Impaired NK cell cytotoxicity in multiple myeloma caused by the homozygous A91V polymorphism in the perforin gene: a case report

Citotoxicidade de células NK prejudicada no mieloma múltiplo causada pelo polimorfismo A91V em homozigose no gene da perforina: um relato de caso

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ABSTRACT

We describe a 58-year-old man diagnosed with IgG/Kappa multiple myeloma (International Staging System III) treated for eight years with polychemotherapy (VAD schee) and autologous peripheral hematopoietic stem cell transplantation. The patient studied was homozygous C272T polymorphism ($PRF1^{272T/T}$) by analysis of perforin gene by direct sequencing. This SNP is considered pathogenic and leads to the substitution of the amino acid alanine for valine in codon 91 of the perforin protein. The cytotoxic lymphocytes (CLs) of the patient and of the healthy wild homozygous individual were evaluated for their cytotoxic capacity. Our results show that $PRF1^{272T/T}$ effector cells had significantly reduced ability to induce specific lysis of K562 cells. The NK cells of the patient had three times less intracellular perforin than observed in the wild-type individual. The gene expression of *PRF1* and *FAS* did not differ between the individuals, however the expression of *GZMB* was approximately 2.5 times higher in the patient. 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. In conclusion, functional analysis of the CLs of the patient revealed a significant decrease in their cytolytic capacity as well as the amount of perforin present in NK cell granules.

Keywords: perforin, granzyme b, multiple myeloma, natural killer cell, case report.

RESUMO

Descrevemos um homem de 58 anos com diagnóstico de mieloma múltiplo IgG/Kappa (International Staging System III) tratado há oito anos com poliquimioterapia (esquema VAD) e transplante autólogo de células-tronco hematopoiéticas. O paciente estudado era homozigoto para o polimorfismo C272T ($PRF1^{272T/T}$) pela análise do gene da perforina por sequenciamento direto. Este SNP é considerado patogênico e leva à substituição do aminoácido alanina por valina no códon 91 da proteína perforina. Os linfócitos citotóxicos (LCs) do paciente e de um indivíduo homozigoto selvagem saudável foram avaliados quanto à sua capacidade citotóxica. Nossos resultados mostram que as células efetoras $PRF1^{272T/T}$ reduziram significativamente a capacidade de induzir lise específica de células K562. As células NK do paciente tinham três vezes menos perforina intracelular do que o observado no indivíduo selvagem. A expressão gênica de *PRF1* e *FAS* não diferiu entre os indivíduos, porém a expressão de *GZMB* foi aproximadamente 2,5 vezes maior no paciente. Também foi observado que a expressão de *T-BET* foi aproximadamente 1,7 vezes maior e a expressão de *IFN-γ* foi 4,5 vezes maior no paciente PRF1272T/T. Em conclusão, a análise funcional dos LCs do paciente revelou uma diminuição significativa em sua capacidade citolítica, bem como na quantidade de perforina presente nos grânulos das células NK.

Palavras-chave: perforina, granzima b, mieloma múltiplo, células natural killer, relato de caso.

1 INTRODUCTION

The *PRF1* gene, located on the human chromosome 10q22, encodes perforin (1). Perforin is a pore-forming protein critical to the functioning of cytotoxic lymphocytes (CLs), comprised primarily of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. 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 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 afflicted 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 caused by the homozygous A91V polymorphism in the perforin gene.

2 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.
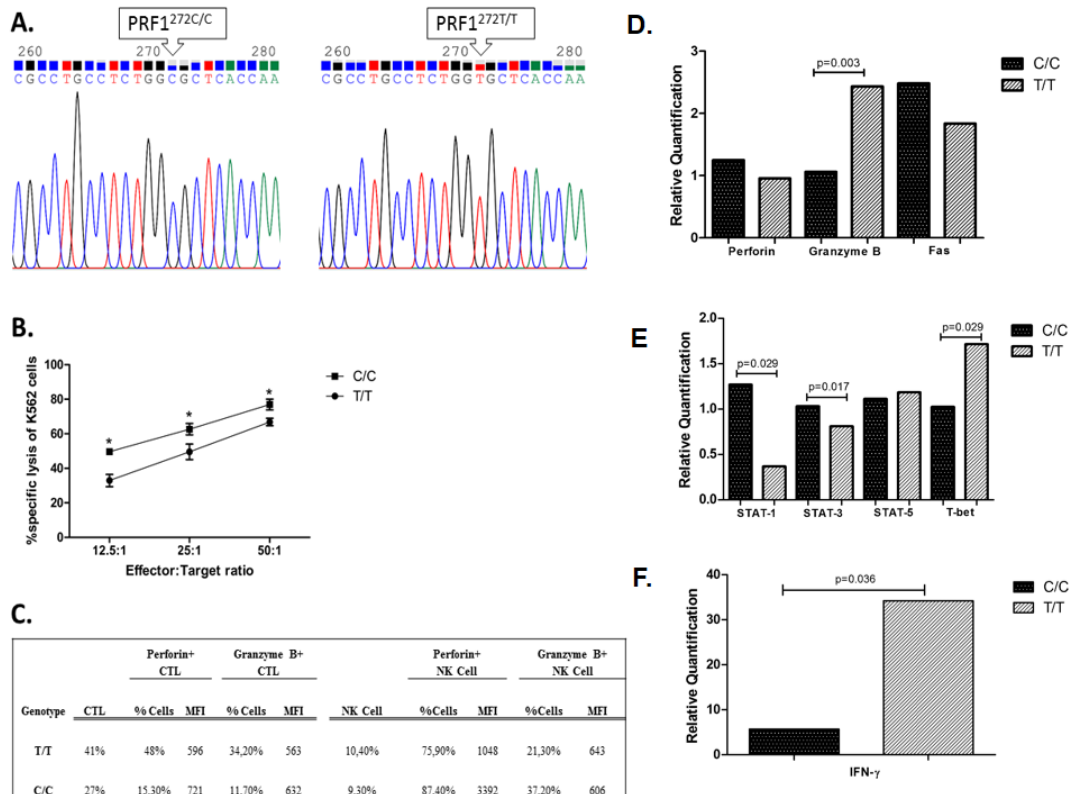
The perforin gene of patient was analyzed by direct sequencing and the homozygous C272T polymorphism (PRF1$^{272T/T}$) was identified. We performed a functional analysis of the CLs of a PRF1$^{272T/T}$ patient and a healthy individual PRF1$^{272C/C}$. Our results show that PRF1$^{272T/T}$ effector cells 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 gene expression of *PRF1* 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).

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.

		Perforin+ CTL		Granzyme B+ CTL			Perforin+ NK Cell		Granzyme B+ NK Cell	
Genotype	CTL	%Cells	MFI	%Cells	MFI	NK Cell	%Cells	MFI	%Cells	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

3 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).

It was noted that the PRF1$^{272T/T}$ patient was diagnosed with advanced stage MM (International Staging System III) at 49 years of age after suffering a femoral fracture and being referred for investigation. This age is considered young as the mean age at diagnosis in Brazil is 61 years (14). In Brazil the diagnosis is often late and approximately 70% of the patients already present stage III disease (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 could be responsible for the lower global 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.

4 SUPPLEMENTARY MATERIAL

4.1 MATERIALS AND METHODS

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

4.1.2 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.

4.13 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.

4.1.4 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.

5 COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest: The authors declare that they have no conflict of interest.

Consent: Written informed consent was obtained from the patient for publication of this case report.

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