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The role of β-catenin and paired-like homeobox 2B (PHOX2B) expression in neuroblastoma patients; predictive and prognostic value

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ABSTRACT

Background: The expression of β-catenin and paired-like homeobox 2B (PHOX2B) expression were assessed in Neuroblastoma (NB) patients as a diagnostic, prognostic and/or predictive markers.

Methods: Bone marrow (BM) samples of 52 NB patients were assessed for the expression of β-catenin by immunohistochemistry (IHC), and PHOX2B by real time PCR (RT-PCR), compared to 12 healthy normal controls (NC). The data were correlated to the clinic-pathological features of the patients, response to treatment and disease relapse.

Results: β-catenin was expressed in 40 (76.92%) patients ($P < .001$). While PHOX2B was expressed in 32/52 (61.5%) patients, with a fold change of 0.29 (0.01–40.0, $P = .096$). β-catenin expression associated significantly with advanced tumor stage, high risk, positive results by MIBG and bone scan ($P = .002$, $P < .001$, $P = .006$, $P = .013$; respectively). Also it associated significantly with synaptophysin expression in the BM biopsy ($P < .001$), with a significant concordance (K = 0.519, P < .001). The expression of β-catenin associated significantly with PHOX2B gene expression [28/32 (87.5%), $P = .04$], and its fold change ($P = .027$), with a significant measure of agreement (K = 0.297, $P = .022$). The fold change of PHOX2B gene expression associated significantly with the high risk of the patients ($P = .04$). Poor response to treatment associated significantly with the expression of neuron specific enolase (NSE), β-catenin and PHOX2B in NB patients ($P = .021$, $P = .019$ and $P = .040$; respectively). The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of synaptophysin for the diagnosis of BM metastasis in NB patients were (69%, 65.2%, 71.4%, 62.5%; respectively, $P = .024$). While with β-catenin (93.1%, 43.5%, 67.5%, 83.3%; respectively, $P = .003$), and PHOX2B expression (65.5%, 34.5%, 59.4%, 50%; respectively, $P = .574$).

Conclusion: β-Catenin could be used as a sensitive and reliable marker for detection of BM metastasis and also a good predictor for resistance to treatment in NB patients. While, PHOX2B gene expression in BM aspirate could be a marker for high risk patients and poor response to treatment.

1. Introduction

Neuroblastoma (NB) is the most common cancer in infants (< 1 year old), it represents about 6% of all cancers in children (Siegel et al., 2019), and 15% of childhood cancer mortality worldwide (Brodeur et al., 2016). Neuroblastoma is arising from neural crest progenitor cells of the sympathetic nervous system which undergo aberrant differentiation and development (Wilzén et al., 2009). It manifest anywhere along the sympathetic nervous system, mainly along the paravertebral sympathetic chain and in the adrenal gland medullary region (Van Arendonk and Chung, 2019). It is a heterogeneous disease classified into three risk groups (low, intermediate and high) according to the age, extent of the disease, histological and cytogenetic abnormalities (Herd et al., 2019). Despite advances in the treatment strategies for NB, still the overall survival (OS) for high risk patients is around 50% at 5 years, with increased incidence of relapses (80%) within 2 years of diagnosis (Van Arendonk and Chung, 2019; Basta et al., 2016).

One of the most important genetic marker of NB aggressiveness is MYCN gene. It is a proto-oncogene which has an essential role early in neurogenesis for survival and differentiation of neural crest stem cells (NCSC) (Sawai et al., 1993). MYCN amplification occurs in about 20%

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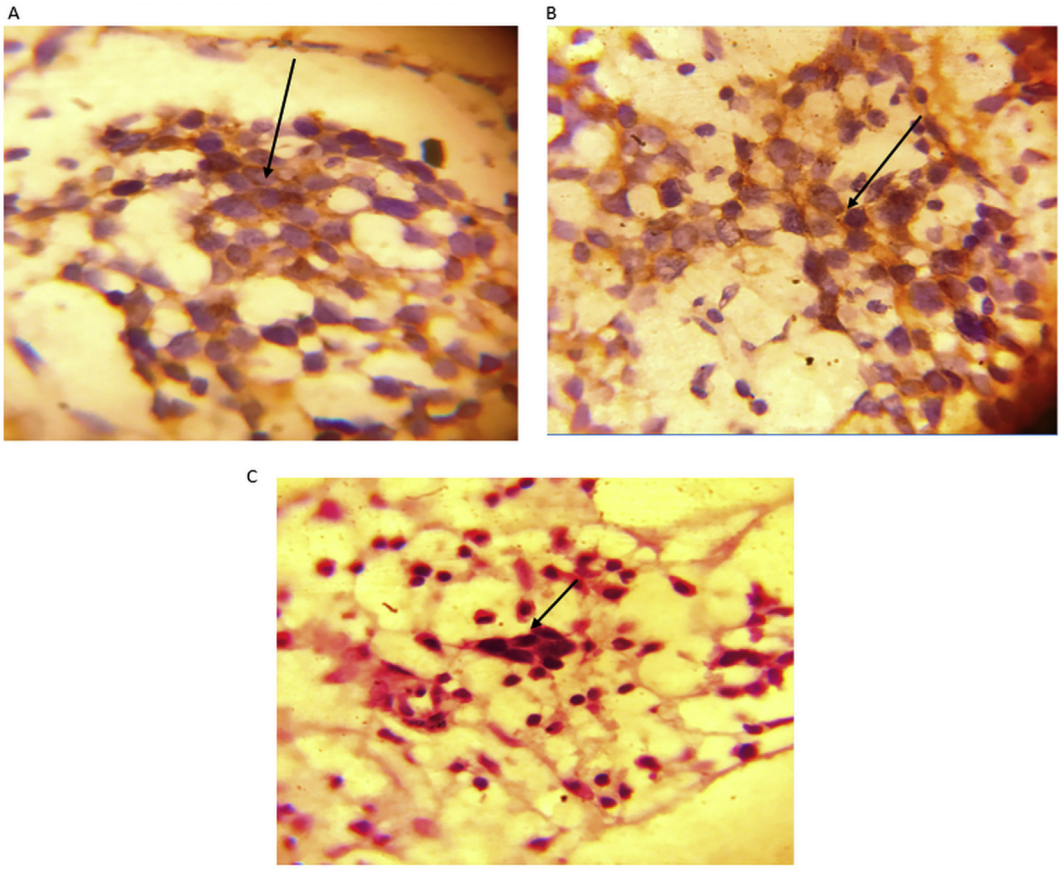

Fig. 1. A) positive immunostaining for β-catenin in neuroblastoma cells giving cytoplasmic and membranous reactivity (*100), B) positive immunostaining for NSE reactivity in neuroblastoma cells showing cytoplasmic reactivity (*100), C) Bone marrow morphology by H&E in the bone marrow biopsy of NB patients showing metastasis (*100).

of NB cases, and its amplification is strongly associated with unfavorable clinical outcomes, being detected in about 50% of patients with high-risk disease (Campbell et al., 2017; Look et al., 1991). However, absence of MYCN amplification in patients with stage IV, and those with age over 18 months old at diagnosis are also categorized as high-risk patients, suggesting the presence of other factors contributing to high-risk NB (Cheung and Dyer, 2013).

Wnt/β-catenin signaling pathway also has an important role in NCSC development and expansion (Lee et al., 2004). Activation of the canonical Wnt/β-catenin pathway results in the stabilization of cytoplasmic β-catenin, with subsequent transcription of Wnt target genes including MYC and cyclin D1 (CCND1) (Hecht and Kemler, 2000; Ilyas, 2005). Moreover, some previous studies reported that absence of MYCN amplification in high-risk NB patients is markedly associated with increased Wnt ligands and β-catenin expression (Liu et al., 2008).

Another factor that take part in the pathogenesis of NB is the paired-like homeobox 2B (PHOX2B) gene, this gene encodes a transcription factor which is essential for the early steps of autonomic nervous system development. Several reports demonstrated the presence of PHOX2B mutation in familial and sporadic cases of neuroblastoma (Mosse et al., 2004; van Limpt et al., 2004). Furthermore, evaluation of *PHOX2B* gene expression is currently used to monitor minimal residual disease in neuroblastoma patients (Stutterheim et al., 2008).

The aim of the current study was to evaluate the role of β-catenin and PHOX2B expression in the diagnosis and prognosis of NB patients through correlation with patients' clinic-pathological features, clinical outcome and response to treatment. Also we assessed their sensitivity and specificity for the possibility of the diagnosis of NB cases.

2. Methods

This is a prospective cohort study included 52 patients with histopathologically confirmed Neuroblastoma (NB), who presented to the Pediatric oncology outpatient clinics of National Cancer Institute (NCI), Cairo University during the period from January 2014 to December 2016.The control group included 12 age and sex matched healthy subjects who were donors for bone marrow transplantation in NCI during this period. The study protocol was approved by the ethical committee of NCI, Cairo University which was in accordance with 2011 declaration of Helsinki. A written informed consent was obtained from all subjects guardians prior to enrollment in the study.

Patients were classified according to the International Neuroblastoma Risk Group (INRG) staging system into; low, intermediate and high risk patients (Cohn et al., 2009). They were subjected to full clinical examination, laboratory work up including serum ferritin, serum neuron specific enolase (NSE), and MYCN amplification by Chromogenic In Situ Hybirdization (CISH). Radiological assessment including meta-iodobenzylguanidine (MIBG) and bone scan.

2.1. Samples

Bone Marrow (BM) Aspirate specimens (1 ml) were collected on potassium ethylene diamine tetra-acetic acid (K-EDTA) for morphologic analysis of hematopoiesis and metastasis. Also, BM Trephine Biopsy was performed at diagnosis for proper staging of NB. Touch imprint was done from the BM core prior to placing in the fixative. Specimens of BM trephine biopsy (BMB) were transported and fixed in formalin, decalcified in Formic acid-Sodium Citrate and processed to paraffin-wax embedding. Sections 3–4 μm thick, were cut and used for Haematoxylin

Table 1
Clinico-pathological features of the assessed Neuroblastoma patients.

Characteristics	Number (%)
Age (mean ± SD)	3.6 ± 1.1 years
Sex	
Male	26 (50)
Female	26 (50)
Stage	
III	21 (40.4)
IV	31 (59.6)
Risk	
Intermediate	19 (36.5)
High	33 (63.5)
MIBG	
Free	23 (44.2)
Positive	29 (55.8)
Bone metastasis	
Negative	22 (42.3)
Positive	30 (57.7)
Serum ferritin	
Median (range) ng/mL	131 (7–3541)
Serum NSE	
Median (range) ng/mL	212 (22–2066)
MYCN amplification	
Over expressed	22 (42.3)
Normally expressed	30 (57.7)
BMB (H&E)	
Positive	28 (53.8)
Negative	24 (46.2)
Synaptophysin	
Positive	28 (53.8)
Negative	24 (46.2)
Mortality	
Live	48 (92.3)
Dead	4 (7.7)
Relapse	
Yes,	5 (9.6)
No,	47 (90.4)
Course	
Stationary	19 (36.5)
Progressive	33 (63.5)

and Eosin (H&E) staining, and also for Immuno-histochemical staining for synaptophysin for assessment of BM metastasis.

2.2. Immuno-histochemical (IHC) staining for β-catenin expression

The IHC staining was performed using DAKO Envision ™ + System, and Horse Radish Peroxidase (HRP) was used as a universal visualization system. Formalin-fixed paraffin-embedded sections were pretreated by heat-induced epitope retrieval in Tris-EDTA buffer solution PH 9.0 (DAKO; S3308) in 90–95 °C. Then, blocking endogenous peroxidase was done. The Monoclonal rabbit IgG Anti-Human β-catenin protein was applied according to manufacturer's protocol (Dako, 71-2700). Isolated tumor cells (ITCs) positive for β-catenin were defined as single cells or small clusters of cells (usually < 5 cells total) with strong cytoplasmic and membranous reactivity, and lacking any reactivity within the nucleus. Cases with ITCs were reviewed by two senior pathologist. False-positive cells as erythroid precursors, osteoblasts and plasma cells were not counted.The specimen is considered positive if there is specific surface and/or cytoplasmic brown coloration in non-haemopoietic cells as positive control sections with or without brown coloration of filamentous material in the infiltrates background (neurofilamints) (Zaher et al., 2011).

2.3. Assessment of PHOX2B expression by real-time (RT-PCR)

Total RNA was extracted from bone marrow cells using QIAamp RNA extraction blood Mini kit (QIAGEN, cat no. 52304) as recommended by the manufacturer's instructions. The purity and the concentration of the extracted RNA was detected using spectrophotometer nano-drop (Quawell, Q-500, Scribner, USA). Retro-transcription (cDNA) was done by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, cat no. 4368814) according to the manufacturer's instructions. The purity and concentration of cDNA was evaluated and then it was stored in – 20 °C till performing quantitative real-time PCR.

PHOX2B mRNA expression was quantified using Taqman Universal PCR Master Mix II (Applied Biosystems, Foster City, CA, USA, cat no. 94404) and Taqman gene Expression Assay for PHOX2B gene (Thermo Fisher Scientific, USA, Hs 00243679). RT-PCR was performed using a total volume of 20 µl, and the thermal reaction conditions were as follows: 95 °C for 10 min (polymerase activation), followed by 40 cycles

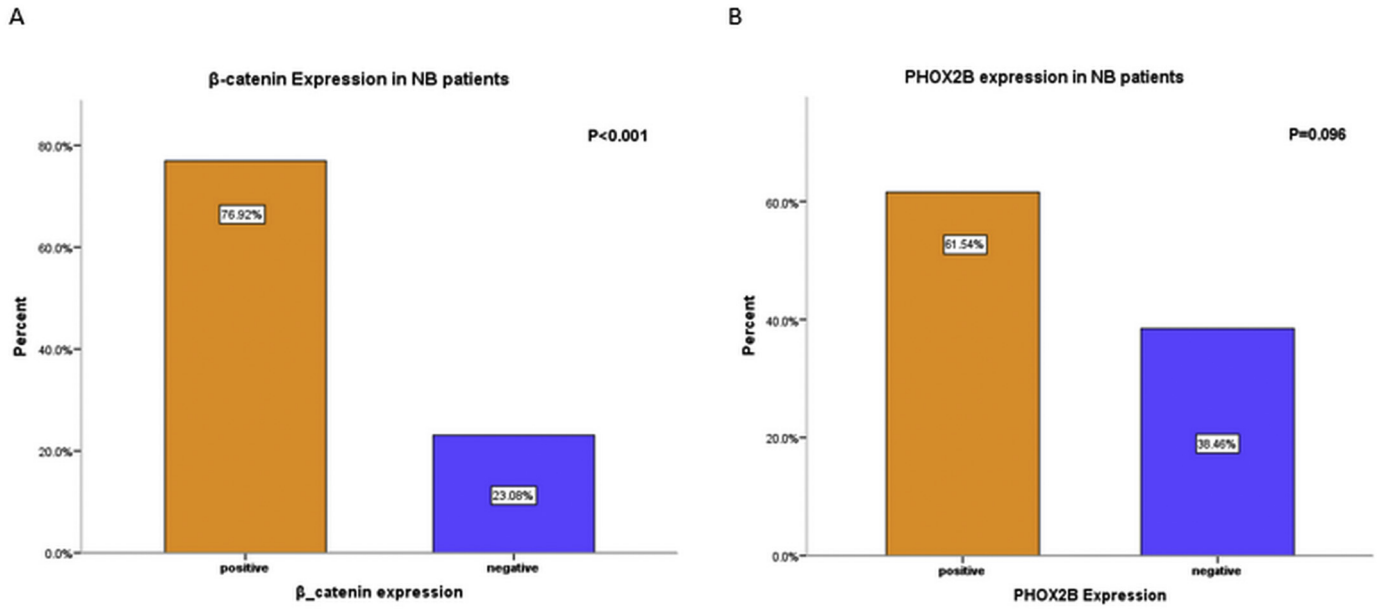

Fig. 2. the expression levels of A) β-catenin, and B) PHOX2B, in the assessed neuroblastoma patients.

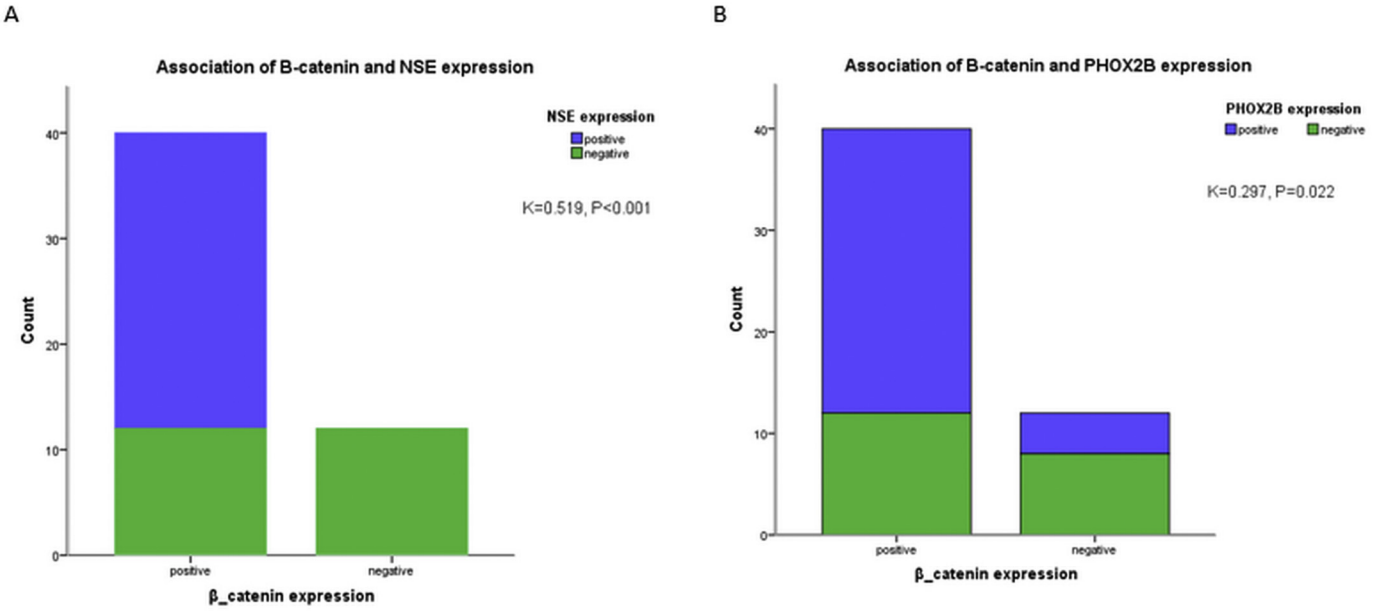

Fig. 3. association of β-catenin expression and A) NSE expression, B) PHOX2B expression in NB cases.

Table 2
Association of B-catenin and the relevant clinic-pathological features of the patients.

	β-catenin		*P* value
	Negative (12)	Positive (40)	
Sex			
Male	9 (75.0%)	17 (42.5%)	0.097
Female	3 (25.0%)	23 (57.5%)	
Stage			
III	10 (83.3%)	11 (27.5%)	**0.002**
IV	2 (16.7%)	29 (72.5%)	
Risk			
High	2 (16.7%)	31 (77.5%)	**p < .001**
Intermediate	10 (83.3%)	9 (22.5%)	
BM biopsy (H&E)			
Negative	12 (100.0%)	12 (30%)	**p < .001**
Positive	0 (0.0%)	28 (70%)	
Synaptophysin			
Negative	12 (100.0%)	12 (30.0%)	**P < .001**
Positive	0 (0.0%)	28 (70.0%)	
Serum Ferritin			
≤300 ng/ml	12 (100%)	29 (72.5%)	**0.05**
> 300 ng/ml	0 (0%)	11 (27.5%)	
Serum NSE			
Median (range)	159.8 (22.7–550)	249 (22–2066)	0.236#
Bone scan			
Negative	10 (83.3%)	12 (30%)	**0.013**
Positive	2(16.7%)	28 (70.0%)	
MIBG			
Negative	10 (83.3%)	13 (32.5%)	**0.006**
Positive	2 (16.7%)	27 (67.5%)	
PHOX2B expression			
Negative	8 (66.7%)	12 (30.0%)	**0.04**
Positive	4 (33.3%)	28 (70.0%)	
PHOX 2- fold change			
Median (range)	0 (0–7.54)	0.05 (0–40)	**0.027**
MYCN amplification			
Normally expressed	6 (50.0%)	24 (60.0%)	0.740
Overexpressed	6 (50.0%)	16 (40.0%)	
Mortality			
Alive	11(91.7%)	37 (92.5%)	1
Dead	1(8.3%)	3 (7.5%)	
Relapse			
Negative	11(91.7%)	36 (90.0%)	1
Positive	1(8.3%)	4 (10.0%)	

Bold numbers indicating significant values < 0.05

of 95 °C for 30s (Denaturation), 60 °C for 60s (annealing and extension), in which fluorescence was acquired and detected by StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Relative expression of PHOX2B gene was analyzed by the comparative Ct method ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001), in which data were expressed as the fold change in PHOX2B gene expression in the patients normalized to the expression levels of the endogenous control gene (B-Actin) and relative to the healthy controls.

2.4. Statistical analysis

The data were analyzed using the statistical package SPSS (version 24 for Windows; SPSS Inc., Chicago, IL, USA). Statistical differences between groups were tested using Chi square test for qualitative variables, Mann Whitney test was used for nonparametric data. Spearman's test was used to detect the strength of correlation between non-parametric variables. A likelihood test was used to detect sensitivity and specificity, and the concordance was measured using Cohen's kappa test of agreement. *P*-values less than or equal to 0.05 were considered statistically significant.

3. Results

3.1. Patients' characteristics

The current study included 52 NB patients with age ranged from 3 months to 16 years and a median of 3.5 years, compared to healthy control with a median of 4 years and range from 7 to 15 years. The male to female ratio was 1:1. There were 21 (40.4%) patients presented with stage III, and 31 (59.6%) with stage IV. Patients had intermediate risk were 19 (36.5%), and high risk were 33(63.5%). The median of serum ferritin was 131 (range; 7–3541) ng/mL, and that of serum neuron-specific enolase (NSE) was 212 (range; 22–2066) ng/mL. Radiological assessment showed that 30 (57.7%) patients had positive bone metastasis by bone scan, and 29 (55.8%) were positive bone and BM metastasis by MIBG. Twenty-two patients (42.3%) had MYCN amplification, while 30 (57.7%) patients had normal MYCN expression. Bone marrow biopsy showed that 28 (53.8%) cases had BM metastasis compared to 24 (46.2%) who were negative by synaptophysin detection as well as hematoxyline and eosin (H&E) staining (Fig. 1). There were 19 (36.5%) patients had a stationary course compared to 33 (63.5%)

Table 3
Association of PHOX2B expression and clinic-pathological features of the patients.

	PHOX2 expression		P value	PHOX2B-fold change	P value
	Negative (20)	Positive (32)		Median (range)	
Gender					
Male	10 (50%)	16 (50%)	1	0.01 (0–36.4)	0.72
Female	10 (50%)	16 (50%)		0.04 (0–40)	
Stage					
III	10 (50%)	11 (34.4%)	0.384	0.01 (0–6.02)	0.086
IV	10 (50%)	21 (65.6%)		0.06 (0–40)	
Risk					
High	10 (50%)	23 (71.9%)	0.144	0.06 (0–40)	**0.04**
Intermediate	10 (50%)	9 (28.1%)		0 (0–6.02)	
HE					
Negative	10 (50%)	14 (43.8%)	0.777	0.02 (0–7.5)	0.269
Positive	10 (50%)	18 (56.2%)		0.05 (0–40)	
Synaptophysin					
Negative	10 (50.0%)	14 (43.8%)	0.777	0.01 (0–7.5)	0.269
Positive	10 (50.0%)	18 (56.2%)		0.05 (0–40)	
Bone Scan					
Negative	10 (50%)	12 (37.5%)	0.403	0.01 (0–40)	
Positive	10(50.0%)	20 (62.5%)		0.05 (0–36.4))	0.179
MIBG					
Negative	10 (50%)	13 (40.6%)	0.574	0.01 (0–40)	
Positive	10 (50.0%)	19 (59.4%)		0.06 (0–36.4)	0.197
β-catenin					
Negative	8 (40.0%)	4 (12.5%)	**0.04**	0 (0–7.54)	**0.027**
Positive	12 (60.0%)	28 (87.5%)		0.05 (0–40)	
MYCN amplification					
Normally expressed	12 (60.0%)	18 (56.3%)	1	0.02 (0–36.4)	0.6
Over expressed	8 (40.0%)	14 (43.7%)		0.04 (0–40)	
Mortality					
Alive	19 (95.0%)	29 (90.6%)	1	0.015 (0–36.4)	0.322
Dead	1 (5.0%)	3 (9.4%)		0.3(0–40)	
Relapse					
Negative	18 (90.0%)	29 (90.6%)	1	0.02 (0–36.4)	0.701
Positive	2 (10.0%)	3 (9.4%)		0.06 (0–40)	

Bold numbers indicating significant values < 0.05

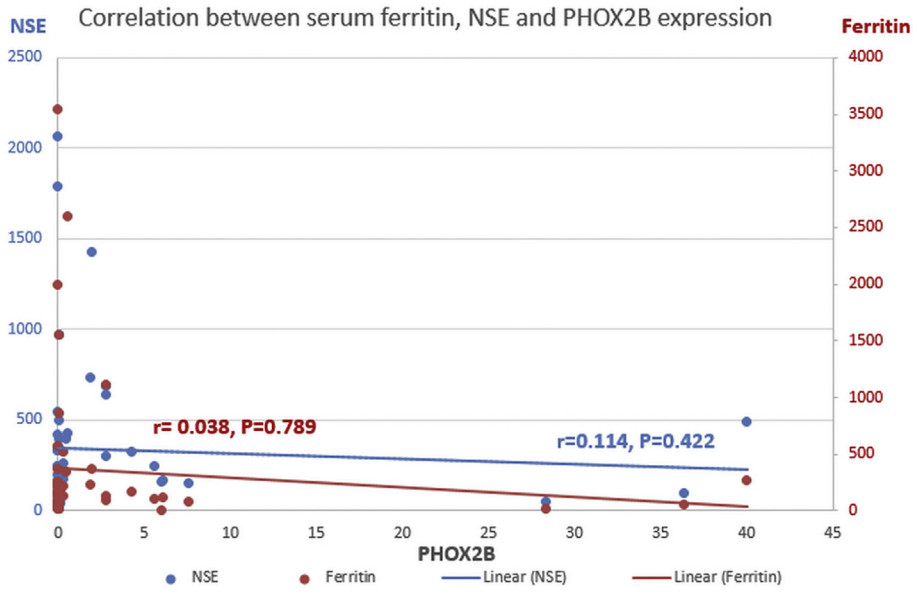

Fig. 4. correlation between the fold change of PHOX2B expression, serum NSE and serum ferritin.

patients with progressive course of the disease, and during the entire period of the study 5 (9.6%) cases relapsed and 4 cases (7.7%) died (Table 1).

3.2. Assessment of β-catenin and PHOX2B expression

β-catenin was expressed in the bone marrow biopsy of 40(76.92%) patients, compared to 12 (23.08%) patients who were negative for it ($P < .001$). While regarding PHOX2B gene expression, there were 32 (61.5%) cases expressing PHOX2B gene, compared to 20 (38.5%) cases

Table 4
Response to treatment.

	Response to treatment		P value
	Not responding (33)	Responding (19)	
Gender			
Male	13 (39.4%)	13 (68.4%)	0.083
Female	20 (60.6%)	6 (31.6%)	
Stage			
III	6 (18.2%)	15 (78.9%)	**P < .001**
IV	27 (81.8%)	4 (21.1%)	
Risk of disease			
High	30 (90.9%)	3 (15.8%)	**P < .001**
Intermediate	3 (9.1%)	16 (84.2%)	
H&E			
Negative	11 (33.3%)	13 (68.4%)	**0.021**
Positive	22 (66.7%)	6 (31.6%)	
Synaptophysin			
Positive	22 (66.7%)	6 (31.6%)	**0.021**
Negative	11 (33.3%)	13 (68.4%)	
β- catenin			
Positive	29 (87.9%)	11 (57.9%)	**0.019**
Negative	4 (12.1%)	8 (42.1%)	
PHOX2B expression			
Positive	24 (72.7%)	8 (42.1%)	**0.040**
Negative	9 (27.3%)	11 (57.9%)	
Serum Ferritin			
≤300 ng/ml	22 (66.7%)	19 (100%)	**0.004**
> 300 ng/ml	11 (33.3%)	0 (0%)	
Serum NSE			
Median (range)	322 (21.5–2066)	114.4 (22.5–399)	**0.003**
Bone scan			
Negative	5 (15.2%)	17 (89.5%)	**P < .001**
Positive	28 (84.8%)	2 (10.5%)	
MIBG			
Positive	27 (81.8%)	2 (10.5%)	***P* < .001**
Negative	6 (18.2%)	17 (89.5%)	
MYCN amplification			
Normally-expressed	18 (54.5%)	12 (63.2%)	0.576
Over expressed	15 (45.5%)	7 (36.8%)	

Bold numbers indicating significant values < 0.05

who did not express it ($P = .096$, Fig. 2). The median of the fold change of PHOX2B gene expression was 0.29 and ranged (0.01–40.0).

3.3. Association of β-catenin and relevant clinico-pathological features of the patients

The expression of β-catenin associated significantly with advanced tumor stage [29/40 (72.5%) in stage IV versus 11/40 (27.5%) in stage III, $P = .002$], high risk patients according to INRG risk stratification [31/40 (77.5%) in high risk patients compared to 9/40 (22.5%) in patients with intermediate risk, $P < .001$]. Also, it associated significantly with positive results by MIBG, since it was expressed in 27/40 (67.5%) patients positive for metastasis, compared to 13/40 (32.5%) in patients who were negative for metastasis ($P = .006$), and similarly with bone scan [28/40 (70.0%) in patients had bone metastasis versus 12/40 (30%) in patients negative for bone metastasis, $P = .013$]. The expression of β-catenin associated significantly with the positive results of BM biopsy by H&E [28/40 (70.0%) in patients with metastasis versus 12/40 (30%) in patients negative for metastasis, $P < .001$], as well as with synaptophysin expression [28/40 (70.0%, P < .001)], with a significant concordance between the expression of β-catenin and synaptophysin (K = 0.519,P < .001, Fig. 3). β-catenin expression had a borderline significance with serum Ferritin level, as all patients who had high serum Ferritin level > 300 ng/ml, were positive for β-catenin11/40 (27.5%, $P = .05$). However it did not associate significantly with gender, MYCN amplification, serum NSE, disease relapse or mortality of the patients ($P > .05$, Table 2).

3.4. Association of PHOX2B expression and the clinico-pathological features of the patients

There was no significant association between PHOX2B gene expression or its fold change and relevant clinico-pathological features of the patients including gender, tumor stage, MYCN amplification, bone scan and MIBG results, BM biopsy results by HE, synaptophysin expression, disease relapse and mortality of the patients ($P > .05$). However, the expression of β-catenin associated significantly with PHOX2B gene expression [28/32 (87.5%), $P = .04$], and PHOX2B fold change ($P = .027$, Fig. 3), with a significant measure of agreement (K = 0.297, $P = .022$). Also, the fold change of PHOX2B gene expression associated significantly with the high risk of the patients (P = .04, Table 3). On the other hand, there was no significant correlation between the fold change of PHOX2B expression and serum NSE ($r = 0.114$, $P = .422$), or serum ferritin ($r = -0.038$, $P = .789$, Fig. 4).

3.5. Response to treatment

Patients included in the study were divided according to their response to treatment into 33 (63.5%) patients who had a progressive disease (PD), and 19 (36.5%) patients who had a stable disease (SD).

Poor response to treatment associated significantly with advanced disease stage [27/33 (81.8%) in patients with stage IV, $P < .001$], high risk patients [30/33 (90.9%), P < .001], patients with positive BM morphology (H&E), as well as positive synaptophysin expression in BM biopsy [22/33 (66.7%, $P = .021$)]. Serum ferritin level > 300 ng/ml was observed in 11/33 (33.3%) in non-responding patients, while it was not increased in responding group ($P = .004$). Serum level of NSE was 322 ng/ml (21.5–2066) in non-responding patients, compared to 114.4 ng/ml (22.5–399) in responding group ($P = .003$). Similarly poor clinical response associated significantly with positive bone metastasis by bone scan [28/33 (84.8%) patients, $P < .001$], and by MIBG [27/33 (81.8%), P < .001]. Patients who had positive synaptophysin expression in BM showed significant poor clinical response [22/33 (66.7%), $P = .021$], as well as in those who had positive β-catenin expression, since β-catenin was expressed in 29/33 (87.9%) in non-responding group compared to 4/33 patients (12.1%) with negative β-catenin expression ($P = .019$). Also PHOX2B gene expression associated significantly with poor clinical response since it was expressed in 24/33 (72.7%) of patients with progressive disease compared to 8/19 (42.1%) in patients who had good response to treatment ($P = .040$). On the other hand, MYCN amplification and gender did not associate significantly with the clinical response to treatment in the assessed patients ($P > .05$, Table 4).

Table 5
Sensitivity, specificity, PPV and NPV of Synaptophysin, B-Catenin and PHOX2B in neuroblastoma patients.

Marker	Sensitivity	Specificity	PPV	NPV	Likelihood ratio	P value[a]
Synaptophysin	69%	65.2%	71.4%	62.5%	6.1	**0.024**
B-Catenin	**93.1%**	43.5%	67.5%	83.3%	10.1	**0.003**
PHOX2B expression	65.5%	34.5%	59.4%	50%	0.44	0.574

Bold numbers indicating significant values < 0.05

3.6. Assessment of the diagnostic power of synaptophysin, B-Catenin and PHOX2B Expression in neuroblastoma patients

The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of synaptophysin for diagnosis of bone marrow metastasis in NB patients were (69%, 65.2%, 71.4%, 62.5%; respectively, $P = .024$). While for β-catenin expression were; 93.1% sensitivity, 43.5% specificity, 67.5% PPV, and 83.3% NPV ($P = .003$). on the other hand, PHOX2B gene expression achieved sensitivity 65.5%, specificity 34.5%, PPV 59.4%, and NPV 50% ($P = .574$, Table 5).

4. Discussion

Neuroblastoma is a biologically and clinically heterogeneous pediatric cancer which comprises about 40% high-risk patients with poor prognosis (Vieira et al., 2015). So that, it is necessary to search for a sensitive and reliable biomarkers used for early detection, diagnosis and/or prognostic stratification of NB patients. Though MYCN amplification is strongly associated with rapid disease progression and poor outcome, however it is not amplified in about 80% of NB cases, and there is another mutational changes that are responsible for NB development and progression (Luo et al., 2018).

The current study showed that β-catenin was expressed (by IHC) in 78.8% of NB patients, it associated significantly with advanced tumor stage and high risk of the disease. These results are in agreement with Zins et al. (2016) and Chen et al. (2019), who reported that FZD2 andTRIM59 knockdown; respectively, inhibit NB cell proliferation by down-regulating the Wnt/β-catenin signaling pathway. Also, In another study done by Dyberg et al. (2016), who concluded that reduced expression of β-catenin associated significantly with low-risk disease and good survival of the patients through increased expression of Prickle1 and Vangl2 mRNA.

Our results also showed significant association between β-catenin expression and positive results of H&E bone marrow morphology, synaptophysin expression (IHC), MIBG and bone scan. However it did not associate significantly with disease relapse, mortality of the patients or MYCN amplification. These data are in concordance with Jansen et al. (2015), who confirmed increased expression of b-catenin in high-risk NB patients without MYCN amplification. Indicating its possible uses as a diagnostic marker for NB patients.

Assessment of germline ALK or PHOX2B mutations has become the standard of care for children with a family history of neuroblastoma, however its clinical significance for the sporadic cases in NB is still unclear (Bosse and Maris, 2016). We found that PHOX2B was expressed in 61.5% of the assessed sporadic NB cases, these data are in agreement with Ke et al. (2015), who demonstrated that Phox2B is an important regulator in NB pathogenesis, as it promotes NB cell proliferation and xenograft tumor growth. Also Pattyn et al. (1999), who performed experimental study on Phox2b knockout mice and concluded that PHOX2B is a master gene of autonomic neuron development and differentiation. Furthermore, our data showed that PHOX2B expression level associated significantly with high risk of the disease and poor clinical response to treatment. These data are in agreement with Yamamoto et al. (2015), who provided evidence that PHOX2B is a sensitive bone marrow marker for detection of the minimal residual disease which is account for the early relapse and tumor metastasis in NB patients.

However our data revealed no significant association between PHOX2B gene expression and other relevant clinico-pathological features including tumor stage, MYCN amplification, serum ferritin, serum NSE, MIBG results, bone marrow morphology, synaptophysin expression, disease relapse and mortality of the patients. While interestingly its expression level associated significantly with the expression of β-catenin with a significant measure of agreement in children with NB. Thus, our data regarding PHOX2B gene expression showed that it could be a possible predictive marker for poor response to treatment and high risk of the disease rather than for diagnosis of bone marrow metastasis in NB cases. This conclusion was confirmed by its low sensitivity (65.5%), specificity (34.5%), PPV (59.4%), and NPV (50%) for detection of BM involvement.

Our data regarding the clinical outcomes of the patients showed that poor response to treatment associated significantly with advanced disease stage, high risk patients, positive metastasis by MIBG, increased serum NSE and serum ferritin levels. These data are in concordance to that reported by Su et al. (2019), who found a significant down-regulation of the serum NSE in NB patients with partial remission (PR) compared to those with stable disease (SD). Also Luo et al. (2018), demonstrated that high levels of serum ferritin (> 142 ng/ml) or NSE (> 100 ng/ml) in patients with NB were shown to be predictive of poorer outcome (Luo et al., 2018). Furthermore, patients who had positive BM biopsy for synaptophysin and β-catenin expression, showed poor response to treatment rather than those who didn't express synaptophysin or β-catenin. Meanwhile, MYCN amplification did not associate significantly with response to treatment in the assessed patients. These results are in agreement with that reported by Alshareef et al. (2017), who reported a significant resistance to treatment in NB cells with high expression of β-catenin.

We assessed the diagnostic power of synaptophysin and β-Catenin expression for detection of BM infiltration in NB patients by using ROC analysis. Our results showed that β-Catenin is more sensitive than synaptophysin for detection of BM metastasis in NB patients in relation to MIBG as it is the most sensitive investigation for detection of skeletal/soft tissue metastasis in NB cases (Luo et al., 2018). Thus our results in the current study demonstrated that β-Catenin could be used as a sensitive and reliable marker for detection of BM metastasis in NB patients, and also a good predictor for resistance to treatment in the patients. While, regarding PHOX2B gene expression in BM aspirate, it could be a marker for high risk patients and poor response to treatment. Therefore, β-Catenin and PHOX2B might be a target for future therapy in children with high risk NB. However these data should be validated on a larger number of patients to confirm its role in the diagnosis and prognosis of neuroblastoma.

Declaration of Competing Interest

All authors declare there is no conflict of interest.

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