J Bras Patol Med Lab. 2021; 57: 1-9.

10.5935/1676-2444.20210042

Flow cytometry as a diagnostic tool in childhood solid tumors

Citometria de fluxo como ferramenta diagnóstica em tumores sólidos da infância

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ABSTRACT

Childhood solid tumors represent about 30% of all pediatric cancers. In recent years, there has been some expansion in the use of flow cytometry (FC) in the diagnosis and monitoring of these diseases, since it is a method that allows for rapid and accurate results, enabling earlier conduct. We performed a literature search for a systematic review of the following terms in the Lilacs, PubMed, and Scielo data platforms: neoplasm, oncology, pediatrics, immunophenotyping, and flow cytometry. Thus, we describe the main findings to date on the use of FC in the differential diagnosis of the five main small round blue cell tumors of childhood: neuroblastoma, Ewing sarcoma, primitive neuroectodermal tumor, Wilms tumor, and rhabdomyosarcoma. In addition, we describe the main advantages and disadvantages of the method and panels that are proposed in the differential diagnosis of these pathologies through the international literature. Through this review, we observed that the use of FC in the diagnosis of solid tumors can be useful for rapid and accurate identification of the disease, as well as for the early initiation of treatment.

Key words: neoplasm; oncology; pediatrics; immunophenotyping; flow cytometry.

RESUMO

Os tumores sólidos da infância representam cerca de 30% de todos os cânceres pediátricos. Nos últimos anos, houve uma expansão no uso da citometria de fluxo (CF) no diagnóstico e no acompanhamento dessas patologias, já que se trata de um método que permite a obtenção rápida e precisa de resultados, possibilitando uma conduta mais precoce. Realizamos esta revisão da literatura com uma pesquisa dos seguintes termos nas plataformas de dados Lilacs, PubMed e Scielo: neoplasia, oncologia, pediatria, imunofenotipagem e citometria de fluxo. Dessa forma, descrevemos os principais achados até o momento sobre o uso da CF no diagnóstico diferencial das cinco principais neoplasias de pequenas células azuis da infância: neuroblastoma, sarcoma de Ewing, tumor neuroectodérmico primitivo, tumor de Wilms e rabdomiossarcoma. Além disso, discutimos as principais vantagens e os inconvenientes do método e dos painéis que são propostos no diagnóstico diferencial dessas patologias por meio da literatura internacional. Observamos por meio desta revisão que a utilização da CF no diagnóstico de tumores sólidos pode ser útil para uma rápida e precisa identificação da patologia, bem como para o início precoce do tratamento.

Unitermos: *neoplasia; oncologia; pediatria; imunofenotipagem; citometria de fluxo.*

First submission on 04/22/20; last submission on 04/29/20; accepted for publication on 05/05/20; published on 10/20/21

RESUMEN

Los tumores sólidos infantiles representan aproximadamente el 30% de todos los cánceres pediátricos. En los últimos años se ha incrementado el uso de la citometría de flujo (CF) en el diagnóstico y seguimiento de estas patologías, ya que es un método que permite obtener resultados rápidos y precisos, posibilitando un manejo más precoz. Realizamos esta revisión sistemática para la búsqueda bibliográfica de los siguientes términos en las plataformas de datos Lilacs, PubMed y Scielo: neoplasma, oncología, pediatría, inmunofenotipificación y citometría de flujo. Así, describimos los principales hallazgos hasta la fecha sobre el uso de CF en el diagnóstico diferencial de los cinco principales tumores de células pequeñas, redondas y azules de la infancia: neuroblastoma, sarcoma de Ewing, tumor neuroectodérmico primitivo, tumor de Wilms y rabdomiosarcoma. Además, describimos las principales ventajas y desventajas del método y paneles que se proponen en el diagnóstico diferencial de estas patologías a través de la literatura internacional. A través de esta revisión, observamos que el uso de CF en el diagnóstico de tumores sólidos puede ser útil para la identificación rápida y precisa de la efermedade, así como para el inicio más temprano del tratamiento.

Palabras clave: *neoplasia; oncología; pediatría; inmunofenotipificación; citometría de flujo.*

INTRODUCTION

Childhood solid tumors are diseases characterized by the abnormal growth of cells in a specific tissue, except those derived from hematopoietic tissue, i.e., leukemias, and lymphomas[1, 2]. These neoplasms represent about 30% of all pediatric cancers; the most common types are brain tumors, neuroblastoma (NB), rhabdomyosarcoma (RMS), Wilms tumor (WT), and osteosarcoma[3].

Clinical manifestations vary according to the histological type of tumor, the primary location, and the patient's age[4]. The diagnosis is made by the correlation between clinical data and the results of laboratory tests, especially the anatomopathological one (histochemical and cytological analysis) which, due to the methodology used, many days can spend between the suspicion and the definition of the diagnosis. This time has implications for the patient and their family, in addition to impacting the cost due to the possible length of stay and prognosis, as it delays is start of treatment[5, 6].

Among solid tumors, there is a subgroup called small round blue cell tumor. This subgroup receives this definition for being composed of primitive cells, therefore, cells that do not yet differentiate and acquire a bluish color when analyzed by the pathologist[7]. Due to its similar appearance between diseases, it needs a thorough analysis of markers for a correct diagnosis. In this case, a careful immunohistochemical study often leads to a long time between tissue biopsy and definitive diagnosis, thus delaying in start of treatment in these children. New methods that allow quick diagnosis without losing the sensitivity and specificity of currently available techniques meet the needs and clinical demands of the moment. Within this context, flow cytometry (FC) is an important tool to aid in a faster and more accurate diagnosis, especially in centers that do not have cytogenetic studies. For this reason, in this review, we will address the use of FC, as well as the use of immunophenotyping in the diagnosis of the five main small round blue cell tumors of childhood: NB, Ewing sarcoma (ES), primitive neuroectodermal tumor (PNET), WT, and RMS.

NB

NB is the most common extracranial solid tumor in childhood; it is responsible for 8%-10% of all neoplasms in this age group[8-10]. This disease corresponds to 25 to 50 cases per million individuals[11]. Its etiology is little-known, however, due to its higher incidence in infants, some authors suggest that preconception factors or gestational events (e.g., gestational diabetes, folic acid deficiency, exposure to drugs, hormones, toxins, or viruses) may have relevance in its development[12-15]. Although several genetic alterations have been observed in these neoplasms, including chromosomal alterations, polymorphism, and genetic amplification, currently, there is no diagnostic pathognomonic alteration[11].

NB originates from neural crest cells and generally develops from the adrenal medulla, and may also occur in sympathetic ganglia[16]. In 70% of cases, the tumor is located in the abdomen (25% in the sympathetic ganglion and 40% in the adrenal medulla[9], 15% in the chest, 5% in the cervical region, and 5% in the pelvic sympathetic ganglion)[17, 18]. Approximately half of them presents localized or regional disease[19], while the other 50%

presents distant metastasis through lymphatic or hematogenous dissemination at the time of diagnosis[20, 21].

Treatment is variable and dependent on several factors that contribute to the classification of the patient's risk group. Most international protocols use the following criteria in defining risk: age at diagnosis, *N-MYC* gene amplification status, deoxyribonucleic acid (DNA) ploidy, histology, and international neuroblastoma staging system (INSS), in addition to genetic abnormalities, which allows classifying the patient as low, intermediate, and high risk[22, 23]. It is also important to assess the elevation of vanillylmandelic acid and homovanillic acid – which can be detected in 90% of individuals with NB[11] –, as well as serum ferritin and lactate dehydrogenase (LDH), which may indicate a worse prognosis[24].

From a molecular point of view, candidate gene sequencing identified mutations in the anaplastic lymphoma kinase (*ALK*) in more than 50% of familial cases, and in 5%-15% of sporadic NB cases[25]. *ALK* is an established oncogenic expander for NB that generates uncontrolled cell proliferation and cell survival properties[24].

Regarding the data currently available in the literature, it is known that amplification of the *N-MYC* gene and DNA ploidy has the most important prognostic implications for the disease[24]. Both the cure rate and the survival rate are above 90%[26] in patients with low and intermediate-risk and localized tumors; in high-risk cases, these rates drop to 40%-50%[27, 28].

From this background, FC can be useful for NB, both in diagnosis, through the analysis of cell surface markers, and in risk stratification, through the evaluation of the DNA index.

NB DETECTION BY FC

Recently, there has been a considerable increase in the use of FC because it is a method that allows for timely and accurate diagnosis of neoplasms, enabling early start of treatment. FC immunophenotyping can be used in the diagnosis of NB and the assessment of the spread of the disease in the peripheral blood (PB) and bone marrow (BM), as well as in the follow-up of treatment through the investigation of minimal residual disease (MRD)[29, 30].

In recent decades, several researchers have tested different combinations of monoclonal antibodies to obtain greater specificity in tumor cells. In 1998, Komada *et al*. (1998)[29] used a combination of CD9/CD56/CD45 to detect residual NB cells in BM and PB. In this study, simultaneous analyzes were performed with different fluorochromes and showed a distinct cell population with the $CD9^+/CD56^+/CD45^-$ phenotype, suggesting the presence of metastatic NB cells. In 2000, Nagai *et al.* (2000)[31] concluded that the combination of $CD81^+/CD56^+/CD45^-$ was more sensitive and specific for detecting MRD for NB. The authors compared the reactivity of CD81 with that of CD9 and observed greater sensitivity of CD81, possibly due to interference of CD9 in platelet labeling.

In 2002, Warzynski *et al.* (2002)[32] used the previously described markers to identify NB cells and studied two new markers: the membrane protein disialoganglioside (GD2) and the intracytoplasmic neuron-specific enolase (NSE) enzyme. With this research, they concluded that NB cells are $CD45^-/CD56^{+strong}/GD2^+/NSE^+$. Therefore, today we can use a combination of immunophenotypic markers with a panel of at least six colors to characterize this disease. For this purpose, monoclonal antibodies conjugated to fluorochromes are used, which allow the identification of the NB antigenic expression profile. The cell population that presents positivity for the markers $CD56^{strong}$, CD81, CD9, CD90, GD2, and negativity for CD45 is specific for NB, as shown in **Figure 1**[31, 33].

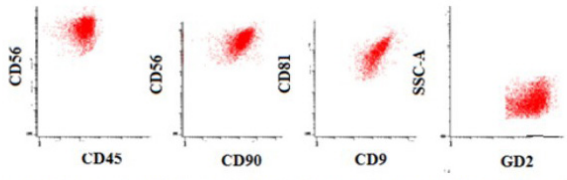

FIGURE 1 – *Dot plot diagrams illustrating the neuroblastoma population, whose phenotype is represented by* $CD45^-/CD56^{+strong}/CD90^+/CD81^+/CD9^+/GD2^+$

Source: Ferreira-Facio et al. *(2013)*[33].

EVALUATION OF NB PLOIDY INDEX BY FC

Ploidy is an important and useful factor for NB patient risk classification and prognosis. This analysis can be established using FC and cytogenetic methods, such as fluorescent *in situ* hybridization (FISH) or microarray[34, 35].

Changes in tumor cell ploidy are the result of an alteration in mitotic function, related to the rate of cell proliferation. They are a prognostic indicator in several types of tumors[36]. When tumors have a DNA index ≤ 1 hypodiploid case, the results are worse than in hyperdiploid cases, where the DNA index is ≥ 1.16[36, 37].

In 1984, Look *et al.* (1984)[36] reported that higher levels of DNA were associated with a better therapeutic response in infants with unresectable tumors. From the analysis of numerous series of patients, we found that children under 1 year of age with NB have a more favorable prognosis compared to older patients. However, the treatment of infants with NB, when hypodiploid, presents a greater chance of early therapeutic failure[38]. A favorable prognostic result is associated with aneuploidy in the stem cell lineage and a low percentage of tumor cells in the S, G2, and M phases of the cell cycle[39]. The influence of ploidy on prognosis seems to disappear after 2 years of age[40].

In 1987, Kaneko *et al.* (1987)[41] showed an association between triploid tumors and a favorable prognosis, while diploid and tetraploid tumors were associated with more advanced stages. Therefore, patients with hyperdiploidy or triploidy usually present low-grade tumors and better therapeutic response, especially if there is no amplification of the *N-MYC* gene[38, 40, 42, 43]. In more advanced stage tumors, diploidy (44-57 chromosomes) and hypotetraplody (81-103 chromosomes) are common[44]. Hyperdiploid neoplasms are more likely to have more apoptosis during anticancer therapy[40].

CF DNA content analysis is widely used to reveal ploidy and estimate cell proliferation through cell cycle distribution in normal and tumor cell populations[45].

For NB identification and DNA analysis, a monoclonal antibody is used and conjugated with a fluorochrome that allows the identification of neoplastic cells present in the sample, in addition to a DNA-binding dye, called propidium iodide (PI), which allows to carry out a DNA content analysis of neoplastic cells, based on the DNA content of normal diploid cells[46].

Comparison of the relative DNA content at the peak of the G0/G1 phases of tumor cells with the content of normal cells allows detection of aneuploidies. The DNA index is calculated by dividing the mean fluorescence intensity (MFI) value of the PI of the tumor population by the MFI of the PI of the reference population (normal cells)[47].

NB HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL DIAGNOSIS COMPARED TO FC

In the histopathological diagnosis, most of these tumors belong to the group "small round and blue cell tumors", as they are characterized by undifferentiated, small, and round cells, usually morphologically difficult to diagnose[48, 49].

In 1999, the International Neuroblastoma Pathology Classification (INPC), based on the first Shimada classification in 1984, added some information related to tumor histology. NB is divided into three subtypes: undifferentiated, poorly differentiated, and differentiated[50]. Conventional histopathological examination, associated with the immunohistochemistry technique, is the gold standard for the diagnosis of NB[33].

The use of FC is a great advance for the diagnosis of solid tumors. In the work by Ferreira-Facio *et al.* (2013)[33], the analysis of NB by FC and immunohistochemistry were in agreement in 100% of the diagnoses.

In 2018, Szánthó *et al.* (2018)[51] carried out a study whose objective was to compare the diagnostic efficacy between FC and morphology/immunohistochemistry in the detection of disseminated tumor cells in BM, as well as the body fluids of patients with solid tumors. Thirty-six samples were analyzed from 16 patients suspected or diagnosed with NB, performed at diagnosis or during treatment follow-up. The agreement between the two methodologies was 65% for the presence of disease. The authors found that detection of disseminated tumor cells is more effective in FC than in immunohistochemistry (100% vs. 86%, respectively). The FC advantage was even more pronounced when they evaluated MRD; the effectiveness was 92% vs. 68%, respectively. Furthermore, another advantage of FC is that it can analyze, even in hypoplastic samples, more cells of a material.

Therefore, FC allows diagnosis agility and sensitivity of NB staging, with more appropriate and early treatment initiation.

ES AND PNET

ES is the tumor whose cells are undifferentiated, and PNET is the disease with cells that present neural differentiation[49, 52, 53]. ES is usually located in long bones and the pelvis; rarely originates in non-osseous tissues; it is uncommon in the spinal epidural space[54]. It is estimated that the ES incidence is approximately 6% to 10% of primary malignant bone tumors, and, therefore, the fourth most frequent tumor in this group of lesions[55]. PNET represents 4% of soft tissue tumors[56].

The differential diagnosis between ES and another PNET is based on patient's history, physical examination, results of imaging tests, in addition to histopathological analysis[53, 57].

FC has been studied more recently as a diagnostic method due to its potential advantages in terms of speed and sensitivity.

IMMUNOPHENOTYPIC PROFILE OF ES AND PNET

Gardner *et al.* (1998)[58] identified the expression of CD56/CD57 in two cases of PNET, suggesting that the expression of CD56, together with that of CD99, in the absence of CD45, could be highly suggestive of PNET.

In 2003, Chang *et al.*[59] reported a case of positive ES for CD56, CD99, CD90, and CD117 by FC. Dubois *et al.*[60], in 2010, described the finding of $CD99^{+}/CD45^{-}$ cells in PB and BM samples from patients with ES. In that same study, they published the use of CD99, CD45, CD14, and CD34. CD14 was used to exclude monocytes and CD34 was used for hematopoietic progenitor cells. This measure made this strategy ideal for detecting ES MRD.

In 2013, Ferreira-Facio *et al.*[33] reported that GD2 and CD271 were the two most useful markers to differentiate NB ($GD2^{strong}$ and $CD271^{negative/weak}$) from other PNET ($GD2^{negative/weak}$ and $CD271^{strong}$). These results support the hypothesis that the strong expression of CD271 observed in PNETs may be associated with the origin of mesenchymal stem cells from these tumors.

The studies mentioned show the potential of using these markers in FC in the diagnosis and differentiation of these neoplasms[61].

WT

WT, also known as nephroblastoma, it is the most common primary renal tumor in childhood; corresponds to 6% of pediatric cancer cases. The average age at diagnosis is 3-5 years[62]. It is estimated that, in Europe, every year, 1000 new patients are diagnosed with this disease[63].

Diagnosis is performed by associating imaging methods, surgical and histological findings[64]. The prognosis of patients with tumors with favorable histology has improved in recent decades, reaching survival rates of 90% in four years.

WT IMMUNOPHENOTYPE

In 2009, Pode-Shakked *et al.*[65] reported the varied expression of some markers of hematopoietic (CD34, CD117, and CD133) and mesenchymal cells (CD105, CD90, and CD44) and those related to cancer (CD133 and MDR1), as well as the association between positivity for NCAM (CD56) and the fraction of "tumor stem cells" in the analysis of WT cells. The authors further suggest that NCAM is also a marker of WT malignant renal progenitor cells.

Royer-Pokora *et al.* (2010)[66] reported the characterization and establishment of five WT lineage cells with *WT1* mutation regarding the expression of genes and proteins that had already been described in mesenchymal stem cells and in paraxial mesoderm (CD73, CD90, and CD105). The results of this study show the limited ability to differentiate Wilms from mesenchymal lineages, as the gene expression profile demonstrates that WT cell lines are very similar to human mesenchymal stem cells, as they have the same surface protein expressions. They also concluded that WT with *WT1* mutations has specific characteristics of the paraxial mesoderm, which is the source of renal stromal cells.

In the study by Ferreira-Facio *et al.* (2013)[33], two patients were diagnosed with WT. Such cases showed populations of tumor cells (coexisting), however, clearly distinct in terms of phenotype; they were positive for CD56 and CD58 and negative for CD45, CD99, GD2, nuMYOD1, nuMyogenin, CD10, and NG2. However, they showed distinct reactivity (negative versus positive expression) toCD90, EpCAM, and CD57. These marker observations are in line with the reported coexistence of epithelium (e.g., $EpCAM^{+}$, $CD90^{-}$) and mesenchymal cellular components ($EpCAM^{-}$, $CD90^{+}$) in WT by histopathology[65, 67, 68]. These data show another potential use of FC: to better understand tumor heterogeneity.

RMS

RMS is a rare soft tissue sarcoma of mesenchymal origin, with evidence of striated muscle cell differentiation. It accounts for 2.9% of all pediatric cancers in the United States and its incidence is 4.5 cases/million children and adolescents per year. It is the third most common extracranial tumor in children, after NB and WT, and approximately 50% of affected patients were under 10 years of age[69, 70].

The most common primary site of RMS in children and adolescents is the head and neck region, followed by the genitourinary tract, extremities, chest, and retroperitoneum. Tumor subsites in the head and neck region include orbit, parameningeal sites (nasopharynx, nasal cavity, paranasal sinuses, temporal bone, pterygopalatine fossa, and infratemporal fossa), and non-parameningeal sites. Tumors that only invade the orbit have a better prognosis[71]. Its diagnosis is based on clinical

history, physical examination, laboratory tests (such as blood count, biochemical profile, and liver enzymes), nasofibroscopy, computed tomography, magnetic resonance, and biopsy with pathological examination[71].

FC IN RMS

Currently, there has been an evolution in the use of FC for the diagnosis of metastases in BM of patients with RMS. Shen *et al.* (2014)[72] evaluated 11 patients with suspected metastasis, comparing morphological analysis (gold standard) with BM aspirate FC. In this study, it was possible to observe the positivity for malignant cells in three patients through the anatomopathological examination and in four patients through FC. All three positive cases in the pathological examination were also positive in FC (which still detected positivity in one more patient). The percentage of positive cells obtained in FC was 29.3%, 12.3%, 6.8%, and 0.35% of the total nucleated cells, showing good sensitivity. The case with negative morphology, but with a cell detection level of 0.35% by FC, led the researchers to carry out a retrospective morphological analysis, which also obtained a positive result for the finding of neoplastic cells. These studies carried out with small numbers of patients suggest the importance of FC as a sensitive method for detecting tumor extension[72, 73].

New possibilities for detecting circulating tumor cells using FC suggest increasing the accuracy of these findings. One of the biomarkers expressed in RMS, the paired BOX gene 3 family (*PAX3*), was quantified by FC in comparison with the real-time polymerase chain reaction (qPCR), obtaining similar or even higher sensitivity in different cell lines for this neoplasm[74].

Currently, the $CD45^-/CD56^+/CD90^+/myogenin^+$ phenotype is used for the diagnosis of RMS. However, there may be a variable expression of CD57[75]. This profile is similar to the NB immunophenotypic profile, however, the use of GD2 aids to differentiate these two neoplasms, as it is only positive in NBs[72].

DISCUSSION

FC is a diagnostic and monitoring method widely used in hematological diseases. It has several advantages over the traditional anatomopathological examination (histopathology and immunohistochemistry), among them, the sensitivity and speed of the method. To ensure the quality and uniformity of the result, the use of appropriate panels for the strains of the pathogen investigated is crucial in the stage of carrying out/completing the laboratory diagnosis. Based on these findings, we developed a panel of tubes to characterize the diseases addressed in this review, as described in **Table**.

Another benefit of FC is the ability to identify individual immunophenotypic cells, even in conditions with little material for analysis due to the sensitivity of the test. In **Figure 2**, we describe the expression of pediatric solid tumors mentioned in our study.

TABLE – Immunophenotypic markers

	Pacific blue	Pacific orange	FITC	PE	PERCP	PE-CY7	APC	APC-H7
Neuroblastoma	CD9	CD45		CD73	GD2	CD56	CD90	CD81
PNET	CD9	CD45	CD99	CD721		CD56	CD117	CD81
Wilms tumor	CD9	CD45	CD90	CD721		CD56	EPCAM	CD81
Rhabdomyosarcoma	CD9	CD45	MYOD1	MYOGENIN		CD56	CD90	CD81
Ewing sarcoma	CD9	CD45	CD99	CD117		CD56	CD90	CD81

PNET: primitive neuroectodermal tumor.

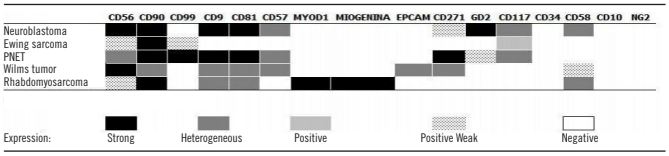

FIGURE 2 – *Immunophenotypic markers*

PNET: primitive neuroectodermal tumor.

Nowadays, we understand that it is essential to implement technologies that accelerate and qualify the diagnostic and therapeutic process in Pediatric Oncology. Such measures have an impact on the length of hospital stay, in the family context, in addition to therapeutic decision-making[5, 6]. In this regard, FC immunophenotyping is a useful tool as a diagnostic method for assessing the spread of tumor cells in BM and body fluids, and for monitoring the assessment of response to treatment through the investigation of MRD of several pediatric tumors[29, 51].

Evidence surrounding the use of phenotypic markers such as CD56 and CD90 demonstrates that, currently, the FC technique is not at a disadvantage in terms of sensitivity and specificity compared to gold standard methods such as immunohistochemistry and conventional histopathological examination. Also, as demonstrated by Almazán-Moga *et al.* (2014)[74], FC seems to have more sensitivity in detecting circulating tumor cells.

CONCLUSION

According to the data presented, we can state that FC is an auxiliary method in the diagnosis, treatment, and monitoring of various malignancies. Therefore it is essential and of great relevance for a quick diagnosis and accurate classification of the disease, in addition to careful monitoring the effectiveness of the treatment.

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