Pioneering Study about the Presence of Paroxysmal Nocturnal Hemoglobinuria Clone during Therapeutic Monitoring of Acute Leukemia

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Estudo Pioneiro sobre a Presença de Clone de Hemoglobinúria Paroxística Noturna durante Acompanhamento Terapêutico de Leucemia Aguda

Estudio Pionero sobre la Presencia de un Clon de Hemoglobinuria Paroxística Nocturna durante el Seguimiento Terapéutico de la Leucemia Aguda

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ABSTRACT

Introduction: The potential for malignant transformation of hematopoietic stem cells carrying mutations in the glycosylphosphatidylinositol class A (PIG-A) gene for acute leukemias, although rare, is already well described in the literature. **Objective**: In this study, however, it was attempted to show for the first time in the literature the emergence or maintenance of paroxysmal nocturnal hemoglobinuria (PNH) clones in patients diagnosed with acute leukemia or even after the beginning of the chemotherapy treatment. **Method**: The search for PNH clones was performed by flow cytometry in blasts, erythrocytes, granulocytes or monocytes of 47 samples of peripheral blood and bone marrow from patients undergoing diagnostic investigation or therapeutic follow-up in two oncological and public hospitals in Belém, from December 2017 to December 2018. **Results**: The presence of PNH clones was observed in 19/47 (40.4%) patient samples, in diagnostic investigation or therapeutic follow-up, who participated of at least one therapeutic follow-up study and still experience the appearance or maintenance of the PNH clone even after the beginning of the chemotherapy treatment. **Conclusion**: Primarily, it was possible to demonstrate the presence of PNH clones in patients diagnosed with acute leukemia both during the diagnostic investigation period and therapeutic follow-up, regardless of cell ontogeny. However, the importance of the presence of these PNH clones for the evolution of the primary disease, prognosis or need for specific treatment was not evaluated yet.

Key words: Hemoglobinuria, Paroxysmal/diagnosis; Hemoglobinuria, Paroxysmal/drug therapy; Leukemia/diagnosis; Acute Disease.

RESUMO

Introdução: O potencial de transformação maligna de células-tronco hematopoiéticas portadoras de mutações no gene glicosilfostatidilinositol classe A (*PIG-A)* para leucemias agudas, embora raro, já é bem descrito na literatura. **Objetivo:** Neste estudo, porém, buscou-se evidenciar pela primeira vez na literatura o surgimento ou a manutenção de clones de hemoglobinúria paroxística noturna (HPN) em pacientes diagnosticados com leucemia aguda ou ainda após o início do tratamento quimioterápico. **Método:** A pesquisa de clones de HPN foi realizada por citometria de fluxo em blastos, hemácias, granulócitos ou monócitos de 47 amostras de sangue periférico e medula óssea de pacientes submetidos à investigação diagnóstica ou acompanhamento terapêutico, provenientes de dois hospitais oncológicos e públicos de Belém, no período de dezembro de 2017 a dezembro de 2018. **Resultados:** A presença de clones de HPN foi observada em 19/47 (40,4%) amostras de pacientes, em investigação diagnóstica ou acompanhamento terapêutico, que realizaram pelo menos um estudo de acompanhamento terapêutico e ainda tiveram o surgimento ou a manutenção do clone de HPN mesmo após iniciado o tratamento quimioterápico. **Conclusão:** Foi possível evidenciar, de forma primária, a presença de clones de HPN em pacientes diagnosticados com leucemia aguda tanto no período de investigação diagnóstica como durante o acompanhamento terapêutico, independentemente da ontogenia celular. Sem, porém, que se possa ainda avaliar a importância da presença desses clones de HPN para a evolução da doença primária, prognóstico ou necessidade de tratamento específico.

Palavras-chave: Hemoglobinúria Paroxística/diagnóstico; Hemoglobinúria Paroxística/tratamento farmacológico; Leucemia/diagnóstico; Doença Aguda.

RESUMEN

Introducción: El potencial de transformación maligna de las células madre hematopoyéticas que portan mutaciones en el gen glicosofosfatidilinositol (GPI) clase A (*PIGA*) para las leucemias agudas, aunque raro, ya está bien descrito en la literatura. **Objetivo:** En este estudio, sin embargo, buscamos mostrar por primera vez en la literatura la aparición o mantenimiento de clones de HPN en pacientes diagnosticados de leucemia aguda o incluso después del inicio de la quimioterapia. **Método:** La investigación de clones de hemoglobinuria paroxística nocturna (HPN) se realizó mediante citometría de flujo en blastos, eritrocitos, granulocitos o monocitos de 47 muestras de sangre periférica y médula ósea de pacientes sometidos a investigación diagnóstica o seguimiento terapéutico de dos hospitales oncológicos y públicos de Belém, durante el período. de diciembre de 2017 a diciembre de 2018. **Resultados:** La presencia de clones HPN se observó en 19/47 (40,4%) muestras de pacientes, en investigación diagnóstica o seguimiento terapéutico, que realizaron al menos un estudio de seguimiento terapéutico y aún tenían la aparición o mantenimiento del clon HPN incluso después de iniciado el tratamiento de quimioterapia. **Conclusión:** Se pudo evidenciar, de forma primaria, la presencia de clones de HPN en pacientes diagnosticados de leucemia aguda tanto durante el período de investigación diagnóstica como durante el seguimiento terapéutico, independientemente de la ontogenia celular. Sin embargo, no podemos todavía evaluar la importancia de la presencia de estos clones de HPN para la evolución de la enfermedad primaria, el pronóstico o la necesidad de un tratamiento específico.

Palabras clave: Hemoglobinuria Paroxística/diagnóstico; Hemoglobinuria Paroxística/tratamiento farmacológico; Leucemia/diagnóstico; Enfermedad Aguda.

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INTRODUCTION

The paroxysmal nocturnal hemoglobinuria (PNH) is a rare clonal disorder of hematopoietic stem-cells resulting from the somatic mutation of the gene glycosylphosphatidylinositol (GPI) class A (PIG-A) whose consequence is the blocking of the GPI-anchor synthesis, which, on its turn, keeps dozens of proteins with specific functions, as anchoring the regulating proteins of the complement system attached to the plasma membrane. The clinical consequence[1-7] of the lack of GPI-anchor in several cellular types is the manifestation of chronic hemolytic anemia, bone marrow failure and thrombosis in unusual sites.

Regarding PNH genesis, more than 180 mutations have already been identified in gene PIG-A, mostly associated with small insertions or deletions of one or more nucleotides which eventually result in frameshift of this gene and for causing the early termination of the transcription and consequent blocking of the normal process of the GPI molecule[1,4-6,8-11] synthesis.

The exact mechanism that causes the clonal expansion of PNH cells is still uncertain. Today, the "two-steps hypothesis" is the mechanism more accepted for the expansion of PNH clone cells: the first step is the occurrence of the PIG-A mutation with no clonal expansion; and in the second, one or more external environment factors pressure selectively in favor of the PNH clone expansion. This clone expansion is also favored by the fact that deficient cells by GPI-anchored proteins appear to be more resistant to the immune attack of T cells and natural killer (NK) in the bone marrow[8,10-14].

Several studies in the literature have been demonstrating that PNH clone carriers present somatic mutations in others than the PIG-A gene and typically are associated with cellular process of growth, cellular differentiation and regulation of apoptosis and are also frequent in the genesis of several hematological neoplasms[11-16]. The main factor associating the PNH clone with the ability of expansion and malignant transformation of these cells is the hypermutability condition of the PNH clone cell[8,9,15-20].

Although rare, this ability of malignant transformation of the PNH clone cells in acute leukemias have been described in the literature[8,14,15]. However, few have portrayed the presence of "dormant" PNH clones in acute leukemias and still less in relation to the persistent ability of these clones even after the therapeutic follow up has commenced. Therefore, the objective of this study was to evidence for the first time in the literature the maintenance of PNH clones in patients diagnosed with acute leukemia even after the chemotherapy treatment has been initiated.

METHOD

Prospective study with peripheral blood and bone marrow of 47 patients, both genders, treated in Hospitals Ophir Loyola (HOL) and "*Oncológico Infantil Dr. Octávio Lobo (HOIOL)*", from December 2017 to December 2018 for diagnostic investigation of acute leukemias or therapeutic follow-up and research of PNH clones by flow cytometry regardless of the presence of hemolysis, thrombosis or any other PNH associated clinical alteration. All these procedures were conducted in a private laboratory in Belém, Pará, upon the approval of the Institutional Review Board of "*Fundação Pública Estadual Hospital de Clínica Gaspar Vianna*", report number 732.668, dated May 22, 2014.

Samples of patients of both genders treated in the oncologic hospitals HOL and HOIOL were included. Necessarily, these patients needed to be in diagnostic investigation or having initiated the chemotherapy treatment (therapeutic follow-up) for acute leukemia. To be enrolled in this study they or their legal guardians signed the Informed Consent Form (ICF). Clotted cells with volume lower than 1 ml and patients diagnosed with other types of onco-hematological diseases or who refused to join the study were excluded.

For all the samples, smears for morphologic analysis and their processing for diagnosis or therapeutic follow-up of acute leukemia were prepared. The processing was performed through the addition of 100 μL of the sample to be investigated in conic tubes added of 7 μL of different combinations of pan-hematopoietic commercial monoclonal antibodies: CD34, CD45, HLA-DR; lymphocytes B: CD19, CD10, CD20, CD22, CD79a, TdT, IgG1, IgG1, IgM, anti-kappa and antilambda; lymphocytes T and NK: CD5, CD7, CD2, CD1a, CD3, CD4, CD8, CD56 or myeloid: CD13, CD33, CD117, CD61, CD14, CD64, CD11b, glycophorin A, CD42a, MPO – marked with FITC, PE, PerCP and APC, plus hemolysis or permeabilization, incubation in dark for ten minutes with two centrifugations and two successive lavages with phosphate buffered saline (PBS). Further, 100 thousand events were acquired and analyzed in flow cytometry BD FACSCalibur™, with BD CellQuest™ Pro *software* (BD, San Jose, CA, USA) for four colors.

For the investigation of PNH clones in patients' samples in diagnostic investigation or in therapeutic follow-up with active disease, the antibodies panel FLAER/CD59PE/CD45Per-Cy5 for the study in populations of leukemic blasts was utilized and the panel CD235FITC/CD59PE/CD45Per-Cy5 for the blood cells study. For the disease-free patients' samples in therapeutic follow-up, the combination of antibodies proposed

by Borowitz et al.[22] was utilized: FLAER/CD15PE/CD45Per-Cy5/CD24APC for granulocytes, FLAER/CD14PE/CD45Per-Cy5/CD64APC for monocytes and CD235FITC/CD59PE/CD45Per-Cy5 for blood cells. All the samples were submitted to acquisition and analysis of 250 thousand events.

Descriptive methods to determine the frequency, maximum, minimum, and median were applied through the software BioEstat 5.0**.**

RESULTS

Of the total cases analyzed, 42/47 (89.4%) consisted of patients' samples in diagnostic investigation that, according to medical decision, were submitted to one or until four sequential studies of therapeutic follow-up and, that, after concurrence of the participants or their legal guardians were submitted to PNH clones investigation; only 5/47 (10.6%) of the samples were from patients who were already in therapeutic follow-up and submitted to sequential study and PNH clones investigations.

From this total, 30/47 (63.83%) of the samples were from males; 17/47 (36.17%), females, and 29/47 (61.7%) of individuals in the age range from zero to 17 years old. Among the most frequent acute leukemias, the pre-pre-B cells lymphoblastic leukemia/lymphoma was observed in 18/47 (38.4%) of the samples and acute myeloid leukemia in 14/47 (29.79%) of the samples investigated (Table 1).

Table 1. Frequency of acute leukemias in relation to the total of patients investigated regardless of age consulted in two oncologic hospitals. Belém-Pará, Brazil, December 2017 to December 2018

Initial Diagnosis	Sample (#)	Percentage (%)
Pre-pre B ALL	18	38.4
Pre B ALL	5	10.2
AML	14	29.9
ALL-T	3	6.5
Bilineage leukemia	2	4.4
Diagnosis not done in this study	5	10.6
TOTAL	47	100

Captions: ALL: Acute lymphoblastic leukemia of B cells or T cells; AML: Acute myeloid leukemia.
(#) absolute value.
(%) percent value.

The presence of PNH clones was observed in 24/42 (57.1%) of the patients' samples in diagnostic investigation, regardless of the type of leukemia (Table 2). The highest frequency of PNH clones was observed among these samples in patients diagnosed with acute myeloid leukemia in 11/14 (78.5%) cases and pre-pre-B lymphoblastic leukemia/lymphoma in 8/17 (47%) cases.

Next, the stratification of the population of patients who participated at least of one sequential study of therapeutic follow-up and of the investigation was performed to verify the presence of PNH clones (Table 1), reaching a total of 19/47 (40.4%) of the samples analyzed. Of these, 15/19 (79%) were of patients in diagnostic investigation who have submitted to sequential study of therapeutic follow up and presented PNH clone in at least one of the follow-up studies and 4/19 (21%) were of samples from patients enrolled in the study even without the investigation of PNH clones in the sequential study of therapeutic follow-up.

Still in relation to patients' samples submitted to therapeutic follow-up study and to investigation of PNH clone, it was observed that 8/19 (42.1%) patients underwent only one therapeutic follow-up study with 15 days after the beginning of chemotherapy (Table 1) and that all who were in diagnostic evaluation presented PNH clones during this evaluation in blasts and red cells (2/8), likewise all the patients who were in therapeutic follow-up, regardless of the presence or not of active disease, presented PNH clones in granulocytes, monocytes and red cells (6/8).

Similarly, it was observed that only 8/19 (42.1%) of the patients were submitted to two therapeutic studies, within 15 and 30 days after the beginning of the chemotherapy, and that PNH clones were found in granulocytes, monocytes, and red cells (Table 1) in the second evaluation (30 days) in all their samples, even without activity of the primary disease. And of these samples, 3/8 (37.5%) had PNH clones at the diagnosis and in the two therapeutic follow-up studies.

Only 2/19 (10.5%) of the patients participated of three therapeutic follow-up studies and for both, their samples had PNH clones in the three periods investigated, regardless of the presence of PNH clones during the diagnostic investigation. Only 1/9 (5.3%) patient was enrolled in four consecutive therapeutic follow-up studies and the presence of PNH clones was observed in three periods of the studies not continuously (Table 1).

Regarding the size of the PNH clone, for those who submitted to only one therapeutic follow-up study (8/19), the PNH clone size ranged between 2.36 – 70.9% in granulocytes, between 7.24 – 89.4% in monocytes and between 2.8 – 62.7% in red cells, regardless of the type of leukemia. For the patients who submitted to two therapeutic follow-up studies (8/19), the size of the PNH clone ranged between 1.38 – 50.9% in granulocytes, 11.6% - 75.7% in monocytes and 3.3 – 81.3% in red cells,

Table 2. Representation of total patients diagnosed with acute leukemia in diagnostic investigation in relation to gender, age (minimum, maximum and median) and presence and size of the PNH clone (>1%) in blasts. Belém-Pará, Brazil, December 2017 to December 2018

Type of leukemia	Patients/ gender	Ages (Min-Max)	Median of ages (years)	Total carriers of PNH clone (#)	Size of the PNH clone (Min%-Max%)
AML	11M/3F	7 – 79	31	11	3.06 – 99.36
ALL-T	3M/1F	5 – 19	14	1	85.81
Pre-pre B ALL	10M/7F	2 – 69	7	8	1.22 – 99.22
Pre B ALL	3M/2F	3 – 76	5	2	5.6 – 90.33
Bilineage leukemia	1M/1F	13 – 32	22.,5	2	8.68 – 97.0
TOTAL	42	--	--	24	--

Captions: ALL: Acute lymphoblastic leukemia of B cells or T cells; AML: Acute myeloid leukemia; M: male; F: female; Min: Minimum; Max: Maximum.
(#) absolute value.
(%) percent value.

Chart 1. Investigation about the presence of the PNH clone and/or activity of the primary disease in patients during the initial diagnosis or in therapeutic follow-up in two oncologic hospitals. Belém-Pará, Brazil, December 2017 to December 2018

Patient	Gender	Age	Initial Diagnosis		1st Therapeutic follow-up		2nd Therapeutic follow-up		3rd Therapeutic follow-up		4th Therapeutic follow-up	
			Diagnosis of acute leukemia	PNH clone	Leukemia in activity	PNH clone	Leukemia in activity	PNH clone	Leukemia in activity	PNH clone	Leukemia in activity	PNH clone
1	M	16	Pre-pre B ALL	-	-	-	-	+				
2	F	7	Pre-pre B ALL	-	-	+						
3	M	3	Pre-pre B ALL	-	-	+	-	+				
4	M	7	Pre-pre B ALL	-	-	-	-	+				
5	M	9	Pre-pre B ALL	+	-	+	-	+				
6	M	14	Pre-pre B ALL	+	+	+	-	+				
7	F	7	Pre-pre B ALL CD33 +	+	-	+	-	+				
8	F	13	Pre-pre B ALL		-	+						
9	M	3	Pre-B ALL	-	-	+	-	+	-	+		
10	F	5	Pre-B ALL	-	-	+						
11	M	7	AML	+	-	+						
12	F	13	AML	+	+	+	-	+	-	+		
13	M	8	AML	-	-	+						
14	M	62	AML	-	+	+						
15	M	13	T ALL	-	-	+	-	+				
16	M	19	T ALL	-	-	+						
17	F	15	T ALL	+	-	+						
18	F	2	ALL*		+	+	+	+	+	-	-	+
19	F	4	ALL*		-	-	-	+				

Captions: ALL: T or B cells lymphoblastic lymphoma/leukemia; AML: acute myeloid leukemia; M: male; F: female.
(-) negative investigation for PNH clone.
(+) grey rows and columns: positive investigation for PNH clone.
(*) Patients without definition of the type of cells B or T lymphoblastic leukemia/lymphoma.

regardless of the type of leukemias. And for the patients who submitted to until three therapeutic follow-up studies (2/19), the size of the PNH clone varied between 3.97 – 10.1% in granulocytes, 40.3% - 64.6% in monocytes and 12.4% - 14.2% in red cells.

DISCUSSION

The potential of malignant transformation of hematopoietic stem-cells bearing mutations of the gene PIG-A for acute leukemias, although rare, is well described in the world literature[5,6,8,9,13,14]. However, Araten and Luzzatto[16], Araten et al.[15] and Inoue el al.[17] suggest that, for this malignant transformation, hematopoietic stem-cells bearing mutations of the gene PIG-A should undergo a second event, that is, additional mutations in other genes, as, for instance, BCR-ABL[19], HMGA[2], JAK2V617F and NRAS[18] in the same cellular population, favoring advantages of the clonal expansion and transformation of the cells bearing mutations of the gene PIG-A[20]

Traulsen et al.[12] and Dingli et al.[13], in their studies suggest still that the second mutation in hematopoietic stem-cells bearing mutations in the gene PIG-A and the consequential leukemic transformation in the PNH are the exception and not the rule in these cellular groups[9].

However, in the present study, it was not attempted to show the potential of malignant transformation of the hematopoietic stem-cells bearing mutations of the gene PIG-A for acute leukemia, but the presence, maintenance or appearance of PNH clones of various types and in several cellular types in patients who had acute leukemia during the diagnostic investigation in blasts and red cells, or after the chemotherapy treatment has been initiated, granulocytes, monocytes or red cells, as also described in another study of the authors[21].

Mon Père et al.[23], utilizing a mathematical model, showed that the size of the PNH clone is directly related not only to the increase of the probability of clonal expansion of the cells bearing mutations of the gene PIG-A, but also it is determinant for the definition of the clinical presentation of the disease in its clinic, sub-clinic forms or extinction of the clone. In this study, the authors propose that the odds of an individual to develop PNH in the clinic form increases with age[24] and depends on the total number of stem-cells bearing mutations of the gene PIG-A (>20%). These results are similar to the findings of the clinical practice.

Nevertheless, in the studies presented herein, even when large PNH in patients' samples at the diagnosis or during the therapeutic follow-up were found, these patients had no clinical evidence of PNH as deep venous thrombosis in uncommon sites, chronic kidney failure, liver failure or bone marrow failure dissociated from leukemia.

The initial hypothesis is that the PNH clones found in the samples of the patients diagnosed or in therapeutic follow-up for acute leukemias are in the form of "silent" or "dormant" clones with no clinical repercussion and are result of the typical condition of hypermutability of leukemias; therefore, in some cases, possibly related to the expansion process of the neoplasms alone. However, this is the first study showing the presence of PNH clones in various phases of the process of therapeutic follow-up of patients with acute leukemias, and, so far, it is not possible to affirm whether for the patients with "silent" PNH clone, it would be necessary the application of therapeutic regimens that incorporate specific drugs to treat PNH.

In the studies of Araten et al.[15], utilizing the PIG-A gene as sentinel for spontaneous somatic mutations, they noticed indications of hypermutability in leukemic blasts of children and found two distinct population of leukemic cells in relation to the PNH phenotype: one with the mutation rate close to the control group and other with mutation 50-fold higher than the control group and hypothetically associated the hypermutability condition of these cells with higher likelihood of these number of mutations to be a critical factor for the onset of leukemia.

In relation to the necessity of treatment of these patients, Lanza et al.[25] showed a case report of a patient with initial diagnosis of myelodysplastic syndrome (MSD) who, during the course of the disease, presented large PNH clone and evolution to diffuse large B-cells lymphoma, being mandatory the treatment of PNH with eculizumab in association with specific chemotherapy. On its turn, Li et al.[26] demonstrated that the presence of the PNH clone in a 52-years old adult patient with chronic myeloid leukemia did not justify the same specific treatment for PNH. So far, consequently, there is no consensus in the literature whether specific treatment for patients with onco-hematologic diseases is needed when PNH clones are present.

CONCLUSION

It was possible to observe for the first time in the literature the presence of PNH clones in patients diagnosed with acute leukemia both in the period of diagnostic investigation and during therapeutic follow-up and the appearance of these clones only after the beginning of the chemotherapy treatment, regardless of the cellular ontogeny, suggestive of the necessity of new studies that are able to demonstrate how the presence of these PNH clones can influence the diagnosis, risk of relapse and whether an additional treatment is required for these patients.

CONTRIBUTIONS

Lacy Cardoso de Brito Junior contributed for the study design and conception, analysis, interpretation of the data, wording, and critical review with intellectual contribution. Eduardo dos Santos Martins Filho, Murilo Chermont Azevedo, Ana Paula Silveira Paixão, Debora Monteiro Carneiro and Matheus Holanda Nascimento contributed for the study conception and design, analysis, and interpretation of the data. All the authors approved the final version to be published.

DECLARATION OF CONFLICT OF INTERESTS

There is no conflict of interests to declare.

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