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Flow cytometry as a diagnostic tool in neuroblastoma

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ABSTRACT

In recent years, there has been an expansion in the use of flow cytometry (FC) immunophenotyping in the diagnosis and monitoring of childhood solid neoplasms. Neuroblastoma (NB), in turn, is the most common extracranial solid tumor in childhood. In the present study, we sought to compare FC and anatomopathological examination (PA) / immunohistochemistry (IHC) of children diagnosed or suspected with NB. The median age was 59 months (minimum 0; maximum 325 months), of these 12 were male (57.1%, 12/21). Forty-eight samples (27 bone marrow (BM), 10 peripheral blood (PB), 8 primary tumors (PT) and 2 liver nodules (HN) and 1 rib fragment (RF)) from 21 patients were evaluated. Twenty-nine samples were from patients with clinical suspicion while 19 samples were from patients with previously confirmed diagnosis. Thirteen samples (7 BM, 5 PT and 1 HN) presented NB when analyzed in FC while 8 (3 BM and 5 PT) samples were positive for NB in the PA/IHC. They were concordant in 88.9% of the cases. No NB cells were identified in any PB. Considering the PA as the gold standard, the FC obtained a sensitivity of 100%, a specificity of 86%, a positive predictive value of 67% and a negative predictive value of 100%. This study demonstrates that FC can be used as a methodology for diagnosis and assessment of NB involvement. In addition, FC has the advantage of allowing a quick diagnosis and accurate classification of the disease, and can also assist in monitoring the treatment.

1. Introduction

Childhood tumors are diseases characterized by the abnormal growth of cells in a specific tissue (Gavhane et al., 2011; Jain et al., 2011). They can be divided into two main groups: leukemias/lymphomas and solid tumors. The latter represent about 30% of all pediatric cancers.The most common types are brain tumors, neuroblastoma (NB), rhabdomyosarcoma (RMS), Wilms' tumor (TW) and osteosarcoma (Kline and Sevier, 2003).

NB is the most common extracranial solid tumor in childhood, accounting for 8–10% of all neoplasms in this age group, corresponding to 25 to 50 cases per million individuals (Brodeur and Maris, 2006; Maris et al., 2007; Davis et al., 1987; Matthay et al., 2016). Its etiology is unknown, however, due to its higher incidence in infants, some authors suggest that preconceptional factors or gestational events may be relevant to their development (Chow et al., 2007; Cook et al., 2004; Harder et al., 2010; Kramer et al., 1987).

The International Neuroblastoma Staging System (INSS) is the most accepted and currently used NB staging and risk classification system. It is based on the extent of the disease at the time of diagnosis, surgical excision and the presence of metastases (Matthay et al., 2016).

Although several genetic alterations have been observed in these neoplasms, including chromosomal alterations, polymorphism and genetic amplifications, there is, until today, no pathognomonic alteration for diagnosis (Matthay et al., 2016). With the data currently available in the literature, it is known that amplification of the MYCN gene and DNA ploidy have important prognostic implications in this pathology (Smith et al., 2010).

The origin of NB occurs in the cells of the neural crest and usually develops from the adrenal medulla, and can also occur in sympathetic ganglia (Pugh et al., 2013). In 70% of cases, the tumor is located in the abdomen (25% in the sympathetic ganglion and 40% in the adrenal medulla (Maris et al., 2007), 15% in the chest, 5% in the cervical region and 5% in the pelvic sympathetic ganglion) (Alexander, 2000; Schulte and Eggert, 2015). Approximately half of them have localized or regional disease (Park et al., 2010), while the other 50% already have

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distant metastases through lymphatic or hematogenous dissemination at the time of diagnosis (Gesundheit et al., 2004; Morandi et al., 2015). The extension of the disease has prognostic implications since both the cure rate and the survival rate are greater than 90% in patients with low and intermediate risks and localized tumors (Newman et al., 2019), while in high-risk cases, these rates drop to less than 50% (Cheung et al., 2001; Cohn et al., 2008).

Another factor that influences the prognosis is ploidy. Neuroblastomas with hyperdiploid cells tend to be associated with the early stages of the disease, respond better to chemotherapy and usually have a more favorable prognosis than diploid cells (Cohn et al., 2008).

Within this context, the use of laboratory methods such as Flow Cytometry (FC), can be useful both in the diagnosis and in the risk stratification of NB, through the phenotypic detection of tumor cells, in the analysis of the DNA index, in the evaluation of the dissemination of the disease in peripheral blood (PB) and bone marrow (BM), as well as in the monitoring of treatment through the study of Measurable Residual Disease (MRD) (Komada et al., 1998; Manrique et al., 2016). In addition to the applications already mentioned for FC, we can consider as its main advantages the diagnostic agility and sensitivity provided by the multiparametric method.

The aim of this study was to use FC as a tool in the evaluation of different specimens of patients with suspected/diagnosed NB and to compare the results found with anatomopathological examination (PA) / immunohistochemistry (IHC), a laboratory method considered the gold standard for the diagnosis of this pathology (Mukherjee et al., 2020; Burchill et al., 2017). We also performed the analysis of the DNA ploidy index by FC.

2. Materials and methods

2.1. Patients and samples

Twenty-one pediatric patients with suspected/diagnosed NB from 3 reference hospitals in Porto Alegre, Brazil, from May 2019 to August 2020 were studied. INSS was used for staging (Table 1) (Brodeur et al., 1993). Forty-eight samples were evaluated, 27 of which were bone marrow, 10 of peripheral blood, 8 of primary tumors (PT), 2 of hepatic nodules (HN) and 1 of rib fragment (RF). Twenty-nine samples were patients with suspected and 19 samples were patients already had a previously confirmed diagnosis.

2.2. Ethical aspects

The study was approved by the ethics committee of the proposing institution, with secondary approvals from the ethics committees of all participating centers (CAAE: 89566018.8.0000.5327; 89566018.8.3001.5330; 89566018.8.3003.5530). All those responsible for the patients signed the Informed consent form (ICF).

The medical records of patients were reviewed for demographic data (sex, date of birth), presentation at diagnosis (date of diagnosis, amplification status of the MYCN gene, DNA ploidy index, histological and staging according to INSS (International Neuroblastoma Staging System).

2.3. Flow cytometry

2.3.1. Collection of samples of tissues and body fluids

The BM and PB samples were collected in tubes with EDTA anticoagulant, while PT, HN and RF were stored in a sterile flask with RPMI (Roswell Park Memorial Institute) to maintain the integrity of the material cells. The samples were transported under refrigeration at 2 to 8 °C and immediately processed when they arrived at the laboratory.

2.3.2. Sample preparation

The preparation and marking of the samples followed the protocols of the EuroFlow consortium (https://euroflow.org/protocols) (*EuroFlow Standard Operating Procedure (SOP) for Sample Preparation and Staining; Version 1.5*, n.d.).

Table 1
The international neuroblastoma staging system.

Stage/Prognostic Group	Description
Stage 1	Localized tumor with complete gross excision, with or without microscopic residual disease. Representative ipsilateral lymph nodes negative for tumor microscopically (i.e., nodes attached to and removed with the primary tumor may be positive).
Stage 2A	Localized tumor with incomplete gross excision; representative ipsilateral nonadherent lymph nodes negative for tumor microscopically.
Stage 2B	Localized tumor with or without complete gross excision, with ipsilateral nonadherent lymph nodes positive for tumor. Enlarged contralateral lymph nodes must be negative microscopically.
Stage 3	Unresectable unilateral tumor infiltrating across the midline, with or without regional lymph node involvement; or localized unilateral tumor with contralateral regional lymph node involvement; or midline tumor with bilateral extension by infiltration (unresectable) or by lymph node involvement. The midline is defined as the vertebral column. Tumors originating on one side and crossing the midline must infiltrate to or beyond the opposite side of the vertebral column.
Stage 4	Any primary tumor with dissemination to distant lymph nodes, bone, bone marrow, liver, skin, and/or other organs, except as defined for stage 4S.
Stage 4S	Localized primary tumor, as defined for stage 1, 2A, or 2B, with dissemination limited to skin, liver, and/or bone marrow (by definition limited to infants younger than 12 months). Marrow involvement should be minimal (i.e., <10% of total nucleated cells identified as malignant by bone biopsy or by bone marrow aspirate). More extensive bone marrow involvement would be considered stage 4 disease. The results of the MIBG scan, if performed, should be negative for disease in the bone marrow.

Source: Brodeur GM, Pritchard J, Berthold F, et al.: Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. J Clin Oncol 11 (8): 1466–77, 1993. 26.

The antibodies used in the immunophenotypic panel for the characterization of NB were adapted from the works by Facio et al. 2013 and Theodorakos et al. 2019. This panel consists of: CD9 Pacific Blue (clone MEM-61, EXBIO), CD45 Pacific Orange (clone HI30, Invitrogen), CD73 PE (clone AD-2, BD - Becton Dickinson), GD2 PerCP-CY5.5 (clone 14. G2a, BD), CD56 PE-CY7 (clone N901, Beckman Coulter), CD90 APC (clone 5E.10, BD) and CD81 APC-H7 (clone JS-81, BD) (Ferreira-facio et al., 2013; Theodorakos et al., 2019).

2.3.3. DNA Ploidy

For the analysis of the DNA index, the CYCLOSCOPE-REAGENT KIT (Cytognos SL, Salamanca, Spain) and the monoclonal antibody CD56 FITC (clone N-CAM, BD) were used.

2.3.4. Acquisition of samples

Immediately at the end of sample preparation, all materials were cell count at low speed using the FACSCanto II Cytometer (BD, San Jose, California, USA).

2.3.5. Analyses of samples

A minimum of 1,000,000 and maximum of 2,000,000 cells were analyzed in each sample.

A minimum of 10 cells with NB phenotype was considered a positive sample (lower limit of detection, LOD) and a minimum of 40 cells for quantification (lower limit of quantification, LLOQ).

Data analysis was performed using Infinicyt™ software (Cytognos SL, Salamanca, Spain) and the NB cells were identified by the immunophenotype: $CD56^{+}/CD81^{+}/CD9^{+}/CD90^{+}/GD2^{+}/CD73^{-}/CD45^{-}$

(Ferreira-facio et al., 2013; Theodorakos et al., 2019), the minimum sensitivity considered was 50 events.

2.4. Statistical analysis

The statistical analyzes were performed using the Statistical Package for the Social Science software version 21.0 (SPSS, Chicago, IL, USA). Central tendency, dispersion and distribution measures were used, depending on the distribution and normality. Data analysis involved Pearson's chi-squared test, P values <0.05 was considered statistically significant.

3. Results

Patients presented a median age of 59 months (minimum 0; maximum 325 months) and 57.1% were male.

Two patients had MYCN amplification. Regarding the INSS staging, 5 patients were clinically classified in stage 3 and 6 patients in stage 4. Of these, none underwent changes regarding staging due to the result of FC.

Of the 48 samples analyzed, 13 samples were positive for NB by FC (Fig. 1) (7 in BM, 5 in PT and 1 in HN) and 8 were positive for PA/IHC (Fig. 2) (3 in BM and 5 in PT).

One sample of BM that was positive in FC was not analyzed by PA/IHC (Table 2). All FC samples for assessing the spread of NB in PB were negative. The methods were consistent in 88.9% of the cases. Considering the PA/IHC as the gold standard, FC obtained a sensitivity of 100%, a specificity of 86%, a positive predictive value of 67% and a negative predictive value of 100%.

Regarding the comparison of results release time between the two methodologies, the median in FC was 30 h and 19 min while the PA / IHC was 94 h and 49 min ($P < 0.05$).

In 4 of the 5 PT samples positive for NB, the analysis of the DNA index was performed which was 0.93, 0.94, 1.03 and 1.04 (almost diploid samples) (Bourhis et al., 1991).

4. Discussion

Pediatric cancers may have similar morphological and histopathological characteristics because they are mainly derived from early

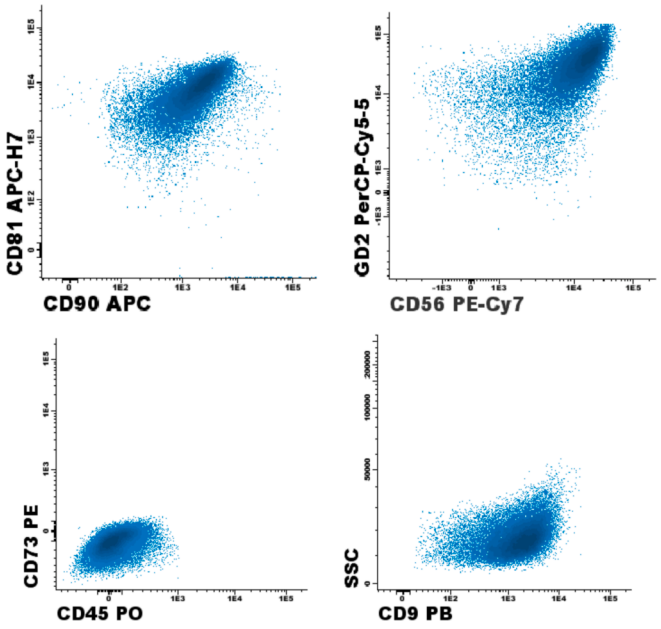

Fig. 1. Dot plot diagrams illustrating the neuroblastoma population in a bone marrow sample by flow cytometry.

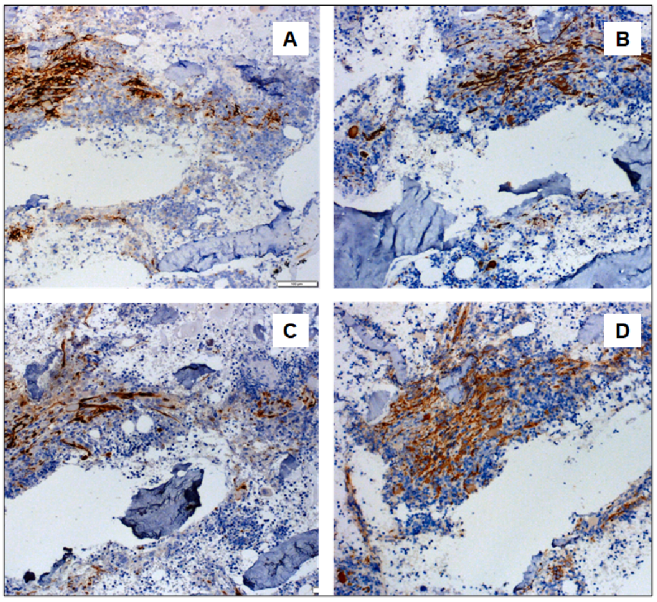

Fig. 2. Positive immunohistochemistry to neuroblastoma. A) Chromogranin. B) Neurofilament. C) S100. D) Synaptophysin.

lymphoid precursors and embryonic mesenchymal and neuroectodermal precursors. Thus, the diagnosis of most of these tumors often requires additional characterization of the neoplastic cells. For these reasons, IHC, FC, molecular biology tests, among other methodologies are extremely relevant (Ferreira-facio et al., 2013; Rushton and López-Terrada, 2010; Triche et al., 2010; Dehner, 1998).

The need for a quick result is crucial in the diagnosis because it influences the patient's treatment protocol for an early start of treatment, with the possibility of a better therapeutic response (Ferreira-facio et al., 2013; Triche et al., 2010; Dang-Tan and Franco, 2007). In this context, the availability of fast and sensitive techniques to accurately track the tumor cell line and establish the relevant differential diagnoses are essential.

In the NB, the investigation of the dissemination of the disease is part of the initial staging, allocation of treatment therapy and reassessment of the disease (Dehner, 1998; Dang-Tan and Franco, 2007).

The panel of antibodies used for our work included immunophenotypic markers characteristic of NB based on published studies (Ferreira-facio et al., 2013; Theodorakos et al., 2019). We add CD73, because mesenchymal cells are immunophenotypically similar to those of NB. The CD73 and CD13 antibodies are the most discriminative because they are highly positive for mesenchymal cells. The proper classification of populations is very important for an accurate classification of NB, especially in MRD (Theodorakos et al., 2019).

Although the sample size of our study is small, the data agree with what is previously described in the literature, where the disease affects mainly infants and children up to 10 years of age, with the majority being diagnosed before 5 years of age with a predominance in males (Lucena et al., 2018; Bom et al., 2014; Cartum, 2011).

In relation to one of the genetic alterations related to NB and mentioned in our research, the MYCN gene, two patients evaluated presented this amplification, both classified in stage 4 of the INSS, that is, of high risk. It is known that the amplification of this gene is related to the degree of aggressiveness of this disease (Brodeur et al., 1984; Morandi et al., 2017).

In 4 samples, 3 BM (0.01; 0.02; 0.27%) and 1 HN (4%), the FC was positive while the PA/IHC was negative. Such data are consistent with what is described in other studies. Brahmi et al. in 2001, they reported that FC is more sensitive, objective and a quantitative methodology when compared to IHC (Brahmi et al., 2001). In 2019, Popov et al. also

Table 2
Characteristics and results of samples obtained by flow cytometry and anatomopathological examination/ immunohistochemistry.

Age (months)	Staging INSS	Type sample	Result FC (%) for NB	Result PA/IHC for NB
52	4	PB	Negative	NA
52	4	BM	8.7	Present
52	4	BM	Negative	Absent
53	4	BM	Negative	Absent
63	4	BM	0.02	Absent
63	4	PB	Negative	NA
16	Investigation	PB	Negative	NA
16	Investigation	BM	Negative	Absent
15	Investigation	BM	0.044	Not done
124	Investigation	BM	Negative	Absent
124	Investigation	BM	Negative	Absent
124	Investigation	HN	Negative	Absent
95	Investigation	BM	Negative	Absent
95	Investigation	BM	Negative	Absent
44	4	PT	52.7	Present
44	4	PB	Negative	NA
50	4	BM	Negative	Absent
50	4	PT	99.0	Present
50	4	PB	Negative	NA
325	Investigation	BM	Negative	Absent
3	Investigation	BM	0.16	Present
3	3	PB	Negative	NA
6	3	PT	75.0	Present
53	Investigation	BM	Negative	Absent
53	Investigation	TP	Negative	Absent
9	3	SP	Negative	NA
9	3	BM	Negative	Absent
9	3	BM	Negative	Absent
209	Investigation	HN	4.0	Absent
1	3	RF	Negative	Absent
65	Investigation	PT	Negative	Absent
65	Investigation	PB	Negative	NA
65	Investigation	BM	Negative	Absent
18	4	PT	16.2	Present
18	4	PB	Negative	NA
18	4	BM	Negative	Absent
18	4	BM	Negative	Absent
165	Investigation	PT	Negative	Absent
165	Investigation	BM	Negative	Absent
165	Investigation	BM	Negative	Absent
89	4	BM	0.01	Absent
51	4	BM	0.0026	Present
6	3	BM	Negative	Absent
0	Investigation	PB	Negative	NA
0	Investigation	BM	Negative	Absent
66	4	BM	0.27	Absent
17	3	PT	62.7	Present
17	3	BM	Negative	Absent

N/A: Not applicable
Discordant resul between methodologies
PA/IHC not done in BM
Concordant resul between methodologies

described a higher sensitivity in FC compared to IHC (Popov et al., 2019), such data are similar to those found in our research. Szántho et al. in 2018, reported that even in hypoplastic/aplastic environments, FC is more effective when compared to IHC, since considerably more cells can be analyzed (Szánthó et al., 2018).

5. Conclusion

Through this study, we can confirm that FC is an applicable, fast and safe methodology in the diagnosis and monitoring of children with neuroblastoma, and can be used in addition to traditional methods such as PA/IHC to increase sensitivity and diagnostic accuracy.

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