reaction (RT-PCR). Then the relationship between PHOX2B level and clinical characteristics including metastasis and prognosis was explored by receiver operating characteristic (ROC) analysis and Kaplan-Meier method. Results We identified the combined expression of PHOX2B in both BM and PB provided a high diagnostic accuracy for metastasis of neuroblastoma patients (AUC = 0.920) with the sensitivity and specificity of 86.7% and 92.9%, respectively. At last, 246 patients were enrolled for prognostic analysis. The median follow-up time was 22 months. Positive expressions of PHOX2B in BM and PB at diagnosis were associated with worse EFS and OS in neuroblastoma patients ($P < 0.05$). What's worse, 19.7% (31/157) and 6.4% (10/157) patients with positive expression of PHOX2B in BM and PB samples in low/intermediate-risk group also had shorter EFS and poor OS ($P < 0.05$). CONCLUSIONS The expressions of PHOX2B in BM and PB were high in patients with unfavorable clinical characteristics. PHOX2B could be an appropriate biomarker for predicting metastasis and prognosis in patients with neuroblastoma.

Background

Neuroblastoma, originating from the peripheral sympathetic nervous system, accounts for 12% of deaths associated with cancer in children [1]. Although patients with low- and intermediate-risk neuroblastoma have favorable outcomes, the prognosis of high-risk patients is still dismal with 5-year overall survival (OS) less than 50% [2]. Nearly one-half of neuroblastoma patients are classified into the high-risk group with metastasis at the initial diagnosis [3]. Positron emission tomography-computed tomography (PET-CT), computed tomography (CT), magnetic resonance imaging (MRI) and iodine-123 metaiodobenzylguanidine (^{123}I-MIBG) scanning are widely used in detecting neuroblastoma patients with distant metastasis. However, repeated CT or ^{123}I-MIBG scans during the long-term treatment expose children to radiation and also unable to detect small primary lesions and metastases accurately. Therefore, new methods should be tested for predicting metastasis in neuroblastoma patients.

Liquid biopsy, including circulating tumor cells (CTCs), cell-free tumor DNAs (ctDNAs), as well as RNAs, proteins, and exosomes, has been introduced as a new method for the diagnosis of cancer metastasis in the past decades [4, 5]. Since the detection of neuroblastoma cells in the blood of patients with disseminated disease by Moss et al in the 1990s [6], some studies have been devoted to the development and improvement of the methods for liquid biopsy in neuroblastoma patients. Paired-like homeobox 2B (PHOX2B) gene, located on chromosome 4p13 [7], is specifically expressed in the nervous system including neuroblastoma cells, but not in normal bone marrow or peripheral blood cells of patients [8, 9]. Previously, some studies showed PHOX2B could be used as a biomarker for minimal residual disease (MRD) and predicting prognosis in neuroblastoma patients [9–12]. In our study, we evaluated the

diagnostic significance of PHOX2B expression in bone marrow (BM) and peripheral blood (PB) for disseminated diseases and patients' outcomes.

Methods

Patients and samples

Paired BM and PB samples were collected from 276 newly diagnosed neuroblastoma patients, who were enrolled in our center from June 1st, 2017 to December 31th, 2018. The study was approved by the Ethical Committee of our Hospital. Informed written consent was obtained from each participant according to the Declaration of Helsinki. The International Neuroblastoma Risk Group Staging System (INRGSS) was used for stratifying patients with different clinical stages at the time of diagnosis before any treatment [13]. We defined cases as patients with metastasis by the evaluation of PET-CT, bone scans, CT, MRI, bone marrow aspirates and trephine biopsies at diagnosis [1]. Each patient had two sites bone marrow aspirates of the sternum and ilium at diagnosis. The patients were treated in different risk groups according to prognostic category [2, 14], and the chemotherapy regimen was guided by BCH-2007-NB [15, 16].

Real-time Polymerase Chain Reaction

The Trizol Reagent was used to extract total RNA from 2 mL BM and PB samples, and then the RNA was reverse transcribed into cDNA [17]. RT-PCR was performed in a ViiA™ 7 Dx System (Applied Biosystems, Carlsbad, USA). The expression of PHOX2B gene was detected by the Applied Biosystems™ TaqMan™ Array Human Hox Genes (Cat.No 4414103, Applied Biosystems, Foster city, CA). Reactions were carried out in 12.5 μL (6.4 μL H_2O, 1 μL forward and reverse primers, 1 μL cDNA (input 80 ng), 6.25μL Mastermix, 0.75μL PHOX2B Assay), started with 10 minutes at 95 °C followed by 50 cycles (15 seconds at 95 °C followed by 1 minute at 60 °C). All RT-PCR experiments were performed in triplicate, and mean values were used. The housekeeping gene GUS was used as the gene for normalization. The quantity of the expression of PHOX2B was normalized to the amount of GUS gene transcripts (normalized Ct [ΔCt] = $Ct_{GUS} - Ct_{PHOX2B}$). Samples with Ct values of PHOX2B above 40 were defined as negative.

Statistical analysis

The primary analytic endpoint was event-free survival (EFS) and overall survival (OS). Time to event was defined as the time from diagnosis until the time of the first occurrence of relapse, progression, death, or until time of the last contact if none of these occurred. Kaplan-Meier method was performed on survival analysis and the log-rank test was used to compare the survival curves between PHOX2B positive and negative subgroups. Hazard ratio (HR) and 95% confidence interval (95% CI) were estimated

from Cox proportional hazard models.

Statistical analyses were performed using SPSS19.0 and Graphpad Prism 5.0 software. The significance of the differences in clinical features between PHOX2B positive and negative patients was determined by the χ^2 test. The efficacy of PHOX2B expression to diagnose metastasis was assessed by receiver operating characteristic (ROC) analysis. The P-value lower than 0.05 was considered as statistically significant.

Results

PHOX2B mRNA expression in BM and PB of patients and Patients' characteristics

A total of 276 patients, aged 1 month to 138 months (median, 35 months) with newly diagnosed neuroblastoma, were enrolled in this study. The clinical and biological characteristics were shown in Table 1. As shown in Table 2, the positive rates of the expression of PHOX2B in BM and PB of neuroblastoma patients at diagnosis were 44.6% and 35.1%. The normalized Ct value ranged from − 18.35 to 3.25 in positive BM samples, and − 21.25 to 0.48 in positive PB samples, respectively. The levels of PHOX2B mRNA in BM and PB were significantly increased in patients with age ≥ 18 months, INRGSS M and MS stages, high-risk group, LDH ≥ 587 U/L, SF ≥ 92 ng/mL, NSE ≥ 100 ng/mL, MYCN amplification, 1p loss, and BM cytology- and immunocytology-positive ($P < 0.05$).

Table 1
Characteristics of the study patients (n = 276)

Characteristics	n (%)	Characteristics	n (%)
Sex		NSE (ng/mL)	
Male	146 (52.9)	< 100	170 (61.6)
Female	130 (47.1)	≥ 100	106 (38.4)
Age, months, median, (range)	35 (1-138)	Bone marrow cytology	
< 18 months	86 (31.2)	Negative	198 (71.7)
≥ 18 months	190 (68.8)	Positive	78 (28.3)
INRGSS stage		Bone marrow immunocytology	
L1	60 (21.7)	Negative	184 (66.7)
L2	82 (29.7)	Positive	92 (33.3)
M	122 (44.2)	Pathology	
MS	12 (4.4)	Neuroblastoma	142 (51.4)
Risk group		Ganglioneuroblastoma	112 (40.6)
Low	60 (21.7)	Unknown	22 (8.0)
Intermediate	105 (38.1)	MYCN	
High	111 (40.2)	No Amplification	223 (84.1)
LDH (U/L)		Amplification	26 (9.4)
< 587	207 (75.0)	Unknown	18 (6.5)
≥ 587	69 (25.0)	1p loss	
Serum ferritin (ng/mL)		No	207 (75.0)
< 92	170 (61.6)	Yes	35 (12.7)
≥ 92	106 (38.4)	Unknown	34 (12.3)

Table 2
The expression of PHOX2B in newly diagnosed NB patients (n = 276)

Characteristics	Bone marrow		Peripheral blood	
	PHOX2B positive %	P	PHOX2B positive %	P
All	44.6 (123/276)		35.1 (97/276)	
Sex		0.639		0.151
Male	45.9 (67/146)		39.0 (57/146)	
Female	43.1 (56/130)		30.8 (40/130)	
Age		0.384		< 0.001
< 18 months	40.7 (35/86)		19.8 (17/86)	
≥ 18 months	46.3 (88/190)		42.1(80/190)	
INRGSS stage		< 0.001		< 0.001
L1	8.3 (5/60)		1.7 (1/60)	
L2	7.3 (6/82)		2.4 (2/82)	
M	84.4 (103/122)		72.1 (88/122)	
MS	75.0 (9/12)		50.0 (6/12)	
Risk group		< 0.001		< 0.001
Low	25.0 (15/60)		13.3 (8/60)	
Intermediate	16.2 (17/105)		3.8 (4/105)	
High	82.0 (91/111)		76.6 (85/111)	
LDH (U/L)		< 0.001		< 0.001
< 587	30.0 (62/207)		18.4 (38/207)	
≥ 587	88.4 (61/69)		85.5 (59/69)	
Serum ferritin (ng/mL)		< 0.001		< 0.001
< 92	21.8 (37/170)		14.1 (24/170)	
≥ 92	81.1 (86/106)	< 0.001	68.9 (73/106)	
NSE (ng/mL)		< 0.001		< 0.001
< 100	20.0 (34/170)		11.8 (20/170)	

Characteristics	Bone marrow		Peripheral blood	
	PHOX2B positive %	P	PHOX2B positive %	P
≥ 100	84.0 (89/106)		72.6 (77/106)	
Pathology		0.025		0.332
Neuroblastoma	47.9 (68/142)		35.2 (50/142)	
Ganglioneuroblastoma	33.9 (38/112)		29.5 (33/112)	
MYCN		< 0.001		< 0.001
No amplification	40.5 (94/232)		31.5 (73/232)	
Amplification	73.1 (19/26)		69.2 (18/26)	
1p loss		< 0.001		< 0.001
No	40.6 (84/207)		30.4 (63/207)	
Yes	80.0 (28/35)		74.3 (26/35)	
Bone marrow cytology		< 0.001		< 0.001
Negative	23.2 (46/198)		13.6 (27/198)	
Positive	98.7 (77/78)		89.7 (70/78)	
Bone marrow immunocytology		< 0.001		< 0.001
Negative	17.4 (32/184)		8.2 (15/184)	
Positive	98.9 (91/92)		89.1 (82/92)	

Correlation between the expression of PHOX2B in BM and PB and metastasis

To evaluate the ability of the PHOX2B mRNA level for predicting metastasis of neuroblastoma patients at diagnosis, ROC curves were utilized. As shown in Fig. 1A and 1B, the expressions of PHOX2B in BM and PB samples of neuroblastoma patients with metastatic disease were higher than patients with localized disease. The calculated area under the ROC curve was 0.906 (95% CI: 0.866–0.938) with the sensitivity and specificity of 83.6% and 93.7% at the value of -17.3 for BM (Fig. 1C), while 0.823 (95% CI: 0.773–0.866) with the sensitivity and specificity of 65.7% and 97.9% at the value of -20.5 for PB (Fig. 1D). Then the calculated area under the curve of surveillance of combined PHOX2B expression in both BM and PB was 0.921 (95%CI: 0.883–0.950) for predicting metastasis of neuroblastoma at diagnosis with the sensitivity and specificity of 86.7% and 92.9%, respectively (Fig. 1E).

Prognosis evaluation of the PHOX2B expression in BM and PB at diagnosis

Among the 276 newly diagnosed patients, 30 patients were lost follow-up, and 246 patients were included in survival analysis, with a median follow-up time of 22 months. By February 15th, 2020, 38 patients had an event. The 2-year EFS was 75.7% for all patents, whereas it was 43.5% and 93.3% for patients with high-risk disease and low/intermediate-risk disease, respectively. The 2-year OS was 86.6% for all patents, whereas it was 70.6% and 95.5% for patients with high-risk disease and low/intermediate-risk disease, respectively. As shown in Fig. 2A – 2B and 3A – 3B, patients with the positive expression of PHOX2B in either BM or PB showed shorter EFS and OS than the negative patients ($P < 0.001$). Then we further analyzed the value of predicting prognostic by PHOX2B expression in patients with different risk subgroups. In the high-risk group, the patients with PHOX2B expression either in BM or PB had poorer EFS than those negative ones ($P < 0.05$, Fig. 2C and 2D). Although there was no statistical difference ($P > 0.05$, Fig. 2C and 2D), the positive BM and PB patients had a poor OS trend. In the Low-/Intermediate-Risk group, 29.0% (9/31) patients with PHOX2B expression in BM had an event occurrence, while only 1.6% (2/126) occurred in patients without PHOX2B expression ($P < 0.05$). Likely, 30.0% (3/10) patients with PHOX2B expression in PB had event occurrence, while only 5.6% (8/147) occurred in patients without PHOX2B expression ($P < 0.05$). Unexpectedly, in Low-/Intermediate-risk group with favorable outcomes, the patients with positive expression of PHOX2B in BM and PB at diagnosis showed worse EFS and OS than the negative patients ($P < 0.05$, Fig. 2E – 2F, Fig. 3E – 3F). The univariate survival analysis showed that age ≥ 18 months, high-risk group, MYCN amplification, chromosome 1p loss, BM and PB positive of PHOX2B, LDH ≥ 587 U/L, SF ≥ 92 ng/mL, and NSE ≥ 100 ng/mL were associated with OS in patients with neuroblastoma ($P < 0.05$). Further analysis using multivariate COX proportional hazards regression model demonstrated that MYCN amplification and the positive expression of PHOX2B in BM were the independent risk factors for the prognosis of patients with neuroblastoma ($P < 0.05$, Table 3).

Table 3
Univariate and multivariate analysis of clinicopathological factors for overall survival

Variables	Univariate			Multivariate		
	HR	95% CI	P	HR	95% CI	P
Risk group	7.46	3.02–18.41	< 0.001	0.41	0.10–1.67	0.216
MYCN status	9.59	4.51–20.37	< 0.001	4.71	1.85–11.99	0.001
1p status	5.58	2.61–11.96	< 0.001	0.80	0.29–2.21	0.659
BM PHOX2B	20.63	4.89–86.98	< 0.001	5.86	1.06–32.38	0.043
PB PHOX2B	9.35	3.79–23.07	< 0.001	1.80	0.56–5.78	0.326
LDH status	10.75	4.72–24.78	< 0.001	2.38	0.78–7.49	0.138
Serum ferritin status	11.43	3.97–32.97	< 0.001	2.50	0.64–9.71	0.186
NSE status	8.08	3.26–20.01	< 0.001	1.39	0.46–4.19	0.556

Discussion

The clinical importance of PHOX2B gene expression examination for predicting metastasis and prognostic evaluation in neuroblastoma patients were evaluated in our study. PHOX2B was first been shown as a highly sensitive and specific biomarker for neuroblastoma in 2008 [8, 18], and it was confirmed not expressed in BM and PB samples of children in the control group without neuroblastoma [8]. But in neuroblastoma patient samples, PHOX2B was detected in 32% cytology-negative and 14% immunocytology-negative BM samples, and in more than 90% of cytology-positive and immunocytology-positive BM samples [8]. Our study revealed PHOX2B was detected in 98.7% BM cytology-positive and 98.9% BM immunocytology-positive patients, and also in 23.2% BM cytology-negative and 17.4% BM immunocytology-negative patients based on the high sensitivity method of RT-PCR. The positive rates of PHOX2B in BM and PB were also higher in patients with unfavorable clinically relevant factors in the present study.

Today, besides CT and MRI, ^{123}I-MIBG and PET-CT have been widely used as more sensitive and specific imaging of neuroblastoma especially in soft tissues and bone sites [1, 19]. However, about 10% of neuroblastomas do not take up radiolabelled MIBG [20]. Even the combined use of ^{123}I-MIBG and PET-CT can not detect all tumor lesions in neuroblastoma patients [19, 21, 22]. So, liquid biopsy like CTCs, ctDNA, ctRNA, and even exosomes which well used in diagnosing metastasis in solid tumors need to be further studied in neuroblastoma when imaging data are ambiguous [23–26]. Neuroblastoma patients with ≥ 3 CTCs per 4 ml of PB were showed with an increased likelihood of having metastasis with the sensitivity and specificity of 88.89% and 78.59%, respectively [27]. The univariate logistic regression results also showed that CTC and NSE levels were associated with metastasis [27]. In another study, the combined 9

circulating miRNAs in newly diagnosed neuroblastoma patients' serum strongly associated with metastatic stage 4 disease, disease burden, and treatment response. Our data found that combined the expression of PHOX2B in both BM and PB for predicting metastasis of neuroblastoma at diagnosis with the sensitivity and specificity high to 86.7% and 92.9%, respectively. That means PHOX2B is a very useful biomarker for predicting metastasis of neuroblastoma patients.

PHOX2B was also revealed to be related to the survival of neuroblastoma patients. The stage 4 neuroblastoma patients with negative expression of PHOX2B in BM at 3 months after diagnosis and after completion of induction chemotherapy had a favorable outcome [28]. However, the expression of PHOX2B in BM at diagnosis did not correlate to survival in patients with stage 4 neuroblastoma [28]. In another research, 19% of high-risk neuroblastoma patients at diagnosis with both high expression of PHOX2B and tyrosine hydroxylase (TH) in PB were identified as ultrahigh-risk, with 5-year EFS and OS rates of unacceptable 0% [29]. In our study, the patients with negative PHOX2B in both BM and PB at diagnosis had a more favorable prognosis than the positive patients in the high-risk group. Our data were consistent with the above studies.

The expression of PHOX2B in BM and PB in low/intermediate-risk patients were also detected in our research. Druy et al revealed the patients with positive expression of PHOX2B/TH in BM at diagnosis had a significant adverse 5-year EFS and OS in total 93 neuroblastoma patients [9]. Unfortunately, 10 patients with the expression of PHOX2B/TH in BM at diagnosis in 69 patients with localized and stage 4S neuroblastoma also had unfavorable 5-year EFS and OS. Moreover, the survival rates of these patients did not differ significantly from patients with metastatic lesions [9]. In our study, the patients with low/intermediate-risk group also had worse EFS and OS with PHOX2B positive expression in BM and PB samples at diagnosis than patients without it. The results of our research and Druy et al supported that patients with PHOX2B expression in BM or PB in the favorable group at diagnosis may suffer from disseminated disease and probably will benefit from upstaging therapy [9].

Conclusion

In conclusion, PHOX2B could be an appropriate biomarker for predicting metastasis in newly diagnosed neuroblastoma patients. The expression of PHOX2B in BM and PB at diagnosis had adverse prognostic significance in the whole group of neuroblastoma patients as well as in patients with low/intermediate-risk group.

Abbreviations

PHOX2B: Paired-like homeobox 2B; BM: Bone marrow; PB: Peripheral blood; RT-PCR: Quantitative reverse transcriptase polymerase chain reaction; ROC: Receiver operating characteristic; EFS: Event free survival; OS: Overall survival; MRD: Minimal residual disease

Declarations

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Authors' contributions

CG and XM were involved in the conception and design of the study. HF and TX conceived, designed, and performed the statistical analysis and wrote the paper. HH, CD and WZ participated in analyzing the data. QZ, XW, CH, YZ, SZ, MJ, YS, and DZ were involved in data acquisition. All authors have read and approved the final version of the manuscript and agree to be 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.

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Availability of data and materials

The raw data are available upon reasonable request from the corresponding authors.

Ethics approval and consent to participate

The study (Clinical trial number: ChiCTR1800017940) was approved by the Ethical Committee of Beijing Children's Hospital (No. 2017-k-54). Informed written consent was obtained from each participant according to the Declaration of Helsinki.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

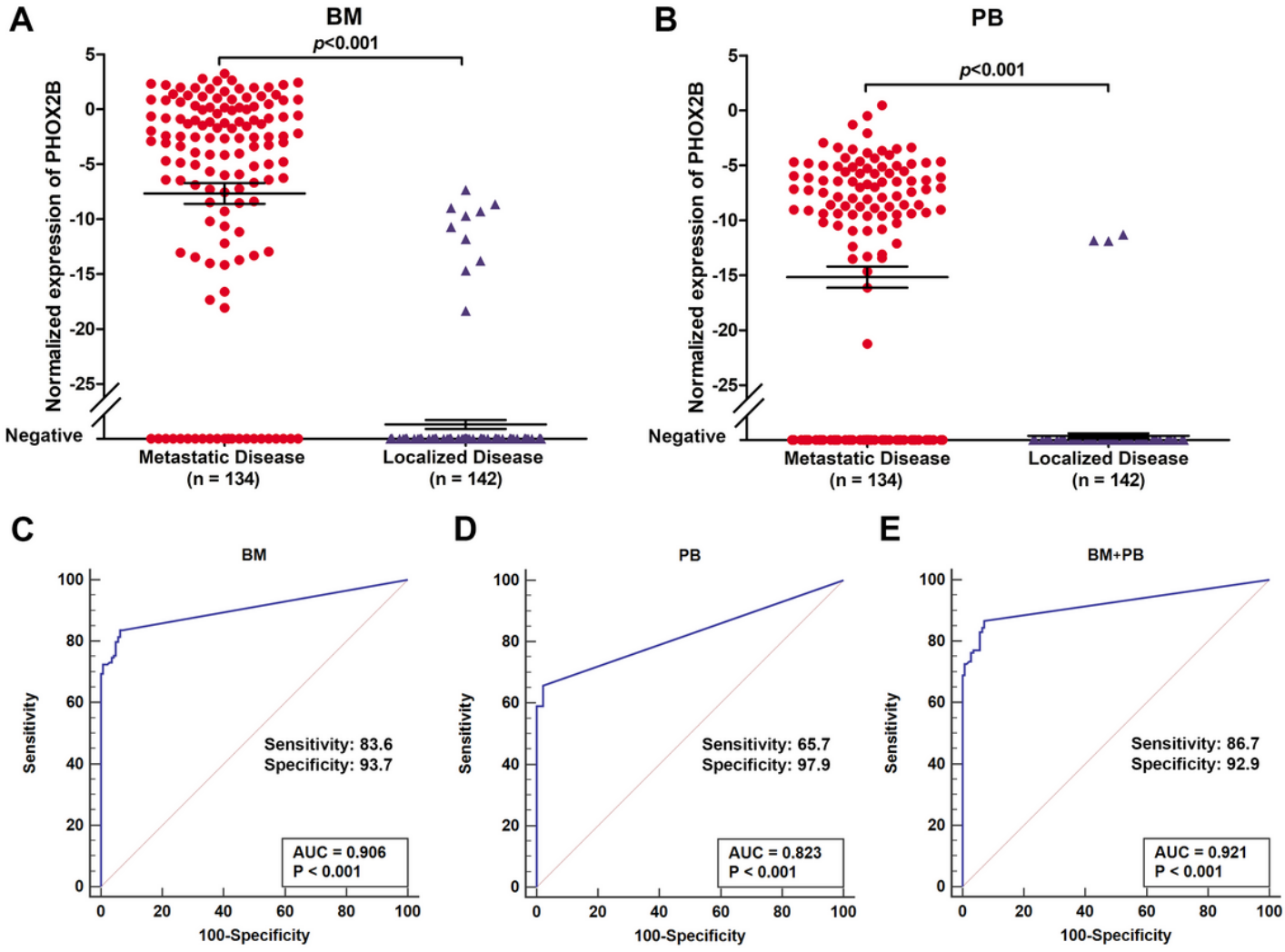

Figure 1

The expression of PHOX2B in BM and PB samples for predicting metastasis of neuroblastoma. The expression of PHOX2B in (A) BM, and in (B) PB of neuroblastoma patient with metastatic disease and localized disease. The ROC curve of the expression of PHOX2B in (C) BM, and (D) PB, and (E) the combined of PHOX2B in BM and PB for discriminating patients with and without metastasis.

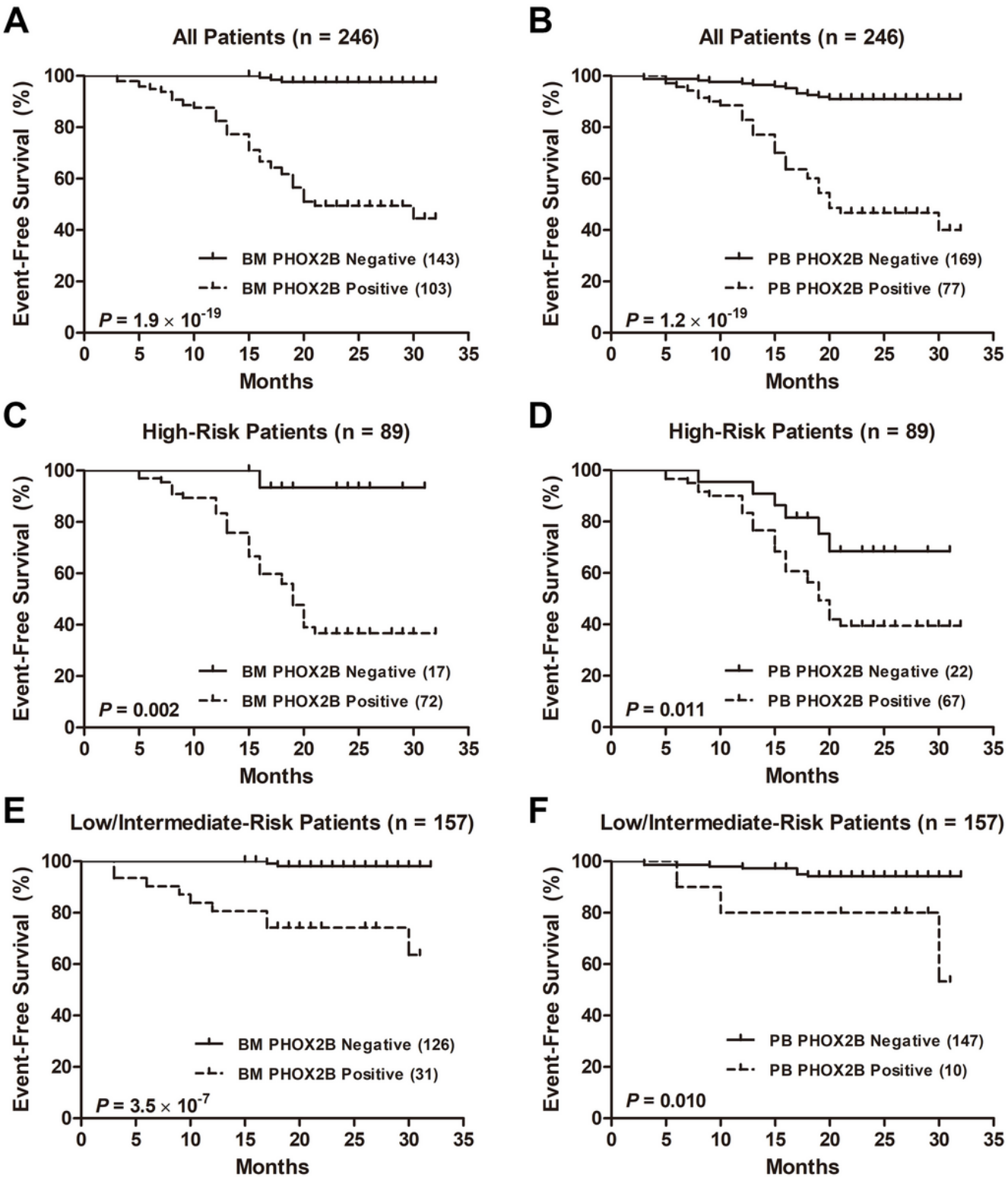

Figure 2

Event-free survival of positive and negative expression of PHOX2B in BM and PB of neuroblastoma patients. EFS of different expression of PHOX2B in BM of (A) all neuroblastoma patients, (C) high-risk patients, and (E) low-/intermediate-risk group. EFS of different expression of PHOX2B in PB of (B) all neuroblastoma patients, (D) high-risk patients, and (F) low-/intermediate-risk group.

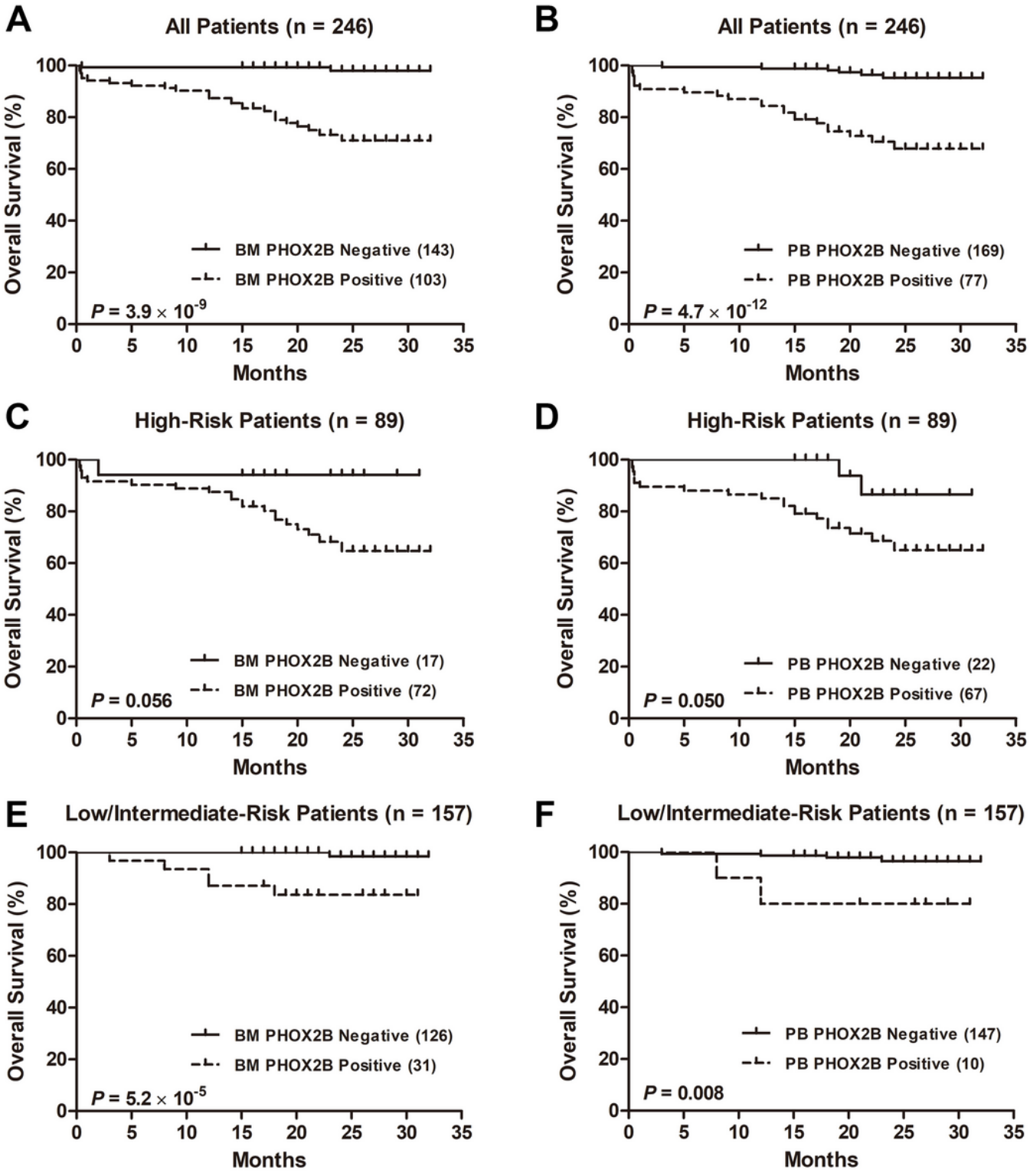

Figure 3

Overall survival of positive and negative expression of PHOX2B in BM and PB of neuroblastoma patients. OS of different expression of PHOX2B in BM of (A) all neuroblastoma patients, (C) high-risk patients, and (E) low-/intermediate-risk group. OS of different expression of PHOX2B in PB of (B) all neuroblastoma patients, (D) high-risk patients, and (F) low-/intermediate-risk group.