UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA: CIÊNCIAS MÉDICAS

POLIMORFISMO DOS GENES KIR E HLA: ESTUDO EM PACIENTES COM DIABETE MELLITUS TIPO 1 E NA POPULAÇÃO CAUCASÓIDE DO RS

Mariana de Sampaio Leite Jobim Wilson

Orientador: Prof. Dr. Rafael Roesler

Tese de doutorado

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Sumário

ABREVIATURAS E SIGLAS

DM1: diabete mellitus tipo 1

HLA: *Human Leukocyte Antigen* - Antígenos Leucocitários Humano

IL: *Interleukin* - Interleucina

KIR: *Killer Immunoglobulin Like Receptor* – Receptor do tipo Imunoglobulina da Célula NK

NK: *Natural Killer Cells* - Células Matadoras Naturais

PCR: *Polimerase Chain Reaction* - Reação em Cadeia da Polimerase

SSP: *Sequence Specific Primers* - Sequência de Primers Específicos

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RESUMO

Nosso estudo teve como um dos objetivos analisarmos a frequência do polimorfismo dos genes KIR na população caucasóide do Rio Grande do Sul e comparamos com resultados de outros Estados do Brasil e também de outros países. Em um segundo momento, avaliar a associação entre os genes KIR em pacientes com diabete Mellitus tipo 1 (DM1) e controles saudáveis. As células *natural killer* (NK) fazem parte do sistema imune inato e reconhecem as moléculas HLA (antígeno leucocitário humano) de classe I em células-alvo através de seus receptores de membrana. Os receptores principais das células NK são conhecidos como receptores killer do tipo imunoglobulina (KIR) e estão localizados no cromossomo 19q13.4. Estão divididos em grupos funcionais inibidores e ativadores. No primeiro artigo, o objetivo era de analisar os genes KIR e HLA -A, -B e -Cw em 200 indivíduos saudáveis do Rio Grande do Sul pela técnica PCR-SSP. A população do Sul do Brasil demonstrou similaridades com Estados mais próximos geograficamente, e diferenças principalmente com o norte e nordeste. O gene KIR2DS5 foi o menos frequente na população estudada, e a interação KIR ⁄HLA foi mais comum na associação 2DS1-⁄2DL1+⁄C2+. No segundo artigo, analisamos 15 genes KIR e os alelos do sistema HLA classe I em 248 pacientes caucasóides brasileiros com DM1 e em 250 controles saudáveis, usando a técnica de PCR com *primers* específicos (PCR-SSP). O genótipo 2DL1/C2+ foi mais comum em controles (p=0.001), assim como o haplótipo KIR2DL2/DR3/DR4+ e KIR2DL2/DR3+ ($p<0.001$; $p<0.001$).

Palavras Chave: genes KIR, células Natural Killer, DM1, população do Sul do Brasil

INTRODUÇÃO

As células "*natural killer*" (NK), assim como os linfócitos T e B, são originadas na medula óssea. As primeiras fazem parte do sistema imune inato, tendo habilidade de destruir células alogênicas, células modificadas por vírus e células tumorais. Além disso, podem secretar citocinas, as quais modulam o sistema imune adaptativo. Estas, através dos seus receptores de superfície, podem ser ativadas ou não, a destruir as suas células-alvo [1,2].

As células NK reconhecem as moléculas de HLA ("*human leucocyte antigen*" ou antígeno leucocitário humano) de classe I, presentes nas células-alvo, por intermédio de uma família de receptores de superfície envolvida na sua atividade citolítica. Os principais receptores das células NK são os KIR ("*killer immunoglobulin-like receptors*") [3].

A atividade citolítica da célula NK depende da integridade do HLA de classe I, expresso na superfície da célula-alvo e de um KIR específico, expresso na célula NK. Quando existir a interação apropriada entre o KIR e o HLA de classe I, acontece a inibição da célula NK, não ocorrendo o ataque da célula alvo. Caso contrário, a célula alvo é destruída [4].

Os genes KIR que codificam estes receptores estão localizados no cromossomo 19q13.4, junto com todos os outros genes do complexo de receptores leucocitários [5]. A família KIR é altamente polimórfica e o seu funcionamento regula a função da célula NK e interfere na fisiopatologia de várias doenças, entre elas a psoríase vulgar, a esclerose sistêmica, a artrite reumatóide, a diabete tipo 1, a imunodeficiência humana adquirida (AIDS), a hepatite C, a bronquiectasia idiopática, os abortos espontâneos de repetição, a endometriose, a doença de Crohn e alguns tumores [6,7,8,9,10,11,12,13,14,15,16,17,18,19].

Portanto o presente estudo tem como objetivo estudar a associação da Diabete Mellitus tipo 1 (DM1) e os genes KIR, mas também avaliar a frequência dos genes KIR na população do Rio Grande do Sul. Este estudo contou com apoio do FIPE-HCPA, CNPq, INCT-TM e SOAD. O estudo foi previamente aprovado pelo Comitê de Ética em Pesquisa.

1 REVISÃO DA LITERATURA

1.1 CÉLULAS NATURAL KILLER

As células NK são linfócitos que diferem das células T e B. Morfologicamente são maiores e apresentam citoplasma granular, representando de 10 a 15 % dos linfócitos do sangue. Estão também presentes, em menor frequência, no pulmão, trato gastrointestinal e útero. As células NK são raras nos linfonodos e medula óssea e não circulam pela linfa [2]. As células NK são precursoras da medula óssea e não dependem do timo para sua maturação.

As células NK fazem parte da resposta imune inata, sendo a primeira linha de defesa do organismo contra vírus, bactérias, tumores e microorganismos. Distinguimos as células NK pela falta do receptor de célula T (TCR) e imunoglobulinas em sua membrana e pela presença de antígenos de superfície CD56 e/ou CD16 [1,2].

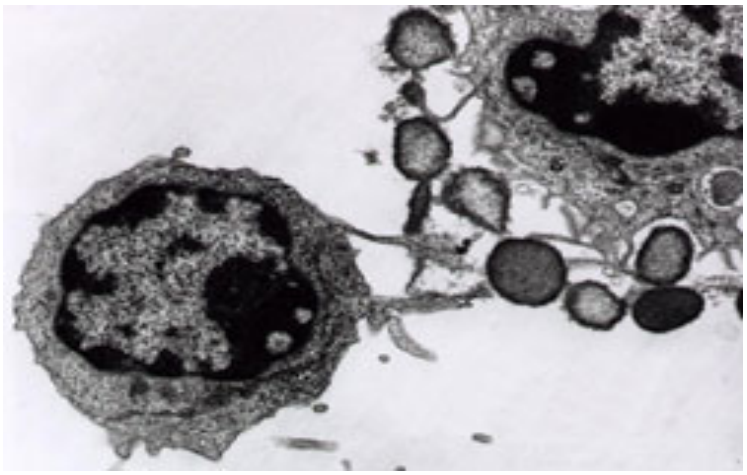

Figura 1: Célula NK lesando a célula tumoral.

A produção de citocinas por estas células é bastante variada, entre elas encontra-se a IL-3, IL-5, IL-10, IL-13, TNF-α, TGF-β1, GM-CSF, INF-γ. A liberação das citocinas regula a atividade do sistema imune, principalmente a função de macrófagos, linfócitos, hemácias e células dendríticas [20].

A ativação das células NK por citocinas, principalmente IL-2, através das cadeias β e γ das células e do receptor (IL-2R) aumentam bastante a atividade citotóxica e de proliferação celular. A IL-15, que é produzida principalmente por macrófagos, também tem um papel importante na ativação celular, aumentando a resposta contra vírus [21,22].

As células NK são capazes de mediar a resposta do sistema imune inato contra células infectadas por vírus e células malignas transformadas. Isso implica que, durante a resposta imune, podem provocar um ataque direto às células-alvo e interagir com as células dendríticas em tecidos periféricos inflamados [1,2]. Para isso, elas usam dois diferentes mecanismos citolíticos. O primeiro é a apoptose mediada por grânulos, no qual

dependem da ação de perforinas e granzimas [23]. O segundo é a apoptose induzida pela interação Fas/FasL [24]. Também pode ocorrer o ataque indireto às células-alvo, através de características da resposta imune adaptativa [25].

A atividade citolítica e a produção de citocinas pelas células NK estão reguladas pela ativação ou inibição de receptores na superfície da célula. Os receptores compreendem famílias distintas: com domínios tipo lectina (CD94/NKG2A, ligante do HLA-E com função inibidora; e NKG2D, ligante do MICA com função ativadora) e com domínios do tipo imunoglobulina (KIR) [26,27]. Os receptores de leucócitos com domínio tipo imunoglobulina ("leukocyte Ig-like receptors" – LILR) são também expressos em células B e T, não sendo específico das células NK [28].

1.2 Receptor KIR

Os receptores KIR são representantes da família das imunoglobulinas presentes na superfície celular, sendo expressos principalmente em células NK e em alguns linfócitos T [29,30]. Os genes KIR que codificam estes receptores estão localizados no cromossomo 19q13.4, junto com todos os outros genes do complexo de receptores leucocitários. A família KIR é altamente polimórfica, existindo atualmente mais de 17 genes [31].

A descoberta do KIR adicionou mais uma importante função as moléculas do HLA, codificados no cromossomo 6. As células NK reconhecem as moléculas de HLA de classe I clássicas (HLA-A, HLA-B e HLA-C) e não clássicas (HLA-E e HLA-G), presentes nas células, por intermédio desta família de receptores de superfície envolvida na sua atividade citolítica [32,33]. Os receptores KIR são resultado da expressão deste sistema genético e estão divididos em grupos funcionais inibidores e ativadores [34,35].

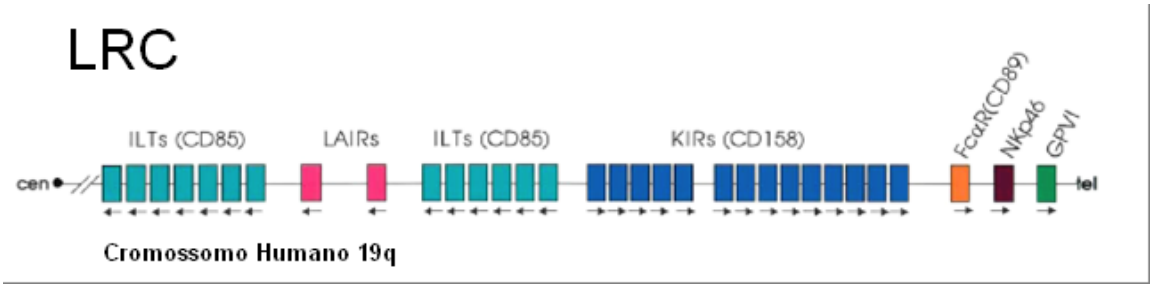

Figura 2: Cromossomo 19

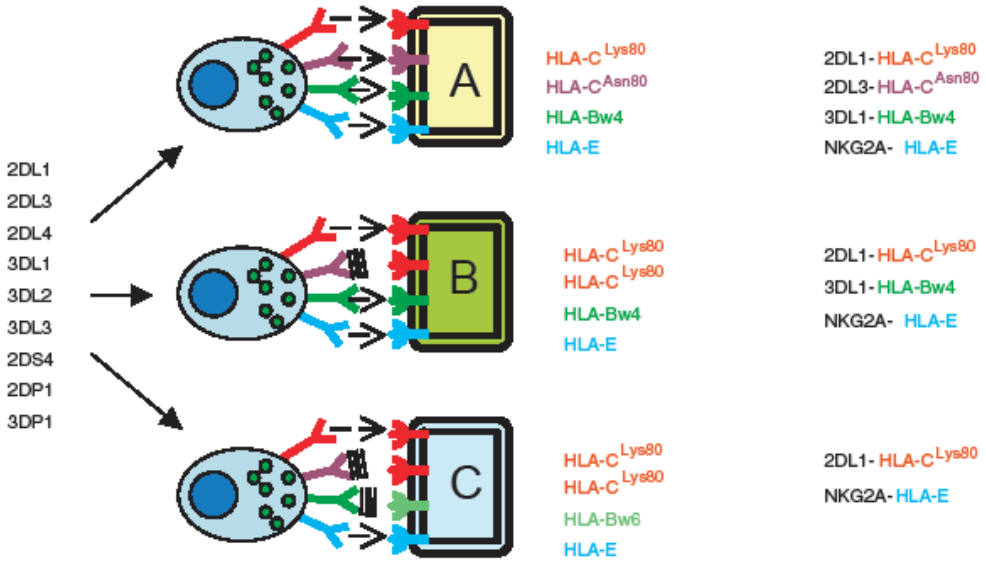

Figura 3: Interação dos receptores KIR com as moléculas de HLA

1.2.1 NOMENCLATURA DOS GENES KIR

A nomenclatura dos receptores das células NK está baseada na sua estrutura protéica extra e intracelular. Essas moléculas apresentam-se semelhantes às imunoglobulinas com dois ou três domínios extracelulares (2D e 3D) usados para ligarem-se ao sistema HLA, uma porção transmembrana e uma cauda intracitoplasmática [36].

Os receptores KIR estão divididos em grupos funcionais inibidores e ativadores. Os receptores com sinal inibitório possuem uma cauda intracitoplasmática longa, por isso recebeu em sua denominação a letra “L” (do inglês “long”). Estes evitam a lise da célula

alvo. A denominação da letra “S” (do inglês “short”) foi descrita para o receptor com cauda curta, possuindo um sinal intracelular ativador (causam a lise da célula alvo) [37,38].

As moléculas com cauda longa apresentam um ou dois imuno-receptores com características inibitórias baseadas em tirosina (ITIM ou *tyrosine-based inhibitory motifs*). Em contraste, os receptores de cauda curta não possuem ITIM, mas sim um aminoácido positivamente carregado na porção transmembrana que permite associação com a molécula acessória, DAP-12, liberando um sinal ativador por intermédio de imuno-receptores ativadores (ITAM ou *immunoreceptors tyrosine-based activating motifs*) [39].

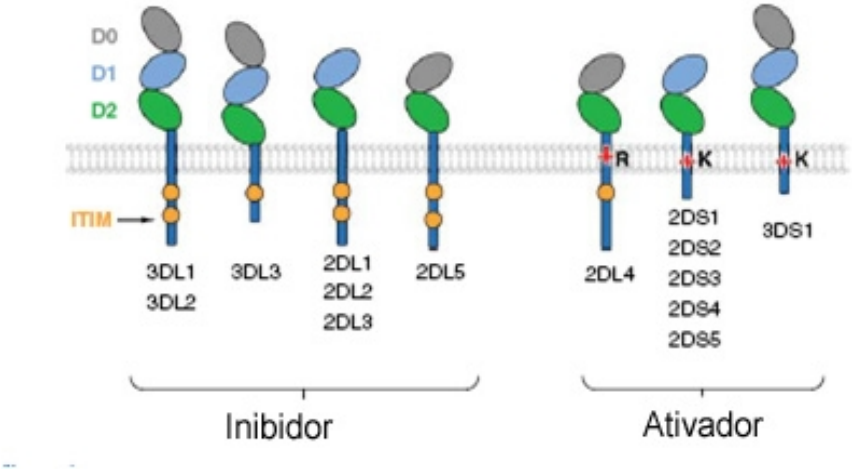

FIGURA 4: Diferenças na estrutura dos genes KIR, baseado na cauda citoplasmática e no número de domínios

1.2.2 DIVERSIDADE HAPLOTÍPICA

Todos os genes KIR estão agrupados na região do complexo de receptores leucocitários (LRC) [34]. Eles formam haplótipos que são um conjunto de genes no mesmo cromossomo e que são passados em bloco de geração a geração. Os haplótipos KIR variam em seres humanos no que diz respeito ao número de genes ativadores e inibidores e as suas formas alélicas [40]. Por causa destas variações, um grande número de haplótipos KIR foi identificado, tendo sido classificados em haplótipos A e B [41,42].

O haplótipo A possui nove genes KIR, e somente um é ativador 2DS4. Geralmente este haplótipo contém cinco genes inibitórios. Em contraste, os haplótipos B possuem uma alta diversidade de genes, tanto ativadores como inibidores. A frequência destes dois haplótipos varia significantemente em diferentes populações [42, 43].

Quatro genes KIR estão presentes em todos (ou quase todos) os haplótipos e são chamados de genes estruturais ou de “moldura”, dentre eles: 3DL3, 3DP1, 2DL4 e 3DL2 [44].

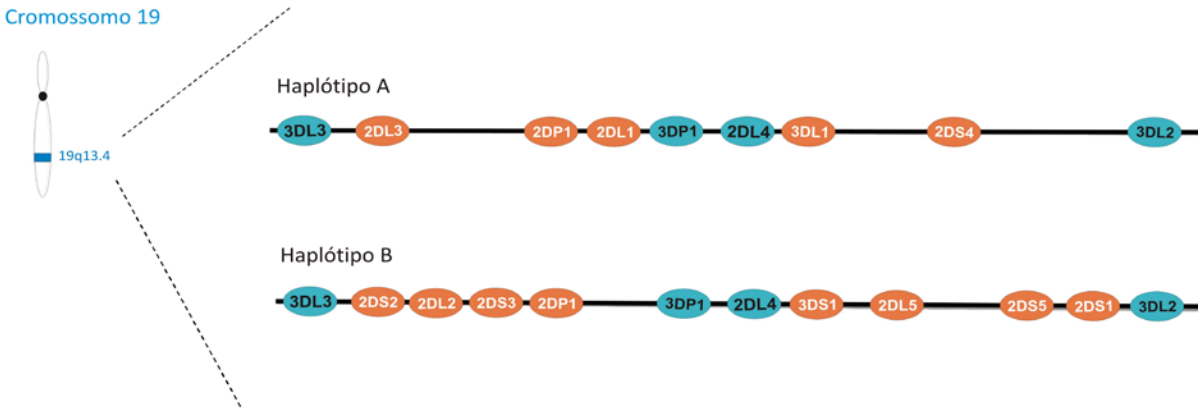

Figura 5: Haplótipos A e B

Os genes KIR3DL1, 2DS4, 2DL5, 2DS5, 3DS1 e 2DS1 localizam-se na região do telômero. Na região do centrômero encontram-se os genes KIR 2DL3, 2DP1, 2DL1, 2DS2, 2DL2, e 2DS3. Enquanto os genes 3DP1 e 2DL4 encontram-se entre as duas regiões, o KIR3DL3 encontra-se ao lado do centrômero e o KIR3DL2 localiza-se ao lado do telômero [45].

1.2.3 Ligantes dos Receptores KIR

As células NK reconhecem uma célula estranha através da ligação dos receptores KIR com as moléculas de HLA de classe I [38, 46].

O dimorfismo nas posições 77 e 80 da sequência do aminoácido define 2 alotipos do HLA-Cw distintos serologicamente. O grupo 1 (C1) tem um resíduo de serina na posição 77 (Ser77) e aspargina na posição 80 (Asn80), e o grupo 2 (C2) tem a presença de um resíduo de aspargina na posição 77 (Asn77) e lisina na posição 80 (Lys80) [47,48,49].

Os receptores KIR também podem ser diferenciados em dois grupos de ligantes. O primeiro grupo possui um resíduo de lisina na posição 44 do domínio D1, e corresponde aos receptores KIR2DS2, 2DL2 e 2DL3. Estes reconhecem o grupo C1. Já os receptores KIR2DS1 e 2DL1 possuem uma metionina nesta posição, reconhecendo o grupo C2 [47,48,49].

O loco HLA-B pode ser dividido em dois grupos, Bw4 e Bw6. O KIR3DL1 e 3DS1 interagem com moléculas HLA-B quando sorologicamente forem Bw4, sendo que se essas apresentarem o aminoácido isoleucina (Ile) na posição 80 acontece uma forte inibição através do KIR3DL1 [50,51]. A molécula KIR2DL4 liga-se com HLA-G, tipo não clássico de HLA, com pouco polimorfismo e expresso em células endoteliais do timo, de trofoblastos fetais e córnea. O KIR3DL2 reconhece os alelos do HLA-A3 e A11 [48].

A interação KIR-HLA é diferenciada pela intensidade da ligação e afinidade entre os receptores e seus respectivos ligantes. Os receptores ligam-se fracamente aos antígenos do grupo C1 e fortemente aos do grupo C2. O receptor inibidor possui mais afinidade do que o receptor ativador. A relevância biológica da baixa afinidade não está totalmente elucidada, talvez exista para atenuar os receptores inibitórios em situações onde a inibição pode não

ser vantajosa ou para evitar a agressão das células NK contra células sadias do organismo [52].

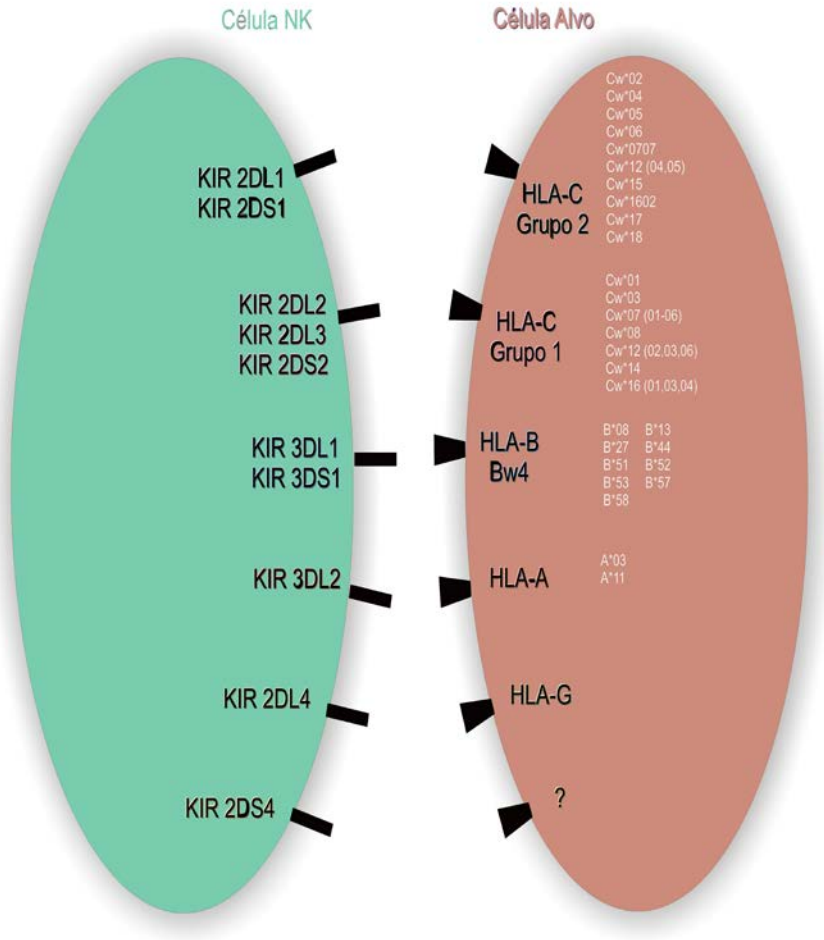

Figura 6: Receptores KIR e seus respectivos ligantes

1.2.4 MECANISMO DE AÇÃO

A atividade da célula NK depende de um determinado antígeno HLA de classe I expresso na superfície das células e de um KIR específico, ativador ou inibidor, expresso na célula NK. A interação de um KIR inibidor com determinado HLA de classe I é um evento protetor, já que evita a auto-agressão mediada pela mesma. Quando a expressão dos HLA de classe I está diminuída ou deficiente, como por exemplo, durante infecção viral ou transformação tumoral, o sinal inibitório é enfraquecido e a célula NK é ativada, subsequentemente levando à morte da célula alvo [4].

Recentemente há uma tendência de avaliar as várias combinações HLA/KIR e reproduzir modelos de ativação e inibição das células NK. Dependendo do genótipo e da presença ou ausência do ligante HLA, indivíduos poderiam ter quatro níveis de resposta celular: excesso de ativação, balanço, excesso de inibição ou indeterminado [9,53].

Na figura abaixo, observa-se a célula NK com seus receptores KIR 2DS2 e 2DL2/2DL3 reconhecendo o ligante C1/C1 e havendo uma inibição. Embora neste caso exista ativação por parte do 2DS2, a sua força é insuficiente e prevalece a inibição, fato que se explica pelo efeito superior dos receptores inibidores. Quando os mesmos receptores não reconhecem os ligantes C2/C2 provocam um sinal ativador vindo do KIR2DS2, levando a ativação. Em contrapartida, quando há presença de heterozigose para os grupos C1 e C2, tanto a inibição, quanto a situação de balanço (neutralidade) são possíveis.

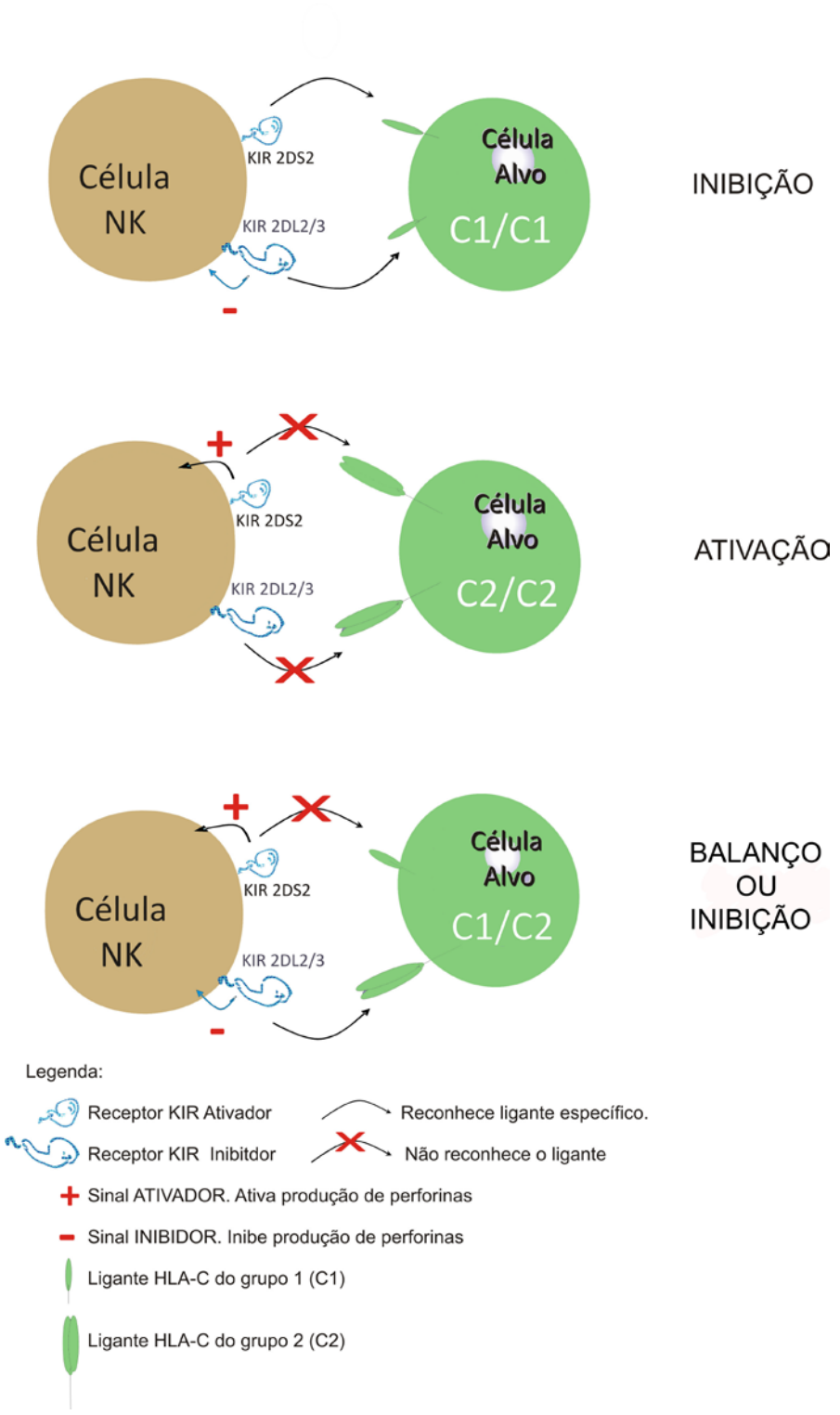

Figura 7: Ativação e inibição da célula NK através do reconhecimento dos seus ligantes.

1.2.5 KIR e Doenças

Devido à especificidade dos diversos receptores KIR para os muitos antígenos HLA de classe I, é razoável imaginar que a variação dos genes KIR afetam a resistência e suscetibilidade a várias doenças de fundo auto-imunes, infeciosas ou tumorais como a psoríase vulgar [6,7], a artrite psoriática [54,55], a artrite reumatóide [56], a DM1 [57,58,59], a doença celíaca [60], a esclerose sistêmica [61], o lúpus [62], os abortos recorrentes [63], o HIV [64], a hepatite C [65] e o citomegalovirus [66].

1.2.6 KIR e Populações

A diversidade da frequência dos alelos KIR e o elevado polimorfismo dos seus haplótipos tem sido observados entre diferentes grupos étnicos [67].

Muitos estudos confirmaram esta diversidade em diversas populações [68,69,70,71,72,73,74], identificaram novos alelos [75], e observaram semelhanças genéticas entre populações de diferentes regiões [67,76,77]. Entretanto, a variabilidade dos genes KIR nas Américas ainda foi pouco estudada [78,79,80].

2 OBJETIVOS

2.1 Objetivos Gerais

Investigar o polimorfismo dos genes KIR em um grupo de pacientes com DM1 e comparar com um grupo controle de indivíduos sadios.

Investigar os polimorfismos dos genes KIR na população caucasóide do Sul do Brasil e comparar com outras populações.

2.2 Objetivos Específicos

Avaliar a frequência dos diversos polimorfismos dos genes HLA através do método de PCR-SSP em pacientes com DM1 e grupo controle.

Avaliar a frequência dos diversos polimorfismos dos genes HLA através do método de PCR-SSP na população caucasóide do Sul do Brasil.

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3 ARTIGO ORIGINAL 1

ASSOCIATION OF KILLER CELL IMMUNOGLOBULIN-LIKE RECEPTORS AND HLA-C GENOTYPES IN SOUTH BRAZILIAN WITH TYPE 1 DIABETES.

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ABSTRACT

Type 1 diabetes mellitus (T1D) is a multifactorial and chronic autoimmune disease caused by the deficiency of insulin synthesis and or by its secretion or action defects. Genetic and environmental factors are known to be involved in its pathogenesis. The human leukocyte antigen complex (HLA) constitutes the most relevant region contributing with 50% of the inherited risk for T1D. Natural killer cells (NK) are part of the innate immune system recognizing class I HLA molecules on target cells through their membrane receptors, called killer immunoglobulin-like receptors (KIR). The aim of our study is to evaluate the association between the KIR genes and HLA alleles in patients with T1D and healthy controls. Two hundred and forty-eight T1D patients and 250 healthy controls were typed for HLA and KIR genes by PCR-SSP. Our results showed an increase of C2 in controls (P=0.002). The genotype 2DL1/C2+ was also more common in controls (P=0.001), as well as haplotype association KIR2DL2/DR3/DR4+ and the combination with only DR3+ (P<0.001; P<0.001). The maximum protection was seen when KIR2DL2/DR3- were absent when the combination of KIR2DL1/C2+ were present (P<0.001) and the maximum risk was observed when KIR2DL2/DR3/DR4+ were present in the absence of KIR2DL1/C2- (P=0.005). Our results confirmed the association of the KIR2DL2/DR3 increasing risk for T1D and suggest a protective role of KIR2DL1/C2.

KEY WORDS: Type 1 diabetes, HLA, Killer cell immunoglobulin-like receptor, Natural killer cell

INTRODUCTION

Type 1 diabetes mellitus (T1D) is an autoimmune disease in which insulin-producing pancreatic beta cells are destroyed by an aberrant T-cell mediated immune response [1]. The presence of antibodies to insulin, glutamic acid decarboxylase (GADA), tyrosine phosphatase IA-2 and, more recently, the zinc transporter (ZnT8) have been found in individuals at risk or who have recently developed T1D [2,3,4,5,6,7].

T1D is a multifactorial and polygenic disease, in which the concordance rate in twins is about 30–50%, demonstrating that genetic susceptibility is relevant to its etiology. One of the most important genetic factors known to be involved in the autoimmune pathogenesis of this disease is the human leukocyte antigen (HLA) [8,9]. HLA class II DR4 and DR3 were shown to be associated with the development of T1D, while the combination of the two susceptible alleles together, DR3/DR4, produced a higher risk [10].

HLA class I molecules are recognized by natural killer (NK) cells through killer immunoglobulin-like receptors (KIR). Inhibitory KIR molecules bind to target cell HLA class I molecules and prevent the attack of NK cells on normal cells [11]. When an activating KIR binds to its ligand, activating signals are generated leading to the destruction of target cells [12].

To date, 17 KIR genes and pseudogenes have been described on human chromosome 19q13.4 [13]. Eight KIR receptors are inhibitory *(*2DL1, 2DL2, 2DL3, 2DL5A, 2DL5B 3DL1,

3DL2 and 3DL3), seven are activating (2DL4, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 2DS5 and 3DS1) and two are pseudogenes (2DP1 and 3DP1). Of these, four KIR genes (3DL3, 3DP1, 2DL4, 3DL2) are always present and are considered framework genes [14,15].

Based on the dimorphism in position 80 (epitope for KIR binding), all HLA-C alleles can be divided into two groups: C1 group carrying asparagine and C2 group carrying lysine at this position. C1 group, consists of HLA- Cw1, -Cw3, -Cw7, -Cw8, -Cw13, -Cw14; and C2 group, consists of HLA-Cw2, -Cw4, - Cw5, -Cw6, -Cw17, -Cw 18. KIR2DL2, 2DL3 and 2DS2 bind HLA-C1 ligands, while KIR2DL1 and 2DS1 bind HLA-C2 ligands. The inhibitory KIR3DL1 recognizes HLA-B Bw4 allotypes and KIR3DL2 binds HLA-A3 and HLA-A11 [16]. However, the HLA ligands for several KIR genes are not yet identified.

This difference in the HLA-C generates different interactions with the KIR receptors. The KIR-HLA interaction is differentiated by the intensity of the connection and similarity between the receptors and their ligands. The receptors are connected weakly to antigens of group C1 and strongly in the group C2 and the inhibitory receptor has more affinity to the HLA receptors [17].

Because of KIR specificity for HLA class I allotypes, and their extensive polymorphisms, it is reasonable to imagine that KIR gene variation affects resistance and susceptibility to several diseases. KIR genotypes and HLA ligand patterns have been recognized for diseases such as hepatitis C [18], psoriasis vulgaris [19,20], psoriatic arthritis [21,22], rheumatoid

arthritis [23], celiac disease [24], ulcerative colitis [25,26], Crohn's disease [27] , HIV [28], recurrent miscarriage [29] and leprosy [30], as well as in T1D [31,32,33,34,35,36,37,38,39].

In the present study, we examined 15 KIR genes and HLA ligands in a group of two hundred and forty-eight T1D patients and compared to 250 healthy controls, aiming at the identification of patterns of KIR genotypes and HLA ligands that could be more associated with susceptibility to this disease. To the best of our knowledge, this is the first study of KIR genes in a Brazilian Caucasian population with T1D.

PATIENTS AND METHODS

Patients

In order to analyze the combination of KIR genotypes and HLA-C ligands, we studied 248 T1D Causasian children from Hospital Nossa Senhora da Conceição, with 0 to 18 years old and 250 unrelated healthy, sex and geographically matched controls, from Hospital de Clínicas de Porto Alegre, Brazil. The diagnosis was based on the concensus on T1D published by the Expert Committee on the Diagnosis for the Classification of Diabetes Mellitus [40].

This study was approved by the Research Ethics Board of Hospital de Clínicas de Porto Alegre (IRB0000921) and all parents signed an informed consent for participating in this study.

Methods

Blood samples were collected into tubes containing EDTA. DNA was extracted using salting-out procedure [41]. DNA samples were genotyped using PCR-SSP for 15 KIR genes (2DS1, 2DS2, 2DS3, 2DS5, 3DS1, 2DS4, 2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 3DL1, 3DL2 e 3DL3, 2DP1). The PCR primers and conditions were based on previous reports [42]. Internal control was included in each PCR reaction. The combination employed to achieve a 10µl volume reaction was 10 ng of genomic DNA, 500 nM specific primers, 2.5 U of Taq polymerase, 0.08µl of PCR buffer, 0.3 µl MgCl and 10 µl of distilled water, which was amplified by the Gene Amp PCR system 9700 (Perkin-Elmer, Norwalk, USA).

Temperature cycling conditions for PCR reaction were as follows: denaturation for 3 min at 94ºC, followed by 4 cycles of 15 s at 94ºC, 15 s at 65ºC, 15 s at 72ºC; 21 cycles of 15 s at 94ºC, 15 s at 60ºC, 30 s at 72ºC; 5 cycles of 15 s at 94ºC, 1 min at 55ºC, 2 min at 72ºC and a final elongation step at 72ºC for 7 min. Resulting products were visualized under ultraviolet light after electrophoresis in 1% agarose gels containing ethidium bromide.

HLA typing Cw epitope C1 (Cw 01, 03, 07 {01-06}, 08, 12 {02, 03, 06}, 14, 16 {01, 03, 04}) and C2 (Cw 02, 04, 05, 06, 0707, 12 {04, 05}, 15, 1602, 17, 18) was done using PCR-SSP, described before by Jones et al., 2006 [25]. HLA-Bw4 was also done using PCR-SSP described by Bunce et al. [43]. HLA-DR was typed by polymerase chain reaction with sequence-specific oligonucleotides (PCR-SSO) from Onelambda (LABType® SSO, Canoga Park, CA).

Statistics

Comparison of the KIR gene frequency with the control group was executed by Pearson Chi-Square with continuity correction and in a few, where the expected difference between the two groups was small, Fisher's exact test was employed. Odds ratio (OR), confidence interval (95%CI) and significance values ($p<0.05$) were calculated using SPSS for Windows version 16.0. Bonferroni correction was used to adjust for the number of genes used.

RESULTS

Individual gene frequencies for the 15 tested KIR loci are shown in Table 1. The frequencies of the KIR genes in our control group were similar to other studies reported for Brazilian populations [44,45,46]. The framework genes KIR2DL4, KIR3DL2 and KIR3DL3 were present in all individuals as expected, and every individual also carried either one or both of KIR3DL1/KIR3DS1 and KIR2DL2/KIR2DL3, which segregate as alleles at the same locus.

Overall, there were no significant differences in the frequency of any of the 15 KIR genes in the patient cohort compared to the control group. HLA-C group 2 were increased in controls when compared to T1D patients (P=0.002). No significant differences in HLA-C group 1 and Bw4 frequencies were detected between T1D patients and controls.

The status of activating genes KIR2DS1 and KIR2DS2 were analyzed in conjunction with the presence of their HLA-C ligand (table 2). However, no significant differences were found. When we analyzed KIR2DL1/C2 ligand, we found a protective factor for T1D when

compared to controls (P=0.001). There was no association of T1D and other combinations of inhibitory KIR genes and corresponding ligands (table 2).

With the intention of observing how KIR genes affect the disease, we stratified our patients and controls for the presence of KIR2DL2 with HLA class II alleles HLA-DR3, -DR4, and -DR3/DR4 in both groups (table 3). We found that individuals carrying KIR2DL2 gene together with HLA class II alleles were more likely to be patients than controls, with exception of HLA DR4, which showed no significant association. The gene HLA DR3 associated with KIR2DL2 was increased in patients when compared to controls, these difference reaching statistical significance (P<0.001). When we analyzed the patients by HLA haplotype DR3/DR4, the association was even greater, showing a higher risk for T1D (P<0.001).

A further analysis was made between the different combinations of inhibitory KIR2DL1 with their corresponding HLA-C ligand and KIR2DL2 with high-risk for T1D HLA class II alleles (table 3). The reason for making this association was to explain the effect of genetic variation at the KIR locus and its ligand in combination with other genes which show disease susceptibility. The presence of activated KIR2DL1/C2+ in the absence of KIR2DL2/DR3- as well as KIR2DL2/DR3/DR4- was increased in controls, inferring protection for T1D (P<0.001; P<0.001). On the other hand, when the combination KIR2DL1/C2- with the 2DL2/DR3/DR4+ and 2DL2/DR3+ was present, we found a higher risk for T1D (P=0.005; P=0.005).

DISCUSSION

A publication from Van der Slik et al. [31] showed that the combination of the activating KIR2DS2 gene, together with its putative HLA ligand, was present more frequently in T1D patients than in controls (P=0.030). Shastry et al. [38] found susceptibility for T1D in the presence of KIR2DL2-C1 and the absence of 2DS1, 2DS2 (P<0.001), while Middleton et al. [33] found that KIR2DS5 was significantly decreased in patients versus controls (P=0.043). A study by Santin et al. [35] observed no association between the KIR gene content and susceptibility to T1D. Park et al. [34] found association in the group A KIR haplotypes.

In a report published on Japanese T1D patients, the authors did not detect any difference in KIR gene frequencies between patients and controls [37]. Still, their results suggest that certain combination of KIR genes might be associated with age at onset of the disease.

Our study failed to identify any association of susceptibility to disease, although we found a protective factor for inhibitory KIR2DL1 and its C2 ligand (P=0.001). When considering only the KIR2DL1 receptor without its respective ligand, statistical significance was not found. HLA and KIR interaction occurs through the innate immune response. This system is the first line of defense against pathogens, working to recognize common components of pathogens so that further immune responses can be signaled in the presence of foreign pathogens. The innate system uses multiple cell types, including macrophages, dendritic cells, NK cells, neutrophils, and epithelial cells, each of which has its own specific function in an innate response. NK cells are involved in destroying target cells, as well as interacting with antigen presenting cells and T-cells [47]. Although a reduced activation of NK cells has

been reported in long standing type 1 diabetes [48], it is unclear whether this alteration is a consequence rather than a cause of disease, since prolonged hyperglycemia could also explain this phenomenon.

Other studies suggest that the balance between innate and acquired immunity is important, so that an imbalance could lead to T1D. Nikitina-Zake et al. [32] found combined association of MICA4 and KIR2DL2. The same author found maximum risk when KIR2DL2 and DR3/DR4 were together. When analyzing the combination KIR2DL2 and HLA DR3, we also found a risk associated with the disease, but we did not find association between KIR2DL2 and HLA DR4. Furthermore, the combination of inhibitory receptor KIR2DL2 in the presence of haplotype DR3/DR4 was highly significant, leading to greater susceptibility to T1D.

Our study found a strong protective factor for gene 2DL1 with its C2 ligand and inhibitory 2DL2 obtained a high risk factor when associated with HLA-DR3/DR4. We observed that this combination has a greater protective factor in the absence of 2DL2 concurrently with HLA DR3 ($P<0.001$) and the haplotype DR3/DR4 ($P<0.001$). Furthermore, when the 2DL2 with HLA DR3 and DR4 are present, the combination 2DL1/C2 failed to protect, and individuals have certain susceptibility for disease. However, when the patients have absence of 2DL1/C2 but showed KIR2DL2 with HLA haplotype DR3/DR4, the susceptibility for disease were higher. Individuals who did not have this haplotype, but showed only the DR3 also had a high risk factor.

Recently, Ramos-Lopez et al. [39] investigated the rs2756923 polymorphism (G/A), which according to the location within the KIR2DL2 gene conferred susceptibility to T1D in a series of individuals from Germany and Belgian. They observed that genotype 'GG' was more frequent in T1D than in healthy controls. Perhaps this mutation in the receptor KIR2DL2 may explain the difference found between our study and others.

Collectively, the above mentioned results, as well as those obtained by other groups, suggest that various genetic factors could be of importance for the development of T1D. Furthermore, several polymorphisms in a given individual may contribute to the individual risk of developing the disease. Our data, combined with other publications, points to a significant association of the KIR gene system with T1D, suggesting that KIR genes may have a pathogenic role in this disease.

CONFLITS OF INTEREST

The authors state no conflict of interest.

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Tables

Table 1. KIR gene frequencies (%) in healthy unrelated individuals (n=250) and T1D (n=248).

KIR gene	Controls		T1D		P-value*
	N	%	N	%	
2DL1	244	97.6	237	95.6	NS
2DL2	136	54.4	122	49.2	NS
2DL3	216	86.4	218	87.9	NS
2DL4	250	100.0	246	99.2	NS
2DL5	124	49.6	139	56.0	NS
3DL1	244	97.6	236	95.2	NS
3DL2	250	100.0	248	100.0	NS
3DL3	250	100.0	248	100.0	NS
2DS1	91	36.4	115	46.4	NS
2DS2	134	53.6	131	52.8	NS
2DS3	83	33.2	84	33.9	NS
2DS4	238	95.2	236	85.2	NS
3DS1	106	42.4	118	47.6	NS
2DP1	250	100.0	248	100.0	NS
2DS5	85	34.0	92	37.1	NS
Bw4	171	68.4	188	75.8	NS
C1	180	72.0	186	76.2	NS
C2	179	71.6	144	58.4	**0.002**

T1D: Type 1 Diabetes Mellitus

*Chi-Square Test or Fischer's Exact Test with Bonferroni correction

C1 group: HLA-Cw 01, 03, 07 (01-06), 08, 12 (02, 03, 06), 14, 16 (01, 03, 04)

C2 group: HLA-Cw 02, 04, 05, 06, 0707, 12 (04, 05), 15, 1602, 17, 18

Bw4: HLA-B 08, 13, 27, 44, 51, 52, 53, 57, 58

Table 2 . KIR combinations and HLA ligands frequencies in healthy controls (250) and T1D (n=248).

	Controls		T1D		P-value*
	n	(%)	n	(%)	
2DL2/ C1	102	(40.8)	94	(37.9)	NS
2DS2/ C1	100	(40.0)	101	(40.9)	NS
2DL3/ C1	153	(61.2)	165	(67.3)	NS
3DL1/ Bw4	166	(66.4)	179	(72.2)	NS
3DS1/ Bw4	67	(26.8)	91	(36.7)	0.018
2DL1/ C2	176	(70.4)	137	(55.6)	0.001
2DS1/ C2	63	(25.2)	67	(27.1)	NS

*Chi-Square Test
T1D: Type 1 Diabetes Mellitus
C1 group: HLA-Cw 01, 03, 07 (01-06), 08, 12 (02, 03, 06), 14, 16 (01, 03, 04)
C2 group: HLA-Cw 02, 04, 05, 06, 0707, 12 (04, 05), 15, 1602, 17, 18
Bw 4: HLA-B 08, 13, 27, 44, 51, 52, 53, 57, 58

Table 3 . KIR and HLA ligands in controls (n=250) and T1D (n=248)

	Controls		T1D		P-value*	OR	95%CI
	(n/x)	%	(n/x)	%			
KIR2DL2+							
DR3	(14/53)	26.4	(39/53)	73.6	*<0.001*	**7.76**	3.02 - 20.21
DR4	(31/68)	45.6	(37/68)	54.4	NS	-	-
DR3 / DR4	(5/30)	16.7	(25/30)	83.3	*<0.001*	**25.0**	5.52 - 122.42
KIR2DL1/C2 -							
KIR2DL2/DR3 **+**	(3/17)	17.6	(14/17)	82.4	*0.005*	**21.78**	2.98 - 183.40
KIR2DL2/DR3/DR4 **+**	(1/11)	9.1	(10/11)	90.9	*0.005*	**100.0**	4.1 - 4743.9
KIR2DL1/C2 +							
KIR2DL2/DR3 **+**	(11/36)	30.6	(25/36)	69.4	*0.008*	**5.17**	1.7 - 15.94
KIR2DL2/DR3/DR4 **+**	(4/19)	21.1	(15/19)	78.9	*0.008*	**14.06**	2.42 - 89.89
KIR2DL2/DR3 **-**	(148/221)	67.0	(73/221)	33.0	*<0.001*	**0.24**	0.16 - 0.36
KIR2DL2/DR3/DR4 **-**	(155/238)	65.1	(83/238)	34.9	*<0.001*	**0.29**	0.19 - 0.42

*Chi-Square Test or Fisher Exact Test
T1D: Type 1 Diabetes ; OR: Odds Ratio ; 95%CI: 95% Confidence Intervals ; n=positive ; x=total

4 ARTIGO ORIGINAL 2

Killer cell immunoglobulin-like receptor gene diversity in a Caucasian population of Southern Brazilian

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Abstract

Killer immunoglobulin-like receptors (KIR) regulate the activity of NK and T cells through interaction with specific HLA class I molecules on target cells. Diversity in KIR gene content, KIR allelic and haplotype polymorphism has been observed between different ethnic groups. However, most population studies on KIR variability have focused on Europe and Asia, while Americas, Oceania, and Africa remain poorly studied. The aim of this study was to analyze the variability of KIR genes in 200 healthy non-related individuals from the

Southern Brazilian population. KIR genes and HLA-A, B and Cw were genotyped using polymerase chain reaction-sequence-specific primers (PCR-SSP). Southern Brazilian population demonstrated several similarities to states that are closer geographically and distinct differences with Northern Brazil in the frequency of genes KIR2DS1, 2DS2, 2DS3, 2DS5, 3DL1, 3DS1, 2DL1 and 2DL2. The activating gene KIR2DS5 was the least frequent locus found in our group. Interaction of KIR/HLA was more common in the 2DS1-/ 2DL1+/C2+ association. This study demonstrated the diversity of KIR genes and of KIR/HLA association in a Caucasian group of Southern Brazil, establishing differences and similarities to other different populations.

Keywords: KIR, HLA class I, Population study, NK cell

Introduction

Human killer cell immunoglobulin-like receptors (KIR) play an important role in controlling natural killer (NK) cell function. Individuals may differ in their susceptibility to autoimmune diseases, infections, and transformed cells, as well as in the outcome of haemopoietic stem cell transplantation, depending on their KIR genes and on the presence or absence of KIR ligands (Ruggeri *et al*., 2004).

This diverse gene family, which consists of 14 genes and two pseudogenes of activating and inhibitory receptors expressed on NK cells and a subset of T cells, is located on the human chromosomic region *19q13.4*, inside the leukocyte receptor complex and has a great genomic diversity among various populations (Dupont *et al*., 1997).

Although considerable variations in allelic polymorphism occur, most KIR haplotypes belong to haplotype groups A and B. The A haplotype is defined as containing the inhibitory genes KIR3DL3, 2DL3, 2DL1, 2DL4, 3DL1, 3DL2 and a single activating gene -2DS4. In contrast, B haplotypes are more variable and characterized by the presence of more than

one activating KIR gene (Hsu *et al*., 2002; Uhrberg *et al*., 1997). Genes KIR3DL3, 3DL2, 3DP1 and 2DL4, called framework genes are found in all haplotypes.

KIR genes are grouped according to whether they have two domains (2D) or three domains (3D) and whether they carry a short (S) or long (L) cytoplasmatic tail. Those with long cytoplasmic tails containing immunoreceptor tyrosine-based inhibition motifs (ITIMs) have an inhibitory function, whereas those with short cytoplasmic tails have a potentially activating function mediated by immunoreceptor tyrosine-based activation motifs (ITAMs)(Moretta *et al*., 1995).

Human leukocyte antigen (HLA) class I molecules are recognized by KIR receptors. Based on the dimorphism in position 80 (epitope for KIR binding), all HLA-C alleles can be divided into two groups: C1 group carrying asparagine and C2 group carrying lysine at this position. KIR 2DL2, 2DL3 and 2DS2 bind HLA-C1 ligands, while KIR 2DL1 and 2DS1 bind HLA-C2 ligands. The inhibitory KIR3DL1 recognizes HLA-B Bw4 allotypes and KIR3DL2 binds HLA-A3 and HLA-A11 (O'Connor *et al*., 2007). However, the HLA ligands for several KIR are not yet identified. (Colonna & Samaridis 1995; Parham 2005).

Inhibitory KIR genes are generally dominant and prevent NK cells from killing autologous cells (Degliantoni *et al*., 1985). However, in certain diseases HLA class I expression may become down-regulated, causing inhibition to be overcome by the generation of activation signal leading to NK-mediated target lysis (Ljunggren & Karre 1990).

Because of KIR specificity for HLA class I allotypes, and their extensive polymorphisms, it is reasonable to imagine that KIR gene variation affects resistance and susceptibility to several diseases with an autoimmune basis (Nelson *et al*., 2004), such as psoriasis vulgaris (Jobim *et al*., 2008), psoriatic arthritis (Martin *et al*., 2002; Williams *et al*., 2005), rheumatoid arthritis (Majorczyk *et al*., 2007), diabetes (van der Slik *et al*., 2007), celiac disease (Santin *et al*., 2007), HIV (Long *et al*., 2008), recurrent miscarriage (Hiby *et al.,* 2008) and leprosy (Franceschi *et al.,* 2008).

Several studies have verified the KIR diversity in different populations (Becker *et al*., 2003; Bontadini *et al*., 2006; Contreras *et al.*, 2007; Gutierrez-Rodriguez *et al.,* 2006; Niokou *et al.*, 2003; Pavlova *et al.,* 2008; Velickovic *et al*., 2009) identified new alleles (Hou *et al.,* 2008), and analyzed the genetic relationships among populations from different geographical areas (Lee *et al.,* 2008; Middleton *et al*., 2008; Single *et al.,* 2007). However, KIR variability in the American continent remains poorly studied (Ewerton *et al*., 2007; Flores *et al*., 2007; Rudnick *et al*., 2008). In this paper, we investigate the frequency of the 15 KIRs genes and their HLA class I ligands in a Southern Caucasoid Brazilian population, and compare the KIR polymorphisms with those expressed in other populations, furthermore evaluating the relationship between inhibitory KIR and their ligands.

Materials and Methods

Study population

The studied population included individuals from Southern Brazil, who were registered as bone marrow donors by the Hospital de Clínicas de Porto Alegre. Two hundred unrelated healthy Caucasoid individuals between the ages of 18 and 55, from both sexes (male, 45%; female, 55%) were selected for this study. Blood samples were collected after obtaining an informed consent and authorization of the ethical committee of the Hospital de Clínicas de Porto Alegre, Brazil, and according to the Declaration of Helsinki.

DNA extraction and KIR genotyping

DNA was extracted using a salting-out procedure (Miller *et al*., 1988). DNA samples were genotyped using polymerase chain reaction–sequence-specific primers (PCR-SSP) for 15 KIR genes (2DS1, 2DS2, 2DS3, 2DS5, 3DS1, 2DS4, 2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 3DL1, 3DL2, 3DL3 and 2DP1). The PCR primers and conditions were based on previous reports (Gomez-Lozano & Vilches 2002). Internal control was included in each PCR reaction. The

combination for 10 µl volume reactions, 10 ng of genomic DNA, 500 nM specific primers, 2.5 U of Taq polymerase, 0.08 µl of PCR buffer, 0.3 µl MgCl and 10 µl of distilled water were amplified by the Gene Amp PCR system 9600 (Perkin-Elmer, Norwalk, CT). Temperature cycling conditions for PCR reaction were as follows: denaturation for 3 min at 94ºC, followed by four cycles for 15 s at 94ºC, 15 s at 65ºC, 15 s at 72ºC; 21 cycles for 15 s at 94ºC, 15 s at 60ºC, 30 s at 72ºC; five cycles for 15 s at 94ºC, 1 min at 55ºC, 2 min at 72ºC and a final elongation step at 72ºC for 7 min. Resulting products were visualized under UV light after electrophoresis in 1% agarose gel containing ethidium bromide. HLA typing A, B and Cw (HLA-Cw*01, *02, *03, *04, *05, *06, *07, *08, *12, *13, *14, *15, *16, *17, *18) alleles was also performed using PCR-SSP, described before (Bunce *et al*., 1995).

Statistical analysis

The observed gene frequency was determined by direct counting, and the differences in frequencies among the populations were evaluated using chi-square tests with *Yates'* correction and *Fisher's* exact test using SPSS 16.0. A value of *p* less than 0.05 was considered significant.

Results and discussion

The distribution of the observed frequencies (*F*%) of each KIR gene for unrelated healthy individuals from Southern Brazil is illustrated in table 1. All 15 KIR sequences tested for were detected. The most frequent genes were the framework loci KIR2DP1, 3DL2, 3DL3 (table 1) which were present in 100%. This group was followed by 2DL4, 3DL1, and 2DL1 which varied in 90%. KIR3DS1, 2DS1, 2DS3 and 2DS5 had a lower frequency (less then 50%) compared to the others. The activating gene KIR2DS5 was the least frequent locus found in this population (35 %).

Table 1. Observed KIR gene and HLA class I frequencies in the Southern Brazil (n=200)

	C1 group	C2 group	Bw4	A3	A11	2DL1	2DL2	2DL3	2DL4	2DL5	3DL1	3DL2	3DL3	2DS1	2DS2	2DS3	2DS4	2DS5	3DS1	2DP1
N	146	136	132	46	28	196	112	168	200	108	197	200	200	76	110	74	191	70	86	200
%	73.0	68.0	66.0	23.0	14.0	98.0	56.0	84.0	100.0	54.0	98.5	100.0	100.0	38.0	55.0	37.0	95.5	35.0	39.6	100.0

C1 group: HLA-Cw 01, 03, 07 (01-06), 08, 12 (02, 03, 06), 14, 16 (01, 03, 04)

C2 group: HLA-Cw 02, 04, 05, 06, 0707, 12 (04, 05), 15, 1602, 17, 18

Bw4: HLA-B 08, 13, 27, 44, 51, 52, 53, 57, 58

Both KIR and their HLA ligands display considerable genetic diversity and segregate independently from each other, resulting in variation in possible KIR and HLA ligand combinations between individuals. For that reason we typed the HLA ligands A, Bw4 and C1 and C2 groups and the results are demonstrated in table 1. The interaction of KIR/HLA is one of the key components of the innate immune system and participates in the early response against infected or transformed cells, through the production of cytokines and direct toxicity (Parham 2005). In table 2 we analyze the interaction KIR/HLA and their combinations in the Southern Brazil population. The association KIR2DS1-/2DL1+/C2+ (62,5%) was the most frequent and KIR2DS2+/2DL2-/2DL3-/C1+ (0,6%) was the least. It is rather difficult to compare KIR/HLA association to different population due to the variety of HLA and KIR polymorphism. However the existence of a stronger or weaker association in some specific population can be the key point to susceptibility or protection to various diseases (Carrington 2006).

It has been proposed that the binding and signaling of HLA ligand to activating KIRs is weaker when compared to their inhibitory counterparts (Biassoni *et al*.,1997). Perhaps the explanation for having a large amount of inhibitory KIR on cell surface and the greater affinity of these receptors is the need of protection, to promote an adequate response against infected or transformed cells. Still it is important to take into account the existence of other receptors on the NK surface, which are also responsible for similar activation (CD94:NKG2A) (Lazetic et al.,1996).

Table 2. Interaction of KIR/HLA in the Southern Brazil (N=200)

	(N)	%
KIR / C1 group (n=146)		
2DS2+ / 2DL2- / 2DL3- / C1+	(1)	0.6
2DS2- / 2DL2+ / 2DL3- / C1+	(2)	1.2
2DS2- / 2DL2- / 2DL3+ / C1+	(51)	34.9
2DS2+ / 2DL2- / 2DL3+ / C1+	(6)	4.1
2DS2- / 2DL2+ / 2DL3+ / C1+	(9)	6.1
2DS2+ / 2DL2+ / 2DL3- / C1+	(14)	9.5
2DS2+ / 2DL2+ / 2DL3+ / C1+	(51)	34.9
KIR / C2 group (n=136)		
2DS1+ / 2DL1- / C2+	(2)	1.0
2DS1- / 2DL1+ / C2+	(85)	62.5
2DS1+ / 2DL1+ / C2+	(48)	35.3
KIR / Bw4 group (n=132)		
3DS1+ / 3DL1+ / Bw4+	(53)	40.1
3DS1+ / 3DL1- / Bw4+	(1)	0.7
3DS1- / 3DL1+ / Bw4+	(76)	57.6
KIR / HLA-A group		
3DL1+ / A3+ / A11+	(3)	1.5
3DL1+ / A3- / A11+	(24)	12.0
3DL1+ / A3+ / A11-	(42)	21.5

C1: HLA-Cw 01, 03, 07 (01-06), 08, 12 (02, 03, 06), 14, 16 (01, 03, 04)

C2: HLA-Cw 02, 04, 05, 06, 0707, 12 (04, 05), 15, 1602, 17, 18

Bw4: HLA-B 08, 13, 27, 44, 51, 52, 53, 57, 58

The main purpose of our research was to compare the KIR frequency and KIR associations between Southern Brazilian populations from the State of Rio Grande do Sul (RS) with those reported in ten other populations (table 3). The observed frequencies proved to be similar to other Caucasian populations like those in Italy, Germany, France and United States.

However, significant differences were observed for some genes between our population and those from other regions: KIR2DL2 (China and South Africa), KIR2DL3 (England and South Africa) KIR3DS1 and KIR2DL5 (South Africa). There was a great variation between the Asian population (Chinese) and the African population reported. KIR3DS1 and 2DS1 frequencies were very low, while KIR2DL2, 2DL5, 2DS2 and 2DS5 were high in the

African populations. In the Asian populations, KIR2DL2, 2DL5, 2DS2 and 2DS3 had lower frequencies (table 3).

Tabela 3. KIR gene frequencies (%) in healthy unrelated individuals from different populations.

KIR gene	Brazil (n=200)	Italy[a] (n=217)	France[b] (n=38)	England[c] (n=136)	United States[d] (n=195)	South Africa[e] (n=50)	Senegal[f] (n=118)	Lebanon[g] (n=120)	Australia[h] (n=50)	China[i] (n=104)	Mexico[j] (n=86)
2DL1	98.0	95.0	100.0	91.0	96.9	96.0	100.0	99.2	84.0	99.0	100.0
2DL2	56.0	53.0	53.0	49.0	49.2	72.0**	55.0	59.2	50.0	17.3*	43.0**
2DL3	84.0	88.0	97.0	92.0**	88.7	64.0*	90.0	88.3	90.0	99.0	100.0
2DL4	100.0	100.0	100.0	100.0	100	100.0	100.0	100.0	100.0	100.0	100.0
2DL5	54.0	NT	55.0	NT	52.8	82.0*	52.0	58.3	NT	NT	49.0
2DP1	100.0	98.0	100.0	NT	97.9	98.0	100.0	NT	NT	99.0	97.0
2DS1	38.0	36.0	34.0	45.0	37.4	10.0*	13.0*	40.8	52.0	33.7	42.0
2DS2	55.0	53.0	55.0	51.0	49.7	64.0	42.0	59.2	50.0	17.3*	44.0
2DS3	37.0	33.0	42.0	24.0**	28.2**	38.0	24.0**	37.5	28.0**	12.5*	17.0*
2DS4	95.5	NT	97.0	96.0	94.9	100.0	100.0	95.0	90.0	94.3	97.6
2DS5	35.0	28.0	34.0	32.0	35.9	62.0*	30.0	30.8	NT	23.0	40.0
3DL1	98.5	96.0	89.0	97.0	94.9	100.0	99.0	95.8	90.0	94.2	99.0
3DL2	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
3DL3	100.0	100.0	100.0	NT	100.0	100.0	100.0	100.0	NT	100.0	100.0
3DS1	39.6	35.0	42.0	42.0	39.5	4.0*	4.0*	35.8	38.0	32.8	42.0

NT: Not tested

*P<0.001 **P<0.05

a- Bontadini et al. 2006
b- Frassati et al 2006
c- Norman et al 2001
d- Du et al. 2007
e- Middleton et al 2008
f- Denis et al. 2005
g- Rayes et al. 2008
h- Witt et al 1999
i- Jiang et al. 2005
j- Contreras et al. 2007

The KIR gene frequencies for this population in RS and from other states in Brazil such as Minas Gerais, Rondônia, Amazonas and Paraná are presented in table 4. The comparison between our population and the above mentioned states verified significant differences. KIR2DL2 and 2DS2 were more frequent in Province Surui, in the state of Rondônia ($p < 0.05$), and KIR2DS1 were far more frequent in Amazonia and Rondônia ($p<0,001$). KIR2DS5 and 2DS3 was increased in Amerindians of the Amazonian state ($p<0,001$; $p < 0.05$). Unfortunately, the other populations from the Northern states of Brazil were not tested for that gene.

Tabela 4. KIR gene frequencies (%) in healthy unrelated individuals from different populations of Brazil

KIR gene	Rio Grande do Sul	Paraná[a]	Belo Horizonte[b]	Amazonia[c]		Rondônia[c]	
				Amerindians	Ticuna	Province Karitiana	Province Surui
	(n=200)	(n=289)	(n=90)	(n=40)	(n=65)	(n=55)	(n=46)
2DL1	98.0	97.2	96.7	93.0	93.8	85.5**	97.8
2DL2	56	47.0	52.2	65.0	39.1	67.3	26.1**
2DL3	84	89.3	94.4	80.0	93.8	85.5	97.8
2DL4	100.0	100	100	100	NT	NT	NT
2DL5	54	52.6	58.9	85.0**	NT	NT	NT
3DL1	98.5	94.1	95.6	65.0*	93.8	81.8*	91.3
3DL2	100	100	100	98.0	NT	NT	NT
3DL3	100	100	100	100	NT	NT	NT
2DS1	38	40.8	37.8	88.0*	61.9**	80.0*	47.8
2DS2	55	47.1	53.3	58.0	39.1	67.3	26.1**
2DS3	37	26.6	38.9	10.0**	NT	NT	NT
2DS4	95.5	93.8	95.6	100	NT	NT	NT
2DS5	35	34.6	32.2	90.0*	NT	NT	NT
3DS1	39.6	39.1	41.1	70.0**	46.0	80.0*	30.4
2DP1	100	96.9	96.7	NT	NT	NT	NT

NT: Not tested
*P<0.001 **P<0.05
a- Rudnick et al. 2008
b- Middleton et al. 2008
c- Ewerton et al. 2007

In table 5, we have a clear view of which genes are presenting dissimilarity, when comparing Rio Grande do Sul to the population of other states. It is interesting to note that the Southern Brazilian population demonstrated several similarities to states that are closer geographically, like Paraná and Minas Gerais. However when we compare RS's population with the North of Brazil's (Amazonia and Rondônia) we see important differences in almost all genes, such as KIR2DS1, 2DS2, 2DS3, 2DS5, 3DL1, 3DS1, 2DL1 and 2DL2.

Four major groups make up the Brazilian population: the local indigenous population; the Portuguese, who colonized the country in the 16th century; Africans brought to Brazil as slaves; and the various other European and Asian ethnic groups who have settled in Brazil as immigrants since the mid-19th century. Intermarriage between the Portuguese and indigenous people or slaves was common in the first centuries of

colonization. While historically the major European ethnic group in Brazil was Portuguese, the subsequent waves of immigration have also contributed to the establishment of a multi-ethnical population (Parra *et al*., 2003). The later European immigrants settled in the Southeast and South of Brazil and had less intermarriage with the indigenous population, which may account for the similarity of the Southern Brazilian's genetic pattern to other Caucasoid groups throughout the world. That would be a plausible explanation for the great difference of the KIR genes between individuals of the same population.

Table 5. Comparison of killer immunoglobulin-like receptor (KIR) frequencies in Porto Alegre with those in other popu

	2DL1	2DL2	2DL3	2DL5	2DS1	2DS2	2DS3	2DS4	2DS5	3DS1	3DL1
RS *vs* Parana[a]											
RS *vs* Rio de Janeiro[b]											
RS *vs* Amazonian Amerindians[c]				P=0.004	$P=1x10^{-7}$		P=0.009		$P=2x10^{-8}$	P=0.003	$P=5x10^{-9}$
RS *vs* Amazonian Ticuna[c]				NT	P=0.009		NT	NT	NT		
RS *vs* Rondônia (Karitana)[c]	P=0.003			NT	$P=6x10^{-7}$		NT	NT	NT	$P=1x10^{-6}$	$P=2x10^{-5}$
RS *vs* Rondonia (Surui)[c]		P=0.003		NT		P=0.006	NT	NT	NT		

	No significance
P-value	Significant (after Bonferroni´s correction)
NT	Not tested

*KIR framework was the same in all populations
a- Rudnick *et al.* 2008
b- Meddleton *et al* . 2008

Intermarriage with the Portuguese brought the indigenous people to the cities and after some generations, they became incorporated as part of the National Brazilian population. Indians who remained in the forest or in distant restricted areas, such as protected Indian National Parks, maintain the indigenous traditions and also the oriental traits and genes. This can be seen in our study, when comparing the oriental Chinese (table 3) with Surui Indians from Rondônia (table 4). Both populations had less KIR2DL2 and 2DS2 than the Caucasian population. The genetic similarity between the Brazilian indigenous groups and that reported by the Chinese authors (Jiang *et al*., 2005) support the theory of migration of Asians through the Artic Cap toward the American continent in the distant

past. Although the Brazilian Indians seem to have their KIR pattern with an oriental influence, during the centuries they developed their own frequencies, owing to the geographical and chronological distance between them. However, they are still very diverse from their Euro-descendant fellow nationals. Comparative studies of geographically isolated people such the South American Indians are very important, as there is a genuine risk of them disappearing as a group in the future.

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CONSIDERAÇÕES FINAIS

As células natural killer fazem parte do sistema imune inato e reconhecem as moléculas HLA de classe I em células-alvo através de seus receptores de membrana. Os receptores principais das células natural killer são conhecidos como receptores KIR e estão divididos em grupos funcionais inibidores e ativadores.

A atividade da célula NK e sua interação com as células T têm importância em uma série de infecções virais e patologias auto-imunes e tumorais, tendo como resultado final a ativação da citotoxicidade com liberação de perforinas, produção de citocinas, INFγ e proliferação das células NK.

Evidências recentes sugerem que a célula NK e os diferentes genótipos KIR/HLA, podem proteger ou contribuir para a auto-imunidade. Diversos modelos foram propostos: um deles seria de que os receptores inibidores seriam mais fortes que os ativadores, ocasionando proteção para a doença. Como as células NK de um mesmo indivíduo podem apresentar diversos receptores ativadores e inibidores, um balanço na ativação e inibição parece ter importância protetora contra a auto-imunidade e outras patologias. No entanto, a falta de representação HLA na superfície de células infectadas ou tumorais estimula as células NK na destruição da célula alvo.

Muitas doenças auto-imunes já evidenciaram receptores KIR ativadores em excesso ou genótipos KIR/HLA com falta de inibidores, entre elas a esclerodermia, a DM1, a artrite

psoriática. Além desses modelos, alguns autores sugerem que a tendência à auto-imunidade ocorra devido à pobre sinalização dos receptores ativadores.

As células NK deixaram de ser conceituadas como apenas células de defesa, tornando-se um elemento-chave para regulação da imunidade inata e, portanto, podendo agir como ativadoras da resposta adaptativa. Na presença de peculiaridades na atividade de células NK, podem ocorrer condições favoráveis ao surgimento de desordens de tipo auto-imune.

A DM1 é uma doença auto-imune multifatorial e poligênica onde as células beta do pâncreas, produtoras de insulina, são destruídas pela resposta imune mediada por linfócitos T. Anticorpos anti-insulina podem ser encontrados antes do desenvolvimento da doença, quase como se fossem marcadores da mesma. A susceptibilidade genética é relevante para sua etiologia, sendo observada a frequente ocorrência da doença entre gêmeos, assim como a presença de alelos HLA-DR3 ou DR4 e a combinação DR3/DR4 está associada com o seu desenvolvimento.

A pesquisa dos genes KIR em pacientes com diabetes tem sido motivo de interesse científico, sendo que analisamos no presente estudo, 248 pacientes e 250 controles caucasóides da região sul do Brasil, tipando os genes KIR, HLA-Cw e alelos DR-3 e 4. Os grupos C1 (HLA-Cw1, Cw3, Cw7, Cw8, Cw13 e Cw14) e C2 (Cw2, Cw4, Cw5, Cw6, Cw17 e Cw18) já foram anteriormente abordados e a importância dos alelos HLA-DR3 e DR4 também já são conhecidas. Esse conjunto de informações permite analisar polimorfismos que podem proteger ou causar susceptibilidade à DM1 na população.

Não identificamos diferenças isoladas entre os genes KIR nas duas populações. Na população controle identificamos que o grupo C2 esteve mais frequente quando comparados com os pacientes (p=0,002). Outro dado observado foi na análise do KIR2DL1/C2, onde 70,4% dos controles apresentavam a combinação desse KIR inibidor com seu ligante HLA contra 55,6% dos pacientes (p=0,001). Quando analisados separadamente, KIR2DL1 e C2 não demonstraram proteção para a doença, havendo a necessidade da combinação KIR e HLA.

Com a intenção de observar como os genes KIR comportam-se na doença, estratificamos os pacientes e controles para a presença de KIR2DL2 com os alelos HLA-DR3, e DR3/DR4. Os achados mostraram frequencia aumentada da associação acima no grupo de pacientes (p<0,001).

Em outra análise mostrou máxima proteção foi vista quando KIR2DL2/DR3- estava ausente na presença da combinação KIR2DL2/C2+ (p<0.001). Já o máximo risco foi observado quando KIR2DL2/DR3/DR4+ estavam presentes na ausência de KIR2DL1/C2- (p=0.005).

Nosso trabalho, assim como o de outros demonstram que os genes KIR e seus ligantes HLA tem importância na proteção e na susceptibilidade ao DM1. Os estudos em outras populações podem identificar novas combinações de genes KIR que protegem ou predispõe para a doença. O polimorfismo, tanto do KIR como de seu ligante HLA facilita a existência de diferenças populacionais, mas sua ação cooperativa deve ser considerada como marco importante no conhecimento da doença. Nesse ponto, a imunidade inata

provavelmente associada à imunidade adaptativa protege ou estimula o surgimento da auto-imunidade como se existisse um balanço entre a saúde e a doença na dependência de fatores genéticos como os expostos, na vigência de estímulos do meio ambiente.

Outro trabalho desenvolvido e publicado versou sobre a diversidade dos genes KIR na população caucasóide do Rio Grande do Sul, analisando-se 200 indivíduos saudáveis e não relacionados. O propósito dessa pesquisa foi o de conhecer o polimorfismo desse sistema genético em nossa população, comparando-o com outras populações de nosso país e do exterior.

O haplótipo A foi o mais comum na nossa população (59%), seguido do haplótipo B (41%). Trinta e dois genótipos foram identificados, sendo que entre os mais frequentes estão o AA (18%), AB (82%), sendo não se encontramos nenhum indivíduo homozigoto BB.

Existe uma diversidade genética considerável entre o KIR e seus ligantes HLA. Por segregarem independentemente um do outro, permitem uma variação de combinações entre indivíduos. Por essa razão foram analisados os genes KIR e os ligantes HLA-A, Bw4 e os grupos C1 e C2 (tabela 1 da publicação). A frequência do grupo C1 foi de 73%, C2 (68%), Bw4 (66%), A3 (23%), A11 (14%), sendo que entre os genes KIR, os mais freqüentes foram: 2DL1 (98%), 2DL3 (84%), 2DL4 (100%), 3DL1 (98,5%), 3DL2 (100%), 3DL3 (100%), 2DS4 (95,5%), 2DP1 (100%). Os menos frequentes foram: 2DL2 (56%), 2DL5 (54%), 2DS1 (38%), 2DS2 (55%), 2DS3 (37%), 2DS5 (35%), 3DS1 (39,6%).

Foi analisada a interação KIR e seus ligantes HLA em nossa população (tabela 2), identificando-se a associação mais freqüente no grupo C1 com o KIR2DS2-/2DL2-/2DL3+/C1+ (34,9%), no grupo C2 com o KIR2DS1-/KIRDL1+/C2 (62,5%). Quando o ligante foi Bw4, a mais freqüente interação foi 3DS1-/3DL1+/Bw4+ (57,6%) e quando o ligante foi o grupo HLA-A encontramos 3DL1+/A3+/A11- (21,5%).

Observamos a frequência e a comparação dos genes KIR entre 11 populações (tabela 3). Diferenças significantes aconteceram entre a nossa população e a de outras regiões: KIR2DL2 – Brasil (56%), China (17,3 %) e África do Sul (72%); KIR2DL3 – Brasil (84%), Inglaterra (92%) e África do Sul (64%). Uma grande variação existe entre as populações Asiáticas (China) e Africanas. KIR3DS1 e 2DS1 são pouco frequentes na África do Sul (4% e 10%), existindo compensação com frequencias mais elevadas no KIR2DL2, 2DL5, 2DS2 e 2DS5. Nas populações Asiáticas encontram-se frequências baixas no KIR2DL2, 2DL5, 2DS2 e 2DS3.

Outras populações brasileiras foram comparadas com a nossa: Minas Gerais, Rondônia, Amazonas e Paraná (tabela 4). O KIR2DL2 e 2DS2 foram menos frequentes na província de Suruí no estado de Rondônia (26,1%, $p<0,05$) e o KIR2DS1 mais frequente nos estados do Amazonas e Rondônia ($p<0,001$). Entre as populações ameríndias o KIR2DS5 foi o alelo mais frequentes (90%) e o 2DS3 o menos encontrado (10%). De uma maneira geral, a população do Rio Grande do Sul apresentou uma similaridade com as dos estados mais ao sul como Paraná e Minas Gerais, existindo diferenças importantes com as populações do norte do país na maioria dos genes.

Nosso estudo possibilitou confirmar a grande diversidade genética da população brasileira, mostrando diferenças e similaridades entre os povos de várias regiões e diferentes nações. Estudos de doenças relacionadas com as células NK e seus receptores e genes KIR poderão acontecer com o conhecimento obtido nesse estudo, antecipando-se diferenças na resposta imune natural entre as populações. Devido ao interesse do estudo KIR no transplante de medula óssea, esse trabalho também permitirá futuros estudos relacionados com a imunogenética desses transplantes e na escolha de melhores doadores para os receptores com doenças hematológicas.

ANEXO

A – PROTOCOLO DE PESQUISA

I – Protocolo de coletas de dados

Projeto: **"Estudo do Polimorfismo dos Genes KIR na Diabete Mellitus tipo 1"**

( ) Diabete Mellitus tipo 1 ou ( ) Grupo Controle

Nome do paciente:_____
Número do paciente:_____

Número do prontuário:_____

Raça: _____

Data de nascimento:_____/_____/_____
Tipagem KIR e HLA:_____

_____

_____

_____

B – Termo de Consentimento Livre e Esclarecido – Pacientes

Prezado Senhor(a),

Este **Termo de Consentimento** pode conter palavras que você não entenda. Peça ao pesquisador que explique as palavras ou informações não compreendidas completamente. O(A) seu(sua) filho(a) tem a doença denominada *Diabetes mellitus tipo 1*. As causas que levam ao aparecimento dessa doença ainda não foram totalmente descobertas, portanto, estamos convidando o(a) seu(sua) filho(a) a nos ajudar a compreender esses problemas genéticos através do estudo que estamos fazendo. A chance de desenvolver o *Diabetes mellitus tipo 1* depende, entre outras coisas, das características genéticas e perfil imunológico de cada indivíduo. Algumas diferenças nos genes aumentam a chance de desenvolver problemas nos pacientes. O objetivo do trabalho é tentar associar os genes KIR em pacientes com Diabetes Mellitus.

Estamos solicitando a sua permissão para análise do DNA de seu filho. Não será necessário fazermos uma coleta de sangue, somente sua autorização para utilização do seu sangue armazenado. Com esse sangue, iremos fazer estudos do DNA (são substâncias que contém todas as informações sobre a formação do corpo humano).

Como os assuntos em medicina evoluem muito rapidamente, o sangue obtido e armazenado para esta pesquisa poderá ser analisado para outros fatores que possam vir a ser considerados relevantes dentro desta linha de pesquisa, após nova avaliação do comitê de ética. O paciente tem a total liberdade de não querer entrar no estudo, ou de sair do mesmo, quando quiser, sem que isso traga prejuízos no seu cuidado. Não há formas de ressarcimento ou de indenização decorrentes da participação na pesquisa. Você tem o

direito de solicitar informações e esclarecer dúvidas sobre a pesquisa que seu (sua) filho(a) venha a participar. A identidade de seu (sua) filho(a) será mantida em sigilo. Os resultados do estudo serão sempre apresentados como o retrato de um grupo e não de uma pessoa. A participação de seu (sua) filho(a) neste estudo é muito importante e voluntária.

O professor Balduino Tschiedel é o médico responsável pelo recrutamento dos pacientes no Hospital Nossa Senhora da Conceição de Porto Alegre e também o responsável pela análise do material cedido pelo paciente. Caso haja a necessidade de maiores explicações, a senhora poderá telefonar para 33627371 ou 33412662 (com Dr. Balduino Tschiedel).

Esse documento se encontra em duas vias de igual conteúdo e valor.

Eu, _____, (responsável legal) abaixo assinado (a), ciente dos termos acima descritos, permito a coleta do sangue para a pesquisa: "***ANÁLISE DE POLIMORFISMOS DOS GENES KIR E HLA EM PACIENTES COM DIABETES MELLITUS"***.

_____ _____

Responsável legal Pesquisador responsável

C – Termo de Consentimento Livre e Esclarecido - Controles

REGISTRO BRASILEIRO DE DOADORES VOLUNTARIOS

DE MEDULA OSSEA – REDOME

TERMO DE CONSENTIMENTO

Eu,_____, abaixo assinado(a) e acima qualificado(a), pelo presente instrumento CONSINTO que os meus dados cadastrais, o resultado de minha tipificaçao HLA e os outros resultados dos exames de histocompatibilidade / Imunogenética sejam incluidos no REGISTRO BRASILEIRO DE DOADORES VOLUNTÁRIOS DE MEDULA OSSEA - REDOME, coordenado pelo Laboratorio de Imunogenética do Instituto Nacional de Cancer — INCA, do Ministério da Saúde. A amostra coletada nesta ocasião poderá ser utilizada em possíveis testes genéticos futuros, desde que de maneira sigilosa.

Nesta data recebi as orientações sobre o que é o transplante de medula óssea e o transplante de células precursoras e estou ciente de que:

O candidato a doador de medula óssea e/ou tecidos hematopoéticos deve encontrar-se em bom estado de saúde.

Na oportunidade de ser selecionado, o doador deverá passar por exames clínicos e laboratoriais que atestem a inexistência de doença, especialmente as infectocontagiosas.

Na oportunidade de ser selecionado para doação de medula óssea, o doador passará por internação hospitalar (hospital/dia) sendo necessário submeter-se a procedimento sob anestesia geral para retirada de não mais que 10% de sua medula óssea.

O procedimento consiste em punção glútea (4 a 8 punções). A medula óssea do doador é espontaneamente restaurada em poucas semanas.

Na oportunidade de ser selecionado para doação de precursores hematopoéticos, após utilizar por via subcutânea uma medicação estimulante de células hematopoéticas, o doador será submetido a procedimento semelhante a doação de sangue sendo este realizado em caráter ambulatorial, não sendo para isso necessários os procedimentos mencionados no segundo item deste termo.

Os riscos para doadores de medula óssea e/ou tecidos hematopoéticos é praticamente inexistente. Nos casos de doação de medula óssea, devido ao procedimento de punção, é comum haver queixa de discreta dor no local da punção.

Tenho também ciência do propósito a que se destina o referido Registro e meu cadastramento nele.

Proponho-me, assim, a ser um eventual doador de medula óssea ou de células precursoras, sabendo que me é reservado o direito de decisão final para doação, mantendo-se a condição de sigilo acima especificada.

Porto Alegre, _____/_____/_____

_____
Nome Legível

_____
Assinatura

Testemunhas:

Nome legível: _____Assinatura:_____

Nome legível:_____ Assinatura:_____