UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
CENTRO DE BIOTECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

Análise de redes de coexpressão de amostras do sangue periférico de pacientes com leucemia mieloide aguda

Tese de Doutorado

Kendi Nishino Miyamoto

Porto Alegre, novembro de 2018

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Tese submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular da Universidade Federal do Rio Grande do Sul (UFRGS), como requisito parcial para a obtenção do grau de Doutor em Biologia Celular e Molecular

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Orientador: Dr. Diego Bonatto

Porto Alegre, novembro de 2018

Esta tese foi desenvolvida no Laboratório de Biologia Computacional e Molecular da UFRGS e no Centro Nacional de Supercomputação (CESUP) da UFRGS, sendo financiada pelo Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)

Dedicatória

献身

Este trabalho científico é dedicado à minha avó Mieko

この科学的研究は私の祖母美枝子に捧げられています.

“Quanto ao mais, irmãos, tudo o que é verdadeiro, tudo o que é honesto, tudo o que é justo, tudo o que é puro, tudo o que é amável, tudo o que é de boa fama, se há alguma virtude, e se há algum louvor, nisso pensai.”

Filipenses 4:8

“We can't have full knowledge all at once. We must start by believing; then afterwards we may be led on to master the evidence for ourselves. “

São Tomás de Aquino

“Há pessoas que desejam saber só por saber, e isso é curiosidade; outras, para alcançarem fama, e isso é vaidade; outras, para enriquecerem com a sua ciência, e isso é um negócio torpe; outras, para serem edificadas, e isso é prudência; outras, para edificarem os outros, e isso é caridade.”

São Tomás de Aquino

Agradecimentos

Durante esse período, houve tantas pessoas que cruzaram pelo meu caminho e que deixaram comigo um pouco da sua bondade em me oferecer atenção, conselhos, carinho e muito amor. Qualquer trabalho se torna mais prazeroso e leve quando somos cercados de pessoas que estão sempre prontas para lhe estender a mão. É impossível trabalhar sozinho, achando que nunca irá depender ou necessitar da ajuda dos outros. Portanto, vou tentar listar aqui abaixo as pessoas pelas quais eu tenho meu mais sincero e profundo agradecimento. Peço perdão de antemão caso eu esqueça do nome de alguém.

Primeiramente, agradeço à minha família, não só pelo apoio em todas as esferas da vida, como também por me ensinar valores inestimáveis a qualquer ser humano: compaixão, carinho, respeito, empatia (tão ausente nos dias atuais) e força e suporte emocional para as mais duras batalhas da vida. Amo vocês e dedico todo esse trabalho a vocês!

Ao meu namorado Igor, eu não saberia nem por onde começar para lhe agradecer o quanto seu carinho, amor e conselhos foram fundamentais para que eu melhorasse como profissional e ser humano. Obrigado pelo ombro sob todas as vezes que tive uma crise de tristeza ou desamparo eu soube que poderia ali me repousar. Esse trabalho tem grande parte da tua contribuição! Te amo eternamente!

Aos meus amigos(as)-irmãos(ãs) do ex-laboratório 219 (Joice, Bruno, Larissa, Bia, Gabrihel, Gabriel, Raquel, Chapola, Itamar), meus mais profundos agradecimentos por serem muito mais que meros colegas de trabalho. Com vocês eu aprendi o real significado de companheirismo, que vai além da questão profissional! Obrigado pelos conselhos e amizade pelos quais serei eternamente grato e guardarei pro resto da minha vida!

Ao professor Diego Bonatto pela orientação, por ter aberto as portas do laboratório e por ter me introduzido ao mundo da Biologia de Sistemas. Agradeço também aos professores Hugo Verli e Márcio Dorn pelo suporte e auxílio em etapas importantes durante o doutorado.

À Silvinha e ao Luciano, profissionais sempre atenciosos e prestativos. Agradeço imensamente pelo carinho e pela boa vontade que sempre tiveram em me auxiliar nas questões burocráticas do PPGBCM.

Ao CNPq pelo suporte financeiro para que esse trabalho pudesse ser executado e ao CESUP/UFRGS pela disponibilidade do uso dos seus clusters para as análises desse trabalho.

Apresentação da estrutura da tese

Esta tese está estruturada em: Introdução, Objetivos, Capítulo I, Capítulo Ii, Resultados Adicionais, Discussão Geral, Conclusões, Perspectivas, Referências, Apêndice e Anexos I e II.

Na introdução, será dissertado acerca dos aspectos básicos sobre leucemias (com ênfase maior em Leucemias Mieloides Agudas - LMA, que é o assunto principal deste trabalho), que vão desde as características clínicas e epidemiológicas até os métodos de diagnóstico e tratamento até o presente momento. Ainda neste capítulo, será abordado um tópico sobre redes biológicas, especialmente redes de coexpressão gênica, bem como suas aplicabilidades para a descoberta de genes/proteínas chaves no desenvolvimento tumoral. Após isso, será apresentada a problemática que justifica a execução deste trabalho, bem como o objetivo geral e específico do mesmo.

No primeiro capítulo será abordada uma revisão bibliográfica, em formato de artigo científico, sobre os principais constituintes na corrente sanguínea (células, pequenas moléculas, exosomas, etc.) que estão diretamente relacionadas com sobrevivência dos blastos em LMA. Este artigo foi submetido no periódico *Current Hematologic Malignancy Reports*.

O segundo capítulo será apresentado na forma de um artigo científico que será submetido para publicação. Este artigo aborda as principais características observadas em uma rede de coexpressão gênica das células de LMA encontradas no sangue periférico.

Na seção de resultados adicionais, será apresentada uma análise adicional de genes diferencialmente expressos das amostras de LMA presentes no sangue periférico em comparação com as de medula óssea com o intuito de verificar de que forma esses diferentes contextos celulares influenciam nas células leucêmicas.

Na discussão geral, serão apresentados dados e comentários adicionais que contemplam a temática das seções previamente citadas, bem como as principais perspectivas do trabalho a partir dos resultados obtidos.

Sumário

Lista de Abreviaturas

ACS – do inglês *American Cancer Society*, Associação norte-americana do câncer
ATRA – do inglês *All trans retinoic acid*, ácido trans-retinoico
CGH – do inglês *Comparative genomic hybridization*, hibridização genômica comparativa
CIVD - Coagulação intravascular disseminada
CN – Cariótipo normal
DNA – do inglês *Deoxyribonucleic acid*, ácido desoxirribonucleico
FAB – do inglês *French-American-British cooperation group*, Grupo de cooperação franco-norteamericano-britânico
FISH – do inglês *Fluorescent in situ hybridization*, Hibridização fluorescente in situ
GDE – Genes diferencialmente expressos
GEO – do inglês *Gene expression omnibus*
GvHD – do inglês *Graft versus host disease*, doença do enxerto contra hospedeiro
ITD – do inglês *Internal Tandem Duplication*, Duplicação em tandem interna
LLA – Leucemia Linfoide Aguda
LLC – Leucemia Linfoide Crônica
LMA – Leucemia Mieloide Aguda
LMC – Leucemia Mieloide Crônica
LPA – Leucemia promielocítica aguda
MAPK – do inglês *Mitogen activated protein kinase*
MRD – do inglês *Minimal residual disease*, doença residual mínima
NOS – do inglês *Non otherwise specified*, não especificada anteriormente
OMS – Organização Mundial de Saúde
PTD – do inglês *Partial Tandem Duplication*, Duplicação em tandem parcial
RC – Remissão completa
RNA – do inglês *Ribonucleic acid*, ácido ribonucleico
SKY – do inglês *Spectral karyotyping*, cariotipagem espectral
SMD – Síndrome Mielodisplásica
TGF – do inglês *Transforming growth factor*, Fator de transformação do

crescimento

TNF – do inglês *Tumor necrosis factor*, Fator de necrose tumoral

TOM – do inglês *Topological overlapping matrix*, matriz de sobreposição topológica

Treg – Linfócito T regulatório

Resumo

A leucemia mieloide aguda (LMA) é um tipo de tumor hematológico caracterizado pela expansão clonal de células imaturas da linhagem mieloide. É um dos tipos de leucemia que possuem uma das maiores taxas de mortalidade em virtude da rápida disseminação no microambiente da medula óssea e facilmente atingindo a corrente sanguínea. Sabe-se que diversos fatores que compõem um ambiente tumoral podem influenciar no desenvolvimento de uma neoplasia. Na LMA, muito tem se estudado sobre o papel das células leucêmicas em modular o microambiente da medula óssea com o intuito de garantir sua capacidade proliferativa. Contudo, pouco se sabe sobre como os fatores que permeiam o ambiente do sangue periférico auxiliam estas mesmas células na sobrevivência e no seu crescimento tumoral. Logo, o objetivo deste trabalho é de observar as principais células e elementos presentes na corrente sanguínea que estão associados à sobrevivência das células leucêmicas neste contexto celular, bem como avaliar, nessas mesmas células, o perfil transcritômico (extraídas do sangue periférico de pacientes com LMA) por meio de análises de redes de coexpressão. Os resultados obtidos indicam que uma alta porcentagem de blastos CD34+ na corrente sanguínea são indicativos de mau prognóstico por modularem a resposta imune, inibindo a ação de células T efetoras e NK, por exemplo, além de promoverem a manutenção do seu estado indiferenciado e sua proliferação.

Abstract

Acute myelogenous leukemia (AML) is a hematological malignancy characterized by the clonal expansion of immature myeloid cells. It is one of leukemia types that has the highest mortality rates, mainly due to its fast dissemination in the bone marrow microenvironments, reaching easily the peripheral bloodstream. It is known that many factors that encompass the tumor environment can influence the tumor development. In AML, much has been studied about the role of leukemic cells in modulating the bone marrow microenvironment to guarantee its growth. However, little is known about how bloodstream factors can help these cells in the survival and proliferation. Therefore, this work aims to describe the main cells and other factors inside the bloodstream that are related to leukemic cells survival in this cellular context, as well as to evaluate, in these same cells, the transcriptomic profile of AML cells (extracted from the peripheral blood of AML patients) through a network coexpression analysis. The results indicate that the high percentage of circulating CD34+ blasts are indicative of bad prognosis due their ability to modulate the immune response, inhibiting NK and/or effector T cells anti-leukemic roles, besides promoting the maintenance of the undifferentiated and their proliferative status.

Introdução

1. Leucemias e leucemia mieloide aguda

1.1. Características gerais e etiologia da LMA

As leucemias são caracterizadas pelo processo de proliferação desordenado de leucócitos formados na medula óssea (ROQUIZ; GANDHI; KINI, 2016). Este crescimento desenfreado tem como resultado o acúmulo de células imaturas que se disseminam pelo organismo do paciente, comprometendo o funcionamento do sistema imune (HOFFBRAND; MOSS, 2013). Por serem de origem hematopoiética, as leucemias podem ser categorizadas de acordo com a linhagem de origem (mieloide ou linfoide) e o grau de agressividade (aguda ou crônica) desta patologia, sendo classificada em 4 principais tipos, sendo estas: 1) Leucemia Linfoide Aguda – LLA, 2) Leucemia Linfoide Crônica – LLC, 3) Leucemia Mieloide Aguda – LMA e 4) Leucemia Mieloide Crônica – LMC.

Especificamente, a LMA, por se tratar de um tipo de leucemia aguda, caracteriza-se pela rápida proliferação e expansão na medula óssea e no sangue. As células leucêmicas originam-se a partir de um processo de malignização de precursores da linhagem mieloide, denominados mieloblastos, ou apenas blastos, como mostra a Figura 1:

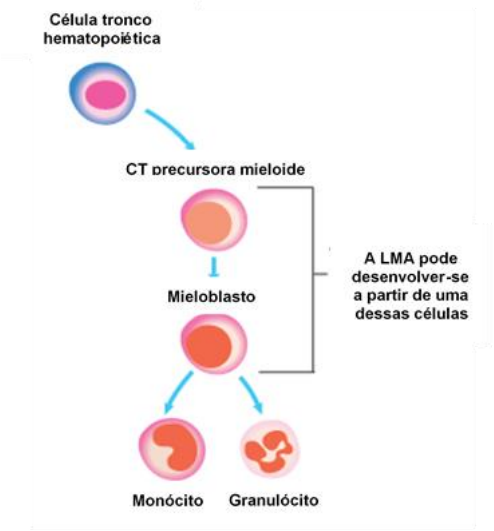

Figura 1 - Estágio de diferenciação de uma célula da linhagem mieloide e de onde potencialmente surgem as células leucêmicas da LMA. Adaptado do site Cancer Research UK (Disponível em: https://www.cancerresearchuk.org/about-cancer/acute-myeloid-leukaemia-aml/about-acute-myeloid-leukaemia)

Em um primeiro momento, a transformação leucêmica dos blastos e sua

posterior expansão clonal torna o espaço da medula óssea incapaz de produzir outros tipos de células hematopoiéticas. Isso se deve à capacidade de os blastos modularem o microambiente medular, tornando-o mais propenso para a ocupação dos nichos celulares pró-leucêmicos em detrimento dos nichos compostos por células tronco hematopoiéticas (COLMONE et al., 2008; SHAFAT et al., 2017; KUMAR et al., 2018). Além disso, esse contexto favorável para o crescimento dos blastos na medula também permite a formação de novos vasos sanguíneos (HUSSONG; RODGERS; SHAMI, 2000; SCHMIDT; CARMELIET, 2011; SHIRZAD et al., 2016).

A modificação do microambiente da medula óssea, aliado com o aumento na formação de capilares na região, permite que as células leucêmicas também migrem para a corrente sanguínea com maior facilidade, causando inúmeros quadros clínicos associados à incapacidade do tecido hematopoiético em produzir componentes necessários para o correto funcionamento do sistema imune e circulatório, bem como todo o processo de hemostasia (WEBERT et al., 2006; ROQUIZ; GANDHI; KINI, 2016).

A LMA é considerada uma doença de etiologia genética. Logo, o processo de transformação leucêmica dos mieloblastos ocorre em virtude das alterações na composição ou na estrutura do material genético. Dentre elas, alterações cromossômicas como eventos translocacionais, inversão, deleção parcial do cromossomo e aneuploidias são frequentemente encontradas em pacientes com LMA. Além disso, alterações moleculares como mutações, ITD e PTD em genes também são observadas (DESCHLER; LÜBBERT, 2006; LORENZI, 2013; DE KOUCHKOVSKY; ABDUL-HAY, 2016). A combinação dessas alterações genotípicas irá determinar qual o grau de severidade da doença e quais alternativas de tratamento para o portador da doença são mais indicadas (ver seção 1.4).

A LMA também pode ser desenvolvida pelo conjunto de 4 principais fatores predisponentes, dentre eles: 1) fatores ambientais ou associados ao estilo de vida, 2) tratamento prévio com quimioterápicos, 3) doenças genéticas e 4) histórico de doenças hematológicas prévias. O primeiro fator está associado à exposição dos indivíduos acometidos a agentes potencialmente mutagênicos e carcinogênicos, como radiações ionizantes e substâncias tóxicas como o benzeno e pesticidas (DESCHLER; LÜBBERT, 2006; TAMAMYAN et al., 2017).

Estudos mostraram que sobreviventes da tragédia das bombas atômicas lançadas nas cidades japonesas de Hiroshima e Nagasaki possuíam uma alta incidência de casos de LMA em decorrência da radiação exposta (LIU et al., 1973; PRESTON et al., 1994; DESCHLER; LÜBBERT, 2006). Além disso, fatores como a obesidade e o tabagismo também são potenciais agentes predisponentes para o desenvolvimento da LMA (DESCHLER; LÜBBERT, 2006; TAMAMYAN et al., 2017).

Outro fator relacionado à incidência de LMA ocorre em pacientes que foram anteriormente submetidos a tratamentos quimioterápicos como inibidores da topoisomerase II (etoposídeo, antraciclinas, etc.) e agentes alquilantes (ciclofosfamida, melfalan, etc.). O uso desses compostos pode causar uma LMA secundária, sendo que a primeira classe de fármacos tende a possuir um período de latência mais curto entre uma doença e outra, em comparação à segunda, que geralmente possui uma latência entre 5 à 6 anos, antecedido sempre por um quadro de mielodisplasia (LORENZI, 2013).

Quanto às doenças genéticas, várias síndromes possuem uma predisposição maior para o desenvolvimento de uma LMA. Paciente com síndrome de Down, por exemplo, tendem a possuir uma incidência maior para a formação de uma LMA, especialmente em crianças portadoras da síndrome. Outras doenças também já reportadas e relacionadas com LMA incluem: Síndrome de Wiskott-Aldrich, Síndrome de Bloom, Anemia de Fanconi, Ataxia Telangiectasia, dentre outros (DESCHLER; LÜBBERT, 2006; KHAN; MALINGE; CRISPINO, 2011; LORENZI, 2013; ROQUIZ; GANDHI; KINI, 2016; SHAND, 2017; TAMAMYAN et al., 2017).

Por fim, pacientes portadores de outras doenças hematológicas prévias podem predispô-los à LMA. Relatos de casos demonstram pacientes com linfomas e até mesmo outros tipos de leucemias desenvolvendo LMA após tratamento da doença anteriormente citada. Contudo, um dos fatores mais relacionados com o desenvolvimento da LMA são as síndromes mielodisplásicas (SMD). Essa síndrome é considerada muitas vezes como um estágio pré-leucêmico que pode levar a uma LMA relacionada à transformação de uma mielodisplasia, afetando principalmente adultos e possuindo um mau prognóstico por possuírem uma baixa taxa de remissão pós tratamento (DESCHLER; LÜBBERT, 2006; SWERDLOW et al., 2017).

1.2. Epidemiologia

Apesar de ser uma doença de baixa prevalência, ao compararmos com os demais tipos de leucemias (cerca de 32,37% do total de casos para essa neoplasia), a LMA possui uma alta taxa de mortalidade, correspondendo a aproximadamente 43,4% do total de óbitos por leucemias, de acordo com a American Cancer Society (ACS). São estimados mais de 19.520 casos novos em 2018, dos quais 10.670 (54,66%) deles resultam em óbito do paciente em território norte-americano (AMERICAN CANCER SOCIETY, 2018). Globalmente, um estudo epidemiológico a partir dos relatórios da Agência Internacional de Pesquisa em Câncer (IARC, do inglês *International Agency for Research in Cancer*) demonstrou que a LMA possui uma taxa de incidência alta em países desenvolvidos, como os EUA, Canadá, países da União Europeia, Reino Unido e Austrália. Nesse mesmo estudo, o Brasil possui uma estimativa de 4 a 5 casos a cada 100 mil habitantes, totalizando cerca de 9.340 casos (MIRANDA-FILHO et al., 2018). Contudo, é importante ressaltar que esses dados são subestimados, haja vista que o relatório não contempla todas as regiões do país e não há um relatório nacional que especifique o número de casos de LMA no Brasil e por estado da federação e/ou região.

Quanto à prevalência de acordo com o sexo, a LMA tende a acometer mais pacientes do sexo masculino em relação ao sexo feminino. Alguns autores hipotetizam, embora sem consenso, que esta diferença pode estar associada ao maior contato de indivíduos do sexo masculino a agentes ambientais predisponentes (como agrotóxicos, radiações ionizantes,etc.) (MIRANDA-FILHO et al., 2018). Com relação à idade, LMA comumente se manifesta em adultos em relação às crianças ou adolescentes de até 15 anos, sendo que a taxa de mortalidade tende a subir à medida em que a idade do paciente é mais avançada (SEKERES et al., 2008; DAVIS; BENJAMIN; JONAS, 2018).

1.3. Classificação dos subtipos de LMA

A classificação dos subtipos de LMA, assim como grande parte das malignidades hematológicas, possui diferenças bastante significativas em

relação a maioria dos tumores sólidos, cuja categorização está atrelada aos estágios de crescimento da massa tumoral e também à sua capacidade de metastização. Na LMA, a categorização é direcionada às propriedades da célula leucêmica, que vão desde suas características morfológicas até as alterações a nível molecular.

Em 1976, um grupo de hematologistas franceses, britânicos e norte-americanos propuseram a divisão dos casos de LMA em sete grupos distintos, baseada no nível de diferenciação em que os blastos se encontram, nomeando-a como classificação FAB (do inglês *French-American-British*, em virtude da nacionalidade dos pesquisadores envolvidos) (BENNETT et al., 1976). Posteriormente, foram adicionadas outras duas categorias: M0, para LMA que possuíam células não diferenciadas; e M7, para leucemia megacarioblástica aguda, totalizando nove subtipos de LMA preconizados por essa classificação, conforme demonstrado na Tabela 1 (BLOOMFIELD; BRUNNING, 1985; LEE et al., 1987). Desde então, a classificação FAB tem sido utilizada amplamente no diagnóstico clínico, uma vez que a subdivisão é útil para determinar qual abordagem terapêutica é mais adequada para o paciente acometido.

Tabela 1- Subtipos de LMA de acordo com a classificação FAB

Classificação FAB	Nome
M0	LMA não diferenciada
M1	LMA com mínima maturação
M2	LMA com maturação
M3	Leucemia promielocítica aguda (LPA)
M4	Leucemia mielomonocítica aguda
M4 eos	Leucemia mielomonocítica aguda com eosinofilia
M5	Leucemia monocítica aguda
M6	Leucemia eritroide aguda
M7	Leucemia megacariobástica aguda

Entretanto, o avanço das técnicas de diagnóstico molecular permitiu identificar alterações mais específicas em LMA, como anormalidades citogenéticas (deleções, translocações, entre outros) e/ou mutações em genes chaves para o crescimento tumoral, permitindo, assim, uma conduta clínica e terapêutica mais eficiente. Tendo em vista este fato, a classificação dos subtipos

de LMA somente pelos aspectos citomorfológicos torna-se, muitas vezes, insuficiente e reducionista demais para definir o prognóstico do paciente e posterior escolha de tratamento (VARDIMAN et al., 2002).

Logo, em 2001, a Organização Mundial de Saúde (OMS) estabeleceu a divisão dos casos de LMA contemplando as alterações moleculares e citogenéticas de relevância clínica, conforme mostra a Tabela 2:

Tabela 2 – Classificação atualizada de 2017 dos subtipos de LMA, conforme a OMS (Adaptado de ARBER et al., 2016 e SWERDLOW et al., 2017)

Leucemia mieloide aguda e neoplasias relacionadas
Leucemia Mieloide Aguda (LMA) com anormalidades genéticas recorrentes:
• LMA com t(8;21)(q22;q22.1); RUNX1-RUNX1T1 • LMA com inv(16)(p13.1q22) ou t(16;16)(p13.1;q22); CBFB-MYH11 • Leucemia Promielocítica Aguda com fusão gênica PML-RARα • LMA com t(9;11)(p21.3;q23.3); MLLT3-KMT2A • LMA com t(6;9)(p23;q34.1); DEK-NUP214 • LMA com inv(3)(q21q26.2) ou t(3;3)(q21;q26.2); GATA2, MECOM • LMA (megacarioblástica) com t(1;22)(p13.3;q13.1); RBM15-MKL1 • LMA com fusão gênica BCR-ABL1
Leucemia mieloide aguda com mutações em genes:
• LMA com mutação no gene NPM1 • LMA com mutação bialélica no gene CEBPA • LMA com mutação no gene RUNX1
Leucemia mieloide aguda relacionada à transformação de mielodisplasia
Neoplasias mieloides associadas a quimioterapia ou radioterapia
Leucemia mieloide aguda, sem outra classificação específica (NOS):
• LMA com diferenciação mínima • LMA sem maturação • LMA com maturação • Leucemia mielomonocítica aguda • Leucemia monoblástica/monocítica aguda • Leucemia eritroide pura • Leucemia megacarioblástica aguda • Leucemia basofílica aguda • Panmielose com mielofibrose aguda

Desde 2001, foram realizadas modificações na classificação com intuito de incluir alterações moleculares e citopatológicas com maior relevância clínica (ARBER et al., 2016). Nesta quarta edição, publicada em 2017, os casos de LMA estão divididos de acordo com as modificações citogenéticas mais recorrentes, com mutações em genes associados ao desenvolvimento da neoplasia

(mencionada pela primeira vez nesta versão), LMA resultantes da transformação de uma Síndrome Mielodisplásica (SMD) e LMA desenvolvidas pós quimioterapia ou radioterapia. Além disso, ela também comporta os critérios estabelecidos na classificação FAB mencionada anteriormente, porém agora descrita como “LMA sem outra classificação específica” (ou NOS, do inglês *“Non otherwise specified”*) (ARBER et al., 2016; SWERDLOW et al., 2017).

Até os dias atuais, a categorização dos casos de LMA de acordo com o preconizado pela OMS tem sido de extrema valia para estabelecer rotinas de diagnóstico e conduta clínica (VARDIMAN et al., 2002). Muitas alterações citogenéticas podem definir imediatamente qual método terapêutico a ser utilizado pelo médico, como é o caso da translocação que origina a fusão gênica PML-RARα, indicativo de uma leucemia promielocítica aguda.

1.4. Sintomas, diagnóstico e tratamento

Inicialmente, as primeiras suspeitas de casos de LMA geralmente ocorrem por meio da sintomatologia do paciente. São bastante comuns quadros de infecções recorrentes, anemias, quadros severos de hemorragia e, em alguns casos, infiltrações de células leucêmicas em tecidos - como as gengivas – e até mesmo no sistema nervoso central (MEENA et al., 2002; HOFFBRAND; MOSS, 2013; FELIX et al., 2017). Outros sintomas mais genéricos incluem: perda de apetite, falta de ar, dificuldade de respirar, febres, cansaço, palidez, dores nas articulações, dentre outros (HOFFBRAND; MOSS, 2013). Por fim, certos subtipos de LMA possuem sintomas bastante específicos. Em uma LMA M3 (LPA), por exemplo, é observado uma alta incidência de coagulação intravascular disseminada (CIVD) nos pacientes, além de uma síndrome hemorrágica em consequência do aumento da taxa de fibrinólise (HOFFBRAND; MOSS, 2013; LORENZI, 2013). Todas essas complicações mencionadas anteriormente podem levar o paciente a óbito meses após o diagnóstico, caso nenhum tratamento seja iniciado (DESCHLER; LÜBBERT, 2006). Pacientes em idade mais avançada tendem a ter uma sobrevida menor ainda, além de serem menos resistente aos ciclos de quimioterapia e/ou radioterapia (SEKERES et al., 2008; TAMAMYAN et al., 2017).

Já nas análises hematológicas laboratoriais, pacientes com LMA possuem um quadro de anemia normocítica e trombocitopenia. Em um esfregaço sanguíneo, observa-se uma grande quantidade de blastos presentes no sangue e/ou em aspirados de medula óssea, sendo necessário que essa quantidade atinja pelo menos 20% do total de leucócitos para ser considerado uma leucemia do tipo aguda, conforme preconizado pela OMS (embora em casos como alterações citogenéticas ou moleculares não necessite esse valor) (O'DONNELL et al., 2017; SWERDLOW et al., 2017). Para o diagnóstico diferencial de LMA, as análises microscópicas geralmente revelam uma estrutura denominada de bastões de Auer nos blastos, conforme ilustra a Figura 2:

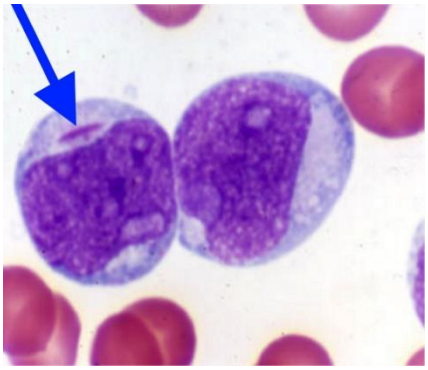

Figura 2 - Mieloblasto com a presença de um Bastão de Auer (indicado pela seta). Imagem retirada no *site* LabCE (Disponível em: https://www.labce.com/spg28990_auer_rods.aspx)

Além da presença de bastões de Auer, outros exames podem ser utilizados com intuito de auxiliar no diagnóstico de LMA. Dentre eles, testes citoquímicos como os ensaios de mieloperoxidase, Sudan Black B e de Esterases não específicas são comumente realizados para a diferenciação de LMA para LLA (HOFFBRAND; MOSS, 2013).

Para a determinação do prognóstico do paciente, bem como a abordagem terapêutica a ser escolhida pelo médico hemato-oncologista, são realizados testes citogenéticos (cariotipagem convencional em banda G, FISH, CGH *array* e SKY) e moleculares (RT-qPCR e sequenciamento de última geração) para determinar a presença de alterações que são indicativos de um prognóstico favorável ou desfavorável ao paciente, conforme a Tabela 3 (MRÓZEK et al., 2002; ZHENG et al., 2009; DEKKING et al., 2010; ZHANG et al., 2014; ILYAS et al., 2015). Um exemplo disso é a detecção da translocação dos cromossomos 15 e 17, fusionando os genes PML e RARα (t(15;17)(q22;q12)), indicativo de uma LMA M3 (LPA), que indica um prognóstico favorável).

Tabela 3 - Estratificação do prognóstico do paciente de acordo com as alterações citogenéticas e moleculares observadas em um paciente com LMA. Adaptado de DE KOUCHKOVSKY; ABDUL-HAY, 2016 e O'DONNELL et al., 2017

Grupo de risco	Somente alterações citogenéticas	Alterações citogenéticas e moleculares
Favorável	t(8:21)(q22;q22)	t(8:21)(q22;q22) sem mutação em c-KIT
	inv(16)(p13;1q22)	inv(16)(p13;1q22), acrescidas de alterações moleculares
	t(15;17)(q22;q12)	t(15;17)(q22;q12), acrescidas de alterações moleculares
		Mutação em FLT3 com ITD (CN)
		Mutação bialélica em CEBPA (CN)
Intermediário	CN	t(8:21)(q22;q22) com mutação em c-KIT
	t(9;11)(p22;q23)	t(9;11)(p22;q23), acrescidas de alterações moleculares
	Anormalidades citogenéticas não incluídas no grupo de prognóstico favorável ou adverso	CN e anormalidades citogenéticas não incluídas no grupo de prognóstico favorável ou adverso
Adverso		Mutação em TP53, independentemente do perfil citogenético
		CN com ITD em FLT3
	inv(3)(q21q26.2)	CN com mutação em DNMT3A
	t(6;9)(p23;q34)	CN com PTD em KMT2A
	Anormalidades em 11q além de t(9;11)	inv(3)(q21q26.2), acrescidas de alterações moleculares
	−5 ou del(5q)	t(6;9)(p23;q34), acrescidas de alterações moleculares
	−7	Anormalidades em 11q além de t(9;11), acrescidas de alterações moleculares
	Cariótipo complexo	−5 ou del(5q), acrescidas de alterações moleculares
		−7, acrescidas de alterações moleculares
		Cariótipo complexo, acrescidas de alterações moleculares

Em relação ao tratamento, as abordagens clínicas estão divididas em: 1) terapia de suporte, 2) terapia de indução, 3) terapia de consolidação, 4) terapia de manutenção e 5) terapia de tratamento de recidivas. O primeiro tipo de terapia visa estabilizar o quadro do paciente, por meio de frequentes transfusões de sangue (e de plaquetas, em casos de LPA), hidratação oral, administração profilática de antibióticos e controle da hiperuricemia. As demais etapas visam atingir a remissão completa (RC), ou seja, a eliminação de quaisquer remanescentes de células leucêmicas (HOURIGAN; KARP, 2013). A chance de uma abordagem clínica atingir uma remissão completa é avaliada durante todo o curso de tratamento, sendo necessário observar as evidências clínicas

apontadas na Tabela 4.

Tabela 4 - Critérios que indicam remissão completa de um paciente com LMA. Adaptado de HOURIGAN; KARP, 2013

Critério de resposta	Evidências
Medula óssea	Menos que 5% de blastos, sem a presença de bastões de Auer
Doença extramedular	Sem evidência residual de doença extramedular
Plaquetas	Menor que 100.000
Contagem de neutrófilos	Menor que 1.000/mcL
Hemoglobina	Livre de transfusões
Clínico	Sem critério

Inicialmente, o tratamento deve controlar ou até mesmo reduzir a proliferação dos blastos. Assim, protocolos com combinação de quimioterápicos são empregados com o intuito de iniciar o declínio no número de células leucêmicas. Um dos protocolos mais utilizados é o protocolo 7+3, na qual são administradas citarabina do 1º ao 7º dia e antraciclinas (como a daunomicina, idarrubicina e mitoxantrona) do 1º ao 3º dia (LORENZI, 2013; TAMAMYAN et al., 2017). Outros protocolos de tratamento de indução também podem ser empregados, dependendo do subtipo de LMA, como é o caso da LPA, na qual é empregado um tratamento com ácido trans-retinoico (ATRA) juntamente com idarrubicina, daunomicina, ou trióxido de arsênico (ESTEY; FREDERICK R. APPELBAUM, 2012; TAMAMYAN et al., 2017).

Após os primeiros sinais de RC, indicando a resposta efetiva do tratamento, é necessário manter os ciclos de tratamento adicionais, aumentando-os caso haja algum indício de recidiva. Esse processo é crucial para consolidar a remissão que, uma vez mantida, aumenta o intervalo de ciclos de tratamento para um mês e redução gradual das doses de quimioterápicos até que não existam mais indícios de células leucêmicas no paciente (LORENZI, 2013). Nesse momento, é de extrema importância o monitoramento de marcadores biológicos que apontem para potenciais recidivas (MAURILLO et al., 2007; HOURIGAN; KARP, 2013).

Já em casos de recidiva, são intensificados os ciclos de quimioterápicos,

com doses e ciclos de aplicação maiores. Além disso, uma outra alternativa é o transplante alogênico de células tronco hematopoiéticas. Por ser bastante agressivo e causar a doença do enxerto contra o hospedeiro (GvHD) caso a compatibilidade não seja adequada, essa abordagem somente é recomendada em casos de recidivas ou em LMA com prognóstico adverso após a primeira remissão (MAURILLO et al., 2007; LORENZI, 2013).

2. Biologia de sistemas: o conceito de redes e a teoria de grafos e redes livres de escala

Alguns fenômenos na natureza possuem uma complexidade a qual é impossível de compreender totalmente analisando elemento a elemento isoladamente, pois fazem parte intricada malha de conexões que permitem o correto funcionamento de um sistema. Logo, é imprescindível que o fenômeno em questão seja abordado de uma maneira holística, isto é, observar o objeto de estudo pelo conjunto de todos os elementos pertencentes atuando dinamicamente, e não somente pela soma de cada parte integrante (BRUGGEMAN; WESTERHOFF, 2007).

Podemos estabelecer uma série de relações em diferentes áreas do conhecimento, que vão desde interações entre moléculas, relações entre espécies em um ecossistema, relações entre indivíduos em uma rede social, entre outros. Essas interações podem ser representadas na forma de nós, que são os elementos de estudo (pessoas, animais, moléculas, etc.), e os conectores, que são as conexões entre dois elementos (grau de familiaridade, interação na cadeia alimentar, interação molecular, etc.), conforme mostra a Tabela 5:

Tabela 5 - Representações de diferentes tipos de redes de interação na natureza

Fenômeno em estudo	Nós	Conectores
Redes sociais	Pessoas	Grau de familiaridade
Cadeia alimentar	Seres vivos	Interações entre seres vivos
Internet	Computadores e servidores	Cabos de transmissão ou sinais wireless
Sistema biológico	Macromoléculas biologicas (DNA,RNA, proteínas, etc)	Interações moleculares (ativação, supressão, etc.)

Assim, para melhor compreendermos como as diversas partes de cada sistema atuam em conjunto, é possível representar cada elemento pertencente de um sistema por nós e a interação entre os mesmos por meio de conectores, resultando em uma rede ou grafo.

Estruturalmente, os grafos possuem níveis de organização distintos, o que permite uma maior funcionalidade de acordo com o sistema envolvido. Basicamente, a organização das redes possui três principais tipos: redes aleatórias, redes hierárquicas ou redes livres de escala (ou *scale-free*, do inglês), mostradas na Figura 3 abaixo:

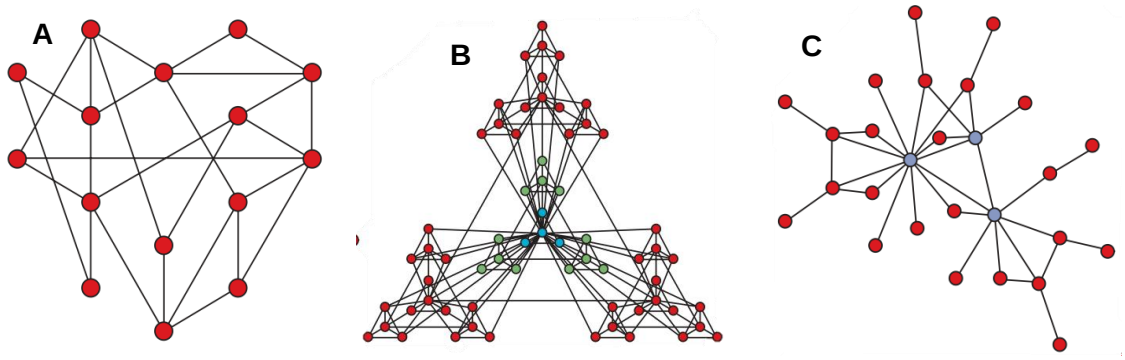

Figura 3 - Representação gráfica de três tipos básicos de redes: Aleatórias (A), hieráriquicas (B) e livres de escala (C). Adaptado de BARABÁSI; OLTVAI, 2004

Esses três tipos de redes possuem diferenças quanto a sua topologia. Redes aleatórias (Figura 5A), por exemplo, possuem como principal característica nós que possuem uma conectividade (ou do inglês *node degree*) seguindo uma distribuição de Poisson, mostrando que grande parte deles tendem a possuir o mesmo número de conexões ou próximo da média geral de conectividade da rede. Já as redes hierárquicas apresentam uma topologia próxima a de uma de livre de escala, porém com a inclusão de regiões com nós altamente conectados entre si, formando módulos (ou do inglês *clusters*) que se comunicam com outros módulos vizinhos através de nós topologicamente relevantes (BARABÁSI; OLTVAI, 2004; POLONI et al., 2014).

Já nas redes de livres de escala, a disposição dos nós segue uma lei de potência, onde a probabilidade de um nó possuir um número k de conexões (P(k)) é representado por $P(k) \sim k^{-\gamma}$, onde γ é o expoente do grau de nó. Em termos estruturais, essa conjectura permite nos inferir que uma rede de livre de escala é composta por uma grande quantidade de nós que possuem poucas conexões, enquanto que uma menor porção de nós possuem uma alta conectividade, denominados *hubs*. Esse tipo de rede é visto em muitos exemplos do nosso cotidiano, como as redes sociais e a *World Wide Web* (BARABÁSI;

OLTVAI, 2004). Porém, um dos exemplos mais observados desse tipo de topologia são as redes de interação biológica, na qual as conexões entre genes e/ou proteínas, por exemplo, seguem essa mesma premissa: muitos elementos compõem uma via que está associada a um determinado processo biológico, enquanto poucos deles são fundamentais para a comunicação entre as diferentes vias de sinalização e/ou manutenção da funcionalidade do processo biológico (BARABÁSI; OLTVAI, 2004; POLONI et al., 2014).

2.1. Redes de correlação ou coexpressão

Uma das maneiras de observar como as relações entre biomoléculas determinam um fenótipo celular associado a uma patologia ou um processo a ser estudado, é verificando como os valores de duas quaisquer biomoléculas estão correlacionados. Conforme mencionado anteriormente, biomoléculas atuam em conjunto, formando vias que desempenharão um papel fundamental na célula. Logo, se analisarmos as correlações entre um grande grupo de genes e/ou proteínas provenientes de um dado ômico, por exemplo, é possível extrairmos informações relevantes sobre quais biomoléculas e, por consequência, quais processos celulares estão mais relacionados a um fenômeno biológico a ser estudado. Para tanto, uma das abordagens em biologia de sistemas que permite obter tais informações é por meio da análise de redes de correlação (ou coexpressão), a qual é representada por uma rede não direcionada ponderada, cujos valores dos conectores são as correlações entre os nós.

Uma rede de correlação possui essas propriedades porque as correlações estão relacionadas ao grau de associação partilhado por ambos os nós envolvidos (e, portanto, não possuem uma direção específica) e também por apresentarem níveis de correlações mais fortes ou fracos (e, com isso possuem um peso – ou força – de conexão). Para melhor ilustrar, suponhamos uma rede simples formada por 4 genes (A, B, C e D). A seguir, estabelecemos os valores de correlação definidos entre os nós (genes), por meio de uma matriz de adjacência, conforme ilustra a Figura 4:

–	A	B	C	D
A	a_{AA}	a_{AB}	a_{AC}	a_{AD}
B	a_{BA}	a_{BB}	a_{BC}	a_{BD}
C	a_{CA}	a_{CB}	a_{CC}	a_{CD}
D	a_{DA}	a_{DB}	a_{DC}	a_{DD}

–	A	B	C	D
A	1	0,6	0,4	0,1
B	0,6	1	0,8	0,2
C	0,4	0,8	1	0,2
D	0,1	0,2	0,2	1

Figura 4 – Matriz de adjacência de uma rede de correlação. Cada célula corresponde aos valores de correlação entre um nó e outro, onde as letras em subscrito da matriz à esquerda corresponde às correlações entre o primeiro nó (primeira letra) e o segundo nó (segunda letra). Exemplo: a_{AB} = Correlação entre o nó A e o nó B.

Logo, a matriz adjacente resultante será uma matriz quadrada n x n (na qual n corresponde ao número de nós envolvidos na rede), onde a diagonal principal é sempre igual a 1, valores esses que são ignorados na rede por não terem nenhum significado biológico. Representando graficamente, a rede final irá resultar em um grafo similar à Figura 5, na qual a espessura dos conectores indica o grau de correlação entre os dois nós.

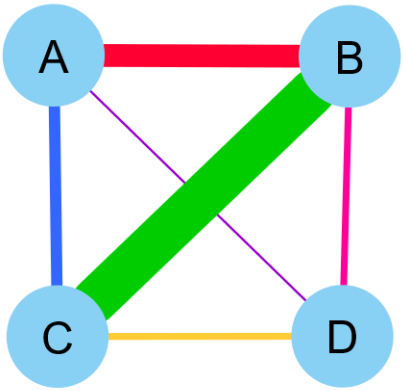

Figura 5 - Representação gráfica de uma rede de coexpressão

Como uma rede de correlação é composta por associações entre todos os nós, resultando em um grafo demasiadamente denso, métodos foram desenvolvidos para dar ênfase às correlações mais relevantes em detrimento das espúrias. Como as correlações negativas dificilmente possuem alguma significância biológica, assim como as correlações positivas de baixo valor (por exemplo, abaixo de 0,5), uma maneira de eliminar tais ruídos é pela simples soma do valor da correlação com 1, dividindo-a em seguida por dois, conforme mostra a Equação 1:

$$a_{ij} = \left| \frac{1+\mathrm{cor}(x_i+x_j)}{2} \right|$$

Equação 1 – Cálculo para a eliminação de correlações de baixa significância biológica

Um outro fator importante a ser considerado é a topologia das redes de correlação. Conforme visto anteriormente, redes biológicas tendem a apresentar uma topologia livre de escala. Logo, para adaptar uma rede de correlação para que a mesma possua uma tendência livre de escala, foi proposto por Zhang e Horvath (2005) o uso de um valor β em cada correlação da rede, conforme mostra a Equação 2:

$$a_{ij} = \left| \frac{1+\mathrm{cor}(x_i+x_j)}{2} \right|^{\beta}$$

Equação 2 – Uso de um valor β para ajustar uma rede de correlação para uma topologia livre de escala

Uma vez estabelecida a rede de correlação, é necessário definir grupos de nós que possuem uma maior interconectividade, formando módulos. Para isso, é necessário estabelecer antes o grau de sobreposição entre um grupo de nós. Logo, Ravasz et al. (2002) propuseram a matriz de sobreposição topológica (ou TOM, do inglês *topological overlapping matrix*), na qual é calculado, a partir dos valores de uma matriz de adjacência, o nível de sobreposição entre um nó

e seus vizinhos. Baseado nessa mesma premissa, Song; Langfelder e Horvath (2012) propuseram uma nova alternativa para o cálculo de TOM e posterior uso para a análise de modularidade.

A partir da formação de uma TOM, é possível então detectar com maior facilidade os módulos de uma rede de coexpressão. Apesar de existi uma grande quantidade de métodos para a detecção de módulos, uma das metodologias mais utilizadas nesse tipo de rede é a de clusterização hierárquica, por se tratar de um método não supervisionado, ou seja, não requer informações prévias para que o método seja aplicado, sendo bastante útil na prospecção de informações biológicas (LANGFELDER; HORVATH, 2008).

3. Justificativa

Apesar de a LMA ser um dos tipos de leucemia mais estudados em toda a comunidade científica, ela ainda permanece como uma das malignidades hematológicas mais letais, como é possível observar nos dados epidemiológicos mencionados anteriormente (ver seção 1.3). Além disso, embora a expectativa de vida dos pacientes com LMA tenha melhorado graças às pesquisas sobre a patologia e, como resultado disso, novas abordagens clínicas tenham sido empregadas; a taxa de remissão completa e duradoura da doença ainda permanece baixa, especialmente em pacientes mais idosos, cuja taxa é de 40–60% (SEKERES et al., 2008).

Uma das causas mais frequentes é em virtude de recidivas oriundas de células leucêmicas residuais pós tratamento. Em alguns casos, a abordagem terapêutica aplicada no paciente é incapaz de atingir a completa remissão da doença. Esse fenômeno é denominado doença residual mínima, ou MRD (do inglês *Minimal Residual Disease*). Os blastos leucêmicos podem permanecer intactos no organismo do paciente mesmo após tratamento, levando a uma potencial chance de recidiva da doença. As possíveis causas de MRD, dentre as principais, são: 1) Resistência ao quimioterápico utilizado; 2) Incapacidade das células oriundas do transplante alogênico de células tronco hematopoiéticas repovoarem o espaço medular e eliminarem completamente a população de células leucêmicas; 3) Presença de mieloblastos ou células tronco leucêmicas circulantes no sangue periférico que sobreviveram pós tratamento e recolonizam a medula óssea (MAURILLO et al., 2007; LIESVELD, 2012; HOURIGAN; KARP, 2013; ZEIJLEMAKER et al., 2016). No que tange à terceira causa, mostrou-se que células leucêmicas na corrente sanguínea possuem células e componentes diferentes da medula óssea, e, por consequência disto, podem responder diferentemente à terapia (PANOSKALTSIS; REID; KNIGHT, 2003; CHEUNG et al., 2009; WHITESIDE, 2013; YANG; XU, 2013; ZAHRAN et al., 2016; HONG et al., 2017).

Grande parte das abordagens terapêuticas tem sido focadas no intuito de combater somente as células leucêmicas. Contudo, o desenvolvimento da LMA depende também da interação dos blastos com o ambiente que os circundam. Conforme já mencionado na introdução, sabe-se que as células leucêmicas são

capazes de modificar todo o ambiente da medula óssea, permitindo assim sua expansão e colonização. Graças a essa descoberta, muitos ensaios clínicos tem sido desenhados com o objetivo de suprimir a expansão dos blastos leucêmicos e/ou da sua capacidade de modificar o microambiente da medula óssea (KONOPLEVA; ANDREEFF, 2007; KONOPLEVA; JORDAN, 2011; RASHIDI; UY, 2015; RASHIDI; DIPERSIO, 2016).

Contudo, pouco se sabe sobre como as células leucêmicas atuam no sangue periférico e de que maneira elas atuam e se comunicam com outras células para sobreviver e manter sua capacidade proliferativa. Conhecer isso é de extrema valia, pois permitirá que futuras abordagens terapêuticas consigam também modular os fatores ambientais da corrente sanguínea com o intuito de minimizar a MRD.

Nesse contexto, o uso de biologia de sistemas, e mais especificamente, de redes de coexpressão, tem sido empregados no estudo do perfil transcritômico de células tumorais, identificando módulos e genes com potenciais alvos terapêuticos e/ou como marcadores biológicos (HORVATH et al., 2006; CLARKE et al., 2013; CHOU et al., 2014; JIA et al., 2014; LI et al., 2018).

4. Objetivos

4.1. Objetivos gerais

Considerando todos os aspectos citados, esse trabalho possui como objetivo avaliar o perfil transcritômico de células leucêmicas do sangue periférico por meio de redes de coexpressão gênica a fim de encontrar módulos e genes chaves associados ao desenvolvimento da LMA.

4.2. Objetivos específicos:

- Descrever as principais células e outros elementos presentes na corrente sanguínea que estão relacionados com a sobrevivência das células leucêmicas nesse contexto celular;
- Construir uma rede de coexpressão gênica a partir de dados transcritômicos de RNAseq de amostras do sangue periférico de pacientes com LMA
- Identificar módulos relevantes com a biologia da doença por meio da técnica de clusterização hierárquica;
- Analisar a representatividade dos módulos obtidos;
- Analisar a relação de módulos e genes com as características clínicas dos pacientes;
- Avaliar a relação do perfil transcritômico entre as amostras de LMA provenientes da medula óssea e do sangue periférico

Capítulo I

Artigo científico de revisão intitulado “*Circulating cells and exosomes in acute myelogenous leukemia and their role in disease progression and survival*”, submetido no periódico *Clinical Immunology*.

Circulating cells and exosomes in acute myelogenous leukemia and their role in disease progression and survival

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Abstract

Acute myelogenous leukemia (AML) is an aggressive hematological malignancy associated with high rates of mortality. This incidence is due to the complexity in which the AML cells interact with other healthy human cells. These phenomena create an environment that favors the expansion of leukemic cells, which will affect the patient's prognosis. An important aspect is the ability of AML cells to evade immune responses via targeting and signaling immune cells to suppress anti-tumor responses. Many studies have reported that associations among components in the peripheral bloodstream might modulate leukemic progression because AML survival is a fundamental step for recolonizing bone marrow after allogeneic hematopoietic stem cell (HSC) transplantation or chemotherapy. Therefore, we collected the most important data about components that circulate with leukemic blasts and contribute to their survival and proliferation. We also discuss clinical approaches that could be conducted to more effectively treat the disease.

Keywords: Acute Myelogenous Leukemia, AML, peripheral blood, hematological malignancies

1. Introduction

Acute myelogenous leukemia (AML) is one of the most aggressive hematological malignancies. It causes around 10,000 deaths per year, according to the latest American Cancer Society report [1]. This high lethality is mainly due to the fast growth and dissemination of immature myeloid blasts in the bone marrow. These cells will later be released into the peripheral bloodstream and, consequently, may be able to invade other tissues and colonize secondary bone marrow sites. Along with this tumorigenic development, severe anemia or hemorrhagic status is commonly observed in affected patients; these major complications can lead to death [2].

Over the years, researchers have discussed the role of auxiliary cells and molecules that surround cancer cells. These factors create an environment that favors tumor cell growth and dissemination by modulating and evading immune responses, as well as the ability to invade secondary sites. Therefore, the study of the composition and role of the tumor microenvironment has provided promising clinical targets for treatment approaches in many cancer types [3].

In AML, like many hematological malignancies, the tumor microenvironment was initially thought to be simpler than in solid tumors. However, leukemic cells can modulate and create niches, besides inducing angiogenesis and other described “cancer hallmarks” in the bone marrow [4–7]. Beyond the bone marrow environment, leukemic cells in the peripheral bloodstream contribute to the AML phenotype. Circulating leukemic cells can invade other tissues and also colonize new bone marrow sites; they also play a crucial role in the minimal residual disease management [8]. However, to do so, an intricate number of auxiliary cells and cytokines are required to conduct proper immune evasion and to prepare for successful infiltration of secondary sites (such as secondary bone

marrows, soft tissues, the central nervous system, etc.). While this environment may not resemble the classical microenvironment composed by a tumor mass with neovessels, as observed in solid tumors and bone marrow niches in leukemias, it has its own complexity and importance with regard to tumor progression.

Many microenvironment-targeted approaches in AML patients have mainly focused on the bone marrow. These endeavors have created drug combinations that block cytokines, molecules that are important for leukemic niche development [9,10]. Regarding the peripheral blood environment, few procedures have targeted cells that might interact with leukemic blasts, particularly because most pathways that underlie the survival and proliferation of such leukemic cells in the bloodstream are largely unknown. However, many studies have demonstrated that in some AML patients, circulating cells have a modified phenotype and/or are detected at a higher number compared to healthy individuals. Among them, changes in circulating innate immune cells (such as natural killer [NK] cells and dendritic cells [DC]) and regulatory T cells (Tregs) are commonly seen as highly correlated with poor disease prognosis. Furthermore, much of this pro-leukemic condition is achieved because AML cells can secrete exosomes that exert immune response modulation. Understanding such features will enable new and promising clinical approaches that, combined with treatment against a leukemic niche in the bone marrow, might dramatically improve complete remission rates, given that many cases of relapse are due to persistent leukemic cells in the bloodstream that recolonize bone marrow sites. Thus, this review aims to gather up-to-date information about these aforementioned components that are found in the bloodstream together with leukemic blasts and play a pivotal role in maintaining the capacity of AML cell survival and proliferation. Furthermore, this review will discuss some clinical perspectives that cover such cells and exosomes.

2. Peripheral blood environment

In the bloodstream, AML cells must overcome the immune surveillance system. They first encounter innate immune response cells, which attack tumor cells and deliver them to adaptive immune-related cells. Several studies reported that AML cells use a myriad of factors to substantially diminish anti-tumoral immune responses [11,12]. Specifically, AML blasts modulate the expression of surrounding cells (mainly related to immunological surveillance), and this action can alter the secretion of cytokines and other molecules, switching the environmental scenario to favor AML blasts [11,13]. Although much has yet to be discovered, some important cells and molecules in the peripheral bloodstream have been found circulating either in higher concentrations or exhibited altered functionality (as compared to healthy patients). Therefore, the main topics that will be discussed in this review focus on circulating exosomes and the following cells: DCs, NK cells, Tregs, circulating endothelial cells (CECs), and circulating endothelial progenitor cells (CEPCs), as depicted in Figure 1. These cells were chosen due to the numerous studies that correlate changes in their number or molecular signature to a worse disease prognosis and/or their potential use as a clinical target; even though there is still no evidence that shows this phenomenon is only exclusive to the bloodstream environment.

3. NK cells

NK cells, along with DCs, are one of the main innate immune cells against tumors circulating in the bloodstream. These cells exert their cytotoxic effects upon the interaction between the NK cell receptor and the target cell ligand. Further, NK cells can

secrete cytokines that modulate the immunological responses [14]. Given the cytotoxic function of these circulating cells, they have been an important object of study for immunotherapy approaches [15]. NK cell activation depends either on the positive balance of activation signals over the inhibitory ones or the absence of major histocompatibility (MHC) class I molecules on target cells ("missing self") [14]. In AML, the levels of circulating activated NK cells are associated with positive outcomes; this population is an efficient immunotherapeutic target for antileukemic responses [16,17]. On the other hand, many studies suggest that NK cells are unable to properly eliminate leukemic blasts due to a weak or absent activation signaling among them, a phenomenon that leads to anergy [18]. Moreover, circulating NK cells also contribute to leukemic progression by hampering the activity of other immune cells against leukemic blasts [19,20].

As stated before, the conduction of NK- based cytotoxicity against leukemic blasts relies on the preponderance of activation signals. Conversely, in many AML cases, NK cells extracted from the peripheral blood have lower expression of activating receptors. For example, circulating AML-derived NK cells have lower expression of a group of proteins called natural cytotoxicity receptors (NCR), namely NKp30, NKp44, and NKp46. This deficit leads to faulty NK-mediated cytotoxicity [21–24]. This event negatively impacts patient survival when compared to AML cases with NK cells that highly express NCRs [22,23]. In addition, other activating receptors can also be underexpressed in NK cells from AML patients, such as the DNAX accessory molecule 1 (DNAM-1) and the NKG2-D type II integral membrane protein (NKG2D). The reduced DNAM-1 expression is caused by chronic exposure to DNAM-1 ligands produced by leukemic blasts. For NKG2D, the deficit is mediated by the transforming growth factor-beta 1 (TGF-β1) pathway [25,26].

Several inactivating receptor genes can be overexpressed in NK cells collected from AML patients. The killer cell immunoglobulin-like (KIR) family of receptors (seven activating and eight inhibitory) are present in multiple combinations on the NK cell surface [27]. The influence of the KIR profile on NK functionality depends on the KIR gene composition in these cells. In a cohort studied by Verheyden et al., there was a predominance of inhibitory KIR receptors, which had a negative impact on KIR-dependent NK cytotoxicity activation [28]. In other studies, like the one conducted by Sandoval-Borrego et al., KIR family genes (CD158a and CD158b) were overexpressed when compared to healthy individuals. In addition, NKG2A, a lectin-like inhibitory receptor, was also overexpressed [21]. This gene expression profile is independent of the NK activation status; these data indicate that blasts might contribute directly to the NK behavior toward AML cell tolerance.

Besides the altered expression of NK receptors, the ligand expression on AML blasts is also crucial for proper NK-based antileukemic activity. Following the same pattern as observed in NK receptors, leukemic blasts express low levels of NK-triggering receptor ligands, and this pattern helpsing them to evade NK cell-based immune responses. Likewise, although NCR ligands were not yet discovered on AML blasts, an elegant 2005 experiment designed by Nowbakht et al., which used dimerized and recombined extracellular domains of NCRs, found that AML cells express low levels of NCR ligands in contrast to mature and healthy myeloid cells [29]. Another classical example is NKG2D ligands, which include the MHC class I polypeptide-related sequence A (MICA/B) and the UL-16 binding proteins 1–5 (ULBP1–5). Typically, both NKG2D ligand types are either underexpressed or absent in AML samples, a phenomenon that impairs NK cell killing mediated by NKG2D triggering, although in some cohorts the expression of each ligand may be different [30–33]. However, some low expression cases

might be associated with ligand shedding from the blasts. Many previous works showed an increased number of the soluble fractions of MICA/B and ULBP2 that causes blast evasion from NK-mediated lysis [31,34]. Thus, blocking the cleavage of NKG2D ligands increases antileukemic cytotoxicity triggered by NK cells [35]. In conclusion, the NK potential to eliminate leukemic blasts depends on the combination of the NK receptors repertoire and the blast ligands that will result in the inactivation of NK-mediated cytotoxicity.

Finally, besides the unfavorable NK activation status in the AML disease context, NK cells can contribute to leukemic progression by modulating, via cytokine secretion and cell-killing, the functions of other immune cells (e.g. DCs). In normal conditions, NK cells are important for triggering antileukemic responses by favoring: 1) a predominance of mature over immature DCs by eliminating the latter; and 2) increasing DC antigen-presenting potential through interferon gamma (IFN-γ) secretion after DC contact, an action that leads to T cell polarization [19,20]. In AML, NK cells are unable to properly eliminate immature DCs; their contact decreases the expression of I-A^d in DCs and inhibits the stimulation of allogeneic T cells. These changes directly impact the DC function as an antigen-presenting cell [19,20].

4. Tregs

Tregs are a T cell subset that control autoimmunity (therefore preserving self-tolerance) and also maintain the balance among T cells varieties by establishing physical contact between them or by secreting cytokines that control their activation [36,37]. They are mainly $CD4^+$ cells (although there are also $CD8^+$ Treg cells) and are separated according to their origin. The thymus-derived natural Tregs (nTregs) are the most

abundant and are $CD4^+$ $CD25^+$ $FOXP3^+$, while induced Tregs (iTregs) are present in the peripheral bloodstream and transformed into $CD4^+$ $CD25^{\pm}$ $FOXP3^{\pm}$ cells by cytokine or antigen presenting cell (APC) stimuli. Increased circulating Treg levels are correlated to a poor prognosis in many solid tumors [38–42]. Following the same pattern, several studies indicated the association between a high level of Tregs in the peripheral blood and an unfavorable outcome in AML cases [39,43–45].

Tregs promote AML blast survival by controlling the responses of several immune cells. This controlling mechanism is mediated through the secretion of cytokines or direct contact. Regarding the latter, cell-to-cell contact-dependent Treg immunomodulation is also an important mechanism in regulating the antileukemic immune responses. Tregs tend to express several receptors that modulate other immune cells. One such example is the expression of programmed cell death protein 1 (PD-1) gene, which encodes a cell receptor that, along with its cognate ligand programmed death-ligand 1 (PD-L1) in target cells, can suppress the antileukemic responses of cytotoxic T cells [44].

At the same time, the cytokine-dependent modulation exerted by Tregs is also fundamental for AML blasts to avoid immune surveillance. The secretion of cytokines, such as interleukin (IL)-35 and TGF-β1, is essential to inactivate the cytotoxic activity of immune cells against blasts. For example, circulating Tregs in AML patients secrete abnormal levels of IL-35 that promote apoptosis suppression and favor the proliferation of AML blasts *in vitro*. In addition, the growth and function of effector T cells are reduced when exposed to this cytokine; this phenomenon will dramatically impact the cellular immune responses mediated by these cells against blasts [46,47]. Another cytokine secreted by Tregs is TGF-β1, which downregulates the expression of the activating NKG2D receptor on NK cells and, consequently, abrogates the ability of these cells to eliminate AML blasts [47,48].

The growth and control of the Treg population can be mediated by many signals provided by AML blasts or other immune cells. For example, Th17 cells can secrete tumor necrosis alpha (TNF-α) and promote the expansion of the Treg population in peripheral blood [45]. One classical example is the expression of indoleamine 2,3-dioxygenase (IDO) by blasts or DCs. This enzyme converts tryptophan to kynurenine and stimulates the proliferation of Tregs, which will subsequently either inhibit the activation of effector T cells or induce apoptosis in these cells [49,50]. Increased IDO expression is correlated with reduced overall survival, probably because it helps to expand the Treg population and lower the effector T cells [51–53]. This scenario is completely reversed when cells are treated *in vitro* with 1-methyl tryptophan (1-MT), an IDO inhibitor [53].

5. DCs derived from AML cells

DCs are one of the main antigen-presenting cells, and thus they play an important role in innate immunity. DCs originate in the bone marrow, where they are later differentiated into two major subgroups: plasmacytoid (pDCs) and myeloid (mDCs) [54]. When activated, the former mainly secretes interferon-alpha (IFN-α), a cytokine responsible for recruiting macrophages and NK cells to attack bacteria, viruses, and tumor cells [55]. mDCs, also called classical DCs (cDCs), are responsible for sensing tissue injuries and presenting antigens to naïve T cells [56,57]. In the AML context, leukemic cells can differentiate into mDCs and pDCs *in vivo* and *in vitro*. Notably, the latter cell type exhibits impaired IFN-α production, a deficit that impacts proper innate immunity response. This phenomenon creates an advantageous environment for AML cells to disseminate in the blood circulation and to other tissues [58,59].

6. CECs and CEPCs

CECs are mature cells that have detached from the blood vessel wall. Under normal physiological conditions, they are almost undetectable in the peripheral blood. CEC levels are elevated in patients who have suffered physical injuries, or inflammatory, autoimmune, or cardiovascular diseases. Thus, this alteration can be used as a good parameter to evaluate the severity of these pathologies [60]. In cancers, this cell type is highly correlated to increased tumor mass and a negative prognosis, which serves as a useful biomarker to assess cancer progression. Furthermore, a higher CEC concentration in the peripheral blood of AML patients is highly correlated with disease severity and chemotherapeutic response. Given that the presence of CECs does not correlate to any specific condition (French-American-British [FAB] classification, age, gender, etc.), their measurement might represent a promising method to evaluate whether a patient has reached complete remission after treatment [61].

The mechanisms by which CECs influence AML progression have not yet been fully elucidated. However, Liesveld and colleagues found that co-cultured endothelial cells promote increased leukemic cell proliferation and their survival by preventing apoptosis mediated by cytarabine, a common drug for AML treatment [62]. This chemoresistance is achieved thanks to the ability of endothelial cells to secrete cytokines and also provide adhesion-related molecules to the leukemic blasts, phenomena that assure the blasts' survival and/or proliferation [62].

CEPCs are derived from the bone marrow—a difference from CECs—and play an important role in vascular tissue regeneration [63]. Initially they were hard to identify due to the lack of a characteristic phenotype, mainly due to the absence of definitive markers. However, earlier functional and molecular phenotyping assays allowed one to

distinguish CEPCs from CECs [63]. One important feature of CEPCs is the expression of surface proteins such as CD133. This protein is a common target for the identification of this cell type, even though this surface marker is also expressed in HSCs. Further, even CEPCs themselves only substantially express CD133 after entering the bloodstream, after which time they lose it [64]. These cells are released into the peripheral blood according to signals that emanate from the bone marrow. Although the exact mechanisms behind the induction of CEPC release are still imprecise, they seem to follow the same signaling factors for releasing hematopoietic cells, namely granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF), and erythropoietin (EPO) [64].

In solid tumors, CEPCs have been related to neovascularization [65]. Their specific role in new vessel formation is still unclear. Some authors suggest that CEPCs can be a constituent of the newly formed vessel, whereas others indicate that they are part of neovascularization signaling [66,67]. It cannot be excluded that both of them occur simultaneously [65,68]. Similar to CECs, the number of CEPCs is directly correlated to the clinical status of AML patients, including for monitoring chemotherapy and allogeneic HSC transplantation responses [61,69]. Thus, CEPCs might contribute to leukemic proliferation by providing and stimulating new vessel formation in bone marrow sites, an action that is fundamental for the establishment of leukemic niches [68].

7. Exosomes

Increasing evidence has recently implicated extracellular vesicles (EV) in cell-to-cell and cell-to-environment communication [70]. Although there is no definitive way to classify all EVs, a specific group of EVs called exosomes are different from the other

vesicles. Exosomes range from 30 to 100 nm in diameter and originate by the intraluminal budding of early endosomes, which generate multivesicular bodies (MVB) that will later fuse with the plasma membrane, releasing the MVB content to the extracellular space. The secretion of such vesicles is observed in many organisms and cell types, such as intestinal epithelial cells and kidney cells, and it is present in body fluids like the blood, urine, and amniotic fluid [71–73]. Furthermore, in a healthy immune system context, exosomes are crucial to drive a proper signal for HSC differentiation and their subsequent fate [74]

Tumors, in a broad sense, also produce exosomes on a large scale; they can stimulate recipient cells that are close or far from the secreted tumor cell. In some cancers, the high secretion rate of exosomes by tumor cells is linked to their malignant potential because they stimulate angiogenesis and blood vessel development [75,76]. This phenomenon is also seen in AML, where AML blast exosomes exert many functions that are favorable to disease progression, depending on the cargo content. In one study, plasma exosomes were more than 60-fold increased compared to healthy patients; they modify the surroundings in order to evade the antileukemic response [77]. The molecular composition of exosomes derived from leukemic blasts is diverse: It contains numerous combinations of cytokines, enzymes, and nucleic acids (DNA, microRNA [miRNA], and messenger RNA [mRNA]) that contribute to leukemia development in the bone marrow, by creating favorable niches for AML blast growth [78], and in the peripheral blood [79,80].

One of the main roles of exosomes secreted by AML blasts in the bloodstream, as mentioned earlier, is to modulate and suppress the antileukemic response. Recipient cells, mainly NK cells, DCs, and Tregs, interact with molecules transported through exosomes, actions that result in the suppression or inactivation of an innate response mediated by

these cells. For example, a group of AML patients possessed blasts that secreted exosomes with TGF-β1; this release was responsible for suppressing NK cell activation by downregulating NKG2D, abrogating the NK-mediated antileukemic response in the bloodstream [26,48,77]. TGF-β1 is also a strong inducer of Tregs, which is inversely associated with increased survival rates in AML patients (for more details about the Treg and NK cell roles, please see sections 4 and 3, respectively) [81]. Furthermore, this same immunosuppressor context exerted by circulating exosomes might interfere with adoptive cell therapies (ACT). This phenomenon was observed in a study where the cargo content misled or inhibited the antileukemic response of transferred NK-92 cells (an FDA-approved NK cell line largely employed in ACT protocols) [11].

Beyond the antileukemic response induced by circulating exosomes that contain miRNAs, they also promote blast proliferation. For example, miR-1246 and miR-155 were found in circulating exosomes derived from AML blasts [13]. Although the specific role in AML has not been completely understood, miR-1246 may have an important role in leukemic expansion: It was found highly expressed in circulating exosomes from AML cells in a murine engraftment model [13]. Moreover, in p53 mutated colorectal cancers, transformed tumor-associated macrophages (TAMs) release exosomes that contain miR-1246. This action promotes Treg expansion in the tumor microenvironment. Thus, this miRNA might have an important role in regulating immune cells related to tumorigenesis [82]. In this same pro-leukemic phenotype context, miRNA-155 is also overexpressed in AML and is mainly related to myeloid proliferation, although in some AML subtypes the role of leukemogenesis depends on its expression levels [83,84].

8. Clinical perspectives

Complete AML remission depends on approaches that target the blasts themselves and focus on the leukemic cell microenvironment. Considering this fact, the peripheral blood environment is crucial for clinical remission: The surviving circulating leukemic blasts can recolonize the bone marrow environment and predict the clinical outcome after chemotherapy and/or allogeneic HSC transplantation [85,86]. Several studies have concentrated on blocking cells and molecules related to a leukemic progression in the bloodstream, a phenomenon that might be promising for future clinical interventions. Some treatment strategies mentioned here comprise already known drugs employed in AML blast control (like azacytidine and cytarabine), but they might have different purposes that could lead to new chemotherapy regimens and/or dosing adjustments to cover both AML blasts and other target cells in the peripheral blood. Therefore, we collected the main approaches that focused on targeting and disrupting the molecular and cellular components that favor AML progression in the peripheral circulation (Table 1).

It is important to evaluate the antileukemic potential of NK cells because immature or non-activated NK cells contribute to a low survival rate or increased relapse rate in AML [87,88]. Therefore, the expression assessment of both circulating NK receptors and ligands on the blast cell surface is critical for predicting the patient's prognosis and the success of an allogeneic HSC transplantation. Indeed, the majority of activating signals favors NK cells to eliminate leukemic blasts in the bloodstream and enhance the graft-versus-leukemia effect [89].

However, if the NK cell activation profile is unfavorable, a possible alternative is heterologous NK cell infusion, which can expand the antileukemic potential in contrast to the pro-leukemic ones found in peripheral blood from AML patients. Another strategy

might by stimulating the expression of activating receptors via cytokines or drugs. A study conducted by Szczepanski and colleagues showed that it is possible to increase the expression of NK activating receptors through IL-15 administration. This treatment enhances the ability of NK cells to eliminate leukemic blasts [90]. In the same way, drugs like decitabine can enhance NK activating receptors and thus augment NKp44 expression [91]. On the other hand, some compounds can alter blasts' ligand expression. Treatment with all-trans retinoic acid (ATRA) or valproic acid causes NKG2D ligand overexpression in blasts through the inhibition of epigenetic mechanisms (by blocking histone deacetylase [HDAC] activity), leading to NK based cytotoxicity [92,93]. In addition, NKG2D ligand shedding can be also mitigated by either azacytidine or decitabine treatment because it increases tissue inhibitor of metalloproteinase 3 (*TIMP3*) expression and, consequently, hampers a disintegrin and metalloproteinase 17 (ADAM17) expression, an enzyme responsible for MICA, MICB, and ULBP2 shedding [35].

When the clinical intervention aims to target Tregs, many strategies can be chosen in order to control their role in maintaining blast survival. One might block the signals that stimulate Treg expansion in the peripheral blood. IDO inhibition with compounds such as 1-MT might represent an interesting strategy to control Treg levels because, as mentioned before, it restores the expansion of effector T cells *in vitro* and increases their ability to promote antileukemic cytotoxicity. However, clinical trials are needed to test the efficiency of such an approach in AML, although in other cancer types it has shown positive results [94].

Given that in most AML cases a low Treg frequency in the bloodstream is correlated to higher chances of clinical remission, the reduction of such cells might be a potential starting point to increase the leukemia-free survival time. Several strategies have

been tested to deplete circulating Tregs. A phase IV clinical trial treatment with histamine dihydrochloride and low-dose IL-2 showed a significant decrease in the Treg counts in many patients, improving their survival rate [95].

DCs that originate from AML cells represent an important target for therapeutics. DC-based vaccines, for example, are useful to control a post-remission relapse. In a cohort of 30 patients, electroporated DCs with Wilms's tumor 1 (*WT1*) mRNA were administered in patients who reached clinical remission, preventing or delaying relapse in 43% of the cases [96]. DCs can also be transformed from leukemic cells *in vitro.* These cells showed an antileukemic profile via T-cell mediated cytotoxicity [97]. Autotransplantation of such cells appears to be generally well tolerated, although it has not presented a sufficient antileukemic response to drive the disease into remission. Another study showed that DC subtypes that originate from non-leukemic cells might have an important role, meaning that a combination of different DC subtypes might be necessary to trigger a proper antileukemic response [98,99]. Notably, DC-derived exosomes in AML might represent an important strategy because, in solid tumor models, these vesicles stimulate cytotoxic T-cell-mediated tumor death or tumor growth suppression via T cells. These findings suggest a possible therapeutic approach [100].

CECs can be a good marker for identifying—or evaluating—post-clinical remission and thus assess the effectiveness of allogeneic HSC transplantation [61,65]. Further, as stated before, due to their ability to protect AML cells from cytarabine cytotoxicity, the combination of a drug that targets CECs enhances the potential of another one that targets blasts. Combined bortezomib and cytarabine treatment *in vitro* revealed that bortezomib helps cytarabine impair the supporting role of CECs in leukemic cell survival [62,101]. Clinical trials with such combinations were tested in elder AML patients (60–75 years old); the results showed a good percentage (65%) of clinical

remission. While this result is promising, more clinical cohorts are needed to guarantee the effectiveness of this treatment [101].

Given that the detection and pathways of exosome formation are not fully understood, specifically in the AML context, targeting blast-derived exosomes to control their immunomodulatory functions remains a challenge. However, even though there are no clinical approaches to inhibit exosome formation or their signaling potential, some authors have suggested potential strategies. One of them is the use of extracorporeal hemofiltration systems to remove these vesicles from the circulation. This system is called the adaptive dialysis-like affinity platform technology (ADAPT^{TM}); it comprises an affinity matrix that captures the exosomes in the bloodstream [102]. Another possible way is to prevent the fusion between the exosome the recipient cell by using diannexin, a phosphatidylserine blocker. All these strategies need to be further analyzed and evaluated in AML patients to confirm their potential value as an alternative to hamper the functions of exosomes in modulating the immune cells toward a pro-leukemic setting in the peripheral blood [103].

9. Concluding remarks

The leukemic setting in peripheral blood is essential for blast survival and proliferation. AML cells can escape from immune surveillance with the help of Tregs, whereas NK cells or DCs are unable to mediate a proper immunological response. Thus, it is clinically important to monitor all these aspects in order to predict a better outcome in patients. This endeavor includes checking, in each cell type, the presence of molecular features related to poor prognosis or that are favorable for AML progression. Furthermore, considering more approaches and clinical trials that include classical blast-targeted chemotherapeutics as well as small molecule or monoclonal antibody therapeutic

that block the peripheral blood cells that contribute for blasts survival will be crucial to increase treatment efficiency and achieve complete remission rates.

Acknowledgments

We thank the Conselho Nacional de Pesquisa e Desenvolvimento (CNPq, grant number 302969/2016-0) funding agency for providing the financial support to perform this work. We also thank Dr. Joice de Faria Poloni for designing Figure 1 and, along with MSc. Igor de Araujo Vieira, for critically reviewing the manuscript.

Competing interests

The authors declare that they have no conflict of interest.

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Table 1 – Treatment alternatives that target cells related to acute myelogenous leukemia (AML) blast survival in the peripheral blood

Therapy target	Methodology approach	Outcome in circulating blasts	References
NK cells	Decitabine treatment in NK cells transformed from HSPCs	Enhances the expression of the NKp44 activating receptor in NK cells treated prior to mouse engraftment	[91]
NK cells and AML blasts	Azacytidine treatment Decitabine treatment	Blasts avoid MICA, MICB, and ULBP2 shedding, allowing the NK-cell-based antileukemic response by activating NCRs	[35]
NK cells	IL-15 treatment	Increases the expression of activation receptors in NK cells	[90]
AML Blasts	Valproic acid treatment ATRA treatment	Increases MICA, MICB, and ULBP1 expression in blasts and renders them more susceptible to NK-mediated cytotoxicity	[92,93]
CECs and AML blasts	Bortezomib + Cytarabine treatment	Enhances cytarabine toxicity in blasts through CEC clearance by bortezomib addition	[62,101]
Dendritic cells	Autotransplantation of transformed AML blasts into dendritic cells	Increased dendritic-cell-based cytotoxicity against AML blasts	[97]
Dendritic cells	Administering a dendritic cell vaccine with *WT1* mRNA	Delays or prevents postremission clinical relapse	[96]
Regulatory T cells	Histamine dihydrochloride and low-dose IL-2	Relapse prevention in the post-consolidation chemotherapy phase	[95]
Regulatory T cells and AML blasts	1-methyltryptophan	Inhibits IDO activity, controls Treg expansion, and enables effector T cell growth and activation	[53,94]
AML blast derived exosomes	ADAPT^{TM} extracorporeal hemofiltration system	Clears the circulation of AML-blast-derived exosomes	[102]

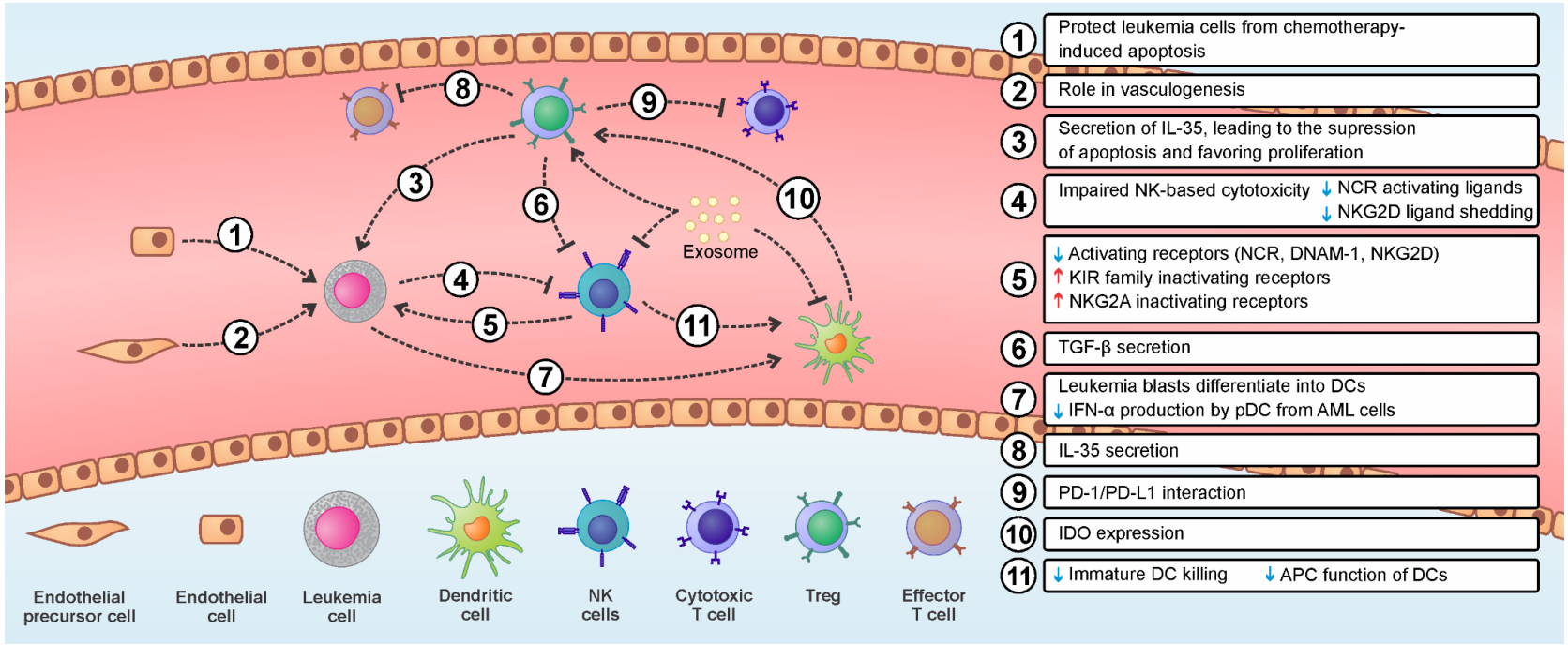

Figure 1 Main cells and exosomes responsible to mediate the leukemic cell survival in the peripheral blood.

Capítulo II

Artigo científico de dados intitulado "*The role of CD34+ cells in the peripheral blood samples of acute myelogenous leukemia through a weighted gene coexpression network analysis approach*", que será submetido a um periódico internacional a ser escolhido.

The role of CD34+ cells in the peripheral blood samples of acute myelogenous leukemia through a weighted gene coexpression network analysis approach

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Abstract

Acute myelogenous leukemia (AML) is a very aggressive hematological malignancy characterized by the rapid clonal expansion of immature myeloid hematopoietic precursors that proliferate in the bone marrow and later disseminates into the bloodstream. Many approaches have been conducted to elucidate the molecular pathways that the leukemic cells modulate in the bone marrow microenvironment, creating new strategies to control the disease progression. However, few approaches have been done to look at the peripheral blood counterparts, since this environment also has a clinical importance due the presence of leukemic cells and other immune cells that can help to recolonize bone marrow sites even after chemotherapy or allogeneic HSC transplantation. Concomitantly, it was found that the worse prognosis is correlated to the high number of circulating CD34+ AML blasts, although their specific role in the disease progression is largely unknown. In order to have a better comprehension of the importance of such cells in AML development, we

designed a weighted gene coexpression network analysis from peripheral blood AML blasts. The results showed 3 modules significantly correlated to CD34+ cells and are related to apoptosis blockage, cell proliferation and cytokine-mediated pathways. These findings open the possibility of looking at potential genes (or their protein products) as targets that might be important to control the AML development mediated by CD34+ cells.

Keywords: AML, leukemia, WGCNA, coexpression, peripheral blood, CD34

1. Introduction

Acute myelogenous leukemia (AML) is one of most aggressive hematological malignancies. According to the American Cancer Society (ACS), it is the most prevalent and lethal among the other leukemia subtypes, accounting for around 34% and 43% of the total leukemia reports, respectively (1). AML is defined by the uncontrolled clonal expansion of myeloid blasts originated in the bone marrow and spread to the bloodstream, infiltrating tissues and developing severe hemorrhagic and immunodeficient conditions. It has been demonstrated that AML cells modify bone marrow microenvironment, modulating HSC expansion, inactivating NK cell innate response towards tumoral cells and modeling other non-hematopoietic cells to accommodate the expanding leukemic niche (2–4).

On the other hand, when leukemic cells get into the bloodstream, it enters in a completely new environment, requiring it to adapt for survival. In addition, those cells are capable of invading other tissues, particularly soft tissues, and other bone marrow sites. Studies demonstrated differences between circulating tumor cells (CTCs) and primary tumor site cells at their transcriptional level, possibly due to the environmental change that these cells were exposed (5,6). On AML context, it's an important feature to take into consideration, due to its fast dissemination over the bloodstream and other tissues (7,8). In this context, one of important features found in the peripheral blood context is the high association of circulating leukemic CD34+ cells and poor prognosis, which it has implications in the conduction of a proper clinical decision (9). However, there is few data about the underlying biological and molecular pathways in the peripheral blood environment, which is an important step for understanding AML biology, providing useful tools for future diagnosis, monitoring, and treatment of this disease.

Biological network analysis has been widely utilized to understand more precisely the complexity of biological molecules interactions. One of approaches is through a weighted gene correlation network analysis (WGCNA), characterized by gene expression correlation coefficients as the connections, resulting in a weighted fashion graph. Hence, when a group of genes are densely

interconnected (termed as modules or clusters) and have a common biological process, it's feasible to assume that modules genes either act actively or have an important role in mediating the highlighted biological function, since the genes are structured on a "guilt-by-association" interaction (10,11). This type of framework could be useful to understand cancer biology since tumor cells have an altered – and complex - cell dynamics, in the broad sense, affecting many pathways and processes to acquire their aggressive and proliferative capacity. For this reason, this methodology has been helpful to look out for interesting genes associated with cancer prognosis, biomarkers, and therapeutic targets (10,12–14). In this sense, this work aims to evaluate gene expression patterns on AML blood samples in order to understand how this environmental context could affect the tumor activity.

2. Material and Methods:

2.1. RNAseq data acquisition and processing

AML blood RNAseq samples were collected at the Gene Expression Omnibus (GEO) database, and are part of the Leucegene project, consisting on five large datasets of many AML patients, which four of them were utilized (GSE49642, GSE52656, GSE62190, and GSE66917; Table S1). These samples reads are unstranded pair-ended type and the cell collection, RNA extraction, and sequencing protocols are described elsewhere (15–17). Since AML has many cellular phenotypes, which can lead to a cellular type-driven bias, it was selected only patient data with non-maturated and M1 status, according to the WHO and French-American-British (FAB) classifications, respectively. Other parameters such as age, sex and karyotype were also evaluated as potential bias drivers. Also, other clinical data from AML patients such as blasts and CD34+ cells percentages were acquired by previous studies (15–17) and also gently provided by Dr. Joseé Hebert, from Centre de Recherche Hôpital Maisonneuve-Rosemont, Montreal, Canada (Table 1). FASTQ files of these samples were submitted to a read quality assessment using the FastQC software version 0.11.2 and

then used as a guide to performing reads trimming using the Trimmomatic tool version 0.32 (18). The resulting data were transcriptome aligned (Ensembl version GRCh38.84), through the quasi-mapping method provided by the Salmon software version 0.7.1 (19). The output gave transcript-level read counts that were converted into gene-level read counts by the Tximport R package (20).

2.2. Data pre-processing

Gene expression estimation by Tximport was read and compiled by the DESeq2 R package, where multiple runs from a single biological replicate were also merged (21). Prior downstream analysis, outlier detection measurement by the Euclidean distance between samples was performed. For each test, it was also measured the sample connectivity and clustering coefficients, as well as possible batch effect potential or covariations through a multivariate analysis of variance (MANOVA), using external patient and RNAseq run data as an input. All these tests were performed by the SampleNetwork R function (22). Finally, processed data were normalized for each one of the experimental designs through DESeq2 functions. Data utilized for coexpression networks were normalized by a non-parametric logarithmized variance stabilizing transformation.

2.3. Weighted Gene Correlation Network Analysis (WGCNA)

A correlation network was created with the WGCNA R package (23) based on normalized expression data. In order to do so, gene-gene correlations were done using a biweight midcorrelation calculus described by Song *et al*. (24). Since negative and low correlations have irrelevant or few biological significance, a signed type network was employed to increase those values from the strongest positive correlations (which have a biological significance, according to the “guilt-by-association” postulate), which simply consists in the correlation coefficient plus one, and divided by two, as the following Equation [1] represents:

$$[1]\ a_{ij} = \left|\frac{1+\mathrm{cor}(x_i+x_j)}{2}\right|^{\beta}$$

Equation [1]: Signed correlation between gene expressions x_i and x_j, raised to a β power

This results in an adjacency matrix that depicts the relationship between genes. However, since biological networks tend to have a scale-free like topology (25), an important WGCNA step is adjusting the network to fit the aforementioned layout. This can be reached by elevating the signed network values to the β power (see equation [1]); emphasizing higher correlations at the expense of lower ones. Thus, β values ranging from 1 to 40 were tested on the networks, and the one who generated a topology closest to a scale-free topology was selected to create the final graph.

2.4. Cluster detection and module to clinical trait relationship:

For cluster detection, the network with the β coefficient chosen was transformed into Topological Overlapping measurements, which it tends to cluster genes that have similar correlations. Based on these values, a hierarchical clustering (HC) and a dynamical branch cutting method were employed to discover graph modules. To give accurate module detection, a Partitioning Around Medoids (PAM) stage was enabled as a secondary clustering method, but always following the HC dendrogram layout created, called only on unsolved branches cases. Each module was represented with a color (except the grey color, which indicates genes that did not belong to any cluster). Module color assignment (except for the grey color) was randomly generated by WGCNA. The gene expression profiles of each module were summarized using a single value decomposition calculation, named as a Module Eigengene (ME) (23). With these values, it was able to assess the relationship between other modules (through distance and correlation measurement between each ME), clinical traits (by evaluating the impact of this particular module on the traits assessed) and genes (correlating the gene expression with the ME, named as module membership, or kME. Genes were considered module hubs (i.e, nodes that have an important role due to their high connectedness

inside the module) those who possess kME values over 0.65. Finally, gene expression from a relevant module were correlated to clinical trait values in order to verify the most significant genes in the module (gene significance, or GS), along with kME values. GS values higher than 0.65 (in modules with positive trait correlation) or lower than -0.65 (in modules with negative trait correlation) were selected as the most relevant. Genes that have hub status and also have relevant GS values are considered as significant hub genes (SHG)

2.5. Module visualization, gene ontology enrichment analysis and gene function prospection:

Module genes were submitted to a gene ontology and pathway enrichment analysis using the TopGO R package, where all the network nodes were used as a background (26–28). Relevant biological processes were defined as the ones who had a corrected *p-value* less than 0.05. Ontology terms search were done at QuickGO database (29) (www.ebi.ac.uk/QuickGO). Gene functions and pathways prospection were done using GeneCards (30) (www.genecards.org), KEGG pathway (31) (www.kegg.co.jp), and Gene Expression Atlas (32) (https://www.ebi.ac.uk/gxa/home) databases.

3. Results:

3.1. Read quality assessment, mapping and pre-processing:

Samples presented an overall good read quality, where the majority of bases had a Phred quality score over 30 (base call accuracy of 99.99%). Five and three prime ends bases who had lower scores (under 20, i.e 99% base call accuracy) were removed (as well as entire low-quality reads) resulting on reads length reaching from 80 to 100 bases. Trimmed reads mapping into the transcriptome provided an average efficiency of 80.46% on blood samples (Table S2). Gene transformed counts analysis by SampleNetwork showed samples one outlier, respectively (BL02H060) (Figure S1). Additionally, MANOVA showed that there was no significant influence of any biological (sex, age, and genotype) or technical (number of runs in a biological replicate and dataset origin) issues on sample variation (Figure S1).

3.2. Weighted gene correlation network and module analysis:

Correlation network from blood samples was created based on normalized gene expression counts and, subsequentially, a beta power estimation was performed to adjust it to a scale-free topology. Thus, a beta power of 22 was defined, resulting on nodes with a mean connectivity of 43.64. (Table S3). After this step, module detection was performed, which 9 clusters were found and 32.12% out of genes had not been assigned to any cluster. The GO enrichment analysis of each module was assessed, which all annotations were included on Table S4.

ME calculation was performed, which allowed the measurement of the module relevance in respect of each clinical trait (CD34+ and Blast percentages), as shown on Figure 1. The black, yellow and green modules showed a significant positive correlation to the presence of CD34+ cells (0.69, 0.59 and 0.76, respectively), whereas the turquoise module showed a strong negative correlation (-0.74) against the same clinical trait.

Regarding these modules, the module membership (kME) values were assessed for each gene and then compared to clinical traits in order to see their significance (which can be seen on Table S5). Although the ME x clinical trait correlation showed the green and black modules as the ones with the highest correlations with the CD34+ cells percentage, the yellow and turquoise modules showed a higher gene significance average, as seen in the Figure 3. Moreover, the black module had the lowest mean GS, including the non-clustered genes (marked with the grey color).

In this sense, we analyzed the relationship of the topological features (kME measurement) and the GS values. Thus, a kME x GS dispersion chart was plotted, as well as the overall correlation of these two measurements, as depicted on the Figure 2. The yellow module, in comparison to the green module, had a higher number of SHGs (176 against 70 genes), besides having a better kME vs GS overall correlation (0.48 in contrast to - 0.065). The turquoise module presented an overall correlation of -0.52 and 445 SHGs, maintaining their negative relationship with the same clinical data. This was not observed in the black module, which initially had a positive correlation of 0.69. However, in the kME vs GS view, the module had an overall correlation of -0.13, and only 5 genes were considered SHGs. Considering these observations, we maintained only the yellow and turquoise modules for posterior analysis.

Next, we looked at the biological processes that these SHGs are part in each module, as seen in the Table 2. In the yellow module, SHG related to CD34+ are mostly associated to response and chemotaxis of immune cells (such as NK, T and B cells, neutrophils, among others), most of them mediated by the HAVCR2 receptor and CCL5 chemokine. Other relevant processes include cell proliferation and apoptotic pathways, represented by the *B2M*, *HAVCR2*, and *PAK1* genes. In the turquoise module, SHGs with negative correlation to CD34+ showed processed related to: a) cell differentiation, b) negative regulation of cell proliferation, c) regulation of activated T cell proliferation and d) negative regulation of chemotaxis. The black module had any significant ontology in which the detected SHGs are important.

4. Discussion

The process of tumor development requires a lot of variables to consider in order to establish the outcome of disease. The acquirement of cancer hallmarks includes many pathways and biological processes that altogether defines the extent and aggressiveness of disease (33). Taking this into consideration, a classical expression abundance measurement sometimes can give few or biased evidence regarding a possible – and larger – biological landscape. This occurs due to the biological oversimplification that this method imposes, since genes and most biomolecules do not act alone, and for this reason, a whole picture of how cells are behaving could turn out to be unclear if we look for one gene at a time (34). However, the information could be more accurate if this kind of data had been embodied into a network view that represents the relationship between genes and their expression profile on given conditions. Therefore, correlation networks have the advantage of integrating all the possible relationships between genes and select the most relevant modules, as well as observe their correlation to any provided clinical trait.

The correlation network created sought to look out the main modules related to three main clinical parameters that is crucial to monitor the AML disease status. As mentioned before, no module had any significative relationship to Blasts %. The reason of this is probably due the low variance of the trait values between samples, which will reflect in the module comparison. In contrast, there were clusters that had a significant correlation with the CD34+ cells percentage data. When we compare the ME vs trait and kME vs GS results for each module, some differences between these measurements are observed probably because of the genes expression heterogeneity in a module. Since ME values summarize the expression data of each sample in the whole module, an oscillating expression pattern could reflect in the ME calculation, resulting in an unreliable measurement. Therefore, it is more accurate to evaluate the relevance of genes by their kME values and look out for their direct relationship to the underlying clinical trait, leaving the ME-based comparison only for an initial prospection of relevant modules. Furthermore, the source of this heterogeneity also could be inherent to the AML disease, since it has multiple origins and genotypes, even inside a

single FAB classification.

The $CD34^+$ cells are a subset of leukemic blasts that have stem cell-like characteristics, often called as Leukemic Stem Cells (LSC) or Leukemia Initiating Cells (LIC), due their ability to promote leukemogenesis in a mice engraftment model (35,36). They are linked to a poor prognosis, with higher risk of developing a minimal residual disease (MRD) in comparison to CD34 negative cells (35,37,38). Moreover, these cells tend to not share the same expression profile like the other cell subtypes, which it impact on their interaction to the whole AML environmental context (39). In addition, differences of gene expression patterns were observed in $CD34^+$ blasts in the bone marrow in comparison to ones found in the peripheral blood context (9). Although it was not well established yet the role of CD34+ cells in this latter environment, our study suggests, based on the results pointed above, some possible functions that these cells may contribute to leukemic cells survival and expansion.

As earlier mentioned, CD34+ blasts are an important subset of cells in the establishment of leukemogenesis. Thus, pathways related to their proliferative potential, as well as their role in modulating the immune responses are crucial for the disease's progression. According to our data, many SHGs are related to the aforementioned processes, which some of them are able to induce multiple pathways. An example of that is the *HAVCR2* gene, which encodes the T-Cell Immunoglobulin Mucin Family Member 3 (Tim-3) expression. It was seen that Tim-3, although it is commonly expressed on other immune cells such as regulatory T cells (Treg), is also expressed on AML blasts. It interacts with its ligand, Galectin-9 (encoded by the *LGALS9* gene), responsible to mediate immunosuppressive pathways, inactivating cytotoxic T and NK cells responses against leukemic blasts (40). It is known that Tim-3 expression in AML cells is mainly related to an increased secretion of Galectin-9 by an autocrine loop, augmenting the functional inhibition of antileukemic immune cells (41). Moreover, this autocrine loop also favors the self-renewal potential of $CD34^+$ cells via the activation of NF-κB and β-catenin pathways (42). Although the galectin-9 gene (*LGALS9*), is not seen in the yellow module (it is marked as part of the blue module), the kME value for this cluster is 0.701, showing that this gene has strong connection to the ontologies from this

particular module, mainly the ones that the *HAVCR2* gene is part of.

Since the Tim-3/Galectin-9 contribute for the expansion and maintenance of undifferentiated cells, besides contributing to inhibit the activation of T cells, we sought the mechanisms behind these pathways and SHGs that might be implicated. The turquoise module presented a high number of SHGs related to cell differentiation and they are negatively correlated to CD34+ cell levels. One of them is the *TNFSF9* gene, which encodes a CD137 ligand (CD137L), a transmembrane protein that interacts with CD137 found in the surface of activated T cells and NK cells (43). These immune cells can promote differentiation of AML cells into dendritic cells trough the CD137-CD137L interaction. This newly-differentiated DC cells, in turn, can induce the T cell activation, which can enhance the antileukemic responses in the bloodstream. Conversely, the blockage of this pathway not only avoid AML cells differentiation, but also enhances its proliferation (43,44).

Another important aspect of CD34+ AML blasts is the interplay with T cells. These immune cells can be either pro or anti leukemic. Since many processes were related to T cells responses in the yellow module, showing that $CD34^{+}$ cells might contribute in modulating the responses from this type of lymphocyte, we looked at the involved SHGs. One of them, the beta-2-microglubulin (*B2M*), is an important marker to predict the disease progression (45). It is a subunit from the HLA class I antigens that is responsible to present the cell to cytotoxic T cells or NK cells. However, as well as HLA molecules, B2M is also prone to suffer shedding from the cell membrane, ending up in the bloodstream. It was shown that high serum levels of B2M is correlated to poor prognosis, probably because of the lower capacity of cytotoxic T cells or NK cells in targeting the leukemic blasts (45). In our data, we sought the HLA type I molecules in the module to confirm this hypothesis. The *HLA-A* and *HLA-C* genes were not part of the mentioned cluster and, although the *HLA-B* gene was found in the yellow module, this gene has no topological relevance (it is not a hub gene) and also does not correlate significantly to *B2M* gene (data not shown).

Since the circulating B2M levels, as well as circulating HLA type I molecules, are sometimes overestimated due the high cellular turnover, we looked at the apoptotic status and the influence of CD34+cells in this context. It has been reported that CD34+ AML cells are more resistant to

apoptosis, increasing this effect according to the percentage of CD34+ cells (46). Our data goes in agreement with this statement, showing genes that are responsible for evading the apoptotic pathways. For example, *PAK1* is a kinase that is responsible of controlling anti-apoptotic pathways and are highly implicated in the tumorigenesis in many cancers. In AML, the apoptosis evasion mechanisms mediated by *PAK1* is by the focal adhesion kinase (FAK) and mitogen activated protein kinase (MAPK) pathway, aiding the nuclear translocation of the STAT5 protein, that will promote *MYC* gene expression. The targets of this gene product are commonly related to escape from the apoptotic machinery of not only regular leukemic cells, but it also include CD34+ LSCs (47). Knockdown or inhibition of *PAK1* expression resulted in increased AML cells death by apoptosis, increasing significantly the patient overall survival.

5. Concluding remarks

In summary, our work showed for the first time the possible role of CD34+ cells in the leukemic development in the peripheral blood context. As observed in the modules and identified SHGs, these leukemic cell type generally participate in the AML development by promoting their own proliferation and their undifferentiated status, besides favoring the inhibition of anti-leukemic T cell responses. Considering the high association of circulating CD34+ cells to lower survival rates in AML, these findings opens a possibility of new therapeutic approaches to control the expression or the activity of aforementioned SHGs, controlling the proliferation of such cells as well as enabling T cells or other immune cells to eliminate AML blasts from the bloodstream.

6. Acknowledgements

We thank the Conselho Nacional de Pesquisa e Desenvolvimento (CNPq, grant number 302969/2016-0) funding agency for providing the financial support to make this work. We also thank the Centro Nacional de Supercomputação (CESUP), from the Universidade Federal do Rio Grande

do Sul, for the RNAseq analysis and other high processing computing analyses, and also to Dr. Joseé Hebert, from Centre de Recherche Hôpital Maisonneuve-Rosemont for kindly provided to us the clinical data utilized in this work.

7. Conflicts of interest

The authors declared that there is no conflict of interest whatsoever.

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Patient	Age	Sex	Blast%	CD34%	WHO	FAB	Karyotype
BL02H060	84	M	92	90	AML without maturation	AML-M1	45,XY,-21[3]/46,XY[22]
BL05H111	83	M	75	80	AML without maturation	AML-M1	46,XY[21]
BL05H163	20	M	86	96	AML without maturation	AML-M1	46,XY[22]
BL05H195	43	F	95	34	AML without maturation	AML-M1	46,XX[20]
BL06H144	71	F	90	2	AML without maturation	AML-M1	46,XX[20]
BL07H135	67	M	97	2	AML without maturation	AML-M1	46,XY[20]
BL08H048	45	M	96	41	AML without maturation	AML-M1	46,XY[21]
BL08H129	22	F	80	12	AML without maturation	AML-M1	46,XX,t(6;11)(q27;q23)[20]
BL09H031	54	F	85	10	AML without maturation	AML-M1	46,XX[20]
BL10H109	65	F	94	0	AML without maturation	AML-M1	45,XX,der(7)?t(7;18)(p12;q12),-18[17]/46,XX[3]
BL11H142	52	F	96	0	AML without maturation	AML-M1	46,XX[21]
BL11H151	61	M	78	5	AML without maturation	AML-M1	46,XY[21]

Table 1 – Peripheral blood AML blasts samples and patient clinical data

Yellow Module		
GO.ID	**Biological process**	**Hubs and CD34 related genes (positive correlation)**
GO:0006915	Apoptotic process	ARL6IP5,BCL2L10,BIN1,CCL5,CD38,ECT2,FIS1,GIMAP8,HCLS1,IRF5,LY86,MTFP1,PAK1,PLAC8,S100B,SH3KBP1,SHISA5
GO:0050855 GO:0002315 GO:0042113	B cell related responses	STAP1,BATF,BST2,CD180,CD38,DOCK11,PPP2R3C
GO:0001816	Cytokine production	B2M,BATF,BST2,DDX60,HAVCR2,IL12RB1,IRF5,NLRC5
GO:0002407 GO:0036336	Dendritic cell migration and chemotaxis	CCL5
GO:0048245	Eosinophil chemotaxis	CCL5
GO:0038094	Fc-gamma receptor signaling pathway	ARPC1B,BRK1,PAK1
GO:0060333 GO:0032729 GO:0034341	Interferon gamma response and production	B2M,CIITA,IRF5,NLRC5,HAVCR2,IL12RB1,BST2,CCL5
GO:0035771 GO:0071353	Interleukin 4 mediated response	IL2RG
GO:0010758 GO:0042116 GO:0010759 GO:0043032	Macrophage associated responses	CCL5,CD93,HAVCR2,STAP1
GO:0045953 GO:0035747	Natural Killer cell associated responses	CCL5, HAVCR2
GO:0030593 GO:0090023 GO:0030223 GO:0043312	Neutrophil associated responses	ALDH3B1,B2M,BST2,CCL5,CD93,CKLF,CTSA,ERP44,GNS,GYG1,JAGN1,LTA4H,MANBA,MLEC,PLAC8,RAC2,SLCO4C1,STOM,TRAPPC1,VCL
GO:0042127	Regulation of cell proliferation	BRK1,CAPNS1,CCL5,CD38,EGFL7,HAVCR2,HCLS1,IL12RB1,KIF20B,NUDT16,PAK1,PLAC8,RAC2,RARRES3,RBPMS2,S100B

GO:0070671	Response to interleukin-12	IL12RB1
GO:0050852 **GO:0042110** **GO:2001188** **GO:0045058** **GO:0042129** **GO:0002827** **GO:0070232** **GO:0010818** **GO:0042102** **GO:0001916** **GO:0042129** **GO:0002827**	T cell associated responses	B2M,BATF,CCL5,CD7,FKBP1A,FUT7,GIMAP8,HAVCR2,IL12RB1,LIME1,PAK1, PSME1,PSME2,RAB29,RAC2
GO:0002224	Toll-like receptor signaling pathway	CD180,HAVCR2
GO:0032760	Tumor necrosis factor production	HAVCR2
	Turquoise Module	
GO.ID	**Biological process**	**Hubs_and_CD34 related genes (negative correlation)**
GO:0030509	BMP signaling pathway	ACVR2B,BMPR2,CHRD,GDF1,ID1,INHA,RGMB,SKI,SLC39A5,ZCCHC12
GO:0030154	Cell differentiation	ABHD5,ACVR2B,ATF3,ATRX,AVPR1A,BMPR2,BTG1,CABYR,CDH3,CELSR1,C HRD,CSPG4,CYP27B1,DCHS1,DCLK2,DDX25,DLG5,DOK6,DPYSL3,DSC2,FARP 1,FGF9,FSCN3,GAB2,GDF1,GDPD5,GPC1,GPR173,H3F3B,HES4,HOXA2,ID1,ID3, IFT172,IGSF22,IGSF9,INHA,JAG1,LMNA,LTBP3,MAP3K5,MELTF,MMP14,NSM F,NUPR1,NYAP2,OBSCN,ONECUT3,OSGIN1,PHLDB1,PLXNA3,POFUT2,PPARG C1A,PRR9,PTPRK,RAB25,RBFOX2,RFX2,RGCC,SEMA3B,SEMA4C,SEMA5A,SK I,SLC39A5,SNAI1,SOX13,SPDEF,SRCIN1,SS18L1,STRC,TCF12,TEAD3,TIPARP,T MEM91,TNFSF9,TNIK,TP53INP2,TSKU,TULP1,ULK1,UST,WHRN,WNT7B,ZNF7 50
GO:0071354	Cellular response to interleukin-6	PPARGC1A
GO:0071560	Cellular response to transforming growth factor beta stimulus	ID1,LTBP1,PPARGC1A,PTPRK,RASL11B,SKI
GO:0042116	Macrophage activation	SPACA3
GO:0008285	Negative regulation of cell proliferation	BMPR2,BTG1,CYP27B1,DLG5,FGFRL1,IFT172,KLF9,LMNA,NUPR1,P3H3,PELI1, PPARGC1A,PTPRK,RGCC,SCG2,SKI,TNS2

GO:0050922	Negative regulation of chemotaxis	ELANE,PLXNA3,SEMA5A
GO:0046426	Negative regulation of JAK-STAT cascade	LRTM2,PODNL1
GO:0030512	Negative regulation of transforming growth factor beta receptor signaling pathway	LTBP1,RASL11B,SKI
GO:0046006	Regulation of activated T cell proliferation	TNFSF9

Table 2 - Hub genes with significant correlation to clinical traits

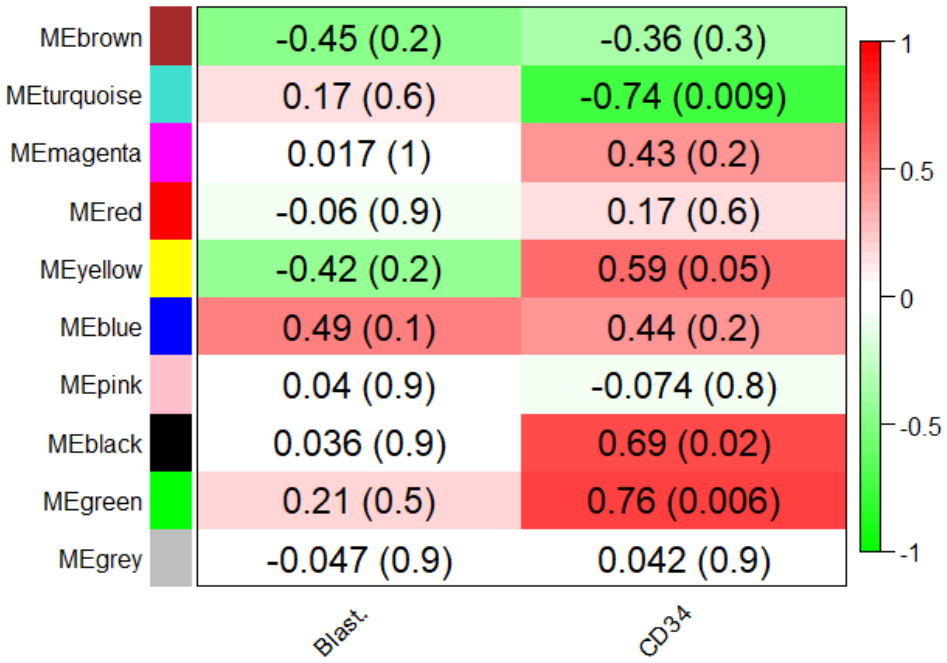


Figure 1 – Module eigengenes and clinical trait relationships (blast and CD34+ cells percentage). The numbers outside of the parentheses are the correlation coefficient between a given module eigengene and a given clinical trait. The values inside the parentheses are the corrected *p*-value of this correlation analysis, which only the ones with $p < 0.05$ were considered as statistically significant for posterior analysis. Table cells with positive ME vs clinical trait correlation are marked with shades of red color, while the ones with negative correlation are marked with shades of green color.

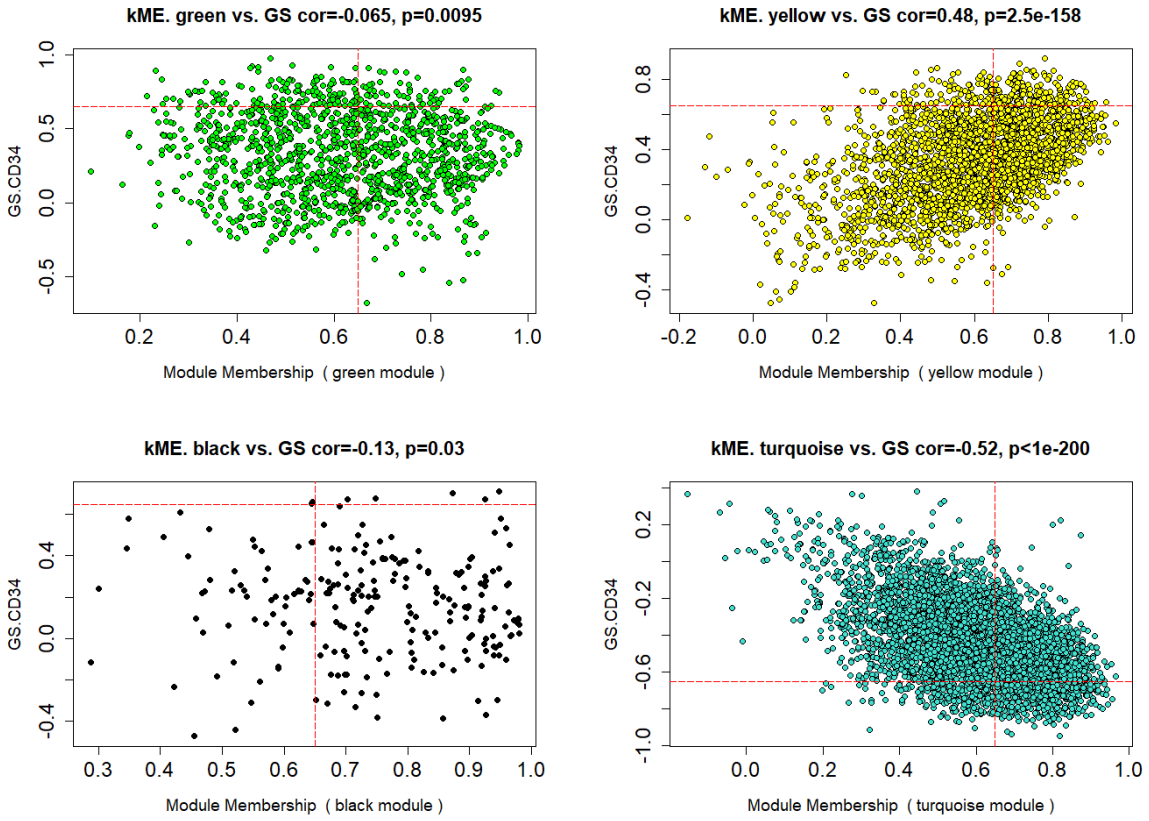


Figure 2 – Module membership (kME) values vs gene significance (GS) measurements and their overall correlation. Each plot corresponds to the previously statistically significant modules selected and the dots are the module genes. The x axis represents kME values whereas the y axis represents the gene significance for CD34+ cells percentage clinical trait. The dashed red lines are the kME and GS threshold values to consider a gene as a SHG (explained at the material and methods section)

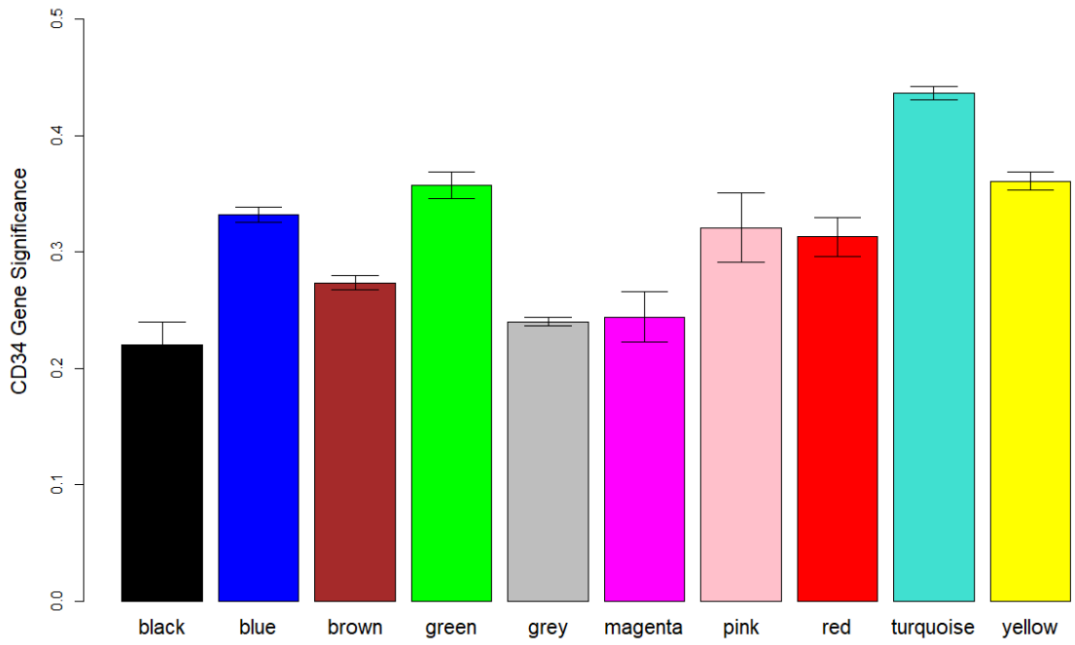


Figure 3 – CD34+ cells percentage gene significance across modules. The ticks indicate the standard deviation of the measurements in all genes from each module.

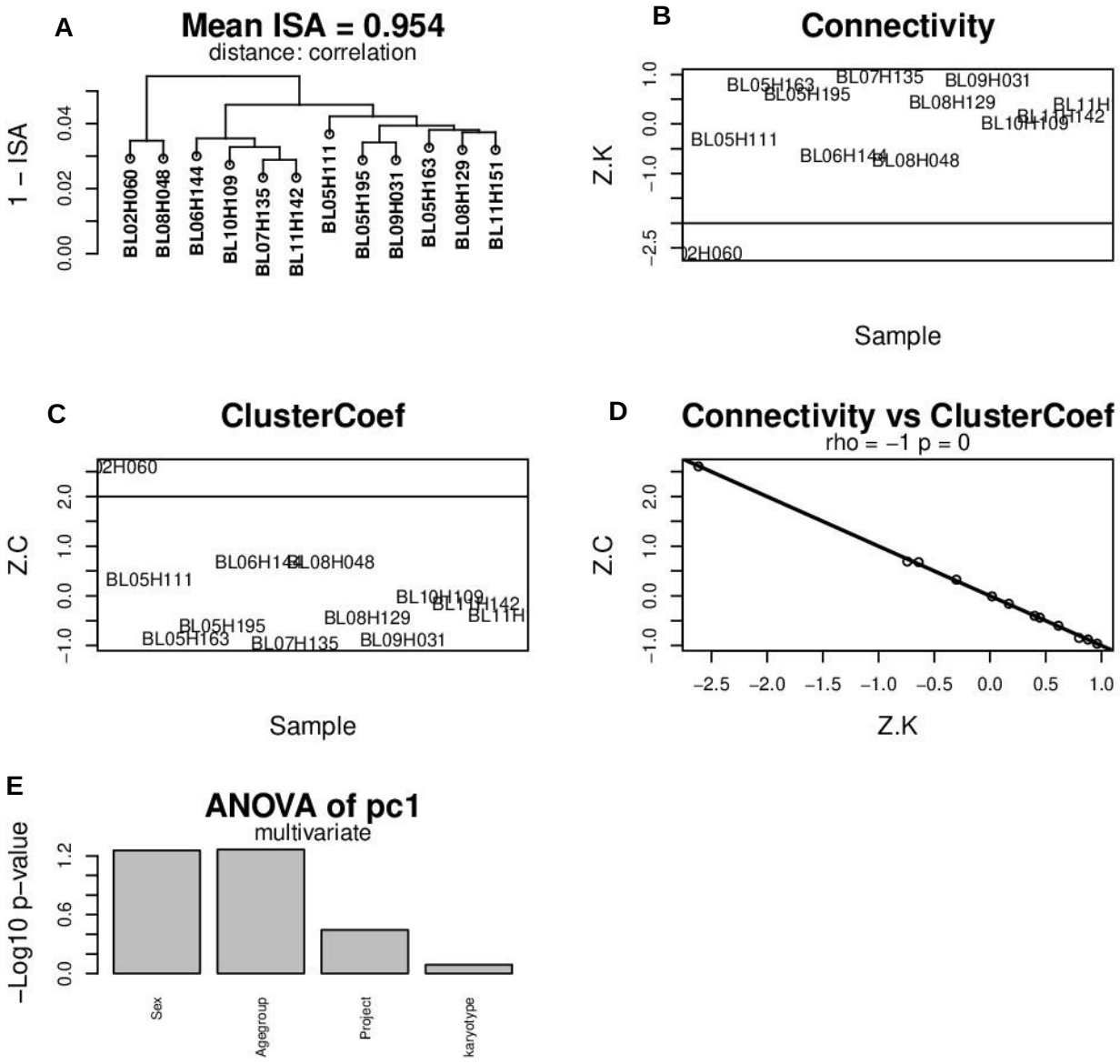


Figure S1 – Outlier detection tests in AML peripheral blood samples using the SampleNetwork R package (22). A) dendrogram produced by the average linkage hierarchical clustering using 1 – ISA (intersample adjacency), B) standarized connectivities (Z.K) of the analyzed samples, where the Z.K values lower than -2.0 were considered as outliers, C) standarized cluster coefficients (Z.C) of the samples, where the ones who had Z.C values higher than 2 were considered outliers, D) Z.K and Z.C parameters comparison, and E) multivariate ANOVA test to measure the influence of covariants (sex, the patient age risk groups - called here as agegroup, the GSE project that the sample belong and the karyotype profile of each sample).

5. Resultados adicionais

5.1. Expressão diferencial de amostras de LMA provenientes do sangue periférico em relação às coletadas na medula óssea

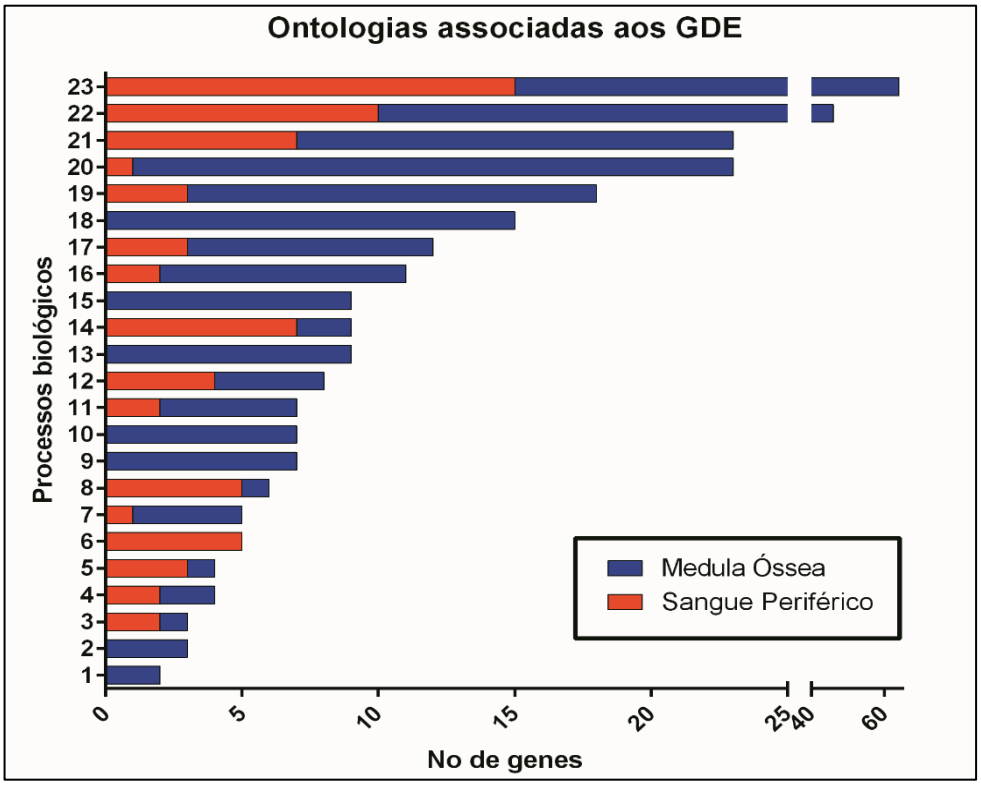
5.1.1. Materiais e métodos

Para verificar a expressão diferencial dos genes nesses dois contextos celulares, dados de RNAseq para as amostras de medula óssea foram coletados no GEO (GSE49642, GSE52656, GSE62190 e GSE66917) e processadas conforme a metodologia já descrita anteriormente (ver Capítulo II, seção 2.1). Para as amostras de sangue periférico, foram utilizadas as mesmas do estudo realizado no Capítulo II. Ambos conjuntos de dados foram submetidos a uma análise de qualidade e detecção de potenciais *outliers* com o pacote *Samplenetwork*, na plataforma estatística R (OLDHAM; LANGFELDER; HORVATH, 2012). O cálculo da expressão diferencial foi realizado através do pacote DESeq2, realizando comparações entre as amostras de sangue periférico contra as de medula óssea empregando o método de modelo linear generalizado negativo binomial acrescido do teste de razão de verossimilhança para eliminar outras fontes de covariação (sexo, idade, etc.) que podem interferir na análise (LOVE; HUBER; ANDERS, 2014). Para determinar o papel desses genes em cada contexto celular, foram realizadas análises de ontologia gênica por meio do pacote TopGO (ALEXA; RAHNENFÜHRER; LENGAUER, 2006).

5.1.2. Resultados

Como resultado, foi obtido um total de 387 genes diferencialmente expressos (GDE), dos quais 137 deles são superexpressos nas amostras de sangue periférico

e 250 deles superexpressos nas amostras de medula óssea. Na Figura 6 e na Tabela 6, observamos que células leucêmicas presentes no sangue periférico estão mais associadas a montagem de nucleossomas e respostas a citocinas como TNF e interferon. Por outro lado, nas amostras de medula óssea, há um grande predomínio de processos biológicos referente a divisão celular, bem como a ativação da via clássica do sistema complemento e migração de leucócitos.



1 - Regulação positiva da via de sinalização mediada por receptores IGF-like
2 - Montagem do cinetócoro
3 - Resposta imune inata na mucosa
4 - Regulação circadiana da expressão gênica
5 - Via de sinalização mediada por adenilato ciclase inibidores de GPCR
6 - Via de sinalização por interferon tipo I
7 - Metabolismo de xenobióticos
8 - Via de sinalização mediada por interferon gama
9 - Citocinese mitótica
10 - Formação da placa metafásica na mitose
11 - Adesão célula-matriz extracelular
12 - Resposta celular mediada por TNF

13 - Ativação do sistema complemento, via clássica
14 - Montagem de nucleossomas
15 - Regulação da transição da metáfase para anáfase
16 - Transição G1 para S do ciclo celular da mitose
17 - Regulação do crescimento celular
18 - Transição G2 para M da mitose
19 - Migração de leucócitos
20 - Regulação positiva da proliferação celular
21 - Regulação negativa da apoptose
22 - Proteólise
23 - Proliferação celular

Figura 6 – Gráfico em barras demonstrando os processos biológicos associados com os GDE. As barras em vermelho e azul representam o número de genes superexpressos no sangue periférico e medula óssea, respectivamente, para uma determinada ontologia.

Tabela 6 - Processos biológicos associados aos GDE encontrados

GO.ID	Processo Biológico	p-value	GDE S. Periférico	GDE Medula Óssea
GO:0000281	Citocinese mitótica	4,60E-06	-	KIF20A, CENPA, KIF4A, CEP55, PLK1, CIT, RACGAP1
GO:0008283	Proliferação celular	2,80E-05	INHBA, IFNB1, SGK1, AXIN2, IFITM1, ZFP36L1, PPP1R16B, HIST1H2AC, MS4A1, RORA, SSTR2, SAT1, SOD2, IRF1, CUL5	PRAME, CCL14, VCAM1, FRZB, PRTN3, ENPEP, ID4, PTGFR, ESM1, IGF2, CTGF, BCAR1, IGFBP3, GPC3, EGFR, DLGAP5, EDNRB, CLEC4G, ASPM, CDC20, KIF15, CENPF, COL8A2, CCNB1, CDC25C, BIRC5, SIX5, IGFBP4, NGFR, AURKB, CDKN3, CCNA2, RARB, TPX2, PLK1, CDK1, TNFSF12, KIF2C, IQGAP3, E2F8, BUB1, UHRF1, MELK, GINS1, FOXM1, WWTR1, RACGAP1, FAM83D, CKS1B
GO:0000086	Transição G2 para M da mitose	0,00034	-	HMMR, CENPF, CCNB1, CDC25C, CCNB2, CCNA2, TPX2, PLK1, CDK1, AURKA, CIT, MELK, NEK2, FOXM1, TICRR
GO:0006958	Ativação do sistema complemento, via clássica	0,00049	-	IGLV1-44, IGKV1D-39, IGKV2-28, IGHV3-13, IGHV3-11, IGHG1, IGHV4-28, IGKV3-20, IGHV1OR15-1
GO:0001558	Regulação do crescimento celular	0,0011	INHBA, SGK1, TSPYL2	FRZB, ESM1, CTGF, BCAR1, IGFBP3, EGFR, PSRC1, IGFBP4, TMEM97
GO:0006508	Proteólise	0,00124	TPSAB1, TPSB2, AXIN2, NRIP3, CELA2B, ENDOU, CD44, YOD1, CTRL, CUL5	IGLV1-44, PRTN3, IGKV1D-39, ENPEP, IGKV2-28, TIMP4, CTGF, IGHV3-13, PBK, IGHV3-11, GPC3, IGHG1, ADAMTSL2, CDC20, AZU1, IGKV3-20, CCNB1, UBE2C, BIRC5, PRICKLE1, NHLRC1, NGFR, AURKB, CCNA2, IGHV1OR15-1, FOXRED2, PLK1, CDK1, USP2, AURKA, CCNF, GTSE1, UHRF1, UFSP1, WWTR1, MAD2L1
GO:0051382	Montagem do cinetócoro	0,00188	-	CENPA, CENPF, CENPE
GO:0006334	Montagem de nucleossomas	0,00287	NAP1L2, HIST1H2BF, HIST1H2BC, TSPYL2, HIST1H2BD, HIST1H1C, HIST1H4I	CENPA, OIP5
GO:0007080	Formação da placa metafásica na mitose	0,00298	-	PSRC1, CCNB1, CDCA8, KIFC1, KIF2C, CENPE, NDC80
GO:0060333	Via de sinalização mediada por interferon gama	0,00531	OASL, NCAM1, GBP2, CD44, IRF1	VCAM1
GO:0060337	Via de sinalização por interferon tipo I	0,00776	IFNB1, OASL, IFITM1, GBP2, IRF1	-
GO:0002227	Resposta imune inata na mucosa	0,0089	HIST1H2BF, HIST1H2BC	RNASE3
GO:0050900	Migração de leucócitos	0,00916	CCL3, P2RY12, CD44	IGLV1-44, CCL14, VCAM1, THY1, IGKV1D-39, EPX, IGKV2-28, LBP, IGHV3-13, IGHV3-11, SDC1, EDNRB, AZU1, IGKV3-20, IGHV1OR15-1
GO:0030071	Regulação da transição da metáfase para anáfase	0,01226	-	DLGAP5, CENPF, CCNB1, UBE2C, PLK1, CENPE, BUB1, MAD2L1, NDC80
GO:0043568	Regulação positiva da via de sinalização mediada por receptores IGF-like	0,01576	-	IGFBP3, IGFBP4

GO:0032922	Regulação circadiana da expressão gênica	0,01686	RELB, RORA	NGFR, USP2
GO:0000082	Transição G1 para S do ciclo celular da mitose	0,02017	INHBA, CUL5	ID4, EGFR, CCNB1, CDC25C, CDKN3, CDK1, AURKA, IQGAP3, GTSE1
GO:0007160	Adesão célula-matriz extracelular	0,02205	CD44, ONECUT1	COL3A1, VCAM1, THY1, CTGF, ECM2
GO:0008284	Regulação positiva da proliferação celular	0, 02479	PPP1R16B	PRAME, CCL14, VCAM1, PRTN3, ID4, PTGFR, ESM1, IGF2, CTGF, EGFR, EDNRB, ASPM, CDC20, CCNB1, BIRC5, CCNA2, RARB, CDK1, TNFSF12, IQGAP3, FOXM1, WWTR1
GO:0043066	Regulação negativa da apoptose	0,02751	PDCD1, EFNA1, CD44, BCL3, CPEB4, PIM3, SOD2	PRAME, PTGFR, CHL1, EGFR, VNN1, EDNRB, HSPB6, AZU1, BIRC5, NGFR, AURKB, RARB, PLK1, CDK1, AURKA, MAD2L1
GO:0007193	Via de sinalização mediada por adenilato ciclase inibidores de GPCR	0,0358	P2RY12, RGS1, SSTR2	ADCY1
GO:0071356	Resposta celular mediada por TNF	0,04482	CCL3, ZFP36L1, RORA, TRAF1	CCL14, VCAM1, NGFR, TNFSF12
GO:0006805	Metabolismo de xenobióticos	0,04809	RORA	FMO2, MARC1, NR1I2, MGST2

6. Discussão geral

O estudo de um câncer hematológico depende de diversos fatores para que o grau de agressividade, e por consequência, o quadro clínico do paciente seja estabelecido. Conforme já demonstrado nos capítulos I e II, blastos leucêmicos interagem diretamente com diversas células presentes na corrente sanguínea, modulando a resposta antitumoral em favor da sua sobrevivência e o seu crescimento neste ambiente celular.

De acordo com as informações apresentadas na revisão contida no capítulo I, células do sistema imune possuem um grande papel no desenvolvimento da doença na corrente sanguínea. Por essa razão, muitas pesquisas em LMA tem sido focadas no estudo da interação dessas células com os blastos leucêmicos para desenvolver estratégias para coibir ou reverter suas atividades pró-tumorigênicas. Dentre elas, células dendríticas e NK merecem uma maior atenção, haja vista suas capacidades como agentes antitumorais diretos contra os blastos leucêmicos (CHRETIEN et al., 2015; WEINSTOCK; ROSENBLATT; AVIGAN, 2017). Apesar disto, compreender de que forma estas células são incapazes de promover citotoxicidade antitumoral na LMA depende também da sinalização exercida entre elas e também pelos blastos leucêmicos ou pelas Tregs (FERLAZZO; MÜNZ, 2004; SANCHEZ-CORREA et al., 2011; LION et al., 2012; ROONEY, 2014). Além disso, o fenótipo pró-leucêmico depende da múltipla combinação destes fatores para determinar com exatidão como cada elemento contribui para que o ambiente na corrente sanguínea permita a expansão e sobrevivência dos blastos leucêmicos. Logo, são necessários mais estudos que elucidem os efeitos em conjunto dessas células na corrente sanguínea em prol da progressão dos blastos leucêmicos.

Nesse mesmo contexto, o prognóstico da doença também é dependente dos vários perfis celulares de cada componente na corrente sanguínea, que irá predizer o comportamento dessas células em relação às células leucêmicas. Um exemplo típico, conforme já mencionado no capítulo I, são as composições dos receptores KIR nas células NK circulantes em pacientes com LMA. Como existe uma grande variedade de receptores KIR (tanto inibitórios quanto ativadores), múltiplas combinações podem ser formadas, cada uma com diferentes implicações na atividade das células NK em promover citotoxicidade contra os blastos leucêmicos.

Diversos estudos de coorte foram realizados com o intuito de mapear os perfis de KIR existentes nos pacientes com LMA (UHRBERG et al., 1997; VERHEYDEN; DEMANET, 2008; VARBANOVA; NAUMOVA; MIHAYLOVA, 2016; BERNSON et al., 2017). Embora haja um consenso que perfis de KIR com predominância de receptores inibitórios em detrimento dos ativadores conferem um mau prognóstico, os dados disponíveis ainda não permitem evidenciar com clareza quais perfis específicos estão mais correlacionados com esse fenótipo (BERNSON et al., 2017). Uma das razões disto é a possível influência de polimorfismos nos genes que expressam os receptores KIR (BABOR; FISCHER; UHRBERG, 2013). Nessa mesma lógica, a composição de receptores de membrana e moléculas ligantes expressos pelos blastos leucêmicos também é bastante diversa, sendo necessário estudos com coortes maiores para que seja avaliado a relação entre cada perfil fenotípico com a progressão da LMA no contexto da corrente sanguínea.

Com relação ao papel dos blastos leucêmicos CD34+ no sangue periférico, algumas observações adicionais são necessárias acerca dos genes mencionados na discussão do capítulo II. Primeiramente, a expressão do gene *HAVCR2* e sua relação direta com a presença de blastos leucêmicos CD34+ pode ser um alvo interessante para terapêutica, visto que células leucêmicas que expressam altos níveis desse gene apresentaram uma maior sensibilidade ao quimioterápico idarrubicina (XU et al., 2017). Logo, analisar a expressão de *HAVCR2* nessas células e verificar sua sensibilidade à idarrubicina pode ser uma abordagem promissora para auxiliar na eliminação dessas células na corrente sanguínea, diminuindo as chances de recolonizar o microambiente da medula óssea pós quimioterapia e/ou transplante alogênico de células tronco hematopoiéticas por esse subtipo de blastos leucêmicos.

Com relação à beta 2 microglobulina, sua implicação com o desenvolvimento da LMA são dependentes da taxa de clivagem dessa proteína, evitando que ocorra a interação da mesma com os receptores de células NK e células T citotóxicas. Logo, é importante verificar que fatores induzem esse evento nas células leucêmicas e também elucidar quais moléculas e vias são responsáveis por tal processo de clivagem. Ademais, a expressão de *B2M* em células leucêmicas CD34+ ainda é desconhecida, necessitando um estudo amplo sobre o impacto da

expressão desse gene e da existência de um processo de clivagem do produto desse gene nesse subtipo de células leucêmicas.

Contudo, apesar de todos os dados revelarem pontos promissores para compreender a biologia da LMA na corrente sanguínea, uma questão ainda necessita ser investigada. Como as células leucêmicas originam-se na medula óssea, a qual possui um microambiente completamente diferente da corrente sanguínea, é importante analisar quão diferente é o contexto da medula óssea quando comparado ao do sangue periférico. Logo, buscamos, a partir dos dados gerados nos resultados adicionais, elucidar essa questão. A partir desses dados, é possível inferir algumas diferenças marcantes acerca desses dois ambientes celulares aos quais as células leucêmicas estão expostas.

Primeiramente, um grande número de GDE associados a diversas etapas do ciclo celular estão mais expressas nas amostras de medula óssea, o que indica uma maior taxa proliferativa em detrimento às amostras de sangue periférico. Dentre as etapas evidenciadas, podemos citar a transição da fase G1 para S e da fase G2 para o início da mitose, a formação da placa metafásica, montagem dos cinetócoros, transição da metáfase para anáfase e, por fim, no processo de citocinese. Com isso, observa-se que os GDE dos processos biológicos recém mencionados atuam em quase todas as instâncias da divisão celular, o que corrobora a alta taxa de multiplicação celular dos blastos leucêmicos na medula óssea. Contudo, apesar de os dados evidenciarem uma taxa proliferativa maior, não é possível inferir de que forma cada elemento que compõe o microambiente da medula óssea pode contribuir para esse fenótipo, embora estudos demonstrem que as diferenças no perfil transcritômico estão diretamente associadas com a influência do microambiente na qual as células leucêmicas estão expostas (CHEUNG et al., 2009).

Em contrapartida, os genes superexpressos no sangue periférico não possuem características relacionadas com a proliferação celular, como ocorre na medula óssea. Nesse ambiente celular, as ontologias evidenciaram GDE relacionados ao controle negativo do ciclo celular, como é o caso do gene AXIN2, cujo produto forma uma proteína homônima que participa na via de sinalização mediada por WNT. Na ausência de WNT, AXIN2 é responsável, juntamente com

GSK3β e APC, pela formação de um complexo que leva à degradação de β-catenina, resultando no controle do ciclo celular (LI et al., 2015). A expressão de AXIN2 é relacionada com diferentes tipos de tumores sólidos e em pacientes com LMA é comumente expresso (LI et al., 2015; BARBIERI et al., 2016). Embora o papel tumorigênico dessa proteína em LMA ainda não estar completamente elucidado, sabe-se que β-catenina é fundamental para promover a proliferação e a colonização do interior da medula óssea (SIAPATI et al., 2011). Logo, em oposição a isso, na corrente sanguínea, a superexpressão de AXIN2 pode indicar uma tendência à degradação de β-catenina mediada por AXIN2, GSK3β e APC, levando à inibição do ciclo celular, e a uma menor taxa proliferativa em comparação ao contexto da medula óssea.

A presença de genes associados à migração de leucócitos foi observada nos dois contextos celulares. No sangue periférico, a quimiocina CLL3, cuja função é de exercer a quimiotaxia de neutrófilos e outras células do sistema imune é responsável por atrair células tronco hematopoiéticas da medula em direção à corrente sanguínea, favorecendo a expansão das células leucêmicas na medula óssea (BABA; MUKAIDA, 2014). Além disso, a secreção de CCL3 está associada com o bloqueio da diferenciação de vários tipos celulares, dentre eles células tronco hematopoiéticas progenitoras e eritrócitos, sendo este último associado a uma das possíveis causas do quadro anêmico em muitos pacientes com LMA, devido à baixa contagem de eritrócitos maduros (WANG et al., 2016).

Na medula óssea, o processo de migração é auxiliado por moléculas como a VCAM1. VCAM1 é um importante fator expresso em células endoteliais para a migração de leucócitos para as regiões vascularizadas por meio da interação dela com VLA-4, comumente expresso em células leucêmicas. Apesar de serem comumente expressos em células endoteliais, *VCAM1* também foi observado em células tronco mesenquimais (CTM) e osteoblastos, contribuindo para o progresso da expansão leucêmica na medula óssea (WU, 2007; JACAMO et al., 2014). Contudo, a expressão desse gene em células leucêmicas foi relacionada com a evasão da resposta imune, provavelmente mediada por linfócitos T $CD8^+$, uma vez que a ausência de VCAM1 em tumores sólidos promove uma maior taxa de apoptose de células tumorais promovidas por linfócitos T $CD8^+$. Além disso, VCAM1

promove a diminuição de infiltrações dessas mesmas células do sistema imune para o interior da massa tumoral (REUSS-BORST et al., 1995; WU, 2007; PINHO et al., 2016). Por fim, outro fator potencialmente relacionado com uma maior taxa de migração de células leucêmicas no microambiente da medula óssea são a expressão de genes relacionados com porção variável da cadeia leve *kappa* das imunoglobulinas (IGKV). Em um estudo realizado por Wang e colaboradores (2015), o repertório de IGKV está diretamente relacionado com a progressão da doença, promovendo não somente uma maior capacidade de migração, mas também uma maior taxa de quimiotaxia (WANG et al., 2015). Contudo, quais vias relacionadas com a expressão dos genes relacionados com IGKV e as características pró-leucêmicas citadas anteriormente ainda necessitam de maiores estudos.

Portanto, os dados obtidos de expressão diferencial permitiram observar dois cenários diferentes na qual os blastos leucêmicos estão presentes. Na medula óssea, grande parte dos GDE estão relacionados com o processo de divisão celular, o que corrobora a tese desse ambiente ser a “residência” dos blastos leucêmicos, conforme proposto por Shafat et al. (2017). Já na corrente sanguínea, essas células, apesar de possuírem uma menor capacidade proliferativa, contribuem para a progressão da doença atuando na indução de sinais que favorecem a migração não somente de células leucêmicas, mas também de células tronco hematopoiéticas, contribuindo com o desenvolvimento da doença.

7. Conclusões gerais:

O presente trabalho atingiram os objetivos propostos neste trabalho, o que nos permitiu inferir as seguintes conclusões acerca do comportamento das células leucêmicas no sangue periférico:

- Células leucêmicas interagem ativamente com outras células do sistema imune, modulando suas respostas celulares e, por consequência, possibilitando sua sobrevivência na corrente sanguínea;
- Células leucêmicas $CD34^+$ são cruciais para aumentar o potencial de sobrevivência no contexto da corrente sanguínea;
- A expressão de *HAVCR2* nessas células $CD34^+$ pode garantir a sobrevivência das células leucêmicas inibindo o potencial antileucêmico por uma via autócrina que leva a expressão de Galectina-9, responsável por inibir a atividade citotóxica de células NK e de linfócitos T $CD8^{+;}$
- A presença de frações solúveis na corrente sanguínea de B2M estão diretamente associadas com uma menor capacidade de morte celular de células $CD34^+$ promovidas por células NK ou linfócitos T citotóxicos;
- Células $CD34^+$ são capazes de escapar da apoptose por meio da expressão de *PAK1*, que auxilia na expressão do gene *MYC*, cujos alvos desse gene estão associados com vias anti-apoptóticas;
- Células leucêmicas da corrente sanguínea e da medula óssea possuem diferenças na expressão de genes associados a proliferação e a migração celular.

8. Perspectivas gerais do trabalho:

- Confirmar a expressão dos genes mencionados na discussão por RT-qPCR
- Analisar a influência das isoformas de mRNA dos genes mencionados na discussão do trabalho;
- Avaliar a influência de fatores de regulação da expressão gênica pós-transcricional, incluindo a avaliação da expressão diferencial de miRNAs e outros RNAs não codificantes, comparando amostras de sangue periférico em relação à medula óssea;
- Avaliar a influência de fatores epigenéticos, principalmente perfil de metilação global, comparando amostras de sangue periférico em relação à medula óssea.

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Anexo I

Capítulo de livro intitulado: “*Human diseases associated with genome instability”*, publicado no livro “*Genome stability: from virus to human application”*

Chapter 26

Human Diseases Associated With Genome Instability

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Chapter Outline

1. INTRODUCTION

The main focus of the following section (Section 2) of this review is to introduce rare genetic diseases associated with different aspects and pathways of DNA repair. Molecular aspects regarding the connection among different aspects of the repair pathways are also considered. In Section 3, we address the main genetic alterations that drive cells to genome instability resulting in acquiring cancerous phenotypes. In addition, we discuss why understanding these phenomena are useful in oncological clinical care. Finally, in Section 4 we discuss epigenetic mechanisms that influence the cell-cycle regulation and the DNA-repair response. Furthermore, the topic also contemplates the most common abnormalities in the epigenetic-regulation mechanisms and their impact on the cell-fate acquisition.

2. RARE GENETIC DISEASES ASSOCIATED WITH DNA REPAIR

The DNA molecule is constantly threatened by a wide range of exogenous and endogenous mutagenic agents, such as reactive oxygen species (ROS), chemical pollutants, drugs, and radiation such as ultraviolet (UV) light [1,2]. However, during evolution, cells have established molecular mechanisms to protect and repair the DNA molecule. These include, but are not limited to, compacting the DNA in the form of chromatin, lowering its contact with the cellular environment, and developing repair mechanisms like the nucleotide excision repair (NER), base excision repair (BER), homologous recombination (HR), DNA interstrand cross-link repair (ICLR), double-strand break (DSB), and mismatch repair (MMR) [1,3]. However, if the damage is not repaired, the cell can undergo apoptosis, senescence, or can lose control of its mitosis and can start an abnormal proliferation and become a tumor [1].

Different consequences can arise from nonrepaired DNA mutations caused by defects in the repair mechanisms, the so-called genetic diseases. In the next two sections, we focus on rare diseases related to defects in the repair machinery: xeroderma pigmentosum (XP), Cockayne syndrome (CS), trichothiodystrophy (TTD), and Fanconi anemia (FA). All four diseases are associated with defects in genes that encode proteins related to DNA repair—XP, TTD, and CS phenotypes

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Genome Stability. http://dx.doi.org/10.1016/B978-0-12-803309-8.00026-4

are derived from mutations in genes that act on the NER pathway, whereas FA is a result from mutations in genes on the ICLR pathway [1,4].

We then focus on the diseases affected by mutations in RECQ family genes, which are Bloom syndrome (BS), Rothmund–Thomson syndrome (RTS), and Werner syndrome (WS).

In the last section, we discuss genetic diseases that are not specific to a single pathway, such as ataxia telangiectasia (AT) and Hutchinson–Gilford progeria syndrome (HGPS). Explanation of the whole spectrum of outcomes and molecular pathways of the listed diseases are complex, hence we focus on the major differences and how they are associated.

2.1 NER-Related Diseases: Xeroderma Pigmentosum, Trichothiodystrophy, and Cockayne Syndrome

In order to comprehend the complexity of CS, TTD, and XP, it is required to understand the functionality of the NER pathway. NER is specialized in removing UV-induced DNA damage, where 6,4-photoproducts (6,4-PP) and cyclobutane pyrimidine dimers (CPDs) are the most common lesions, although there are other types of UV-induced lesions [1]. NER is divided in two sub-pathways: the global genome NER (GG-NER), which is responsible for the removal of DNA lesion in nonactive genes, heterochromatin, and transcribed strands of active genes, and the transcription-coupled NER (TC-NER), responsible for removing DNA damage only from transcribed strands of active genes [5,6]. Molecularly, the main difference in both sub-pathways is that in TC-NER RNA polymerase is hindered in the lesion site with the aid of specific factors, the DNA-dependent ATPases (CSA, CSB) and the pre-mRNA splicing factor XAB2 that bind to the lesion where RNA polymerase is stalled, whereas in GG-NER the lesion is recognized by the *xeroderma pigmentosum, complementation group C* (XPC)-HR23B (RAD23B) heterodimer or the DDB-complex (composed by the DNA-binding proteins DDB1 and DDB2, and the ubiquitin–ligase complex CUL4A and ROC1). XPC-HR23B have a high affinity for 6,4-PP lesions and the DDB complex for CPD lesions, but it is known that the DDB complex recruits XPC–HR23B to the site once the damage is recognized [5]. After the damage recognition, both pathways follow a core NER reaction of damage excision as follows: (1) the recruitment of the TFIIH helicase complex to open the damaged site; (2) recruitment of XPA–RPA heterodimer to form a platform of protein–protein interaction; (3) DNA-damage excision by the endonucleases XPF and XPG; and (4) synthesis of a new DNA strand [5] (Fig. 26.1). XPF forms a heterodimer with the ERCC1 protein, and it is still debatable whether XPG is recruited to the excision complex or it is a subunit of the TFIIH complex [7].

CS, TTD, and XP are autosomal diseases characterized by hypersensitivity to sunlight, premature aging, and a shorter life span, but differ in the extension of other symptoms. XP was the first NER-related discovered disease, described in 1874 by Moriz Kaposi [1]. XP affects 1:250,000 individuals in Western countries and 1:45,000 in Japan and North Africa, where individuals show severe risk to develop skin cancer and sunburns, in which skin neoplasms can appear during childhood [1,2]. They also present ocular degeneration in the lids, cornea, and conjunctiva [8]. Neurological symptoms are less common, but can appear in some cases [1,2].

Moreover, CS was the second NER-related disease, discovered 62 years later, in 1936, by Edward Alfred Cockayne, and 44 years later, in 1980, TTD was described by Price [1]. In contrast to XP, CS, and TTD individuals commonly present cognitive impairments and neurological degeneration, cachectic dwarfism, skeletal and muscular defects as well for a facial characteristic called "bird-like" face, defined by deep sunken eyes and preeminent ears [2,9,10]. Some cases of CS can develop cerebro-oculo-facial-skeletal (COFS) syndrome, a disorder that can cause neurological and visual deficiencies, whereas TTD patients can present decreased fertility and osteosclerosis, combined with more aggressive neurological symptoms, such as tremors, low IQ, and incomplete myelination of nervous fibers [2,5,9]. It is also interesting to highlight that CS individuals do not exhibit skin cancer predisposition, although they are hypersensitive to sunlight, indicating that the TC-NER pathway is not required to prevent skin cancer.

The differences in each syndrome are related to the genes affected. As seen in Fig. 26.1, three proteins are specific for TC-NER, CSA, CSB, and XAB2, where mutations in CSA and CSB are responsible for the CS phenotypes. Mutations in CSA are related to Type I (classical) form of CS, where manifestations occur around the first years of life, and to Type III (mild), where individuals show a greater life span than other types and retain basic cognitive function such as walking and speaking [10]. On the other hand, mutations in CSB can manifest themselves as any type of CS, including Type II (severe), where individuals have a maximum life span of 7 years and display strong mental retardation and loss of basic cognitive functions [10].

In XP, the differences lie on which XP gene was compromised. There are seven XP genes in NER (XPA–XPG) (Fig. 26.1); mutations in any of those genes provoke an XP phenotype and in case of XPB, XPD, XPF, and XPG, some manifestations show a CS-like characteristic, such as neurological abnormalities [2,10].

Different from XP and CS, the core origin of the TTD phenotype lies in mutations in the helicases XPB and XPD, with XPD mutations being the major cause [9]. Thus, TTD phenotype appears to be related to the TFIIH complex activity more

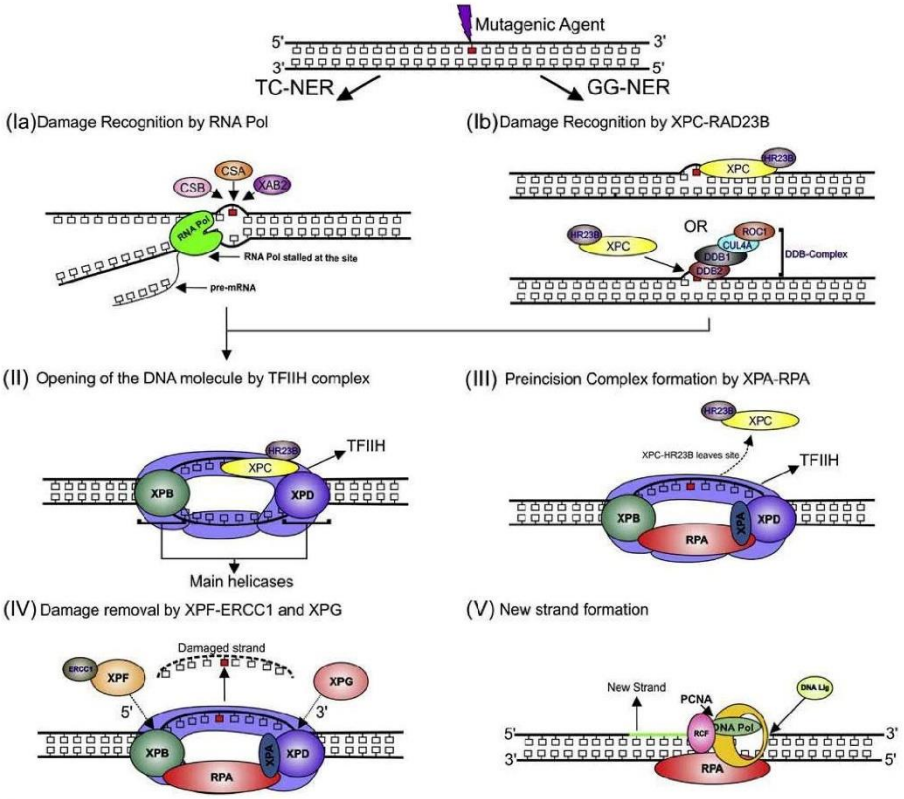

FIGURE 26.1 **The NER pathway divided by its fundamental steps.** It begins after DNA damage and one of the two sub-pathways is triggered. (Ia) TC–NER pathway is triggered if the damage site is in an active gene that is currently being transcribed. RNA Pol II is stalled at the lesion with the aid of CSA and CSB, that bind to the lesion and help in the recruitment of other factors, such as XAB2. (Ib) GG–NER can be triggered in any case, since it can act on heterochromatin, nonactive genes, and euchromatin. In this case, the damage is recognized by the XPC–HR23B heterodimer or the DDB complex. This separation is required to understand that it is a case of affinity: both complexes can recognize different damages, but they have higher specificity for a given substrate. XPC–HR23B have higher affinity for 6,4-PP while the DDB complex has it for CPDs. Nonetheless, XPC–HR23B is recruited by the DDB complex after it recognized the damage. After the initial recognition, both pathways converge in the core NER steps. (II) XPC–HR23B recruits the helicase complex TFIIH, where the 3′-helicase XPB and the 5′-helicase XPD act on opening the damage site. (III) XPC–HR23B leaves the site and the XPA–RPA heterodimer binds to unwounded DNA to allow a protein–protein platform. (IV) The 3′-endonuclease XPF and the 5′-endonuclease XPG are recruited to excise the damaged strand. (V) Finally, all proteins leave the site except for RPA which is required for the final polymerization step that recruits PCNA, DNA Polδ, and RCF to create a new strand of DNA. DNA ligase connects the new strand with the ends of the old strand.

than any other molecular aspect. The severity of TTD manifestation depends on what residue was mutated in the XPD protein, where the R112H, R592P, D673G, and R722W mutations are the cause of a severe phenotype, and R658C, R658H, and A725P result in a mild form of TTD [9].

It is interesting to observe that XP, TTD, and CS share the core NER reaction of damage excision, and the mutations in the XP genes related to this step can provoke similar phenotypes. Moreover, although all three diseases are associated with NER, the regular phenotypes are distinct, indicating that the proteins derived from the mutated genes are probably acting on other pathways beside DNA repair.

One explanation is that XP proteins, shared in both pathways, are interacting with a broad range of other proteins [11]. Taking the XP genes related to XP/CS/TTD phenotypes for example: XPB and XPD helicases are part of the TFIIH complex, where both exert structural role in interconnecting other subunits of the complex [11]. The TFIIH helicase complex is also required for regular transcription, and its deregulation can affect a broad range of cellular processes. In addition, XPB and XPD interact with proteins related to DNA repair such as p53, and to RAD52, where they would have a role in HR pathway [11]. Additionally, XPG is associated with the BER pathway by interacting with the protein NTH1. NTH1 plays a role in repairing thymine glycol mutations, and its affinity for the lesion is increased by XPG [11]. Finally, XPF interacts with RAD51 and RAD52 of the HR-repair pathway and with TRF2, a telomere elongation factor and with the Fanconi anemia, complementation group A (FANCA) protein, which is discussed later in more detail [11]. These relations clearly indicate that affecting XP proteins may disrupt a variety of mechanisms besides NER.

2.2 Fanconi Anemia

FA was discovered by Guido Fanconi in 1927. FA is distinct from XP, TTD, and CS, since the genes involved in the FA are mainly connected to the ICLR pathway instead of NER. ICLs are DNA lesions that covalently links paired strands of DNA, preventing the separation of the strands and the formation of the replication/transcription fork [12].

FA is mainly a hematopoietic disease that ultimately causes bone-marrow failure [12]. Individuals do not show most of the symptoms that appear in XP or CS, although they can present short stature and facial deformities [13]. Although the whole ICLR pathway consists of more than 30 genes, there are 16 FA genes related to ICLR, where 8 of them compose a multisubunit ubiquitin E3 ligase complex (FA core), and mutation in any of those 16 genes leads to FA [4,12–14]. A summary of the ICLR pathway can be found in Fig. 26.2. Broadly, the ICLR pathway can be divided into five stages: (1) damage recognition, (2) FA core recruitment, (3) complex assembly, (4) translesion polymerase activation, and (5) HR pathway triggering [4,12–16] (Fig. 26.2).

One interesting aspect of this pathway is its link to NER, since XPF is one of the main proteins that act on the pathway, and one of the responsible proteins for the XP/CS phenotype [2,10,15]. Remarkably, a 2013 work from Kashiyama et al. [17] described a patient who showed phenotypes associated with XP, CS, and FA [17]. The XPF–ERCC1 heterodimer is extremely important to proper removal of damaged sites, and it is known that ERCC1 mutant mice display neurodegeneration, whereas mutation in XPF results in a genetic disease called XPE progeroid syndrome, which is characterized by premature aging and aging related-diseases [15,17]. These studies indicate that there is a connection between ICLR and NER that may result in a combined phenotype of the three diseases, although this association is yet to be established. It is possible that this association was evolutionarily selected to enhance the response to DNA damage.

2.3 RECQ-Related Diseases: Rothmund–Thomson Syndrome, Werner Syndrome, and Bloom Syndrome

Another syndrome associated with the DNA-repair pathways, like BS, RTS, and WS arise from mutations in the RECQ helicase family. In this sense, before we discuss each syndrome individually, a broad view of the RECQ role on DNA repair is necessary [18].

The human RECQ helicases family consists of five proteins, RECQL1, WRN, BLM, RECQL4, and RECQL5, all of which play a crucial role in DNA damage–sensing and –repair pathways, either for helping other proteins to assemble the repair machinery or to recognize and unwind specific DNA rearrangements (Fig. 26.3) [18]. They interact with DSB repair–pathway proteins at different stages, and when DSB repair is initiated by HR, they are important at the initial DSB recognition, further disassembly of RAD51–ssDNA nucleoprotein filaments during recombination, and in resolving double Holliday junctions (DHJ) (Fig. 26.3-IV) at the branch migration phase [18]. On the other hand, in nonhomologous end joining (NHEJ) pathway, RECQ helicases act by modulating protein complexes like the DNA-dependent protein kinase catalytic subunit (DNA-PKCS), the Ku70/80 heterodimer, which detect DNA damage, and the XRCC4/ligase IV, responsible for DNA end ligation [18]. RECQ family, especially WRN, also mediates base lesion targeting in BER pathways [19], which is discussed later. Additionally, the role of RECQ helicases in the NER pathway remains poorly understood. Finally, RECQ proteins are also important to replication events; they are recruited at stalled or collapsed replication forks, interact with replication repair–machinery proteins, mainly RPA, guiding the DNA-damage fixing, and further replication restart [19]. This is an interesting fact, since RPA is also a close partner of XPA during NER (Fig. 26.1-III) [1,2].

Since the RECQ family is essential for genome maintenance, mutations in these genes could cause defects in many repair pathways, leading to genome instability. For this reason, diseases like WS, BS, and RTS, all caused by mutations in members of the RECQ family, are characterized by a wide range of symptoms and cancer development. We address each syndrome individually.

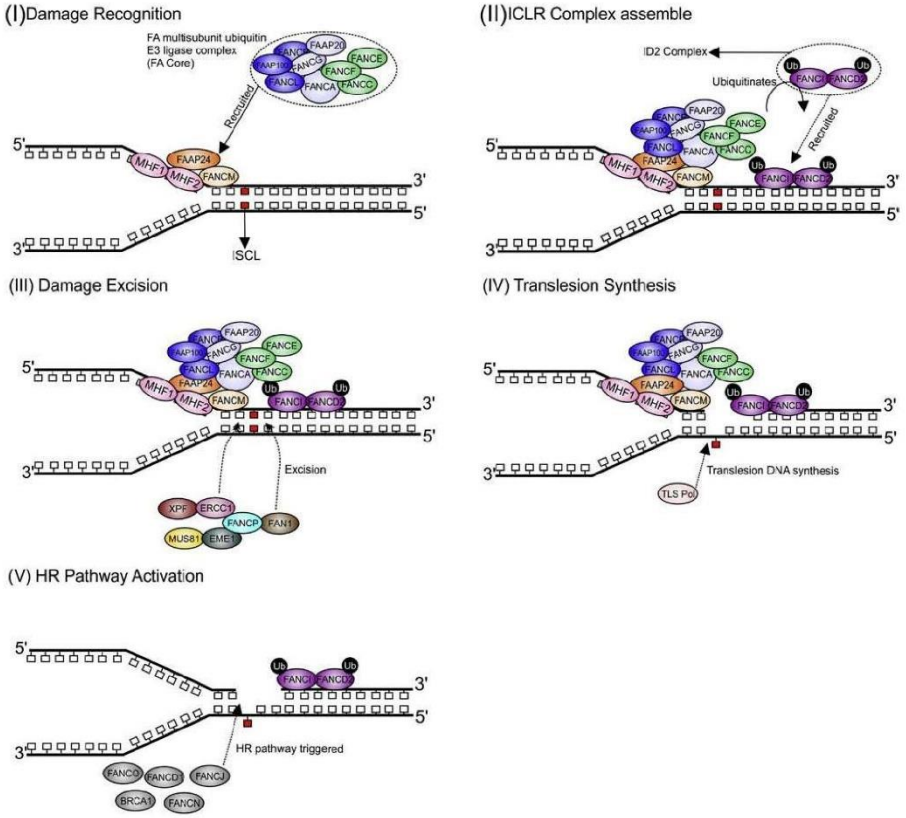

FIGURE 26.2 **The ICLR pathway divided by its fundamental steps.** (I) It begins with the ICL recognition by the proteins FANCM, MHF1–MHF2, and FAAP24 that bind to unwounded DNA and recruit the FA core. The FA core is composed by three subcomplexes, one composed of FANCL, FAAP100, and FANCB (blue); the other composed of FANCG, FANCA, and FAAP20 (light gray); and the third composed of FANCF, FANCC, and FANCE (green). (II) The FA core then ubiquitinates the ID2 complex, which is composed of FANCI and FANCD2 and binds to the unwounded DNA. With the complex formed, the excision machinery composed of XPF–ERCC1, MUS81–EME1, FANCP, and FAN1 excise the damaged region. (III) TLS polymerase than adds nucleotides to the removed strand. (IV) Finally, the HR machinery is triggered by FANCO, BRCA1, BRCA2, FANCD1, and FANCJ.

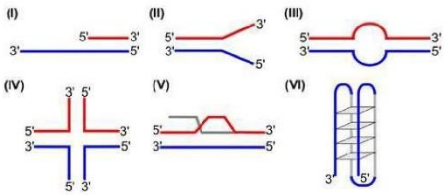

FIGURE 26.3 **Types of DNA arrangements that are substrates of the RECQ family.** (I) 3′-tailed DNA. (II) Forked DNA. (III) "Bubble" structured DNA. (IV) Holliday junction. (V) D-loop. (VI) G-quadruplex.

RTS is an autosomal recessive disease first described in 1868 by August von Rothmund, and then redescribed, with addition of phenotype variances by Matthew Sydney Thomson in 1926 [20]. However, the term Rothmund–Thompson was just coined in 1967 by William Taylor [20]. RTS major symptoms are epidermis-related tissue malformation (eg, hair, skin, and nails), short stature derived from skeletal malformations, cataracts, and cancer predisposition, especially osteosarcomas and spinocellular carcinomas [20].

RTS is caused by mutations in the RECQL4 protein, and ATP-dependent helicase, that is the only RECQ helicase present in both nucleus and mitochondria [21]. RECQL4 is necessary for the initiation of DNA replication and studies with RTS patients have shown that RECQL4 is also associated with sister chromatid separation, DSB, and BER repair pathways and telomere replication [18,20,21]. This protein can unwind forked duplexes, Holliday junctions, G-quadruplex structures, "bubble" structures, and D-loops, but cannot unwind normal duplex DNA (Fig. 26.3) [22]. Interestingly, RECQL4 shows little DNA-unwinding activity, when compared to other RECQ proteins, and seems to be more prone to anneal DNA [21]. Another fact to be observed is that mutations in RECQL4 are also related to two other syndromes: (1) Baler–Gerold syndrome (BGS), characterized by craniosynostosis and radial hypoplasia, together with short stature, and (2) the radial hypoplasia, patella hypoplasia and cleft or arched palate, diarrhea and dislocated joints, little size and limb malformation, slender nose and normal intelligence (RAPADILINO) syndrome [20].

Since RECQL4 is the only of the RECQ family to be present in the nucleus and the mitochondria, further studies focusing on understanding of RTS relationship with mitochondrial function are necessary. Additionally, the fact that RECQL4 appears to be colocalized with XPA after UV irradiation in the nucleus suggests a possible role for RECQL4 in NER that might show promising explanations for the RTS phenotype and possibly for XP [22].

Another autosomal recessive disease related to defects in genes of the RECQ family is Bloom syndrome, which was discovered by David Bloom in 1954 [23]. BS individuals present morphological abnormalities, such as long limbs, short stature, and the bird-like features similar to CS and TTD, as well as low subcutaneous fat content and dermatological conditions like photosensitivity and poikilorderma [24]. In addition, patients show high predisposition to cancers (eg, breast, larynx, skin, and colorectal cancers), lymphoma, and leukemia [24].

BS is another disease caused by a mutation in a gene of the RECQ family—in this case, the ATP-dependent RECQL2 known as BLM [18,25]. BLM can unwind the same DNA structures as RECQL4 plus 3′-end of the DNA, but shows preference for unwinding G-quadruplex DNA (Fig. 26.3), [25]. Similarly to RECQL4, BLM also has an ss-DNA annealing capacity, although the full mechanism by which it can promote strand annealing is not fully understood [25]. BLM also repairs centers of collapsed or stalled replication forks, where it appears to promote fork regression [18]. Thus, the loss of BLM function is related to a broad range of chromosomal aberrations and cancer formation [24,25]. It is not a surprise that BS individuals show high levels of sister chromatids exchanges, chromosomal breakage, translocation, and chromosomal quadri-radials [24,25].

One interesting molecular aspect of BLM is the fact that it appears to interact with FANCM, one of the proteins that comprises the FANC complex [24], showing that there might be a connection between the molecular pathways that lead to BS and FA. FANCM-deficient cells show high levels of sister chromatids exchange, similar to BS-derived cells [26]. A study made by Hoadly et al. [26] suggests that FANCM binds to the damage site and recruits the BLM complex (composed of BLM, topoisomerase IIIα, and the RMI1/RMI2 heterodimer) through its interaction with the RMI1/RMI2 heterodimer [26].

Chromosomal maintenance is a complex process that involves a wide variety of proteins and pathways, and understanding the interplay between BLM and these other mechanisms might help improve the knowledge about BS.

Finally, the last disease related to mutations in the RECQ family is WS, an autosomal recessive disease originated from mutations in WRN gene, which encodes for RECQL3/WRN protein. WS was described for the first time in 1904 by Carl Wilhelm Otto Werner. Its clinical manifestations are extensive, although most of the presented symptoms are aging related, such as atherosclerosis, diabetes mellitus type 2, osteoporosis, and cataracts, among others [27]. Nonaging-like symptoms include hypogonadism, reduced fertility, low height, and others. In addition, WS patients are also susceptible to development of tumors, especially sarcomas [28]. However, even though this could represent an important risk factor, most WS-affected individuals decease by a myocardial infarction between their fourth and fifth decades of life [28].

WRN protein has a helicase domain which has specificity for certain DNA structures (Fig. 26.3), especially G-quadruplex (Fig. 26.3-VI.) and Holliday junction (Fig. 26.3-IV.), DNA structures found mainly in telomeric DNA and in the recombination process, respectively [19,29]. In addition, WRN has a unique 3′–5′ exonuclease activity that digests 3′-recessed termini or blunt DNA duplexes that contain structures like bubbles (Fig. 26.3-III), forked duplexes (Fig. 26.3-II), Holliday junctions, and DNA–RNA heteroduplexes. This exonuclease acts coordinately with a helicase domain through the DNA duplexes size reduction, allowing proper helicase-unwinding role, although both domains have also independent functions [28].

WRN interacts with many regulating proteins related to DNA-repair pathways. For example, in the long-patch BER (LP-BER) pathway, WRN interacts with NEIL1, a formamidopyrimidine lesion glycosylase, DNA polymerase β, responsible for base replacement at the lesion site, and with FEN1, an endonuclease that removes the 5′-overhanging flap [19]. In DSB repair, WRN plays an active role in NHEJ by interacting with KU70/80, DNA-PKCS and XRCC4/ligase IV complexes. During HR, WRN interacts with proteins like RAD51 and RAD52, which are fundamental during strand invasion and annealing [28]. Moreover, WRN is important in telomere replication and maintenance pathways, recognizing D-loops (Fig. 26.3-V), G-quadruplex structures in telomeric DNA, and interacting with the shelterin complex proteins, such as TRF1, TRF2, and POT1 [29]. Conversely, WS cells present characteristics that are directly associated with impaired DNA repair, including telomeric erosions, oxidative DNA damage, and a defective DNA interstrand cross-link removal, which can contribute to develop aging-related symptoms and tumorigenic processes like sarcomas in WS patients [29].

2.4 Ataxia Telangiectasia

Different from all the previously discussed diseases, AT is not caused by defects in a specific DNA-repair pathway. AT is a rare autosomal recessive disorder described originally in 1926, but the term "ataxia telangiectasia" was suggested by Elena Boder and Robert P. Sedgewick in 1957 [30]. The clinical characteristics of AT disorder are progressive neurological dysfunctions, which includes oculocutaneous telangiectasia and cerebellar ataxia. In addition, individuals with this disorder show cancer predisposition, susceptibility to bronchopulmonary disease, and multisystem abnormalities, such as immunodeficiency, radiosensitivity, infertility, and endocrine dysfunctions [30,31].

AT is caused by mutations in ataxia telangiectasia–mutated gene (ATM), located at chromosome 11q22–23, which encodes to an ATM serine/threonine kinase [30]. The ATM protein is a member of the phosphoinositide 3-kinase (PI3K)-related protein kinase (PIKK) family, which is able to induce a DNA-damage response [30,31].

ATM is activated by different biological processes, such as cell-cycle checkpoint and DNA damage [30]. Indeed, many ATM substrates are cell-cycle regulators with important roles in DNA-damage response, such as p53, CHK2, and BRCA1 [31,32]. During DNA-damage repair, a complex composed of MRE11–RAD50–NBS1 (MRN complex) recognizes DSB and leads to ATM activation, as well as performs an adaptor role to subsequent phosphorylation of downstream ATM substrates [30–32]. In response to the MRN-complex signaling, ATM undergoes autophosphorylation at serine 1981, and is converted from inactive multimeric to an active monomeric kinase [30,31]. Once activated, ATM orchestrates a signaling cascade in response to DSB that coordinates cell-cycle arrest, DNA repair, or the cell-apoptosis process [30,31]. The response capacity of ATM to DNA damage is the primary in vivo function of this kinase and is intrinsically related to phenotypes of AT disorder [31].

In this sense, the role of ATM in DNA repair involves the phosphorylation of specific repair factors, like KRAB-associated protein 1 (KAP-1), which relaxes chromatin structure and allow the accessibility of repair proteins (the chromatin role in DNA repair is discussed later) [31]. Another target of ATM phosphorylation is the FANCD2, a protein whose defects leads to FA; in response to DNA damage, FANCD2 is phosphorylated at Ser222 [32].

2.5 Hutchinson–Gilford Progeria Syndrome

HGPS is a progeroid syndrome described at first time by Jonathan Hutchinson in 1886 and by Hastings Gilford in 1897 [33]. However, this syndrome was described in greater detail in 2003, when the molecular basis of the disease was discovered [33,34].

Despite HGPS patients born with normal appearance and weight, the clinical symptoms appear within 12 months and progress rapidly [35]. The best described characteristic of HGPS patients is the development of age-related diseases, such as cardiovascular pathologies, prominent superficial veins, skin complications, and alopecia. In addition, these individuals show disturbed growth, lipodystrophy, joint abnormalities, and osteolysis [35]. This disease is caused by a single nucleotide substitution on the gene *LMNA* that encodes the A-type nuclear lamin proteins [33,34]. Differential alternative splicing generates A-type lamin proteins, with the most abundant being lamins A and C; however, mutated *LMNA* leads to aberrant splicing that results in the deletion of 50 amino acid residues from C-terminal region of prelamin A [33,34,36]. This aberrant splicing produces a mutant protein called "progerin," which accumulates in a farnesylated form, affecting the nuclear organization, chromatin dynamics, epigenetic regulation, and gene expression, causing genomic instability, premature senescence, and telomeres disruptions [34,36].

It was observed that progerin modifies the composition and mechanical properties of nuclear lamina, which are related to abnormal nuclear morphology [34,36]. This occurs due to the high affinity of progerin to nuclear envelope and by immobilization of A-type lamins in the nuclear lamina induced by progerin. In addition, high levels of γH2AX phosphorylation are an

indicative of activated DNA-damage response and cellular accumulation of DNA damage [34,36]. Furthermore, HPGS cells show reduced survival and proliferation, besides sensitivity to DSB and delayed recruitment of repair proteins [34,36].

Among the proteins involved in this impaired recruitment are the components of MRN complex, which are crucial for HR [36]. Furthermore, another DNA-repair defect observed in HPGS cells is the mislocalization of XPA to DSB that can be associated with the delay activation of DNA-repair proteins, such as the MRN complex [36,37]. Thus, HGTS molecular pathways have an interplay with the ATM related–molecular mechanisms that may lead to AT, as well as with NER-related proteins, such as XPA.

2.6 Rare Genetic Diseases: Summary

In conclusion, the understanding of DNA-repair diseases is crucial to boost the knowledge of the DNA-repair machinery and the consequences of its defects. Moreover, the phenotypes that result from diseases described earlier go from a broad range of anatomical abnormalities to neurodegeneration and cancer development, indicating that they regulate much more than DNA repair. For example, XP proteins are also targets of different post-translational modifications and have different protein–protein interaction sites that may answer how they are regulated and to what proteins they may be connected [5]. Other examples are CSA and CSB, that are also known to regulate cellular redox balancing, where cells lacking CSA and CSB show increased level of ROS [38]. These proteins are also connected to BER and the maintenance of the stability of mitochondrial DNA [38]. CSB is associated with biological processes, such as cell growth, angiogenesis, proliferation, and cell death [39]. The same logic goes for the proteins related to FA, BS, RTS, WS, AT, and HGTS which comprise large complexes and are connected to multiple proteins.

3. CANCER AND GENOME INSTABILITY

It is impossible to discuss genetic diseases without mentioning cancer, since it is intimately associated with DNA-repair defects and is a common outcome of the rare genetic diseases described before. Cancer onset begins when precancerous cells acquire uncontrollable growth, sustain angiogenesis, and become able to invade different tissues [40]. These are the main factors that contribute to the extent of cancer malignancy. However, since the human organism has redundant and self-regulating pathways to maintain homeostatic conditions, an extensive set of genes must be affected to reach conditions necessary to carcinogenesis. This change in genome profile can be achieved if DNA damage–repair systems and/or replicating machineries work improperly, or when cells are exposed to mutagenic or genotoxic agents, such as tobacco smoke, UV light, and ionizing radiations, among others. The increased mutation rate that changes drastically the genome landscape deregulates basal expression and surpasses genome integrity, and cell-cycle surveillance promotes cells transformation. It is important to reinforce the idea that genome instability makes cells more susceptible to carcinogenesis [41]. Since there are many pathways associated with cancer development, the focus of this section is to describe the basis of the different types of genome instability, as well as its relationship with cancer development and how understanding these phenomena can be useful in clinical practice and therapy.

Genome instabilities can vary from a single nucleotide mutation to a whole chromosome structure modification (clastogenesis). These changes alter cell homeostasis in many ways, depending on which genes are affected. Single nucleotide mutations arise usually due to the high cell exposure to DNA-damaging agents or when DNA-repair genes involved—for example in NER, BER, and MMR—are mutated, although a nucleotide-deficient environment could also promote such imbalance (Fig. 26.4-I) [42,43]. For example, XP patients are highly susceptible to sunlight UV-induced carcinogenesis (about 2000–10,000 times higher than a healthy person) due to the accumulation of nucleotide mutations that are caused by a deficient NER from one or more mutated XP proteins [8]. Some regions of the genome, containing repeated nucleotide sequences—one to six nucleotides repeated multiple times—called microsatellites, suffer more extensive modifications caused mainly by an inefficient MMR system, leading to insertions and/or deletions (indels) in these regions during the S phase of cell cycle (Fig. 26.4-II). This microsatellite instability (MSI) promotes frameshifts in coding sequences of genes resulting in truncated or nonfunctional proteins. MSI is present in some cancers, mainly in colorectal tumors, which corresponds to 15% of these cases [44].

Chromosomal instability (CIN), especially chimeric chromosomes and/or aneuploidies, on the other hand, is a common genome instability in most cancers. Since the discovery of Philadelphia chromosome, formed by the chromosome 9 and 22 translocation, much has been done to understand the importance of CIN events in tumor progression. In this sense, defective DNA-repair mechanisms can induce multiple chromosomal fragmentations. For example, DSB-repair failures contribute to CIN generation, creating a chromothripsis phenomenon characterized by multiple chromosome breaks and rearrangements (Fig. 26.5-I) [45]. In addition, other factors also contribute to the acquisition of an abnormal karyotype

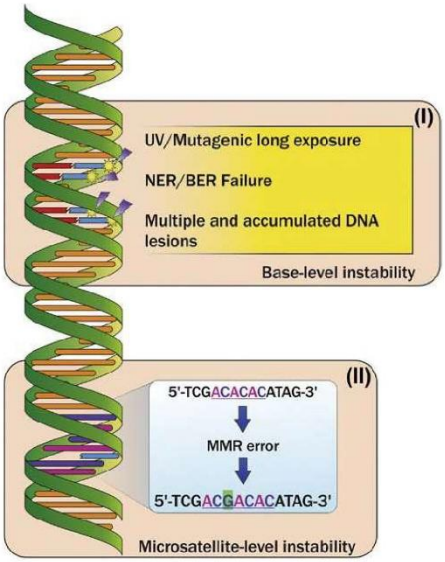

FIGURE 26.4 **Cancer-related DNA mutations that cause genome instability.** (I) Base-level accumulated mutations, caused by high exposure to mutagenic agents and/or defective NER/BER pathways. (II) Indel events in microsatellite regions caused by defective MMR systems.

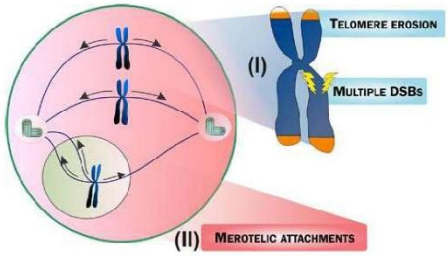

FIGURE 26.5 **Cancer-related events that lead to chromosome instability and clastogenic phenomenon.** (I) Chromosomal breakage and rearrangements by the progressive telomere shortening and/or double-strand breaks. (II) Multiple microtubules attached to one kinetochore (merotelic attachments) that causes spindle pole asymmetry and incorrect chromosome segregation.

in cancer cells. Telomere shortening or loss is one of the main driving forces of chromosome fragmentation. These six tandem repeated sequences at the end of chromosomes, along the protein complex called shelterins, are crucial to protect chromosomes from fusing to each other during cell cycle and generating aberrant chromosomes (Fig. 26.5-I). When cells naturally cease to express telomerase, they die due to a telomeric erosion condition in which cells either enter a replicative senescence state or begin to generate multiple chromosome fusions [46]. This process is tightly controlled in the cell and when they are impaired, the cells are able to proliferate, leading to uncontrolled cell growth. Curiously, tumor cells can even restabilize their genome through the reexpression of telomerase or via a homologous recombination alternative lengthening of telomere (ALT) mechanism, although the triggering mechanisms remain unclear, especially for ALT [47–49].

Finally, another CIN-inducing event is the incorrect segregation of chromosomes during cell cycle. Mutated proteins responsible for chromosome organization and cell structure can compromise the sister chromatids separation (karyokinesis)

or cells separation (cytokinesis) resulting in aneuploidies [50]. Centrosome dynamics during mitosis, for example, is a very coordinated process that requires a myriad of signaling proteins. Thus, any change in the velocity of chromatids separation during the M phase of the cell cycle can induce CIN. This could make the chromosome's kinetochore attach to microtubules coming from both spindle poles, instead of just one of them, forming merotelic attachments (Fig. 26.5-II). This creates a spindle asymmetry that compromises the correct chromosome segregation and creates a lagging chromosome that will result in aneuploidic daughter cells [51].

One of many hypotheses that tried to explain the origins of genome instability is the mutator phenotype, which suggests that mutations in genes involved in the genome-maintenance pathways, known as caretaker genes, makes cells more prone to DNA lesions or replication failures that facilitate the cancer development [52]. In hereditary cancers, mutations in caretaker genes mostly drive cells to genome instability, as observed in the Lynch syndrome (in which MSI caused by MMR failure triggers oncogenesis) as well as other inherited DNA-repair gene mutations, such as mutations in the FANC family and in the BRCA1 gene. However, as demonstrated in many high throughput–sequencing studies, these types of mutations are unlikely to occur in sporadic cancers, and even if they do, it requires that both alleles must be affected to drive cells to genome instability [40]. In these nonhereditary cancers, the most accepted hypothesis is that the same altered pathways that activated oncogenes also drives deacceleration or stalling of replication fork progression, especially in regions denominated as common fragile sites, creating chromosome breakage at these locations. This favors the selection of defective tumor-suppressor genes, such as TP53, and genome amplification of other oncogenes, leading to cancer development through the escape from apoptosis and senescence [40,53–55].

Understanding how complex patterns of genome instability events contribute to cancer has many implications in the clinical health care. In cancer diagnosis, detection of chromosome instabilities is important to determine tumor aggressiveness and patient's prognosis. For example, MSI-containing colorectal cancers are considered to have more favorable prognosis as compared to stable microsatellite colorectal cancer types. Some authors hypothesized that translation frameshifts caused by MSI generate novel peptides at C-terminus region that are immunogenic and stimulate an inflammatory response against tumor cells [44].

Also, the comprehension of how cancer begins and develops is crucial for new chemotherapeutics drug design. One strategy is inducing mitotic catastrophe by small molecules that act on the kinetochore and spindle poles assembly proteins, such as aurora kinase inhibitors [56]. However, since many of these potential chemotherapeutics are toxic to the bone marrow, many types of DNA damage–response inhibitors were tested as adjuvants to maximize genomic instability in cancer cells, promoting mitotic catastrophe and apoptosis and avoiding potential drug resistance [57,58].

Therefore, cancer development can be accelerated by genome instability. High-proliferative capacity, sustained angiogenesis, cell cycle–checkpoint evasion, for example, are part of so-called "Cancer Hallmarks," and most of these features are acquired by multiple events of genome instability [59,60]. Although more studies are required to understand the complex relationship between cancer and genome rearrangements, especially in sporadic tumors, there is still plenty of information available that can help oncologists in clinical care to establish patient prognosis and to search for potential anticancer targets.

4. EPIGENETIC REGULATION OF CELL CYCLE AND DNA REPAIR IN CANCER

The transformation of healthy cell toward a cancerous cell occurs gradually by a series of factors, including genetic and epigenetic modifications. Proper maintenance of epigenetic marks is essential to healthy cells and is associated with cell-fate acquisition [61–63]. Epigenetic alterations can change chromatin structure to loose state, which is transcriptionally active (called euchromatin) or to compact state, resulting in a transcriptionally inactive configuration (called heterochromatin) (Fig. 26.6-I) [64]. In this sense, chromatin alterations changes DNA accessibility and are responsible for modulation of the gene expression by affecting the interaction of DNA with transcriptional complexes, resulting in activation or inhibition of different signaling pathways (Fig. 26.6-I) [61,63]. Furthermore, histone modifications may affect DNA–histone or histone–histone interactions, or recruit nonhistone proteins to chromatin, creating a binding site for specific proteins that can act as regulatory factors [62,65]. In addition, different biological processes such as transcription, cell cycle, DNA repair, and replication are regulated by posttranslational histone modifications [62,65].

During cell cycle, checkpoints are surveillance systems that have the capacity to interrupt cell-cycle progression [66]; however, abnormalities in checkpoints and signaling pathways associated with proliferation are commonly observed in cancerous cells [66]. An example of signaling pathway disturbed in cancerous cells that is addressed in this chapter involves the retinoblastoma tumor-suppressor protein (RB) [66]. RB is a tumor suppressor whose activity is associated with different biological processes, such as differentiation, apoptosis, DNA-damage response and repair, DNA replication, and cell cycle [67]. During cell cycle, RB binds to the transcription factor E2F and prevents the transcriptional activation of E2F target

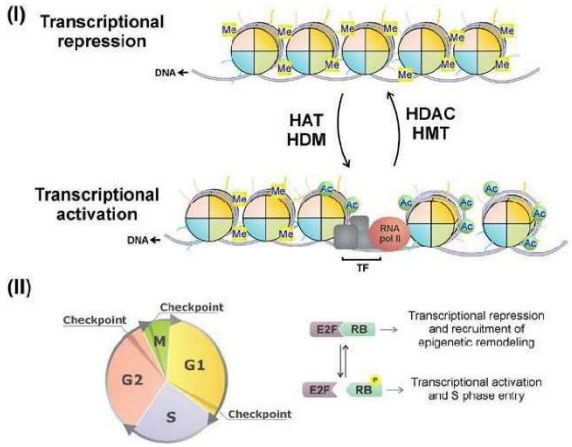

FIGURE 26.6 **(I) Simplified schematic of a transcriptionally inactive configuration of chromatin changing to an active chromatin configuration.** This transition is regulated by modifications in histone tails, where methylation and acetylation are most commonly observed. The proteins responsible for this transition include histone acetyltransferases (HAT), histone demethylases (HDM), histone methyltransferases (HMT), and histone deacetylases (HDAC). (II) Cell-cycle representation and RB-associated transcriptional-regulation mechanism. RB interacts with E2F, preventing the transcription of E2F target genes and recruiting chromatin remodelers to lead the transcriptional repression of genes associated with the cell-cycle progression. Phosphorylated RB is unable to interact with E2F, allowing the transcription and cell-cycle progression.

genes (Fig. 26.6-II) [67]. E2F is a transcription factor that regulates the expression of different genes associated with DNA synthesis and cell-cycle progression from G1 to S phase [66,68]. The RB activity is linked to the inhibition of cell cycle by interacting with E2F, leading to the down-regulation of specific cell cycle–related genes (Fig. 26.6-II) [66,68]. To make this inhibition more efficient, RB also recruits chromatin remodelers (Fig. 26.6-II), such as the co-repressor SIN3 transcription regulator family member B (SIN3B) that promotes lysine deacetylation from histone tails by recruiting histones deacetylases 1 and 2 (HDAC1 and 2) [68]. Lysine acetylation is correlated to transcriptional activation, and its deacetylation leads to a more compacted chromatin structure and consequently, transcriptional repression of E2F-target gene promoters [61,68]. Furthermore, histone methyltransferases (HMT), DNA methyltransferase 1 (DNMT1), and heterochromatin protein 1 (HP1) also are chromatin remodelers recruited by RB stimulation, promoting methylation in promoter region of genes regulated by E2F, contributing to its transcriptional repression [67]. Accordingly, RB is a crucial tumor suppressor, and it is necessary to ensure proper cell-cycle progression, one that promotes the silencing of genes that regulate cell-cycle progression and DNA replication [67].

RB mutations have been associated with reduced H4K20 trimethylation on the N-terminal tail [67,68]. Methylation state of H4K20 has an important role in cell cycle, and has been associated with cell-cycle progression, transcription, chromosome condensation, and origin firing for DNA replication [68]. It is observed that mono- and dimethylation are related to DNA repair and DNA replication, whereas trimethylation of H4K20 is associated with silenced heterochromatin formation and with cell-cycle arrest [68,69]. In addition, aberrant methylation in RB promoter leads to decrease in RB gene expression, and these abnormalities have been observed in different cancers, such as retinoblastoma, bladder cancer, neuroblastoma, and gastric carcinoma, among others [70].

Cell growth and division are both processes regulated tightly by a set of coordinated proteins that monitor cell-cycle progression and DNA integrity. The loss of cell-cycle control and DNA-damage propagation has emerged as the main inducer of cancer and other diseases. In this sense, modifications in the DNA sequence may alter gene products or lead to a loss of gene function. To prevent such genetic deregulation, DNA-damage response is activated, leading subsequently to cell-cycle arrest, recruitment of DNA-repair machinery, and damage correction or apoptosis [71]. Defective activity of epigenetic regulators can also lead to gene expression deregulation and, consequently, cell transformation [72]. As a result, proto-oncogenes expression can be activated by promoter hypomethylation, whereas the expression of tumor suppressors may be silenced by its promoter hypermethylation [72].

Thus, proper DNA repair is necessary for the coordination between chromatin modifications, cell cycle, and DNA-repair machineries [65]. In this sense, different proteins are able to mediate the communication among chromatin and repair, such as ATM, whose activation occurs in response to chromatin structure changes, like formation of DSB [65]. In addition to ATM, DNA-dependent protein kinase (DNA-PK) and/or Rad3-related protein (ATR) mediate the phosphorylation on the variant histone H2AX, which creates a binding motif to mediator of DNA damage–checkpoint protein (MDC1), that recruits other DNA-repair proteins, such as E3 ubiquitin–protein ligase (RNF8) and Nijmegen breakage syndrome 1 (NBS1) [65]. H2AX phosphorylation is the histone modification in response to a DNA break, but it is viewed to act as a broad signal in response to DNA damage, although primarily in the form of DSBs, as well as a triggering pathway in response to stalled replication forks (Fig. 26.7-I) [73].

At DNA-damage sites, the ubiquitin ligase Rnf20/Rnf40 mediates the ubiquitylation of H2B [65]. H2BK123 ubiquitylation is necessary to H3K4 and H3K79 methylation, being these modifications are required to alter the chromatin structure and allow the access of proteins involved in DNA repair (Fig. 26.7-I) [65,73].

Nonetheless, in DNA-damage region, methylated H4K20 (Fig. 26.7-I) acts as a binding platform to the P53-binding protein (53BP1), providing a stable 53BP1–chromatin association [69]. In human cancer–derived cells, the decreasing H4K20 trimethylation has been proposed as a common hallmark related to cell transformation [74]. This transformation can be associated with the fact that low H4K20 methylation avoids the repression of genes that regulate cell-cycle progression [74].

Another histone modification is the acetylation of H3K56 (Fig. 26.7-I) that occurs in response to replication fork damage [73]. During DSB repair, H3K56 acetylation is necessary for Rad52-dependent repair and promotes sister chromatids recombination [73].

Furthermore, in response to UV irradiation, H3K9 (Fig. 26.7-I) is acetylated during the NER process [73]. H3K9 acetylation regulates two pathways, the recruitment of histone acetyltransferase GCN5 to DNA lesions and the coordination of the activity of tumor suppressor p53 and acetyltransferase p300 [73]. In addition, ING2 activity is required to enhance the p53 and p300 interaction to induce the histone acetylation and to mediate a relaxation in chromatin structure; it is also required to recruit the XPA protein to the DNA lesions caused by UV irradiation [62,75]. In this sense, ING2 promotes the chromatin remodeling, which is adequate to DNA-damage repair and to the proper NER [75]. Many aspects of tumor biology, including cancer invasion and metastasis, are associated with deficiency in ING activity [75].

The events described earlier illustrate the importance of chromatin modifications and remodeling, acting in the DNA damage–response signaling and allowing formation of the complexes that mediate the DNA repair. In this sense, according to histone modifications, different chromatin–protein interactions are allowed, interfering with the propagation of repair signaling and turnover of factors involved in repair signaling. In addition, once repair process finishes, histone modifications are frequently reversed, allowing to establish a "prior to DNA damage" chromatin state.

Chromatin remodeling during cell cycle and DNA repair is a mechanistic step that allows or impairs the access to the specific DNA regions. Different chromatin remodelers and histone modifications act as signaling messengers, promoting the recruitment of proteins responsible for different DNA-repair pathways or cell-cycle progression. In addition, epigenetic inactivation of different genes is associated with increased genetic instability and with abnormal cell growth. In this sense, interference with the establishment of histone modifications, changes in chromatin accessibility, or silencing of chromatin are intrinsically associated with tumorigenesis. Thus, epigenetics has become an area of increased interest for the development of therapy and clinical strategies against cancer.

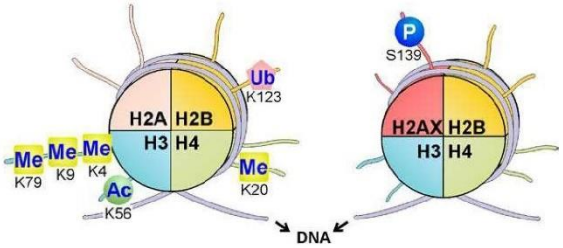

FIGURE 26.7 Nucleosome representation with the most described modifications in histone tails related to DNA-repair response.

GLOSSARY

Ataxia telangiectasia (AT) Is a disorder caused by mutations in the ATM gene. This disease is characterized by neurological dysfunctions, cancer predisposition, immunodeficiency, radiosensitivity, infertility and endocrine dysfunctions, among others.

Bloom syndrome (BS) Autosomal recessive genetic disease characterized by the lack of the BLM protein activity. Individuals affected by this disease show morphological abnormalities, photosensitivity, poikiloderma, and high predisposition to cancers.

Cerebro-oculo-facial-skeletal (COFS) syndrome Autosomal recessive genetic disease characterized by an intrinsic inability of the global genome nucleotide excision repair machinery to remove DNA lesions. Individuals affected by this disease present developmental delay, facial abnormalities, microcephaly, cataracts, and microphthalmia, among other symptoms.

Chromothripsis An event of multiple chromosomal rearrangements in a single event.

Clastogenesis A process defined by the loss, addition, or any rearrangement of chromosomes.

Cockayne syndrome (CS) Autosomal recessive genetic disease characterized by an intrinsic inability of the transcription-coupled nucleotide excision repair machinery to remove DNA lesions. Individuals affected by this disease present impaired neurodevelopment photosensitivity progeria, among other symptoms. However, unlike XP, individuals bearing the CS phenotype do not display high predisposition to skin cancers.

Common fragile sites Encountered in the majority of individuals, they are specific locations that are prone to chromosomal rearrangements.

Fanconi anemia Inherited blood disorder caused by intrinsic defects on the interstrand cross-link–repair machinery. Individuals affected by this disease shows severe predisposition to develop myelogenous leukemia and bone-marrow failure, in addition to numerous morphological abnormalities.

Hutchinson–Gilford progeria syndrome (HGTS) Genetic disease caused by a mutation in the LMNA gene. It is a progeroid syndrome characterized by age-related diseases, such as cardiovascular pathologies, skin complications, alopecia, lipodystrophy, joint abnormalities, and osteolysis.

Indels Base insertion and/or deletion.

Lynch syndrome (formerly known as hereditary nonpolyposis colorectal cancer) An inherited condition that makes carriers susceptible to develop certain types of cancer (especially colorectal cancers). They are characterized by mutations in mismatch repair genes.

Merotelic attachments Characterized when a centromere is attached to microtubules coming from both spindle poles.

Mutator phenotype A carcinogenesis hypothesis which postulates that cells acquire cancerous features (see Cancer Hallmarks) due to defective genes responsible for the maintenance of the genome stability.

Philadelphia chromosome A translocation of chromosomes 9 and 22, resulting in a shorter 22 chromosome and a BCR–ABL gene fusion. This phenomenon is present in some hematological malignancies such as acute lymphoblastic leukemia and chronic myelogenous leukemia.

Rothmund–Thomson syndrome (RTS) Autosomal recessive genetic disease characterized by the lack of the RECQL4 protein activity. Individuals affected by this disease show epidermis-related tissue malformation, morphological abnormalities, and cancer predisposition.

Trichothiodystrophy Autosomal recessive genetic disease characterized by an intrinsic inability of the global-genome nucleotide excision repair machinery to remove DNA lesions. Individuals affected by this disease present neurological impairments, brittle hair, and short stature, but do not show photosensitivity.

Werner syndrome (WS) A rare progeroid autosomal recessive disease defined by mutations in WRN gene. Clinical manifestations are aging-like symptoms such as diabetes mellitus type-2, osteoporosis, and cataracts, among others. The affected individuals are also more susceptible to develop cancers (especially sarcomas) and cardiovascular diseases.

Xeroderma pigmentosum (XP) Autosomal recessive genetic disease characterized by an intrinsic inability of the global-genome nucleotide excision repair machinery to remove DNA lesions. Individuals affected by this disease present with high predisposition of skin cancers, skin hyperpigmentation, and, in some cases, can develop neurological impairments, progeria, and cataracts.

LIST OF ACRONYMS AND ABBREVIATIONS

53BP1 P53-binding protein
6,4-PP 6,4-Photoproducts
ALT Alternative lengthening of telomeres
AT Ataxia telangiectasia
ATM Ataxia telangiectasia mutated
ATR Rad3-related protein
BER Base excision repair
BGS Baler–Gerold syndrome
BLM/RECQL3 Bloom syndrome, RecQ helicase-like
BRCA1 Breast cancer gene 1
BS Bloom syndrome
CHK2 Checkpoint kinase 2
CIN Chromosome instability
CPD Cyclobutane pyrimidine dimmers
COFS Cerebro-oculo-facial-skeletal syndrome
CSA Cockayne syndrome WD repeat protein CSA
CSB Cockayne syndrome protein CSB

CS Cockayne syndrome
CUL4A Cullin 4A
DDB1 and DDB2 Damage-specific DNA-binding protein 1 and 2
DNA-PK DNA-dependent protein kinase
DNA Lig DNA ligase IV
DNMT1 DNA methyltransferase
DNA Pol DNA polymerase (delta)
DSB Double-strand break
E2F E2F transcription factor
ERCC1 Excision repair cross-complementation group 1
FA Fanconi anemia
FANC family Fanconi anemia complementation group (composed by many proteins)
FEN1 Flap structure-specific endonuclease 1
GCN5 Histone acetyltransferase GCN5
GG-NER Global genome-nucleotide excision repair
H2AFX H2A histone family, member X
H2BK123 Lysine 123 of histone H2B
H3K4 Lysine 4 of histone H3
H3K9 Lysine 20 of histone H3
H3K79 Lysine 79 of histone H3
H4K20 Lysine 20 of histone H4
HAT Histone acetyltransferase
HDAC1 and 2 Histones deacetylase 1 and 2
HDM Histone demethylase
HGPS Hutchinson–Gilford progeria syndrome
HMT Histone methyltransferase
HP1 Heterochromatin protein 1
HR Homologous recombination
HR23B/RAD23B XP-C repair complementing protein
ICLR Interstrand cross-link repair
ING2 Inhibitor of growth family, member 2
KAP1 KRAB-associated protein 1
KU70 and KU80 X-ray repair cross-complementing protein 6 and 5
LP-BER Long-patch base-excision repair
LMNA Lamin
MDC1 Mediator of DNA-damage checkpoint
MMR Mismatch repair
MRE11 Meiotic recombination 11 homolog 1
MSI Microsatellite instability
NBS1 Nijmegen breakage syndrome 1
NEIL1 Nei endonuclease VIII-like 1 (*Escherichia coli*)
NER Nucleotide excision repair
NHEJ Nonhomologous end joining
NTH1 Nth endonuclease III-like 1
P53 Tumor protein p53
P300 E1A-binding protein P300
PCNA Proliferating cell nuclear antigen
PIKK Phosphoinositide 3-kinase(PI3K)-related protein kinase family
POT1 Protection of telomere 1
PR-Set7 Lysine N-methyltransferase
RAD51 RAD51 recombinase
RAD52 RAD52 homolog (*Saccharomyces cerevisiae*)
RAD53 Serine/threonine-protein kinase RAD53
RAPADILINO *RA*dial hypoplasia, *P*atella hypoplasia and cleft or *A*rched palate, *DI*arrhea and dislocated joints, *LI*ttle size and limb malformation, slender *N*ose and n*O*rmal intelligence syndrome
RB Retinoblastoma tumor suppressor protein
RCF Replication Factor C
RECQL4 RecQ protein-like 4
RMI1/RMI2 RecQ-mediated genome instability 1 and 2
RNA Pol RNA polymerase II

Rnf20/Rnf40 Ring finger protein 20/40 complex
RNF8 Ring finger protein 8
ROC1 Regulator of cullins 1
ROS Reactive oxygen species
RPA Replication protein A
RTS Rothmund–Thomson syndrome
SIN3A SIN3 transcription regulator family member A
SIN3B SIN3 transcription regulator family member B
ssDNA Single-stranded DNA
TC-NER Transcription-coupled-nucleotide excision repair
TFIIH Transcription factor II human
TRF1 and 2 Telomeric repeat binding factor 1 and 2
TTD Trichothiodystrophy
UV Ultraviolet light
WRN/RECQL2 Werner syndrome, RecQ helicase like
WS Werner syndrome
XP Xeroderma pigmentosum
XPA to XPG Xeroderma pigmentosum complementation group A to G
XAB2 XPA-binding protein 2
XRCC4 X-ray repair cross-complementing protein 4

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Anexo II

Curriculum vitae

Kendi Nishino Miyamoto

Curriculum Vitae

Novembro/2018

Kendi Nishino Miyamoto

Curriculum Vitae

Nome civil

Nome: Kendi Nishino Miyamoto

Dados pessoais

Filiação: Norio Miyamoto e Kyoko Nishino Miyamoto
Nascimento: 09/02/1987 - Santa Maria/RS - Brasil

Endereço residencial Rua São Manoel 1191, ap 404
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Endereço profissional Universidade Federal do Rio Grande do Sul, Centro de Biotecnologia
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Formação acadêmica/titulação

2013 – 2018 Doutorado em Biologia Celular e Molecular.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título: Análise de redes de coexpressão gênica de pacientes Leucemia Mielóide Aguda em amostras de sangue e medula óssea
Orientador: Diego Bonatto
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico

2011 - 2013 Mestrado em Biologia Celular e Molecular.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título: Análise proteômica comparativa de Listeria monocytogenes exposta a concentrações subletais de nisina, Ano de obtenção: 2013
Orientador: Adriano Brandelli
Co-orientador: Henrique Bunselmeyer Ferreira
Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

2007 - 2011 Graduação em Biomedicina.
Universidade Franciscana, UFN, Santa Maria, Brasil
Título: Identificação de sorovariedades de Salmonella sp. por diferentes metodologias
Orientador: Roberto Christ Vianna Santos

2006 Graduação interrompido(a) em Engenharia Civil.
Universidade Federal de Santa Maria, UFSM, Santa Maria, Brasil
Ano de interrupção: 2006

Formação complementar

2016 - 2016 Curso de curta duração
Genome Engineering 4.0. (Carga horária: 15h).
Broad Institute, BROAD, Cambridge, Estados Unidos

2015 - 2015 Curso de curta duração
2nd Annual Network Analysis Course. (Carga horária: 30h).
University of California, Los Angeles, UCLA, Los Angeles, Estados Unidos

2015 - 2015 Curso de curta duração
Introdução a Linguagem Python. (Carga horária: 15h).
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

2014 - 2014 Curso de curta duração
Introduction to transcriptional network analysis. (Carga horária: 15h).
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

2010 - 2010 Curso de curta duração
Resistência Bacteriana de impacto clínico. (Carga horária: 5h).
Universidade Feevale, FEEVALE, Novo Hamburgo, Brasil

1998 - 2001 Inglês Básico. .
Curso de idiomas Wizard, WIZARD, Brasil

Atuação profissional

1. Universidade Federal do Rio Grande do Sul - UFRGS

Vínculo institucional

2013 - Atual - Vínculo: Bolsista , Enquadramento funcional: Bolsista de doutorado CAPES, Regime: Dedicação exclusiva

2011 - 2013 - Vínculo: Estudante de Mestrado, Enquadramento funcional: Bolsista - CAPES/REUNI, Carga horária: 40, Regime: Dedicação exclusiva

2. Universidade Franciscana - UFN

Vínculo institucional

2010 - 2010 - Vínculo: Estagiário em Pesquisa no Mestrado em Nanociências, Enquadramento funcional: Pesquisa , Carga horária: 20, Regime: Parcial
2010 - 2010 - Vínculo: Estágio de pesquisa, Enquadramento funcional: Iniciação Científica em Microbiologia Clínica , Carga horária: 20, Regime: Parcial
2009 - 2010 - Vínculo: Monitoria em microbiologia clínica, Enquadramento funcional: Monitor em Microbiologia Clínica , Carga horária: 10, Regime: Parcial
2007 - 2007 - Vínculo: Tutoria em química básica, Enquadramento funcional: Tutor de Química Básica , Carga horária: 10, Regime: Parcial

3. Hospital Casa de Saúde - HCS

Vínculo institucional

2010 - 2011 - Vínculo: Estagiário , Enquadramento funcional: Estágio em Análises Clínicas , Carga horária: 30, Regime: Parcial

Revisor de periódico

1. REVISTA DE INFORMÁTICA TEÓRICA E APLICADA: RITA

Vínculo

2017 - Atual - Regime: Parcial

2. Disciplinarum Scientia. Série Ciências da Saúde

Vínculo

2013 - Atual - Regime: Parcial

Áreas de atuação

1. Biologia de Sistemas
2. Genética Humana e Médica
3. Cancerologia
4. Transcriptomica
5. Proteomics
6. Bacteriologia

Idiomas

Inglês: Compreende Bem , Fala Bem , Escreve Bem , Lê Bem

Espanhol: Compreende Bem , Fala Razoavelmente , Escreve Pouco , Lê Bem

Português: Compreende Bem , Fala Bem , Escreve Bem , Lê Bem

Prêmios e títulos

2017: Aluno Gabriel Baldissera - Destaque na sessão de bioinformática do XIX Salão de Iniciação Científica da UFRGS, com indicação ao prêmio jovem pesquisador, UFRGS

2016: Aluno Gabriel Baldissera - Destaque na sessão de bioinformática do XVIII Salão de Iniciação Científica da UFRGS, com indicação ao prêmio jovem pesquisador, UFRGS

2015: Aluno Gabriel Baldissera - Destaque na sessão de bioinformática do XVII Salão de Iniciação Científica da UFRGS, com indicação ao prêmio jovem pesquisador, UFRGS

Producão

Produção bibliográfica

Artigos completos publicados em periódicos

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Comparative proteomic analysis of Listeria monocytogenes ATCC 7644 exposed to a sublethal concentration of nisin. Journal of Proteomics (Print). , v.119, p.230 - 237, 2015.

2. **MIYAMOTO, KENDI NISHINO**; MARIANTE MONTEIRO, KARINA; DA SILVA CAUMO, KARIN; RODRIGUES LORENZATTO, KARINA; BUNSELMEYER FERREIRA, HENRIQUE; BRANDELLI, ADRIANO
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4. GARCIA, L. F. M.; VARINI FILHO, C. A.; **MIYAMOTO, K. N.**; OLIVEIRA, L. G. R.; NASCIMENTO, K.; CAPELETO, D. M.; SILVEIRA, R. A.; SCHUH, G. M.
Prevalência de anemia em crianças de 0 a 12 anos em uma unidade de pronto atendimento em Santa Maria - RS. DISCIPLINARUM SCIENTIA. , v.12, p.1 - , 2011.

5. DELIBERALI, B.; MIYAMOTO, K. N.; WINCKLER NETO, C. H. D. P.; PULCINELLI, R.; AQUINO, A. R. C.; SANTOS, R. C. V.
Prevalência de Bacilos Gram Negativos Não-Fermentadores de Pacientes Internados em Porto Alegre-RS. Jornal Brasileiro de Patologia e Medicina Laboratorial (Impresso). , v.47, p.529 - 534, 2011.

Capítulos de livros publicados

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Human Diseases Associated With Genome Instability In: Genome Stability.1 ed. : Elsevier, 2016, p. 447-462.

Trabalhos publicados em anais de eventos (completo)

1. OLIVEIRA, D. C.; MIYAMOTO, K. N.; ZANETTE, R. A.; DA SILVA, A.; ROSSATO, L.; DA ROCHA, M. P.; HERRMANN, G. P.; NEVES, V. L.; ALVES, S. H.
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II Simpósio Internacional de Microbiologia Clínica. , 2010.

Trabalhos publicados em anais de eventos (resumo expandido)

1. BALDISSERA, G.; **MIYAMOTO, K.N.**; BONATTO, D.
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Anais da 2a Escola Gaúcha de Bioinformática. , 2017.

Apresentação de trabalho e palestra

1. **MIYAMOTO, K. N.**; BONATTO, D.
Acute Myeloid Leukemia gene co-expression networks and differential expression analysis in blood and bone marrow samples,, 2016. (Congresso,Apresentação de Trabalho)

2. BALDISSERA, G.; **MIYAMOTO, K. N.**; BONATTO, D.
Influence of a high-fat diet in the cerebellar tissue of Cockayne Syndrome mice, 2016. (Congresso,Apresentação de Trabalho)

3. MARTINS, F. P.; MIYAMOTO, K. N.; GARCIA, L. F. M.; BORIN, D. B.; VIEIRA, G. B.; ZAGO, A. M.; SANTOS, R. C. V.
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