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ORIGINAL ARTICLE – CLINICAL ONCOLOGY

Prognostic value of initial bone marrow disease detection by multiparameter flow cytometry in children with neuroblastoma

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Abstract

Purpose Multicolor flow cytometry (MFC) is widely available, fast and has an easy-to perform approach for finding neuroblastoma (NB) cells among normal bone marrow (BM) hematopoietic cells. Aim of the study was to investigate prognostic significance of initial MFC tumor cells' detection in BM of children with NB.

Methods 51 patients (24 boys and 27 girls) aged from 6 days to 15 years (median age 1 year 3 months) with NB were included in the study. BM samples at the time of diagnosis were obtained from 2 to 5 aspiration sites per patient. CD45(−)CD56(+) CD81(+)GD2(+)-cells were evaluated by MFC.

Results NB cells were detected in BM by FC more frequently compared to conventional cytomorphology (49.0% and 29.4% patients, respectively, $p = 0.043$). Patients with NB cells detected in BM by MFC had significantly worse event-free survival and cumulative incidence of relapse/progression [0.24(0.08) and 0.60(0.10), respectively] compared to children with negative result of immunophenotyping [0.85(0.07) and 0.12(0.06), respectively, $p < 0.001$ in both cases]. BM involvement detection by MFC maintained its prognostic significance in various patients groups. In multivariate analysis, immunophenotyping proved to be an independent prognostic factor when analyzed jointly with other NB risk factors. In 42 patients BM involvement was also studied by RQ-PCR for *PHOX2B* and *TH* genes expression. Within groups of patients divided by RQ-PCR positivity, MFC-positivity retained prognostic significance.

Conclusions Thus flow cytometric BM involvement detection has very strong prognostic impact even stronger than RQ-PCR. It could be used in combination with other parameters for the treatment strategy choice in patients with NB.

Keywords Neuroblastoma · Flow cytometry · Bone marrow · Prediction of outcome

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Introduction

The clinical diversity of neuroblastoma (NB), childhood malignant tumor of sympathetic ganglia and adrenal gland are paradigms of pediatric oncology. Up to half of all patients suffering from neuroblastoma present with disseminated disease and these patients, with the exception of stage 4S patients, have dismal outcome (Cotterill et al. 2000; Hartmann et al. 1999; Monclair et al. 2009; Stutterheim et al. 2008). Bone marrow (BM), one of the most frequent metastatic sites, is very well suited to be studied by biopsies (Cotterill et al. 2000; Hartmann et al. 1999; Monclair et al. 2009; Stutterheim et al. 2008).

Detection of BM involvement is an essential aspect for the correct staging and allocating patients to risk groups. Conventional cytomorphological examination of BM routinely used for this purpose has limited accuracy and

analytical sensitivity (less than 1×10^{-3}) (Burchill et al. 2017; Cheung and Cheung 2001; Monclair et al. 2009). Thus more precise and sensitive tools for detection of BM involvement were developed in the past decades. These techniques include immunocytological investigation of BM slides, reversed transcriptase polymerase chain reaction (PCR) or multiparameter flow cytometry (MFC). Immunocytology (IC)—a robust and well-standardized method for precise detection of tumor BM infiltration (Burchill et al. 2017; Corrias et al. 2008)—is costly and laborious. PCR-based detection of tumor-associated gene transcripts (most frequently *PHOX2B* and *TH*) demonstrates comparable sensitivity to IC (up to 0.0001%), high reproducibility and robustness (Stutterheim et al. 2008; Trager et al. 2003; van Wezel et al. 2016). In the recent publications it is one of the most frequently used methods for minimal metastatic or residual disease detection in neuroblastoma patients (Beiske et al. 2009; Druy et al. 2018; van Wezel et al. 2016). Analogously to the IC, quantitative PCR (RQ-PCR) was standardized for BM involvement detection in multicenter clinical trials (Burchill et al. 2017).

Small tumor populations' detection by MFC is widely used in oncohematology, but it is not routinely applied for searching of BM micrometastases of solid tumors. Nevertheless in the recent decade there were several studies showing that cytometric evaluation of NB cells in BM aspirate could be fast and a relatively cheap alternative for IC and PCR-based techniques (Ifversen et al. 2005). Although detection of CD45(−)GD2(+)CD56(+)CD81(+) NB84(+) cells (Bomken et al. 2006; Bozzi et al. 2006; Ferreira-Facio et al. 2013; Gautam et al. 2008; Okcu et al. 2005; Swerts et al. 2004; Warzynski et al. 2002) by MFC reached a rather limited sensitivity of 0.01% (Ifversen et al. 2005; Swerts et al. 2004; Tsang et al. 2003), it could be applicable for initial BM involvement assessment in NB patients (Bozzi et al. 2006; Tsang et al. 2003; Warzynski et al. 2002). Nevertheless, prognostic significance of BM MFC results in NB is currently not well established. Only for stage 4 patients inferior outcome is shown for initially MFC-positive cases (Bozzi et al. 2006).

The aim of this present study was the investigation of the prognostic significance of initial cytometric BM disease detection in patients with neuroblastoma.

Patients and methods

The study was approved by the Ethic Committees of the Ural State Medical University and Regional Children's Hospital 1. The informed consent for the sample collection and investigation was obtained from patients' parents or legal representatives in all cases.

Patients

All consecutive patients ($n = 51$) with histologically verified neuroblastoma (43), ganglioneuroblastoma (5) and ganglioneuroma (3), that were admitted to Pediatric oncology and hematology center from October 2005 till December 2011, were enrolled into the trial. All patients were treated or observed according to the NB2004 GPOH protocol(Berthold et al. 2017). Median of age at diagnosis was 15 months (range 0.2–180 months), stage distribution was as follows: stage 1–14 cases (27.5%), stage 2–2 (3.9%), stage 3–12 (23.5%), stage 4–18 (35.3%), stage 4S–5 (9.8%). 20 (39.2%) patients were younger than 12 months. In 11 (21.6%) cases *MYCN* gene amplification was present. After complex clinical and laboratory examination 23 patients (45.1%) were allocated to the observation group according to NB2004 treatment schema, 6 patients (11.8%)—to the intermediate risk group and 22 (43.1%)—to the high-risk group. Taking into account the possibility of a focal or patchy metastatic process, BM biopsy was taken from two to five different anatomic sites in each patient. In 42 out of 51 analyzed patients BM involvement was assessed in synonymous samples by flow cytometry and by detection of *PHOX2B* and *TH* genes expression using RQ-PCR.

Cytomorphological evaluation

BM aspirates (in EDTA) were smeared on at least two slides and investigated by conventional cytology after staining with azure and eosin.

Flow cytometry

According to cellularity of the sample 50–100 µl of BM aspirates was stained with appropriate volume of directly conjugated monoclonal antibodies (MoABs). After 15 min incubation at 4 °C in the dark erythrocytes were lysed with FACS Lysing solution [Becton & Dickinson (BD), San Jose, CA]. Subsequently samples were washed in phosphate buffer saline (PBS) and resuspended in 0.5 ml of PBS. Antibodies were labeled with fluorescein isothiocyanate (FITC), R-phycoerythrin (PE), peridinin chlorophyll protein (PerCP), allophycocyanin (APC) and tandem conjugates of PE and APC with Cyanine7 (Cy7). Until end of 2006 two-color staining for NB84 (NB84a-FITC, Novocastra, UK) and CD45 (2D1-PE, BD) was applied. Since 2006, this MoABs combination was complemented and then substituted by the following four-color panel: CD81 (JS-81-FITC, BD), GD2 (14.Ga2-PE, BD), CD45 (2D1-PerCP, BD), and CD56 (NCAM16.2-APC, BD). Performed in parallel, both approaches led to

fully concordant results. MFC was performed on dual-laser FACS Canto and FACS Canto II flow cytometers (both from BD). At least 300 000 events were acquired. Cells' immunophenotype was analyzed using FACS Diva 6.1 software. CD45-NB84 + and CD45-CD56 + CD81 + GD2 + cells were gated on bifluorescent dot plots as previously described (Swerts et al. 2004; Warzynski et al. 2002) with obligatory backgating on the forward vs side scatter (FSC and SSC, respectively) plot. Duplets were excluded. According to rules of cytometric rare event analysis (Hedley and Keeney 2013) at least 30 clustered cells with tumor-associated immunophenotype and appropriate light-scatter properties were required to define MFC-positivity. BM samples were considered MFC-positive if NB cells represented more than 0.01% among all nucleated cells (also gated on FSC/SSC plot). Examples of various levels of tumor infiltration in the BM are shown on Fig. 1.

RQ-PCR

Tumor positivity of a BM sample by RQ-PCR was defined as previously described (Druy et al. 2018). *PHOX2B* gene expression at any detectable level was considered as a sign of presence of NB cells. As an additional criterion *TH* gene expression was applied. To avoid the false positivity based on possible background presence of *TH* mRNA (Stutterheim et al. 2008; Druy et al. 2018) the threshold level of normalized *TH* expression was established. *ABL* was used as a housekeeping gene. The difference of the RQ-PCR threshold cycles of *ABL* and *TH* genes exceeding − 16.535 was considered as a criterion for BM sample positivity (Druy et al. 2018).

For all methods BM was scored positive if at least one site revealed neoplastic cells or expression of tumor-associated genes.

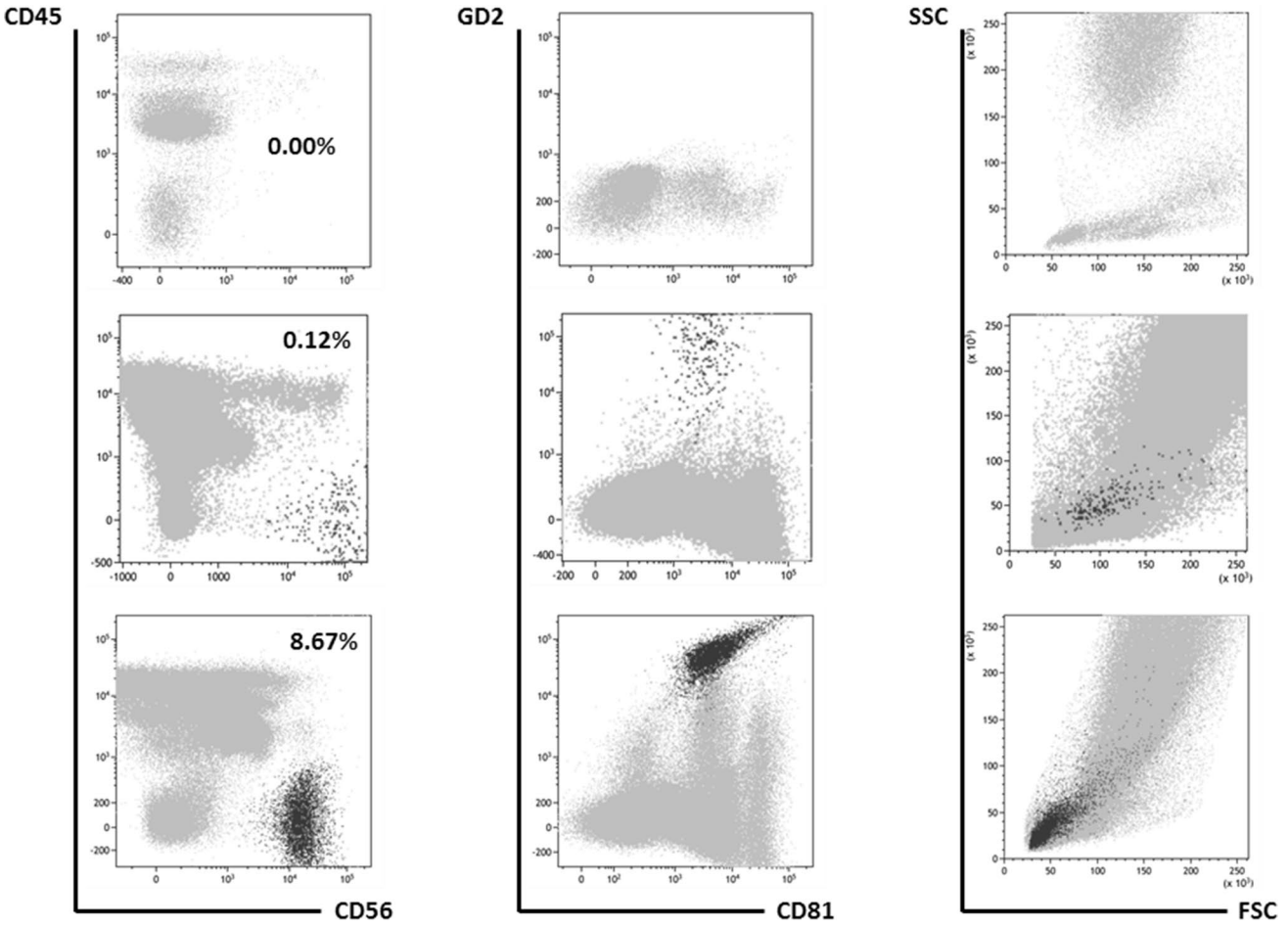

Fig. 1 Typical antigen-expression patterns and light-scatter properties of bone marrow cells of patients with neuroblastoma. NB cells are painted black while other BM cells are grey. The upper row represents MFC-negative case, medium row—MFC-positivity at the submicroscopic level, while in lower row a patient with abundant BM involvement is shown

Statistical analysis

Event-free survival (EFS) was defined as time from diagnosis to first event, i.e., refractory disease, progression, relapse, death from any cause, or secondary malignancy. Observation periods were censored at time of last contact when no events were reported. EFS curves were estimated with the Kaplan–Meier method and standard errors were calculated according to Greenwood. Differences in outcome between groups were compared with the log-rank test. Cumulative incidence of relapse/progression (CIRP) curves were estimated adjusting for competing risks of the other pertinent events and compared with Gray's test. Median of follow-up time was 6.17 years, ranging from 3.50 to 9.41 years. Analysis of prognostic correlation of MFC results in combination with other known risk factors with an unfavorable event (Hazard ratio) was studied using the COX model and the Wald test. All tests were two sided. Analysis was performed using XLSTAT-2016 and SPSS 23.0.

Results

Among 51 studied patients 28 (54.9%) were in complete continuous remission (CCR). Eighteen children (35.3%) suffered from a relapse or tumor progression, while five patients (9.8%) died without disease progression: three due to severe infections and two because of complications related to hematopoietic stem cell transplantation. Total 5-year EFS was 0.55 (0.07) and CIRP 0.35 (0.07).

Presence of tumor cells in BM was demonstrated by flow cytometry in 25 (49.0%) of 51 studied patients. Only 13 (25.5%) of them had BM disease detected by conventional cytology. Conversely, in 2 (3.9%) patients BM involvement was detected by cytomorphological examination only and not by flow cytometry. The incidence of tumor cells' detection in BM by flow cytometry was significantly higher than by cytology (49.0% and 29.4%, respectively, $p = 0.043$). Notably, among 28 patients with localized neuroblastoma (stages 1–3) 9 (32.1%) demonstrated BM disease detected by flow cytometry. The treatment outcome in 25 MFC-positive patients was significantly worse compared to MFC-negative group ($n = 26$): 5-year EFS 0.24 (0.08) and 0.85 (0.07), respectively, $p < 0.001$; CIRP 0.60 (0.10) and 0.12 (0.06) correspondingly, $p < 0.001$ (Fig. 2).

The correlation of adverse outcome and BM disease, detected by immunophenotyping, was seen both in the favorable and unfavorable groups of patients, defined by relevant clinical and biological criteria (Table 1).

Multivariate Cox regression model on the EFS was first performed for investigating the role of MFC-positivity on top of the most important single prognostic variables (Cotterill et al. 2000; Monclair et al. 2009): stage 4, age at the time of diagnosis above 12 months and *MYCN* gene amplification. In this analysis (Table 2, model 1), MFC data were the most important prognostic factor: positivity was related approximately to a sevenfold increase in the risk of treatment failure, as compared to patients with undetectable NB cells. When conventional risk factors were substituted by risk groups of NB2004 stratification scheme (Table 2, model II), MFC-positivity still retained its significance.

Interestingly, survival rates of patients with localized disease (stages 1–3) and BM involvement detected by flow cytometry ($n = 9$) did not differ significantly from those who had diagnosed metastatic neuroblastoma (stage 4, $n = 18$): 5-year EFS 0.44 (0.17) and 0.28 (0.11), respectively, $p = 0.624$.

In 42 children, BM was studied in parallel by MFC and RQ-PCR. In this group prognostic impact of MFC data remained significant. Treatment outcome in 25

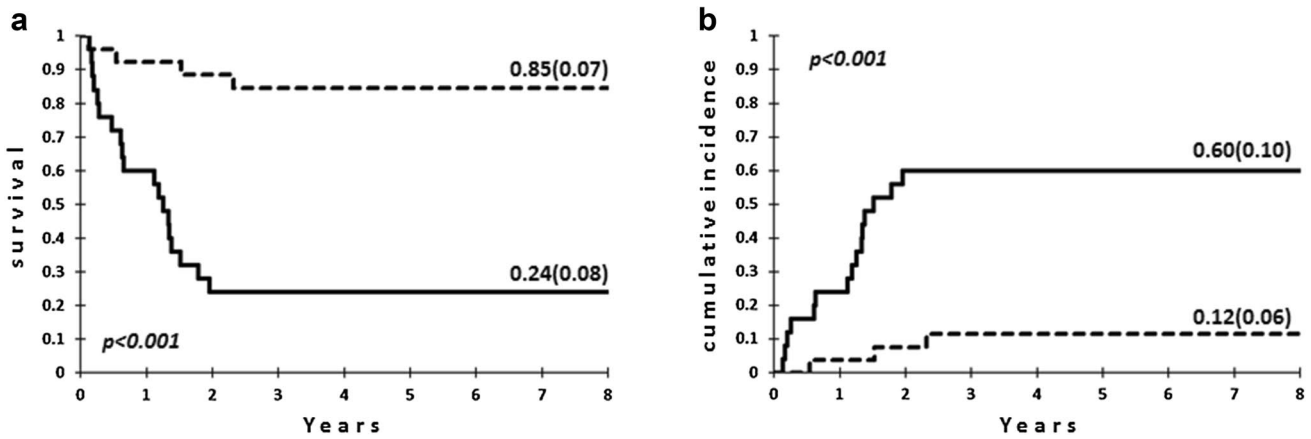

Fig. 2 Event-free survival (**a**) and cumulative incidence of relapse/progression (**b**) in 26 MFC-negative (dashed line) and 25 MFC-positive (solid line) patients with neuroblastoma

Table 1 Prognostic significance of BM involvement detected by flow cytometry in different groups of neuroblastoma patients

Results of flow cytometry	Number of patients	5-year event-free survival			Cumulative incidence of relapse or progression		
		EFS	SE	*p*	CIRP	SE	*p*
Entire cohort							
Negative	26	0.85	0.07	< 0.001	0.12	0.06	< 0.001
Positive	25	0.24	0.08		0.60	0.10	
Patients with *MYCN* single copy							
Negative	22	0.91	0.06	< 0.001	0.09	0.06	0.003
Positive	18	0.33	0.11		0.50	0.12	
Patients without BM lesion detected by CM							
Negative	24	0.83	0.08	0.006	0.12	0.07	0.011
Positive	12	0.42	0.14		0.50	0.14	
Patients below 12 months of age							
Negative	10	1.00	–	0.004	0.00	–	0.012
Positive	10	0.40	0.15		0.50	0.16	
Patients with localized neuroblastoma (stages 1–3)							
Negative	19	0.89	0.07	0.006	0.10	0.05	0.009
Positive	9	0.44	0.17		0.56	0.17	
Stage 4 patients							
Negative	6	0.67	0.19	0.029	0.17	0.18	0.060
Positive	12	0.08	0.08		0.67	0.14	
Observation group							
Negative	14	0.93	0.07	0.025	0.07	0.07	0.098
Positive	9	0.56	0.17		0.33	0.16	
High-risk group							
Negative	9	0.67	0.16	0.002	0.22	0.14	0.018
Positive	13	0.00	–		0.77	0.12	

Table 2 Impact of different prognostic factors on the EFS in Cox regression model

Covariate	Number of patients	Number of events	Model 1			Model 2		
			Hazard ratio	95% CI	*p*	Hazard ratio	95% CI	*p*
Results of BM immunophenotyping								
Negative	26	4	1		0.001	1		< 0.001
Positive	25	19	7.210	2.269–22.918		7.736	2.546–23.509	
Stage								
1–3, 4S	33	10	1		0.556	–	–	–
4	18	13	1.327	0.517–3.409		–	–	
Age at the time of diagnosis								
< 12 months	20	6	1		0.258	–	–	–
> 12 months	31	17	1.782	0.655–4.847		–	–	
MYCN status								
Single copy	40	14	1		0.044	–	–	–
Amplification	11	9	2.502	1.023–6.116		–	–	
Risk group								
Observation and medium-risk groups	29	7	–	–	–	1		0.004
High-risk group	22	16	–	–		3.818	1.518–9.604	

MFC-negative patients [5-year EFS 0.84(0.07) and CIRP 0.12(0.06)] was better than in 17 MFC-positive ones [5-year EFS 0.24(0.10) and CIRP 0.71(0.11), $p < 0.001$ for both comparisons]. 21 cases had no detectable BM involvement as measured by both techniques, while in 11 children concordant positive results were obtained. In 6 patients BM was MFC-positive but RQ-PCR negative, and in 4 cases BM disease was found only as *PHOX2B*/*TH* expression (Druy et al. 2018). Furthermore, results of flow cytometry allow distinguishing patients with different outcome in groups with positive and negative BM assessed by RQ-PCR (Fig. 3).

Discussion

In this prospective study we demonstrated the correlation of BM invasion detected by flow cytometry in diagnostic BM samples and outcome in a cohort of 51 patients with neuroblastoma. BM involvement is routinely detected by conventional cytological investigation of BM smears and histological examination of BM biopsies (Burchill et al. 2017; Cheung and Cheung 2001; Monclair et al. 2009). Both methods have limited analytical sensitivity and are very subjective (Cheung and Cheung 2001).

Our MFC results split patients into two almost equal groups. Those who have BM involvement detected by MFC had significantly worse outcome compared to patients with intact BM. Cumulative incidence of relapse or progression also was higher in patients harboring BM disease. Adverse prognostic significance of BM positivity by flow cytometry remained in favorable (patients with localized tumor, single copy of *MYCN* gene, younger 12 months of age or belonged to the observation group) as well as in unfavorable groups (patients with stage 4 and belonged to the high-risk group). Despite the absence of tumor cells in patient's BM as defined by conventional cytology, flow cytometry could reveal patients suffering from metastatic tumor which correspondingly had dismal outcome. Furthermore, among patients with MFC-positive BM, neither BM positivity by conventional cytology, nor presence of other metastatic sites

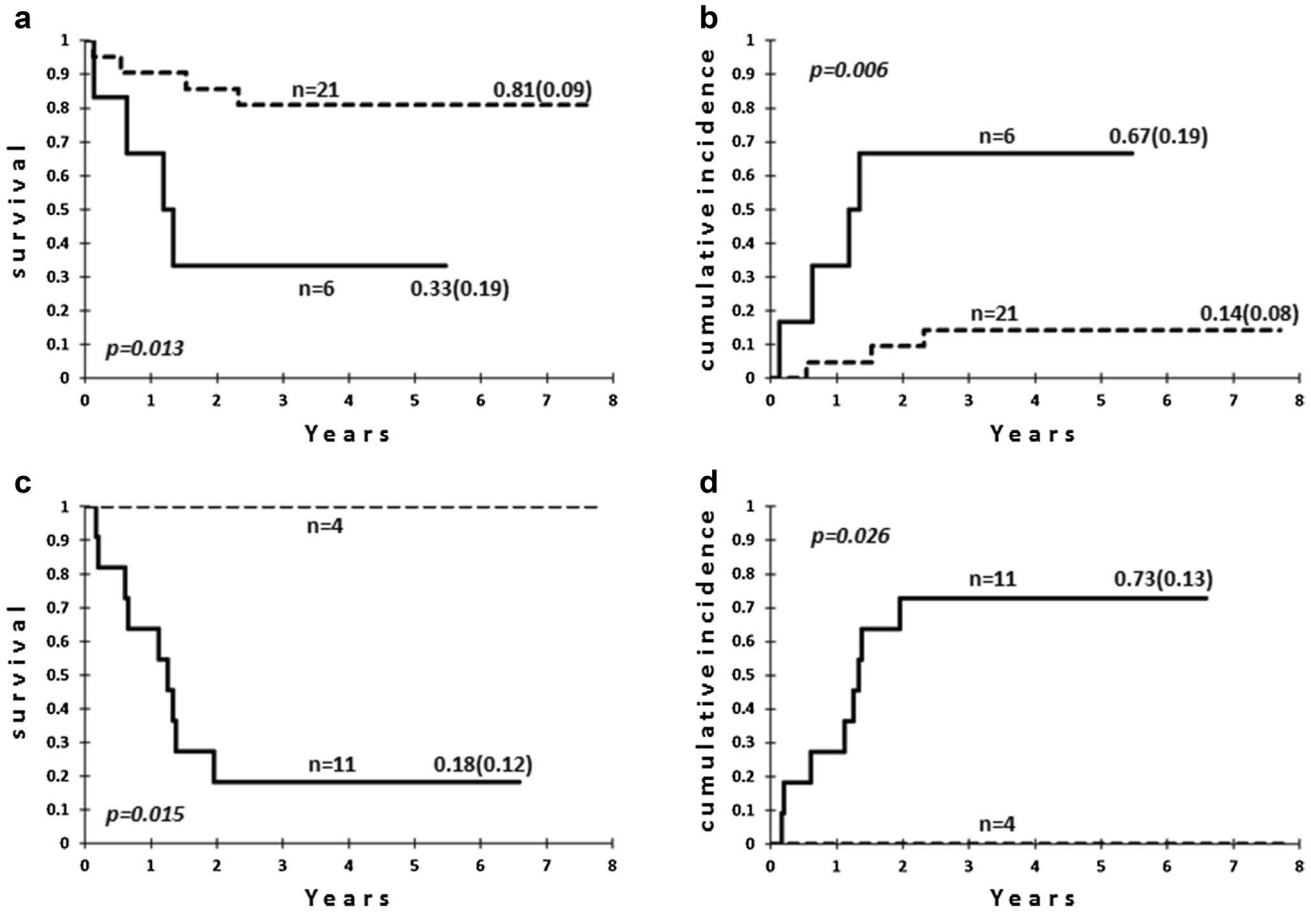

Fig. 3 Event-free survival (**a, c**) and cumulative incidence of relapse/progression (**b, d**) in MFC-negative (dashed line) and MFC-positive (solid line) patients with neuroblastoma. The upper row represents patients with no BM disease as assessed by RQ-PCR, while lower row—patients with positive RQ-PCR results

had an additional prognostic significance. This indicates that flow cytometry allows very precise distinguishing of patients with metastatic spread. Moreover, both of MFC-negative patients, who were defined to have BM disease according to cytomorphology, are present in complete continuous remission.

Moreover MFC-positivity has shown independent negative prognostic significance in multivariate analysis together with very powerful adverse prognostic factor—*MYCN* gene amplification. Even after model adjustment for high-risk group criteria of the NB2004 protocol stratification MFC data remained its independent prognostic value. This could be the most useful finding, as MFC-positive patients from observation and intermediate risk groups probably should be upstaged and treated adequately.

In past decades MFC techniques for BM micrometastases evaluation had developed from rather rough two-color assays (Ifversen et al. 2005; Warzynski et al. 2002) to very precise 4–5-color approaches (Swerts et al. 2004) using even NB-specific antibodies (Bozzi et al. 2006; Ifversen et al. 2005). Not further available in fluorochrome-conjugated format NB84 was substituted by anti-GD2 (Warzynski et al. 2005) and complemented with CD81, CD56, and CD9 (Swerts et al. 2004; Warzynski et al. 2002). The modern MFC technique could be successfully used both for initial BM metastases detection (Bozzi et al. 2006; Swerts et al. 2004) and for disease monitoring (Cai et al. 2012). In a meantime MFC is fast, relatively cheap, informative and reliable tool for NB staging. Nevertheless, MFC still has several disadvantages. The key weaknesses are limited sensitivity (Ifversen et al. 2005; Okcu et al. 2005; Tsang et al. 2003), subjective MFC data interpretation and lack of absolute NB-specific immunophenotypes (Bozzi et al. 2008; Ferreira-Facio et al. 2013; Shen et al. 2013).

Another, more sensitive technique for BM involvement evaluation is PCR-based detection of neuroblastoma molecular markers. Recent studies demonstrated high diagnostic test performance values for *PHOX2B, TH, DDC, CHRNA3*, and *GAP43* genes (Stutterheim et al. 2009). Real-time PCR with reverse transcription is characterized by high sensitivity, reproducibility, and labor and cost effectiveness (Burchill et al. 2017; Stutterheim et al. 2008). BM positivity for the molecular markers both at the time of initial diagnosis and during the treatment leads to decreased survival rates (Corrias et al. 2012; Druy et al. 2018; Stutterheim et al. 2008).

Published studies on direct comparison with ICC and RQ-PCR showed lower sensitivity of MFC (Ifversen et al. 2005; Okcu et al. 2005; Swerts et al. 2004; Tsang et al. 2003), although results concordance improved significantly when the 0.01% threshold of positivity was applied (Ifversen et al. 2005; Tsang et al. 2003). Unfortunately mentioned flaws of MFC became a serious obstacle for its implementation in clinical studies. Moreover, a very few data about prognostic significance of MFC BM investigation results are currently published (Bozzi et al. 2006). So, although MFC cannot achieve sensitivity comparable with ICC and RQ-PCR, it could be a reliable tool for NB cells detection if they represent at least 0.01% of all BM cells. Furthermore, in this study although in a small subset of patients, the clinical correlation with poor outcome of MFC-positivity in RQ-PCR-negative samples was demonstrated. Comparison of BM involvement detection results by MFC and by assessment of *PHOX2B*/*TH* genes expression by RQ-PCR revealed its incomplete concordance. Discordant specimens were both RQ-PCR-positive but MFC-negative and vice versa. Thus these discrepancies can not be explained mechanistically by superior analytical sensitivity of RQ-PCR. Probably, enlargement of the RQ-PCR markers panel will improve concordance rate (Stutterheim et al. 2009). Adverse prognostic significance of *PHOX2B*/*TH* transcripts presence in the BM of primary neuroblastoma patients is widely described (Corrias et al. 2012; Druy et al. 2018; Stutterheim et al. 2008; van Wezel et al. 2016). Interestingly, MFC results allowed distinguishing patients with good and poor outcome both in cases with and without BM disease detected by RQ-PCR. This fact indicates usefulness of the BM immunophenotyping in addition to more sensitive RQ-PCR.

Considering rather high analytical sensitivity of the MFC method, correspondent higher frequency of BM MFC-positivity, compared to conventional cytology, and independent prognostic value, immunophenotyping can be recognized as robust tool for BM disease detection. It could be implemented in clinical practice as an addition to other risk factors, both clinical and laboratory.

Conclusions

BM involvement detection at the time of initial diagnostics by MFC was identified as a useful and reliable tool for evaluation of high-risk patients with neuroblastoma. Those who harbored BM disease had significantly poor outcome even in cases of favorable clinical features of the tumor. BM positivity defined by flow cytometry demonstrated independent powerful prognostic significance in the multivariate analysis. Concordance of MFC and RQ-PCR results was not absolute while MFC was able to divide patients with unequal prognosis in both RQ-PCR-positive and -negative cases.

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Compliance with ethical standards

Conflict of interest Authors declare no relevant conflicts of interests.

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