ORIGINAL ARTICLE

NADPH Oxidase 5 upregulation is associated with lymphoma aggressiveness

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SUMMARY

OBJECTIVES: *Lymphomas are a heterogeneous set of malignant neoplasias of lymphoid B and NK/T mature and immature cells at various stages of differentiation. Genetic and molecular biology tools are used to appropriately classify the type and prognosis of the lymphomas, which have implications in therapeutic effectiveness. Among them, the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase (NOX5) enzymes have been explored. This study analyzed the expression of NADPH oxidase 5 in lymphoma tissue according to the degree of tumor aggressiveness.*

METHODS: *Slides from 64 patients with lymphoma who had paraffin-embedded tissue available were reviewed by two independent, experienced pathologists. They classified tumors according to the WHO classification (2017). NOX5 expression in tissues was assessed by immunohistochemical staining using a tissue microarray. The assay was interpreted using a scoring system of 0, 1, 2, and 3, for cytoplasmic staining of NOX5 corresponding to negative, weak, intermediate, and strong staining, respectively. We compared the expression of NOX5 in patients with aggressive versus non-aggressive lymphomas.*

RESULTS: *NOX5 expression was positive in 100% (27/27) of aggressive lymphomas and in 19% (7/37) of non-aggressive ones. The seven patients with positive expression of NOX5 presented intermediate staining (2); strong staining (3) was observed only in tissues of aggressive lymphomas, and negative and weak staining (0 and 1) were observed only in non-aggressive lymphomas.*

CONCLUSIONS: *Aggressive lymphomas overexpress NOX5 protein. The higher NOX5 expression in aggressive lymphomas can suggest an involvement of this enzyme on the acquisition of an aggressive phenotype in lymphoid neoplasia.*

KEYWORDS: *Lymphoma. NADPH Oxidase 5. Reactive oxygen species. Immunohistochemistry.*

INTRODUCTION

Lymphomas have a clinical behavior that ranges from the most indolent to the most aggressive human malignancies[1,2]. This heterogeneity is also observed in morphological, immunophenotypical, and genotypical aspects[1,3]. Clinical and biological markers of prognosis for both Hodgkin and non-Hodgkin's lymphomas have been sought in order to decide on the best appropriate treatment for each patient[4-8]. Specific markers

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could also improve the diagnosis and contribute to the development of therapeutic targets to be used in personalized therapy[9].

The NOX family is composed of seven members, NOX1-NOX5 and DUOX1/2, which are differentially expressed among tissues[10]. NOX are transmembrane proteins, which transport electrons across biological membranes in order to reduce molecular oxygen to superoxide anion or hydrogen peroxide.

Oxidative stress is characterized by a cellular redox imbalance and is involved in several steps related to the carcinogenic process due to increased reactive oxygen species (ROS) availability. The initiation step can be triggered by deoxyribonucleic acid (DNA) oxidation, leading to changes in the pattern of gene expression. In addition, ROS can alter cellular signaling pathways, leading to changes in cellular proliferation, apoptosis, angiogenesis, among others[11]. ROS, such as superoxide and hydrogen peroxide (H2O2), can be formed by xanthine-oxidase, cytochrome P-450, or mitochondrial electron transport chain, as a by-product, or directly by the NOX family of enzymes[12].

NOX overexpression promotes mutagenesis[13], chromosomal aberrations[14], proliferation[15], avoids mitotic control by inactivating tyrosine phosphatases[16], stimulates angiogenesis[17] and contributes to the acquisition of an invasive and metastatic phenotype[18]. Furthermore, increased expression of the NOX enzymes has been documented in a wide range of neoplasias, such as prostate tumors[19], and in tumor cell lines of various types[20].

NOX5 was the last member of the NOX family to be identified and compared with the other NOXs; little is known about its regulation and function in human physiology and diseases. NOX5 is highly expressed in testis, uterine smooth muscle, and in lymphocyte-rich areas of the spleen and lymph nodes, but several other cell types express this enzyme in a less expressive way[21]. The majority of cellular models show that NOX5 is present in intracellular membranes, but its presence in the cellular plasma membrane has already been reported[22]. Calcium is essential for the activity of NOX5 that contains two pairs of 4 EF-hands in its N-terminal region[23]. Antony et al.[24] showed by tissue microarray analysis that NOX5 is overexpressed in several human cancers when compared to their adjacent non-tumor tissues, such as breast, lung, prostate, brain, ovary, colon, malignant melanoma, and non-Hodgkin lymphoma.

Some studies have shown NOX5 as an important source of ROS in cancer cells and during the carcinogenic process. Li et al.[25] have shown that the activation of NOX5 after exposure to bile acid can cause DNA damage in Barrett's human adenocarcinoma cell line, FLO-123. Interestingly, the authors suggest that high levels of ROS derived from NOX5 could contribute to the progression of the disease from Barrett's esophagus to esophageal adenocarcinoma. In addition, the inactivation of NOX5 by RNA interference protects human primary fibroblasts from DNA damage induced by ionizing radiation, reinforcing the role of NOX5-derived ROS in genetic instability[26]. Another key point related to tumorigenesis is the disruption of physiological mechanisms related to proliferation, migration, and survival. Shigemura et al.[27] showed that NOX5 was upregulated in adult T-cell leukemia (TLA) compared to normal peripheral blood T cells. Since human T-cell leukemia virus type 1 (HTLV-1) infection is associated with human peripheral blood T-cell transformation and ATL development, the authors evaluated the expression of NOX5 in HTLV-1 transformed cell lines and observed a marked increase in their expression after infection, which was linked to increased cell growth, migration, survival, and tumorigenicity. ATL is a very aggressive form of leukemia/lymphoma. Very similar results were observed in prostate carcinoma cells in which shRNA-mediated silencing of NOX5 impaired cell proliferation and increased apoptosis of the cells studied[28].

The objective of this study was to analyze the expression of NOX5 in a greater sample of different types of lymphoma, and its association with the degree of tumor aggressiveness.

METHODS

Study design and setting

This was a retrospective study of specimens from patients with lymphomas diagnosed between January 1981 and December 2012 in Clementino Fraga Filho University Hospital, from the Federal University of Rio de Janeiro. All patients diagnosed with lymphoma of any type were eligible. Patients with available paraffin-embedded blocks were included.

Slides from the diagnosis period were reviewed by two independent and blinded pathologists who classified the lymphomas according to the criteria defined by the World Health Organization[1]. They were then divided into two groups: aggressive and non-aggressive.

The paraffin-embedded blocks were tested for NOX5 using the tissue microarray technique (TMA). A manual instrument (Beecher Instruments, Sun Prairie, WI, USA) was used to include at least two spots of the blocks, which were selected by the same pathologists during the slide review process. Paraffin sections were dehydrated and dewaxed according to standard procedures. Immunohistochemical processing was performed using the NOX5 antibody (rabbit polyclonal antibody raised, SANTA CRUZ BIOTECHNOLOGY, INC) in a 1:200 dilution, with overnight incubation. The secondary antibody used was EnVision®+Dual Link/Peroxidase (Dakocytomation®). Heat-induced antigen retrieval was then performed.

The assay was interpreted according to the criteria described by Antony et al.[24], which consists of a scoring system (0-3) for cytoplasmic staining corresponding to negative (0), weak positive (1), moderate positive (2), and strong positive (3) staining, respectively. Negative and weak staining were considered "negative", while intermediate and strong staining were considered positive.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (version 5.01, GraphPad Software Inc., San Diego, USA). All results were expressed as mean ± standard error of the mean (SEM). The histological quantification of intensity and proportion was analyzed using Fisher's exact test, and $p < 0.05$ was considered statistically significant.

RESULTS

Sixty-four patients were included, of which 27 (42,2%) had aggressive lymphomas [diffuse large B-cell lymphoma (18/27) 66,6% and Burkitt lymphoma (9/27) 33,4%] and 37 (57,8%) had non-aggressive lymphomas [follicular lymphoma (1/37) 2,7%, Hodgkin Lymphoma Mixed Cellularity (5/37) 13,5%, Hodgkin Lymphoma Nodular Sclerosis I (22/37) 59,5%, Hodgkin Lymphoma Nodular Sclerosis II (8/37) 21,6%, and Hodgkin Lymphoma Lymphocyte-Depletion (1/37) 2,7%].

The expression of NOX5 was positive in 27/27 (100%) tissues of aggressive lymphomas and 7/37 (19%) of non-aggressive lymphomas.

The positivity of Hodgkin's lymphoma was evaluated in the Reed-Sternberg cell and its variants. The inflammatory background was not considered for the evaluation. The cases of aggressive lymphomas were usually marked diffusely and, interestingly, all the cases were positive for NOX5 staining, but only 19% of non-aggressive scored as positive. (Figures 1 and 2)

The staining proportion was scored using a range from 0 to 3 for cytoplasmic staining. Negative and weak staining was considered negative, while intermediate and strong staining was considered positive. (Figure 3)

FIGURE 1

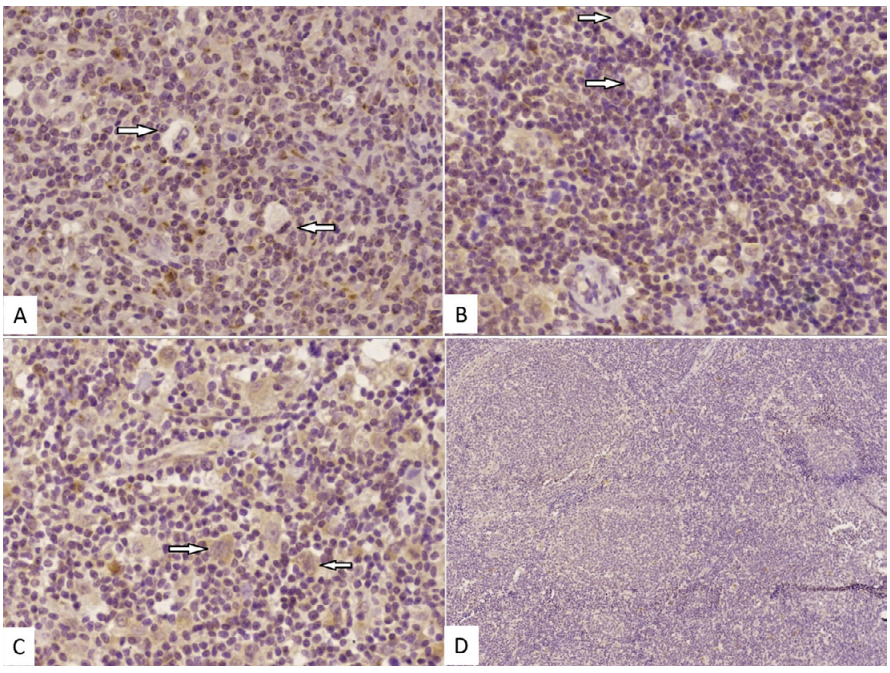
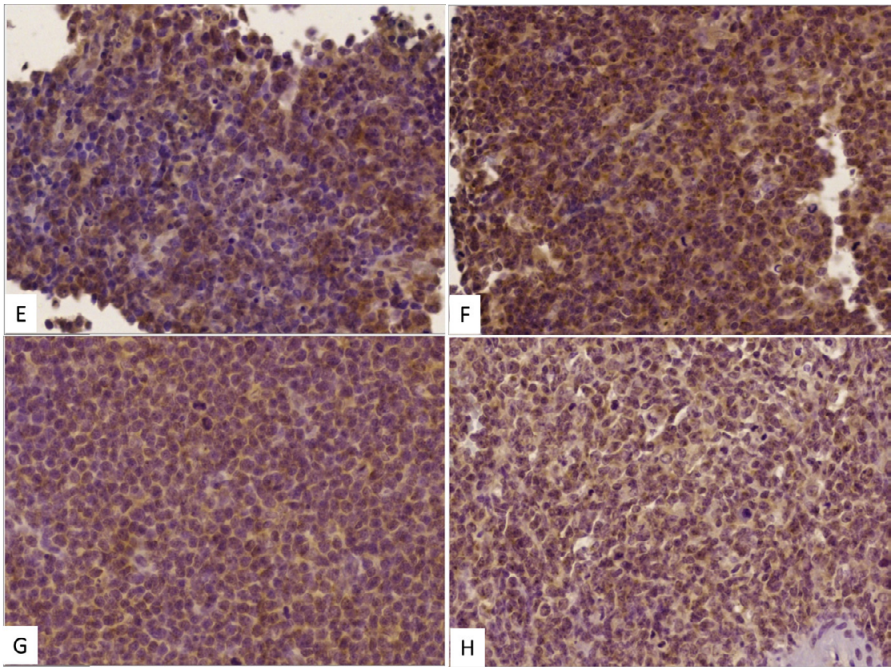


FIGURE 2

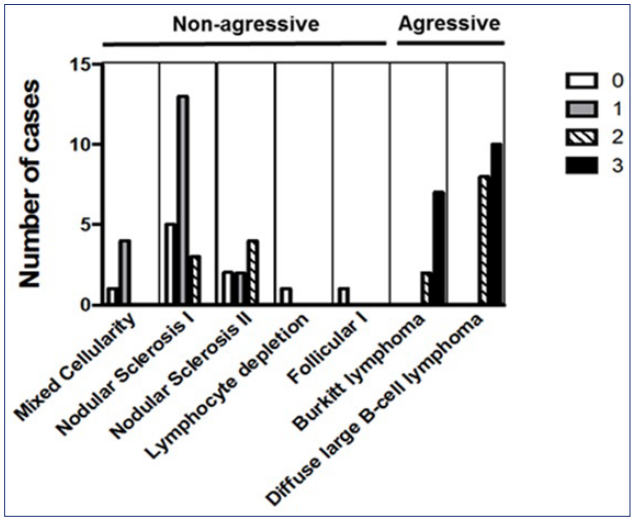


DISCUSSION

In this study, we have shown that strong staining (3) of NOX5 in Hodgkin's and non-Hodgkin's lymphomas can identify aggressive lymphomas and that the absence of staining or weak staining (1) can identify non-aggressive lymphomas.

Only a few articles have studied the expression of NOX in lymphomas. Antony et al.[24] showed for the first time, by tissue microarray analysis, that NOX5 is overexpressed in several human cancers when compared to their adjacent non-tumor tissues. Forty-three samples of non-Hodgkin Lymphomas were analyzed; 24 (56%) were negative (intermediate expression), while 19 (44%) were positive[24]. In this study, there was no distinction between the types of non-Hodgkin lymphomas. In our study, we separated cases that clinically presented as indolent lymphomas from another group with aggressive lymphomas.

FIGURE 3



Carnesecchi et al.[29] demonstrated that ROS derived from NOX5 are involved in blocking apoptosis of anaplastic large cell lymphoma cell lines positive for anaplastic lymphoma kinase (ALK) cells, the authors detected NOX5 mRNA only in ALK + ALCL cells, but not in any other Hodgkin's or non-Hodgkin's lymphoma. In our study, we found 17% and 97, 3% positivity in Hodgkin's and non-Hodgkin's lymphomas, respectively.

Burkitt's lymphoma, another aggressive lymphoma, has been little explored in the literature on NADPH oxidase derivatives. Klingerberg et al.[30] explored the therapeutic response of the NADPH oxidase 4 inhibitor imipramine-blue in the Burkitt cell line and observed decreased viability of cancer in vitro and in vivo in the cells. All 9 cases of Burkit's lymphoma present in our study showed NOX 5 positivity.

To the best of our knowledge, this is the first study that analyzes NOX5 expression in a set of different lymphoma types, according to their aggressiveness. Our data opens new perspectives that could be useful for the prognosis and future treatment of lymphomas.

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Conflicts of Interest

None

Ethics

The study was approved by the institutional research ethics review committee (CAAE 516504415.9.0000.5257).

Author's Contributions

João dos Santos Gonçalves: Selection of samples, morphological evaluation, immunohistochemistry, and text writing; Fabiano Lacerda Carvalho: Selection of samples, morphological evaluation, immunohistochemistry, and text writing; Igor Cabral do Rego Coutinho: Selection of samples, morphological evaluation, immunohistochemistry, and text writing; José Carlos Oliveira Morais: Morphological evaluation, immunohistochemistry, and text revision; Rodrigo S Fortunato: Selection of samples, morphological evaluation, immunohistochemistry, and text writing; Cristiane Bedran Milito: Selection of samples, morphological evaluation, immunohistochemistry, and text revision.

RESUMO

OBJETIVOS: *Os linfomas são um grupo heterogêneo de neoplasias malignas de células linfoides B e NK/T maduras e imaturas em vários estágios de diferenciação. Ferramentas de biologia molecular e genética são usadas para classificar adequadamente o tipo e o prognóstico dos linfomas, os quais têm implicações na eficácia terapêutica. Entre eles, as enzimas nicotinamida adenina dinucleótido fosfato oxidase (NADPH) oxidase (NOX5) foram exploradas. Este estudo analisou a expressão da NADPH oxidase 5 em linfomas de acordo com o grau de agressividade tumoral.*

MÉTODOS: *As lâminas de 64 pacientes com linfoma, que tinham tecido embebido em parafina disponível, foram revisadas por dois patologistas experientes independentemente. Eles utilizaram a classificação da OMS (2017). A expressão de NOX5 nos tecidos foi avaliada por coloração imuno-histoquímica utilizando microarray de tecido. O ensaio foi interpretado com um sistema de pontuação de 0, 1, 2 e 3, para coloração citoplasmática de NOX5 correspondente à coloração negativa, fraca, intermediária e forte, respectivamente. Comparamos a expressão de NOX5 em pacientes com linfomas agressivos versus não agressivos.*

RESULTADOS: *A expressão de NOX5 foi positiva em 100% (27/27) dos linfomas agressivos e em 19% (7/37) dos linfomas não agressivos. Os sete pacientes com expressão positiva de NOX5 apresentaram coloração intermediária (2); coloração forte (3) foi observada apenas em tecidos de linfomas agressivos, e negativos e fracos (0 e 1) observados apenas em linfomas não agressivos.*

CONCLUSÕES: *Linfomas agressivos superexpressam a proteína NOX5. A expressão aumentada de NOX5 em linfomas agressivos pode sugerir um envolvimento dessa enzima na aquisição de um fenótipo agressivo na neoplasia linfoide.*

PALAVRAS-CHAVE: *Linfoma. NADPH oxidase 5. Espécies reativas de oxigênio. Imuno-histoquímica.*

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