Universidade Federal do Triângulo Mineiro

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Análise da resposta imune citotóxica de linfócitos T CD8+ e células NK de pacientes com linfoma não-Hodgkin e mieloma múltiplo

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Orientador: Prof. Dr. Helio Moraes de Souza
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DEDICATÓRIA

Aos meus pais Antônio (*in memoriam*) e Abadia que me inspiraram e sempre incentivaram a busca pelo conhecimento.

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“A verdadeira viagem de descobrimento não consiste em procurar novas paisagens, mas em ter novos olhos”. (Marcel Proust)

RESUMO

Os linfócitos T citotóxicos (LTCs) e células *natural killer* (NK) são responsáveis pela defesa contra células tumorais e compartilham a via de morte mediada por perforina e granzimas como um dos seus mecanismos citotóxicos principais. Este estudo teve como objetivo identificar polimorfismos no gene da perforina (*PRF1)* e quantificar os níveis de expressão gênica e proteica de perforina e granzima B em pacientes com linfoma não-Hodgkin (LNH) e mieloma múltiplo (MM). Os resultados referentes à expressão destas proteínas foram correlacionados com a sobrevida dos pacientes e a análise funcional dos polimorfismos não-sinônimos em homozigose foi realizada. Em relação ao perfil linfocitário presente no sangue periférico, observamos que os pacientes com LNH apresentaram um maior número de LTCs ativados e que expressavam perforina, no entanto, a quantificação intracelular de perforina nas células NK foi significativamente reduzida em relação aos indivíduos saudáveis. Os pacientes com MM apresentaram um maior número de LTCs que expressavam perforina e granzima B, e, apesar de apresentarem aumento na porcentagem de células NK em comparação aos indivíduos saudáveis, não foram observadas diferenças em relação à quantidade de células NK que expressavam perforina e granzima B em pacientes com MM e indivíduos saudáveis. Não houve diferença significativa entre a frequência de polimorfismos na região codificadora do gene *PRF1* de pacientes com LNH e MM em comparação ao grupo controle, contudo, a maior expressão gênica de perforina e granzima B foi associada a maior sobrevida de pacientes com MM e LNH. Apesar de não diferir estatisticamente a frequência do polimorfismo A91V nos pacientes com MM foi mais do que o dobro da frequência encontrada nos indivíduos saudáveis. Contudo, acreditamos ser necessário um estudo futuro com maior número de pacientes para que se possa esclarecer melhor a associação deste polimorfismo com o MM. Apenas um paciente foi identificado como homozigoto de uma mutação não-sinônima; este foi diagnosticado com MM, era portador do polimorfismo A91V e suas células efetoras tiveram a capacidade significativamente reduzida de induzir a lise específica de uma linhagem tumoral. Também observamos que este paciente apresentava níveis intracelulares de perforina reduzidos nos grânulos das células NK. Considerando estes resultados, acreditamos que a expressão gênica de perforina e granzima B possam ser um potencial marcador prognóstico em LNH e MM.

Palavras-chave: Linfoma não Hodgkin. Mieloma múltiplo. Linfócitos T Citotóxicos. Células natural killer. Perforina.

ABSTRACT

Cytotoxic T lymphocytes (LTCs) and natural killer cells (NK) are responsible for defending against tumor cells and share the perforin and granzymes-mediated death pathway as one of their major cytotoxic mechanisms. This study aimed to identify polymorphisms in the perforin gene (*PRF1*) and to quantify perforin and granzyme B gene and protein expression levels in patients with non-Hodgkin's lymphoma (NHL) and multiple myeloma (MM).The results concerning the expression of these proteins were correlated with patient survival and the functional analysis of the non-synonymous polymorphisms in homozygotes was performed. We did not observe differences between the frequency of polymorphisms in the coding region of the PRF1 gene of patients with NHL and MM compared to healthy individuals. However, the greater gene expression of perforin and granzyme B was associated with longer survival of patients with MM and NHL. Regarding the lymphocyte profile in the peripheral blood, we observed that NHL patients had a higher number of activated CTLs that expressed perforin, however, the intracellular quantification of perforin in NK cells was significantly reduced compared to healthy individuals. MM patients had a higher number of perforin and granzyme B-expressing CTLs, and although there was an increase in the percentage of NK cells compared to healthy individuals, no differences were observed regarding the amount of NK cells expressing perforin and granzyme B in patients with MM and healthy individuals. There was no significant difference between the frequency of polymorphisms in the *PRF1* coding region of patients with NHL and MM compared to the control group; however, higher gene expression of perforin and granzyme B was associated with longer survival of patients with MM and NHL. Although not statistically different, the frequency of A91V polymorphism in MM patients was more than double the frequency found in healthy subjects. We believe that a future study with a larger number of patients is needed to better clarify the association of this polymorphism with MM.

Only one patient with MM was identified as homozygous for a non-synonymous mutation. This patient was carrying the A91V polymorphism and its effector cells showed significantly reduced ability to induce specific lysis of a K562 tumor line. We also observed that this patient has had reduced intracellular perforin levels in NK cell granules. Considering these results, we believe that the gene expression of perforin and granzyme B may be potential prognostic markers in NHL and MM.

Keywords: Non-Hodgkin's lymphoma. Multiple myeloma. Cytotoxic T lymphocytes. Natural killer cells. Perforin.

LISTA DE ILUSTRAÇÕES

INTRODUÇÃO

ARTIGO 1

ARTIGO 2

ARTIGO 3

LISTA DE ABREVIATURAS E SÍMBOLOS

APAF-1: fator-1 apoptótico ativador da peptidase

ATP: adenosina trifosfato

CAR: *Chimeric Antigen Receptor* – receptor de antígeno quimérico

ICAD: inibidor da caspase ativada DNase

IFN-ɣ: interferon-gamma

Ig: imunoglobulina

IL-10: interleucina 10

KIRs: *Killer Immunoglobulin-like Receptors*

LHF: linfohistiocitose hemofagocítica familiar

LNH: linfoma não-Hodgkin

LTC: linfócitos T citotóxicos

MHC: *major histocompatibility complex* - complexo de histocompatibilidade principal

MM: mieloma múltiplo

NK: *Natural Killer*

PARP: proteína poli ADP-ribose polimerase

STAT: transdutores de sinal e ativadores das proteínas de transcrição

TGF-B: fator de crescimento transformador-β

TNF: fator de necrose tumoral

Treg: T reguladora

SUMÁRIO

1 INTRODUÇÃO

Durante o século XX, o Brasil e o mundo passaram por profundas alterações em seus perfis epidemiológicos e demográficos. Concomitante ao declínio das taxas de mortalidade por doenças infecciosas houve um aumento substancial na expectativa de vida da população e na proporção de mortes por doenças crônicas. Estas transformações têm ocorrido ao longo das últimas décadas com padrões e intensidades distintas entre as regiões geográficas e os estratos sociais dos países (BARRETO E CARMO, 1995; OPAS, 2002).

No Brasil, desde o ano 2000, as neoplasias correspondem à segunda causa de morte, atrás apenas das doenças do aparelho circulatório e superam o total de óbitos por causas externas (MINISTÉRIO DA SAÚDE, 2016).

As altas taxas de incidência e de mortalidade por câncer no mundo no início do século XXI, somadas à expectativa de acréscimo nos seus valores para as próximas décadas apontam para a necessidade urgente de se intensificar pesquisas e ações para o melhor entendimento desta enfermidade e da resposta imune contra as células tumorais.

1.1 NEOPLASIAS LINFOPROLIFERATIVAS

As neoplasias hematológicas são um grupo de doenças que afetam principalmente a medula óssea, sangue periférico, linfonodos, baço e fígado. São categorizadas em leucemias, linfoma Hodgkin, linfoma não-Hodgkin (LNH) e mieloma múltiplo (MM) (SMITH et al., 2011).

O LNH e o MM são considerados neoplasias linfoides por acometerem linfócitos das linhagens T, B ou células NK que sofrem proliferação clonal decorrente da transformação maligna em um ponto específico do seu processo de maturação, acometendo células mais imaturas ou mais maduras, o que determinará o quadro clínico agudo ou crônico da doença. Dentre as linfoproliferações clonais agudas temos as leucemias linfoides agudas e os linfomas agressivos, que se originam de precursores linfoides primitivos. Já as linfoproliferações clonais crônicas derivam de linfócitos mais diferenciados e compreendem as leucemias linfoides crônicas, os linfomas indolentes e o MM (VERRASTRO et al., 2005).

O LNH abrange um espectro diverso de cânceres do sistema imune que são decorrentes da malignização de um linfócito localizado nos gânglios linfáticos ou linfonodos. É a malignidade hematológica mais comum no mundo, correspondendo a aproximadamente 5,1% de todos os cânceres (BOFFETTA, 2011).

A primeira descrição de tumores originados do tecido linfático foi realizada em 1832, por Thomas Hodgkin. Posteriormente, percebeu-se que alguns tumores dos tecidos linfáticos apresentavam características morfológicas diferentes da doença descrita por Hodgkin, recebendo então a denominação de LNH para distingui-los daqueles originariamente descritos (AISENBERG, 2000).

Conceitualmente, o LNH difere-se do linfoma de Hodgkin pela ausência das células mononucleares de Hodgkin e multinucleadas de Reed-Sternberg, por um menor número de células inflamatórias e por não possuir um tipo celular característico, com expressiva heterogeneidade morfológica, imunofenotípica e genética. Em aproximadamente 85% dos casos a célula maligna possui fenótipo de célula B, mas também pode apresentar fenótipo de célula T ou NK, o que é determinado por imunofenotipagem e/ou estudos de rearranjo gênico (VAN DER WAAL et al., 2005; ARMITAGE et al., 2017).

Os fatores de risco envolvidos no desenvolvimento do LNH são inespecíficos e não explicam a maioria dos casos. No entanto, estudos epidemiológicos apontam associação do LNH com distúrbios imunológicos (como artrite reumatoide, Síndrome de Sjögren e lúpus eritematoso sistêmico), infecções (HIV, Helicobacter pylori, Epstein-Barr e hepatite C), estilo de vida (tabagismo), genética (polimorfismos nos genes que codificam a IL-10 e TNF) e fatores ocupacionais (exposição a pesticidas, benzeno e derivados do petróleo) (ZINTZARAS et al. 2005; BOFFETTA 2011; MORTON et al. 2014; CERHAN et al. 2015).

O MM é uma doença causada pela proliferação desregulada e clonal de plasmócitos na medula óssea que produzem e secretam imunoglobulina (Ig) monoclonal anômala ou fragmentos dessa (MANGAN, 2005). Representa 1% das neoplasias malignas e 1% de todas as mortes por câncer nos países ocidentais, sendo a segunda neoplasia hematológica mais comum no mundo (aproximadamente 10% dos casos), atrás apenas dos linfomas (HUSSEIN et al., 2002). É ligeiramente mais frequente em homens e sua prevalência é maior a partir da quinta década de vida, com idade média de 65 anos ao diagnóstico. A sobrevida média dos indivíduos acometidos é de 6 a 7 anos, mas pode variar bastante de acordo com a carga tumoral, anormalidades citogenéticas e resposta terapêutica (KYLE et al., 2003; RAJKUMAR, 2016).

As consequências fisiopatológicas e as manifestações clínicas ocorrem em decorrência da infiltração de órgãos por plasmócitos neoplásicos, principalmente os ossos, pela produção de imunoglobulinas em excesso e pela supressão da imunidade normal. Estes processos levarão à destruição óssea, falência renal, supressão da hematopoiese e maior risco de infecções (KYLE et al., 2003).

A disfunção imune em pacientes com MM é causada por diversos fatores e inclui a disfunção de células dendríticas induzida por TGF-β (BROWN et al., 2001), desequilíbrio entre a proporção de células T reguladoras e células T helper 17 (FAVALORO et al., 2014), geração de células Treg adquiridas por trogocitose (BROWN et al., 2012) e aumento do número de células supressoras derivadas da linhagem mielóide (FAVALORO et al., 2014b)

Além disso, os clones de células T em pacientes com MM podem constituir até 50% de todos os linfócitos encontrados no sangue periférico e *in vitro* podem ser considerados hipo-responsivos (BRYANT et al., 2013). No entanto, sua presença está relacionada a maior sobrevida de pacientes com MM e a superação da hiporresponsividade dessas células é hoje uma estratégia de imunoterapia promissora que busca reestabelecer a resposta imune do paciente com MM (SUEN et al., 2016).

1.2 RESPOSTA IMUNE MEDIADA POR GRÂNULOS CONTRA AS CÉLULAS TUMORAIS

Os linfócitos T citotóxicos (LTC) e células natural killer (NK) são linfócitos efetores que apresentam mecanismos citotóxicos necessários para a defesa contra células tumorais ou infectadas por vírus. Ambos destroem seus alvos celulares por dois mecanismos que requerem contato direto entre a célula efetora e o alvo. No primeiro mecanismo, grânulos citoplasmáticos contendo proteínas citolíticas, predominantemente uma proteína disruptora de membrana denominada perforina e uma família de serino-proteases (granzimas), são secretadas por exocitose e juntas induzem a apoptose da célula-alvo (SMYTH E TRAPANI, 1995). O segundo mecanismo envolve a ligação de receptores de morte da célula-alvo, como Fas (CD95), com seu ligante (FasL) na membrana da célula efetora, resultando na apoptose através da via das caspases clássica (Figura 1) (VAN ELSAS et al., 2001).

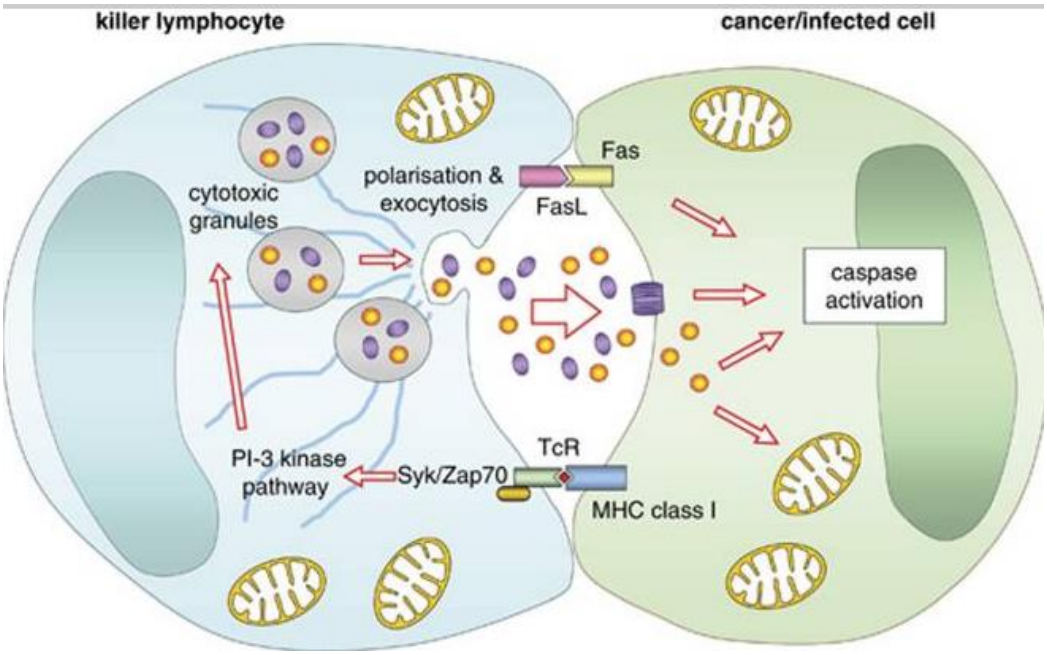


Figura 1. Mecanismos de ação do linfócito citotóxico contra uma célula tumoral.
Fonte: TRAPANI, 2012.

O processo de liberação dos grânulos proteicos citotóxicos se inicia quando os receptores de antígenos das células efetoras (LTC e células NK) reconhecem os peptídeos associados ao complexo de histocompatibilidade principal (MHC) na célula-alvo. O citoesqueleto destas células é reorganizado de tal modo que o centro de organização dos microtúbulos é deslocado para a área próxima do contato com a célula-alvo. Então, os grânulos do citoplasma concentram-se nessa região e a membrana do grânulo funde-se com a membrana plasmática. A fusão da membrana resulta em exocitose do conteúdo dos grânulos, perforina e granzimas, em uma região que é denominada sinapse imune (TRAPANI et al., 2006).

O gene *PRF1*, que codifica a perforina, está localizado no cromossomo humano 10q22 e tem um tamanho aproximado de 6.000 pares de bases (pb) (FINK et al., 1992). É constituído por três éxons e dois íntrons, sendo que apenas o segundo e o terceiro éxon codificam os aminoácidos que irão compor a proteína ativa (LICHTENHELD e PODACK, 1989). A perforina foi descrita pela primeira vez em 1984 e caracterizada como uma proteína citolítica formadora de poros, com massa molecular aproximada de 67 KDa (PODACK e KONIGSBERG, 1984).

No interior dos grânulos linfocitários, a perforina permanece armazenada na forma de monômeros e quando é liberada na fenda sináptica, os monômeros entram em contato com altas concentrações de cálcio extracelulares e sofrem polimerização (KAGI et al., 1994;

LOWIN et al., 1994). Esta polimerização ocorre preferencialmente na membrana plasmática da célula alvo, onde a perforina polimerizada forma um poro de 5 a 20nm. Estudos apontam quatro mecanismos de deflagração de morte celular orquestrados pela perforina; o primeiro mecanismo descrito na década de 1980 mostra a perforina como molécula lítica capaz de formar poros que levam a célula alvo ao colapso osmótico (Figura 2A); com o passar dos anos, o segundo mecanismo descrito mostrou que os poros formados pela perforina também permitiam a difusão passiva de moléculas de granzimas para o citoplasma da células alvo, onde estas proteínas pró-apoptóticas poderiam acessar seu substrato (caspases) (Figura 2B); o terceiro mecanismo coloca o receptor de manose 6-fosfato como responsável pela internalização de granzima B - no entanto, esta proteína é endocitada, sendo necessário pequenas doses de perforina que atuarão em sinergismo com granzimas e induzirão a apoptose da células alvo (Figura 2C); o quarto mecanismo propõe a formação de um complexo macromolecular, onde se associam perforina, granzima B e serglicina para serem endocitados através do receptor de manose-6-fosfato (Figura 2D) (TRAPANI e SMYTH, 2002).

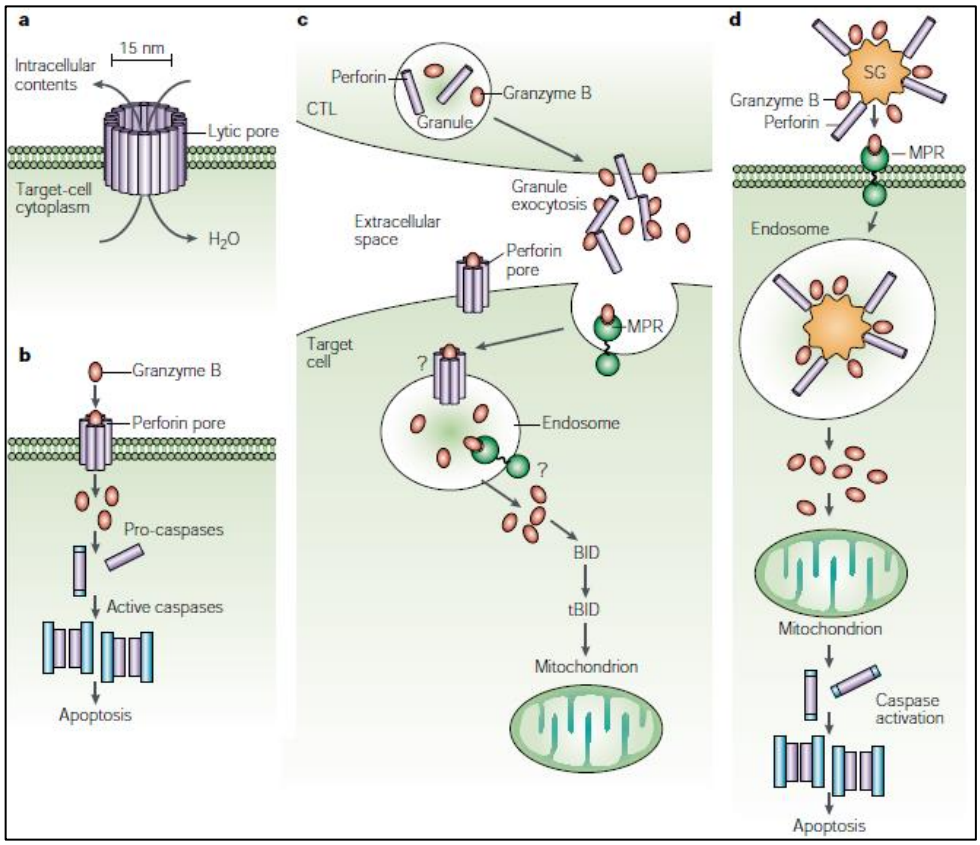


Figura 2: Mecanismos de morte celular orquestrados pela perforina.
Fonte: Trapani & Smyth 2002.

As granzimas compreendem uma família de serino proteases que permanecem armazenadas na forma ativa, em grânulos citoplasmáticos de LTCs e células NK (MCGUIRE et al., 1993). Estas proteínas são caracterizadas por uma tríade catalítica His-Asp-Ser e em humanos foram subdivididas em cinco tipos, A, B, H, K e M (KAM et al., 2000).

A granzima B, codificada pelo gene *GZMB* é o subtipo mais abundante em LTCs e células NK e atua como uma potente molécula pró-apoptótica, principalmente por sua capacidade em clivar resíduos de ácido aspártico. Esta proteína induz a rápida fragmentação do DNA e condensação da cromatina na célula-alvo (Figura 3) (STASSI e DE MARIA, 2002).

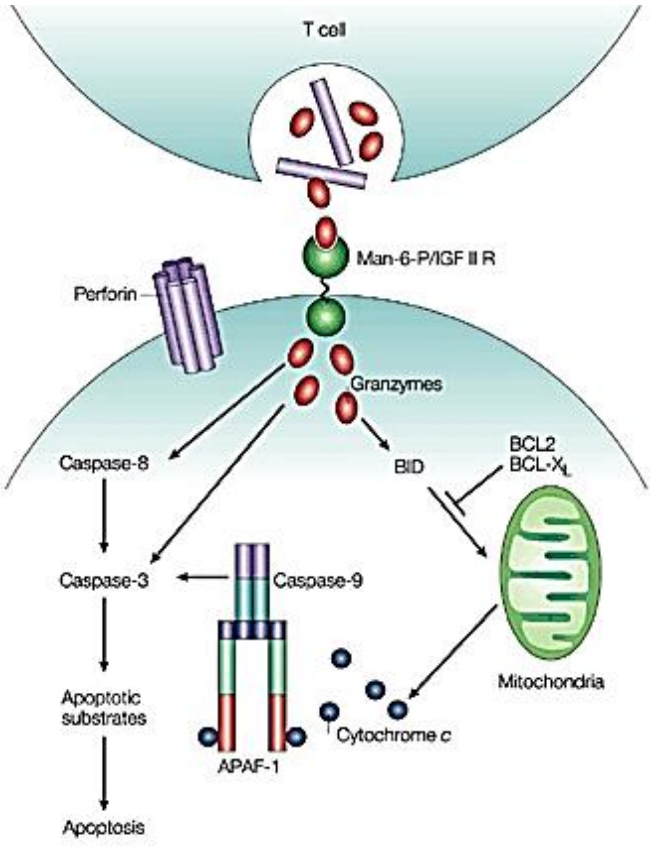


Figura 3: Indução de morte por apoptose mediada pela granzima B.
Fonte: STASSI & DE MARIA. 2002

Posteriormente, foi demonstrado que a granzima B age no interior da célula induzindo a morte celular por apoptose através de duas vias diferentes, a via caspase dependente (DARMON et al., 1995) e a via caspase independente (HEIBEIN et al., 1999).

Na via caspase dependente, a granzima B pode clivar as pró-caspases-3 e -7 resultando na formação de caspases ativas. Uma vez ativa, a caspase-3 pode clivar a proteína

poli ADP-ribose polimerase (PARP) e o inibidor da caspase ativada DNase (ICAD), levando à fragmentação do DNA e apoptose (DARMON et al., 1995).

Na via caspase independente, a molécula Bid é clivada proteoliticamente em gtBid, que resulta em sua translocação para a membrana externa mitocondrial e na oligomerização de moléculas Bax/Bak, facilitando a liberação do citocromo c e a formação de um complexo de alto-peso molecular (BARRY et al., 2000). Este complexo contendo citocromo c, fator-1 apoptótico ativador da peptidase (APAF-1) e caspase-9, é denominado apoptossoma e, na presença de adenosina trifosfato (ATP) induz a formação da caspase-9 ativa, que por sua vez ativa as pró-caspases executoras -3, -6 e -7 desencadeando o processo apoptótico (BLEACKLEY e HEIBEIN, 2001). Assim, a apoptose mediada por granzima B é dependente do processamento proteolítico de caspase-3 e da permeabilização da membrana mitocondrial externa (ADRAIN et al., 2005).

Estudos em camundongos deficientes de granzima B demonstraram uma susceptibilidade aumentada para citomegalovírus e *Ectromelia poxvirus* (MULLBACHER et al., 1999), além de uma capacidade reduzida, mas não nula, para eliminar tumores alogênicos transplantados (SHRESTA et al., 1999; REVELL et al., 2005). No entanto, em vários outros modelos, camundongos nocautes para o gene *GZMB* apresentam citotoxicidade residual e continuam a ser competentes na eliminação de tumores (VOSKOBOINIK et al., 2015).

Ao contrário do que foi observado em modelos animais deficientes de GZMB, estudos em camundongos nocaute para o gene *PRF1* demonstraram claramente que a perforina está intimamente envolvida na defesa contra patógenos virais e células transformadas. Estes animais perdem a função citotóxica dos LTCs e células NK e se tornam susceptíveis a vários patógenos. Além disso, foram até 1.000 vezes mais susceptíveis que animais imunocompetentes para malignidades transplantadas e induzidas, predominantemente as de origem hematológica (VAN DEN BROEK et al., 1996; SMYTH et al., 2000). Destaca-se o fato de que os linfomas de células B desenvolvidos espontaneamente em camundongos nocaute para o gene da perforina foram transplantados e rejeitados avidamente em camundongos nocaute para o gene da granzima A e B (SMYTH et al., 2003). Em humanos, enquanto a deficiência congênita de perforina está bem caracterizada, nenhuma doença é relacionada à deficiência de granzima B (VOSKOBOINIK et al., 2015).

Mutações no gene *PRF1* foram inicialmente associadas à linfohistiocitose hemofagocítica familiar (LHF) (STEPP et al., 1999), uma doença rara e rapidamente fatal que se manifesta nas primeiras décadas de vida, caracterizada pela incapacidade de LTC ativados eliminar os alvos em células apresentadoras de antígeno e pela ativação e proliferação

excessiva de linfócitos e macrófagos. Foi também constatado que pacientes com LHF com mutações bialélicas no gene *PRF1* apresentam grave comprometimento da atividade citotóxica de LTC e células NK (GORANSDOTTER et al., 2001).

A combinação de alterações genéticas, como mutação no gene da perforina associada à mutação no gene Fas, foi relatada inicialmente por Clementi e colaboradores (2004). O caso descrito foi de um indivíduo com 27 anos de idade e diagnóstico de síndrome linfoproliferativa auto-imune e linfoma de grandes células B, com duas mutações heterozigóticas, uma no gene da perforina herdada da mãe, e outra no gene Fas herdada do pai. O quadro do paciente progrediu com curso agressivo e inesperado da doença evoluindo ao óbito (CLEMENTI et al., 2004).

Em 2005, Clementi e colaboradores investigaram mutações no gene da perforina em vinte e nove pacientes diagnosticados com linfoma, com evidências de hemofagocitose ou com curso agressivo da doença. Destes, quatro apresentavam mutações bialélicas e outros quatro mutações monoalélicas. Assim, levantou-se a seguinte questão: mutações no gene da perforina poderiam atuar como fatores de risco hereditário que, associado a outras variações genéticas (somáticas ou constitucionais) e/ou na presença de fatores ambientais, poderiam predispor ao desenvolvimento de linfomas? (CLEMENTI et al., 2005)

Em 2007, Cannella e colaboradores pesquisaram mutações no gene da perforina em pacientes com linfoma anaplásico de grandes células. O autor investigou 44 pacientes por sequenciamento direto do gene da perforina e 400 indivíduos saudáveis. De forma global, a incidência de mutações no gene da perforina foi significantemente maior em pacientes com linfoma. A substituição C272T, que resulta na mudança de aminoácido A91V, foi alteração mais frequente nestes pacientes.

Por outro lado, a alteração A91V no gene *PRF1* também é a variante mais comum encontrada na população caucasiana, relatada em uma frequência entre 3% e 17% nestes indivíduos, considerada relativamente alta. Devido a este fato, estudos sugeriram durante algum tempo que A91V seria um polimorfismo neutro. No entanto, através de estudos experimentais foi verificado que este polimorfismo afeta a estrutura tridimensional da proteína e sua estabilidade na célula efetora e como resultado reduz grandemente a sua atividade citolítica intrínseca (BRENNAM *et al*. 2010). Somadas as controvérsias em relação ao polimorfismo A91V, outros estudos não encontraram associação entre polimorfismos no gene *PRF1* e o aumento da susceptibilidade a diversos tipos de câncer, incluindo as neoplasias hematológicas (MEHTA et al. 2006, TRAPANI *et al*. 2013)

Apesar da falta de consenso entre os autores citados acima, Voskoboinik e Trapani (2013) usaram pela primeira vez o termo perforinopatias para denotar uma ampla gama de manifestações clínicas causadas pela deficiência funcional de perforina. Asim, as perforinopatias agudas e frequentemente fatais, cursariam com manisfestação precoce na infância e seria resultado da desregulação da homestase imune, levando à hipercitocinemia pela estimulação crônica de linfócitos T CD4+, T CD8+ e células NK. Já as manifestações crônicas ocorreriam na idade adulta e seriam decorrentes principalmente da ineficiência em eliminar células pré-malignas, sendo as neoplasias hematológicas ou a associação entre mais de um câncer primário um resultado comum da deficiência de perforina (VOSKOBOINIK e TRAPANI 2013; TRAPANI et al., 2013).

Na literatura há apenas um estudo sobre polimorfismos do gene *PRF1* em pacientes com MM. Este trabalho, realizado anteriormente por nosso grupo de pesquisa, investigou a presença do polimorfismo -398C/T na região promotora do gene *PRF1* em pacientes com neoplasias hematológicas e não encontrou diferença significativa entre a frequência desta alteração em pacientes e indivíduos saudáveis (GARCIA et al., 2011).

Apesar de mutações no gene da perforina ainda não terem sido associadas ao desenvolvimento do MM, o papel chave da perforina na resposta imune contra esta enfermidade já é bem conhecido. Estudo realizado em modelo animal mostrou que a resposta imune contra uma linhagem celular de MM transplantada foi mediada principalmente por células NK e linfócitos T CD8+ através das vias de perforina e interferon-gama (IFN-ɣ) (GUILLEREY et al., 2015). Ainda neste sentido, um estudo sobre imunoterapia com LTCs avaliou o dano causado pela perforina purificada na membrana de células tumorais e observou que os plasmócitos do MM são altamente susceptíveis à lise mediada por essa proteína (AZUMA et al., 2004).

A despeito dos diversos mecanismos utilizados pelas células de defesa, sabemos que as células tumorais possuem mecanismos de escape com o objetivo de evasão do sistema imune. Como exemplo podemos citar a inibição da atividade de célula NK através da expressão de receptores KIRs (Killer Immunoglobulin-like Receptors) inibitórios, como p70 (KIR3DL/1) e p140 (KIR3DL/2). Estes receptores foram identificados anteriormente em linfomas cutâneos e atuam inibindo seletivamente as células NK (KAMARASHEV et al., 2001). Da mesma forma, moléculas inibitórias como PD-L1, CTLA4 e TIM-3 podem causar a ineficiência de LTCs impedindo o processo de ativação e contribuindo para a anergia e hiporresponsividade das células citotóxicas, como já observado em tumores sólidos, linfomas

e leucemias mielóide (YANG et al. 2012, SPRANGER et al. 2013, MIYOSHI et al. 2016, CHEN et al. 2017, REN et al. 2018, , ZHAO et al. 2018)

1.3 JUSTIFICATIVA

Apesar do grande número de trabalhos já publicados avaliando a associação de polimorfismos na região codificadora do gene *PRF1* com linfomas, nada encontramos sobre esta associação com o MM.

Em trabalho preliminar realizado por nosso grupo de pesquisa, foi realizada a análise da região promotora do gene *PRF1* em uma coorte de pacientes com diversas neoplasias hematológicas e verificamos que a frequência do polimorfismo -398C/T no mieloma múltiplo foi uma das mais elevadas dentre as doenças estudadas (GARCIA et al., 2011).

Na literatura encontramos resultados contraditórios de trabalhos que visam esclarecer o papel da perforina na imunovigilância e desenvolvimento de neoplasias linfoproliferativas (ZUR et al., 2004; SANTORO et al., 2005; MEHTA et al., 2006; CETICA et al., 2016)

Assim, é de suma importância a análise da estrutura de genes, transcritos de RNA mensageiro e quantificação de proteínas intracelulares envolvidas na resposta imune e função LTCs e células NK. Estas são estratégias úteis que poderão auxiliar na compreensão dos mecanismos imunológicos de resposta à células tumorais e na influência de fatores genéticos envolvidos na patobiologia do LNH e MM.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Este estudo teve como objetivo a avaliação da resposta imune mediada por grânulos de LTCs e células NK, identificando os polimorfismos no gene da perforina (PRF1) e quantificando os níveis de expressão gênica e proteicos de perforina e granzima B em relação a sobrevida de pacientes com LNH e MM

2.2 OBJETIVOS ESPECÍFICOS

2.2.1 Objetivos específicos do artigo 1

1. Quantificar a expressão dos genes *PRF1* e *GZMB* em pacientes com LNH sem histórico de tratamento quimioterápico e indivíduos saudáveis.
2. Quantificar a produção intracelular de perforina e granzima B nas células NK e LTCs de pacientes com LNH sem histórico de tratamento quimioterápico e indivíduos saudáveis.
3. Correlacionar os níveis proteicos e de expressão gênica com a sobrevida dos pacientes com LNH.
4. Investigar a presença de polimorfismos na região codificadora do gene *PRF1* de pacientes com LNH e indivíduos saudáveis

2.2.2 Objetivos específicos do artigo 2

1. Investigar a presença de polimorfismos na região codificadora do gene *PRF1* de pacientes com MM e indivíduos saudáveis
2. Quantificar a expressão dos genes *PRF1* e *GZMB* em pacientes com MM sem histórico de tratamento quimioterápico e indivíduos saudáveis.
3. Quantificar a produção intracelular de perforina e granzima B nas células NK e LTCs de pacientes com MM sem histórico de tratamento quimioterápico e indivíduos saudáveis.
4. Correlacionar os níveis proteicos e de expressão gênica com a sobrevida dos pacientes com MM.

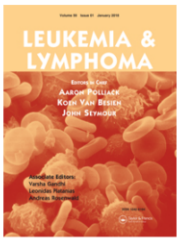
2.2.3 Objetivos específicos do artigo 3

1. Relatar o caso de um paciente com MM portador do polimorfismos A91V em homozigose.
2. Avaliar a capacidade dos linfócitos citotóxicos deste paciente em induzir a lise específica de uma linhagem tumoral.
3. Quantificar os níveis proteicos de perforina e granzima B nos LTCs e células NK deste paciente.
4. Quantificar a expressão dos genes PRF1, GZMB, FAS, STAT-1, STAT-3, STAT-5, T-bet e IFN-γ.

3 RESULTADOS

A seção de materiais e métodos, resultados e discussão serão apresentados sob a forma de três artigos científicos, produtos deste projeto de pesquisa.

3.1 ARTIGO 1



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Evaluation of the cytotoxic response mediated by perforin and granzyme B in patients with non-Hodgkin lymphoma

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ORIGINAL ARTICLE: RESEARCH

Evaluation of the cytotoxic response mediated by perforin and granzyme B in patients with non-Hodgkin lymphoma

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ABSTRACT

This study quantified the perforin and granzyme B in patients with non-Hodgkin lymphoma (NHL) at the time of diagnosis. Protein quantification was performed by flow cytometry. NHL patients had a higher number of cytotoxic T lymphocytes (CTLs) expressing perforin as well as a greater number of activated CTLs than the control group. However, intracellular perforin levels in natural killer cells were lower in the NHL patients compared to the control group. Quantitative real time PCR showed that patients had more expression of perforin and granzyme B transcripts compared to the control group. In addition, patients who had expression of both genes below the median found for the NHL group had lower survival rates. Considering this, we believe that perforin and granzyme B are potential prognostic markers in NHL and thus it is fundamental to pay attention to their expressions in these patients.

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KEYWORDS
Perforin; granzyme B; non-Hodgkin lymphoma

Introduction

Cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells are responsible for defense against tumor cells or cells infected by viruses. The main mechanism of death of cell targets is the release of cytoplasmic granules containing cytolytic proteins such as perforin and granzyme B secreted by exocytosis. Together they induce target cell death by rupturing the plasma membrane and/or by induction of apoptosis [1].

Deficiencies in the structure and production of these molecules have already been studied and correlated with the susceptibility to and onset of oncohematological diseases and hereditary inflammatory disorders [2–6]. Knockout mice for the *PRF1* gene had reduced cytotoxic function of their effector cells and were susceptible to various pathogens. Furthermore, they are up to 1000 times more susceptible to transplanted and induced malignancies (predominantly of hematological origin) than immunocompetent animals [7,8]. However, the most intriguing observation is that most knockout mice for perforin spontaneously developed highly aggressive and disseminated B-cell lymphoma [9].

Granzyme B-deficient mice demonstrated increased susceptibility to cytomegalovirus and Ectromelia poxvirus [10], as well as a reduced ability, albeit not complete inability, to eliminate allogeneic transplanted tumors [11,12]. In humans, while a congenital deficiency of perforin is well characterized, no disease has been linked to granzyme B deficiency [13].

Although there are studies that correlate the presence of mutations in the perforin gene with the development and severity of the disease, data on the role of these genetic alterations are still scarce, both in respect to the amount of mRNA expressed and the amount of protein produced. Changes in the quantity of these proteins could have direct consequences on immunological surveillance of tumors in humans. This study aimed to evaluate the expression of the genes encoding perforin and granzyme B, to perform intracellular quantification of these proteins in the cytoplasmic granules of CTLs and NK cells in patients diagnosed with non-Hodgkin lymphomas (NHL) as well as to investigate whether the presence and severity of the malignancy influences the production of these proteins.

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Table 1. Patients' characteristics.

Age	Gender	Histological subtypes	Ann Arbor	Clinical features	Clinical outcome	Survival (months)
71	F	Anaplastic large cell lymphoma	I	Progressive inguinal adenopathy Missing B symptoms	Complete Alive	28
68	M	MALT lymphoma	IV	Weight loss	No indication of treatment Alive	40
76	M	Left orbital MALT lymphoma	IE	Pruritus and hyperemia of the left eye exophthalmos	Loss of follow-up	N.I
61	F	MALT lymphoma	IIE	Hemifacial edema 4 years ago	Partial response Alive	35
49	M	Diffuse large B-cell lymphoma	IE	Epigastralgia with 3 years of duration Weight loss	Complete Alive	57
75	M	Diffuse large B-cell lymphoma	IVX	Abdominal pain for 4 months Weight loss	Complete Alive	39
31	M	Diffuse large B-cell lymphoma	I	Recent onset cervical adenopathy and fever	Complete Alive	36
70	F	Cutaneous T-cell lymphoma (Mycosis fungoides)	[a]	Disseminated and pruritic cutaneous lesions Progressive inguinal and axillary adenopathies	Death from refractory disease	9
57	M	Follicular lymphoma (3a grade)	IV	Axillary and cervical adenopathy Weight loss Night sweats	Death from disease progression to refractory diffuse large B-cell lymphoma	5
63	M	Mantle cell lymphoma	III	Splenomegaly during investigation of hyperferritinemia	Chemosensitive disease Death in autologous HSCT	15
83	F	Diffuse large B-cell lymphoma	IV	Abdominal distension with ascites Progressive supraclavicular and cervical adenopathy	Death from progressive disease	5
74	F	Diffuse large B-cell lymphoma, leg type	I	Vesicles on thighs that have become nodules	Death due to febrile neutropenia	3

N.I: not informed.
[a]Tumor-node-metastasis-blood (TNMB) staging system IVa.
When treated, patients received CHOP-like polychemotherapy and intrathecal chemotherapy when indicated, except patients 4 and 10 who were treated with FCR and Hyper-CVAD schemes, respectively.

Materials and methods

Participants

Twelve patients with NHL (seven male and five female; mean age 65 years) were included in this study (Table 1). All were attended in Clinics Hospital of Triângulo Mineiro Federal University and accepted to participate in this study before starting chemotherapy.

A control group was formed of 20 individuals with no history of neoplasms, or autoimmune or chronic infectious diseases (14 male and six female; mean age 61 years). The individuals of the two groups were matched for gender and age (p value $=.7026$ and .4367, respectively).

This research was approved by the Ethics Committee of the Federal University of Triângulo Mineiro (protocol # 2677) and all participants gave their written consent.

Real-time polymerase chain reaction

Gene expression was assessed using quantitative real-time polymerase chain reaction (qPCR). RNA was isolated from the peripheral blood using the QIAamp® Mini RNA Blood kit (Qiagen, Hilden, Germany) and then complementary DNA was generated by reverse transcription using the GoScript™ Reverse Transcription System (Promega, Madison, WI). The following inventoried TaqMan® assays (Applied Biosystems, Foster City, CA) were performed: perforin (Hs00169473_m1), granzyme B (Hs01554355_m1), and β-actin (Hs99999903_m1). The values were normalized using the endogenous β-actin gene and the levels of gene expression were calculated using the ΔΔ cycle threshold (ΔΔCT) method. The t-test was employed in the statistical analysis with differences in the p value of less than .05 being considered statistically significant.

To evaluate the impact of perforin and granzyme B deficiency on the survival of patients with NHL, a threshold value was established from the median of the group. The comparison between the survival curves of the two groups was performed using the log-rank test, with differences in the p value of less than .05 being considered statistically significant.

Flow cytometry

Quantification of the intracellular expression of perforin and granzyme B was performed by flow

cytometry using 2×10^5 peripheral blood mononuclear cells collected in a tube containing heparin and separated by the density gradient technique using Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden).

The labeling of CTLs and NK cells used CD3, CD8, CD56 antibodies (BD Pharmingen™, San Jose, CA). In addition, the CD69 marker was used to indicate T-cell activation. Fifty thousand events were performed on a FACSCalibur cytometer (Becton Dickinson, San Jose, CA) to count cells labeled with the perforin-PE (Clone δ G9) or Granzyme B-FITC (Clone GB11) intracellular antibodies (BD Pharmingen™, San Jose, CA) and to calculate the mean fluorescence intensity (MFI) of each antibody.

After verifying the sample distribution using the Kolmogorov–Smirnov test, the data were analyzed employing the *t*-test. Differences were considered statistically significant when the *p* value was less than .05.

Sequencing

In order to better understand the results from the quantification of the gene expression and intracellular expression of perforin, the region responsible for the active protein sequence (exons 2 and 3) of the *PRF1* gene (Gene ID: 5551) was coded. The amplification of the sequences to be investigated by polymerase chain reaction (PCR) was performed using primers specific to each exon (Invitrogen Life Technologies, São Paulo, Brazil), for exon 2, forward (F2) 5′-CCTTCCATGTG CCCTGATAATC-3′ and reverse (R2) 5′-GAAGCAGCCT CCAAGTTTGATTG-3′; and for exon 3, forward (F3) 5′-CAGTCCTAGTTCTGCCCACTTA-3′ and reverse (R3) 5′-CTAATGGGAATACGAAGACAGCC-3′; following the technique described by Martínez-Pomar et al. [14]. Sequencing was performed using the PCR products with the same amplification primers and the ABI Prism BigDye™ Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA) in an ABI Prism 3100xl DNA sequencer (Applied Biosystems, Foster City, CA) following the manufacturer's instructions.

Allele frequencies of the polymorphisms observed were compared between patients and controls using Fisher's exact test. Differences were considered statistically significant when the *p* value was less than .05.

Results

Gene expression

The results obtained by qPCR showed that the gene expressions of perforin and granzyme B were almost twice as high in patients with NHL compared to the control group (RQ = 1.8; *p* value = .0051 and RQ = 1.6; *p* value = .0076 – Figure 1).

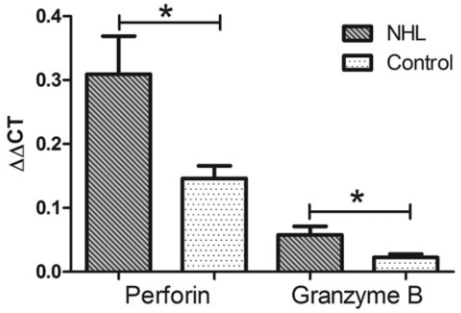

Figure 1. Perforin and granzyme B gene expression in patients with non-Hodgkin lymphoma and healthy individuals. Patients had significantly higher levels of perforin and granzyme B gene expression than the control group (*t*-test: perforin $p = .0051$ and granzima B $p = .0076$).

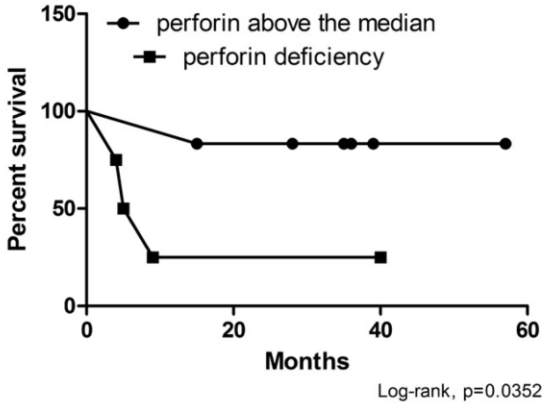

Figure 2. Survival of patients with non-Hodgkin lymphoma who had normal or deficient levels of perforin gene expression.

When survival curves were correlated with expression of the PRF1 and GZMB genes, it was noted that patients with perforin and granzyme B expression below the median had a poor survival ($p = .0352$, for both) (Figure 2).

Intracellular quantification

Cytometry showed that the number of CTLs (25.38% versus 21.86%; *p* value = .3507) and NK cells (14.30% versus 13.85%; *p* value = .8204) did not differ significantly between the patients with NHL and the controls. However, the number of CTLs expressing perforin was higher in the patients with NHL (63.55% versus 49.50%; *p* value = .0206 – Figure 3(A)).

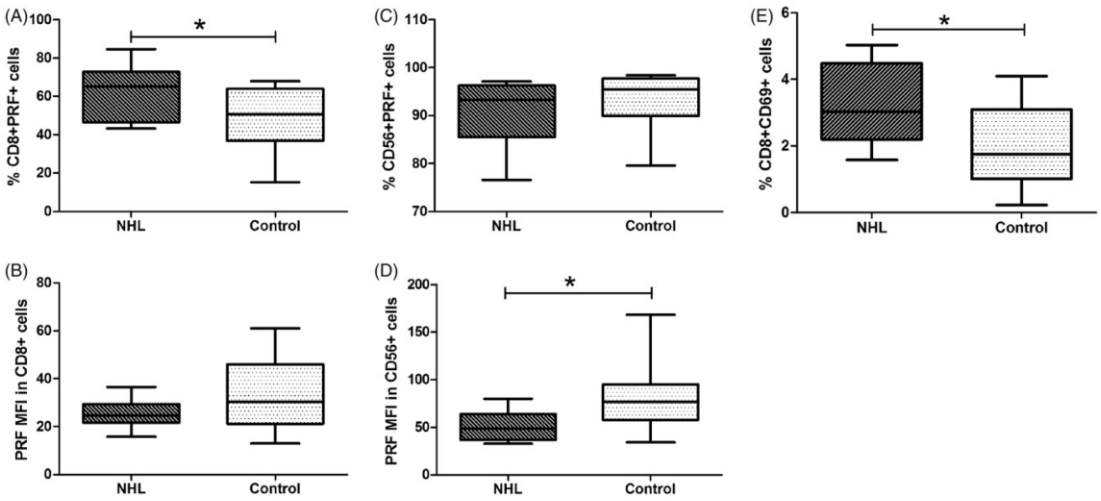

Figure 3. Intracellular quantification of perforin in CTLs and NK cells of patients with non-Hodgkin lymphoma and healthy individuals. (A) Percentage of CTLs expressing perforin. (B) Mean fluorescence intensity (MFI) of perforin in CTLs. (C) Percentage of NK cells expressing perforin. (D) Mean fluorescence intensity of perforin in NK cells. (E) Percentage of activated CTLs. The number of CTLs activated ($CD69^{+}$) and those expressing perforin was higher in the patients than the controls ($p = .01$ and .0206, respectively). The MFI was lower in NK cells of patients (50.37 versus. 82.19, $p = .0077$). CTLs: cytotoxic T lymphocytes; NK: natural killer.

Despite the greater number of CTLs expressing perforin in patients, the MFI of this protein did not differ significantly between the NHL and control groups (p value $= .1233$ – Figure 3(B)).

Furthermore, no difference was observed between the percentage of NK cells expressing perforin in the patients with NHL and the controls (p value $= .3025$ – Figure 3(C)). However, the amount of perforin expressed per cell, assessed using the MFI, was significantly lower in the NK cells of the patients with NHL (50.37 versus 82.19; p value $= .0077$ – Figure 3(D)).

No significant difference was found in the percentages of CTLs (51.71% versus 45.67%; p value $= .4165$) or NK cells (63.15% versus 66.30%; $p = .6129$) expressing granzyme B in the NHL patients and the controls. Additionally, the MFI of granzyme B in the CTLs and NK cells of the patients with NHL did not differ significantly from that of the control group (p value $= .5052$ and .3044, respectively).

Activation of the CTLs was measured by the co-expression of CD8 and CD69; the number of CTLs activated in the NHL patients (3.26%) was significantly higher than in the control group (1.9%; p value $= .01$ – Figure 3(E)).

Direct sequencing

Direct sequencing of the *PRF1* gene was performed in all patients and controls enrolled in this study to investigate possible causes of post-transcriptional changes of perforin. Four synonymous single nucleotide polymorphisms (SNPs) were found in exon 2, G96A (K32K), G435A (V145V), C444T (A148A), and G462A (A154A), as were two non-synonymous SNPs, G11A (R4H) and C272T (A91V), all in heterozygosis (Table 2). As for exon 3, two synonymous SNPs were found: C822T (A274A) and C900T (H300H) (Table 3). The allelic frequencies of all polymorphisms were similar between the NHL patients and the healthy individuals.

Discussion

In this study, we found that the expressions of the proteins that correlate with effector cytotoxic response differ between the NHL patients and the healthy individuals. It should be remembered that the patients were analyzed at the time of diagnosis, which excludes any interference of medications or therapies targeting their immune response. This allowed us to analyze them during the active phase of the disease, with the results found attributed exclusively to the response of the organism to the disease. In addition, the expression of perforin, a key protein in the induction of target cell death, differs between CTL and NK cells in the NHL patients with its production correlating to the aggressiveness of the disease.

We ascribed the greater gene expression of perforin and granzyme B in the NHL patients to the response of effector cells to tumor cells, that is, greater cell

Table 2. Allelic frequency of the polymorphisms found in exon 2 of the gene *PRF1*.

		Allelic frequency		
		Wild allele	Mutated allele	*p* Value
G11A R4H (rs35418374)	P	23/24 (0.96)	1/24 (0.04)	.37
	C	40/40 (1.0)	0/40 (0.0)	
G96A K32K (rs2228018)	P	23/24 (0.96)	1/24 (0.04)	.37
	C	40/40 (1.0)	0/40 (0.0)	
C272T A91V (rs35947132)	P	23/24 (0.96)	1/24 (0.04)	1.00
	C	38/40 (0.95)	2/40 (0.05)	
G435A V145V (rs115281140)	P	23/24 (0.96)	1/24 (0.04)	.37
	C	40/40 (1.0)	0/40 (0.0)	
C444T A148A (rs61737403)	P	23/24 (0.96)	1/24 (0.04)	.37
	C	40/40 (1.0)	0/40 (0.0)	
G462A A154A (rs116554195)	P	23/24 (0.96)	1/24 (0.04)	.37
	C	40/40 (1.0)	0/40 (0.0)	

Allele frequencies of the polymorphisms were compared using Fisher's exact test.
P: patients; C: controls.

Table 3. Allelic frequency of the polymorphisms found in exon 3 of the gene *PRF1*.

		Allelic frequency		
		Wild allele	Mutated allele	*p* Value
C822T A274A (rs885821)	P	20/24 (0.83)	4/24 (0.17)	.41
	C	37/40 (0.92)	3/40 (0.08)	
C900T H300H (rs885822)	P	21/24 (0.87)	3/24 (0.13)	1.0
	C	35/40 (0.87)	5/40 (0.13)	

Allele frequencies of the polymorphisms were compared using Fisher's exact test.
P: patients; C: controls.

activation and a consequent increase in the expression of these genes.

Some studies have shown that the expression of perforin can be used as an important prognostic biomarker. In cases of advanced-stage lung cancer, the larger number of CTLs expressing perforin was positively associated with increased survival [15] and the impairment of the immune response of patients with diffuse large B-cell lymphoma has been correlated with a decreased number of effector T lymphocytes and a worse prognosis [16]. We observed that the shorter survival of patients is clearly related to diminished gene expression of perforin and granzyme B, demonstrating that the impaired immune response in these individuals was responsible for the worse evolution of the disease.

Contrary to what we observed in the CTLs of NHL patients, the NK cells had a lower expression of perforin. We believe that despite the greater gene expression of perforin, the activity of NK cells against tumor cells would consume the protein present in the granule through degranulation of these cells, thus reducing the amount of stored perforin. In agreement with our research, other studies have also reported a reduction in the expression of perforin and granzyme B in effector cells of patients who evolved with NHL after lung transplantation [17] and in other types of neoplasia, such as squamous cell carcinoma of the head and neck [18].

On the other hand, NK cell activity can be modified in the tumor microenvironment by the expression of Killer Immunoglobulin-like Receptors (KIR), such as p70 and p140 that have been reported in cutaneous lymphomas [19]. The reduction in cytotoxicity and defective maturation of NK cells have been associated with hematological dysplasias and attributed to lower expressions of perforin and granzyme B and an increase in the expression of the KIR-NKG2A subtype [20]. Escape mechanisms similar to these could inhibit NK cells from interfering in the cytotoxic response.

In the literature, studies have reported that mRNA and perforin protein levels are not always associated in NK cells or in CTLs, with cytokine stimulation and cell activation being the main mechanisms affecting this balance [21,22]. Perforin expression seems to be regulated by transcriptional and post-transcriptional mechanisms [23]. Despite the mechanisms of activation, whether *via* the T cell receptor (TCR) or through stimulation of IL-12 and IL-2 cytokines that act to increase gene expression related to effector molecules, to date it has been demonstrated that only IL-2 can regulate gene expression after transcription, thus increasing the stability of perforin and granzyme B mRNA [24]. In the present study, it was not possible to correlate the gene expression with the intracellular quantification of perforin in NK cells because the quantification of transcripts was performed in whole blood. This is a limitation of the work and the association between the methodologies should be done cautiously. Despite this, the results of gene expression are clearly correlated to the shorter patient survival.

Non-synonymous polymorphisms can affect protein stability and lead to conformational and functional changes. Regarding the two non-synonymous SNPs identified in this study, R4H and A91V, both had already been reported as having pathological significance. In one case report, R4H was associated with aplastic anemia [3] and A91V has been associated to the susceptibility of developing type 2 familial hemophagocytic lymphohistiocytosis and NHL [25]. However, no difference was found in the frequencies of mutations between the NHL patients and the healthy individuals.

The A91V polymorphism was already reported in strong linkage disequilibrium with the H300H polymorphism, with the presence of the A91V polymorphism in approximately 40% of individuals who were homozygous for c.900C and not being found in homozygous for c.900T [26]. Although our study did not perform the analysis of linkage disequilibrium between these polymorphisms, we observed that of the three individuals with the A91V polymorphism, two were homozygous for c.900C and one heterozygous for c.900C/T.

In this context, the results of this study suggest the importance of treatment strategies aimed at recovering and potentiating NK cell cytotoxicity; drugs such as lenalidomide and other thalidomide derivatives act by increasing IL-2 production. The Food and Drug Administration (FDA) approves the use of lenalidomide only in the treatment of multiple myeloma, 5q syndrome, and relapsed/refractory mantle cell lymphoma. Even so, its efficacy, whether administered in isolation or in combination with monoclonal antibodies such as rituximab, has stimulated researchers to advocate the use of this drug as a first-line treatment for patients with B-cell NHL [27–29].

In conclusion, we observed that despite the higher gene expression of perforin and granzyme B, the NK cells of the NHL patients presented lower intracellular levels of perforin. The difference in the patterns of perforin expression in CTLs and NK cells underscores the importance of these cells of the innate immune system antitumor response, as the greater number of CTLs expressing perforin and the increased activation of these cells by TCR are not sufficient to prevent the progression of the disease. The continuation of this study evaluating the overall and disease free survival may allow us to conclude that a lower expression of perforin is associated with worse prognoses.

Disclosure statement

Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article online at https://doi.org/10.1080/10428194.2017.1341978.

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3.2 ARTIGO 2

INCREASE IN CYTOTOXIC LYMPHOCYTES AND CORRELATION OF PERFORIN EXPRESSION AND GRANZYME B WITH IMPROVED SURVIVAL OF PATIENTS WITH MULTIPLE MYELOMA

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ABSTRACT

Multiple myeloma (MM) is an incurable and progressive neoplasm of plasma cells that causes immune dysfunction due to several factors, including the hyporesponsiveness of cytotoxic lymphocytes. To evaluate the main cell death pathway used by cytotoxic lymphocytes and natural killer cells in defense against tumor cells, our study investigated the presence of polymorphisms in the coding region of the *PRF1* gene and quantified the levels of gene expression and intracellular protein of perforin and granzyme B in 58 patients with MM and in 78 healthy individuals. We did not observe differences between allele frequencies of polymorphisms in the coding region of *PRF1* and in perforin and granzyme B expression between patients with MM and healthy individuals. However, patients with MM had significantly more cytotoxic T lymphocytes (CTLs) expressing perforin and granzyme B, in addition to a greater number of natural killer (NK) cells, compared to that in healthy subjects. In addition, the lower expression of perforin and granzyme B genes was associated with poorer survival in these patients. Therefore, even if inherited *PRF1* polymorphisms are not associated with MM, the elevated expression of genes encoding these cytolytic proteins may be associated with better patient prognosis, demonstrating the critical role of immunovigilance in the control and progression of MM. Future studies should clarify whether this expansion of CTLs expressing perforin and NK cells occurs at the expense of

senescent dysfunctional cells, thus tracing the functional profile of these effector cells in patients with MM.

INTRODUCTION

Multiple myeloma (MM) is a progressive B-cell lymphoproliferative neoplasm caused by deregulated and clonal proliferation of plasma cells in the bone marrow, which produce and secrete anomalous monoclonal immunoglobulin. The pathophysiological consequences and clinical manifestations occur as a consequence of the infiltration of organs by neoplastic plasmocytes, especially the bones, by the production of excess immunoglobulin, and by the suppression of normal humoral immunity. These processes lead to bone destruction, renal failure, suppression of hematopoiesis, and increased risk of infections [1]. Despite recent therapeutic advances, MM is still considered an incurable disease and the life expectancy of patients with MM does not exceed 5 years [2].

The interaction of malignant plasma cells with cytotoxic T lymphocytes (CTLs) or natural killer (NK) cells is able to modulate the growth and differentiation of these effector cells, with T cells being responsible for a key role in controlling the progression of monoclonal gammopathies [3]. After being activated, CTLs and NK cells exert their immunovigilance activity mainly through the exocytosis of granules containing perforin and granzyme B [4]. Perforin undergoes polymerization in the membrane of the target cell, forming pores that lead to osmotic imbalance and, together with granzymes, which activate the caspase cascade, triggering the apoptotic pathway, provoke the death of the neoplastic cell [5].

The *PRF1* gene encodes perforin and is located on human chromosome 10q22 [6]. Studies in knockout mice involving this gene have demonstrated a reduction in the cytotoxic function of CTLs and NK cells, rendering the animals up to 1000 times more susceptible to transplanted and/or induced malignancies [7,4]. In humans, changes in the *PRF1* gene were initially associated with familial hemophagocytic lymphohistiocytosis (LHF), a rare and rapidly fatal disease that occurs in the first decades of life [8]. Individuals afflicted with the disease and who have biallelic mutations in the *PRF1* gene present severe impairment of the cytotoxic activity of CTLs and NK cells [9]. Other lymphoproliferative disorders have been associated with mutations in *PRF1*, such as acute lymphoblastic leukemia [10] and non-Hodgkin's lymphoma [11, 12]. In contrast to that observed for *PRF1*, cases of neoplasia

related to the presence of mutations in the granzyme B gene (*GZMB*) have not been reported to date.

Despite the large number of published papers evaluating the association of polymorphisms in the coding region of the *PRF1* gene with lymphomas, we found no such association with MM. Among the few investigations that analyzed perforin-mediated immune responses against MM, we identified an animal model study that showed that the immune response against a transplanted myeloma cell line was mediated primarily by NK cells and CD8+ T lymphocytes through the pathways of perforin and interferon gamma (IFN-γ) [13]. A previous study evaluated the damage caused by purified perforin in the membrane of tumor cells and observed that MM plasma cells are highly susceptible to perforin-mediated lysis [14].

Due to the lack of information regarding the effector mechanism of CTLs and NK cells in patients with MM, this study aimed to evaluate the granule-mediated pathway of cell death by investigating the presence of polymorphisms in the coding region of the *PRF1* gene. It is also proposed to quantify the levels of mRNAs and perforin and granzyme B proteins to assist in understanding the immunological mechanisms of responses to tumor cells and the influence of genetic factors involved in the pathobiology of MM.

MATERIALS AND METHODS

Research Subjects

This study included 58 patients with MM, of which 32 were male and 26 were female, with a median age of 63 years (34–86 years) visiting the Clinical Hospital of Universidade Federal do Triângulo Mineiro and the Faculdade de Ciências Médicas da Santa Casa de São Paulo (FCMSCSP). The control group consisted of 78 individuals with no history of neoplasia, chronic or autoimmune infectious disease, of which 55 were male and 23 were female, with a median age of 46 years (23–76 years).

This research was approved by the Ethics Committee of the Universidade Federal do Triângulo Mineiro (protocol n ° 2677) and all participants gave written consent.

Polymorphism search in the coding region of the *PRF1* gene

A search for polymorphisms in the *PRF1* coding region was performed by direct sequencing. DNA was extracted from leukocytes obtained from total peripheral blood using the FlexiGene Mini DNA isolation kit (Quiagen, Hilden, Germany) according to the

manufacturer's instructions. Amplification of the sequences to be investigated was performed by polymerase chain reaction (PCR) using specific primers (Invitrogen Life Technologies, São Paulo, Brazil), for exon 2: forward (F2) 5′-CCTTCCATGTGCCCTGATAATC-3′ e reverse (R2) 5′-GAAGCAGCCTCCAAGTTTGATTG-3′; and for exon 3: forward (F3) 5′-CAGTCCTAGTTCTGCCCACTTA-3′ e reverse (R3) 5′-CTAATGGGAATACGAAGACAGCC-3′, following the technique described by Martínez-Pomar [15]. Sequencing was performed using the PCR products, the same amplification primers, and the ABI Prism Big Dye ™ Terminator Cycle Sequencing Ready kit (Applied Biosystems, Foster City, USA), on an ABI Prism 3100xl DNA sequencer (Applied Biosystems) following the manufacturer's instructions.

Gene Expression Analysis

Quantitative real-time PCR (qPCR) was used for the quantification of *PRF1* and *GZMB* gene expression. Total RNA was extracted from peripheral blood using the QIAamp RNA Blood Mini (Qiagen, Hilden, Germany) kit and complementary DNA (cDNA) was generated by reverse transcription using GoScript ™ Reverse Transcription System (Promega, Madison, USA). For amplification by qPCR, we used the inventoried TaqMan® assays: perforin (Hs00169473_m1), granzyme B (Hs01554355_m1) and β-actin (Hs99999903_m1) and the GoTaq® qPCR Master Mix kit (Promega). Gene expression values were normalized to that of the endogenous β-actin gene (*ACTB*), and the expression levels of *PRF1* and *GZMB* were calculated by the Δ cycle threshold (ΔCT) method using the $2^{-\Delta\Delta CT}$ formula.

Immunophenotyping of CD8+ T and NK cells

Quantification of intracellular expression of perforin and granzyme B was performed by flow cytometry of 2×10^5 peripheral blood mononuclear cells from each individual, collected in a tube containing heparin, and isolated by density gradient separation using Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden). Identification of CTLs and NK cells was performed using anti-CD3 (APC), anti-CD8 (Pe-Cy5), and anti-CD56 (Pe-Cy7) antibodies (BD Pharmingen ™, New Jersey, USA). CD69 (PE) marker (BD Pharmingen ™) was used to identify T cell activation. For the quantification of intracellular protein levels of perforin and granzyme B, anti-perforin (PE) (Clone dG9), and anti-Granzyme B (FITC) (Clone GB11) (BD Pharmingen ™) were used. Fifty thousand events were acquired on a FACSCanto II cytometer (Becton Dickinson, New Jersey, USA) and the results were analyzed using FlowJo 10 software (TreeStar, Ashland, USA).

Statistical analysis

Data analysis was performed using GraphPad Prism version 6.01 software (San Diego, USA). Comparison of the allele frequencies of the polymorphisms observed in patients and controls was performed using the Chi-squared test.

In relation to the results of protein quantification and gene expression by qPCR, after verifying the sample distribution through the Kolmogorov-Smirnov test, statistical analysis was performed using Student's *t*-test (for parametric distributions) or by the Mann-Whitney test (for non-parametric distributions). Survival analyzes were performed using the log-rank test to evaluate the impact of perforin and granzyme B deficiency in patients. Differences were considered significant when the p value was < 0.05.

RESULTS

Frequency of *PRF1* gene polymorphisms

Six single nucleotide polymorphisms (SNPs) on exon 2 and three SNPs on exon 3 of the *PRF1* gene were identified, five of which were synonymous (G96A, G435A, G462A, C822T, and C900T) and four non-synonymous polymorphisms (G11A, C272T, C310T, and G781A). The alleles 96A, 435A, and 462G were observed only in healthy individuals (allelic frequency of 0.01, 0.03, and 0.03, respectively), but without significant difference between groups analyzed ($p = 0.39$, $p = 0.08$, and $p = 0.16$, respectively). In exon 3, 822T and 900T alleles were observed in both MM and controls, and allelic frequency did not differ between them (0.1 versus 0.09, $p = 0.54$; and 0.5 versus 0.4, $p = 0.09$).

Among the non-synonymous SNPs identified, one was found with a higher frequency in relation to the others. The altered *PRF1* allele, C272T, was observed at a frequency of 0.07 in patients with MM, with patients being heterozygous or homozygous. This frequency was more than double that found in the group of healthy individuals, however, the difference was not statistically significant ($p = 0.16$). The other non-synonymous polymorphisms, G11A, C310T, and G781A, were found only in the heterozygous state, with allele frequencies that did not differ between the groups studied (Table 1).

Lower expression of perforin and granzyme B correlated with lower survival of patients with MM

To avoid possible interference in our analysis, only patients who had not started chemotherapy treatment, and control subjects matched by gender and age, underwent

quantification of gene expression and intracellular expression of perforin and granzyme B. Thus, the techniques of qPCR and flow cytometry were used in 15 patients with MM and in 15 healthy individuals matched by gender and age (Table 02).

The values obtained by qPCR analysis demonstrated that there was no statistically significant difference in *PRF1* and *GZMB* expression between patients with MM and healthy individuals (p = 0.6778 and p = 0.2808, respectively) (Figure 1A-B). However, we observed that patients with expression of *PRF1* or *GZMB* above the median had significantly longer survival time compared to patients who had deficiency expression of both proteins (p =0.0462) (Figure 1C). We also observed a significant correlation between *PRF1* and *GZMB* gene expression in both MM (p = 0.0005, r = 0.7870) and healthy subjects (0.0412, r = 0.5321) (Figura 2)

Increased number of activated lymphocytes expressing perforin and granzyme B

Flow cytometric analysis showed that the percentage of CTLs (CD3+ CD8+) in patients with MM and controls did not differ significantly. Regarding the expression of perforin and granzyme B, a significant increase was observed in the percentage of CTLs expressing granzyme B (Figure 3B p = 0.0070) and perforin (Figure 3C, p = 0.0310) in patients with MM. Patients with MM also had a significant increase in the percentage of NK cells (Figure 4B, p = 0.0342), but the proportion of these cells expressing granzyme B and perforin was not statistically different compared to healthy individuals (Figure 4C–D). In addition, the activation of lymphocytes was evaluated through CD69 marker expression. Patients with MM had significantly more activated lymphocytes than healthy individuals (p = 0.0186) (Figure 5).

DISCUSSION

Among the polymorphisms found in this study, all had already been described and two were previously considered pathogenic, or with conflicting interpretations in this respect [16, 17]. One of the SNPs that we identified, and which was initially considered pathogenic according to the literature, was G11A (rs35418374), which causes exchange of the amino acid arginine for histidine in codon four of the perforin protein. In our study, this change was identified only in the heterozygous state, with no significant difference in allelic frequency between the two groups studied. There are conflicting interpretations regarding the pathogenicity of this polymorphism, since only a single study published in the literature

reported an association of this alteration in a patient with aplastic anemia [18]. However, recent clinical studies that have performed more refined analyses for confirming the clinical significance of this variation, according to the Sherloc methods and ACMG (American College of Medical Genetics) guidelines, which point to this polymorphism as being benign [19, 20].

The second pathogenic SNP that we identified and the only one found in the homozygous state was C272T (rs35947132), which leads to substitution of the amino acid alanine for a valine in codon 91 (A91V) of the perforin protein. In our study, although the frequency of the altered allele was twice as high among patients with MM, this difference was not considered statistically significant. This is a hypomorphic variant most commonly found in the *PRF1* gene, with up to 8% frequency in caucasians [21]. Data available from the National Center for Biotechnology Information and raised through the genome project show that the C272T SNP is predominantly identified in populations of European or mixed-race American origin, with frequencies of the mutated allele ranging from 0.02 to 0.04 [17].

Although we found no differences in both the frequency of polymorphisms and the gene expression of perforin, our study identified that longer survival of patients with MM is positively correlated with elevated expression of *PRF1* and *GZMB*. An earlier study by our research group also found that the expression of these genes correlated positively with the survival time of patients with non-Hodgkin's lymphoma [22]. Thus, even if inherited polymorphisms in the *PRF1* gene have not been associated with MM, deficient cytotoxic responses may be associated with poorer prognosis for these patients, demonstrating that the immune system plays a critical role in controlling the progression of, and response to, MM treatment.

Polymorphisms located in the coding region of the perforin gene may impact gene expression or protein quantification of cytoplasmic granules. Non-synonymous polymorphisms can generate lower availability of tRNA for the altered codon, or may cause incorrect folding (misfolding) of the mutated protein. This phenomenon may lead to decreased cleavage to the active form, rendering the protein more unstable, increasing its degradation, and consequently leading to a decrease in intracytoplasmic protein levels without interfering in the gene expression process [23-26].

In our study, we observed significant increases both in the percentage of CTLs expressing perforin and granzyme B, and in the percentage of NK cells, results similar to those of several previous studies [27-33]. Raitakari et al (2000) identified that the majority of T-cell expansions have the phenotype of cytotoxic T cells (CD8+CD57+ and perforin+) [28].

Reinforcing the key roles of CTLs and NK cells, which are the main cells expressing perforin and granzyme B, an animal model study demonstrated that the tumor burden in MM mice was inversely correlated with the number of CD8+ and NK T cells present in the bone marrow [13].

The T cell clones in MM patients can constitute up to 50% of all blood lymphocytes and despite the expression of cytolytic proteins, these cells are considered hyporesponsive in vitro. However, the presence of these cells is still correlated with better survival. [29-31]. The best understanding of the normal CD8+ lymphocyte response to antigen and the factors that lead to abnormal continued expansion in certain disease states will be essentials to comprising the pathogenesis of chronic antigenic stimulation [34]. Overcoming a hypo-responsivity of these cells is currently a promising therapeutic strategy [35].

CONCLUSION

In the present study, no differences were observed between the frequency of polymorphisms in the coding region of the *PRF1* gene and in perforin and granzyme B gene expression in patients with MM and healthy individuals. Patients with MM had higher numbers of CTLs expressing perforin and granzyme B, in addition to greater numbers of NK cells, compared to healthy individuals. In addition, deficiency perforin and granzyme B expression was associated with lower survival in these patients. New studies should seek to clarify if this expansion of CD8+ T lymphocytes expressing perforin and NK cells occurs at the expense of senescent dysfunctional cells, which could make it possible to trace the functional profile of these effector cells in patients with MM.

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Tables

Table 1. Non-synonymous polymorphisms found in the coding region of the *PRF1* gene in patients with multiple myeloma and in healthy individuals.

		Allele frequency		
		Reference allele	**Variant allele**	***p-value***
G11A R4H	P	115/116 (0.99)	1/116 (0.01)	0.47
(rs35418374)	C	153/156 (0.98)	3/156 (0.02)	
C272T A91V	P	108/116 (0.93)	8/116 (0.07)	0.16
(rs35947132)	C	151/156 (0.97)	5/156 (0.03)	
C310T R104C	P	115/116 (0.99)	1/116 (0.01)	0.24
(rs547723649)	C	156/156 (1.0)	0/156 (0.0)	
G781A E261K	P	114/116 (0.98)	2/116 (0.02)	0.24
(rs758110629)	C	154/156 (0.99)	2/156 (0.01)	

P: patients; C: controls (healthy individuals), Chi-square test: there was no significant difference in allele frequencies between patients and controls.

Table 2: Patient`s characteristics.

Patient	Gender	Age	Classification	Clinical Features	Durie Salmon	International Staging System	Therapeutic outcome	Survival time (months)
P24	M	43	IgG-Lambda	Low back pain and asthenia	IIIA	III	Very good partial response	54
P26	M	69	IgG-kappa	Left hip pain	IIA	I	Very good partial response	90
P28	F	64	IgG-Kappa	low back pain	IA	I	No treatment (asymptomatic multiple myeloma)	56
P33	M	74	IgA-Lambda	Dyspnea and edema of lower limbs	IIIB	IIIA	Death - no treatment	Not informed
P34	F	68	IgA-Kappa	Acute renal failure, anemia and thrombocytopenia	IIIB	III	Death - no treatment	1
P44	M	76	Unrealized	Not informed	Not informed	Not informed	Death - no treatment	1
P93	F	55	IgA-lambda	Right arm pain lasting 4 months	IA	II	Complete therapeutic response	30
P99	F	76	IgA-Lambda	Lower limb pain, asthenia, and weight loss	IA	III	Very good partial response	27
P100	F	71	IgG-Lambda	Adynamia, weakness, lower limb pain and dyspnea on exertion	IIIB	III	Very good partial response	21
P103	F	59	IgA-Kappa	Pathologic fracture of the left upper limb	IIIA	III	Very good partial response	21
P104	F	83	IgA-Kappa	Asymptomatic patient with detection of moderate anemia in preoperative cholecystectomy	IIIA	III	Very good partial response	17
P106	M	55	Kappa	Low back pain and weight loss	IIIA	II	Partial response	17
P107	F	73	IgG/Kappa	Inability to walk and tumor in left clavicle	IIIA	II	Very good partial response	16
P108	F	68	IgG/Kappa	Asthenia and adynamia associated with episodes of forgetfulness and lower limb pain	IIIA	III	Partial response	17
P115	M	38	Lambda	Presentation with diffuse tumor masses with initial evaluation motivated by headache and strabismus caused by skull base tumor	IIIA	Not informed	Death due to febrile neutropenia	3

Figures

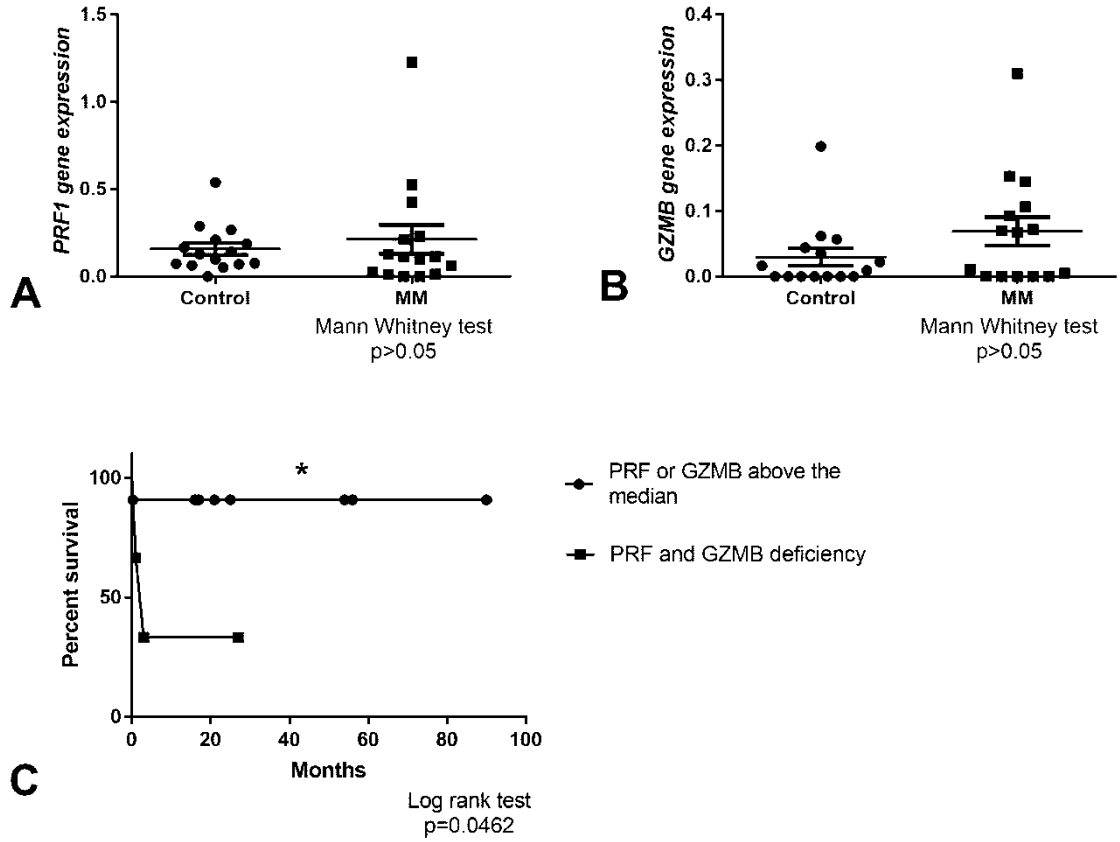

Figure 1: Quantification of perforin and granzyme B expression and correlation with survival of multiple myeloma patients and controls. **A.** *PRF1* gene expression relative to ACTB; **B.** *GZMB* gene expression relative to ACTB; **C.** Comparison between survival curves of patients with *PRF1* or *GZMB* expression above the median and patients with deficiency expression of both proteins (gene expression below the group median).

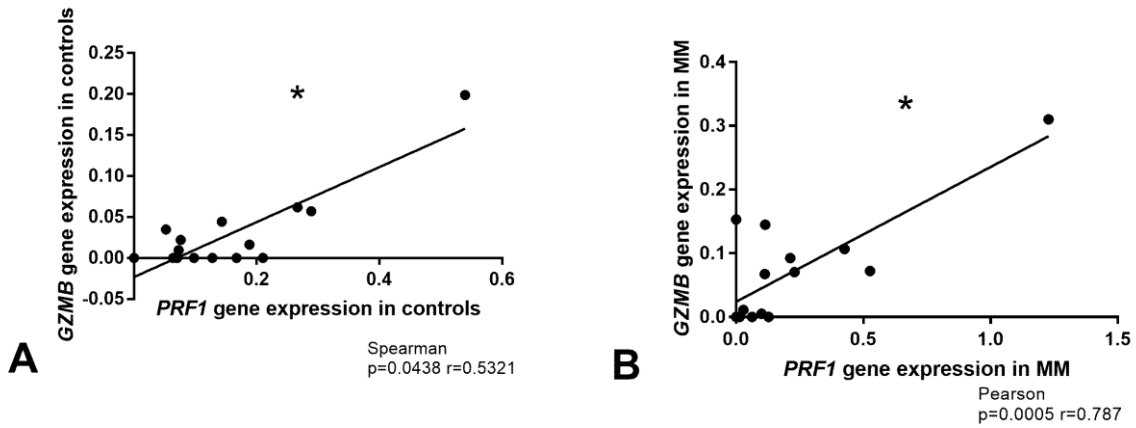

Figure 2: Correlation between perforin gene expression and granzyme B. **A.** controls; **B.** healthy individuals.

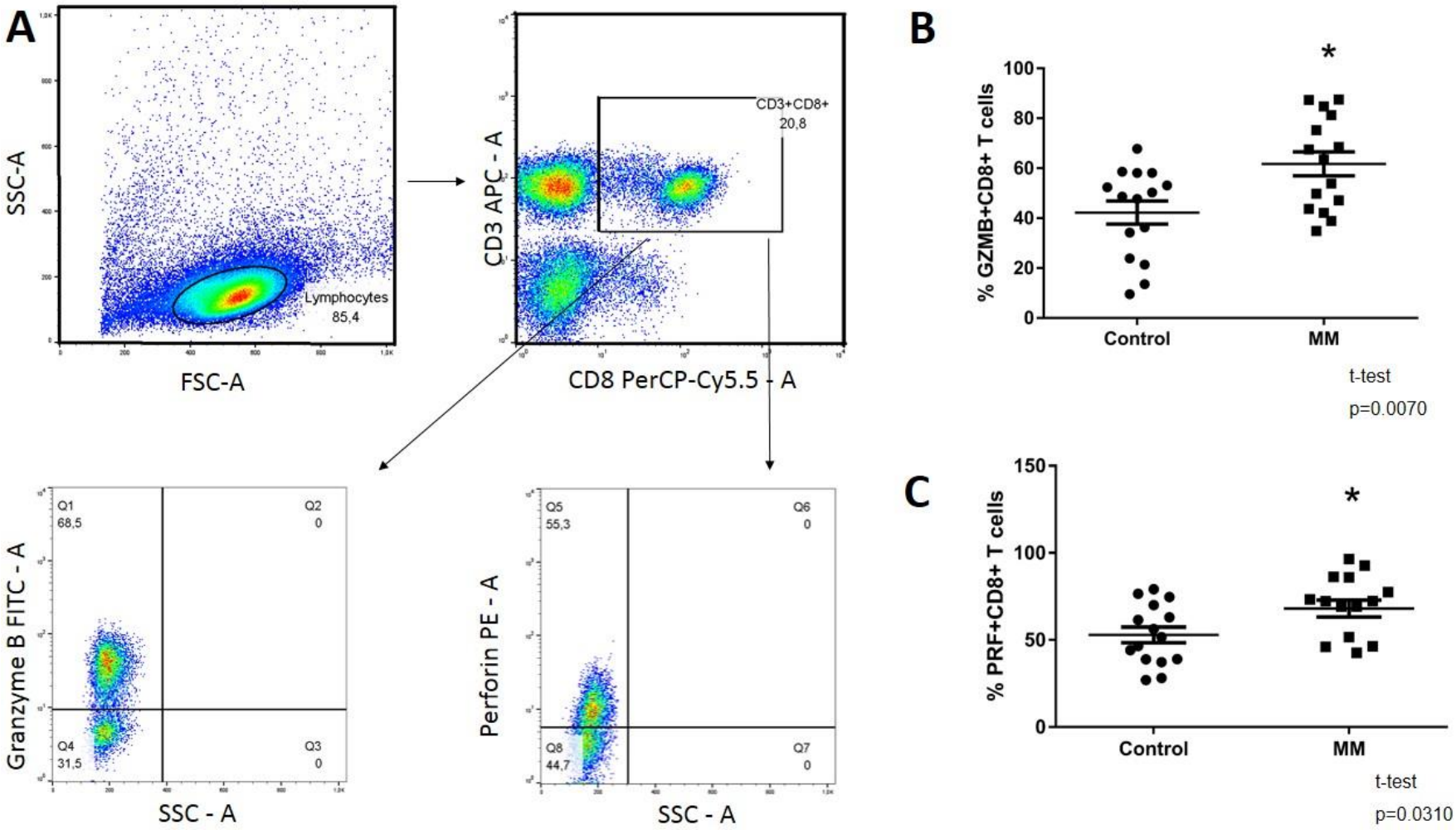

Figure 3: Strategy for gating and quantification of subpopulations of CD8+ T lymphocytes expressing perforin and granzyme B. **A.** Gating strategy for separation of the lymphocyte population, separation of CD8+ T lymphocytes; and quantification of the percentage of cells expressing perforin and granzyme B. **B.** Quantification of CD8+ T lymphocytes expressing granzyme B. **C.** Quantification of CD8+ T lymphocytes expressing perforin.

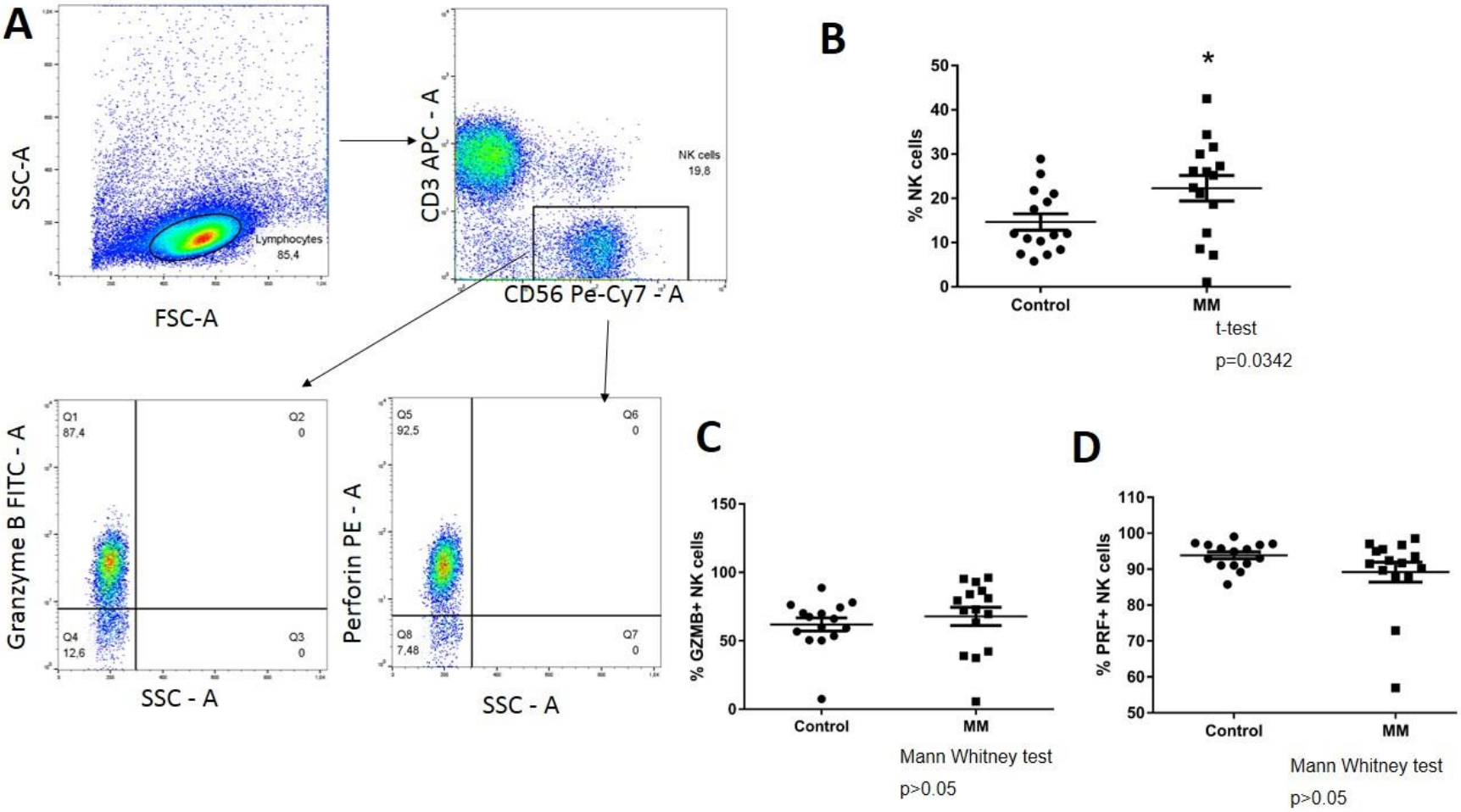

Figure 4: Gating strategy and population quantification of NK cells expressing perforin and granzyme B. **A.** Gates strategy for separation of the lymphocyte population; separation of the NK cell population and quantification of the percentage of cells expressing perforin and/or granzyme B. **B.** Quantification of NK cells. **C.** Quantification of NK cells expressing granzyme B. **D.** Quantification of NK cells expressing perforin.

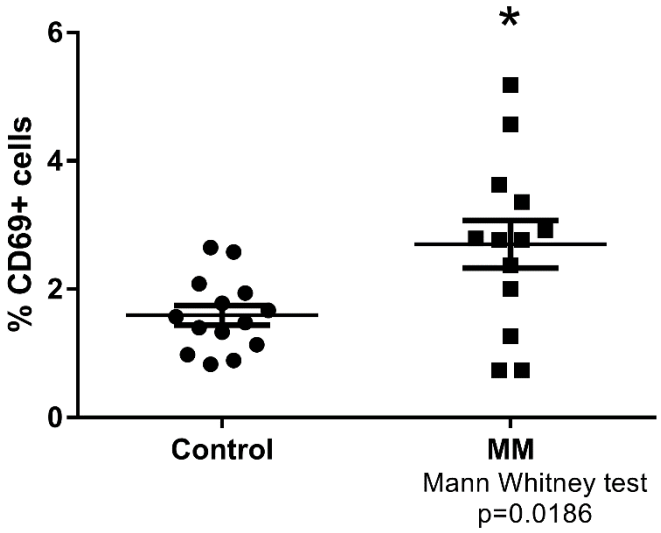

Figure 5: Quantification of activated lymphocytes in patients with multiple myeloma.

3.3 ARTIGO 3

Case report

Title: Impaired NK cell cytotoxicity in multiple myeloma associated with homozygous A91V polymorphism in the perforin gene.

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Introduction

The perforin gene (*PRF1*), located on the human chromosome 10q22, encodes perforin, a pore-forming protein critical to the functioning of cytotoxic lymphocytes (CLs), comprised primarily of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells [1]. In conjunction with granzymes, perforin induces target cell apoptosis and is essential in the immune response against intracellular pathogens and tumor cells [2].

Studies in knockout mice for this gene have demonstrated a reduction in the cytotoxic function of CTLs and NK cells, making the animals up to 1000 times more susceptible to transplanted and/or induced malignancies [3]. In addition, 60% of knockout mice that were not challenged with pathogens and/or carcinogens spontaneously developed highly aggressive and disseminated B-cell lymphomas before 12 months of age [4].

In humans, changes in the *PRF1* gene were first associated with familial hemophagocytic lymphohistiocytosis, a rare and potentially fatal disease occurring within the first decades of life [5]. Individuals with this disease who have biallelic mutations of the *PRF1* gene present severe impairment of the cytotoxic activity of CTLs and NK cells [6]. Subsequently, other lymphoproliferative disorders were associated with mutations in this gene such as acute lymphoid leukemia [7] and non-Hodgkin lymphoma [8, 9].

The aim of this report is to show a case of a multiple myeloma patient who presented impaired cytotoxicity associated with homozygous A91V polymorphism in the perforin gene.

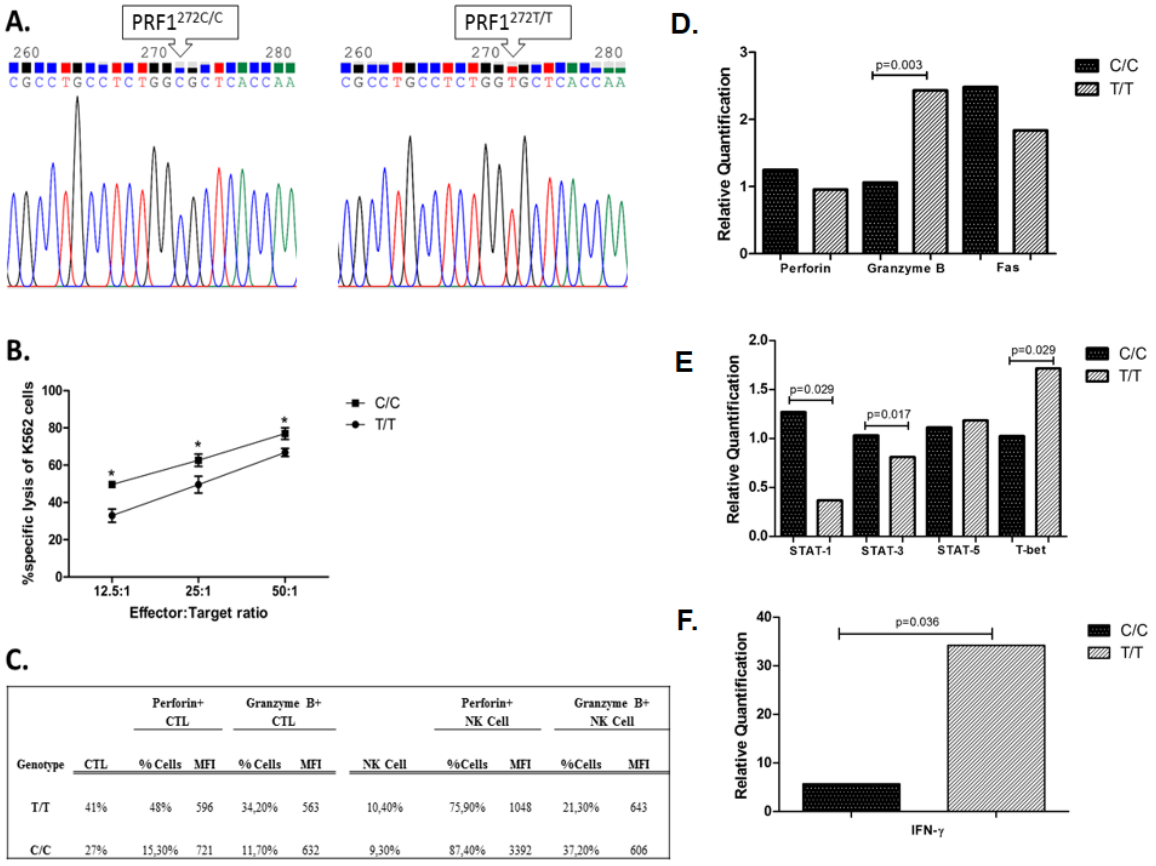
Case report

A 58-year-old male diagnosed with IgG/Kappa multiple myeloma (International Staging System III) treated eight years ago with VAD polychemotherapy (vincristine/doxorubicin/dexamethasone) and autologous peripheral hematopoietic stem cell transplantation and in follow-up with indication of re-treatment for progression of disease due to hip pain, anemia and increase of monoclonal component.

The *PRF1* gene of patient was analyzed by direct sequencing and the homozygous C272T polymorphism ($PRF1^{272T/T}$) was identified. A C>T change at position 272 in exon 2 of *PRF1* replaces alanine with valine at position 91 (A91V) in the protein. We performed a functional analysis of the CLs of a $PRF1^{272T/T}$ patient and a healthy individual $PRF1^{272C/C}$. Our results show that $PRF1^{272T/T}$ effector cells had significantly reduced ability to induce specific lysis of the tumoral K562 cells (ATCC® CCL-243™) (Figure 1A). The highest number of effector cells was not able to restore the cytotoxic capacity of this patient, since even at the effector/target cell ratio of 50:1, $PRF1^{272T/T}$ CLs continued to eliminate significantly fewer tumor cells than the $PRF1^{272C/C}$ CLs (60% versus 77%; p-value = 0.0292).

It was observed that the NK cells of the $PRF1^{272T/T}$ patient had three times less perforin than observed in the wild-type individual (MIF 1048 *vs.* 3392, respectively) and the percentage of NK cells expressing perforin was also slightly reduced (75.9% *vs.* 87.4%, respectively) (Figure 1B).

The *PRF1* gene expression did not differ between the individuals, however the expression of granzyme B was approximately 2.5 times higher in the $PRF1^{272T/T}$ patient (p-value = 0.003) (Figure 1D). It was also observed that the T-bet expression was approximately 1.7-fold higher and IFN-γ expression was 4.5-fold higher in the $PRF1^{272T/T}$ patient (p-value = 0.029 and p-value= 0.036, respectively) (Figure 1E-F).



C.

Genotype	CTL	Perforin+ CTL %Cells	Perforin+ CTL MFI	Granzyme B+ CTL %Cells	Granzyme B+ CTL MFI	NK Cell	Perforin+ NK Cell %Cells	Perforin+ NK Cell MFI	Granzyme B+ NK Cell %Cells	Granzyme B+ NK Cell MFI
T/T	41%	48%	596	34,20%	563	10,40%	75,90%	1048	21,30%	643
C/C	27%	15,30%	721	11,70%	632	9,30%	87,40%	3392	37,20%	606

Figure 1: Results of perforin gene (*PRF1*) sequencing, cytotoxicity assay, perforin/granzyme B intracellular quantification and gene expression quantification. (A) Electropherograms of the PRF1$^{272T/T}$ patient and the PRF1$^{272C/C}$ healthy individual obtained by direct sequencing of *PRF1*. (B) Specific lysis of K562 target cells at an effector:target ratio of 12.5:1, 25:1 and 50:1 in the cytotoxicity assay. (C) Percentage of cytotoxic T lymphocytes (CTL) and natural killer (NK) cells expressing perforin and granzyme B and the mean fluorescence intensity (MFI) by flow cytometry. (D) Perforin, granzyme B and Fas gene expression quantification. (E) STAT1, STAT3, STAT5 and T-bet gene expression quantification. (F) Interferon-gamma gene expression quantification.

Discussion

Previous studies have reported that the reduction in cytotoxicity caused by the C272T SNP can affect the cytotoxicity of CLs by up to 50%. This fact is explained by the misfolding of the mutated protein that causes a decrease in its cleavage compared to the active form and makes it more unstable, increasing its degradation without interfering in the process of gene expression [10-13].

The patient was diagnosed with advanced stage MM (International Staging System III) at 49 years of age after a femoral fracture and being referred for investigation. The manifestation of the disease in this age is considered precocious, since the average age of diagnosis in Brazil of 61 years [14].

A reduction in LC cytotoxicity caused by missense or frame-shift mutations may result in decreased immune surveillance against tumors and increased susceptibility to cancer [10]. In homozygosis, the C272T alteration has been associated with familial hemophagocytic lymphohistiocytosis, non-Hodgkin lymphoma and aplastic anemia [5, 8, 9, 15]. However, we did not find any published studies that correlated this alteration with MM.

Although early studies had considered C272T as a neutral polymorphism because of its high frequency in the general population, a recent study showed that even in heterozygosis this polymorphism causes a decrease in NK cell cytotoxicity in healthy individuals [16].

An interesting observation can be made regarding the greater gene expressions of GZMB, T-bet and IFN-γ in the $PRF1^{272T/T}$ patient compared to the wild homozygous individual. A study published by Jenkins et al. 2015 showed that perforin deficiency increases immunological synapse dwell time by up to 5-fold, resulting in increased secretion of inflammatory cytokines such as IFN-γ, IL-2 and TNF by CTLs and NK cells and activation of macrophages [17].

T-bet is a transcription factor that promotes the activation of other transcription factors, such as STAT1 and STAT3 [18]. Due to the higher T-bet expression in the $PRF1^{272T/T}$ patient, we expected to find greater expressions of STAT1 and STAT3, however we found the opposite. These reductions in STAT1 and STAT3 also could be responsible for the lower expression of perforin, since STAT proteins are transcription factors that are involved in the regulation of the perforin gene [19]. Further studies are essential to elucidate the mechanism involved in inhibiting the expression of these STATs.

Studies have used IL-2 treatment as a strategy to recover the function of perforin-deficient CLs and have reported increased cytotoxic potential at levels similar to the normal cells evaluated [20,21]. Currently, different therapeutic strategies, such as lenalidomide and other thalidomide derivatives that stimulate IL-2 production, used to treat MM seek to recover and potentiate the cytotoxicity of CLs [22-24].

In conclusion, the functional analysis of the CLs of a $PRF1^{272T/T}$ patient revealed a significant decrease in their cytolytic capacity as well as the amount of perforin present in NK cell granules.

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SUPPLEMENTARY MATERIAL

Materials and Methods

Direct Sequencing

DNA was extracted from total peripheral blood and subsequently, the sequences to be investigated were amplified by polymerase chain reaction (PCR) using specific primers for each region as previously described in the literature [10]. Sequencing was performed using the PCR products with the same amplification primers and the ABI Prism BigDye™

Terminator Cycle Sequencing Ready kit (Applied Biosystems) following the manufacturer's instructions with the ABI Prism 3100xI DNA Sequencer (Applied Biosystems).

Cytotoxicity Assay

A commercial tumor line derived from a human erythroleukemia named K562 (ATCC® CCL-243™) and peripheral blood mononuclear cells (PBMC) of the subject were used for the functional evaluation of CLs. For the assay, the target cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) and co-cultured with the PBMC of the patient containing effector cells in the proportions of 12.5:1, 25:1 and 50:1. After four hours of incubation, the cells were recovered and labeled with propidium iodide (PI). Specific lysis of K562 cells was calculated by the frequency of double-positive K562 cells for CFSE and PI labeling, subtracted from spontaneous lysis.

Flow Cytometry

Quantification of the intracellular expressions of perforin and granzyme B was performed by flow cytometry in 2 x 10^5 PBMC, which were maintained in culture to perform the cytotoxicity assay.

The labeling of LTCs and NK cells was achieved using CD3, CD8 and CD56 antibodies (BD Pharmingen ™, CA, USA). Fifty thousand events were acquired on a FACSCanto II cytometer (Becton Dickinson, CA, USA) to quantify cells labeled with the perforin-PE or Granzyme B-FITC (BD Pharmingen ™, CA) intracellular antibodies and to calculate the mean fluorescence intensity (MFI) of each antibody.

Quantitative Real-time Polymerase Chain Reaction (qPCR)

Quantification of the gene expression was performed by the quantitative real-time PCR technique (qPCR). RNA was isolated from the separated PBMC and then complementary DNA (cDNA) was generated by reverse transcription. The following inventoried TaqMan® assays were performed: perforin (Hs00169473_m1), granzyme B (Hs01554355_m1), fas (Hs00236330_m1), STAT1 (Hs01013996_m1), STAT3 (Hs00374280_m1), STAT5 (Hs00234181_m1), T-bet (Hs00203436_m1), INF-γ (Hs00989291_m1), and β-actin (Hs99999903_m1). The values were normalized with the endogenous β-actin gene and the levels of gene expression were calculated by the ΔΔ cycle threshold (ΔΔCT) method.

Statistical analysis was performed using the ExpressionSuite Software v1.1, Applied Biosystems®, the differences being considered statistically significant when the p-value was less than 0.05.

4 CONSIDERAÇÕES FINAIS

A imunoterapia nos dias de hoje se faz cada vez mais uma estratégia terapêutica de sucesso. O aprimoramento das células T CAR e as terapias com células NK ganham cada vez mais espaço no cenário onco-hematológico. Nossa perspectiva é de que possamos continuar os estudos nesta linha de pesquisa através de ensaios funcionais para avaliar o potencial citotóxico das células T CD8+, T CD4+ e células NK em neoplasias linfoproliferativas.

5 CONCLUSÕES

- Não foram observadas diferenças entre a frequência de polimorfismos na região codificadora do gene *PRF1* de pacientes com LNH e MM em comparação a indivíduos saudáveis.
- Houve maior expressão gênica de perforina e granzima B nos pacientes com LNH em comparação aos indivíduos saudáveis, o que não foi observado nos pacientes com MM.
- Os pacientes com LNH apresentaram maior número de LTC ativados e que expressavam perforina, no entanto a quantificação intracelular de perforina nas células NK foi significativamente reduzida nestes pacientes em relação aos indivíduos saudáveis. Os pacientes com MM também apresentaram maior número de LTCs que expressavam perforina e granzima B, contudo ao contrário do que observamos no LNH os pacientes com MM mostraram aumento na porcentagem de células NK em comparação aos indivíduos saudáveis.
- Os pacientes com MM e LNH que apresentavam expressão gênica de perforina e granzima B acima da mediana do grupo tiveram o tempo de sobrevida significativamente maior em comparação aos pacientes que apresentavam deficiência na expressão destes genes.
- As células NK de um paciente com MM, que foi identificado como homozigoto para o polimorfismo A91V no gene *PRF1*, tiveram a capacidade significativamente reduzida de induzir a lise específica das células K562, independentemente da proporção entre o número de células efetoras e o número de células alvo utilizadas. Também observamos que apesar de não haver diferenças na expressão do gene *PRF1* este paciente apresentava níveis intracelulares de perforina reduzidos nos grânulos das células NK.

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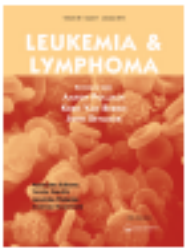
ANEXO A – COMPROVANTE DE PUBLICAÇÃO



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Evaluation of the cytotoxic response mediated by perforin and granzyme B in patients with non-Hodgkin lymphoma

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ANEXO B – APROVAÇÃO DO PROJETO DE PESQUISA PELO COMITÊ DE ÉTICA

UNIVERSIDADE FEDERAL DO TRIÂNGULO MINEIRO - UFTM

PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: ANÁLISE DO GENE DA PERFORINA EM LINFÓCITOS CITOTÓXICOS DE PACIENTES COM NEOPLASIAS LINFOPROLIFERATIVAS

Pesquisador: Helio Moraes de Souza

Área Temática: Genética Humana:
(Trata-se de pesquisa envolvendo Genética Humana que não necessita de análise ética por parte da CONEP;);

Versão: 3

CAAE: 46009115.2.0000.5154

Instituição Proponente: Universidade Federal do Triângulo Mineiro - MG

Patrocinador Principal: Universidade Federal do Triangulo Mineiro

DADOS DO PARECER

Número do Parecer: 2.555.435

Apresentação do Projeto:

Segundo o pesquisador: "Os linfomas representam 22% e 16% de todos os cânceres entre as pessoas com idade entre 20 a 24 e 25 a 29 anos, respectivamente (BLEYER et al., 2006a), e o linfoma não-Hodgkin (LNH) é responsável por 6% de todas as neoplasias entre 20 a 29 anos (BLEYER et al., 2005; BLEYER et al., 2006b). Ao longo dos últimos 20 anos, as taxas de incidência de linfoma entre os jovens adultos com idades entre 20 a 29 anos têm aumentado a um ritmo mais rápido comparados com aqueles entre as pessoas menores de 20 anos (BLEYER et al., 2006a).

Linfomas não-Hodgkin são um grupo heterogêneo de doenças malignas clonais que resultam de mutação somática na célula linfóide progenitora. A célula maligna possui fenótipo de célula B, T ou natural killer (NK), o que é determinado por imunofenotipagem e/ou estudos de rearranjo gênico (VAN der WAAL et al., 2005). Pelo menos dois terços dos casos de LNH apresentam linfonodomegalia extranodal persistindo por mais de dois meses. Sinais e sintomas sistêmicos incluem febre de origem desconhecida, inexplicável perda de peso, sudorese noturna, dor visceral e mal-estar, que coletivamente são conhecidos como sintomas B, e estão relacionados com pior prognóstico (MAWARDI et al., 2009).

O prognóstico de crianças e adolescentes com LNH tem melhorado desde a última década e, atualmente, 70%-90% das crianças são curadas com protocolos de quimioterapia intensiva,

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adaptada de acordo com a classificação de risco (PATTE, 1998). Atualmente, grande interesse tem sido dedicado a um subgrupo mais raro de crianças, nas quais as doenças linfoproliferativas (DLP) ocorrem na presença de imunodeficiência primária ou secundária.

Imunodeficiências e imunossupressão pós-transplante predispõem crianças a desenvolver neoplasias, especialmente as DLP, consideradas como grave complicação (GRANOVSKY et al., 1998; KNOWLES, 1999). Sabe-se que pacientes submetidos a transplante de órgãos sólidos têm risco aumentado para desenvolver neoplasias, particularmente linfomas (PENN, 1994).

Na imunodeficiência primária, a incidência de DLP é pouco conhecida, sendo estimada entre 1,4% e 24% dependendo do tipo de imunodeficiência (FRIZZERA et al., 1980). O tipo histológico da DLP varia de hiperplasia reativa não-específica até linfoma (ELENITOBA-JOHNSON e JAFFE, 1997). Dentre os linfomas, a maioria dos casos relatados é constituída de linfoma de células B de alto grau, frequentemente LNH de grandes células difusas com envolvimento extranodal (MUELLER, 1999), sendo que os linfomas de Burkitt, LNH de célula T periférica e linfoma de Hodgkin são raros (GRANOVSKY et al., 1998; FRIZZERA et al., 1980; ELENITOBA-JOHNSON e JAFFE, 1997)

O prognóstico para crianças com DLP e imunodeficiência primária ou secundária é considerado ruim. Nos pacientes submetidos ao transplante de órgãos sólidos, a redução nas drogas imunossupressoras parece ser benéfica (PINKERTON et al., 2002).

A.1.2. Resposta imune contra as células tumorais

Linfócitos T citotóxicos (Cytotoxic T Lymphocytes - CTLs) e células NK são linfócitos efetores que apresentam mecanismos citotóxicos semelhantes, necessários à defesa contra células transformadas (tumorais) ou infectadas por vírus. Ambos destroem seus alvos celulares por dois mecanismos principais que requerem contato direto entre a célula efetora e o alvo.

No primeiro mecanismo, grânulos citoplasmáticos contendo proteínas citolíticas, predominantemente uma proteína disruptora de membrana denominada perforina e uma família de serino-proteases (granzimas), são secretadas por exocitose e juntas induzem a apoptose da célula-alvo (SMYTH e TRAPANI, 1995). O segundo mecanismo envolve a ligação de receptores de morte da célula-alvo, como Fas (CD95), com seu ligante (FasL) na membrana da célula efetora, resultando na apoptose através da via das caspases clássica (VAN ELSAS et al., 2001).

O mecanismo principal da citólise mediada por estas células consiste na liberação de grânulos protéicos citotóxicos contra a célula-alvo que foi reconhecida (HENKART, 1985). Quando os receptores de antígenos dos CTLs e das células NK reconhecem os peptídeos associados ao complexo de histocompatibilidade principal (MHC) na célula-alvo, o citoesqueleto destas células é

reorganizado de tal modo, que o centro de organização dos microtúbulos é deslocado para a área próxima do contato com a célula-alvo. Os grânulos do citoplasma concentram-se nessa região e a membrana do grânulo funde-se com a membrana plasmática. A fusão da membrana resulta em exocitose do conteúdo dos grânulos na superfície da célula-alvo (TRAPANI et al., 2006).

A.1.3. Perforina

O gene que codifica perforina (PRF1) está localizado no cromossomo humano 10q22 e tem um tamanho aproximado de 6.000 pares de bases (pb) (FINK et al. 1992). Ele é constituído de três éxons e dois íntrons e sua região promotora tem 1.409 pb (LICHTENHELD & PODACK, 1989).

A perforina foi descrita pela primeira vez em 1984 e caracterizada como uma proteína citolítica formadora de poros, com massa molecular aproximada de 67 KDa (PODACK e KONIGSBERG, 1984). Perforinas de várias espécies mostraram pouca similaridade de aminoácidos com qualquer outra proteína conhecida e ainda são altamente conservadas entre espécies tão diferentes como os seres humanos e peixes (HWANG et al., 2004). Um nível tão elevado de conservação ao longo de pelo menos 300 milhões de anos de evolução e ausência de isoformas conhecidas de perforina sugere que ela tenha propriedades biológicas fundamentais.

No interior dos grânulos linfocitários, a perforina permanece armazenada na forma de monômeros e quando é liberada dos grânulos na fenda sináptica, os monômeros entram em contato com altas concentrações de cálcio extracelulares e sofrem polimerização (KAGI et al. 1994; LOWIN et al. 1994). Esta polimerização ocorre preferencialmente na membrana plasmática da célula alvo, onde a perforina polimerizada forma um poro de 5 a 20nm (SAUER et al. 1991). Quando um número suficiente destes canais é reunido, a célula se torna incapaz de eliminar íons e água, o que determina a morte celular. Em geral, a perforina pode ser descrita como uma proteína complexa constituída por vários domínios discretos, que atuam em conjunto para fornecer suas propriedades biológicas distintas.

Estudos em ratos nocaute para o gene PRF1 demonstraram claramente que a perforina está intimamente envolvida na defesa contra patógenos virais e células transformadas. Estes animais perdem a função citotóxica dos CTLs e células NK e se tornam susceptíveis a vários patógenos. Além disso, seu sistema imune não consegue evitar o rápido crescimento e a disseminação de tumores experimentais (LEE et al. 2006).

Em humanos, mutações e substituições missense no gene PRF1 estão associados a uma síndrome chamada linfohistiocitose hemofagocítica familiar, doença imune rara e rapidamente fatal caracterizada pela incapacidade de CTLs ativados eliminar os alvos em células apresentadoras de

antígeno (STEPP et al. 1999; VOSKOBOINIK et al. 2005). Outros defeitos no gene PRF1 já foram descritos e correlacionados com doenças como anemia aplástica (SOLOMOU et al., 2007), linfoma infantil (CANNELLA et al., 2007), linfoma não-Hodgkin e doença de Hodgkin (CLEMENTI et al., 2005).
Análises da incidência estimada do câncer em pacientes com imunodeficiências primárias confirmam a antiga suspeita de que os defeitos genéticos associados à imunodeficiência figuram entre os principais fatores de risco para o desenvolvimento de neoplasias em seres humanos (ONG et al., 1998). Anormalidades na resposta imunológica específica, tanto celular quanto humoral, têm sido detectadas em cerca de 80% das crianças após o tratamento convencional para neoplasias malignas (SMITH et al., 1995). A doença de base e o próprio tratamento promovem importante imunossupressão, que pode permanecer mesmo após a recuperação dos valores leucocitários no sangue periférico (MACLENNAN et al., 1992).
Na literatura encontramos escassos trabalhos que visam à compreensão dos mecanismos envolvidos na resposta imune nos pacientes com linfomas. Assim, torna-se de suma importância a realização de estudos sobre este aspecto, como a análise da expressão de genes envolvidos na resposta contra células tumorais nestes pacientes, para colaborar na compreensão dos mecanismos imunológicos de resposta às neoplasias hematológicas.

A.2. IMPACTO SOCIAL DO TEMA

Durante o século XX, o Brasil e o mundo passaram por profundas alterações em seus perfis epidemiológicos e demográficos. Concomitante ao declínio das taxas de mortalidade por doenças infecciosas, houve aumento substancial na expectativa de vida da população e na proporção de mortes por doenças crônicas. Estas transformações têm ocorrido ao longo das últimas décadas com padrões e intensidades distintas entre as regiões geográficas e os estratos sociais dos países (BARRETO E CARMO, 1995; OPAS, 2002).
A Organização Mundial de Saúde estimou que em 2005 ocorreram 35 milhões de mortes por doenças crônicas no mundo, sendo que aproximadamente 7,6 milhões, ou 21,7%, corresponderam às neoplasias (WHO, 2005). Os linfomas correspondem ao terceiro tipo de câncer na infância mais comum em países desenvolvidos (LI et al., 2008). Já nos países em desenvolvimento, correspondem ao segundo lugar, ficando atrás apenas das leucemias. Dentre os linfomas, o mais incidente na infância é o linfoma não-Hodgkin (MINISTÉRIO DA SAÚDE, 2008a).
Caso medidas para o controle do câncer não sejam tomadas, 84 milhões de pessoas irão morrer nos próximos dez anos por esta causa. A tendência também é de acréscimo na incidência das

neoplasias. Um aumento de 50% num prazo de quinze anos é estimado, chegando-se a um total de 15 milhões de novos casos em 2020 (WHO, 2003). No Brasil, desde o ano 2000, as neoplasias correspondem à segunda causa de morte, atrás apenas das doenças do aparelho circulatório e superam o total de óbitos por causas externas (MINISTÉRIO DA SAÚDE, 2008b).

Entre 1993 e 1999 houve uma variação percentual anual na mortalidade por câncer no Brasil de 2,68% entre as mulheres e de 2,79% entre os homens. Estes valores são superiores aos observados nos períodos 1979-1985 e 1986-1992 (MINISTÉRIO DA SAÚDE, 2009).

A.3. CONTEXTUALIZAÇÃO DO TEMA

Pacientes com distúrbios hematológicos atendidos na rede pública de Uberaba e região são encaminhados aos ambulatórios de Hematologia do HC-UFTM, onde são atendidos pelo médico Hematologista.

De acordo com o registro estatístico do HC-UFTM no ano de 2010 o referido ambulatório atendeu 3.870 pacientes e este número é crescente, sendo que em 2012 foram atendidos 6.460 pacientes.

O registro de câncer do hospital mostra que, entre os anos de 2009 e 2011, 54 pacientes foram diagnosticados com linfoma, destes 59,25% eram do gênero masculino e 40,75% do gênero feminino. Caso o diagnóstico de linfoma seja confirmado, o médico encaminhará o paciente até uma sala reservada, na qual a aluna responsável pela aplicação do termo de consentimento explicará sobre a pesquisa e responderá a todas as dúvidas e questionamentos feitos pelo paciente.

A.4. LACUNAS NO CONHECIMENTO SOBRE O TEMA

As altas taxas de incidência e de mortalidade por câncer no mundo no início do século XXI, somadas à expectativa de acréscimo nos seus valores nas próximas décadas apontam para a necessidade urgente de se intensificar pesquisas e ações para o controle desse agravo.

Devido ao fato de que análises da incidência estimada do câncer em pacientes com imunodeficiências primárias confirmam a antiga suspeita de que os defeitos genéticos associados à imunodeficiência figuram entre os principais fatores de risco para o desenvolvimento de neoplasias em seres humanos, torna-se de suma importância a realização de estudos que visam à compreensão dos mecanismos envolvidos na resposta imune (principalmente sobre a resposta mediada por células citotóxicas como os CTLs e as células NK) como a análise da expressão de genes e proteínas envolvidos na resposta contra células tumorais nestes pacientes, para colaborar na compreensão dos mecanismos imunológicos de resposta às neoplasias hematológicas.

Em trabalho anterior, nosso grupo investigou a presença de polimorfismos de base única (SNPs) na região promotora do gene PRF1 e nos exons 2, 3 e 5 do gene da granzima B em grupos populacionais brasileiros, em associação com o Centro Regional de Hemoterapia de Ribeirão Preto/USP. Neste estudo, encontramos três novos polimorfismos na região promotora do gene da perforina e, baseado na associação dos polimorfismos encontrados, foram obtidos dez novos haplótipos. Foram realizados ensaios funcionais in vitro dos sete haplótipos mais prevalentes utilizando-se técnica de clonagem e in vivo, pela quantificação da proteína nos três haplótipos mais frequentes. Os resultados revelaram que os haplótipos apresentam diferenças significativas de expressão do mRNA e da proteína perforina (dados enviados para publicação).
A próxima etapa desta linha de pesquisa será a verificação de polimorfismos na região codificadora do gene PRF1. Também iremos verificar a expressão deste gene e associá-la a quantificação da proteína perforina dos CTLs e das células NK de pacientes com linfoma, comparando com controles saudáveis.
Visto que a resposta imune contra doenças tumorais está relacionada às células NK e CTLs, esperamos encontrar uma associação entre polimorfismos no gene PRF1 e linfoma, também esperamos encontrar uma menor expressão do gene PRF1 e uma menor quantidade de perforina nos grânulos citoplasmáticos das células de pacientes com linfomas. Ademais, a quantificação da expressão gênica de PRF1 associada aos dados de quantificação da proteína nos guiará a entender se este processo ocorre devido a mecanismos regulatórios pré ou pós-transcricionais.
Assim, estes resultados poderão auxiliar na compreensão dos mecanismos imunológicos de resposta aos linfomas".

Objetivo da Pesquisa:

Segundo o pesquisador: "1. Quantificar a expressão do gene da perforina em pacientes com linfomas e comparar com a expressão deste gene em indivíduos controles saudáveis.
2. Quantificar a produção de perforina nas células Natural Killer (NK) e CTLs de pacientes com linfomas e comparar com a quantificação desta proteína em indivíduos controles saudáveis.
3. Associar os dados de expressão gênica e quantificação da proteína perforina nos pacientes com linfomas.
4. Investigar a presença dos principais polimorfismos já descritos na literatura na região codificadora do gene PRF1.
5. Correlacionar os resultados encontrados com a resposta terapêutica e evolução dos pacientes".

Avaliação dos Riscos e Benefícios:

O presente trabalho não trará desconforto e risco aos sujeitos da pesquisa, além daqueles

inerentes à punção venosa periférica. Quanto à confidencialidade os resultados só serão informados aos próprios pacientes e controles, que serão sempre identificados por números. Os dados deste projeto só serão publicados em conjunto e não de forma individual, não permitindo dessa forma a identificação dos sujeitos.

Como benefícios, destacamos a vantagem de definir novos polimorfismos que poderão estar associados ao desenvolvimento do linfoma, e também correlacionar os resultados de expressão gênica com a quantificação da proteína perforina.

Portanto, de acordo com os resultados, haveria um melhor entendimento sobre a patobiologia do linfoma principalmente no que se diz respeito ao controle da expressão gênica no gene PRF1. A.5.1. Para os sujeitos da pesquisa

Identificação de polimorfismos na região codificadora do gene PRF1 que podem estar associados ao linfoma e ao prognóstico do paciente.

Criação de novas estratégias de condutas terapêuticas individualizadas de acordo com as características genotípicas de cada paciente.

A.5.2. Para a sociedade

Identificação de polimorfismos na região codificadora do gene PRF1 que podem estar associados ao linfoma e ao prognóstico positivo ou negativo dos pacientes.

Melhor compreendimento da resposta imune citotóxica frente às células tumorais, principalmente no que se diz respeito ao controle da expressão gênica no gene PRF1.

Criação de novas estratégias de condutas terapêuticas".

Comentários e Considerações sobre a Pesquisa:

Trata-se de emenda para alteração do vínculo da pesquisadora Dra. Vânia Tiestche de Moraes Hungria, portadora do CPF 034.487.848-18, com a CLÍNICA MÉDICA SÃO GERMANO S/S LTDA, CNPJ 59.843.888/0001-77, onde atualmente funciona seu centro de pesquisa.

Exclusão da parceria interinstitucional firmada com o Dr. Angelo Maiolino, vinculado ao Hospital Universitário Clementino Fraga Filho, da Universidade Federal do Rio de Janeiro, uma vez que, por motivos de logística em relação ao envio das amostras que seriam coletadas, esta parceria não foi efetivada. E a alteração do título do projeto original para que passasse a constar: "ANÁLISE DO GENE DA PERFORINA EM LINFÓCITOS CITOTÓXICOS DE PACIENTES COM NEOPLASIAS LINFOPROLIFERATIVAS". Assim, solicito que este título também seja alterado na Plataforma Brasil".

Considerações sobre os Termos de apresentação obrigatória:

Todos os termos necessários foram apresentados adequadamente.

Conclusões ou Pendências e Lista de Inadequações:

De acordo com as atribuições definidas na Resolução CNS 466/12 e norma operacional 001/2013, o colegiado do CEP-UFTM manifesta-se pela aprovação do protocolo de pesquisa proposto, situação definida em reunião do dia 16/03/2018.

Considerações Finais a critério do CEP:

A aprovação do protocolo de pesquisa pelo CEP/UFTM dá-se em decorrência do atendimento à Resolução CNS 466/12 e norma operacional 001/2013, não implicando na qualidade científica do mesmo.

Conforme prevê a legislação, são responsabilidades, indelegáveis e indeclináveis, do pesquisador responsável, dentre outras: comunicar o início da pesquisa ao CEP; elaborar e apresentar os relatórios parciais (semestralmente) e final. Para isso deverá ser utilizada a opção 'notificação' disponível na Plataforma Brasil.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_1070992_E1.pdf	08/02/2018 13:16:15		Aceito
Outros	EMENDA_08_02_2018.docx	08/02/2018 13:13:22	Bruna Maria Bereta de Souza	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_ATUAL.docx	08/02/2018 13:12:48	Bruna Maria Bereta de Souza	Aceito
Folha de Rosto	Folha_de_rosto.pdf	08/02/2018 13:03:34	Bruna Maria Bereta de Souza	Aceito
Projeto Detalhado / Brochura Investigador	Projeto detalhado ajustado.doc	11/08/2015 10:46:13		Aceito
Projeto Detalhado / Brochura Investigador	Projeto detalhado digitalizado - 2677.pdf	11/08/2015 10:43:11		Aceito
Parecer Anterior	parecer de aprovação do projeto proto2677.pdf	07/08/2015 10:10:51		Aceito
Outros	2677.pdf	12/06/2015 16:19:42		Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

UBERABA, 21 de Março de 2018

Assinado por:
Alessandra Cavalcanti de Albuquerque e Souza
(Coordenador)

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