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CARACTERIZAÇÃO MOLECULAR E AVALIAÇÃO PROGNÓSTICA DE BIOMARCADORES EM LINFOMA DIFUSO DE GRANDES CÉLULAS B

FORTALEZA
2023

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Tese apresentada ao Programa de Pós-Graduação em Biotecnologia - RENORBIO da Universidade Federal do Ceará para obtenção do título de Doutor em Biotecnologia. Área de concentração: Biotecnologia em Saúde.

Orientador: Silvia Helena Barem Rabenhorst.

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RESUMO

O Linfoma Difuso de Grandes Células B (LDGCB) é o subtipo mais comum entre os linfomas, apresentando comportamento clínico agressivo e resposta terapêutica heterogênea. Propôs-se, assim, a subdivisão do LDGCB em dois subtipos principais a depender da célula de origem da neoplasia, o subtipo centro germinativo (CG) e não-centro germinativo (nCG), apresentando vias patogênicas distintas e aparente evolução clínica diferente. Adicionalmente, biomarcadores como a expressão das oncoproteínas MYC e BCL2 e a presença de alterações cromossômicas envolvendo os genes *MYC*, *BCL2* e *BCL6* têm demonstrado importância na estratificação prognóstica nos LDGCB. Alterações genéticas no gene *TP53*, importante supressor tumoral, e no gene *EZH2,* que codifica uma subunidade do complexo *PRC2 (polycomb repressive complex 2)* atuando como uma metiltransferase, parecem estar associadas à patogênese dos LDGCB, embora o significado clínico dessas alterações seja incerto. Por outro lado, diferentes padrões de expressão de microRNAs, como miR-125b e miR-155b, são descritos por influenciar o comportamento biológico desses linfomas, entretanto, a associação desses miRs com os genes descritos é pouco relatada. Outro aspecto relevante nestes tumores é a presença do vírus de Epstein-Barr (EBV), que parece também determinar diferentes padrões prognósticos a depender das células de origem. Assim, o objetivo deste trabalho foi avaliar a importância patogenética e prognóstica das translocações de *MYC*, *BCL2* e *BCL6*, da expressão proteica de BCL2, MYC, TP53 e EZH2, do perfil de expressão de miR-155b e miR-125b e da presença de EBV, considerando a estratificação dos LDGCB pela célula de origem. O estudo dos rearranjos cromossômicos foi realizado por Hibridização *in situ* com fluorescência (FISH), das expressões proteicas por imuno-histoquímica e da expressão de miRNAs e presença EBV por Hibridização *in situ* com estratégias diferenciadas. Cento e trinta e nove casos fizeram parte desse estudo, sendo o subtipo CG discretamente mais frequente que o nCG (53,9% e 46,1%, respectivamente). Em uma primeira abordagem, EZH2 foi significativamente associado à expressão de MYC e TP53 (p=0.0002 e p=0.0000, respectivamente) e a elevado índice de proliferação celular (Ki-67>70%, p=0.0082). O subtipo nCG apresentou as associações observadas na amostra geral, enquanto apenas a expressão de TP53 foi associada à de EZH2 no grupo CG. Nas análises relacionadas ao miRs, foi observado que a detecção de miR-125b correlacionava-se positivamente com o índice de Ki-67 (p= 0.035) nos nCG. Considerando o subtipo CG, além da associação da positividade de miR-125b com Ki-67>70% (p= 0.043), a porcentagem de células positivas foi também correlacionada tanto à expressão de MYC quanto à dupla expressão de MYC/BCL2 (p= 0.047 e p= 0.049, respectivamente). Analisando os aspectos clínicos, nos pacientes com LDGCB nCG, a porcentagem e a intensidade da marcação de miR-155b, assim como o

escore de *Allred*, foram correlacionados à progressão da doença (p= 0.038, p=0.057 e p= 0.039, respectivamente). Na análise multivariada, o fenótipo CG demonstrou-se como fator prognóstico independente, associado com maior sobrevida geral (*p*=0.007). Considerando o grupo nCG, embora não significante, as expressões de TP53, miR-125b e miR-155b demonstra uma tendência estatística a funcionar como potenciais biomarcadores de prognóstico nesses tumores. Levando em consideração a presença de EBV, este foi positivo em 7,9% dos pacientes. Apesar da baixa frequência, foi observado que a maioria dos casos EBV(+) apresentavam alto índice de proliferação (72,7%), independentemente do subgrupo. Interessantemente, miR-125b foi observado em praticamente todos esses casos, enquanto miR-155b em apenas dois. Embora a expressão de BCL2 tenha sido frequente nos casos EBV(+), a presença de translocação envolvendo BCL2 foi vista em apenas dois casos. Similarmente, a expressão e a translocação de MYC também foram raramente observadas, enquanto a expressão de TP53 foi relativamente comum (45,4%). Em conclusão, as associações significativas entre EZH2, MYC e TP53, em diferentes padrões de acordo com a célula de origem, indicam potenciais vias patogênicas nesses subtipos. A expressão de miR-125b está relacionada a MYC no subtipo nCG. Adicionalmente, TP53, miR125b e miR-155b parecem representar fatores prognósticos potenciais no subgrupo nCG.

Palavras-chaves: linfoma; biomarcadores; microRNA; EBV

ABSTRACT

Diffuse large B-cell lymphoma (DLBCL) is the most common lymphoma in adults, often with aggressive behavior and representing a clinically and genetically heterogeneous disease. Hence, it has been proposed to classify DLBCL in two subsets by the cell-of-origin into germinal center (GC) and non-germinal center (nGC) subtypes, with apparent distinct pathogenic pathways and differences with respect to clinical course. Additional biomarkers, such as MYC and BCL2 expression and chromosomic translocations involving MYC, BCL2 and BCL6, have been given attention as DLBCL prognostic stratification. Alterations involving TP53, an important tumor suppressor gene, and EZH2, which codifies a polycomb repressive complex 2 (PRC2) subunit and acts as a methyltransferase, seem to be associated to DLBCL pathogenesis, but their clinical impact is not well understood. On the other hand, different microRNA expression patterns, such as miR-125b and miR-155b, are described as key influencers on DLBCL biological behavior, however their association with the aforementioned genes are little reported. Another relevant aspect regarding these tumors is the presence of Epstein-Barr Virus (EBV), which also seem to imply on distinct prognostic patterns depending on the cell-of-origin classification. The aim of this study was to evaluate the pathogenetic and prognostic importance of MYC, BCL2 and BCL6 translocations, BCL2, MYC, TP53 and EZH2 protein expression, miR-125b and miR-155b expression profile and the presence of EBV in a series of DLBCL considering the cell-of-origin classification. Chromosomic rearrangements were carried out by fluorescent in situ hybridization (FISH), protein expressions by immunohistochemistry and microRNA detection, as well as the presence of EBV, by in situ hybridization, with special strategies. Among 139 DBCL cases, the GC subtype was slightly more frequent than the nGC (53.9% and 46.1%, respectively). In a first approach, EZH2 positivity was associated to MYC and TP53 expression (p=0.0002 and p=0.0000, respectively) and to high proliferative index (Ki-67>70%, p=0.0082). The nGC DLBCL presented the associations observed in the general sample; however, only TP53 immunodetection showed associations to EZH2 expression in the GC subtype. When we analyzed the microRNAs, miR-125b detection was positively correlated to the Ki-67 index (p= 0.035) in the nGC. Considering the GC subgroup, the percentage of miR-125b positive cells was also correlated to both MYC and MYC/BCL2 double expression (p= 0.047 and p= 0.049, respectively). When it comes to the clinical aspects, in the nGC patients, miR-155b percentage and intensity, as well as Allred score, were positively correlated to disease progression (p= 0.038, p=0.057 and p= 0.039, respectively). In a multivariate analysis, GC phenotype was a significant independent factor associated with higher overall survival (*p*=0.007) and, considering the nCG group, although not significant, the expression of TP53, miR-125b and miR-155b showed statistics trends to be regarded as potentials prognostic biomarkers in

these tumors. Taking in consideration EBV, 7.9% of cases were EBV(+)-DLBCL. Although the number of EBV (+) cases is not high, most of the tumors (72.7%) had a high proliferative index (Ki-67>70%) independently on being GC or nGC subtype. miR-125b was detected in almost all of these cases, and conversely, only two cases were miR155b positive. It is noteworthy that BCL2 expression was observed in most cases, although *BCL2* translocation was a rare event, being detected in only two cases. Similarly, MYC expression and MYC translocation were rarely observed, and TP53, on the other hand, was commonly expressed (45.4%). In conclusion, the significant associations among EZH2, MYC and TP53, with different patterns depending on cell-of-origin classification, indicate potential pathogenetic pathways in these subtypes. miR-125b expression is related to MYC in nGC-DLBCL. In addition, TP53, miR-125b and miR-155b seem to represent potential prognostic factors in the nGC subtype.

Key words: lymphoma; biomarkers; microRNA; EBV

SUMÁRIO

1 INTRODUÇÃO

Os linfomas não-Hodgkin (LNH) são malignidades histologicamente e molecularmente heterogêneas originadas dos linfócitos. Dentre estes tumores, o subtipo mais comum é o Linfoma Difuso de Grandes Células B (LDGCB), perfazendo aproximadamente 31% dos diagnósticos de LNH, segundo o estudo multicêntrico (THE NON-HODGKIN'S LYMPHOMA CLASSIFICATION PROJECT, 1997). Estimativas mundiais de 2020 mostram que ocorreram, aproximadamente, 540 mil casos novos de linfoma não-Hodgkin, o equivalente a 3% do total dos casos novos estimados. Destes, 304 mil casos novos ocorreram em homens, com um risco estimado de 7,3 por 100 mil, e, nas mulheres, houve 240 mil casos novos, com um risco estimado de 5,2 por 100 mil. As maiores taxas de incidência encontram-se na Austrália, na Nova Zelândia, na América do Norte e nos países do Norte da Europa, em ambos os sexos (GLOBOCAN, 2020). O LDGCB acomete discretamente mais homens que mulheres (relação 1,2:1), com média de idade ao diagnóstico de 64 anos, embora qualquer idade possa ser acometida (JAFFE *et al*, 2017). No Brasil, os linfomas LNH correspondem à oitava neoplasia mais frequente nos homens e à nona neoplasia mais frequente em mulheres, com aproximadamente 12 mil novos casos diagnosticados por ano. Por razões ainda desconhecidas, a incidência desses linfomas dobrou nos últimos 25 anos, principalmente entre pacientes acima de 60 anos. A mortalidade por esta doença é alta, sendo de, aproximadamente, 39% nos cinco primeiros anos do diagnóstico (INCA, 2020).

O LDGCB apresenta comportamento clínico agressivo e, embora uma boa parcela dos pacientes diagnosticados com LDGCB possa ser curada com o esquema quimioterápico padrão baseado em rituximab, ciclofosfamida, doxurrubicina, vincristina e prednisona (R-CHOP), a resposta terapêutica pode ser imprevisível, com muitos pacientes apresentando desfecho desfavorável (LENZ *et al*, 2008). Na prática clínica, o Índice Internacional Prognóstico (IPI) tem sido usado largamente como a ferramenta primária para predizer prognóstico nos pacientes com LNH agressivos e com resposta negativa aos tratamentos, sendo baseado em cinco critérios: idade, estadiamento, níveis sérios de Lactato Desidrogenase (LDH), Performance Status (PS) e número de sítios de acometimento extranodal (LARRABEITI-ETXEBARRIA *et al ET AL*, 2019). Embora seja um sistema reprodutível e facilmente aplicado para estratificação clínica, o IPI não considera as

características biológicas do tumor necessárias para abordagem individualizada dos pacientes (CAO *et al*, 2016).

1.1 Etiologia

A maior parte dos pacientes com LDGCB não apresenta fatores de risco definidos. Raros casos estão relacionados a síndromes de imunodeficiências primárias ou adquiridas, transplante de órgãos ou uso de medicações, como metotrexato e fludarabina. Adicionalmente, uma minoria de casos extranodais pode estar associada à inflamação ou irritação crônica (JAFFE *et al*, 2017).

A maioria dos LDGCB desenvolve-se *de novo*, no entanto, alguns casos são transformações de um linfoma de baixo grau de base que progrediu, como o linfoma folicular, leucemia linfocítica crônica/linfoma linfocítico de pequenas células, linfoma da zona marginal, linfoma linfoplasmocítico ou linfoma de Hodgkin com predominância linfocitária nodular. Raramente, alguns casos de LDGCB podem ainda ocorrer sincronicamente ou metacronicamente com linfoma de Hodgkin clássico (FREEDMAN, 2019).

1.2 Características clínicas

Pacientes com LDGCB frequentemente apresentam uma massa sintomática de crescimento rápido, geralmente um aumento nodal no pescoço ou abdome ou, no caso de linfoma mediastinal primário de grandes células B, no mediastino, mas podem apresentar uma massa em qualquer parte do corpo. O local mais comum de doença primária extranodal é o trato gastrointestinal, especialmente o estômago, mas a doença pode surgir em praticamente qualquer tecido, incluindo testículo, osso, tireoide, glândulas salivares, pele, fígado, mama, suprarrenais, rins, cavidade nasal, anexos oculares, seios paranasais, colo uterino, vagina e sistema nervoso central (FREEDMAN; FREIDBERG; ASTER, 2022).

Sintomas sistêmicos "B" (ou seja, febre, perda de peso, sudorese noturna intensos) são observados em, aproximadamente, 30% dos pacientes, estando normalmente associados à doença agressiva, e a LDH está elevada em mais da metade deles (FREEDMAN; ASTER, 2020). Aproximadamente 60% dos casos são diagnosticados em estágios avançados (III/IV), cujo estadiamento é realizado

baseando-se no sistema de *Ann Arbor*, que leva em consideração o número de cadeias linfáticas e a localização como principais critérios classificatórios. Apesar do tamanho do tumor não determinar alteração no estadiamento, massas iguais ou maiores a 10cm avaliadas por tomografia computadorizada são chamadas de massa "bulk" e parecem estar relacionadas a pior prognóstico (AJCC, 2018). A determinação da *Performance Status (PS)* da *Eastern Cooperative Oncology Group (ECOG)* é outro aspecto clínico relevante, que descreve o nível de funcionalidade do paciente em termos de habilidades de autocuidado, atividade diária e integridade física (Tabela 1) (OKEN *et al*, 1982).

LDGCB é ainda uma doença definidora de AIDS. Quando comparado com o linfoma na população HIV-negativa, o linfoma na população HIV-positiva é caracterizado por sintomas B mais frequentes, doença extranodal, envolvimento de locais incomuns (como cavidades corporais, reto, partes moles) e estágio avançado ao diagnóstico (FREEDMAN, 2019).

Tabela 1 – Descrição dos graus de PS da ECOG

Grau da PS - ECOG	**Descrição**
1	Assintomático.
2	Sintomático, mas capaz de realizar todas suas atividades normalmente.
3	Sintomático, restrito ao leito <50% do dia.
4	Sintomático, restrito ao leito >50% do dia.
5	Morto

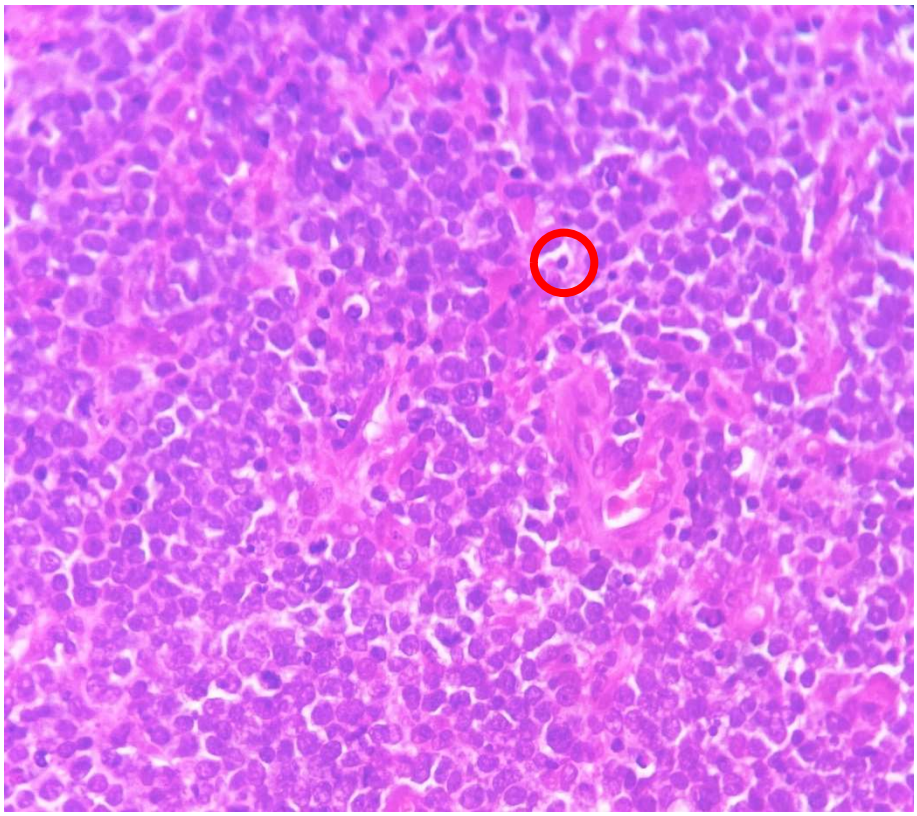
Fonte: elaborada pelo autor.

1.3 Diagnóstico

Os LDGCB são heterogêneos dos pontos de vista clínico, morfológico e biológico (NOGAI; DÖRKEN; LENZ, 2011). Essa entidade é histopatologicamente definida como uma proliferação linfoide atípica difusa formada por células de

tamanho igual ao de um núcleo de histiócito ou maior que o tamanho do núcleo de dois linfócitos normais (Figura 1). Essa definição ampla abrange, consequentemente, um grupo de diversos padrões morfológicos (JAFFE *et al*, 2017). O diagnóstico patológico do LDGCB é, assim, baseado na morfologia e na imunofenotipagem. A imunoexpressão de marcadores pan-B, como CD20 e CD79a, é suficiente para estabelecer o diagnóstico em muitos casos, mas um conjunto muito mais amplo de marcadores imuno-histoquímicos pode ser necessário em casos com características morfológicas atípicas.

Figura 1 – Corte histológico corado em hematoxilina-eosina (HE) representando caso de LDGCB (40x). Note a comparação do tamanho das células neoplásicas com o linfócito normal circulado em vermelho.

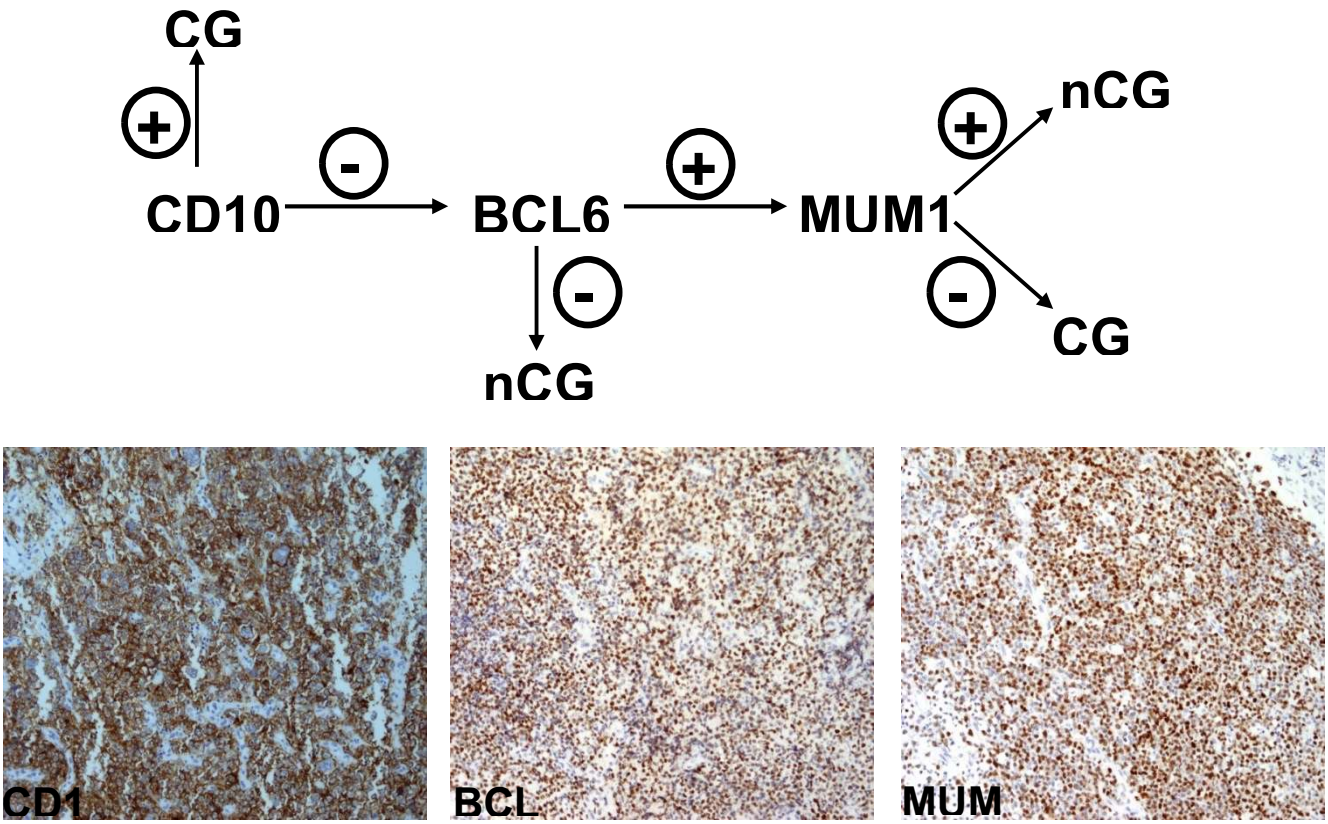


Fonte: elaborada pelo autor.

Nos últimos anos, estudos de perfis de expressão gênica foram realizados (ALIZADEH *et al*, 2000; CAO *et al*, 2016; LENZ *et al*, 2008) e dividiram os LDGCB em três subgrupos, de acordo com a célula de origem: os LDGCB de origem em centro germinativo (GCB), LDGCB de origem em células ativadas (ABC) e Linfoma B difuso Primário do Mediastino (LBPM). Essas subdivisões sugerem que a célula

originária do tumor deriva de células B em diferentes estágios de maturação e apresentam comportamento clínico significativamente diferente (NOGAI; DÖRKEN; LENZ, 2011), justificando o alto índice de mortes decorrentes dessa neoplasia apesar do tratamento, motivando estudos para a compreensão da patogênese dos LDGCB e para a identificação de marcadores preditivos dentro desses subgrupos. A aplicação de técnicas de sequenciamento genético para traçar esses perfis de expressão gênica, entretanto, está restrita a grandes centros, tornando esse método de classificação pouco aplicável na prática diagnóstica. Nesse sentido, protocolos de perfis de expressão proteica, avaliados por imuno-histoquímica, têm sido desenvolvidos como alternativa. Dentre eles, um dos mais usados é o algoritmo de Hans, que se baseia no perfil de expressão de CD10, BCL6 e MUM1 (Figura 2) e subdivide o LDGCB em tipo centro germinativo (CG) e não-centro germinativo (nCG), demonstrando boa correlação com os subtipos moleculares GCB e ABC, respectivamente. O valor prognóstico da utilização desses algoritmos, entretanto, ainda é contraditório (JAFFE *et al*, 2017).

Figura 2 – Esquematização do algoritmo de Hans



Fonte: elaborada pelo autor.

1. 4 Biomarcadores prognósticos

1.4.1 Rede MYC e TP53

Um avanço significativo na compreensão da patogênese dos LDGCB foi obtido em casos nos quais os rearranjos do gene *MYC* estão presentes. *MYC* é um fator de transcrição que atua em várias vias celulares, incluindo a proliferação celular, sendo rearranjado entre 5% a 15% dos casos de LDGCB. Frequentemente, a translocação envolvendo *MYC* é associada a outras translocações, sendo a mais frequente com a de *BCL2*, gene chave na regulação da apoptose e, em menor frequência, com *BCL6*, fator de transcrição atuante no processo de maturação linfocitária em centro germinativo. Linfomas com recorrentes quebras cromossômicas ativando múltiplos oncogenes, um dos quais o *MYC*, são referidos como linfomas *"double-hit"* ou *"triple-hit"*, incluídos na atualização de 2016 da classificação da Organização Mundial da Saúde (OMS) das doenças hematológicas (JAFFE *et al*, 2017; SWERDLOW *et al* , 2016).

A expressão imuno-histoquímica de MYC também tem sido explorada nos LDGCB, sendo detectada entre 30 e 50% dos casos, com a concomitância da expressão com BCL2 encontrada entre 20 e 35% dos casos. Entretanto, a maioria desses tumores não possui alterações cromossômicas de *MYC* ou de *BCL2*, sendo chamados, então, de "linfomas duplo-expressores", apresentando melhor comportamento do que os linfomas *"double-hit"*, porém, pior sobrevida em comparação ao LDGCB padrão. Essas observações sugerem que a dupla expressão de *MYC* e de BCL2 deve ser considerada indicador prognóstico nos LDGCB, embora não defina nova categoria diagnóstica (JOHNSON; SLACK; SAVAGE, 2012; SWERDLOW, 2014, 2016). A definição dessas alterações, entretanto, é ainda insuficiente para explicar a heterogeneidade clínica da doença, de modo que a avaliação de outros marcadores de promissor valor prognóstico e terapêutico é fundamental.

Interessantemente, observa-se que os mecanismos regulatórios envolvendo *MYC* incluem repressão transcripcional relacionada a complexos proteicos nos quais a proteína EZH2 é subunidade enzimática. EZH2 é uma metiltransferase que leva à trimetilação da lisina 27 da histona 3 (H3K27me3), uma modificação pós-tradução que culmina com a repressão dos complexos proteicos associados às vias de *MYC*. Adicionalmente, a expressão de EZH2 é uma característica comum de várias

neoplasias hematológicas, mas, em LDGCB, os mecanismos pelos quais essa expressão é regulada e suas associações com características clínico-patológicas e com a resposta terapêutica ao R-CHOP ainda são mal compreendidos (TIAN *et al*, 2016; TIAN; DORFMAN, 2019).

Estudos recentes envolvendo sequenciamento de última geração identificaram diversas mutações somáticas comuns em todos os grupos de LDGCB, mas também perfis de alterações genéticas diferentemente representados nos subtipos GCB e ABC. Entre as anomalias comumente observadas em ambos os subtipos, destacam-se as alterações em *TP53* (PASQUALUCCI; DALLA-FAVERA, 2015; SWERDLOW, 2016). Mutações no gene *TP53* e imunorreatividade para a proteína TP53 ocorrem entre 22% e 40% dos casos de LDGCB, respectivamente, e não há uma correlação estrita entre os dois fenômenos. O papel de *TP53* na gênese dos LDGCB, entretanto, é pouco compreendido (JAFFE *et al*, 2017; PASQUALUCCI; DALLA-FAVERA, 2015; SWERDLOW, 2016).

O supressor tumoral *TP53* age regulando diversos genes-alvo, coordenando as principais defesas celulares contra o crescimento tumoral e promove reparo do DNA, apoptose, controle do ciclo celular e senescência. Assim, mutações em *TP53* levam à desregulação do ciclo celular, à instabilidade genômica e à perda do controle da proliferação celular. Interessantemente, mutações em *TP53* que alteram a função da proteína TP53 têm sido descritas em mais de 50% dos tipos de câncer (TOLEDO; WAHL, 2006; VENTURA *et al*, 2007; XUE *et al*, 2007). Nesse sentido, uma coorte multicêntrica envolvendo 506 pacientes demonstrou que mutações na região codificante de *TP53* estão relacionadas à pior sobrevida em pacientes com LDGCB tratados com esquema R-CHOP tanto do subtipo CGB quanto ABC, sugerindo que a avaliação do *status* de *TP53* pode ser útil para estratificar pacientes em tratamento com R-CHOP em grupos prognósticos distintos (XU-MONETTE *et al* , 2012).

1.4.2 MicroRNAs

Entre os biomarcadores estudados na última década com potencial prognóstico em LDGCB, incluem-se os microRNAs (miRNA). Essas moléculas são sequências curtas de 20 a 24 nucleotídeos não-codificantes que podem controlar a expressão gênica principalmente a nível pós-transcrição. Assim, o miRNA tem um

papel crucial em diversos processos biológicos como a diferenciação celular e a homeostase, o que pode se estender para a iniciação e progressão tumoral e resposta ao tratamento. A desregulação dos miRNA está presente em muitos cânceres, incluindo os LDGCB, podendo agir como oncogene ou supressora tumoral dependendo do tipo celular ou do microambiente (JØRGENSEN *et al* , 2015; NI *et al,* 2016).

Em 2009, Li e colaboradores, utilizando gene chip de microRNA como método para analisar a expressão tecidual de 59 pacientes com LDGCB e de 26 tipos de linhagens celulares de LDGCB, identificaram 63 microRNAs envolvidos no desenvolvimento e na progressão desse linfoma. O miR-155 foi encontrado significativamente superexpresso em LDGCB, onde pacientes LDGCB ABC possuem maior expressão se comparados aos GCB. O papel central de miR-155 na patogênese e na agressividade dos LDGCB tem sido relacionado à ativação das vias de *MYC*, já que a expressão de antagonistas dessa via é diretamente inibida por miR-155 (LAWRIE, 2013). Adicionalmente, estudos apontam a regulação da expressão do gene *TP53* por miRs. Dentre os estudados, o miR-125b parece inibir diretamente a ação de *TP53* quando superexpresso em LDGCB (KUMAR *et al*, 2011; LAWRIE *et al*, 2009). Outro estudo nesse sentido, associou o miR-125b à patogênese de neoplasias hematológicas em modelos de camundongos (ENOMOTO *et al*, 2011) (Figura 3).

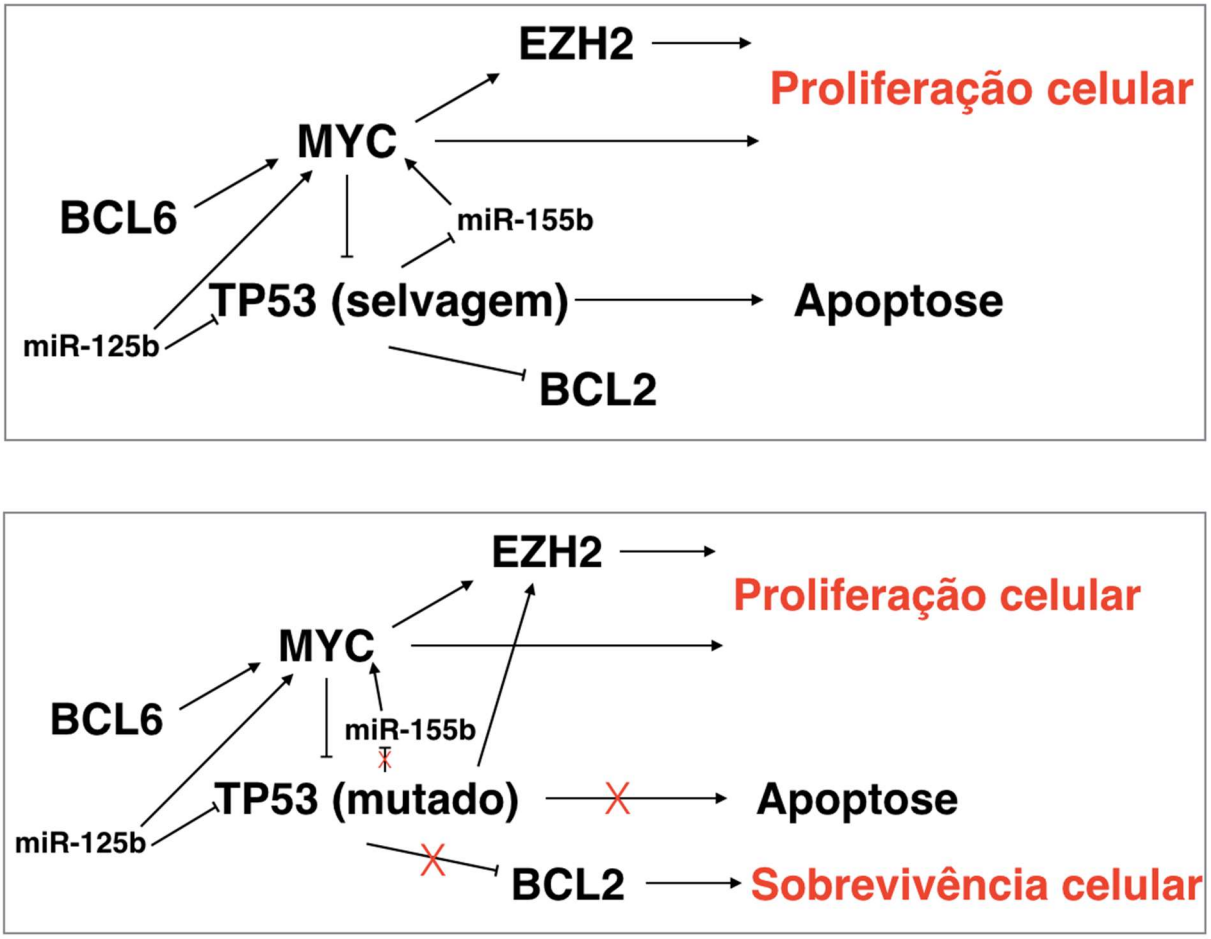


Figura 3 - Esquema representativo da interação dos biomarcadores citados.
Fonte: elaborada pelo autor.

1.4.3 *Vírus de Epstein-Barr (EBV)*

O vírus de Epstein-Barr (EBV) foi descrito há, aproximadamente, 50 anos em LNH e tem sido demonstrado que 90% da população adulta é portadora do vírus na circulação sanguínea de forma assintomática. Em LDGCB, a presença de EBV está fortemente associada a pacientes imunossuprimidos (JAFFE *et al*, 2017). Embora descrita como baixa em pacientes imunocompetentes, a prevalência de EBV afeta principalmente idosos e é variável geograficamente, com estudos europeus apontando uma positividade de aproximadamente 2% e estudos latino-americanos com taxas próximas às de estudos asiáticos (9%) (HOFSCHEIER*et al* , 2011; HOELLER *et al*, 2009; UNER *et al*, 2011; PARK *et al*, 2007). Interessantemente, a

positividade de EBV tem sido associada à deterioração clínica mais rápida, à resposta terapêutica pobre e à menor sobrevida global e sobrevida livre de doença nesses pacientes (PARK *et al*, 2007). Estratificando os subtipos de LDGCB de acordo com a célula de origem, um estudo coreano apontou ainda uma pior sobrevida global nos pacientes com o subtipo ABC quando associados à positividade para EBV (PARK *et al*, 2007). Estudo recente em pacientes imunossuprimidos pós-transplante demonstrou, inclusive, que o perfil mutacional de *TP53* em LDGCB varia de acordo com a presença ou não de EBV, o que aponta a necessidade de um melhor entendimento da influência viral na patogênese desses linfomas (COURVILLE *et al*, 2016).

Diante do exposto, fica clara a importância de se estudar as alterações em LDGCB de acordo com os subtipos. Frente à necessidade de marcadores prognósticos, o objetivo deste estudo foi investigar a expressão de EZH2 e o perfil de expressão de miR-155 e miR-125b, além de suas associações com as apresentações imunofenotípicas, incluindo a expressão de MYC, BCL2 e TP53 e os rearranjos cromossômicos envolvendo *MYC*, *BCL2* e *BCL6*, características clínico-patológicas e resposta terapêutica a R-CHOP em uma série de LDGCB.

2 HIPÓTESE

As translocações de *MYC, BCL2* e *BCL6*, a expressão proteica de TP53, EZH2, BCL2 e MYC, o perfil de expressão dos miRNAs miR-155b e miR-125b e a presença de EBV determinam padrões prognósticos diferenciados nos diferentes tipos moleculares de LDGCB.

3 OBJETIVO GERAL

Avaliar a importância prognóstica das translocações de *MYC, BCL2* e *BCL6*, da expressão proteica de TP53, EZH2, BCL2 e MYC, do perfil de expressão de miR-155b e miR-125b e da presença de EBV nos diversos subtipos moleculares de LDGCB.

4 OBJETIVOS ESPECÍFICOS

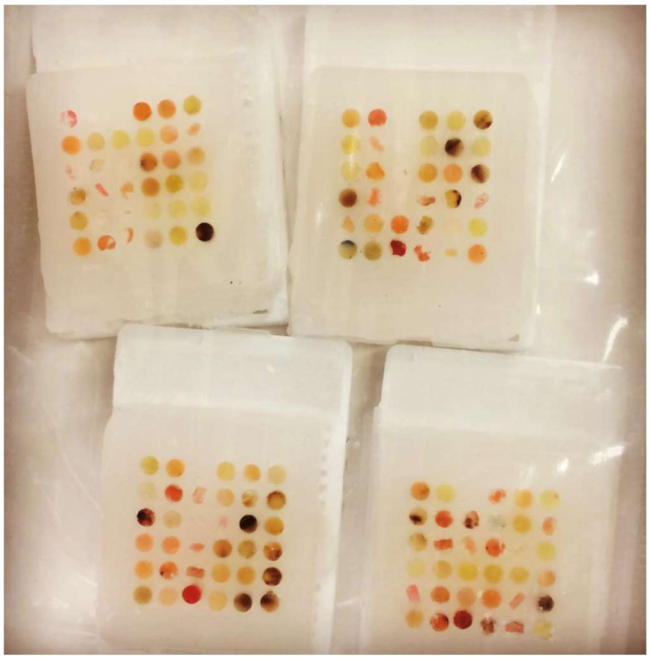
1. Associar a expressão proteica de MYC, BCL2 e EZH2 nos casos de LDGCB de acordo com a célula de origem e com a presença das translocações envolvendo *MYC*, *BCL2* e *BCL6*;
2. Analisar a expressão proteica de TP53 de acordo com os dados clínicos e histopatológicos;
3. Traçar o perfil de expressão de miR-125b e miR-155 nos casos de LDGCB;
4. Determinar a frequência da infecção por EBV nos casos de LDGCB, correlacionando os dados clínico-patológicos com os dados encontrados;
5. Avaliar a importância prognóstica e o potencial terapêutico dos dados encontrados.

5 METODOLOGIA

Trata-se de um estudo longitudinal, retrospectivo, não-intervencionista, cuja população de estudo constou inicialmente de 263 pacientes acompanhados do Instituto do Câncer do Ceará (ICC) com diagnóstico de LDGCB entre os anos de 2011 e2017, com média de seguimento de 130 meses, aprovado no comitê de ética e pesquisa local sob CAAE 66308017.8.0000.5528. Foram excluídos do estudo os pacientes cujo material anátomo-patológico não estivesse arquivado no Serviço de Patologia do ICC ou cuja amostragem fosse exígua, totalizando 139 casos disponíveis para estudo. Cortes histológicos do espécime cirúrgico corados em hematoxilina-eosina (HE) foram utilizados para avaliação histopatológica, sendo aplicada a classificação revisada da OMS de 2016 e estabelecido o estadiamento clínico de *Ann Arbor* (SWERDLOW, 2014, 2016). Adicionalmente, foi aplicado o algoritmo definido por Hans para distinção entre os subtipos moleculares de acordo com a célula de origem (JAFFE *et al*, 2017). Para avaliação dos marcadores estudados, foram construídos blocos de microarranjo de tecido (TMA, *tissue microarray*), no qual áreas representativas de cada caso foram selecionadas microscopicamente com um *punch* de 2,0mm usando o sistema *Manual Tissue Microarrayer Quick-Ray™ system* (UNITMA Co., Bokjeong-ro, Korea), de acordo com orientações do fabricante (Figura 4). A maioria dos pacientes foi submetida a pelo menos quatro ciclos de R-CHOP ou a protocolos R-CHOP-símiles (R-

miniCHOP e R-COP). A resposta primária ao tratamento (RC, resposta completa; RP, resposta parcial; DE, doença estável e PD, progressão de doença) e o seguimento dos pacientes foram avaliados de acordo com os critérios recomendados (CHESON, 2007).

Figura 4 – Foto representativa dos blocos de TMA construídos para realização das análises de biomarcadores



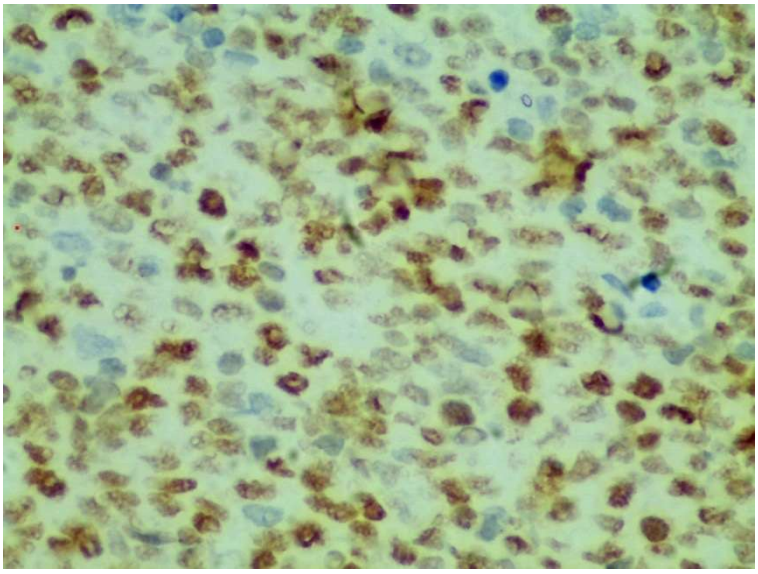
Fonte: elaborado pelo autor.

5.1 *Expressão de* MYC, BCL2, TP53 e EZH2

A expressão desses marcadores foi avaliada centralmente por imuno-histoquímica. Essa técnica foi realizada em cortes histológicos de 2µm dos blocos de TMA usando EZH2 (*Clone* 11, *dilution* 1:50, *Cell Marque*™, Darmstadt, Germany), *MYC* (*Clone* Y69, *dilution* 1:50, *Abcam*, Shanghai, China), TP53 (DO-7, *ready-for-use*, *DAKO*™, Glostrup, Denmark) e BCL2 (*Clone* 124, *ready-for-use*, *DAKO*™). Após desparafinização e recuperação de epítopos utilizando *PT Link*™ *DAKO*, os cortes foram incubados através do *AutostainerLink*48™ *DAKO* e visualizados utilizando polímero *Envison Flex*™ *DAKO*. Controles positivos e negativos foram utilizados para atestar a fidelidade das reações.

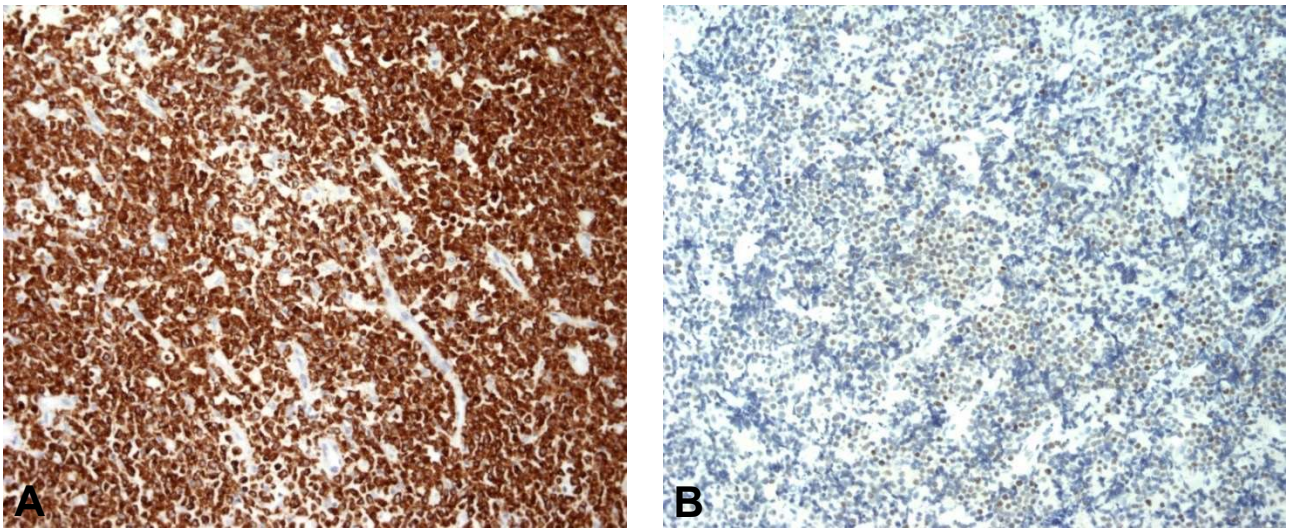
Os valores de positividade foram os seguintes: MYC ≥ 40%; BCL2 e TP53 ≥ 50%; e EZH2 ≥ 70% (Figuras 5 e 6), como previamente descrito (LIU, 2017; XU-MONETTE, 2012). Quando positivas, a intensidade de marcação de TP53 e EZH2 foram classificadas em fraca, moderada e forte (Figura 7) (FEDCHENKO; REIFENRATH, 2014).

Figura 4 – Corte histológico representativo de caso positivo para a expressão de EZH2 (40x)



Fonte: elaborada pelo autor.

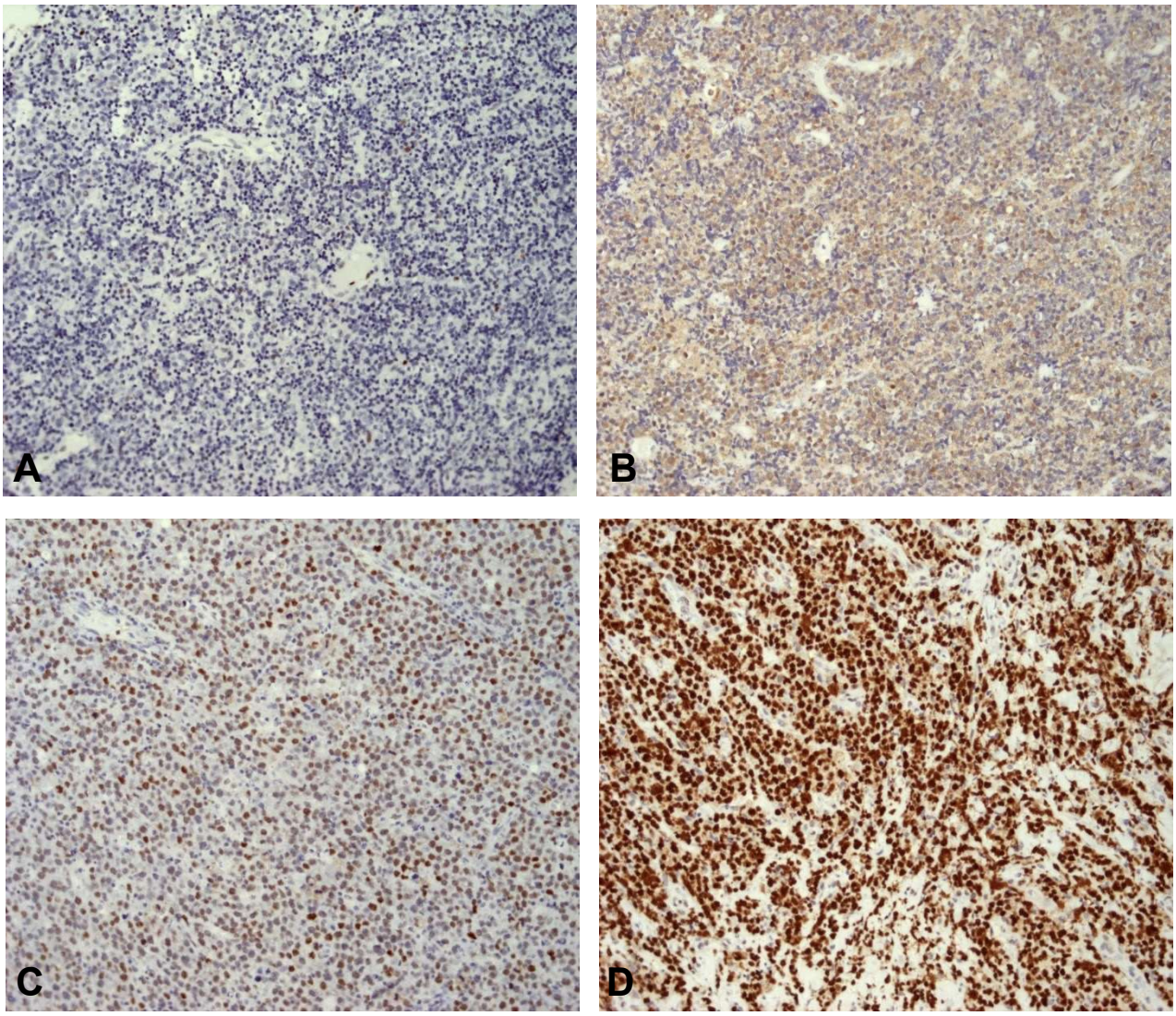
Figura 5 – Corte histológico representativo de caso positivo para a expressão de (A)



BCL2 e (B) MYC (20x)

Fonte: elaborada pelo autor.

Figura 7 – Corte histológico representativo de casos com marcação (A) negativa, (B)



fraca, (C) moderada e (D) forte para a expressão de TP53 (20x)

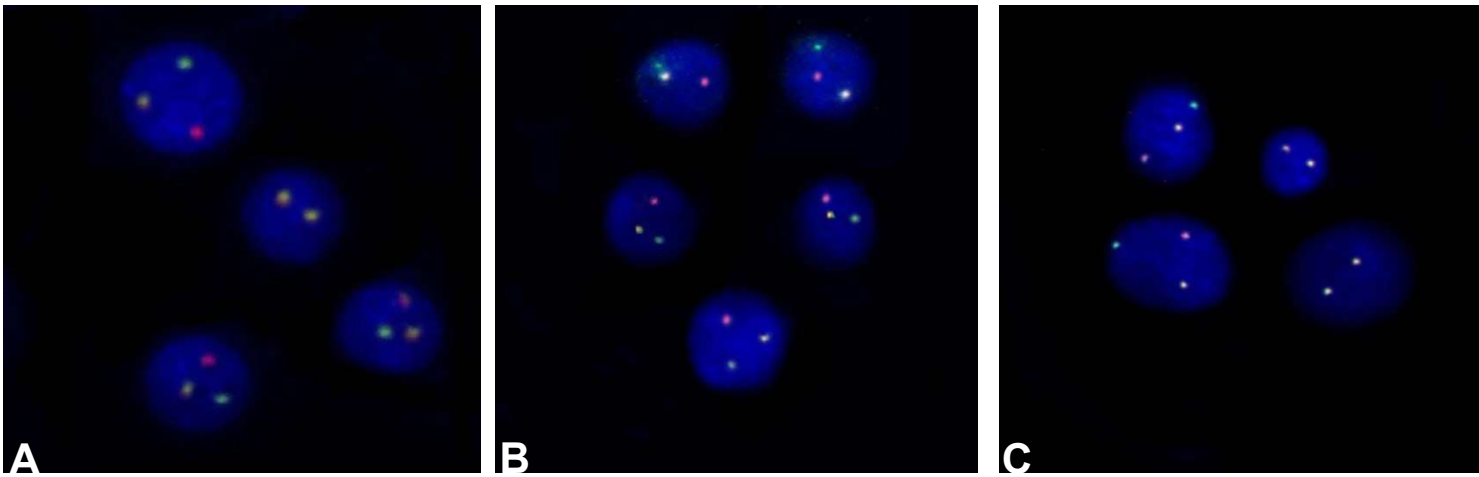
Fonte: elaborada pelo autor.

5.2 *Translocações em BCL2, BCL6 e MYC*

As translocações envolvendo *MYC*, *BCL2* e *BCL6* foram identificadas por Hibridização *in situ* Fluorescente (FISH). Essa técnica foi realizada em cortes de 2,0µm dos blocos de TMA usando sondas de dupla coloração do tipo *break-apart* (MYC/8q24 e BCL6/3q27) e *dual-color fusion* (BCL2/18q21) (*Abbott Laboratories*, Des Plaines, IL) como previamente descrito (HORN *et al*, 2013). As células foram hibridizadas com sondas de *MYC*, *BCL2* e *BCL6* e os núcleos foram contracorados

com 4',6-diamidino-2-fenilindol *antifade*. A fluorescência foi detectada utilizando-se de microscópio de fluorescência *Olympus* BX41 (*Olympus*™, Japão) com filtros de excitação para 4',6-diamidino-2-fenilindol (260 nm) e rodamina (570 mn). Para cada caso, até 200 núcleos em intérfase foram analisados através do sistema *ASI image analysis* (*Applied Spectral Imaging*™, Israel) (Figura 8).

Figura 8 – Corte histológico com fluorescência representativo de casos positivos para translocação em (A) MYC, (B) BCL2 e (C) BCL6



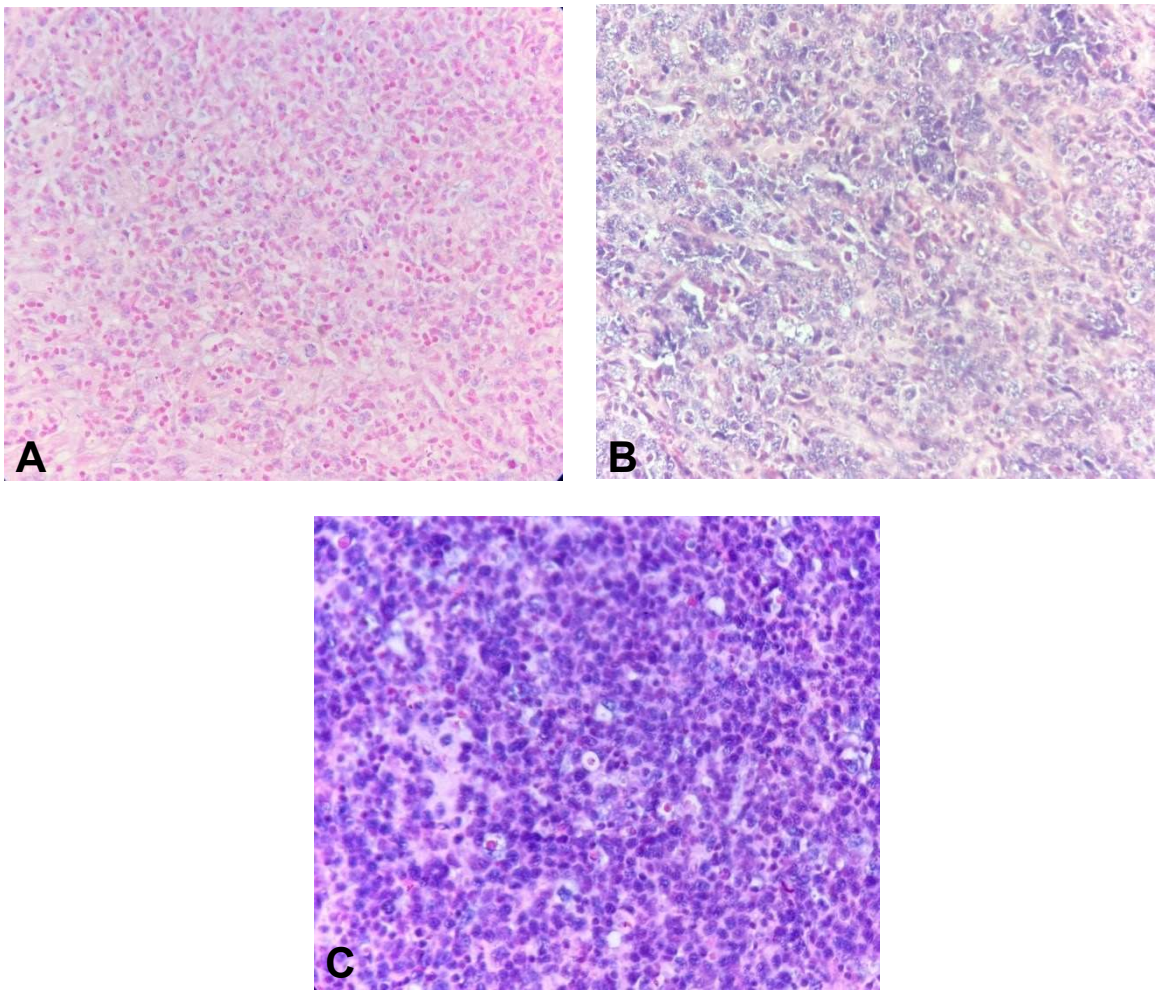
Fonte: elaborada pelo autor.

5.3 Determinação da expressão de miR-125b e miR-155

A expressão dos miRNAs estudados foram avaliadas por Hibridização *in situ*, utilizando-se diferentes sondas de detecção de miRNA de ácido nucleico bloqueado com mercúrio (LNA) (*Exiqon*, Vedbaek, Dinamarca). Essa metodologia é pouco utilizada na pesquisa de expressão de miRNAs, porém é facilmente aplicável à rotina de um laboratório de anatomia patológica e a tecnologia das sondas de LNA garante especificidade de detecção (FAN, 2012). Resumidamente, cortes de 2,0µm dos blocos de TMA foram submetidos a tratamento com proteinase K (15mg/mL) por 10 minutos e posteriormente à hibridização a 53ºC durante 90 minutos com sondas hsa-miR-125b-5p (40nM em um tampão ISH livre de formamida), hsa-miR-155-5b (60nM em um tampão ISH livre de formamida), sonda U6 (1nM, como controle positivo) e sonda "scramble miRNA" (pronta para uso, como controle negativo) marcadas com digoxigenina (DIG). Posteriormente, realizaram-se lavagens rigorosas com tampões de SSC a 53ºC e à temperatura ambiente. Todos os cortes

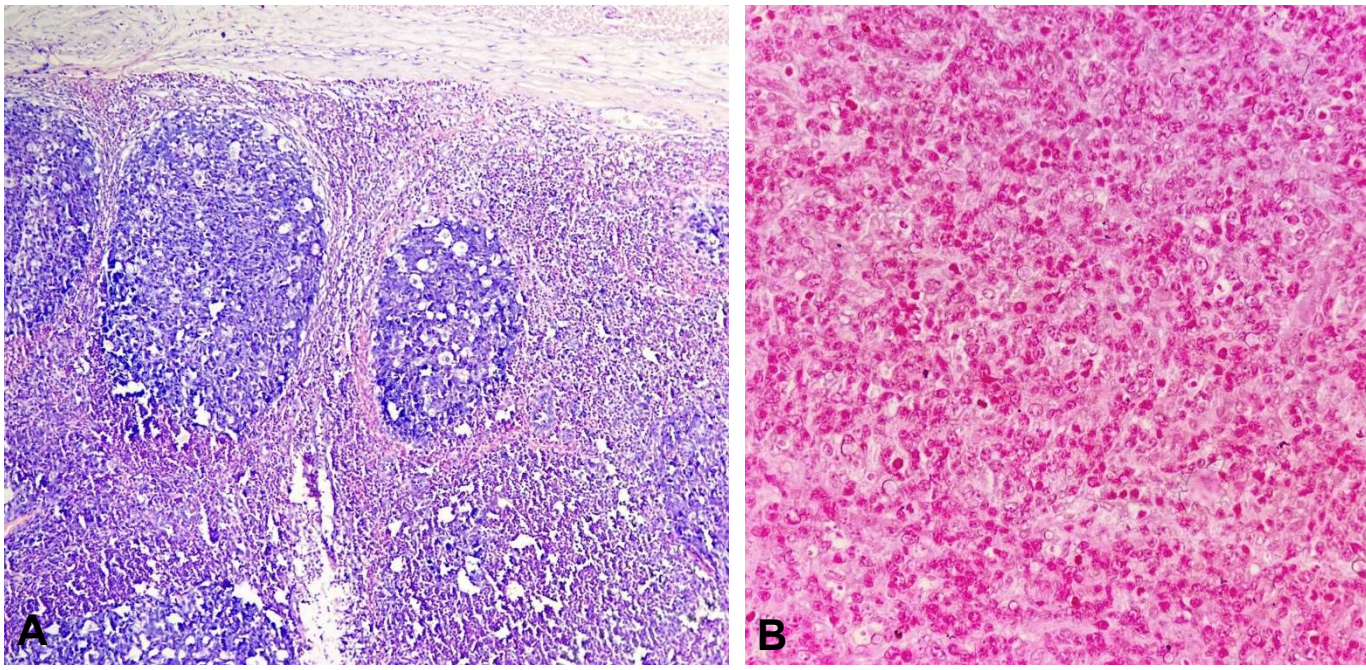
foram incubados com anticorpos anti-DIG (Roche, Alemanha) e submetidos ao método da fosfatase alcalina para revelação com NBT-BCIP e contracoloração com *Fast-Red*, conforme descrito previamente (MUNCH-PETERSEN *et al*, 2015). Consideraram-se positivos os casos com pelo menos 30% das células marcadas de azul em contraste com a contracoloração avermelhada e a intensidade da marcação foi classificada como fraca, moderada ou intensa (Figura 9). Tecido linfoide não neoplásico (tonsila) de quatro pacientes diferentes foi incubado com sondas U6 e "*scramble* miRNA" e utilizado como controle positivo e negativo, respectivamente, das reações (Figura 10). Adicionalmente, as sondas hsa-miR-125b-5p e hsa-miR-155-5b também foram testadas nestes casos a título de comparação com a marcação em amostra neoplásica. Avaliou-se, ainda, o escore de *Allred* para cada caso, como previamente descrito (SARIKOC *et al*, 2013).

Figura 9 – Corte histológico representativo de casos com marcação (A) fraca, (B) moderada e (C) forte para a expressão de miR-125b (40x)



Fonte: elaborada pelo autor.

Figura 10 – Corte histológico representativo de tonsila incubada com (A) sonda U6 (controle positivo) e (B) “scramble RNA” (controle negativo) (20x)

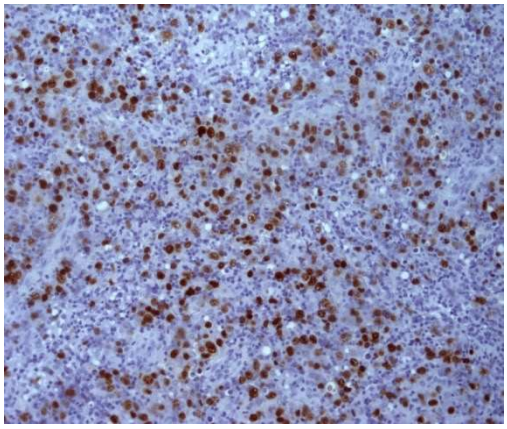


Fonte: elaborada pelo autor.

5.4 Identificação da presença de EBV

A presença de EBV foi avaliada através da técnica de Hibridização *in situ* do RNA usando sondas biotiniladas de 30bp (5’-AGACACCGTCCTCACCACCCGGGACTTGTA-3') complementares ao RNA EBER1, de forma a demonstrar o transcrito de EBV em grande quantidade nos núcleos de células latentemente infectadas, conforme previamente descrito (Figura 11) (FERRASI *et al*, 2010).

Figura 11 – Corte histológico representativo de caso positivo para a expressão de EBER1 (20x**)**



Fonte: elaborada pelo autor.

5.5 Análise estatística

A análise dos dados foi avaliada através do software *SPSS statistics* 17.0 (*SPSS Inc.*, Chicago, IL, USA). Diferenças estatisticamente significativas foram analisadas pelo teste do χ2 e pelo teste exato de Fisher. Curvas de sobrevida foram analisadas pelo método de Kaplan-Meier e a análise de regressão de Cox foi utilizada para avaliação do valor prognóstico. Adicionalmente, os dados não-paramétricos foram analisados pelo coeficiente de correlação de Spearman. A significância estatística foi dada por $p<0,05$.

6 RESULTADOS

6.1 População de estudo

Cento e trinta e nove casos fizeram parte da análise, sendo 52,5% do sexo masculino (73/139) com idade mediana de 61,0 anos, variando de 21 a 98 anos. A maioria dos pacientes apresentou doença nodal (64,0%, 89/139) e, entre aqueles com acometimento extranodal, o trato gastrointestinal foi o sítio mais frequente (34%, 17/50). Levando em consideração a classificação de Hans para a célula de origem, o subtipo centro germinativo foi mais frequente que o não-centro germinativo (53,9%, 75/139 e 46,1%, 64/139, respectivamente). Adicionalmente, EBV foi detectado em onze pacientes (7,9%). A distribuição de características clínicas na população de estudo está resumida na TABELA 2.

Tabela 2 – Distribuição das características clínicas de acordo com o subtipo.

Idade			0,142
> 60	34 (45,3%)	37 (57,8%)	
≤ 60	41 (54,7%)	27 (42,2%)	
Estágio (Ann Arbor)			0,807
I-II	35 (59,3%)	43 (61,4%)	
III-IV	24 (40,7%)	27 (38,6%)	
Gênero			0,583
Feminino	34 (45,3%)	32 (50,0%)	
Masculino	41 (54,7%)	32 (50,0%)	
Performance status (ECOG)			0,270
0-1	31 (64,6%)	24 (53.,3%)	
≥ 2	17 (35,4%)	21 (46,7%)	
LDH			0,887
Normal	24 (38,7%)	22 (40,0%)	
Aumentado	38 (61,3%)	33 (60,0%)	
Acometimento **extranodal**			0,707
> 1	7 (9,3%)	7 (11,3%)	
≤ 1	68 (90,7%)	55 (88,7%)	
Sintomas B			
Presentes	29 (42,6%)	21 (38,2%)	0,616
Ausentes	39 (57,4%)	34 (61,8%)	

Massa *Bulky*			0,309
Presente	43 (61,4%)	31 (52,5%)	
Ausente	27 (38,6%)	28 (47,5%)	

Fonte: Elaborada pelo autor.

A avaliação da frequência e distribuição dos marcadores e as análises de sobrevida encontram-se nos capítulos 1, 2 e 3.

7 CAPÍTULO 1

Artigo: EZH2 expression is dependent on MYC and TP53 regulation in diffuse large B-cell lymphoma

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EZH2 expression is dependent on MYC and TP53 regulation in diffuse large B-cell lymphoma

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EZH2 is an important epigenetic regulator, but its role in diffuse large B-cell lymphoma (DLBCL) pathogenesis and its relationship with MYC, BCL2, and TP53 expression, chromosomal rearrangements, and clinical features are still poorly understood. So, we investigated EZH2 expression and its associations with the immunophenotypic presentations, including MYC, BCL2, and TP53 expression, *MYC*, *BCL2*, and *BCL6* translocation status, clinicopathological features, and therapeutic response to R-CHOP in a series of 139 DLBCL cases. EZH2 positivity was associated with MYC and TP53 expression ($p = 0.0002$ and $p = 0.0000$, respectively) and to high proliferative index (Ki67>70%, $p = 0.0082$). No associations were found among EZH2 expression and chromosomal translocation status. The non-germinal center (nGC) DLBCL presented most of associations observed in the general sample; however, only TP53 immunodetection showed associations with EZH2 expression in the germinal center (GC) DLBCL. EZH2 expression had no impact on therapeutic efficacy in R-CHOP-treated patients. In conclusion, EZH2 seems to be upregulated by MYC, to rely on *TP53* alterations, and is associated with high proliferative tumors in DLBCL, which might be dependent on GC or nGC subclassifications. Furthermore, it is not a therapeutic efficacy marker to R-CHOP in our series.

Key words: Diffuse large B-cell lymphoma; EZH2; MYC; TP53; R-CHOP.

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Diffuse large B-cell lymphoma (DLBCL) is the most common non-Hodgkin lymphoma in adults, accounting for up to 35% of B-cell malignancies, representing a clinically and genetically heterogeneous disease (1). In recent years, gene expression profiling (GEP) has identified at least two distinct subsets of DLBCL with clear phenotypic differences with respect to clinical characteristics and disease course, namely germinal center (GC) versus activated B cell (ABC) (2). Subsequently, immunohistochemical signatures were developed to capture the distinction between these subgroups and to be used as surrogates to GEP in the daily practice, notably Hans algorithm which classifies DLBCL by the cell of origin into germinal center (GC-DLBCL) and non-germinal center (nGC-DLBCL) subtypes (3, 4). This classification, nevertheless, is insufficient to address DLBCL complexity, suggesting that a variety of molecular pathways with distinct interactions may be involved in DLBCL. Understanding of the molecular mechanisms that contribute to this clinical heterogeneity is relevant to identify patients with more aggressive behavior and an initial step for design alternative therapeutic strategies.

Alterations in *MYC* gene and protein expression in DLBCL define a subset with particularly aggressive behaviors (5, 6). These alterations have been found frequently associated with other oncogenic abnormalities, such as *BCL2* or *BCL6* gene translocations and protein overexpression, which are associated with poor performance status, rapid

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evolution, poor response to current therapies, and a median survival of less than 1 year (5, 7).

The regulatory mechanisms involving *MYC* include transcriptional repression due to polycomb repression complexes 1 and 2 (PRC1 and PRC2, respectively) both in *MYC* autoregulation and in general *MYC*-mediated repression (8). EZH2, an enzymatic subunit of the PRC2, has been reported to play a significant role in DLBCL tumorigenesis (9), functioning as a methytransferase that leads to trimethylation of lysine 27 of histone H3 (H3K27me3), a post-translational modification that leads to transcriptional repression of PRC2 target genes (10). The mutation or overexpression of *EZH2* has been identified in several types of cancers and has been shown to correlate with metastatic progression and poor survival (11). EZH2 expression is a common feature of several hematological neoplasms, but in DLBCL, the mechanisms by which EZH2 expression is regulated and its associations with clinicopathological characteristics and therapeutical response to the standard chemotherapy protocol based on rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) are still poorly understood (10, 12, 13). Furthermore, alterations in the tumor suppressor gene *TP53*, an important mediator of cell-cycle arrest, DNA repair, and apoptosis, are an independent marker of poor prognosis in patients with DLBCL and appear to influence EZH2 regulation by poorly understood mechanisms (14, 15).

Hence, the aim of this study was to investigate EZH2 expression and its associations with the immunophenotypic presentations, including MYC, BCL2, and TP53 expression and *MYC*, *BCL2*, and *BCL6* translocation status, clinicopathological features, and therapeutic response to R-CHOP in a series of DLBCL.

MATERIALS AND METHODS

Case selections

Samples of 139 lymphoma cases were obtained from the files of the Department of Pathology, Instituto do Câncer do Ceará (Cancer Institute of Ceará), Fortaleza-Brazil, from 2012 to 2017, with the institutional internal review board's approval. The pathological diagnoses were established according to the criteria of the 2016 World Health Organization Classification, based on morphological and immunohistochemical (IHC) findings. Diagnostic slides from all cases were reviewed by two hematopathologists (EHCNF and CGH). Determination of the germinal center (GC) or non-GC phenotype was based on the Hans algorithm (16). Most patients (n = 90) underwent at least 4 cycles of R-CHOP or R-CHOP-like regimens. Treatment responses (CR, complete response; PR, partial response; SD, stable disease; and PD, progressive disease) and follow-up were evaluated according to the recommended criteria (17).

Tissue microarray preparation, immunohistochemistry, and analyses

The representative tumor areas in each case were selected by microscopic examination to make a tissue microarray (TMA) with 2.0 mm punch using Manual Tissue Microarrayer Quick-Ray™ system (Unitma Co., Bokjeong-ro, Korea), according to the manufacturer's instructions. IHC was performed on 2-μm tissue microarray sections using EZH2 (Clone 11, dilution 1:50, Cell Marque™, Darmstadt, Germany), MYC (Clone Y69, dilution 1:50, Abcam, Shanghai, China), TP53 (DO-7, ready-for-use, DAKO™, Glostrup, Denmark), BCL2 (Clone 124, ready-for-use, DAKO™), CD10 (Clone 56C6, ready-for-use, DAKO™), BCL6 (Clone PG-B6P, ready-for-use, DAKO™), MUM1 (Clone Mum1P, ready-for-use, DAKO™), and Ki-67 (Clone MIB-1, ready-for-use, DAKO™). All immunoreactions were stained using a fully automated Auto Stainer (DAKO™), in which antigen retrieval was processed through the PTLink (DAKO™) system with the FLEX Plus (DAKO™) amplification system according to the manufacturer's instructions. The cutoff values for positivity were as follows: CD10, BCL6, and MUM1, ≥30%; MYC, ≥40%; BCL2 and TP53, ≥50%; and Ki-67 and EZH2, ≥70%, as described previously (Fig. 1) (13, 14). When positive, both TP53 and EZH2 stain intensity were classified as weak, moderate, and intense (18). All samples were independently evaluated by two experienced hematopathologists (EHCNF and CGH) who were blinded to the clinical information. Conflicting results were properly discussed, and a consensus was achieved for all cases.

Fluorescence in situ hybridization

FISH was performed on 2-μm tissue microarray sections using dual-color break-apart probes (MYC/8q24 and BCL6/3q27) and dual-color fusion translocation probes (BCL2/18q21) (Abbott Laboratories, Des Plaines, IL) as previously described (7). Cells were hybridized with *MYC, BCL2*, *and BCL6* probes, and nuclei were counterstained with 4',6-diamidino-2-phenylindole antifade. Fluorescence was detected using a Olympus BX41 fluorescence microscope (Olympus™, Japan) with excitation filters for 4',6-diamidino-2-phenylindole (260 nm) and rhodamine (570 mn). For each case, up to 200 interphase nuclei were analyzed using an ASI image analysis system (Applied Spectral Imaging™, Israel) (Fig. 2). The spots in nuclei were scored as previously described (7).

Statistical analyses

Analyses were carried out using the statistics software SPSS version 17.0 (Chicago, IL, USA). Statistically significant differences were evaluated by the chi-squared test. Correlations were analyzed by Spearman's rank correlation coefficient. The results were considered statistically significant when p values were < 0.05.

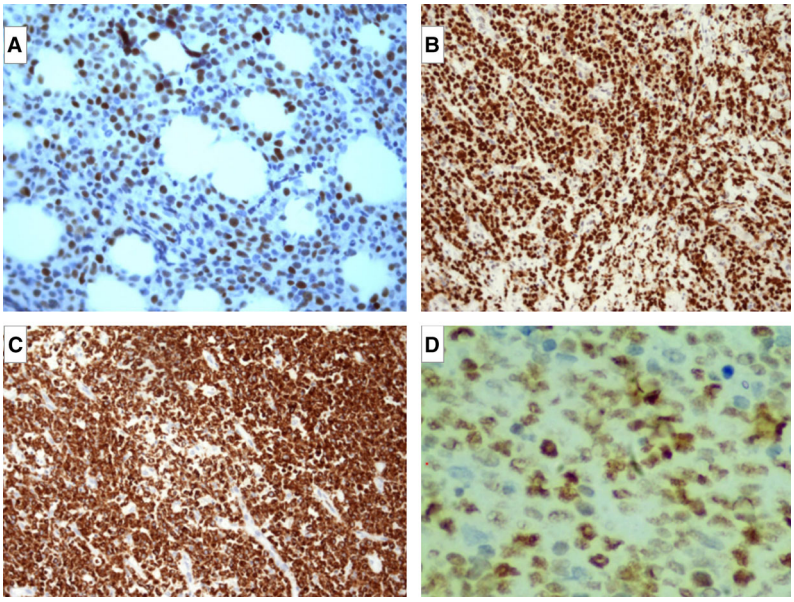

Fig. 1. Immunohistochemical staining of representative cases of (A) MYC, (B) TP53, (C) BCL2, and (D) EZH2 [Colour figure can be viewed at wileyonlinelibrary.com]

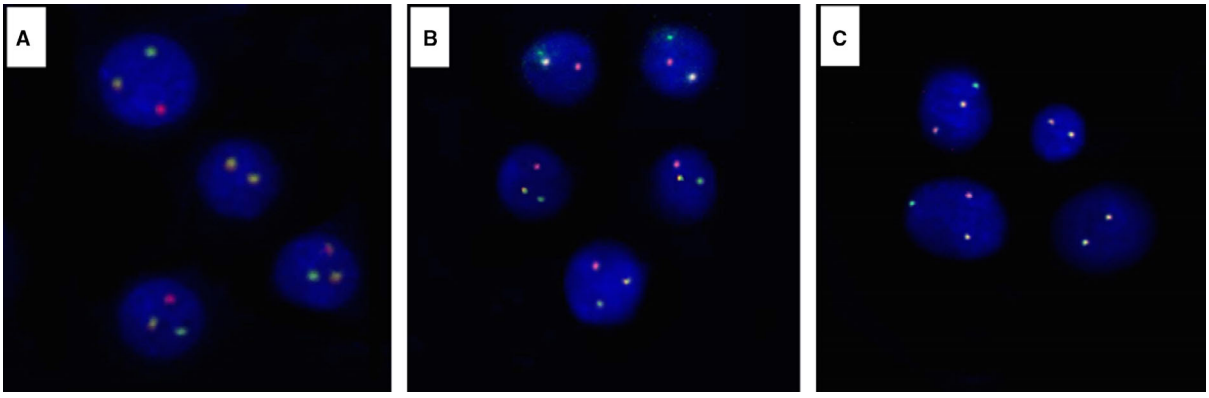

Fig. 2. FISH representative cases of rearranged (A) MYC, (B) BCL2, and (C) BCL6 [Colour figure can be viewed at wile yonlinelibrary.com]

RESULTS

Study population

Among the 139 analyzed cases, 52.5% (73/139) were males. The median age was 61.0 years, ranging from 21 to 98. Most of the patients had nodal disease (55.4%, 77/139), and among those with extranodal lymphoma, the gastrointestinal tract was the most common site (25.8%, 16/62). When the cell-of-origin classification according to Hans algorithm was applied, the germinal center subtype (GC-DLBCL) was slightly more frequent (53.9%, 75/139) than the non-germinal center subtype (nGC-DLBCL) (46.1%, 64/139).

EZH2, MYC, BCL2, and TP53 expression and MYC, BCL2, and BCL6 translocation status

All samples were successfully available for immunohistochemistry reactions of MYC, BCL2, and TP53, and 137 samples were available for EZH2 evaluation. BCL2 was the most frequently expressed (67.6%, 94/139), followed by TP53 (32.3%, 45/139), EZH2 (28.5%, 39/137), and MYC (10.0%, 14/139). Nine cases (6.4%) harbored both MYC and BCL2 immunoexpression.

EZH2 positivity was significantly associated with MYC and TP53 expression ($p = 0.0002$ and 0.000, respectively). Moreover, when the EZH2 immunostaining intensity was considered, a positive

Table 1. Relationship among EZH2, MYC, BCL2, and TP53 expression according to Hans algorithm

	GC-DLCBL			nGC-DLCBL		
	EZH2+	EZH2−	p	EZH2+	EZH2−	p
MYC+	2 (11.1%)	2 (3.6%)	0.2	8 (38.1%)	1 (2.4%)	0.0004
MYC−	16 (88.9%)	54 (96.4%)		13 (61.9%)	41 (97.6%)	
BCL2+	12 (66.7%)	39 (69.6%)	1	13 (61.9%)	30 (71.4%)	0.6
BCL2−	6 (33.3%)	17 (30.4%)		8 (38.1%)	12 (28.6%)	
TP53+	11 (61.1%)	11 (19.6%)	0.0002	12 (57.1%)	9 (21.4%)	0.01
TP53−	7 (38.9%	45 (80.4%)		9 (42.9%)	33 (78.6%)	
MYC/BCL2 +	2 (11.1%)	1 (1.8%)	0.1	5 (23.8%)	1 (2.4%)	0.01
MYC/BCL2−	16 (88.9%)	55 (98.2%)		16 (76.2%)	41 (97.6%)	
MYC/TP53+	1 (55.6%)	1 (1.8%)	0.4	6 (28.6%)	0 (0%)	0.0008
MYC/TP53−	17 (94.4%)	55 (98.2%)		15 (71.4%)	42 (100%)	
BCL2/TP53+	7 (38.9%	9 (16.1%)	0.08	9 (42.9%)	8 (19.0%)	0.08
BCL2/TP53−	11 (61.1%)	47 (83.9%)		12 (57.1%)	34 (81.0%)	
Ki67 ≥ 70%	12 (66.7%)	32 (57.1%)	0.6	21 (100%)	26 (61.9%)	0.0006
Ki67 < 70%	6 (33.3%)	24 (42.9%)		0 (0%)	16 (38.1%)	
*MYC*t+	3 (16.7%)	15 (26.8%)	0.5	7 (33.3%)	5 (11.9%)	0.08
*MYC*t−	15 (83.3%)	41 (73.2%)		14 (66.7%)	37 (88.1%)	
*BCL2*t+	3 (16.7%)	7 (12.5%)	0.6	5 (23.8%)	8 (19.0%)	0.9
*BCL2*t−	15 (83.3%)	49 (87.5%)		16 (76.2%)	34 (81.0%)	
*BCL6*t+	1 (55.6%)	8 (14.3%)	0.4	1 (4.8%)	0 (0%)	0.3
*BCL6*t	17 (94.4%)	48 (85.7%)		20 (95.2%)	42 (100%)	
Double-hit+	1 (55.6%)	7 (12.5%)	0.6	3 (14.3%)	3 (7.1%)	0.4
Double-hit−	17 (94.4%)	49 (87.5%)		19 (85.7%)	39 (92.9%)	

correlation with MYC expression ($r = 0.460$; $p = 0.000$) and with TP53 expression and stain intensity was found ($r = 0.472$; $p = 0.000$ and $r = 0.446$; $p = 0.000$, respectively). In addition, a significant relationship between EZH2 positivity and MYC+/TP53+ double expressors was observed ($p = 0.0006$). Although no relationship between EZH2 and BCL2 was found, 77.8% (7/9) of MYC+/BCL2+ and 87.5% (7/8) of TP53+/BCL2+ cases were statistically associated with EZH2 expression and, also, correlated to EZH2 intensity ($r = 0.396$; $p = 0.001$ and $r = 0.246$; $p = 0.05$, respectively).

Taking in account the proliferation index evaluated by Ki67 expression, EZH2 positivity was related to Ki67>70% ($p = 0.0082$). Additionally, a positive correlation between Ki67 index and EZH2 expression and stain intensity showed up ($r = 0.369$; $p = 0.003$ and $r = 0.394$; $p = 0.001$, respectively). MYC and TP53 were also associated with Ki67>70% ($p = 0.003$ and 0.002, respectively). Worth noting, MYC+/TP53+, MYC+/BCL2+, and TP53+/BCL2 + were consistently related to Ki67>70% ($p = 0.023$, 0.023, and 0.012, respectively).

Considering the translocation status, *MYC* was the most frequently rearranged gene (21.6%, 30/139), followed by *BCL2* (16.5%, 23/139) and *BCL6* (7.2%, 10/139). Double-hit translocations were observed in 7.9% (11/139) considering *MYC*/*BCL2* and in 2.2% (3/139) considering *MYC*/*BCL6*. No triple-hit alterations were observed in our data. No relationship between EZH2 expression and the presence of chromosomal translocations involving any studied gene was found.

Cell-of-origin classification and EZH2 analyses

Although no associations were observed considering EZH2 expression and DLBCL subtypes according to Hans algorithm, EZH2 immunostaining was significantly associated either with the lack of CD10 expression ($p = 0.019$) or, conversely, with MUM1 positivity ($p = 0.039$). Noteworthy, MYC was statistically more frequent in the nGC-DLBCL group ($p = 0.042$) and, similarly to EZH2, tended to be associated with the CD10 negativity ($p = 0.070$) and with MUM1 positivity ($p = 0.054$).

The data were also analyzed taking in consideration the Hans algorithm subtypes. The nGC-DLBCL cases had similar results to the general sample, as shown in Table 1. On the other hand, in the CG-DLBCL group, EZH2 expression was only associated with TP53 expression (Table 1) and correlated to TP53 stain intensity ($r = 440$; $p = 0.000$). No relationship between EZH2 and proliferation index in the CG-DLBCL cases was found.

Although in the general sample no relationship was observed between EZH2 expression and the presence of chromosomal rearrangements, when we considered the nGC-DLBCL cases, an association between EZH2 expression and *MYC* rearrangement tended to show up (Table 1).

Table 2. Distribution of EZH2 expression according to clinical response to R-CHOP therapy

	EZH2 +	EZH2−	p
cR	14 (58.4%)	43 (67.2%)	0.6
pR	6 (25.0%)	8 (12.5%)	0.2
SD	2 (8.3%)	10 (15.6%)	0.4
P	2 (8.3%)	3 (4.7%)	0.6
cR + pR	20 (83.3%)	51 (79.7%)	1
SD + P	4 (16.7%)	13 (20.3%)	1

EZH2 expression and clinicopathological associations

No association was found between EZH2 expression and LDH levels, ECOG status, presence of B symptoms, Bulky mass, or disease staging in the general sample. Among the nGC-DLBCL cases, EZH2 tended to be associated with advanced ECOG status (2–4) ($p = 0.05$).

The expression of EZH2 was also evaluated according to the primary response to R-CHOP-based therapy. We were able to analyze this parameter in 90 patients, which is summarized in Table 2. No associations between therapeutical response and EZH2 expression in the either the general sample or the Hans subtype groups were found in the analyzed series.

DISCUSSION

Alterations involving *MYC*, *BCL2*, and *BCL6* are well-established prognostic and therapeutical markers in DLBCL, with currently some routine applications (5). In addition, *TP53* status has been pointed as an additional prognostic feature (14). However, these alterations are insufficient to address DLBCL complex molecular landscape and to fully explain its prognostic heterogeneity. So that, EZH2 appears to be a potential biomarker, as it has been shown to be a common DLBCL feature and it seems to be a key part of *MYC* and *TP53* regulatory network (8, 19). In this study, we observed that EZH2 expression is a common event in a DLBCL series, being expressed in about 28% of the cases, similar to a Korean series (20), and we demonstrated a relationship among EZH2, MYC, and TP53, corroborating with proposals of some authors for EZH2 regulation (15, 21, 22). This is, to our knowledge, the first epidemiological evaluation of EZH2 expression in DLBCL in the Brazilian population.

Overexpression of MYC is a common event in lymphoma, including DLBCL. However, it does not appear to be a frequent feature of Brazilian DLBCL patients, as highlighted in our results (10.0%) and in Oliveira et al.'s (15.7%) (23). In this study, MYC overexpression was significantly associated with EZH2 expression and staining intensity. There are very few studies associating EZH2 and MYC, highlighting Tian et al.'s (10, 12). In their first evaluation, they found that EZH2 expression was an important feature of aggressive B-cell neoplasms and its expression was associated with MYC overexpression in Burkitt and double-hit lymphomas, but not in DLBCL (12). They proposed that EZH2 is involved in different regulation cascades according to its different types and confirmed the previous cited findings in a subsequent study (10). However, they had a lower number of cases than our series, which may lead to an underestimation of possible associations.

The results of the present study also corroborate Liu et al.'s, in which they evaluated EZH2 and MYC expression in gastrointestinal DLBCL (13) and showed that EZH2 tends to be overexpressed in a high number of tumor cells, reflecting its role as an oncoprotein and its importance in tumor development.

According to our results, among the 13 MYC+ cases, 10 showed EZH2 overexpression, what evidences that when MYC is detected, EZH2 tends to be expressed. Indeed, the regulations involving MYC and EZH2 are complex. Sander et al. suggested that EZH2 regulation by MYC might depend on microRNA modulation in lymphoma (24). Sun et al., studying ovarian cancer, also suggested that MYC promotes EZH2 expression through miRNA regulation and might play an important role in cell survival (25). These and our results suggest that MYC overexpression plays a function in upregulating EZH2 expression.

In addition to MYC, TP53 positivity was significantly associated with EZH2 expression, either considering immunodetection or immunointensity. These findings are corroborated by several studies, which have analyzed TP53 expression and *TP53* mutational status in solid tumors (15, 26, 27). Likewise, Tang et al., in a in vitro study on cell lines, proposed a novel *TP53*-mediated cell-cycle arrest pathway through *TP53*-dependent EZH2 repression (19). Although *TP53* genetic alterations have not been evaluated in our study, TP53 immunoexpression has been widely correlated to *TP53* mutations (15, 28, 29), including in DLBCL (14), as mutated *TP53* leads to protein stability in comparison with the wild-type form, which has a shorter half-life. So that, our findings suggest that *TP53* impaired transcriptional activity may have a role in EZH2 upregulation in DLBCL.

Furthermore, in this series of DLBCL, EZH2 expression correlated with proliferation rate assessed by Ki-67 staining, which is typically associated with aggressive tumors in B-cell non-Hodgkin lymphoma, as has been previously reported in a

range of human cancers and different hematological neoplasias (12, 23, 30).

In our series of DLBCL, no relationship between EZH2 and BCL2 expression was found, which is in accordance with the literature (13, 30). The fact that MYC+/BCL2+ and TP53+/BCL2+ cases were statistically associated with EZH2 expression was probably due to the interactions with MYC and TP53. *MYC*, *BCL2*, and *BCL6* translocation status individually or combined (double-hits) was also not associated with EZH2 expression. Although Liu et al. (13) have not evaluated *BCL6* rearrangements, they found similar results in gastrointestinal DLBCL for *MYC* and *BCL2* translocations.

No association between EZH2 expression and cell-of-origin group was found in our data. This is consistent with recent studies reporting that abundant EZH2 expression was common in DLBCL regardless of cell of origin (13, 31). Nevertheless, when the analyses were carried out considering each marker involved in Hans algorithm, we found that EZH2 expression was statistically more frequent in CD10-negative and in MUM1-positive cases, which would suggest a non-germinal center-like phenotype.

Considering the cell-of-origin classification and the relationship of EZH2 expression and the other markers, only TP53 immunodetection and stain intensity showed associations with EZH2 expression in the GC-DLBCL. Although *TP53* mutations might result in distinctive molecular programs harbored by GC and nGC-DLBCL (14), according to our results, *TP53* impairment estimated by TP53 overexpression seems to upregulate EZH2 expression regardless of DLBCL subtype. On the other hand, EZH2 upregulation by MYC expression seems to be a restricted feature to the nGC-DLBCL cases, as in the GC-DLBCL group no relationship between MYC and EZH2 expression was observed. Also, EZH2 expression tended to be associated with *MYC* translocation in the nGC-DLBCL. This may possibly be explained by a direct correlation of *MYC* translocation and MYC expression in this group. These results suggest that, considering these genes, EZH2 upregulation is dependent on MYC overexpression rather than on *MYC* rearrangements.

In the present study, no association between EZH2 expression and well-known prognostic factor including poor performance status, elevated LDH, B symptoms, and disease staging was noted. Considering the nGC-DLBCL group, however, a poor performance status defined by ECOG scores ≥ 2 showed a borderline association with EZH2 expression, which is consistent with Liu et al. study (13). Other authors studying trimethylated H3K27 (H3K27me3) expression, a direct consequence of EZH2 upregulation in many cancers, found that its overexpression is also associated with an ECOG status 2-4 (10, 30, 32). Furthermore, Oh et al. (30) suggest that H3k27me3 expression is associated with poorer survival specially in the nGC subtype, what might explain that, in our cases, a EZH2-associated poor performance status in nCG-DLBCL is due to H3k27me3 effect. To identify these possible mechanisms, however, further studies should follow.

EZH2 expression, in this study, had no impact on therapeutic efficacy in R-CHOP-treated patients. Oh et al. (30) also failed on finding EZH2 expression as a therapeutic factor. However, the current literature regarding these aspects is limited and controversial. As a matter of fact, Deng et al. (33, 34), in China, found that patients with EZH2 low expression usually responded well to R-CHOP regimen in the short-term or long-term survival in comparison with those of patients with higher expression, and Lee et al. (32), in Korea, found that high-level expression of EZH2 was associated with superior overall survival compared to low expression. Our study may be limited as it is from a single institute. Further study, especially multicenter-based studies, should be followed for validating the present results.

In conclusion, EZH2 seems to be upregulated by MYC expression, to rely on TP53 alterations, and is associated with high proliferative tumors in DLBCL, which might be dependent on GC or nGC subclassifications. In addition, although EZH2 was associated with poor performance status in nGC-DLBCL, it is not a therapeutic efficacy marker to R-CHOP in our series.

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CAPÍTULO 2

Artigo: Prognostic impact of miR-125b and miR-155b and their relationship with MYC and TP53 in diffuse large B-cell lymphoma: cell-of-origin classification matters.

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PROGNOSTIC IMPACT OF MIR-125B AND MIR-155B AND THEIR RELATIONSHIP WITH *MYC* AND *TP53* IN DIFFUSE LARGE B-CELL LYMPHOMA: CELL-OF-ORIGIN CLASSIFICATION MATTERS.

Running title: miR-125b and miR155b detection in diffuse large B-cell lymphoma

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ABSTRACT

Tumoral microRNAs, such as miR-125b and miR-155b, are important gene expression regulators with complex pathogenetic mechanisms. However, their role in DLBCL, especially when cell-of-origin classification is considered, are still to be elucidated. In a series of 139 DLBCL cases considering germinal center (GC) *versus* nonGC subtypes, we investigated miR-125b and miR-155b expression by *in situ* hibridization and their association with some immunophenotypic presentations, including MYC, BCL2 and TP53 expression, *MYC*, *BCL2* and *BCL6* translocation status, as well as clinicopathological features and outcomes. miR-125b detection was positively correlated to the Ki-67 index (p= 0.035) in the nGC. Considering the GC subgroup, the percentage of miR-125b positive cells was also correlated to either MYC and MYC/BCL2 double expression (p= 0.047 and p= 0.049, respectively). When it comes to nGC patients, miR-155b percentage and intensity, as well as Allred score, were positively correlated to disease progression (p= 0.038, p=0.057 and p= 0.039, respectively). In a multivariate analysis, GC phenotype was a significant independent factor associated with higher OS (*p*=0.007) and, considering the nGC group, although not significant, the expression of TP53, miR-125b and miR-155b seems to be potential prognostic biomarkers in these tumors. This study demonstrated different pathways based on cell-of-origin classification and highlighted different clinical outcomes. miR-125b, miR-155b and TP53 expression may also represent potential prognostic factors in nGC-DLBCL.

KEY WORDS: Diffuse Large B-Cell Lymphoma, miR-125b, miR-155b, MYC, TP53, R-CHOP.

1. INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most common non-Hodgkin lymphoma in adults, accounting for up to 35% of B-cell malignancies, representing a biologically heterogeneous disease with unpredictable clinical outcomes. Despite the standard chemotherapy protocol based on rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone (R-CHOP), 20% to 30% of patients still experience relapse or refractory disease [1]. DLBCL has been defined by genetic expression profiling (GEP) in mainly two distinct subsets named germinal center (GC) and activated B cell (ABC) with notable phenotypic differences with respect to clinical characteristics and disease course, but still with very limited application in clinical practice [2]. Subsequently, immunohistochemical protocols were developed to be used as surrogates to GEP. Notably, Hans algorithm, which classifies DLBCL in the cell-of-origin into germinal center (GC-DLBCL) and in non-germinal center (nGC-DLBCL) subtypes, showing a good correlation with GEP defined entities and potential prognostic impact [3]. This classification, nevertheless, is insufficient to address DLBCL complexity, suggesting that a variety of molecular pathways with distinct interactions may be involved in DLBCL.

Several biomarkers have been shown to predict the outcome of DLBCL, but only a few have clinical applicability, reinforcing the necessity to identify their pathways. In a previous publication [4], changes in the interaction of *MYC*, *BCL2*, *BCL6*, and *TP53* may imply in loss of cell proliferation and apoptotic control, impaired DNA-repair mechanisms and B-cell maturation disturbance. Furthermore, these intricate interactions seem to vary upon GC versus nGC subgroups, with different molecular pathways between both subtypes with variable clinical outcomes, suggesting that GC and nGC phenotypes and their characteristic biological profile should be considered as two different entities.

In the last few years, changes in noncoding RNAs have been shown relevant in many cancers, addressing the importance of epigenetic alterations and aberrant expression. In this context, microRNAs (miRs) came up as a group of biomarkers with potential prognostic impact on DLBCL, as an endogenous gene expression regulator [5, 6]. For instance, miR-125b became relevant in cancer research, being often associated to cell proliferation, apoptosis inhibition and loss of tumor suppres-

sive functions [7]. Although miR-125b is aberrantly expressed in a variety of tumors and has either oncogene and tumor suppressor activity, the expression, function, and clinical relevance of miR-125b in both DLBCL GC and nGC subgroups are still unclear. In addition, miR-155b has been demonstrated to have a key role in hematopoiesis and immune response, acting as an oncomiR in many malignancies, especially in lymphoma [8]. Indeed, some studies have pointed miR-155b as a marker of malignancy in B-cell lymphoproliferative disorders, and even as a potential tool to differentiate ABC from GCB-DLBCL, but its prognostic role ~~i~~s still controversial [9, 10].

The aim of this study was to investigate the association of miR-125b and miR-155b with MYC, BCL2 and TP53 expression, *MYC*, *BCL2* and *BCL6* translocation status along with clinicopathological features, therapeutic response to R-CHOP and clinical outcomes in DLBCL, focusing in the differences between GC and nGC subtypes.

2. MATERIALS AND METHODS

2.1 *Case selections*

Samples of 139 lymphoma cases were obtained from the files of the Department of Pathology, Instituto do Câncer do Ceará (Cancer Institute of Ceará), Fortaleza-Brazil, from 2012-2017, with the institutional internal review board's approval. The pathological diagnoses were established according to the criteria of the 2022 World Health Organization classification, based on morphological and immunohistochemical (IHC) findings. Diagnostic slides from all cases were reviewed by two hematopathologists. Determination of the germinal center (GC) or non-GC phenotype was based on the Hans' algorithm [3]. Most patients (n = 92) underwent at least 4 cycles of R-CHOP or R-CHOP–like regimens and only these cases were considered in univariate and multivariate analyses. All other patients (n = 47) were submitted to heterogeneous treatment regimens and were not considered in such analyses. Treatment response~~s~~ (CR, complete response; PR, partial response; SD, stable disease and PD, progressive disease) and follow-up were evaluated according to the recommended criteria [11].

2.2 *Tissue microarray preparation and immunohistochemistry analyses*

The representative tumor areas in each case were selected by microscopic examination to make a tissue microarray (TMA) with a 2.0 mm punch using Manual Tissue Microarrayer Quick-Ray™ system (UNITMA Co., Bokjeong-ro, Korea), according to the manufacturer's instructions. IHC was performed on 2-µm tissue microarray sections using MYC (Clone Y69, dilution 1:50, Abcam, Shanghai, China), TP53 (DO-7, ready-for-use, DAKO™, Glostrup, Denmark), BCL2 (Clone 124, ready-for-use, DAKO™), CD10 (Clone 56C6, ready-for-use, DAKO™), BCL6 (Clone PG-B6P, ready-for-use, DAKO™), MUM1 (Clone Mum1P, ready-for-use, DAKO™) and Ki-67 (Clone MIB-1, ready-for-use, DAKO™). Immunoreactions were stained with a fully automated Auto Stainer (DAKO™), in which antigen retrieval was processed through PTLink (DAKO™) with FLEX Plus (DAKO™) amplification system according to the manufacturer's instructions. The cutoff values for positivity were considered as follows: CD10, BCL6, and MUM1, ≥ 30%; MYC, ≥ 40%; BCL2 and TP53, ≥ 50%; and Ki-67, ≥ 70%, as described previously [4]. When positive, TP53 staining intensity was classified as weak, moderate and intense [12]. All samples were independently evaluated by two experienced hematopathologists who were blinded to the clinical information. Conflicting results were properly discussed and a consensus was achieved for all cases.

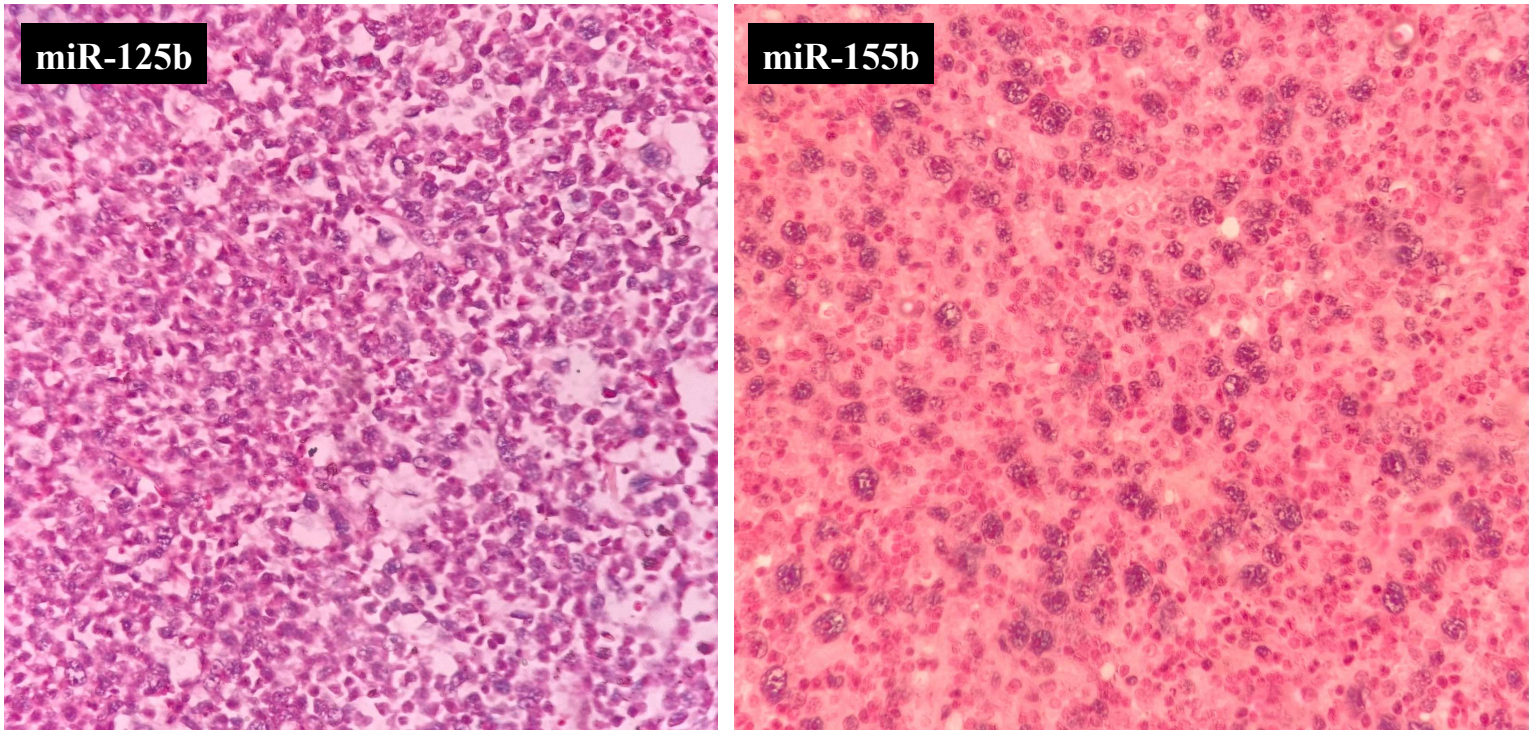
2.3 *Fluorescence in-situ hybridization*

FISH was performed on 2-µm tissue microarray sections using dual-color break-apart probes (MYC/8q24 and BCL6/3q27) and dual-color fusion translocation probes (BCL2/18q21 and IGH/MYC) (Abbott Laboratories, Des Plaines, IL) as previously described [13]. Cells were hybridized with *MYC, BCL2 e BCL6* probes and nuclei were counterstained with 4',6-diamidino-2-phenylindole antifade. Fluorescence was detected with a Olympus BX41 fluorescence microscope (Olympus™, Japan) with excitation filters for 4',6-diamidino-2-phenylindole (260 nm), and rhodamine (570 mn). For each case, up to 200 interphase nuclei were analyzed using an ASI image analysis system (Applied Spectral Imaging™, Israel). The spots in nuclei were scored as previously described [13].

2.4 *miRNA in-situ hybridization*

For miR-125b and miR-155b we performed ISH using specific LNA probes (hsa-miR-125b-5p miRCURY and hsa-miR-155b-5p miRCURY LNA miRNA detection probes, respectively, at 40nM for both probes (Exiqon^{TM}, Vedbæk, Denmark) as previously described [14]. Briefly, 2-µm tissue microarray sections were submitted to a proteinase-K treatment at 15 mg/mL for 10 minutes and hybridization at 53°C for 90 minutes with the aforementioned DIG-labeled locked nucleic acid probes. Stringent washes were performed at 53°C and room temperature for both probes using SSC buffers. All slides were incubated with a DIG-blocking reagent (Roche, Mannheim, Germany), followed by alkaline phosphatase-conjugated anti-DIG (Roche), and NBT-BCIP substrate for 1 hour to elicit the blue ISH signal. All sections were counterstained with nuclear fast red (Vector Labs., Burlingame, CA) and mounted. Non-neoplastic lymphoid tissue (tonsil) incubated with U6 snRNA probe and a scramble probe were used as positive and negative controls, respectively. The cutoff for positivity was ≥ 30% of blue-stained neoplastic cells in contrast to the red colored background as previously described [14] (Figure 1). When positive, the stain intensity was classified as weak, moderate and intense and the Allred score was calculated accordingly [15].

Figure 1 - ISH representative case of positive miR-125b and miR-155b, highlighting



blue-stained neoplastic cells in contrast to the red colored inflammatory background

2.5 *Statistical analyses*

Data was analyzed with SPSS version 17.0 (Chicago, IL, USA). Statistically significant differences were evaluated by the chi-square or Fisher exact test. Correlations between miRNA continuous variables such as positive cells percentage, Allred score and staining intensity and clinicopathological features were analyzed by Spearman's rank correlation coefficient. Overall survival (OS) was calculated from the date of diagnosis until whether death from any cause or the last follow-up appointment date. Survival rates were plotted in Kaplan-Meier curves and compared by the log-rank test. Univariate and multivariate analyses were performed using the Cox proportional hazard regression model. A $p<0.05$ was considered significant.

3. RESULTS

3.1 **Study population**

Among the 139 cases, 52.5% (73/139) were males. The median age was 61.0 years, ranging from 21 to 98. Most of the patients had nodal disease (64.0%, 89/139) and, among those with extranodal lymphoma, the gastrointestinal tract was the most common site affected (34%, 17/50). When the cell-of-origin classification according to Hans' algorithm was applied, the germinal center subtype (GC-DLBCL) was slightly more frequent (53.9%, 75/139) than the non-germinal center subtype (nGC-DLBCL) (46.1%, 64/139).

3.2 *MYC, BCL2* and *TP53* expression, MYC, BCL2 and BCL6 translocations status and miR-125b and miR-155b *detection*

3.2.1 Distribution by cell-of-origin classification

The data of the biomarker's distribution according to cell-of-origin classification are summarized in Table 1. In the nGC group, MYC expression was significantly more frequent (p=0.044), along with MYC/TP53 double expression (p=0.048). Interestingly, BCL2 expression was associated to the presence of *BCL2* chromosomic rearrangement only in this subset of cases (p=0.005). Conversely, *BCL6* translocation (p=0.018) was almost exclusively present in the GC subgroup. Association of MYC immunoexpression with *MYC* translocations were observed in both subgroups (p<0.001 for the nGC and p=0.014 for the GC).

Among the 139 samples, 135 cases were successfully available for microRNA ISH detection, being miR-125b detected in 58.5% (79/135) and miR-155b in only 24.4% (33/135). No statistical difference was observed in the cell-of-origin subgroups for neither miRs detection. In the nGC patients, miR-125b detection was positively correlated to the Ki-67 index (r= 0.267; p= 0.035). Considering the GC subgroup, besides a miR-125b association to Ki-67>70% (p= 0.043) (supplementary table), the percentage of miR-125b positive cells was also correlated to either MYC and MYC/BCL2 double expression (r=0.234; p= 0.047 and r= 0.234; p= 0.049, respectively) when we analyzed by Spearman's rank coefficient. No associations were observed between miR-125b and the studied chromosomic rearrangements, nor between miR-155b and any of these parameters. Supplementary table with biomarkers distribution and clinical features according to miR-125b and miR-155b positive *versus* negative status is available.

In a previous study, we described different association patterns among EZH2, MYC and TP53 depending on the cell-of-origin classification, as being an EZH2/MYC/TP53 pathway a nGC feature and, conversely, an EZH2/TP53 pathway a GC characteristic [4]. Despite the small number of studied cases, we analyzed miR-125b and miR-155b distribution according to these different pathways. In the EZH2/MYC/TP53 patients, positivity for miR-125b was observed in 66.6% (4/6) of the cases, while for miR-155b in 33.3% (2/6). In the EZH2/TP53 patients (GC subgroup), 45.4% (5/11) were positive for miR-125b and 27.3% (3/11) for miR-155b expression. Considering the MYC/TP53 cases, miR-125b was detected in 75.0% (6/8) and miR-155b in only 25.0% (2/8). No statistically significant associations were found among any of these groups.

Table 1 - GC versus nGC classification and biomarkers detection

	GC (53.9%, 75/139)	**nGC (46.1%, 64/139)**	***p***
Age (median)	58.3 (21-98)	61.7 (22-90)	0.142
Nodal disease	50 (66.7%)	39 (61.0%)	0.483
MYC expression	4 (5.3%)	10 (15.6%)	**0.044***
BCL2 expression	51 (68.0%)	43 (67.2%)	0.919
BCL6 expression	**69 (92.0%)**	**31 (48.4%)**	**0**
TP53 expression	23 (30.7%)	22 (34.4%)	0.641
Ki-67 index > 70%	45 (60.0%)	48 (75.0%)	0.061

	GC (53.9%, 75/139)	nGC (46.1%, 64/139)	*p*
MYC/BCL2 expression	3 (4.0%)	6 (9.4%)	0.967
MYC/TP53 expression	2 (2.7%)	7 (11.1%)	**0.048***
MYC translocation	18 (24.0%)	12 (18.7%)	0.453
IgH/MYC translocation	**4 (5.3%)**	**1 (1.6%)**	**0. 373**
BCL2 translocation	10 (13.3%)	13 (20.3%)	0.270
BCL6 translocation	9 (12.0%)	1 (1.6%)	**0.018***
Double translocation	8 (10.7%)	6 (9.4%)	0.801
miR-125b detection	39 (54.2%)	40 (63.5%)	0.243
miR-155b detection	15 (20.5%)	17 (27.0%)	0.402

3.2.2 *Clinicopathological associations*

LDH levels, ECOG status, presence of B symptoms, Bulky mass, extranodal disease and staging were evaluated as clinical features in association to the studied biomarkers. In the GC subgroup, miR-155b detection was significantly associated to the absence of B symptoms (p= 0.006) (supplementary table). In fact, both the percentage and the intensity of miR-155b staining as well as Allred score were negatively correlated to the presence of B symptoms (r= -0.377; p= 0.002, r= -0.333; p= 0.0060 and r= -0.358; p= 0.003, respectively) when we analyzed by Spearman's rank coefficient. Similar results were observed for the ECOG status regarding to the same

staining parameters for miR-155b using Spearman's rank coefficient (r= -0.404; p= 0.021, r= -0.406; p= 0.006, r= -0.384; p= 0.009 and r= -0.394; p= 0.007, respectively).

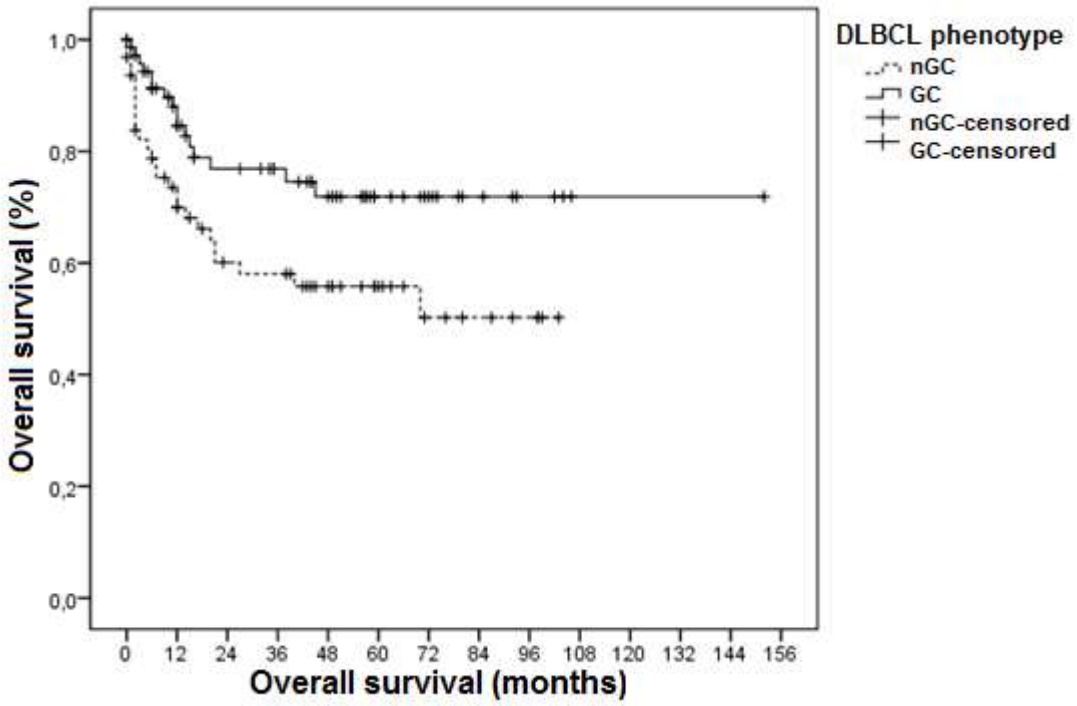
In the nGC subgroup, miR-125b detection was significantly associated to nodal disease (p= 0.009) by the chi-square test, and miR-125b percentage and Allred score were also correlated to nodal disease (r= 0.303; p= 0.016 and r= 0.266; p= 0.035, respectively) when we applied the Spearman's rank coefficient. TP53 expression showed association with B symptoms (p=0.031) and advanced ECOG status (2-4) (p=0.044), while BCL2 was associated to the presence of bulky mass. Neither MYC expression nor the presence of any of chromosomic rearrangements were statistically associated to these features. Regarding the presenting age of patients, BCL2 expression was consistently higher in those older than 60, either in the GC and nGC subgroups (p=0.015 and p=0.026, respectively). Supplementary table with clinical features according to miR-125b and miR-155b positive *versus* negative status is also provided.

3.2.3 ***Primary response to R-CHOP and prognostic analyses***

Biomarkers detection was also evaluated according to the primary response to R-CHOP based therapy, which we were able to analyze in 92 patients. In nGC patients, miR-155b percentage and intensity, as well as Allred score, were positively correlated to progressive disease (PD) (r= 0.317; p= 0.038, r= 0.293; p=0.057 and r= 0.317; p= 0.039, respectively). Additionally, association between BCL2 expression with either stable disease (SD) and stable disease plus progressive disease (SD+PD) was observed (p=0.018 and p=0.016, respectively).

Kaplan-Meier survival curves for OS rates for both GC and nGC subtypes are outlined in Figure 2. OS rates of the GC and nGC patients were 78.1% and 58.7%, respectively, with nGC-DLBCL having poorer OS rates compared with GC-DLBCL (p=0.023).

Figure 2 - Survival curves for OS rates with respect to the GC (n=49) and nGC (n=43) status



3.2.4 ***Univariate analyses of prognostic factors for OS***

Univariate Cox proportional hazard regression analysis was used to assess ~~the~~ prognostic factors for OS, as described in table 2. In the general sample, the GC phenotype showed a significant positive prognostic impact. Considering cell-of-origin classification, in the nGC-DLBCL group we found that age, Ann Arbor stage, extranodal involvement, LDH level, ECOG performance status and bulky mass were significant negative prognostic factors for OS in our patients cohort. However, in the GC subgroup, only extranodal involvement, stage and the presence of B symptoms were negatively significant. In addition, MYC expression, MYC(+)/TP53(+) double-expression, and MYC(+)/BCL2(+)/TP53(+) triple-expression were consistently associated to poorer OS rates in GC-DLBCL. No significant OS rates comparing miR-125b and miR-155b detection was observed in any subgroup.

Table 2 - Prognostic factors that affect OS (univariate analysis)

Risk Factor	**GC**		nGC	
	RR (95% CI)	*p*	RR (95% CI)	*p*

Age (>60 vs </=60)	78.24 (60.05-96.42)	0.906	85.39 (71.51-99.28)	***<0.001***
Stage (I-II vs III-IV)	90.18 (78.91-101.45)	***0.048***	72.86 (57.49-88.22)	***0.039***
Extranodal disease (>1 vs </=1)	32.43 (7.40-57.46)	***0.010***	11.07 (2.02-20.12)	***0.001***
LDH level (elevated vs normal)	109.21 (83.79-134.63)	0.160	44.48 (27.98-60.98)	***0.028***
ECOG PS (>/=2 vs <2)	60.92 (34.08-87.77)	0.052	34.89 (14.93-54.85)	***0.003***
MYC (positive vs negative)	21.00 (3.37-38.63)	***0.037***	54.06 (23.88-84.24)	0.577
BCL2 (positive vs negative)	123.52 (105.61-141.43)	0.108	45.30 (31.38-59.22)	**0.003**
TP53 (positive vs negative)	78.01 (54.61-101.41)	0.883	56.86 (35.83-77.89)	0.501
MYC/BCL2 (positive vs negative)	44.60 (15.15-74.05)	0.334	28.70 (4.15-53.25)	0.422
MYC/TP53 (positive vs negative)	11.00 (11.00-11.00)	***0.013***	61.57 (26.07-97.08)	0.990
MYC/BCL2/TP53 (positive vs negative)	11.00 (11.00-11.00)	***0.013***	15.50 (0.00-31.52)	0.090

MYC translocation (positive vs negative)	73.82 (50.46-97.19)	0.310	45.85 (29.78-61.91)	0.560
BCL2 translocation (positive vs negative)	45.50 (21.37-69.63)	0.303	34.63(17.84-51.43)	0.531
BCL6 translocation (positive vs negative)	47.80 (29.61-65.99)	0.854	0	0.439
Double-hit translocation (positive vs negative)	52.43 (29.48-75.37)	0.592	36.17 (12.97-59.36)	0.742
miR-125b (positive vs negative)	107.36 (82.68-132.04)	0.472	66.94 (51.94-81.95)	0.341
miR-155b (positive vs negative)	86.13 (75.02-97.24)	0.156	50.41 (33.98-66.85)	0.850

3.2.5 ***Multivariate analyses of prognostic factors for OS***

The clinicopathological features and biomarkers presence were also included in a multivariate analysis of survival, as described in table 3. In this analysis, GC phenotype was a significant independent factor associated to higher OS (HR= 0.14; CI 95% 0.04 - 0.6; *p*=0.007). Considering the nGC group, Ann Arbor stage, extranodal involvement and ECOG PS were consistently independent factors related to lower OS. Interestingly, although not significant, the expression of TP53, miR-125b and miR-155b seems to be potential prognostic biomarkers in these tumors. No significant OS rates comparing any variables was observed in the GC subset.

Table 3 - Prognostic factors that affect OS (multivariate analysis)

	GC		nGC	
Risk Factor	HR (95% CI)	*p*	HR (95% CI)	*p*
Age (>60 vs </=60)	12.10 (0.27-53.27)	0.197	1.08 (0.95-1.23)	0.235
Stage (I-II vs III-IV)	1.45 (0.00-1.51)	0.219	4.67 (1.11-19.71)	**0.036**
Extranodal disease (>1 vs </=1)	6.41 (0.00-1.03)	0.191	4.13 (1.91-8.92)	**0.028**
LDH level (elevated vs normal)	4.67 (0.00-8.27)	0.180	0.48 (0.04-5.65)	0.561
ECOG PS (>/=2 vs <2)	1.00 (0.00-22.15)	0.219	13.55 (2.22-82.80)	**0.005**
MYC (positive vs negative)	1.51 (0.00-15.70)	0.915	3.60 (0.00-1.55)	0.900
BCL2 (positive vs negative)	1.00 (0.00-10.99)	0.175	16.78 (0.45-622.19)	0.126
TP53 (positive vs negative)	17.80 (0.00-28.29)	0.790	2.61 (0.90-7.60)	0.078
MYC translocation (positive vs negative)	2.30 (0.00-1.61)	0.527	1.00 (0.00-3.94)	0.906
BCL2 translocation (positive vs negative)	9.10 (0.00-3.65)	0.866	0.05 (0.00-2.72)	0.144

BCL6 translocation (positive vs negative)	6.16 (0.00-3.04)	0.755	0.00 (0.00-9.62)	0.965
Double-hit translocation (positive vs negative)	1.00 (0.00-10.98)	0.797	2.85 (0.14-5.96)	0.147
miR-125b (positive vs negative)	1.13 (0.78-1.62)	0.525	0.95 (0.91-1.00)	0.065
miR-155b (positive vs negative)	0.84 (0.43-1.66)	0.620	1.03 (1.00-1.07)	0.084

4. DISCUSSION

The DLBCL categorization in GC and nGC subgroups has clinical relevance as it implies in different carcinogenic pathways and potential prognostic impact. In order to better understand the interaction of some DLBCL most studied biomarkers and their impact on DLBCL outcome, we focused our analyses on distinguishing the main features of GC from nGC phenotypes.

4.1 Differential detection of the studied biomarkers according to cell-of-origin phenotype and their interactions

Our results showed that MYC expression was significantly associated to the nGC group, corroborating with Wight et al. findings in which MYC overexpression was a nGC feature [16]. In nGC DLBCL patients, the mechanism described in which MYC is overexpressed is mainly BCR and NF-κB induced, while in the GC subtype they are caused by the translocation of *MYC* [17]. However, in this study *MYC* chromosomal translocation was nearly equivalent in both subtypes, with MYC expression consistently associated to its translocation, suggesting that *MYC* translocation, in our series, is a molecular mechanism that leads to MYC expression regardless the cell-of-origin. In addition, among the 30 cases with translocated *MYC,* only 14 were positive

for MYC expression, which highlights that *MYC* translocation itself is not always sufficient to result in its own overexpression. Copie-Bergman *et al* investigated *MYC* translocation partners (immunoglobulin loci - Ig *vs.* non-Ig) in a large series of DLBCL and found that high MYC protein expression was indeed significantly more frequent in the MYC-Ig subgroup compared to MYC-non-Ig DLBCL [18]. So that, we next analyzed among the 30 *MYC* rearranged patients which cases carried IgH/MYC translocation using a specific probe. Interestingly, only one out of all five IgH/MYC positive cases showed MYC expression. Studying Burkitt lymphoma, Wilda et al. analyzed the influence of breakpoint locations within the MYC gene on MYC transcript levels and demonstrated that the highest MYC mRNA levels occur when MYC expression is directly triggered via its cryptic P3 promoter within the first intron, which may explain why although our cases show MYC rearrangements, even IgH/MYC translocations, and not necessarily result on MYC expression [19]. Although we have not studied MYC translocation partners in this study, the presence of a great number of non-Ig partners may explain our observations. Interestingly, despite ~~the~~ small differences in the *BCL2* translocation frequency between GC-DLBCL (13%) and nGC-DLBCL (20%), only nGC tumors –demonstrated to have a relationship between this translocation and BCL2 expression. As well as MYC expression, BCL2 expression was not caused by translocation in most cases, suggesting that other mechanisms might be involved. We have also found that the MYC/TP53 double expression was more frequent in the nGC subtype. Several studies have shown that MYC overexpression *per se* is insufficient to promote neoplastic grown as it induces apoptosis via *TP53*-related pathway [20, 21]. However, as TP53 expression is highly associated to mutated *TP53*, its expression may enhance adverse effects of MYC overexpression on DLBCL as demonstrated by some authors [21, 22, 23], indicating that MYC/TP53 double expression is also a nGC feature.

It has been shown that *BCL6* is frequently affected by chromosomal translocations, occurring more frequently in nGC than in GC DLBCL and leading to a deregulated BCL6 expression by preventing its silencing at the conclusion of the germinal center response [24]. Conversely, In our series, *BCL6* translocation was almost exclusively observed in the GC subgroup. That is interesting as in this subgroup BCL6 expression was supposed to be positive as a result of Hans algorithm itself, which may suggest that in our cases, chromosomal translocation accounted for such overexpression. BCL6 translocation involves several non-Ig partners, whose expression

may be regulated by some BCL6 regulatory regions, and the expression of some of these partner genes may influence the biology of the DLBCL [25]. We speculate that in our cases differential translocation partners may lead to BCL6 overexpression without activating a complete post germinal center genetic machinery, keeping the lymphoma cell-of-origin with a GC phenotype.

When it comes to gene expression, miRNA demonstrated to have an important role In DLBCL pathogenesis. miR-125b and miR-155b have been described to be crucial in regulating important pathogenetic pathways, especially involving *MYC* and *TP53*, which may lead to cell proliferation, disruption of apoptosis and DNA repair mechanisms [6, 10]. In this study, although neither miR-125b nor miR155b were related to any DLBCL subtype, it is noteworthy that miR-125b was associated to high proliferation index independently on cell-of-origin subgroups. The ectopic expression of miR-125b in B-cell lymphoma results in an enhanced NF-κB activity, thereby facilitating cell proliferation and cancer progression in malignant B-cell lymphoma [5, 7]. Likewise, Kim et al demonstrated that miR-125b constitutively activates NF-κB pathway, regardless of the molecular subtype, which may promote tumor cell proliferation and regulate apoptosis [26]. In fact, for both, GC and nGC DLBCL, miR-125b was positively correlated to Ki-67. As a GC characteristic, miR-125b was associated to either MYC or MYC/BCL2 expression. As a matter of fact, Manfè et al. suggest a mechanism in cancer cells in which miR-125b targets the MYC antagonist MAD4 [27]. In addition, miR-125b is likely to up-regulate Wnt/β-catenin activity by down-regulating β-catenin degradation targets, thereby allowing more downstream targets such MYC to be expressed [5].

4.2 Clinical impact of the studied biomarkers according to cell-of-origin phenotype

We also analyzed clinical features and their associations with the studied biomarkers considering the cell-of-origin classification. It is interesting to note that miR-155b is associated to the absence of B symptoms and to a lower ECOG status in our GC subgroup. In contrast, a Turkish study found that higher levels of miRNA-155 in patients were associated to the presence of B symptoms, involvement of extranodal sites, and high ECOG performance scores in DLBCL [28]. In a mantle-cell lymphoma study, however, He et al. found no associations between miR-155b and these parameters at all [29]. Both studies did not consider GC *versus* nGC classification on

their analyses, and they used RT-PCR to evaluate miR-155b expression rather than ISH, which may explain our conflicting findings. Considering the nGC subgroup, we found that mi-125b expression was consistently associated to nodal disease, as opposed to Zhan et al., who found no association between miR-125b expression and clinical parameters [30].

With regards to nGC-DLBCL, we found that TP53 expression was associated to B-symptoms and advanced ECOG status. That is in accordance with a Russian cohort of *de novo* DLBCL patients, whose B-symptoms, splenomegaly and bone marrow involvement were *TP53* mutation correlated [31]. However, in this study, the authors have not stratified their patients by cell-of-origin classification. BCL2 expression, on the other hand, seems to be associated to higher disease onset age regardless of DLBCL molecular subtype. Again, this does not seem to be a general observation, as Iqbal et al., also considering cell-of-origin classification, has not found such result [32].

Next, we evaluated clinical outcomes of each biomarker according to molecular subtype. As presumed, the GC phenotype showed an independent positive prognostic impact as highlighted by the multivariate analysis. Most DLBCL recapitulate the differentiation and maturation mechanisms observed in the two main subtypes previously defined by GEP: the GCB DLBCL subtype has a GEP related to a GC cell of origin, with gene mutation instrumental for GC development, GC dark and light zone transitions and microenvironmental interactions, whereas the ABC subtype, which derives from cells of GC exit or post GC origin, with either germinal center-exit or early plasmablastic phenotype, and is characterized by being BCR and NFκB dependent. In fact, although Hans algorithm may present concordance issues with GEP, it has being widely used in routine practice and is recommended by the World Health Organization on its last edition [2, 33].

Interestingly, when analyzing primary response to R-CHOP scheme, we found that miR-155b expression was associated to progressive disease in the nGC subgroup. This suggests that in this subgroup, miR-155b is more likely to indicate a poor prognostic feature. Although it was not statistically significant, its borderline *p*-value in multivariate analyses may be interpreted as a potential prognostic marker as result of our limited sample size. Quantifying miR-155b plasmatic levels by RT-PCR, Ahmadvand et al. found a negative impact of miR-155b high levels on DLBCL clinical outcomes, however they did not consider molecular subgroups on their analyses [8].

Similarly, Zhong et al. found that low miR-155b levels were related to complete response to R-CHOP therapy and, conversely, miR-155b was an independent prognostic factor in DLBCL [34]. Nevertheless, this observation is heterogeneous in literature. Contradictorily, Jung and Aguiar observed that high expression of miR-155 was associated to improved prognosis exclusively within the ABC subgroup [35]. More studies, with a large sample size, are needed to confirm the prognostic impact of miR-155b in DLBCL and in its subtypes.

On the other hand, also considering nGC cases, miR-125b tended to be an independent positive prognostic factor. miR-125b signalling has an important functional significance in the regulation of lymphoma tumor development and resistance of lymphoma cells to chemotherapy due its interactions with different pathways [36], but the literature is scarce when it comes to evaluate miR-125b as a potential prognostic marker in DLBCL [37, 38]. Yuan et al. found that high levels of miR-125b were associated to R-CHOP resistance and therefore to poor prognosis, as patients with higher miR-125b levels had a shorter overall survival [39]. Nevertheless, they analyzed serum miRNA rather than direct tumor miRNA and they did not stratify their cases in DLBCL subtypes, which may explain our conflicting results. As previously discussed, we found that miR-125b is associated to MYC only among GC-DLBCL, which suggests that miR-125b role in fact vary upon DLBCL subtype. It would be more accurate to evaluate miR-125b expression as a prognostic marker considering GC and nGC subgroups as different diseases rather than DLBCL as a single entity. Our analyses are limited due a small number of patients and further investigation is needed.

Also, in the nGC subgroup TP53 expression showed a trend of negative prognostic factor in the multivariate analyses. The TP53 protein is so-called the "genome guardian"", a central tumor suppressor that mediates cell-cycle arrest, DNA repair, apoptosis and senescence. Alterations in the *TP53* gene may disrupt the protein functions and has been implicated on lymphomagenesis and disease progression [21, 22]. Although *TP53* genetic alterations have not been evaluated in our study, TP53 immunoexpression has been strongly correlated to *TP53* mutations in DLBCL [40, 41], as mutated *TP53* leads to protein stability in comparison to the shorter half-life wild type form. For instance, a classic study leaded by Xu-Monette et al. demonstrated that *TP53* mutations predict poor survival rates in all DLBCL patients. Albeit both molecular subtypes OS were affected by TP53 mutation, ABC-DLBCL patients treated with R-CHOP have significantly inferior OS and PFS com-

pared to GCB-DLBCL patients both in the entire DLBCL cohort [40]. As a matter of fact, the distinctive molecular programs harbored by GCB- and ABC-DLBCL might explain the differences in treatment response and in the impact of *TP53* mutations. Interestingly, Peroja et al found that overall *TP53* mutations was not of prognostic impact, but specific mutations involving the DNA-biding domain were associated to a shorter survival in DLBCL [41]. We have previously demonstrated that TP53 overexpression may reflect specific *TP53* mutations in gliomas [42] and here we hypothesize that in the nGC-DLBCL the genetic alterations culminating in TP53 immunoexpression are crucial to derange protein functions and to indicate worse survival.

In conclusion, the present study validates Hans algorithm as an alternative approach to subclassify DLBCL. We have demonstrated different pathways based on GC *versus* nGC classification and highlighted different clinical outcomes. In addition, miR-125b, miR-155b and TP53 expression might represent potential prognostic factors in nGC-DLBCL. Further investigation, multi-institutional, with a larger number of subjects are needed to confirm this data and to achieve a better understanding of DLBCL molecular landscape.

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COMPETITION INTERESTS

None.

ETHICS APPROVAL STATEMENT

The present study was approved by the hospital ethics committee of the Instituto do Câncer do Ceará under reference approval number 66308017.8.0000.5528.

CONTRIBUTORSHIP STATEMENT

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by EHCNF, SBZ, PGBS, RMRB and SHBR. The first draft of the manuscript was written by EHCNF and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Supplementary table - biomarkers distribution and clinical features according to miR-125b and miR-155b positivity.

	GC-DLBCL						nGC-DLBCL					
	miR-125b+	**miR-125b -**	***p***	**miR-155b+**	**miR-155b -**	***p***	**miR-125b+**	**miR-125b -**	***p***	**miR-155b+**	**miR-155b -**	***p***
Age >60	17	15	0.85	6	27	0.73	23	13	1	9	28	0.78
Age <60	21	15		9	28		16	10		8	18	
Stage I-II	23	16	0.88	10	31	0.18	20	14	0.50	8	26	0.59
Stage III-IV	16	9		3	22		17	7		8	16	
>1 Extranodal disease	3	4	0.69	2	5	0.63	5	2	0.70	3	4	0.38
≤1 Extranodal disease	36	26		13	52		33	21		14	40	
Elevated LDH levels	20	17	0.71	9	28	0.53	23	10	0.39	10	23	1
Normal LDH levels	11	13		4	20		12	9		6	15	
B symptoms +	22	19	0.94	4	36	**0,006**	20	10	0.84	6	24	0.29

B symptoms -	15	11		11	16		17	11		10	18	
ECOG PS ≥ 2	14	7	0.16	7	21	0.13	14	10	0.54	4	20	0.54
ECOG PS <2	8	12		1	16		15	6		6	15	
MYC +	3	0	0.24	0	3	1	8	2	0.30	2	8	0.71
MYC -	36	33		15	54		32	21		15	38	
BCL2 +	26	23	0.98	11	38	0.76	25	17	0.51	11	31	1
BCL2 -	13	10		4	19		15	6		6	15	
TP53 +	12	9	0.94	4	17	1	15	7	0.77	6	16	1
TP53 -	27	24		11	40		25	16		11	30	
MYC/BCL2 +	2	0	0.49	0	2	1	6	1	0.40	2	4	0.65
MYC/BCL2 -	37	33		15	55		35	22		15	42	
MYC/TP53 +	1	0	1	0	1	1	5	2	1	2	5	1
MYC/TP53 -	38	33		15	56		35	21		15	41	

MYCt +	10	8	1	2	16	0.32	10	2	0.18	3	9	1
MYCt -	29	25		13	41		30	21		14	37	
BCL2t +	6	4	0.74	1	9	0.67	9	5	1	2	11	0.48
BCL2t -	33	29		14	48		32	18		15	35	
BCL6t +	4	6	0.49	1	8	0.67	1	1	1	0	1	1
BCL6t -	35	28		14	49		39	23		17	45	
Double-hit +	4	4	1	1	7	1	4	2	1	0	6	0.17
Double-hit -	35	29		14	50		36	21		17	40	
Ki-67 ≥ 70%	28	16	**0.04**	11	32	0.25	32	15	0.31	12	35	0.90
Ki-67 < 70%	11	17		4	25		8	8		5	11	

Note: chi-square or Fisher exact test was used to compare miRNA positive and negative groups.

CAPÍTULO 3

Artigo: Letter to the Editor - Molecular profile of EBV-DLBCL in a Brazilian population.

Autores: **Eduardo Henrique Neves Filho, Silvia Helena Rabenhorst**

Ano de confecção: 2023.

LETTER TO THE EDITOR

MOLECULAR PROFILE OF EBV-DLBCL IN A BRAZILIAN POPULATION.

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INTRODUCTION

Epstein-Barr virus (EBV)+ diffuse large B-cell lymphoma not otherwise specified (EBV-DLBCL) is a rare entity, especially in Western countries, with only few studies considering the Brazilian population [1, 2]. EBV-DLBCL consists of a group of highly heterogeneous tumors with different clinicopathological characteristics, genetic alterations, responses to therapy and prognosis, in which lack of uniform criteria and heterogenous geographic distribution may account for differences reported in molecular features, especially variations in microRNAs expression among lymphoma patients due to EBV infection [3, 4]. So that, the aim of this study was to analyze the presentation profile of commonly studied biomarkers in DLBCL, such as *MYC*, *BCL2*, *BCL6* chromosomic rearrangements and MYC, BCL2, EZH2 and TP53 expression in a series of EBV-DLBCL. In addition, we also considered miR-125b and miR-155b detection in these cases.

MATERIALS AND METHODS

Samples of 139 DLBCL cases were obtained from the files of the Cancer Institute of Ceará, Fortaleza-Brazil, from 2012-2017, with the institutional internal review board's approval. The pathological diagnoses were established according to the criteria of the 2016 World Health Organization classification, and determination of the germinal center (GC) or non-GC phenotype was based on the Hans algorithm. EBV detection was analyzed by RNA *in situ* hybridization (ISH) using complementary probes to the transcript EBER1 as previously described and EBV positivity was considered when at least 80% of neoplastic nuclei were stained [3, 5]. The representative tumor areas in each case were selected by microscopic examination to make a tissue microarray (TMA) with 2.0 mm punch using Manual Tissue Microarrayer QuickRay™ system (UNITMA Co., Bokjeong-ro, Korea), in which immunohistochemistry was performed on 2-μm tissue microarray sections using EZH2 (Clone 11, dilution 1:50, Cell Marque™, Darmstadt, Germany), MYC (Clone Y69, dilution 1:50, Abcam, Shanghai, China), TP53 (DO-7, ready-for-use, DAKO™, Glostrup, Denmark), BCL2 (Clone 124, ready-for-use, DAKO™) and Ki-67 (Clone MIB-1, ready-for-use, DAKO™) as previously described. FISH dual-color break-apart probes (MYC/8q24 and BCL6/3q27) and dual-color fusion translocation probes (BCL2/18q21) (Abbott

Laboratories, Des Plaines, IL) were used for translocation detection as previously describe [6]. For miR-125b and miR-155b we performed ISH using specific LNA probes (hsa-miR-125b-5p miRCURY and hsa-miR-155b-5p miRCURY LNA miRNA detection probe, concentration at 40nM for both probes, Exiqon™, Vedbæk, Denmark) as previously described [7]. As we had a very limited sample (see results section), all our findings were presented descriptively, with no statistic analyses.

RESULTS

EBV was positive in 7.9% (11/139) in the series of selected cases. Although the number of EBV-positive cases is not high, this series of DLBCL, composed of 63.6% nGC subtype, showed peculiar aspects related to the studied markers (Table 1). Interestingly, the majority of nGC patients presented extranodal disease (5/7) and, conversely, only one CG case was extranodal (1/4).

Most of the tumors (72.7%; 9/11) had a high proliferative index (Ki-67>70%) independently on being GC or nGC subtype. miR-125b was detected in almost all of these cases, and conversely, only two cases were miR155b positive. It is noteworthy that BCL2 expression was observed in most cases (81.8%; 9/11), although *BCL2* translocation was a rare event (18.2%; 2/11). Similarly, MYC expression and *MYC* translocation was observed in only one and two patients, respectively. TP53, on the other hand, was commonly expressed (45.4%; 5/11) regardless the molecular subtype.

DISCUSSION

EBV-related DLBCL is rare in immunocompetent patients and its frequency varies geographically, comprising as low as 2% of cases in Europe *versus* approximately 9% in Latin American or Asian studies [3, 8]. The prevalence of EBV in this study was high considering western population but is similar to another Brazilian study (8.5%) [1, 9]. To our knowledge, this is the first description of a Northeastern Brazil series of immunocompetent EBV-DLBCL with biomarker expression profile.

Dojcinov et al [10] and Zhou et al [8] pointed EBV-DLBCL as a mainly extran-

odal disease. We found similar results considering the nGC cases, although in the general sample no difference was observed (6 extranodal x 5 nodal). However, our series presented more CG cases and these authors did not consider the molecular subtypes on their analyses, which may suggest that extranodal disease is a nGC EBV-DLBCL feature. As demonstrated by Bourbon et al [3], BCL2 was widely expressed. Interestingly, this does not seem to happen due *BCL2* translocation, as it is a rare event in our series, but probably due a virus intrinsic mechanism. For instance, as discussed by Fu et al (2013), EBV nuclear and membrane proteins, specially LMP1, may induce BCL2 overexpression by *BCL2* upregulation as the main immortalization effect on B-lymphomas [11].

Draws attention that miR-125b was detected in almost all high proliferative cases independently on CG versus nCG classification. Several studies have reported that miR-125b activates NF-κB pathway in lymphoma [12, 13]. In addition, EBV might also mediate NF-κB pathway through LMP1, resulting in high proliferative B-cell disorders [3, 10]. Interestingly, in our series, only two cases showed miR-155b expression. The literature is contradictory in EBV-induced changes of miR-155b expression in lymphoma. Although some authors points miR-155b as one of the most frequently expressed microRNA in DLBCL [14, 15], Imig et al. (2011), in accordance to our findings, showed that miR-155b was down-regulated by more than two folds in EBV-DLBCL compared to EBV-negative DLBCL patients [16]. It is noteworthy that these studies were performed on *in vitro* samples, in different populations and mostly applied gene sequencing and RT-PCR on their analyses, which albeit are more sensitive techniques, are less specific than ISH, what may explain our diverging results. As demonstrated in a previous study, EZH2 expression is consistently associated with high proliferative index in DLBCL [6]. Although only four cases were EZH2-positive in these selected EBV patients, it is interesting to note that in miR-125b negative cases with high Ki-67 index, EZH2 was expressed and, conversely, in miR-125b negative cases with low proliferative index, EZH2 is either negative. A few studies have shown that chronic activation of NF-kB can also induce EZH2 expression in B-cell malignancies [17, 18]. This pathway might provide a mechanism through which tumor induced NF-kB can inhibit tumor suppressor functions and promote tumorigenesis. Furthermore, the association miR-125b/EZH2 positive could be an additive factor.

This study is limited to a small sample size due the rarity of EBV-DLBCL

among the Brazilian population; nevertheless, it is representative of the EBV-DLGBL cases frequency treated at the Ceará Cancer Institute in a relatively short period of time (2012-2017). Others studies, multi-institutional, with a larger number of subjects are needed to a better understanding of EBV-DLBCL molecular landscape.

CONFLICTS OF INTEREST

The authors have no conflict of interest to declare.

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TABLE 1 - Distribution of the studied biomarkers expression profile over the 11 EBV-DLBCL cases

Patient	Subtype	MYC	BCL2	TP53	*MYC*t	*BCL2*t	*BCL6*t	EZH2	miR125b	miR155b	Ki-67
1	CG	Negative	**Positive**	Negative	Negative	Negative	Negative	Negative	**Positive**	**Positive**	70%
2	CG	Negative	**Positive**	Negative	Negative	Negative	Negative	**Positive**	Negative	Negative	70%
3	CG	Negative	**Positive**	**Positive**	Negative	**Positive**	Negative	Negative	**Positive**	Negative	85%
4	CG	Negative	**Positive**	**Positive**	**Positive**	Negative	Negative	Negative	**Positive**	Negative	70%
5	nCG	Negative	**Positive**	**Positive**	Negative	Negative	Negative	**Positive**	**Positive**	Negative	80%
6	nCG	Negative	**Positive**	Negative	Negative	Negative	Negative	Negative	Negative	Negative	30%
7	nCG	Negative	**Positive**	**Positive**	Negative	Negative	Negative	Negative	**Positive**	Negative	60%
8	nCG	Negative	**Positive**	Negative	negativo	**Positive**	Negative	**Positive**	**Positive**	**Positive**	85%

9	nCG	**Positive**	Negative	**Positive**	Negative	Negative	Negative	**Positive**	Negative	Negative	70%
10	nCG	Negative	**Positive**	Negative	Negative	Negative	Negative	Negative	Negative	Negative	20%
11	nCG	Negative	Negative	Negative	**Positive**	Negative	Negative	Negative	**Positive**	Negative	90%

CG - germinal center; nCG - non-germinal center; *MYCt* - *MYC* translocation; *BCL2t* - *BCL2* translocation; *BCL6t* - *BCL6* translocation

10 CONCLUSÕES

Objetivo 1- Associar a expressão proteica de MYC, BCL2, TP53 e EZH2 nos casos de LDGCB de acordo com a célula de origem e com a presença das translocações envolvendo MYC, BCL2 e BCL6.

Conclusão:

A) a expressão de MYC foi associada ao subtipo nCG. Neste subgrupo, a expressão de EZH2, frequentemente observada nos casos de LDGCB avaliados, foi associada à expressão de MYC e TP53 e a um alto índice. Adicionalmente, a translocação de BCL2 foi associada à expressão de BCL2 somente no subtipo nCG.

B) nos LDGCB tipo CG, a translocação de BCL6 parece ser uma característica marcante. Neste subgrupo, entretanto, a expressão de EZH2 está associada apenas à expressão de TP53;

C) a translocação de MYC está associada à expressão de MYC em ambos os tipos moleculares e está envolviada na linfomagênese independente das células de origem.

D) os diferentes padrões de expressão dependendo da classificação pela célula de origem, assim, descrevem uma via molecular envolvendo EZH2/MYC/TP53 característica do subgrupo nCG e, por outro lado, uma via EZH2/TP53 no subtipo CG.

Objetivo 2- Traçar o perfil de expressão de miR-125b e miR-155 nos casos de LDGCB.

Conclusão:

A) nos LDGCB, a expressão de miR-125b foi frequente, sendo associada à elevada proliferação celular independentemente do subtipo molecular analisado. No subgrupo CG, entretanto, sua expressão foi associada à expressão de MYC;

B) a expressão de miR-155b foi baixa e não apresentou nenhuma associação com os demais biomarcadores avaliados ou com o subtipo molecular.

Objetivo 3- Determinar a frequência da infecção por EBV nos casos de LDGCB, correlacionando os dados clínico-patológicos com os dados encontrados.

Conclusão:

A) o EBV foi raramente expresso em LDGCB na nossa população;

B) os casos de LDGCB associados ao EBV normalmente apresentam alto índice proliferativo e alta frequência da expressão de miR-125b.

Objetivo 4- Avaliar a importância prognóstica e o potencial terapêutico dos dados encontrados.

Conclusão:

A) o fenótipo CG demonstrou ser fator prognóstico positivo independente, estando associado a maior sobrevida global;

B) EZH2 não apresentou associações com a resposta primária ao R-CHOP ou com a sobrevida global dos casos de LDGCB avaliados;

C) A expressão de miR-155b foi associada à progressão de doença em pacientes tratados com R-CHOP no subgrupo nCG e a expressão de BCL2 à ausência de resposta nesses pacientes;

D) A expressão de TP53, miR-125b e miR-155b demonstraram tendência estatística como potenciais biomarcadores prognósticos no subgrupo nCG.

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