Characterization of the immunogenetic markers and the impact of their polymorphisms on susceptibility to immune-related diseases in the Saudi population

Awad Elsid Osman

A doctoral report and portfolio submitted in partial fulfilment of the requirements of the University of Sunderland for the degree of PhD by Existing Published Work

PhDEx **May 2025**

The work presented in this Thesis was performed at Pathology and the Clinical Laboratory Management, King Fahad Medical City, Riyadh, Saudi Arabia. The KFMC Intramural Research Fund sponsored the entire research work that led to publications presented in this Thesis.

Contents

Abstract

Specific immune mechanisms regulate the immune system to protect the host against environmental risk factors and clear abnormal immunological responses. All immune cells originate from Hematopoietic stem cells, which differentiate into several sub-types and can communicate with each other. Human leukocyte antigen (HLA) is a group of genes encoding particular proteins responsible for immune system regulation. Killer immunoglobulin receptors (KIRs) are expressed on the surface of NK cells and work with specific HLA ligands. At the same time, cytokines can influence the immune mechanism through signalling pathways among various immune cells.

Genetic studies on immunogenetic markers provide significant information for stem cell transplantation, on disease susceptibility and on infections. Stem cell transplantation is a complicated procedure that needs specific precautions pre-transplantation. Accordingly, I have characterized in detail human leukocyte antigen (HLA), Killer immunoglobulin-like receptor (KIRs), and short tandem repeats (STRs) genes among Saudi individuals to establish reference data that may help in the transplantation process and be used as a control for disease association studies. The results demonstrated some similarities and differences for alleles and haplotypes compared to other national and international populations.

I have also focused on markers for susceptibility to type 1 diabetes mellitus and investigated the possibility of linkage disequilibrium among them. Based on that, I published three studies exploring KIRs and their HLA class I ligands, HLA-DRB1 and SNPs within the pro-inflammatory cytokines/toll-like receptor genes polymorphisms and DRB1, DRA1, DQB1 and anti-inflammatory cytokines polymorphisms. Multiple associations with T1DM that are not linked to HLA genes were observed, and the stratifications of the genes that have shown significant differences demonstrated specific models of associations that I reported for the first time.

In conclusion, data on immunogenetic markers in the Saudi population have been obtained and used for a study on genetic markers for type 1 diabetes mellitus.

Acknowledgements

The presented PhD thesis is the fruit of a long journey of my experience that initially began in Sudan through the United Kingdom and Saudi Arabia. During this trip, I passed through many stations. I was exposed to different scientific environments that helped me establish a solid base to stand on during this journey to accomplish the current marvelous and sweet achievement.

First, I would like to thank the University of Sunderland for accepting me to do a PhD by published work. I am sincerely grateful to my supervisor, Dr Noel Carter, who initially evaluated my publications and took it forward for more judgment by the University subcommittee. Also, I thank him for his kind guidance, instruction and motivation that allowed this thesis to reach the level to be presented for gaining a PhD degree through published work. Also, I would like to thank my co-supervisor, Dr Aimee Kibondo, for her help and support.

I want to thank King Fahad Medical City (KFMC), specifically the Research Center, for their generous funding for my research projects, which led to numerous published papers in peer-reviewed journals that are a part of this thesis. Also, my thanks to Pathology and Clinical Laboratory Management for their kind support and help. I feel I am lucky as I work in institutions such as KFMC that encourage their staff to conduct research besides clinical work. With the KFMC's sponsorship for these research projects, the present thesis is now possible.

I would happily acknowledge Prof. Benjamin Bradley (University of Bristol; National University of Ireland, Ireland) for his valuable supervision and support during my MSc degree; the period put me in the right direction in the research field and constituted a solid base to stand on during my research journey. His continuous support through the collaboration in my research activities at KFMC has led to multiple published scientific papers, and I happily included them in this thesis. Also, I thank Prof. Derek Middleton (Royal Liverpool and Broadgreen University Hospital, Liverpool, UK) for his meticulous contribution to a couple of papers that helped me understand the genetics of human leukocyte antigens (HLA) and killer immunoglobulin-like receptors (KIRs). I want to thank

Prof. Mohamed Mubasher, who patiently sat with me for long hours, explaining specific points and providing excellent advice on research biostatistics. Also, I thank him for his critical reviews of HLA, KIRs and STRs papers that directly impacted the qualities of these publications.

Thanks to all serology/immunology and HLA staff members at King Fahad Medical City (KFMC) who provided valuable help while conducting my research. I want to thank the previous directors of our laboratory for their encouragement and for allowing me to carry out my research work in the laboratory while I was doing the clinical parts. Specifically, I thank Dr Geyhad Elghazali (previous director) for his encouragement and for seeking valuable international connections to secure collaboration. Also, I thank Dr Abdullah Aljurryan (previous director) and Dr Ahmed Alyami (the current director) for their kind support in facilitating difficulties and keeping a suitable environment for work.

I thank Dr. Raga Rajalingam (University of California at Los Angeles, USA) for his kind help in the KIRs genotype paper. Also, I thank Dr Faviel F. Gonzalez-Galarza (University of Liverpool, UK) for his support in statistical analysis for the HLA alleles frequencies paper and for reviewing the article. My thanks also extend to Dr Habiba Alsafar (Sheikh Khalifa Medical City, UAE), Dr Michael H. Crawford (University of Kansas, USA) and Dr Jasem BJM Theyab (Kuwait University, Kuwait) for their valuable advice. I thank Dr Imad Brema (Obesity, Endocrine and Metabolism Center, KFMC, Riyadh), Dr Muaawia A Hamza (Research Center, KFMC, Riyadh), Dr Alshahid Fadil (Orthopedic department, KFMC, Riyadh), Dr Abdulwahid Al-Dehaimi (director of PCLM, KFMC, Riyadh) and Dr AlTahir Sabiel for their valuable supports and helps.

My deepest sympathy to my uncle, Professor Awad Elsid Mustafa (May Allah bless him). He passed away some time ago, but he played a significant role in my academic journey when he was alive. I miss him and love him always.

Lastly, thanks to my wife Rania and my daughters Fatima and Nusayba. I am delighted to share this PhD journey with them, and I appreciate their long-standing support, help, and spare much time for me to fulfill this marvelous achievement.

Declarations

Statement of original authorship

I, Awad Elsid Osman, certify that the submitted work is my own work and that I have not obtained a degree elsewhere on the basis of the research presented in this submitted thesis.

Awad Elsid Osman Date

Abbreviations:

ASP	Affected sibpair
APCs	Antigen-presenting cells
CLPs	Common lymphoid progenitors
CLIP	Invariant chain peptides
CSF	Colony-stimulating factors
DCs	Dendritic cells
ETP	Early thymic progenitor
ER	Endoplasmic reticulum
GD	Gene Diversity
GVHD	Graft-versus-host disease
GAD	Glutamic acid decarboxylase
HLA	Human leukocyte antigen
HSC	Haematopoietic stem cells
HWE	Hardy–Weinberg equilibrium
Ii	Invariant chain
ILCs	Innate immune lymphocytes
IFN-γ	Interferon-gamma
IL-1	Interleukin-1
IL-1R1	Interleukin-1 receptor 1
IL-1RN	Interleukin-1 receptor antagonist
IL-2	Interleukin-2

ICA	Islet-cell antibodies
IAA	Insulin autoantibodies
IRB	Institutional Review Board
IL-1	Interleukin-1
IL-12	Interleukin 12
ILC	Innate lymphoid cells
Ig	Immunoglobulin
KIRs	Killer immunoglobulin receptors
LD	linkage disequilibrium
LGL	Large granular lymphocytes
MZ	Marginal zone
MP	Matching probability
MHC	Major histocompatibility complex
MDS	Multidimensional scaling
NK	Natural killer
Pre-BCR	Pre-B cell receptor
PD	Power of discrimination
PIC	Polymorphic information content
PE	Probability of exclusion
PCA	Principal component analysis
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
SSOP	Sequence-specific oligonucleotide probe

SOT	Solid organ transplant
SA	Saudi Arabia
STRs	Short Tandem Repeats
SNPs	Single-nucleotide polymorphisms
SBT	Sequence-based typing
TDT	Transmission/disequilibrium test
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-alpha
T1DM	Type 1 Diabetes Mellitus
TLRs	Toll-like receptors
TCR	T cell receptor
UAE	United Arab Emirates

List of Publications

Publication 1: HLA-A, -B, -C, -DRB1, and -DQB1 Allele Lineages and Haplotype Frequencies among Saudis. *Immunology and Immunogenetics Insights* 2014:6 1–6. doi:10.4137/III.s16796.

Awad E. Osman *(Corresponding Author)*, Faviel F. Gonzalez-Galarza, Mohamed Mubasher, Hanan Al-Harthi, Nezar Eltayeb Elsheikh, Noureddine Berka, Derek Middleton and Gehad Elghazali.

Publication 2: Characterization of human killer immunoglobulin-like receptors (KIRs) among healthy Saudis. *Human Immunology* 75 (2014): 536–540.

Awad E. Osman *(Corresponding Author)*, Mohamed Mubasher, Nezar E. ElSheikh, Hanan AlHarthi, Ahmed S. Al Yami, Raja Rajalingam, Abdulwahid Al-Dehaimi, Derek Middleton, Gehad ElGhazali.

Publication 3: Autosomal Short Tandem Repeat (STR) Variation Based on 15 Loci in a Population from the Central Region (Riyadh Province) of Saudi Arabia. *Journal of Forensic research* 2015, 6:1 (http://dx.doi.org/10.4172/2157-7145.1000267).

Awad E Osman *(Corresponding Author)*, Habiba Alsafar, Guan K Tay, Jasem BJM Theyab, Mohamed Mubasher, Nezar Eltayeb-El Sheikh, Hanan AlHarthi, Michael H. Crawford and Gehad El Ghazali.

Publication 4: Investigation of activating and inhibitory killer cell immunoglobulin-like receptors and their putative ligands in type 1 diabetes (T1D). *Human Immunology* 77 (2016) 110–114)

Awad E. Osman *(Corresponding Author)*, Nezar Eltayeb-ELSheikh, Mohamed Mubasher, Hanan Al Harthi, Sahar Alharbi,Muaawia A. Hamza, Gehad ElGhazali.

Publication 5: Association of single-nucleotide polymorphisms in tumour necrosis factor and human leukocyte antigens genes with type 1 diabetes*. Int J Immunogenetics*. 2021; 00:1–*10 (DOI: 10.1111/iji.12535).* ***Awad Elsid Osman*** *(Corresponding author)*, Imad Brema, Alaa AlQurashi, Abdullah Al-Jurayyan, Benjamin Bradley and Muaawia Ahmed Hamza.

Publication 6: Single nucleotide polymorphism rs 2070874 at Interleukin-4 is associated with increased risk of type 1 diabetes mellitus independently of human leukocyte antigens; *International Journal of Immunopathology and Pharmacology.* 2022; Volume 36: 1–10 (DOI: 10.1177/03946320221090330). ***Awad E Osman*** *(Corresponding author)*, Imad Brema, Alaa AlQurashi, Abdullah Al-Jurayyan, Benjamin Bradley and Muaawia A Hamza.

Chapter 1. Introduction

Immune cells and cellular immune mechanisms

The immune system acts as the first line of defence against invading pathogens in the human body. This system comprises multiple biological structures, including highly specialized cells, and can differentiate between self and non-self-antigens[1]. The balance of cell components (white blood cell subpopulations) is driven by both genetic and environmental factors that influence the control of innate cell types, adaptive B and T lymphocytes, and memory cells. The normal immune system functions under specific immune mechanisms to achieve host protection against environmental risk factors and to clear abnormal immunological responses [2]. B and T cells are the two main types of lymphocytes produced from the hematopoietic stem cells in the bone marrow. B lymphocytes are associated with humoral immune response, and T lymphocytes are associated with cell-mediated immune response. The cytotoxic T (CD8) cells identify the antigens only coupled to human leukocyte antigen (HLA) class I molecules, whereas the T helper (CD4) identifies HLA class II. The γδ are the minor subtype of T cells that promote the inflammatory responses of lymphoid and myeloid lineages and recognize antigens or non-peptidic compounds without instant HLA assistance [3].

B cell-specific antigen receptors are antibodies expressed on the surface of the B cells, and they identify native antigens without processing. B cell antigen receptors represent all antibodies the host produces, but each B cell lineage produces specific antibodies. Clonal selection is a term used for T or B cells when each produces similar targets for specific encountered antigens [4].

T lymphocyte cells (T cells) are produced from c-kit+Sca1+ haematopoietic stem cells (HSC) in the bone marrow. They are defined as common lymphoid progenitors (CLP) that differentiate into T, B, or natural killer (NK) cells to control the variable immunological functions and shapes [5]. CLPs that migrate through the bloodstream into the thymus are early thymic progenitor (ETP) or double-negative cells expressing CD4−CD8−CD44+CD25−ckit+ [6].

CD2, CD5, and CD7 expression can identify the double negative thymocytes, and then CD34 expression will stop, and the CD1 expression will initiate in this stage of development [7].

T cell development results in mature T cells expressing T cell receptors (TCR). Each cell expresses one specific TCR, which is composed of alpha and beta chains. On each mature T cell, the TCR is selected by specialist cells within the thymus in a process known as positive selection. This process involves a range of self-peptides-MHC protein complexes within the thymus so that the mature T cell repertoire complements available HLA proteins. Negative selection helps ensure that the ability of mature T cells to react to self-antigens is limited [7][8].

B lymphocytes are the main generators of the adaptive humoral immune system, which involves the production of antigen-specific immunoglobulin (Ig) to defend the body against microbes [4].

Disulfide bonds join heavy and light chains to form the Ig, and the rearrangement of the heavy chain is an initial step during B lymphocyte development that starts with D-J recombination in the common lymphoid progenitors (CLPs). The pre-pro B cells are subsequently recombined with V to form V-DJ in the large pre-B lymphocytes [9]

The recombined heavy chain is associated with the surrogate light chains to form the pre-B cell receptor (pre-BCR) on the cell surface. Forming pre-B lymphocytes followed by V-J rearrangement of the Ig light chain leads to mature BCR with solely specific molecules expressed on the surface of immature B lymphocytes known as IgM [10]. Immature B lymphocytes that express IgM migrate from bone marrow to the spleen, where they can differentiate into long-lived mature follicular (FO) or marginal zone (MZ) B lymphocytes. Another mature B lymphocyte known as the B1 cell originates from the fetal liver and is found in the spleen, intestine, peritoneal cavity, and pleural cavities [11].

When mature B lymphocytes encounter antigens, they receive a signal from T helper cells to carry out plasmacytic differentiation, develop extrafollicular plasmablasts, and develop IgM-secreting plasma cells. These cells have a limited life span and interact in a quick manner with antigens [12].

Establishing the germinal centre is the second developmental process in which B cells conduct proliferation associated with affinity maturation. Class switching is a vital mechanism for the B cell diversity response in which immunoglobulin switches to IgG, IgA and IgE and is functionally matched to a suitable immunological reaction [13].

Natural Killer (NK) cells are a subtype of lymphocytes functioning within the innate immune system and control several types of tumours and virally infected cells. Also, NK cells can be detected in the placenta, which is considered an indicator of playing a role in pregnancy [14].

NK cells are classified as group 1 innate immune lymphocytes (ILCs) and belong to the same family of T and B lymphocytes originating from the common progenitor cells. NK cells can release cytokines, e.g., interferon-gamma (IFN-γ) and tumour necrosis factor (TNFα), that act on macrophage and dendritic cells to consolidate their immune response [15] [16]. NK cells play a surveillance role in the human body to detect and eliminate abnormal blood cells. The killing mechanism depending on the fine balance between the signals arising from activating and inhibitory receptors that identify the antigens expressed on the surface of abnormal cells [15].

Cytokines are a group of multiple small glycoproteins that affect immune response behavior, through which lymphocytes, inflammatory cells and haematopoietic cells communicate with each other to achieve specific roles in the human body [17]. Also, cytokines were demonstrated to have a direct role in cell differentiation and cells mediated migration. T helper and macrophages are the two main secretors for cytokines that affect innate and adaptive immune responses [18].

Cytokines are not always in a soluble form and sometimes are anchored on the cell membrane, making it difficult to differentiate between the role of cytokines and cytokine receptors [19]. In general, cytokines contribute to cell activation, division, apoptosis, or cell movement that signal to their receptors in different ways, including acting as autocrine, paracrine, and endocrine messengers. Cytokines have various names such as those secreted by leukocytes are called interleukins, those with chemoattractant role are termed chemokines, and others are known as colony-stimulating factors (CSF) because of their role in cell differentiation and proliferation, whereas interferons named for their interaction with viral replication [20].

Antigen presentation

HLA class-II proteins are expressed specifically on the surface of professional antigen-presenting cells (APCs), including DCs, B cells, and thymic epithelial cells. Also, they can be induced on monocytes and macrophages due to gamma interferon (IFN-γ) mediated activation [21]. Peptides attached to HLA class II are processed in endosomal/lysosomal (antigen processing compartments) by proteolysis of antigens utilizing different mechanisms including *receptor-mediated endocytosis*, *macropinocytosis*, *phagocytosis*, and *autophagy* [22]. On the other hand, the major histocompatibility complex (MHC)-II molecules associated with invariant chain (Ii) protein can enter antigen processing compartments shortly after biosynthesis in the endoplasmic reticulum (ER) [23].

Ii protein supports HLA class II with three functions:

1) Acts as a chaperone to help in ideal folding and movement of the HLA class II/Ii complex to Golgi apparatus via ER

2) Protects peptides and unfolded proteins found in the ER attach to nascent MHC-II molecule

3) Directed HLA class II/Ii complex to antigen processing compartments through targeting signals in its cytoplasmic domain [22].

These signals are composed of a couple of dileucine-based internalizing motifs that interact with clathrin-associated adaptor proteins to deliver the HLA class II/Ii complex into the endocytic pathway [24].

HLA class II proteins cannot react with antigenic peptides unless the Ii is proteolytically degraded and separated from the HLA class II/Ii complex. This complex degradation takes place in a series of steps utilizing different proteinase enzymes to liberate invariant chain peptides (CLIP). The CLIP attaches to the MHC II binding groove [25], then will be removed by catalytic action to make space for lysosomal-generated peptides by an accessory protein known as DM. DM also acts as a peptide editor for HLA class II to accelerate the generation of high affinity immunodominant epitopes [26]. DO is another protein on the cell surface of B cells, thymic cells and Langerhans cells and contributes to the HLA class II processing involving DM [27].

The role of HLA class I proteins is mainly in presenting peptides derived from viral proteins by proteolysis in the proteasome and subject to more processing by cytosolic aminopeptidase enzymes before being delivered into the ER lumen by a transporter associated with antigen processing (TAP) [28]. ERAP1 and ERAP2 are resident aminopeptidases present in the ER that to carry out further processing to suitable peptides (8 to 10 amino acid length) before reacting with HLA class I protein [29].

Macropinocytosis is an extracellular capturing process responsible for the uptake of varied materials from tiny molecules to larger intact bacteria and protozoans. Macropinosomes are products of plasma membrane ruffles, folded together then, fused with the plasma membrane, then with early endosome delivering materials to the endolysosomal pathway (commonly used for antigen acquisition by dendritic cells (DCs)) for antigens processing [30].

Receptor-Mediated Endocytosis

Multiple receptors found on the surface of APCs, such as Fc-γ in DCs and macrophages, can bind to immune complexes and pass to antigen-processing compartments [31]. The others are Lectin receptors such as mannose and DEC 205 that identify the carbohydrate residues and aim them to receptor-mediated phagocytosis [32].

Phagocytosis is a process to internalize insoluble antigens such as necrotic/apoptotic cells, bacteria, and viruses into macrophages and DCs using specific receptors including Fc receptors, complement receptors, and C-type lectin receptors. After receptor engagement and engulfment, the internalized particle is delivered to a specialist organelle, the phagosome. Fusion with lysosomes occur and the pathogens are killed by proteolysis and release of toxic reactive oxygen species [33] [34].

Genes relevant to antigen presentation

Human leukocyte antigens (HLA) genes

Genes encoding the HLA are located on the short arm of chromosome 6 (region 6p21.3.1). The genes encode numerous proteins and are highly polymorphic (Figure 1). HLA is classified into three classes: HLA class I, II, and III. HLA class I is divided into HLA-

A, HLA-B, and HLA-C and presents endogenous peptides (8-10 amino acids in length) to T cells, which are known as CD8-positive cells or cytotoxic T cells [35]. HLA class II is divided into DP, DM, DO, DQ, and DR. This class presents exogenous peptides to T cells known as CD4-positive T cells, which ultimately stimulate antibody-producing B cells to produce specific antibodies [36]. HLA class III is located between HLA class I and HLA class II and includes specifically the genes of complement component C2, C4 and B factor and other genes such as tumour necrosis factors (TNFs) and heat shock proteins [37].

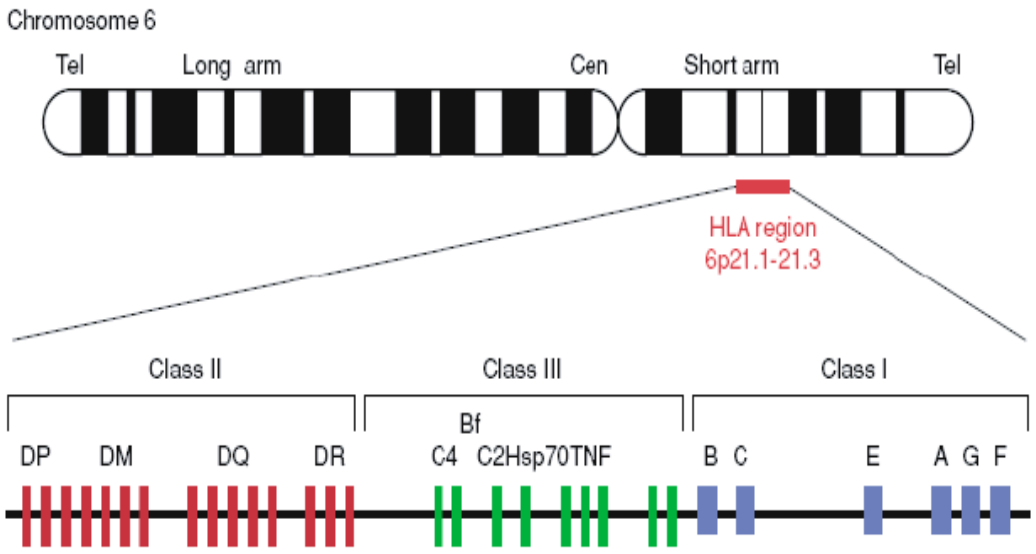

Figure 1. Location and distribution of the HLA structural gene clusters [35].
HLA region is located on 6p21.1-21.3, which stretches on 3 Mbp lengths and is classified into 3 different classes. 1. HLA class I (Shown in blue) includes A, B, C, E, F and G loci. 2. HLA class II (Shown in red) includes DM, DP, DQ and DR loci. 3. HLA class III (shown in green) and contains C4, C2, Hsp70 and TNF genes.

Killer immunoglobulin like receptors (KIR) genes

The KIR locus includes a group of 16 genes, as shown in Figure 2, and will be discussed in detail in Chapter 2. This locus extends around 1 Mb within the leukocyte receptor complex (LRC) and comprises 2 or 3 Ig-like domains (KIR2D and KIR3D) that bind to HLA Class-I molecules on the extracellular domains. A delicate balance between activating and inhibitory KIR receptors governs NK cell effector functions. In the presence of tumour or viral infection, NK cells act to remove tumour or virally infected cells and regulate downstream responses of the innate and acquired immune systems [38][39].

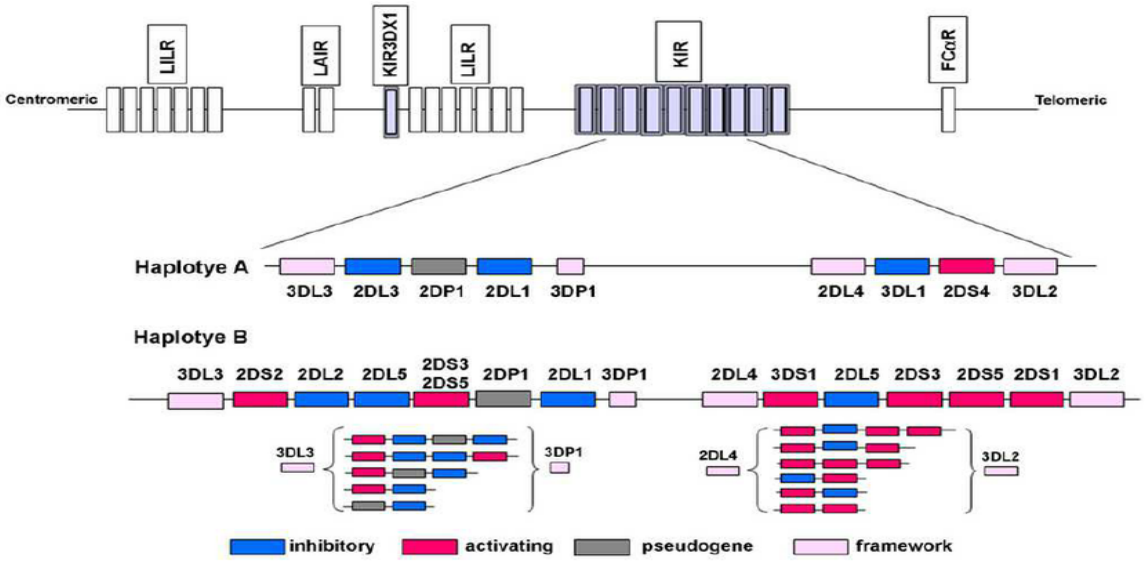

Figure 2, The structural variation of KIR gene in haplotype A and B [40].

The KIR gene cluster is located at 19q13.4 and contains 16 KIR genes including KIR2DL1, -2DL2, -2DL3, -2DL4, -2DL5, -3DL1, -3DL2, -3DL3, -2DS1, -2DS2, -2DS3, -2DS4, -2DS5, -3DS1, -2DP1 and -3DP1 genes. These genes encode inhibitory (blue colour), activating (red colour), pseudogene (grey dark) or framework genes (purple). Haplotype A is composed of KIR2DL1, -2DL3, -3DL1, -3DL2, -3DL3 and -2DL4 (inhibitory), KIR2DS4 (activating) and two pseudogenes KIR2DP1 and -3DP1. Haplotype B is determined mainly by the presence or absence of the genes that are not part of the haplotype A. KIR3DL2, -3DL3 and -2DL4 (framework genes) that divided the KIR locus into centromeric and telomeric parts and are present in both the A and B haplotypes

Aims of the studies described in this thesis and methods used

Aims

The geographical location of the Arabian Peninsula, which accommodates the Arab population, including the Saudis, may have affected the genetic pool of these populations. Self-peptides associated with β-cell are targeted by autoimmune T-cell clones whose evolution is facilitated by an excess of pro-inflammatory cytokines (e.g., TNF-α) and an absence of anti-inflammatory cytokines (e.g., IL-4 or IL-10); both scenarios facilitating β-cell destruction. KIRs and their corresponding HLA class I ligands are crucial in NK cell activation/suppression, reflected in autoimmune diseases. The first three publications aimed to determine allele and haplotype frequencies for HLA, KIRs, and short tandem repeats (STRs), compare the results with those of other populations, and provide a frequency pool reference to assist researchers in anthropological analysis and

immunogenetics disease studies. In the last three publications, I aimed to investigate the KIRs, their HLA ligands and pro-inflammatory and anti-inflammatory cytokines in T1DM to identify significant differences in gene frequencies between patients and controls.

Experimental Design and Genotyping Methods Justification

In this thesis, I include 6 publications resulting from multiple research projects that were approved by the Institutional Review Board (IRB) and funded by the Intra-Mural Fund (IRF) program in King Medical City (KFMC). Only Saudi nationals (whose parents and grandparents lived in Saudi Arabia) were recruited, and blood samples in EDTA were collected from patients or healthy controls. Sample sizes were calculated based on implementing Fisher's exact test, and the level of significance was adjusted at 5%.

Our research utilized two known methods for DNA extraction: 1. An automated nucleic acid purification utilizing the MagNa pure compact instrument (Roche Diagnostics GmbH, Mannheim, Germany) that uses a proven magnetic beads technology. 2. Spin-column (Roche Diagnostics) methods that utilize glass fibre fleece immobilized in a special plastic filter tube. The extracted DNA was measured for quality and quantity using the NANODROP 2000 (Thermo Scientific), which reads the absorbance of diluted samples at 260 nm and 280 nm wavelengths.

I utilized three methods for genotyping (Table 1), including Sequence-specific oligonucleotide probe (SSOP), TaqMan® MGB probes technique and The AmpFlSTR™ Identifiler for STRs, as detailed in Table 1.

The SSOP method was used for HLA and KIR genotypes utilizing the Luminex 100 system as a platform for data acquisition. We implemented predesigned reverse group-specific primers from One Lambda (One Lambda, San Diego, USA) to amplify exon 2 and exon 3 of the HLA loci. I implemented reverse group-specific primers to amplify exons 3, 4, 5, 7, 8 and 9 of KIR genes for KIR genotyping. The biotinylated PCR products for HLA and KIR were denatured and re-hybridized to complementary DNA probes conjugated to fluorescently coded microspheres. Then, the complementary DNA was labelled with R Phycoerythrin-conjugated Streptavidin (SAPE). For this genotyping procedure, I utilized

proficiency testing samples for quality control, validated by the University of California Los Angeles (UCLA) and the College of American Pathologists (CAP) for quality assurance. Multiple DNA samples with a known KIR genotype were run in parallel with our study samples for additional quality assurance. Our protocols always resulted in the expected calling of all controls.

We used commercially available TaqMan® MGB probes (Life Technologies) for SNP genotyping, and amplification was performed using the Lightcycler 480 (Roche Diagnostics). (I used the Minor Groove Binder (MGB) technology with TaqMan probes, two unlabeled forward and reverse PCR primers, a VIC dye-labelled hydrolysis probe, and a FAM dye-labelled hydrolysis probe.) I validated the TaqMan assay by triplicating ten samples to genotype each SNP before carrying out the whole genotype and consistently obtained the same results (https://www.genome.gov/10002404). Using the AmpFlSTR™ Identifiler™ to amplify for STRs, we amplified 15 tetra-nucleotide repeat loci (on different chromosomes) and the Amelogenin (gender-determinant marker) in a single PCR tube. The combination of the 15 loci that were globally recommended were genotyped, and several dyes, including 6-FAM™, VIC™, NED™ and PET™, were used. The AmpFlSTR™ Identifiler™ Allelic Ladder and positive control DNA 9947A were included in the assay. Multiple DNA samples with known combined STR genotypes were run in parallel with this study samples, and typical results were obtained for these control samples on every occasion.

Table 1

A summary of the methods used in the publications in the thesis

No	Method	Gene	Primer	Amplified exon	Instrument
1	SSOP Bead-Based Multiplexing	HLA	R	2 and 3	luminex 100 system
		KIRs		3, 4, 5, 7, 8 and 9	
2	TaqMan® MGB probes	SNPs	F and R		lightcycler 480 roche
3	AmpFlSTR Identifiler	STRs	16-locus multiplex primers	15 tetranucleotide repeat loci	3130 xl genetic analyzer

We used various statistical and data analysis software tools, including the SAS program, SNPStats, and SPSS version 22.0. SAS software is widely used software for statistical analysis and data visualization, and it is only available for Windows operating systems [41]. SPSS statistics have been used by researchers in different fields, including health researchers. IBM developed this software for data management, advanced analytics, and multivariate analysis (https://www.ibm.com/spss?). SNPStats software has been designed to analyze genetic epidemiology by focusing on multiple genetic parameters such as allele and genotype frequencies, Hardy-Weinberg equilibrium, Linkage disequilibrium, and Haplotype frequency estimation (https://www.snpstats.net/start.htm).

The Hardy–Weinberg equilibrium (HWE) for the expected and observed distribution of allele frequencies for the control data was analysed by implementing the $\chi 2$ distribution (degree of freedom = 1). The D′ statistic and *p-value* tests were used to analyse the linkage disequilibrium (LD) among the genetic markers. Principal components analysis (PCA) was used to determine the genetic differences between these study samples and other populations. Fisher's exact test and the log of the odds (logit) were calculated to determine the significant differences at an overall level of $p < .05$. The Bonferroni correction test was applied whenever necessary to address multiple comparisons.

Chapter 2. Immune-related gene frequencies in the Saudi population

Introduction and Background

Hematopoietic stem-cell transplantation (HSCT) is an infusion of multipotent hematopoietic stem cells from a donor to a patient with a specific life-threatening haematological disease including leukemia, lymphoma, multiple myeloma, and myelodysplastic syndromes. Also, HSCT is used for other diseases such as neuroblastoma, Ewing sarcoma and brain tumors [42]. There are three types of stem cells including peripheral blood stem cells, bone marrow stem cells, and umbilical cord stem cells. Based on the origin of stem cells, HSCT is divided into three categories: a) autologous transplantation, when stem cells are collected from the same patients before giving chemotherapy. b) allogeneic transplantation, when stem cells are collected from related or unrelated donors. c) Syngeneic transplantation is the origin of stem cells from identical twins [43].

HSCT is a complicated procedure, and specific precautions should be considered before transplantation. Graft-versus-host disease (GVHD) is considered a serious problem that develops post-SCT and causes damage to the skin, liver, intestines, and many other organs [44]. GVHD has two different types: acute, which develops in the first three months post-transplant, and chronic, which develops after three months post-transplantation. GVHD could be minimized by selecting an HLA-matched donor, using immune-suppression drugs, or removing T cells from donated stem cells before transplantation, known as the T cell depletion process [45].

According to the Consensus Opinion of the Blood and Marrow Transplant Clinical Trials Network (BMT CTN): a fully HLA-matched sibling is the best choice for allogeneic Hematopoietic cell transplantation (HCT). The patient and related donor (parent or sibling) should at least meet a 6/6 match for HLA-A and HLA-B at intermediate resolution DNA genotype and HLA-DRB1 typed at high resolution. HLA-C and -DQB1 loci may be considered. The choice for graft in case a full match-related donor is unavailable is considering one HLA antigen mismatch with a 7/8 match at HLA-A, -B, -C, and -DRB1 or a haploidentical donor sharing a single HLA haplotype. There should be at least a 4/8 match at HLA-A, -B, -C and -DRB1 loci with only one mismatch per locus. However, the risk of graft failure is high in the HLA mismatch-related donor when HLA antibodies are detected

in the patient. For bone marrow transplantation (BMT)-unrelated donors, HLA-A, -B, -C, and HLA-DRB1 genotypes at high resolution with 8/8 HLA matches will increase the survival rate for patients. A single locus mismatch should be considered if an 8/8 HLA match is unavailable. When multiple choices for unrelated donors are available, HLA-DPB1, DQB1 and DRB3/4/5 should be considered when selecting donors with minimal mismatches [46]. HLA-DQB1 mismatch only does not affect the survival rate, but if the mismatches are associated with 7/8 or 6/8 of HLA-A, B, C, and DRB1, then poor survival will consequently occur[47]. Also, Tie et al. concluded that HLA-DQB1 will be better controlled than HLA-A, B, C, and DRB1 loci in GVHD [48].

Haploidentical donors within the families have increased the BMT (this term is used when cells are collected from bone marrow) opportunities for patients who do not have HLA identical siblings. Cyclophosphamide post-transplantation is essential to reduce the risk of GVHD caused by HLA mismatches and destroy alloreactive T cells [49]. The haploidentical donors may be fathers or mothers, which is always suitable for pediatric patients as adult patients are not always available. Siblings and children are often considered second-degree related donors, but this could be possible [50]. The contact of the maternal fetus during the pregnancy period may cause tolerance against mother antigens that the fetus does not inherit. These antigens are known as non-inherited maternal antigens (NIMAs) [51]. NIMAs are defined as acceptable HLA mismatch; therefore, the opportunities for HLA alleles that are acceptable for potential BMT patients will increase [51].

Establishing a population appropriate HLA genotyping within the hospital service was critical to finding a better match between donor and recipient for HSCT **(Publication 1)**. Immune-genetic differences between patient and recipient lead to many complications post-transplantation. The establishment of improved HLA allele matching between patient and unrelated donor improves the chance of graft survival and reduces transplant-related risks.

Specific HLA class I antigens govern the role of the NK cells in the immune system, and the type of KIRs expressed on the surface of NK cells induce activation or inhibition signals **(Publication 2).** The simultaneous presence of the two receptors in one individual may block the effect of the KIR-HLA ligand. This preliminary work establishing the ability to

genotype KIRs in donors and patients was critical to its clinical use. KIRs genotyping has a positive impact in transplanted patients with AML if a donor is KIRs mismatched with the patient, but this was not the case for recipients with ALL. Consideration of KIR mismatching in the transplantation practice reduces disease relapse and has improved the disease-free survival of patients [52].

The condition of mixed DNA chimerism of donor and recipient post-transplantation is expected in nonmalignant diseases such as haemoglobinopathies. The mixed chimerism could transform into graft failure if the chimerism is not controlled by inducing a tolerance initiated by regulatory T cells.

The success of allogeneic SCT depends on the efficiency of the procedure and the proper time of stem cell engraftment. Also, protection against infection and graft-versus leukaemia induced by donor cells against the residual malignant cells of patient origin are other factors for transplant success. Currently, the STRs test is a gold standard method in routine clinical practice to identify genetic variation depending on multiple base pair lengths among individuals **(Publication 3).**

HLA genotyping is the first step in determining patient and donor histocompatibility in transplantation. The STR genotype is a crucial post-transplantation test that helps physicians follow engraftment and make the right decision at the right time. Notably, the role of KIR genes was positively demonstrated in transplanted patients with specific haematological diseases.

In this chapter, I include three published papers in which I was the main generator throughout the publication process. In these publications, we investigated HLA, KIRs, and STR allele frequencies and characterized the genetic components. The aim was to provide high-quality work and accumulate experience in the research field to provide better service for BMT patients.

Publication 1. HLA allele frequencies in Saudi individuals

Significance and Background

The Major histocompatibility complex (MHC) is a protein family in vertebrates, and the HLA is specifically present in humans is synonymous with MHC. The HLA region includes numerous genes and is considered a highly polymorphic region in the human genome**;** these variations have a crucial impact on various clinical outcomes related to the human immune system. HLA proteins are expressed on the cell surface of human cells except for RBCs, and their main function is to present foreign antigens to T cells. Many HLA gene loci and their related alleles have been detected so far, but the most important ones include A, B, and C antigens for HLA class I, and DRB1 and DQB1 for HLA class II. We utilized a sequence-specific oligonucleotide probe (SSOP), a Luminex-based method and the Lab Type kit to genotype HLA class I and class II in this study. To the best of our knowledge, it was the first time this technology was implemented in HLA genotyping in the Saudi population, and we were also able to recruit a large sample compared to previous studies in this population at the time of publication.

The structure of HLA is explained in Chapter 1 (Figure 1); the HLA genes encode numerous proteins and are highly polymorphic. Variations in some of these genes (Class I, II and III) on chromosome 6 have a crucial impact on various clinical outcomes such as solid organ transplant (SOT), hematopoietic stem cell transplantation (HSCT), pharmacogenetics, and HLA-associated diseases [53] [54]. Matching HLA between patients and the potential donor could decrease the possibility of rejection in solid organ transplantation and minimize the graft-versus-host disease in bone marrow transplantation [55]. Also, the importance of HLA genes was demonstrated in the field of pharmacogenomics, where certain drugs such as abacavir and carbamazepine were found to cause serious immunological reactions by binding to specific HLA proteins and leading to T-cell mediated hypersensitivity. Studying HLA alleles and/or haplotypes provides useful genetic information for different populations [56] [57].

Saudi Arabia (SA) is the largest Arab country in the Arabian Peninsula. Most Saudis are of Arab origin and have lived in this part of the world for thousands of years [58]. Riyadh

and Al Gaseem are in the middle part of SA and the population in these areas is more stable than in the other parts of the Kingdom [59].

In Saudi Arabia, consanguineous marriage is considered higher than in other parts of the world, a report in a multicenter study demonstrated the overall rate of consanguineous marriage was about 56% whereas, the rate in Riyadh was estimated to be around 62.8%. The Arabian Peninsula is composed of seven countries including Bahrain, Kuwait, Oman, Qatar, the United Arab Emirates (UAE), Yemen, and Saudi Arabia which is the largest one. In the time between the 7th and 8th centuries during the Islamic state power, Muslims were able to distribute their Arabic language and their culture outside the Peninsula that extended to North Africa and the middle of Asia [60] [61].

In the last 30 years, different methods to genotype HLA genes have allowed numerous studies to be performed worldwide. However, only a few studies have been done on HLA gene frequencies in Saudi Arabia [62] [63].

In this study we genotyped HLA-A, -B, -C, -DRB1, and -DQB1 genes; the purpose was to characterize HLA allele and haplotype distribution in this population and compare the data with other available Arab-related studies.

Results

A strong LD was found between the B-C pair, followed by the DRB1-DQB1 pair. A*02 was found to be the most frequent allele for the HLA-A locus then, followed by A*24, A*26, A*03, and A*01 with no presence for A*25, A*43, and A*80 in this study sample. The B*51 and the B*50 were the most common in the B locus, followed by B*08 and B*07alleles. Whereas the more frequent alleles in the HLA-C were observed are C*07 and C*06, then C*15 and C*04. HLA class II, located on the centromeric end of the 6p21.3.1 region, is less polymorphic than class I. DRB1*07 and DRB1*03 were found at higher frequencies, followed by DRB1*13 and DRB1*15 alleles.

The A.C.B haplotype calculation revealed that 02.06.50 was the most frequent, followed by 02.07.07, 26.07.08 (3.34%), and 31.15.51. For the DRB1.DQB1 of class II, 07.02, 03.02, and 04.03 were the common haplotypes. The overall calculation for class I and II loci (A.C.B.DRB1.DQB1) showed 424 haplotypes (see Table 2 for the top 50 haplotypes), the most common of which are 02.06.50.07.02, 26.07.08.03.02, and 02.07.07.15.06.

Table 2

The top 50 haplotypes out of 424 for the overall calculation for class I and II loci (A.C.B.DRB1.DQB1).

No	A.B.C.DRB1.DQB1	*2n=940*	*HF (2n=940)	HF(%) 2n=(940)
1	02.50.06.07.02	63	0.0665	6.65%
2	26.08.07.03.02	23	0.0245	2.45%
3	02.07.07.15.06	21	0.0224	2.24%
4	31.51.15.13.06	18	0.0191	1.91%
5	24.08.07.03.02	17	0.0183	1.83%
6	02.51.15.04.03	15	0.0163	1.63%
7	33.58.03.03.02	13	0.0138	1.38%
8	33.50.06.07.02	12	0.0128	1.28%
9	68.08.07.03.02	11	0.0112	1.12%
10	02.07.07.03.02	10	0.0102	1.02%
11	31.50.06.07.02	9	0.0099	0.99%
12	23.50.06.07.02	9	0.0096	0.96%
13	01.41.17.07.03	9	0.0096	0.96%
14	33.14.05.01.05	8	0.0083	0.83%
15	02.51.14.15.06	8	0.0083	0.83%
16	02.51.16.11.03	8	0.0082	0.82%
17	26.51.15.04.03	8	0.0081	0.81%
18	11.52.12.15.06	7	0.0079	0.79%
19	24.15.07.13.06	7	0.0075	0.75%
20	30.13.06.07.02	7	0.0075	0.75%
21	24.35.04.13.06	7	0.0075	0.75%
22	26.38.12.13.06	7	0.0074	0.74%
23	02.07.07.07.02	6	0.0067	0.67%
24	68.50.06.07.02	6	0.0066	0.66%
25	29.07.07.15.06	6	0.0064	0.64%
26	68.08.07.15.06	6	0.0064	0.64%
27	24.35.04.16.05	6	0.0061	0.61%
28	01.51.15.15.06	6	0.0061	0.61%
29	02.50.06.04.03	5	0.0056	0.56%
30	02.51.16.16.05	5	0.0056	0.56%
31	31.51.15.07.02	5	0.0055	0.55%
32	68.51.15.11.03	5	0.0053	0.53%
33	29.07.15.10.05	5	0.0053	0.53%
34	30.53.04.13.06	5	0.0053	0.53%
35	32.50.06.07.02	5	0.0053	0.53%
36	03.51.15.11.03	5	0.0053	0.53%
37	02.35.04.04.03	5	0.0052	0.52%
38	02.52.12.15.06	5	0.0050	0.50%
39	03.50.06.07.02	4	0.0047	0.47%
40	03.08.07.03.02	4	0.0047	0.47%
41	68.08.07.04.03	4	0.0043	0.43%
42	31.51.15.03.02	4	0.0043	0.43%
43	03.53.04.04.03	4	0.0043	0.43%
44	31.15.01.13.06	4	0.0043	0.43%
45	03.15.04.13.06	4	0.0043	0.43%
46	02.49.07.11.03	4	0.0043	0.43%
47	03.51.16.04.03	4	0.0043	0.43%
48	68.51.15.13.06	4	0.0043	0.43%
49	03.58.03.13.06	4	0.0042	0.42%
50	68.51.15.04.03	3	0.0036	0.36%

* HF means haplotype frequency

Principal component analysis (PCA) was performed in order to look at the distributions of A, B and DRB1, comparing my data to the available data from other previous Arab studies that included Saudi Arabians, Jordanians, Omanis, Moroccans, Palestine, Tunisians, Algerians, and the United Arab Emirates (Publication no 1; Figure 1). PCA for A locus demonstrated a clustering data of the three Saudi studies including ours, Omani and United Arab Emirates. For B locus, only Saudi studies were found clustering together, while Omani and United Arab Emirates were a little far but still were closer than the others. The PCA for the DRB1 locus, demonstrated a cluster area composed of two Saudi studies including this report, Tunisia, Algeria, and Oman.

Commentary and Impact

Graft-versus-host disease (GVHD) is the most critical issue in allogeneic hematopoietic stem cell transplantation. HLA matching between patient and donor is a crucial factor preventing this disease [64]. Loren *et al*. explained the benefit of HLA matching between the donor and recipient [65].

The large sample size, the method we used and the number of HLA loci investigated in this study provided a clinically significant data which is distinct from the other two previous studies conducted in this population [62], [66]. We identified similarities and differences in allele frequencies and carried out comparisons with other local and regional studies that have provided a clear understanding of the extent of variation among the various ethnic groups.

I demonstrated that alleles A*02 and B*51 were at higher frequencies. We suggested that blood banks should observe patients with platelet refractoriness for possible novel antibodies due to multiple transfusions. The B*51 allele is known to be associated with Behçet's disease [67].

One of the crucial variations I detected was the absence of the A*43. This allele was observed more frequently in African people [68]. An increase in homozygosity of various alleles with non-conformation to HWE was also detected by Hajeer *et al*. [69]. This non-conformation to HWE in the homozygous alleles may be due to the high rate of

consanguineous marriage among the Saudi people [61] or to the Wahlund effect that happens because of the subpopulation structure [70].

Numerous haplotypes were identified using the maximum likelihood estimation test. The finding demonstrated similar frequencies in the A*02.C*06.B*50 haplotype (the highest in our study) and North African populations, namely the Chaouya population from Morocco and Tunisia, which are attributed to their Arab [71], [72].

PCA analyses test on HLA-A, -B, and -DRB1 alleles data retrieved from http://www.allelefrequencies.net for Arabs-related ethnic studies to portray the genetic components have shown similarities and differences among the tested populations.

One of the key limitations of this study was the HLA genotyping method we used, which was at a low-resolution level (only two digits). In the light of this, we strongly recommend future studies with larger sample sizes and higher HLA resolution. These studies could provide more comprehensive and detailed insights into the genetic components of the tested populations.

This study is part of a broader set of recent literature looking at HLA allele frequencies in different populations. A study from Iran focused on the people of Mashhad who live in the northeast region of Iran. The authors demonstrated the allele frequencies for HLA-A and HLA-B alleles. They found that HLA-A *24 and HLA-B*35 were the most frequent alleles in this population [73]. At the same time, an Italian study demonstrated HLA haplotypes provided remarkable differentiation in Sardinia populations compared to other parts of the country [74]. Also, a study of HLA-A, -B, -C, and -DRB1 loci that was conducted in the Kinh population of Vietnam showed HLA-A*11:01, -B*15:02, -C*08:01 and -DRB1*12:02 were the most frequent alleles in their report [75]. It is worth noting that we implemented the result of this study in our daily routine work to sort out some ambiguous HLA results based on alleles frequencies for this publication.

Publication 2; The distribution of Killer Immunoglobulin-like receptors in the Saudi population

Significance and Background

Human natural killer (NK) cells are an effector type of lymphocyte subpopulation that acts within the innate immune system mechanism against various tumours and virally infected cells. Killer immunoglobulin receptors (KIRs) are a family of highly polymorphic cell receptor proteins that express on the surface of NK cells and work with certain types of HLA ligands on the surface of the target cells. KIR molecules can control NK cell behavior through the activation and suppression mechanism. In **Publication 2**, we studied and characterized the frequency distribution of 16 KIR genes using the sequence-specific oligonucleotide probe (SSOP) method that amplified seven different exons within the KIRs genes. Also, I compared the study outcome with other population studies in the region and worldwide.

A quick release of interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α) post-cell activation can induce NK cells to interact quickly and start destroying their targets, activating other inflammatory molecules [76].

KIRs are a family of 16 combined genes present on chromosome 19 (19q13.4) and encode the inhibitory and activating surface receptors present on the NK [76]. These genes are highly polymorphic and characterized into several types, including KIR2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 3DL1, 3DL2, 3DL3, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 3DS1, 2DP1 and 3DP1 (see figure 2). In addition, most of these genes can encode proteins that play a role in the immune mechanism.

KIR genes are divided into two groups of haplotypes based on the gene content:

1. Haplotype A has simple and constant genes content, including 2DL1, 2DL3, 3DL1, 3DL2, 3DL3 and 2DL4 (the inhibitory genes), KIR2DS4 (activating genes) and KIR2DP1 and -3DP1(pseudogenes). KIR2DL3 and -2DL1 are present in the variable centromeric region, while -3DL1 and -2DS4 are in the telomeric part.
2. B haplotypes are characterized by a specific distinct feature that includes at least one of the following three: a) including several activating KIR genes, b) lacking at least one inhibitory KIR gene and c) containing 2DL5 gene [77].

The 3DL2, 3DL3, and 2DL4 are framework genes found in A and B haplotypes. Full-length sequencing of the KIR haplotypes demonstrated two structured regions, a proximal centromeric ("C") region containing 2DL3, 2DL1, 2DP1, 3DL3 and 3DP1 and a distal telomeric ("T") region that has 3DL1, 2DS4, 2DL4 and 3DL1 genes. A haplotype is a conservative with a constant uniform difference from the B haplotype; the latter demonstrated various centromeric ends, including 2DS2, 2DL2, 2DL5B, 2DS3, 2DS5, 3DL3 and 3DP1 genes, while the telomeric part for B haplotype contains 3DS1 and 2DS1 genes [78].

KIRs genes have also been classified according to the number of extracellular immunoglobulin domains expressed on the surface of the NK cells and the length of the cytoplasmic tails. The long cytoplasmic tail confers the inhibitory function with two immunoreceptor tyrosine-based inhibitory motifs (ITIM), such as in 2DL and 3DL. In contrast, the activation function is present in the short cytoplasmic tail genes (2DS, 3DS) [79].

The specificity and affinity of the KIRs ligands' capacity, determined by the genetic coding sequence that leads to protein production, may be linked to susceptibility and progression for specific diseases [80]. In addition, the role of the KIR genes in the immune system may affect the susceptibility to infectious/autoimmune diseases and the outcome of transplantation. Distribution of the KIRs genes/haplotype frequencies was demonstrated in considerable differences in ethnic groups among various populations worldwide [81][82][83].

HLA class I-specific ligands include HLA-C group 1 (C1), HLA-C group 2 (C2) and HLA-Bw4 (Bw4) motifs that span residues 77–83 of the alpha1 domain, but HLA-C is the most important ligand for NK cell regulation [84]. Also, haplotype A is known as HLA-C-specific genes due to its C1 and C2 epitope's function, C1 is identified by 2DL1 and 2DS2 while C1 is identified by 2DL2 and 2DL3 [85].

The dispersion out of Africa hypothesis is one of the most exciting models scientists use to track the distribution of the modern human journey from East Africa to other parts of the world. However, the fact was different when looking at the middle part of the Peninsula because of the limitation of the immigrant settlement that contributed to the stability of the Arab gene pool [58][86].

In this study, we investigated and characterized the KIR genes and KIR genotype content in healthy Saudi individuals and compared the outcome with other studies in the region and worldwide.

Results

As shown in Table 1 (**publication 2**), allele frequencies were determined, and the framework genes (2DL4, 3DL2, 3DL3 and 3DP1) were observed at 100% frequency as expected. The lowest frequencies were observed in 3DS1, 2DS3 and 2DS5 (inhibitory genes), while the highest frequencies were observed in 2DL1 and 3DL1 (activating genes). Also, comparing my data with other populations showed similarities and significant variations in allele frequencies, as in Table 2 (**publication 2**). Remarkable differences were observed between the Saudi population and Australian Aborigines on one side and between the Saudi and Senegalese/Indians Kanikar on the other. In contrast, minimum differences were shown between our study and the other Arab-related populations. A sum of thirty-one KIR genotype groups was demonstrated, as explained in Figure 1 (**publication 2**).

The AA genotype was found in a frequency of 18.1% of the total observed genotypes, while the Bx genotype with ID number 5 was observed at 12.1% frequency and the Bx with ID 6 at 10.1% frequency. Other genotypes were observed at frequencies less than 10% along the obtained result. Subsets of the Bx genotype gene clusters C4T4, C4Tx, CxT4 and CxTx were observed at frequencies between 19% and 31.4%. Additionally, portraying the PCA showed the clustering of the Arab populations to the middle of the plot between Africans and Asians, as shown in Figure 2 (**publication 2**).

Commentary and Impact

This study characterized KIR genes and individual genotype components. The activating KIR genes were observed at frequencies higher than the inhibitory ones.

Based on 2DL1, 2DL3, 2DS4, and 3DL1 frequencies, I found higher frequencies among the Senegalese population, and the lowest were observed in Australian Aborigines. The Arab-related origin, including this study, was found between these two populations. Also, significant variations between the Saudi population and Australian Aborigines were found across most KIR gene frequencies, explaining the differences between them.

Similarities of culture and language, speaking Arabic as the mother tongue, among Arab populations may explain why their Arab genes frequencies are similar. However, the observed differences among the Arab gene frequencies may be attributed to ethnic admixture that occurred over time.

A group of studies characterizing the KIRs genes in different populations has been published recently. A study that analyzed KIR gene frequencies in unrelated individuals from Malay and Malaysian Chinese has shown the Malay population is more diverse than the Malaysian Chinese [87]. Cardozo et al. identified that haplotype A frequency among the Brazilian population is around 27.4%, which is more frequent than we identified among the Saudi population [88]. At the same time, a German study demonstrates links between KIR haplotype structures and allele group frequencies [89].

Publication 3. Variation of STRs in population from the central region of Saudi Arabia

Significance and Background

In this study, we characterized fifteen autosomal STR markers used in forensic and paternity testing to demonstrate genetic variations in this Saudi sample from Riyadh Province. Short Tandem Repeats (STRs) are a pattern of short nucleotide sequences arranged in 2 to 6 nucleotides along the human genome. They vary from one person to another (polymorphic). The STR DNA is estimated to be around 3% of the entire genome and repeats in every ten thousand nucleotides. The principal amount of STRs is present in the non-coding region, whereas only less than 10% is found in the coding areas [90] [91]. Based on repeat units, STRs are grouped according to their: a) Length that extends from one to six repeats of nucleotides, but the most common STRs in humans are in dinucleotide form. b) A repeating structure in simple repeats form includes one nucleotide unit or compound repeats that include multiple repeats [92].

DNA sequences outside of STR locations demonstrate a very low mutation rate along the genome in each generation, but in the STRs, the rate is highly increased [93]. STRs can cause frame-shift mutation in particular genes leading to amino acid sequence changes. Such changes when they involve immune-related genes can affect the ability of the individual to deal with pathogens.

Multiple DNA-based methods were used to demonstrate variation in the human population worldwide. However, the STR technique is one of the most popular ones utilized in studies on human diversity for many reasons, including the small size of the genetic markers, low rate of mutations occurrence and high distribution along the human genome [94].

Genetic studies focused on DNA sequence variations, including the STRs test, are excellent for detecting genetic polymorphisms that make differentiation among human populations possible.

More than 85% of the Saudi population are Arabs who have lived in this area for thousands of years, which may show how far or close divergence from the typical human ancestor. People of Saudi Arabia are rarely mixed with other ethnic groups, in addition to an increased rate of consanguineous marriage compared with other populations [61].

Based on all these facts, Saudis may have specific genetic components that identify them from different populations in near geographical areas or other parts of the world.

Accordingly, we characterized the autosomal STR markers used in forensic and paternity testing to demonstrate genetic variations in this Saudi sample from Riyadh Province.

Results

We characterized 15 STR genetic markers in Saudis, polymorphic loci were demonstrated, and the number of alleles was found to vary from one locus to another. D18S51 was found at greater polymorphisms (17 alleles) and the TPOX was found at the lower polymorphism (5 alleles). Allele 10 of the TPOX and allele 12 of D13S317 were found at greater frequencies than the other alleles. D2S1338, D19S433, and FGA loci were demonstrated as the most polymorphic STR markers based on the high PD values.

Deviation from HWE was observed in three different loci (TH0, D5S818, and FGA), which were ascribed to high rates of consanguineous marriage among the Saudis or to the Wahlund effect due to a high rate of homozygosis (**Publication 1**, **Publication 2**). HWE values were compared with other Arab-related published studies, as shown in Table 2 of **Publication 3**. I observed significant deviation from HWE in the current study and Tunisians, while no deviation was detected in Omanis or Syrians. Also, we applied multi-dimensional scaling (MDS) to explain the genetic distances between 14 different populations, including Caucasians, Asians, Africans, and Arabs, as shown in Figure 1 of **Publication 3**. Arabs were found clustering in the near middle of the plot

Commentary and Impact

The allele frequencies of 15 STR markers and genetic/forensic parameters were analyzed, and results have been shown. The degree of polymorphism at each locus can also be expressed in terms of heterozygosis along with the PIC [95]. The polymorphic nature of the tested loci was found sufficient to allow the differentiation of Saudi individuals by specific genetic markers, including D21S1, THO, D2S1338, D19S433, D18S5, D5S818, and FGA.

A low degree of polymorphism was observed among Iraqis compared to Saudis and Tunisians [96] as seen in the heterozygosis values for this population, which ranged

between 0.621 and 0.869. Lower values of homozygosis may be attributed to random mating or less consanguinity among specific populations. Although the rate of consanguineous marriage is more frequent among Saudis [61], we demonstrated seven different markers that could be used in their identification. Consequently, this method can be used in clinical practice for Bone Marrow Transplant (BMT) patients to demonstrate engraftment post-transplantation.

Variant alleles such as 34.2 (D21S11), 16.2 (D18S51), 12.2 (D16S539), 29 and 22.2 (FGA), and 7.3 and 8.3 (THO1) that were observed at low frequencies in previous studies were also found at low frequencies in this study. Also, similarities and differences in allele frequencies between the current and previous population studies were observed, including Slovenians [97], Bolivians [98], Wallachians in South Romania [99], and in the Adaima community from Egypt [100].

This study has been followed by several recent publications that characterize STRs in many populations. Chen et al. studied the link between the number of STR repeats and the regulation of gene transcription and how the nearby SNPs are required for STR function. They concluded that the C allele of rs35767 provides a binding site for CCAAT/enhancer binding protein δ (C/EBPD) [101]. In 2018, Saini et al. demonstrated a SNP+STR haplotype reference panel that allows the imputation of STRs from SNP (Single Nucleotide Polymorphisms) array data [102]. Fernández-López studied 25 STR loci of genetic markers in the population of Bahia, Northeastern Brazil [103]. SE33, D21S11, and FGA were observed as the most polymorphic loci in this population, while in this study, I found that D18S5, FGA, and D21S11 were the most polymorphic ones.

Copies of Publications for Chapter 2

Publication No: 1

My contribution included obtaining IRB approval and the approval under my name. I contributed equally with the senior author in the research design and sample selection; I generated the bulk of the laboratory work and analyzed the raw data. I drafted the manuscript, communicated with the other co-authors for review, acted as a corresponding author, and responded to all reviewer comments throughout the publication.

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Immunology and Immunogenetics Insights

HLA-A, -B, -C, -DRB1, and -DQB1 Allele Lineages and Haplotype Frequencies among Saudis

Awad E. Osman[1], Faviel F. Gonzalez-Galarza[2], Mohamed Mubasher[1], Hanan Al-Harthi[1], Nezar Eltayeb ElSheikh[1], Noureddine Berka[3], Derek Middleton[4] and Gehad ElGhazali[5]

[1]King Fahad Medical City, Riyadh, Saudi Arabia. [2]Institute of Integrative Biology, University of Liverpool, Liverpool, UK. [3]University of Calgary, Calgary, Canada. [4]Transplant Immunology, Royal Liverpool and Broadgreen University Hospital, Liverpool, UK. [5]Mafraq Hospital and Sheikh Khalifa Medical City, Abu Dhabi, United Arab Emirates.

ABSTRACT: There are few reported studies on Saudi population for human leukocyte antigens (HLA) genes. We investigated allele lineages (two-digit) and haplotype frequencies of HLA-A, -B, -C, -DRB1, and -DQB1 loci in 499 healthy unrelated individuals, selected from potential bone marrow transplant (BMT) families' donors at King Fahad Medical City (KFMC), Saudi Arabia (SA). Genotyping was performed by Sequence Specific Oligonucleotide Probe (SSOP) utilizing a Luminex-based method. Allele lineages and haplotype frequencies were evaluated along with principal component analysis (PCA) to compare findings with previously reported data on Arab related populations. A total of 18 allele lineages for HLA-A, 28 for -B, 14 for -C, 13 for -DRB1, and 5 for -DQB1 were detected. High values for linkage disequilibrium indicators were found for B:C ($D' = 0.86599$) and DRB1:DQB1 ($D' = 0.89468$) loci. Additionally, PCA results confirmed previous findings on this population, but also indicated some genetic distances from other Arab related populations. The present study helps in further investigations of this population in anthropological analysis and HLA-associated disease studies.

KEYWORDS: HLA, allele, frequencies, Saudis

CITATION: Osman et al. HLA-A, -B, -C, -DRB1, and -DQB1 Allele Lineages and Haplotype Frequencies among Saudis. *Immunology and Immunogenetics Insights* 2014:6 1–6 doi:10.4137/III.S16796.

RECEIVED: May 11, 2014. **RESUBMITTED:** July 7, 2014. **ACCEPTED FOR PUBLICATION:** July 8, 2014.

ACADEMIC EDITOR: Darren Flower, Editor in Chief

TYPE: Original Research

FUNDING: This study was fully supported by King Fahad Medical City, Riyadh, Saudi Arabia.

COMPETING INTERESTS: Authors disclose no potential conflicts of interest.

CORRESPONDENCE: awadelsid@yahoo.com

This paper was subject to independent, expert peer review by a minimum of two blind peer reviewers. All editorial decisions were made by the independent academic editor. All authors have provided signed confirmation of their compliance with ethical and legal obligations including (but not limited to) use of any copyrighted material, compliance with ICMJE authorship and competing interests disclosure guidelines and, where applicable, compliance with legal and ethical guidelines on human and animal research participants.

Introduction

Major histocompatibility complex (MHC) molecules in humans, also known as human leukocyte antigens (HLA), are encoded by several genes on the short arm of chromosome 6 in region 6p21.3.1. HLA genes are highly polymorphic and have a significant impact on the outcome of organ transplant (SOT), hematopoietic stem cell transplantation (HSCT), and pharmacogenetics.[1–6] In HSCT, serious complications such as graft rejection and graft-versus-host disease could be minimized by matching HLA alleles of donor and recipient prior to transplant.[7] In the case of pharmacogenomics, several drugs such as abacavir and carbamazepine may cause diverse reactions, as these drugs appear to react with the antigen-binding groove of certain HLA alleles causing T-cell mediated hypersensitivity.[8] Analysis of alleles and/or haplotypes of the HLA molecules also provides useful means to study the genetic diversity among different populations and within individuals of the same ethnic group.[9–11]

Most of the population in Saudi Arabia (SA) are of Arab origin who have lived in this region for thousands of years.[12] The central areas of SA such as Riyadh and Al Gaseem have more population stability than other parts.[13] The rate of consanguineous marriage is relatively higher among the Saudis, and for the central area of Riyadh province, it is estimated to be around 62.8%.[13,14] SA is part of the Arabian Peninsula, which also includes other countries such as Oman and the United Arab Emirates. During the Islamic state era (between the 7th and 8th centuries AD), the Muslim Arabs spread

their language and culture to North Africa and the middle of Asia.[13,15]

Frequency and geographical distribution of HLA genes/alleles have been well studied over the last two decades; however, few studies were performed among the Saudi population.[16–18] In this study, we investigated HLA-A, -B, -C, -DRB1, and -DQB1 allele lineages and haplotype frequencies among healthy and unrelated families of bone marrow donors from Riyadh province. To our knowledge, the sample size of individuals recruited in this study is relatively larger than the previous HLA studies in Saudi population. Our aim was to determine allele and haplotype frequencies in the Saudi population and compare the outcome with other Arab related populations and also to provide a frequency pool reference to assist researchers in anthropological analysis and HLA-associated disease studies.

Materials and Methods

Samples. Ethylenediaminetetraacetic acid (EDTA) blood samples were collected from 499 healthy unrelated individuals randomly selected from donor-related Bone Marrow Transplant (BMT) patients list (one donor from each family) whose parent and grandparent were living in the Riyadh Province of SA. There were 275 males with an average age of 33.3 years and 224 females with an average age of 29.5 years at the time of sample collection. According to King Fahad Medical City (KFMC) hospital records, the selection of study subjects precluded having any two individuals or their mothers/fathers descending from the same parents. Thus, first or second cousins are not included in the study. The study was approved by the institutional review board of KFMC, and informed consent from study participants was obtained.

Allele lineage typing. Genomic DNA was extracted using a MagNa pure compact instrument (Roche Diagnostics GmbH, Mannheim, Germany). HLA-A, -B, -C, -DRB1, and -DQB1 genotyping was performed by Sequence Specific Oligonucleotide Probe (SSOP) utilizing a Luminex-based method using the Lab Type kit (One Lambda, San Diego, USA), procedure details are available at http://www.onelambda.com. The genotyping procedure at KFMC is continuously monitored for quality assurance by the University of California Los Angeles (UCLA) and by the College of American Pathologists (CAP).

Statistical analysis. Allele lineages for HLA-A, -B, -C, -DRB1, and -DQB1 loci were calculated by a direct counting method, the exact testing method was used to evaluate Hardy–Weinberg equilibrium (HWE), and also the Ewens–Watterson (EW) homozygosity neutrality test, linkage disequilibrium (LD) tests among different loci as well as haplotype frequencies were all evaluated using the PyPop software.[19] Principal component analysis (PCA) was conducted using the SAS software[20] to determine and portray the genetic differences and similarities between our study sample and other available data on Arab related populations.

Results and Discussion

The frequencies for HLA-A, -B, -C, -DRB1, and -DQB1 allele lineages are shown in Tables 1 and 2. All previously described allele lineages for the HLA-A locus were observed in this study, except A*25, A*43, and A*80. The highest frequency was observed for A*02 (28.9%), followed by A*68 (10.1%), A*24 (8%), and A*26 (7.4%). Similar frequencies of A*02 have been reported in other SA studies (Riyadh city) (30.4%) and Guraiat and Hail areas (27.9%) and also in other related Arabs populations such as United Arab Emirates (25.2%).[16,18,21,22] The allele lineage A*43 has been mainly reported in populations of African descent,[23] which may explain its absence in our Saudi population. For HLA-B locus, 28 allele lineages were identified in this study with B*51, B*50, and B*08 representing more than 45% of the alleles frequency. The B*51 allele lineage, which has been associated with Behçet's disease,[24] demonstrated the highest frequency among Saudis (19.3%). In contrast, B*46, B*48, B*54, B*56, B*59, B*67, B*78, and B*83 allele lineages were not observed in our study population. There is nothing remarkable about finding previously reported results. The highest observed frequencies for HLA-C were C*07 (24.9%), C*06 (20.2%), and C*15 (12.6%); whereas for HLA-DQB1 were DQB1*02 (36.1%), DQB1*03 (25.1%), and DQB1*06 (24.6%). Lastly, DRB1*07, DRB1*04, DRB1*03, and DRB1*13 were the most predominant allele lineages for the HLA-DRB1 locus with 20.2%, 16%, 15.8%, and 14.5% frequencies, respectively. It is worth noting that our study is in agreement with previously reported Saudi studies in terms of allele lineage frequencies for HLA-A, -B, and -DRB1.[16,21] However, little variation from our study was indicated in Guraiat and Hail area reports that may explain the possible differences from one area to another within one country.

As displayed in Table 3, the HWE deviation was not observed for the heterozygote component of the HLA-A, -B, -C, -DRB1, and -DQB1 loci data. However, that was not the case for the homozygote component of these genetic markers except for HLA-A locus; the high frequency of A*02 (28.9%) allele suggests that this population may have been positively selected.[4] Excess homozygosity was observed in this dataset and Hajeer et al[18] dataset. This deviation from HWE can be attributed to the high rate of consanguinity among the Saudi population and particularly in Riyadh province or to Wahlund effect that is caused by subpopulation structure.[14]

Estimation of multiple-locus haplotypes by the maximum likelihood method revealed 20 common (>1%) haplotype groups for HLA-A~C~B loci combinations (Table 4). The most common haplotypes were A*02~C*06~B*50 (7.3%), A*02~C*07~B*07 (4.2%), and A*26~C*07~B*08 (3.3%). The A*02~C*06~B*50 haplotype has been mainly found in two North African populations, Morocco and Tunisia,[9,25] probably because of their Arabic descent. For HLA-DRB1 and -DQB1 haplotypes, 11 common haplotypes were found (Table 4). The most predominant haplotypes were

Table 1. Allele lineage frequencies of HLA-A, -B, and -C loci in Saudis.

HLA-A*	COUNTS	AF	GF (%)	HLA-B*	COUNTS	AF	GF (%)	HLA-C*	COUNTS	AF	GF (%)
01	70	0.070	13.6	07	81	0.081	15.3	01	13	0.013	2.6
02	288	0.289	49.3	08	100	0.100	19.5	02	26	0.026	5.1
03	72	0.072	13.2	13	14	0.014	2.6	03	53	0.054	10.8
11	24	0.024	4.6	14	20	0.020	4	04	104	0.106	18.7
23	39	0.039	7	15	48	0.048	9.4	05	23	0.023	4.7
24	80	0.080	15	18	20	0.020	3.8	06	198	0.202	34.7
26	74	0.074	14.4	27	18	0.018	3.4	07	245	0.249	44.1
29	32	0.032	6.4	35	70	0.070	13.1	08	4	0.004	0.8
30	45	0.045	8.6	37	8	0.008	1.6	12	61	0.062	11.8
31	66	0.066	12.6	38	18	0.018	3.6	14	28	0.028	5.7
32	37	0.037	7.4	39	19	0.019	3.8	15	124	0.126	22.5
33	50	0.050	9.6	40	15	0.015	3	16	61	0.062	12
34	1	0.001	0.2	41	34	0.034	6.6	17	39	0.040	7.7
36	1	0.001	0.2	42	10	0.010	1.8	18	5	0.005	1
66	6	0.006	1.2	44	21	0.021	4.2	**Total**	984	1.0	
68	100	0.101	18.4	45	6	0.006	1.2				
69	3	0.003	0.6	47	3	0.003	0.6				
74	10	0.010	2	49	15	0.015	2.8				
Total	998	1.0		50	162	0.163	28.9				
				51	192	0.193	33.3				
				52	22	0.022	4				
				53	35	0.035	6.2				
				55	6	0.006	1.2				
				57	13	0.013	2.6				
				58	40	0.040	8				
				73	2	0.002	0.4				
				81	3	0.003	0.6				
				82	1	0.001	0.2				
				Total	996	1.0					

Abbreviations: AF, allele frequency; GF, gene frequency.

Table 2. Allele lineage frequencies of HLA-DRB1 and -DQB1 loci in Saudis.

HLA-DRB1*	COUNTS	AF	GF (%)	HLA-DQB1*	COUNTS	AF	GF (%)
01	34	0.035	6.6	02	346	0.361	58.1
03	154	0.158	29.4	03	240	0.251	42.8
04	156	0.160	28.8	04	18	0.019	3.8
07	196	0.202	34.1	05	118	0.123	22.2
08	9	0.009	1.6	06	235	0.246	40.5
09	7	0.007	1.4	**Total**	957	1.000	
10	32	0.033	6.4				
11	88	0.090	15.8				
12	1	0.001	0.2				
13	140	0.145	25.9				
14	9	0.009	1.8				
15	106	0.109	20.3				
16	41	0.042	7.6				
Total	973	1.0					

Abbreviations: AF, allele frequency; GF, gene frequency.

Table 3. Observed and expected heterozygosity/homozygosity and HWE significance for HLA-A, -B and -DRB1 in Saudis.

LOCUS	OBSERVED HETEROZYGOTE COUNT	EXPECTED HETEROZYGOTE COUNT	*P*-VALUE	OBSERVED HOMOZYGOTE COUNT	EXPECTED HOMOZYGOTE COUNT	*P*-VALUE
A	422	434.66	0.5437	77	64.34	0.1145
B	428	450.48	0.2896	70	47.52	0.0011
C	404	421.17	0.4028	88	70.83	0.0413
DRB1	391	421.85	0.1331	97	66.15	0.0001
DQB1	323	350.69	0.1393	156	128.31	0.0145

DRB1*07~DQB1*02 (18.9%), DRB1*03~DQB1*02 (14.4%), and DRB1*04~DQB1*03 (13.6%). Finally, the analysis of HLA-A: C:B: DRB1:DQB1 loci revealed 424 haplotypes with 10 haplotypes having frequencies >1%. The highest observed frequencies among these allele lineages were for combinations A*02~C*06~B*50~DRB1*07~DQB1*02 (6.7%), A*26~C*07~B*08~DRB1*03~DQB1*02 (2.4%), and A*02~C*07~B*07~DRB1*15~DQB1*06 (2.2%). The most common haplotype in our study (A*02~C*06~B*50~DRB1*07~DQB1*02) was also reported as one of the highest in the Tunisian population with a frequency of 3%.[25]

The LD values for A-B, A-C, A-DRB1, A-DQB1, B-C, B-DRB1, B-DQB1, C-DRB1, C-DQB1, and DRB1-DQB1 pairs were calculated (Table 5), the highest D' values were observed between B-C (0.86599) and DRB1-DQB1 (0.89468) loci pairs.

PCA analyses (Fig. 1) were performed to portray the genetic distances of the HLA-A, -B, and -DRB1 allelic distributions between our study population and other Arab related origin, available at http://www.allelefrequencies.net.[21] Except for the study in Guraiat and Hail areas of HLA-DRB1 locus, other Saudi reports were very close in genetic distance to our study population. Similarities with our data were also indicated for United Arab Emirates, and Oman in HLA-A locus, and for Moroccan, Tunisians, and Algerians for HLA-DRB1. Interestingly, our previous data demonstrated close

Table 4. Most common haplotypes for HLA-A~C~B, HLA-DRB1~DQB1, HLA-A~C~B~DRB1~DQB1 in Saudis (frequencies >1%).

HLA-A~C~B	HF (2n = 982)	HLA-DRB1~DQB1	HF (2n = 956)	HLA-A~C~B~DRB1~DQB1	HF (2n = 940)
02~06~50	0.0735	07~02	0.1894	02~06~50~07~02	0.0665
02~07~07	0.0421	03~02	0.1445	26~07~08~03~02	0.0245
26~07~08	0.0334	04~03	0.1367	02~07~07~15~06	0.0224
31~15~51	0.0322	13~06	0.1349	31~15~51~13~06	0.0191
02~15~51	0.0263	15~06	0.1046	24~07~08~03~02	0.0183
68~07~08	0.0256	11~03	0.0784	02~15~51~04~03	0.0163
23~06~50	0.0218	16~05	0.0387	33~03~58~03~02	0.0138
02~16~51	0.0215	01~05	0.0335	33~06~50~07~02	0.0128
24~07~08	0.0196	10~05	0.0324	68~07~08~03~02	0.0112
68~15~51	0.0184	04~02	0.0195	02~07~07~03~02	0.0102
24~04~35	0.0179	07~03	0.0125		
02~14~51	0.0163				
01~17~41	0.0162				
03~06~50	0.0150				
33~03~58	0.0131				
33~06~50	0.0109				
30~04~53	0.0102				
11~12~52	0.0102				
31~06~50	0.0101				
33~05~14	0.0100				

Abbreviation: HF, haplotype frequency.

Table 5. LD of 2-locus pairs in Saudis.

LOCUS PAIR	D	D′
A:B	0.00660	0.46857
A:C	0.00772	0.38416
A:DRB1	0.00714	0.33670
A:DQB1	0.00815	0.22860
B:C	0.02008	**0.86599**
B:DRB1	0.01198	0.55092
B:DQB1	0.01723	0.47409
C:DRB1	0.01566	0.47103
C:DQB1	0.01700	0.35877
DRB1:DQB1	0.04654	**0.89468**

Note: LD based on Hendricks's statistic (*D′*), highest values are in bold.
Abbreviation: LD, linkage disequilibrium.

affinities between the Saudis and Omani, Tunisian, Palestinian (Gaza), and Moroccan populations in killer immunoglobulin-like receptors study.[15] The noted variable degree of genetic distances between our study and other Arab populations (Fig. 1) may be attributed to admixture with populations from other ethnic origins.

Although one of the potential limitations of our results is that the analyses were performed using a two-digit resolution typing level, this study provides basic data for anthropological analysis, HLA-associated disease studies, and organ transplantation (eg renal transplantation). Larger sample size studies using higher resolution typing methods are also recommended.

Acknowledgments

Our thanks and appreciation is extended to the BMT donors who participated in this study. We also thank Dr Abdulwahid

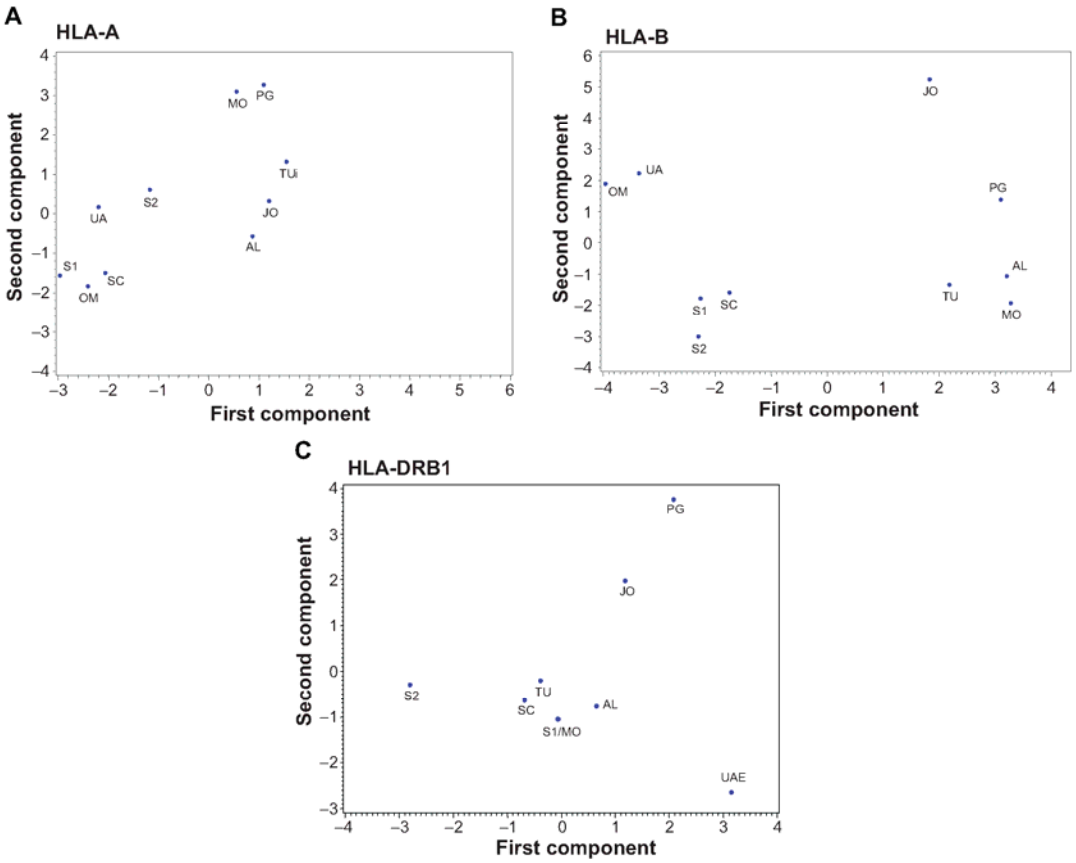

Figure 1. PCA of (**A**) HLA-A, (**B**) -B, and (**C**) -DRB1 allele frequencies data of 10 Arab related populations. Saudis Riyadh[16] (S1), Saudis Guraiat and Hail[21] (S2), Saudi current study (SC), Jordanians[21] (JO), Omanis[21] (OM), Moroccans[9] (MO), Palestine[21] (PG), Tunisians[25] (TU), Algerians[21] (AL), and United Arab Emirates[22] (UA). First component represents 68.5% of the total genetic variation and second component represents 17.5%.

Aldehaimi and members of the immunology and serology laboratory at KFMC for their help and support.

Author Contributions

AO and GE conceived and designed the experiments. AO, HA, and NE performed the experiments. FG and MM analyzed the data. AO and HA contributed reagents/materials/analysis tools. AO, FG, and MM wrote the paper. DM, GE, and NB supervised the study. All authors reviewed and approved of the final manuscript.

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Publication No: 2

My contribution to this study included obtaining IRB approval and the approval under my name. I equally contributed to the research design and sample selection with a senior author. I generated the bulk of laboratory work, analyzed the raw data, drafted the manuscript, communicated with the other co-authors for review, acted as the corresponding author and responded to all reviewer comments throughout the publication.

Human Immunology 75 (2014) 536–540

Contents lists available at ScienceDirect

journal homepage: www.elsevier.com/locate/humimm

Brief Communication

Characterization of human killer immunoglobulin-like receptors (KIRs) among healthy Saudis

Awad E. Osman [a,*], Mohamed Mubasher [a], Nezar E. ElSheikh [a], Hanan AlHarthi [a], Ahmed S. Al Yami [a], Raja Rajalingam [b], Abdulwahid Al-Dehaimi [a], Derek Middleton [c], Gehad ElGhazali [d]

[a] King Fahad Medical City, Riyadh 11525, Saudi Arabia
[b] UCLA Immunogenetics Centre, Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at UCLA, University of California at Los Angeles, United States
[c] Transplant Immunology, Royal Liverpool and Broadgreen University Hospital, Liverpool, UK
[d] Mafraq Hospital and Sheikh Khalifa Medical City, Abu Dhabi, United Arab Emirates

ARTICLE INFO

Article history:
Received 2 October 2013
Accepted 24 February 2014
Available online 6 March 2014

Keywords:
Killer immunoglobulin-like receptor
Haplotype
Saudi

ABSTRACT

Genes encoding KIRs vary in frequency among different populations and ethnic groups. This study investigated the KIR gene frequency distribution in 148 healthy unrelated Saudi subjects and compared the results with other published findings. All inhibitory and activating KIR genes were present at variable frequencies, with A haplotype-associated genes (KIR2DL1, -2DL3, -3DL1, and KIR2DS4) being observed at higher frequencies (88.9–99.5%) than B haplotype-associated genes (KIR2DS1, -2DS2, -2DS3, -2DS5, -2DL5 and -2DL2) (31.1–70.1%). Thirty-one different KIR genotypes were observed, and AA genotypes displayed the highest frequency (18.2%). This Saudi population possesses similar KIR gene distributional characteristics to those reported in other neighboring populations (e.g., Lebanese) and shows disparities in certain genes and gene contents from other populations (e.g., Australian Aborigines). These findings can be used as a reference control in future studies evaluating the functional significance of the KIR genes and their associations with specific diseases.

1. Introduction

Human natural killer (NK) cells are a subset of lymphocytes that have the ability to react with cells lacking human leukocyte antigen (HLA) class I without prior immunological exposure [1]. NK cell function is determined by killer immunoglobulin receptors (KIRs) and the type of HLA ligand on the surface of target cells [2]. Binding of KIRs to putative ligands can induce suppression or activation of NK cells. NK cell-mediated cytotoxicity depends on a fine balance between the inhibitory and activating signals induced by KIR molecules on the NK cell surface [3,4].

KIR genes are highly polymorphic and are located on chromosome 19q13.4 [5]. In humans, 16 KIR genes have been identified, defined as the KIR2DL1, -2DL2, -2DL3, -2DL4, -2DL5, -3DL1, -3DL2, -3DL3, -2DS1, -2DS2, -2DS3, -2DS4, -2DS5, -3DS1, -2DP1 and -3DP1 genes. With few exceptions, each KIR gene typically encodes either an inhibitory or activating molecule [6]. Two groups of KIR haplotypes have been defined, designated A and B based on their KIR gene content [7]. The A haplotype is composed of six inhibitory KIR genes: KIR2DL1, -2DL3, -3DL1, -3DL2, -3DL3 and -2DL4; one activating KIR gene: KIR2DS4; and two pseudogenes KIR2DP1 and -3DP1. The B haplotype differ in its gene content, which is determined mainly by the presence or absence of the genes that are not part of the A haplotype. [5]. The framework KIR3DL2, -3DL3 and -2DL4 genes are present in both the A and B haplotypes, and most activating KIR genes are found within the B haplotype [8].

Full-length sequencing of KIR haplotypes showed that the framework regions divide the KIR locus into two parts: centromeric (C) and telomeric (T) segments that differ in their gene contents [5,9,10]. The centromeric part of the A haplotype contains KIR2DL3, -2DL1 and -2DP1, in addition to 3DL3 and -3DP1,while the telomeric part contains the KIR3DL1 and -2DS4 genes, in addition to KIR2DL4 and -3DL1. Unlike the A haplotype, which is fixed, uniform and conserved, the B haplotype is polymorphic and exhibits a variable centromeric end that includes the KIR2DS2, -2DL2, -2DL5B, -2DS3 and -2DS5 genes, in addition to KIR3DL3

Abbreviations: NK, natural killer; KIR, killer immunoglobulin-like receptor; BMT, bone marrow transplant; SSOP, Sequence-Specific Oligonucleotide Probe.

* Corresponding author.
E-mail address: awadelsid@yahoo.com (A.E. Osman).

http://dx.doi.org/10.1016/j.humimm.2014.02.023
0198-8859/

and -3DP1, while the telomeric part of the B haplotype contains the KIR3DS1 and -2DS1 genes, in addition to KIR2DL4 and -3DL1 [10,11]. Therefore, diversity is generated in the KIR locus by the content of group A and group B haplotype genes and the allelic variation that occurs within both the centromeric and telomeric regions [12]. KIR allelic products have been shown to differ in their specificity and affinity regarding ligand binding [13], and these allelic differences may contribute to disease susceptibility and progression [14]. The frequencies of KIR genes and haplotypes show great variation among different populations, and the crucial function of KIRs in immunity may have an impact on susceptibility to infection, autoimmune diseases and transplantation outcomes among different ethnic groups [10,15,16].

Saudi Arabia covers 80% of the Arabian Peninsula, mainly in the central arid region. The remaining 20% comprises Yemen, Oman, the United Arab Emirates, Qatar, Bahrain and Kuwait. Some reports have provided support for the "out-of-Africa" hypothesis of human dispersion, which indicates that immigrants followed a southern pathway along the tropical coast of the Arabian Peninsula, India, Southeast Asia and Australia before pursuing the Levantine pathway [11,17]. The geographical location of the Arabian Peninsula played an important role in trade, cultural exchange and warfare following the appearance of the Old World civilizations as well as the emergence of Islam and the subsequent cultural expansion, all of which are factors that may have affected the Arab gene pool. Unlike the population of the coastal area, the central part of the Peninsula has been stable and is less susceptible to migration-related factors [17,18]. The present study aimed to investigate and characterize the KIR genes frequencies and KIR genotype contents of unrelated healthy Saudi subjects and compare the results with other populations.

2. Materials and methods

From the available list of potential bone marrow transplant (BMT) family donors, 148 subjects met the inclusion criteria of being healthy and unrelated. This entailed the exclusion of siblings showing identical HLA matching to the patient. In the event of finding more than one eligible sibling per family, only one sibling was randomly selected for this study. The sample size of 148 afforded the statistical ability to detect at least a 1.5-fold significant difference in the odds ratio vs. other populations in at least one KIR gene. The study received ethical approval from the Institutional Review Board of King Fahad Medical City (KFMC), and informed consent was obtained prior to the time of blood collection. Sequence-Specific Oligonucleotide Probe (SSOP) kits from One Lambda (San Diego, CA, USA) were used for KIR genotyping, and for quality assurance purposes, multiple DNA samples with a known KIR genotype were run in parallel with our study samples. The same exact results were always obtained for these controls.

2.1. Assigning haplogroup-based genotypes

Individuals carrying one or more of the KIR2DL2, -2DL5, -3DS1, -2DS1, -2DS2, -2DS3 and -2DS5 genes were considered to be of group B haplotypes, and conversely, those who did not carry any of these genes were considered to be of group A haplotypes. Haplotype group ID profiles and the assignment of each individual to an AA or Bx genotype (x can be either an A or B haplotype) were obtained according to http://www.allelefrequencies.net [19].

2.2. Centromeric and telomeric genes cluster classification

Based on the presence or absence of the centromeric (C) and telomeric (T) clusters, Bx genotypes are classified into four subsets: C4T4 (presence of both C and T), C4Tx (presence of C and absence of T), CxT4 (absence of C and presence of T) and CxTx (absence of both) [5].

2.3. Statistical analysis

Using the SAS program [20], the frequencies of positive individuals for each of the 16 KIR genes were determined using an algorithm based on a direct counting method. Comparisons with other populations were based on nine KIR genes (KIR2DL1, -2DL3, -2DS4, -3DL1, -2DL2, -2DS1, -2DS2, -2DS3 and -3DS1). For this purpose, Fisher's exact test and logistic regression methodology were used. To determine significant differences between our study population and other populations, the log of the odds (logit) for the presence of each KIR gene was modeled as a function of each population. The overall significance level was set at 0.05. Principal components analysis (PCA) was conducted using the SAS software [20] to determine and portray the genetic differences and similarities between our study sample and other populations.

3. Results and discussion

3.1. KIR gene frequencies

As explained in Table 1, the framework genes were observed at a frequency of 100% in our sample and all of the compared populations (data obtained from http://www.allelefrequencies.net), except for 3DL3 in the Moroccan Chaouya population (97%). Additionally, the inhibitory KIR genes were observed at variable but high frequencies, except for 2DL2 and 2DL5, which were observed at proportionally lower frequencies. Notably, this case for all of the compared populations shown in Table 1, except for the

Table 1
The frequencies of KIR genes in our study population compared to previously published data for Arabs and non-Arab-related populations.

Population	No	2DL1%	2DL3%	2DS4%	3DL1%	2DL2%	2DL5%	2DS1%	2DS2%	2DS3%	2DS5%	3DS1%	2DP1%	2DL4%	3DL2%	3DL3%	3DP1%
Saudis-2 (current)	148	98	83.1	92.5	94.4	68.2	64.9	43.9	73	43.2	43.9	35.8	98	100	100	100	100
Saudis-1	162	96.3	91.4	93.8	95.7	56.2	56.2	33.3	57.4	38.3	27.8	34.6	96	100	100	100	100
Jordanians Palestine	105	83	85	88	88	62	63	44	64	37	27	39	N/R	100	100	N/R	N/R
Lebanese	120	99.2	88.3	95	95.8	59.2	58.3	40.8	59.2	37.5	30.8	35.8	N/R	100	100	N/R	N/R
Omani	99	98	87.9	94.9	96	50.5	59.6	32.3	49.5	30.3	39.4	29.3	98	100	100	100	100
Moroccans Chaouya	67	95.5	73.1	100	100	70.1	67.2	25.4	65.7	52.2	32.8	25	100	100	100	97	100
Tunisians	114	99.1	91.2	96.5	100	59.6	60.5	22.8	59.6	38.6	23.7	23.7	N/R	100	100	100	N/R
Iranians- Arab	76	100	89.5	98.7	85.5	63.1	67.1	42.7	56.3	50	35.5	42.1	98.7	100	100	100	100
Indians Paravar	77	97	79	84	84	69	83	65	75	49	66	60	97	100	100	100	100
Indians Kanikar	35	97	77	80	80	74	86	60	74	63	60	69	94	100	100	100	100
Gabonese	54	100	79.6	100	100	64.8	70.4	18.5	57.4	35.2	33.3	7.4	100	100	100	100	100
Senegalese	118	100	90	100	99	55	52	13	42	24	30	4	100	100	100	100	100
Australian Aborigines	67	72	68	52	55	79	N/R	82	84	81	N/R	78	N/R	N/R	N/R	N/R	N/R

Australian Aborigines. Worldwide, the frequencies of KIR2DL2 and KIR2DL5 are reported to be high among the population of Papua New Guinea (95.5% and 86%, respectively), whereas much lower values are observed for KIR2DL2 among Japanese individuals, at 8.5%, and for KIR2DL5 among Indian Asians from the West Midlands, England, at 26% [19]. In accordance with previous reports, KIR3DL1 and KIR3DS1 in this study were ascertained to be either positive, or one positive while the other negative [1]. The selected populations for comparison in this study were either of Arab origin or part of the populations that followed the southern pathway along the tropical coast of the Arabian Peninsula and India, ending in Australia, according to the out-of-Africa dispersion theory.

As shown from the determination of the significant differences for our study population versus others in Table 2, the Australian Aborigines significantly segregated themselves across seven out of the nine examined KIR genes frequencies (all p-values $\leqslant 0.001$). Furthermore, the Senegalese and Indians Kanikar (as well as the Indians Paravar) displayed significant differences in five and four KIR genes, respectively. The Arab-related populations (Saudi-1, Lebanese, Jordanian Palestine, Omani, Moroccan Chaouya, Tunisian and Iran Arab) exhibited statistically significant differences in one to three KIR genes. It is not surprising that the gene frequencies observed in our study population are closer to those found in populations with similar cultures and the same mother tongue. The reason for some of the disparities observed in the frequencies of a few KIR genes for these populations from the current study is not yet clear, but this heterogeneity may reflect the ethnic admixture of these populations.

Table 2
Statistical significance (p-value) testing of the KIR genes that were commonly reported across all of the populations compared in this study versus our study population (statistically significant values at the <0.5 level are indicated in **bold**).

Population	2DL1	2DL2	2DL3	2DS1	2DS2	2DS3	2DS4	3DL1	3DS1
Saudis-1	0.38	**0.03**	0.11	0.07	**0.05**	0.44	0.82	0.85	0.81
Jordanians Palestine	**<.001**	0.29	0.91	0.91	0.12	0.38	0.19	0.06	0.59
Lebanese	0.43	0.12	0.36	0.69	**0.02**	0.41	0.41	0.63	0.99
Omanis	0.54	**0.01**	0.45	0.08	**<.001**	0.06	0.45	0.62	0.28
Moroccan Chaouya	0.32	0.77	0.06	0.01	0.28	0.18	0.98	0.98	0.13
Tunisians	0.46	0.15	0.11	**<.001**	0.02	0.51	0.18	0.97	**0.04**
Iranians- Arab	0.98	0.44	0.31	0.87	0.01	0.29	0.09	**0.03**	0.35
Indians Paravar	0.41	0.92	0.12	**0.002**	0.7	0.43	0.03	**0.01**	**0.001**
Indians Kanikar	0.76	0.48	0.31	0.07	0.87	**0.03**	0.03	**0.01**	**<.001**
Gabonese	0.97	0.64	0.41	**<.001**	**0.03**	0.35	0.98	0.98	**0.001**
Senegalese	0.96	**0.02**	0.21	**<.001**	**<.001**	**<.001**	0.97	0.07	**<.0001**
Australian Aborigines	**<.001**	0.11	**<.001**	**<.001**	0.09	**<.001**	**<.001**	**<.001**	**<.001**

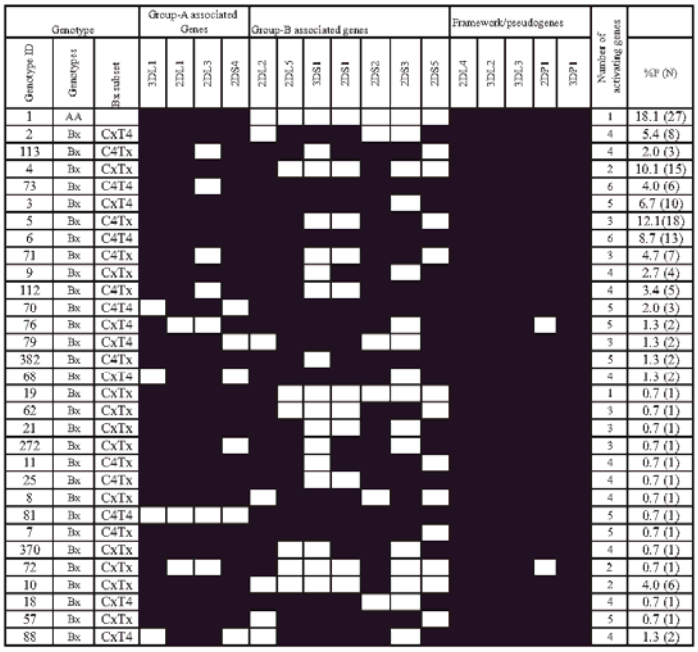

Fig. 1. Presents the KIR gene diversity in our study population. This figure includes the genotype IDs, A and Bx haplotype-associated groups, framework/pseudogenes and the activating KIR gene frequencies. Black areas indicate positive genes, and blank areas indicate negative ones.

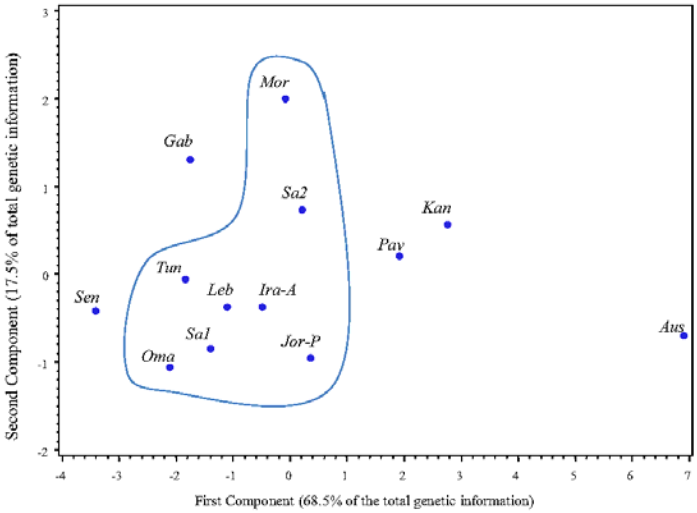

Fig. 2. PCA of nine variable KIR genes (2DL1, 2DL2, 2DL3, 2DS1, 2DS2, 2DS3, 2DS4, 3DL1 and 3DS1) that were commonly reported or typed across all of the populations compared in this study, while we excluded other genes that were not reported in certain studies. Saudis-1(*Sa1*), Saudis 2(*Sa2*), Palestinian Jordanians (*Jor-P*), Lebanese (*Leb*), Omanis (*Oma*), Moroccan Chaouya (*Mor*), Tunisians (*Tun*), Iranian Arab (*Ira-A*), Indian Paravar (*Pav*), Indian Kanikar (*Kan*), Gabonese (Gab), Senegalese (*Sen*) and Australian Aborigines (*Aus*).

3.2. KIR genotype frequencies

KIR genotypes are a set of KIR genes found in one person and are considered to be more informative markers for differentiating human ethnic groups than the gene frequencies [21]. As indicated in Fig. 1, a total of thirty-one different KIR genotype groups were observed. The most prevalent were 3, 6, 4, 5 and 1, in accord with what has previously been reported [1]. Published reports indicate that lower frequencies of these genotypes are observed among North Asian Indian populations, such as Kanikar and Paravar (2.9%-5.5%), while higher frequencies are found in Japanese and Chinese Han populations (55.2%-58.7%) [5]. Genotype 6, which includes all of the inhibitory and activating KIR genes, was observed at a frequency of 7.9% in this study and at frequencies of 2.8%-5.9% among populations from Iran and Palestine [5,7]. All subsets of Bx genotype gene clusters C4T4, C4Tx, CxT4 and CxTx were observed at frequencies of 19%-31.4% in our study. An absence of one or two of these genotype subsets has been reported in some other populations; for instance, C4Cx, which is observed at frequencies of 40.5% among Iranian Azeri and 31.6% among Iranian Arab, was not detected among American Natives [5].

3.3. Principal components analysis (PCA)

In consideration of the limitations of various typing methods as a reason for unexpected groupings [2], the Arab populations are clustered approximately in the middle between Africans and Asians, as illustrated in Fig. 2. This may support the out-of-Africa theory of migration, which provides some historical perspective regarding the genetic relationships between Arabs and both Africans and Asians. It can also be noted in Fig. 2 that the spatial distance of Australian Aborigines from the Arab populations suggests an absence of any conceivable genetic link. It is worth noting that our HLA data (not yet published) revealed close affinities among Saudi, Omani, Tunisian, Palestinian (Gaza) and Moroccan populations.

In conclusion, this study was able to characterize and describe the KIR gene distributions among healthy, non-blood-related Saudis. These results were consistent with those found in other neighboring populations, but they also reflected disparities compared to other non-Arab populations, such as Australian Aborigines. These findings can be used as a reference control in future studies evaluating the functional significance of the KIR genes and their associations with specific diseases.

Acknowledgments

This study was fully supported by King Fahad Medical City, Riyadh, Saudi Arabia. Our thanks and appreciations are extended to the BMT donors who participated in this study, and we also thank members of the immunology and serology laboratory at KFMC for their help and support.

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Publication No: 3

My contribution to this study included obtaining the IRB approval, which was under my name. I contributed equally with a senior author in the research design and sample selection. I generated the bulk of laboratory work and analyzed the raw data, drafted the manuscript, communicated with the other co-authors for review, acted as the corresponding author, and responded to all reviewers' comments throughout the publication.

Forensic Research

Osman, et al., J Forensic Res 2015, 6:1
http://dx.doi.org/10.4172/2157-7145.1000267

Research Article Open Access

Autosomal Short Tandem Repeat (STR) Variation Based on 15 Loci in a Population from the Central Region (Riyadh Province) of Saudi Arabia

Awad E Osman[1]*, Habiba Alsafar[2], Guan K Tay[3], Jasem BJM Theyab[4], Mohamed Mubasher[1], Nezar Eltayeb-El Sheikh[1], Hanan AlHarthi[1], Michael H. Crawford[5] and Gehad El Ghazali[6]

[1]*PCLM, King Fahad Medical City, Riyadh 11525, Saudi Arabia*
[2]*Khalifa University of Science Technology and Research, Abu Dhabi, United Arab Emirates*
[3]*Centre for Forensic Science, The University of Western Australia, Crawley, Western Australia*
[4]*Department of Sociology and Social Work and Anthropology, Kuwait University, Kuwait*
[5]*Laboratory of Biological Anthropology, University of Kansas, USA*
[6]*Sheikh Khalifa Medical City, PaLMS, SEHA, Abu Dhabi, United Arab Emirates*

Abstract

Introduction: The small size of Short Tandem Repeats (STRs), their ubiquitous genome-wide distribution and polymorphic nature enhances their value in human forensic/population genetics applications.

Objectives: This study aims to investigate the short tandem repeat variation based on 15 loci in a population from the central region of Saudi Arabia.

Methods: Allele frequency variation for 15 Short Tandem Repeat (STR) loci was examined in 190 unrelated Saudi volunteers.

Results: This study summarizes the allele distribution in the Saudi population and compares them to other populations located in Asia, Africa, the Middle East and Europe. The standard forensic parameters of Observed Hetrozygosity (Ho), Expected Heterozygosity (He) and Gene Diversity Index (GD) were determined for the following 15 STR loci: D8S1179, D21S1, D7S820, CSF1PO, D3S1358, TH0, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S5, D5S818 and FGA. The most frequent alleles in the Saudi population were: 8 repeats (0.558) at TPOX, 12 (0.411) at D13S317, 12 (0.385) at CSF1PO, 11 (0.382) at D16D539 and 10 (0.358) at D7S820. The 15 markers utilized in this study are highly informative as evidenced by their high power of discrimination (PD) values with D2S1338, D19S433 and FGA having the highest PD values. The relationship between the Saudi population and other geographically distributed populations, assessed by a Multidimensional Scaling (MDS) plot, showed that the Saudi population clustered with groups from Yemen, Iraq, Qatar, Oman and Bahrain.

Conclusion: TPOX, D13S317, CSF1PO, D16D539 and D7S820 markers were found suitable for forensic analysis, paternity testing and can also be used for chimerism study after allogenic bone marrow transplantation for Saudi population. On the other hand, the population admixture with other ethnic origins might explain the variable degree of genetic distances of this population and other Arab-related groups.

Keywords: Short tandem repeat; Saudi arabia; Allele frequencies

Abbreviations: STR(s): Short Tandem Repeat(s); MDS: Multidimensional Scaling; HWE: Hardy–Weinberg Equilibrium

Background

Short Tandem Repeats (STRs) are nucleotides sequences with repeat motifs of variable lengths (2 to 8bp) that are polymorphic (i.e. number of repeats varies between individuals) [1,2]. STRs represent about 3% of human DNA and occur approximately one time in every 10,000 nucleotides. STRs are an invaluable tool and their unique sequences used in genetic finger printing during forensic investigations involve significant biological evidence. Their high degree of polymorphisms makes them informative [3], especially when considering multiple loci simultaneously. The new STR kits now contain over 20 STR loci that are amplified in a single multiplex reaction. Additionally, STR markers have been widely used in medical applications such as for the assessment of allogenic bone marrow transplantation engraftment in calculating the ratio of donor/patient DNA presence and in the study of population genetics. The use of STRs depends on the allele frequencies distributions that vary between various populations [1-4].

Various DNA-based techniques have been used to identify the genetic differences in human populations. STR loci are useful and preferred because of their small size, relatively low incidence of mutation and wide spread distribution [5]. Genetic studies and in particular those based on STR applications have been very important towards developing an appreciation of the extent of genetic polymorphisms that exists between different populations.

Throughout history, society had been stratified on the basis of caste, class, clan, race, region, religion, ethnicity, gender, age and socioeconomic status. It is ethnicity and racial discrimination that distinguishes one nation from the other. Ethnicity is, as defined by Macionis; "a shared cultural heritage and people define themselves or others as members of an ethnic category based on common ancestry, language or religion that gives them a distinctive social identity" [6]. The

***Corresponding author:** Awad E Osman, PCLM, King Fahad Medical City, Riyadh 11525, Saudi Arabia, Tel: 966-1-2880000; Fax: 966-1-2881215; E-mail: awadelsid@yahoo.com

Received December 22, 2014; **Accepted** January 20, 2015; **Published** January 25, 2015

Citation: Osman AE, Alsafar H, Tay GT, Theyab JBJM, Mubasher M, et al. (2015) Towards a Forensic Psychological Evaluation of Juvenile Fire Setters: Parent Power. J Forensic Res 6: 267. doi:10.4172/2157-7145.1000267

same is the case within the Arab world, which has maintained a unique ethnographic identity, historical background, ancestry, cultural traits, social norms, moral values, religious beliefs and genealogy. At present, the total Arab population is estimated at about 325 million, increasing at a rate of approximately 2.3% annually. The Arab population of the Middle East and North Africa is distributed throughout 17 different countries, namely: Algeria, Bahrain, Egypt, Iraq, Jordan, Kuwait, Lebanon, Libya, Morocco, Oman, Palestine, Qatar, Saudi Arabia, Syria, Tunisia, the United Arab Emirates, and Yemen.

In Saudi Arabia, approximately 87% of the population is of Arab descent, a group of people who have inhabited this region for thousands of years. The remaining 13% of inhabitants of this region migrated into this country during the last, 400 years [7]. The rate of consanguineous marriage is relatively high and has been estimated to at around 57.7% [8]. The Saudi population can be characterized by a specific ethnic subset which presumably reflects a unique set of allele frequencies and genotypes and that could differentiate them from other populations in near geographical areas and worldwide i.e. the alleles frequencies will be different from other populations and the genetic distances between these populations will be variable depending on the time of divergence from the common human ancestor.

Therefore, this study was conducted to investigate the genetic variation of populations from Saudi Arabia by using 15 autosomal STR markers - currently used in forensic and paternity testing. DNA was collected from a sample of 190 unrelated volunteers residing in the central region (Riyadh Province) of Saudi Arabia. The allele frequencies of the 15 STR markers were calculated using gene counting methods and compared with frequencies from other populations previously studied.

Methods

Population

A total of 190 healthy unrelated individuals from the central region (Riyadh Province) of Saudi Arabia were randomly recruited at King Fahad Medical City (KFMC) (Riyadh, Saudi Arabia) from the donors of patients under preparation for Bone Marrow Transplantation. According to KFMC hospital record system, the selection of the study subjects precluded having any two individuals or their father/mother descending from the same parent. Thus, first and second cousins are not included in the study. The age of the study population ranged between 10 and 45 years (median of 25) at the time of blood collection. There were 131 (68.9 %) males and all were of Saudi origin. The sample size was selected to provide sufficient analytical power in terms of degrees of freedom to determine allele frequencies that will allow testing of the Hardy Weinberg Equilibrium (HWE) assumption based on a Fisher's Exact Test. The study received ethical approval from the Institutional Review Board at KFMC.

DNA extraction, PCR and Fragment analysis for STR markers

Genomic DNA was extracted from whole blood samples in EDTA anticoagulant using a MagNa pure compact instrument (Roche Diagnostics GmbH, Mannheim, Germany). PCR amplification was performed according to manufacturer instruction on GeneAmp® PCR System **9700 Thermal Cycler** (Applied Biosystems, Foster City CA, USA) using AmpFlSTR® Identifiler® PCR amplification kit (Applied Biosystems, Foster City CA, USA) that include D8S1179, D21S1, D7S820, CSF1PO, D3S1358, THO, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S5, D5S818, FGA loci. The PCR product was separated on the 3130xL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and analyzed using GeneMapper® Software v3.2 (Applied Biosystems, Foster City CA, USA). For quality assurance purposes, multiple DNA samples with known combined STR genotypes were run in parallel with our study samples and appropriate results were obtained for these control samples on every occasion.

Statistical analysis

The allele frequencies were calculated based on the number of the detected alleles for each specific locus. To assess departures from Hardy-Weinberg Equilibrium (HWE) (observed heterozygosity (Ho), expected heterozygosity (He), *p*-value; using Fisher's Exact test) and Gene Diversity indices (GD), the Arlequin software version 3.5.1.3 was used [9]. Forensic and population genetic parameters including power of discrimination (PD), Polymorphic Information Content (PIC), Matching probability (MP) and probability of exclusion (PE) were derived utilizing Powerstats software version 1.2 [10]. An overall significance level for testing HWE hypothesis was set at 0.05 and adjusted for multiple comparisons using Bonferonni Criteria. Genetic relationships between groups were assessed through multidimensional scaling (MDS) plot of the genetic distance matrix using NTSYS 2.1. Software to determine the genetic differences and similarities between this study sample and published data from other populations [11].

Results and Discussion

The 15 autosomal STR loci from all samples tested were amplified successfully. A total of 150 alleles were identified for the 15 STR loci. Their corresponding frequencies were calculated (Table 1). The highest allelic frequencies observed were: allele 10 of TPOX (55.8%) and allele 12 of D13S317 (41.1%) with the most polymorphic loci observed in this study being D18S5, FGA, D21S11--defined by 17, 15 and 15 alleles respectively. However, the Polymorphic Information Content (PIC) of the 15 loci were greater than 0.57 through all loci that suggesting that the markers are highly polymorphic and would be useful as informative markers for differentiating individuals of Saudi descent. The degree of polymorphism at each locus can also be expressed in terms of heterozygosity along with the PIC value [12]. The highest values were observed for the D19S433 locus (He=0.86977; PIC=0.85) while the lowest values were identified in TPOX (He=0.6212; PIC=0.57).

After applying the Bonferonni corrections to the Fisher's exact test a deviation from Hardy–Weinberg equilibrium (HWE) was detected for the TH0, D5S818 and FGA loci. A deviation from HWE was also detected in a previous Saudi study of HLA allele frequencies [13]. This deviation was attributed to the high rates of consanguineous marriage among Saudis or to the Wahlund effect resulting in the reduction of heterozygosity. Findings in a previous Saudi study observed that 2 out of 8 STR loci did not conform to HWE which is consistent with our data [14]. As shown in Table 2, the *p* values for HWE of the available STR loci data that were obtained from previous Arab related populations reports were compared with the data from this study. It is worth noting that some alleles such as 34.2 (D21S11), 16.2 (D18S51), 12.2 (D16S539), 29 and 22.2 (FGA) and 7.3 and 8.3 (THO1) that were considered to be variant alleles in previous reports were also identified in frequencies ranged between 0.3 to 1.6% within the Saudi population studied in this project.

This study was based on a random sample of unrelated healthy volunteers of Arab descent who reside in the Riyadh district of Saudi Arabia. Allelic frequencies of 15 STR markers and their population genetic/forensic parameters were analyzed and results showed that the polymorphic nature of loci examined were sufficient to allow the

allele	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539	D2S1338	D19S433	vWA	TPOX	D18S51	D5S818	FGA
5.3						0.032									
6				0.004		0.284									
7			0.016			0.195									
7.3						0.003									
8			0.134			0.15	0.113	0.008				0.558		0.008	
8.3						0.003									
9	0.008		0.137	0.008		0.232	0.053	0.158				0.187		0.068	0.003
9.3						0.097									
10	0.063		0.358	0.294		0.005	0.029	0.071				0.092	0.005	0.087	
10.2													0.003		
11	0.084		0.187	0.246			0.182	0.382		0.021		0.161	0.028	0.226	
12	0.132		0.134	0.385			0.411	0.229		0.095		0.003	0.188	0.384	
12.2								0.003		0.003					
13	0.208		0.021	0.029			0.155	0.124	0.003	0.161	0		0.268	0.195	
13.2										0.061					
14	0.244		0.003	0.008	0.045		0.058	0.026	0.008	0.205	0.08		0.108	0.026	
14.2										0.076					
15	0.205				0.192					0.121	0.2		0.149	0.005	
15.2										0.166					
16	0.066				0.305				0.068	0.032	0.25		0.095		
16.2										0.05			0.005		
17	0.008				0.269				0.216	0.003	0.24		0.055		
17.2										0.008					
18					0.129				0.092		0.16		0.045		0.003
19	0.003				0.32				0.164		0.07		0.016		0.053
20					0.008				0.198		0		0.008		0.082
21									0.038						0.213
21.2															0.003
22									0.027				0.011		0.116
22.2															0.003
23									0.047				0.005		0.134
24									0.078				0.008		0.187
24.2															0.003
25									0.047				0.003		0.105
26									0.003						0.079
27		0.013													0.003
28		0.105													
29		0.289													0.016
29.2		0.005													
30		0.211													
30.2		0.008													
31		0.079													
31.2		0.074													
32		0.011													
32.2		0.111													
33		0.008													
33.2		0.066													
34.2		0.008													
36		0.003													
38		0.008													
Ho	0.9	0.815	0.758	0.6705	0.7368	0.774	0.7474	0.7579	0.7869	0.8053	0.72	0.621	0.845	0.653	0.811
He	0.834	0.834	0.782	0.713	0.7685	0.797	0.7566	0.7579	0.8596	0.8698	0.81	0.621	0.844	0.752	0.863
p-value	0.0739	0.004	0.457	0.23	0.0827	0.0002	0.0933	0.0279	0.0037	0	0.08	0.407	0.005	0.0008	0.0003
GD	0.834	0.829	0.763	0.6835	0.7684	0.797	0.7566	0.7579	0.8336	0.8698	0.81	0.621	0.832	0.752	0.864
MP	0.061	0.057	0.082	0.136	0.095	0.079	0.097	0.102	0.042	0.041	0.07	0.194	0.048	0.098	0.041
PD	0.939	0.943	0.918	0.864	0.905	0.921	0.903	0.898	0.958	0.959	0.93	0.806	0.952	0.902	0.959
PIC	0.81	0.81	0.75	0.66	0.73	0.76	0.72	0.72	0.84	0.85	0.78	0.57	0.82	0.71	0.85
PE	0.795	0.627	0.524	0.384	0.488	0.551	0.505	0.523	0.575	0.609	0.46	0.317	0.685	0.359	0.619

Table 1: Allele Frequency Distribution for the 15 STR loci in a Sample of Saudi population (n=190)

Population	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539	D2S1338	D19S433	vWA	TPOX	D18S51	D5S818	FGA
Saudis (current)	0.0739	**0.0036**	0.4573	0.23	0.0827	**0.0003**	0.0933	**0.0279**	**0.0037**	**0**	0.0806	0.4069	**0.005**	**0.0008**	**0.0004**
Saudis [9]	N/A	**N/A**	N/A	0.037	N/A	**0.094**	N/A	**N/A**	**N/A**	**N/A**	0.858	0.821	**N/A**	**N/A**	**N/A**
Tunisians [4]	**0.0134**	**0.0414**	N/A	N/A	0.0764	0.3144	N/A	N/A	0.1897	**0**	**0.009**	N/A	**0.001**	N/A	**0.0116**
Jordanians [14]	0.997	0.997	0.75	0.874	0.277	0.421	0.066	0.108	N/A	N/A	0.673	0.952	0.275	0.471	**0.004**
Palestinians [7]	0.865	0.424	0.794	0.173	0.141	0.157	**0.019**	0.081	N/A	N/A	**0.006**	0.474	0.455	**0.009**	0.285
Iraqis [12]	0.06	0.135	0.393	0.533	0.824	0.936	0.66	0.25	0.473	0.344	0.058	0.377	0.399	0.45	0.71
Omanis [13]	0.282	0.333	0.32	N/A	0.56	N/A	0.082	N/A	N/A	N/A	0.254	N/A	0.204	0.644	0.533
Egyptians [11]	0.139	0.598	0.527	N/A	0.695	N/A	0.08	N/A	N/A	N/A	**0.031**	N/A	0.067	0.52	0.223
Moroccans [15]	0.144	N/A	0.441	0.692	0.304	0.111	0.109	0.903	N/A	N/A	**0.002**	0.449	N/A	0.414	0.848
Syrians [15]	0.781	0.094	0.423	0.712	0.349	0.703	0.788	0.74	N/A	N/A	0.735	0.242	0.775	0.89	0.6

Table 2: Comparison p values of HWE for STRs data for nine Arab related populations. *Bold indicates a significant difference between the observed and the expected heterozygosity.

characterization of individuals from Saudi Arabia (markers D21S1, THO, D2S1338, D19S433, D18S5, D5S818 and FGA). This study showed that D2S1338, D19S433 and FGA were the most polymorphic markers as evidenced by their high PD values. It is worth noting that the 4 markers with high PD values (vWA, THO1, CSF1PO and TPOX) were also identified by a previous study that used 8 STR loci to study unrelated subjects in Saudi Arabia (14).

In other studies, the most frequent allele for forensic STR loci can vary depending on the population, for example 12 (0.4093) at D5S818 in Slovenian population [15], 15 (0.4143) at D3S1358 in Bolivians [16], 8 (0.4890) at TPOX among Wallachians in South Romania [17] and 8 (0.424) at TPOX in Adaima community from Egypt [18]. In this study the most frequent alleles were 8 (0.558) at TPOX, 12 (0.411) at D13S317, 12 (0.385) at CSF1PO, 11(0.382) at D16D539 and 10 (0.358) at D7S820. The most polymorphic marker in this study was D18S51; spanning 17 tandem repeat alleles. Allele 13 of this locus was the most predominant one with a frequency of 0.268. Interestingly, a study of Tunisians found markers D19S433 and D21S11 to be the most polymorphic; each spanning 18 alleles [4]. In contrast, a lesser degree of polymorphism was reported for Iraqi individuals as compared to Saudis and Tunisians [19] due to less admixture.

For the sample used in this study, the observed values of heterozygosity ranged from 0.621 at TPOX to 0.869 at D19S433. This lower degree of homozygosity suggests presence of a random mating in the study population and less consanguinity. Despite prolific rates of consanguineous marriage in the Saudi population [8], this study was able to clearly identify seven markers that can be used for characterizing the genetic makeup among individuals of Arab descent in Saudi Arabia.

Furthermore, it can also be argued that Independent Inheritance property could apply to two cases for markers collocated on the same chromosome, namely CSF1PO and D5S818 on chromosome 5, and TPOX and D2S1338 on chromosome 2. This was reflected by the disparate statistical values regarding HWE assumption that rendered in each case one marker as statistically significant while the other one was not.

It is also interesting to note that variant alleles of 34.2 (D21S11), 16.2 (D18S51), 12.2 (D16S539), 29 and 22.2 (FGA) as well as 7.3 and 8.3 (THO1) have also been identified by other studies. The 15 markers studied here were highly informative as illustrated by their high PD values (Table 1). Consistently higher PD values were also reported studies that examined populations from South Romania at locus FGA (PD=0.970), Tunisian at locus D19S433 (PD=0.976), sub-population of Botswana at locus D21S11 (PD=0.974) and Turkish at locus D18S51 (PD=0.979) [3,4,17,20].

A Multidimensional Scaling (MDS) plot was constructed to illustrate the genetic distances between 14 populations (Figure 1). The analysis of these populations showed clustering into four groups: (1) the Asian subpopulation, (2) the Caucasians, (3) African and (4) Middle East subpopulations. The Saudi group in this study clustered with populations from Yemen, Iraq, Qatar, Oman and Bahrain. In contrast, African populations like the Hutu and Kenyans as well as the Pakistani, Bangladeshi, Punjabi and those of Caucasian origin like the Belgium, Croatians and Georgians were located furthest from the samples of this study.

Conclusions

TPOX, D13S317, CSF1PO, D16D539 and D7S820 markers were found suitable for forensic analysis, paternity testing and can also be used for chimerism study after allogenic bone marrow transplantation for Saudi population. On the other hand, the variable degree of genetic distances of this population and other Arab related groups might be explained by the populations admixture with other ethnic origins. Further analyses of Arab population are needed to understand the interrelationship between the different ethnic groups of the region.

Acknowledgements

We gratefully acknowledge the volunteers whose cooperation made this study possible. We also would like to thank Dr Abdulwahid Aldehaimi the head of the Pathology and Clinical laboratory at KFMC for his kind support and encouragement and also our thanks extend to members of staff of immunology and serology laboratory for their specific technical support. Funding for this project was provided by King Fahad Medical City.

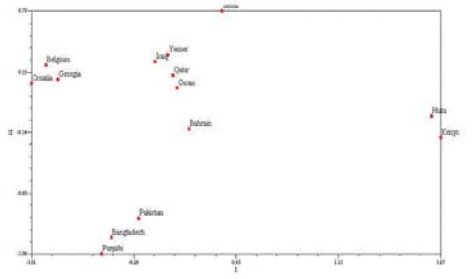

Figure 1: Multidimensional Scaling plot of genetic distance between 14 populations: Saudi Arabia, Yemen, Iraq, Qatar, Oman, Bahrain, Pakistan, Bangladesh, Punjabi, Belgium, Croatia, Georgia, Hutu and Kenya. The Saudi population studied grouped with other populations from the Middle East.

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Citation: Osman AE, Alsafar H, Tay GT, Theyab JBJM, Mubasher M, et al .(2015) Towards a Forensic Psychological Evaluation of Juvenile Fire Setters: Parent Power. J Forensic Res 6: 267. doi:10.4172/2157-7145.1000267

Chapter 3. The role of genetic variants in T1DM development among Saudi population.

Introduction and background

Type 1 Diabetes (T1D) is an autoimmune disease characterized by the progressive destruction of insulin-producing pancreatic beta-cells through T lymphocytes and macrophages that can infiltrate and destroy the islet cells of Langerhans and eventually lead to insufficient secretion of insulin in the blood circulation [104][105].

Three critical conditions that cause the failure of beta cells include the activation of beta cell-reactive T cells, the creation of factors including production of pro-inflammatory cytokines/chemokines and the loss of control by the immune regulatory system [106]. However, the exact immunological mechanisms controlling disease initiation and progression remain to be fully clarified. An experimental study on animals and patients with T1DM showed an essential role of pro-inflammatory cytokines and cell-mediated immunity in the pathogenesis of beta cell destruction [107]. Similarly, several clinical trials were conducted using targeted immunotherapy directed towards cytokines and adaptive lymphocytes, such as anti-IL-1 (canakinumab or anakinra), anti-TNF-α (etanercept), anti-CD3 (teplizumab or otelixizumab, to block T cell function), anti-CD20 (rituximab, for B cell depletion) and CTLA4-Ig (abatacept, to block T-cell activation) [108][109][110]. Recurrence of beta cell loss was found in most patients post-treatment, and the pathogenic lymphocytes remained autoreactive after the immunomodulatory intervention, so this approach to date has not been very successful.

The inflammation of the islet cells depends on the interaction between the immune cells, pancreatic beta cells and the mediation of cytokines and chemokines. Expression of cytokines such as interleukin (IL-1) and tumour necrosis factor (TNF-α) have been demonstrated in the islet cells of patients with T1DM [104]. In addition, Toll-like receptors (TLRs) are involved in the pathogenesis of various autoimmune diseases [105][111][112].

T1DM is considered a multifactorial autoimmune disease with proven genetic susceptibilities that interact with multiple environmental factors, determining the potential progression of the disease [113]. Environmental Determinants of Diabetes in the Young (TEDDY) study is a prospective international study that has followed children from 5 to 15 years through a coordinated protocol. This study aimed to identify the relationship between environmental factors and genetic susceptibility in T1DM patients [114][115]. At least two

marked stages exist before the derangement of the beta cell function occurs and blood glucose begins to rise. The first stage is the presence of autoantibodies against islet cells, during which endogenous insulin production is still subclinical. In the second stage, blood glucose levels rise, and cytotoxic T-cell infiltration occurs. The persistent islet autoimmunity assessment was checked every three months, and the primary outcome of TEDDY was reported based on the American Diabetes Association criteria for T1DM diagnosis [116].

The HLA is a critical genetic locus for susceptibility to autoimmune diseases such as T1DM [117][118]. Although HLA is regarded as a risk factor for approximately 50% of type 1 diabetes Mellitus (T1DM) cases, there are other residual genetic factors outside the HLA region, such as single nucleotide polymorphisms (SNPs) that may be associated with the risk of T1DM development [119]. Furthermore, non-genetic (environmental) factors could contribute to disease pathogenesis since the concordance rate between monozygotic twins is significantly less than 100%, particularly for T1DM, estimated to be between 13–65% [120].

The HLA is a critical genetic locus for susceptibility to many autoimmune diseases. Natural killer (NK) cells are a type of large granular lymphocytes (LGL) that are essential to the innate immune system and belong to the innate lymphoid cells (ILC); also see **Publication 2**. NK cells were demonstrated as crucial regulators and inducers for autoimmune diseases, including T1DM; they act against the target cells through direct contact to destroy the pancreatic islets of Langerhans [121].

A SNP is defined as a DNA sequence variation at single nucleotide position of A, T, G or C that can be involved in critical genetic variations among individuals. Some of these variations are associated with susceptibility to diseases, such as autoimmune diseases [122]. Additionally, SNPs are considered recent units in genetic variation and have single base-pair variations in the DNA sequence that occur with high frequencies along the human genome and are used as genetic markers for specific genetic regions [123]. SNPs are present in many human populations, and each SNP has two alleles in which the lower frequency allele is known as the mutant or variant form. SNPs may have a functional role in causing amino acid changes, by affecting the mRNA transcript stability or changes to transcription factor binding affinity [124]. SNPs are used as genetic markers in disease

association studies to demonstrate which part of the human genome may be involved. Specific studies identified some SNPs in coding and regulatory regions of specific genes that may be implicated in disease development. Non-synonymous SNPs that lead to an amino acid change in the protein sequence product are of significant interest since amino acid substitutions account for half of the known gene defects responsible for human genetic diseases [125].

Genome-wide association studies (GWAS) involve analysis of SNPs throughout the genome by use of high throughput genotyping methods using microarray technology. GWAS truly investigates the role of significant SNPs associated with specific genomic risk loci [126]. Over the last few years, GWAS has been used to measure and analyze DNA sequence variations across the human genome to identify a wide range of genetic risk factors for common population diseases [127][128].

An early GWAS study identified the Complement Factor H gene as a common risk factor for age-related macular degeneration. Later, the Type 1 Diabetes Genetics Consortium conducted a GWAS study in TEDDY participants carrying high-risk HLA genotypes and demonstrated that the PTPN22, ERBB3, SH2B3, and INS genes are associated with a significant risk of T1DM [120]. A large sample size to provide sufficient statistical power and linkage disequilibrium (LD) reliability is considered critical for GWAS [129].The relatively small sample size collected for the studies in this thesis did not allow a formal GWAS to be performed and instead we investigated the relevance of HLA and certain specific SNP genotypes on T1DM risk. This approach is consistent with the view that low levels of anti-inflammatory cytokines facilitate autoimmunity to β-cells of the pancreas.

In this chapter, I include three published papers. **Publication 4** investigated the role of KIRs and HLA class I ligands in T1DM patients. Accordingly, we studied KIRs and their corresponding HLA class I ligands in Saudi T1DM patients and compared them to healthy controls. In **Publication 5**, the focus was on the role of HLA-DRB1 alleles frequencies and SNPs within the cytokine genes regulating the autoimmune responses that include toll-like receptors (TLR-2, TLR4), tumour necrosis factor (TNF), interleukin-1 (IL-1), interleukin-1 receptor 1 (IL-1R1), interleukin-1 receptor antagonist (IL-1RN), interleukin-2 (IL-2) and

interleukin 12 (IL-12). The goal was to demonstrate possible significant differences between T1DM and healthy controls and identify linkages between SNPs and HLA.

Finally, in **Publication 6**, I investigated rs1800896, rs1800871, rs1800972, rs2070874 and rs1801275 SNPs that located within IL-4, IL4R and IL-10 genes and, HLA-DRB1, DQA1, and DQB1 loci in T1DM and healthy controls. The purpose was to identify possible significant differences between the two groups and detect any link with the onset of T1DM for patients above the age of 13 or below this age.

Publication 4, Killer Immunoglobulin-like receptor, and their corresponding HLA class I ligands in T1DM

Significance and Background

Type 1 Diabetes Mellitus (T1DM) is a T cell-mediated autoimmune disease developed due to beta cell malfunction [130]. Direct viral infection, environmental toxin or immune invasion against B-cell-specific antigens may lead to islet cell destruction. B-cell antigens and antigen-presenting cells such as macrophage and dendritic cells can activate T helper lymphocytes. For example, macrophages release interleukin 12 (IL12), and the activated CD4 positive T cells secret IFN γ; the latter cytokine activates resting macrophages to produce IL-1β, TNF-α and other free molecules that lead to beta cell toxicity [107].

Natural killer (NK) cells are a type of large granular lymphocytes (LGL) that are essential to the innate immune system. They belong to the innate lymphoid cells (ILC) family and are found in between 5% and 20% of the circulating lymphocytes in the human body [131][132] (see also **Publication 2**). NK cells can easily detect stressed cells caused by infection or malignancy and react to release cytotoxic granules or death receptor ligands, which consequently produce cytokines such as IFN-γ and TNF-α [133]. NK cells were demonstrated as crucial regulators and inducers of autoimmune diseases, including T1DM. They act against the target cells through direct contact to destroy the pancreatic islets of Langerhans [121].

Killer immunoglobulin-like receptors (KIRs) are expressed on the surface of NK cells and interact with the HLA class I-specific ligands on target cells (see also Publication 2). The activation mode for these cells is controlled by a specific balance between activating and inhibitory signals received from the KIRs [82].

The HLA class I ligand on the human healthy cell reacts with the inhibitory KIRs to protect these cells from killing. If the NK cell cannot identify a perfect HLA ligand, it will be activated to destroy the target [134]. HLA ligands that interact with KIRs were grouped into three distinct types, including HLA-C group 1 (C1), HLA-C group 2 (C2) and HLA-Bw4 (Bw4) motifs that present with the alpha 1 domain within the amino acid residues number 77-83. HLA-C plays a more significant role in NK cell regulation than the HLA-Bw4 motif [135].

The presence of the KIR genes on chromosome 19 and the HLA genes on chromosome 6 gives a property of independent genetic segregation during cell division. Hence, their inheritance in everyone is necessary to maintain a functional interaction in the immune system [136]. Different studies demonstrated the association of specific genetic models for HLA class 1 ligands and the corresponding KIR genes with autoimmune diseases, including T1DM [130] [121] [82][137][138].

To the best of our knowledge, this study was the only study investigating the role of KIRs and HLA class I ligands in T1DM patients in the Saudi population. Accordingly, we aimed to investigate these genes in T1DM patients and compare them to healthy controls.

Results

No significant differences were observed regarding the KIR genes between patients and control or in A and Bx haplotype groups, as shown in Table 1 and Figure 1 of **Publication 4**. Forty-six different KIR genotype groups were observed in this study. The frequency distribution of HLA-C alleles is shown in Table 3, and subsequently, we calculated C1 and C2 ligands as shown in Table 4. The homozygous C1C1 ligands were found at higher significant frequencies in T1DM patients, while the C2C2 ligands and Bw4 motif were at higher significant frequencies in the control group. There is no difference between patients and control in A*03/11alleles. For KIRs and HLA class 1 ligands, KIR2DL2-C1C1, KIR2DS2-C1C1 and KIR2DL3-C1C1 were observed at significant frequencies among patients. In contrast, the KIR2DS1-C1C1 combination was significantly greater in the control group.

2DL2/3-C1C1 (A), 3DL1-Bw4 (B), and 2DL1-C2C2 (C) were stratified as shown in Table 3, **Publication 4**. The model positive for A, negative for B and C; and that positive for A and B, negative for C were demonstrated high significant frequencies in T1DM patients. On the other hand, the model positive for B, negative for A and C; and the model positive for B and C, negative for A were found to be greater in the control group.

Commentary and Impact

There was no effect for the presence of the KIR genes alone in T1DM, as shown by two previous studies carried out in the Han Chinese and Spanish Basque populations [139][139], which agreed with our findings. However, this was not the case in a British study, in which the frequency of the KIR2DL3 gene was observed at a significant difference between patients and control [139]. KIRs can modulate NK cell and T-cell function through their HLA class 1 ligand, which is present on the surface of the target cell [140]. The binding of the inhibitory KIR with the suitable immune-receptor tyrosine-based inhibitory motif (ITIM) leads to the gathering of tyrosine phosphorylated and protein tyrosine phosphatases and consequently, to the initiation of the inhibition mechanism [141].

In this study, we demonstrated the C1C1 genotype as a risk factor for T1DM, while the C2C2 genotype and Bw5 motif may act as a protective factor for T1DM in Saudis. Our study did not show significant effects on the heterozygous C1C2 genotype, but C1C2 was observed to be substantial in the Han population (China)[142].

HLA class 1 molecule shapes T cell repertoire and presents antigens for CD8 cytotoxic cells to act against beta cells in the pancreas. Accordingly, specific combinations are suggested to affect the destruction process in the pancreas [143]. KIR2DL2 and KIR2DS2 have one HLA-C ligand (C1); these genes are found in strong linkage disequilibrium [144]. The presence of these receptors with their ligand was considered a risk factor for T1DM in our population; this was also observed in Dutch and Latvian people [121][145]. The presence of the homozygous 2DL1 with its cognate C2 ligand increases the number of NK cells to almost double [75], which may indicate a protective role against immunological reactions. This may explain the high frequency of the KIR2DL1-C2C2 combination was observed in the control group. Interestingly, we observed 2DL1-C2C2 and 3DL1-Bw4 without KIR2DL2/3-C1C1 increased protection against T1DM fivefold.

In conclusion, the presence of certain KIR genes alone has no role in T1DM, but when combined with genotype for HLA ligands this changes. A combination of different KIR genes and HLA class 1 ligand was demonstrated to be a risk or protective factor for T1DM.

Many recent publications investigated a link between KIR gene polymorphisms and T1DM. A meta-analysis paper published in 2017 demonstrated a negative association between the KIR2DL1 polymorphism and susceptibility to T1DM in the overall population [146]. Yang *et al*. discussed the role of NK cells in T1DM development and concluded that NK cells can be involved in various classic autoimmune diseases such as rheumatoid arthritis (RA), multiple sclerosis (MS), systemic lupus erythematosus (SLE) and type 1 diabetes mellitus (T1DM) [147]. Most recently, Gunavathy et al. demonstrated the role of KIRs and their corresponding HLA class I ligands among south Indian populations and that KIR3DL1 in the presence of HLA-B Bw4 Ile80 has a protective role against T1DM [148].

Table 3

HLA-C allele frequencies in T1DM patients and controls.

T1DM patients				**Healthy control**			
HLA-C*	2n =212	AF (2n=212)	AF (%) 2n=212	HLA-C*	Counts	AF (2n=288)	AF (%) 2n=288
C*01	2	0.009	0.9	C*01	1	0.003	0.3
C*02	6	0.028	2.8	C*02	7	0.025	2.5
C*03	8	0.038	3.8	C*03	11	0.038	3.8
C*04	26	0.123	12.3	C*04	32	0.112	11.2
C*05	4	0.019	1.9	C*05	7	0.025	2.5
C*06	27	0.127	12.7	C*06	37	0.128	12.8
C*07	54	0.255	25.5	C*07	72	0.25	25
C*08	1	0.005	0.5	C*08	1	0.003	0.3
C*12	12	0.057	5.7	C*12	17	0.059	5.9
C*14	8	0.038	3.8	C*14	15	0.052	5.2
C*15	45	0.212	21.2	C*15	59	0.205	20.5
C*16	10	0.047	4.7	C*16	15	0.052	5.2
C*17	7	0.033	3.3	C*17	12	0.041	4.1
C*18	2	0.009	0.9	C*18	2	0.007	0.7

HLA alleles in bold italic with red color mean C1 ligands and HLA alleles in bold only mean C2 ligands

Table 4

KIR HLA class I ligand frequencies in cases and controls

Ligands	T1DM n=106 (%)	Control n= 136 (%)	OR (95%)	P value
C1C1	48 (45.3)[a]	23 (16.9)[a]		
C1C2	43 (40.5)	64 (47.1)	3.0 (1.7, 5.8)	0.0004
C2C2	15 (14.2)[a]	49 (36)[a]	7.5 (3.2, 14.6)	0.0002

[a]Slight variations from the values appeared in Publication 5 but did not affect the statistical power.

Publication 5; Single-nucleotide polymorphisms (SNPs), pro-inflammatory cytokines and human leukocyte antigens (HLA) with type 1 diabetes mellitus (T1DM)

Significance and Background

We hypothesized that in T1DM, the 'self' peptides associated with pancreatic islet cells are targeted by autoimmune clones normally directed down tolerogenic pathways. Instead, they are directed down immune pathways, where they become amplified into self-destructive clones under the influence of pro-inflammatory cytokines. To further assess the relevance of polymorphisms in the adaptive and innate immune systems to T1DM risk in the Saudi population, we investigated HLA-DRB1 alleles and SNPs within the cytokine genes regulating autoimmune responses, including toll-like receptors (TLR-2, TLR4), tumour necrosis factor (TNF), interleukin-1 (IL-1), interleukin-1 receptor 1 (IL-1R1), interleukin-1 receptor antagonist (IL-1RN), interleukin-2 (IL-2), and interleukin 12 (IL-12). The aim was to demonstrate possible significant differences between T1DM cases and healthy controls and in the frequencies of individual and combined genotypes in these genes.

Results

Allele frequencies and codominant, dominant, and recessive models were derived and presented in Table 3 of **Publication 5**. The presence of the TNF rs361525G-G genotype in our study indicates a susceptibility risk for T1DM about fivefold more than the rs361525A-G or rs361525A-G ones, and the rs1800629G:361525A haplotype was observed more frequently in the controls and considered to be conferring protection against the disease.

The sum of allele frequencies for DRB1*03 and -*04 among T1DM patients was about 73%, which was highly significant compared with the controls and considered to be risk factors for T1DM. DRB1*07, -*11, -*13 and -*15 alleles were found at higher frequencies in the control group compared with cases.

Stratification of T1DM patients for DRB1*03, -*04, -*07 and -*13 alleles with rs1800629 and rs361525 revealed a significant difference between T1DM patients and controls when

comparing individuals positive for DRB1*07 in the presence of rs1800629A/G genotype against those positive for DRB1*07 and negative for rs1800629G/G. No other meaningful results were demonstrated in this context. A significantly increased frequency was observed in patients with both DRB1*07 and DRB1*03/04 genotypes compared with controls, and in patients positive for DRB1*07 and rs18006629 A/G genotypes. But there was no difference between patients and controls when comparing individuals positive for DRB1*07, DRB1*03/*04 and rs1800629A/G genotypes in the two groups.

Commentary and Impact

After adjusting our significance values SNP frequencies between patients and controls using the Bonferroni correction test, only rs361525 genotypes of the TNF-α demonstrated statistical differences. The result agreed with the Croatian study that found over-transmission of this SNP allele from parents to T1DM patients' children [149] and with the Caucasian study in T1DM [150]. However, this was not the case with Kaidonis's study investigating sight-threatening diabetic retinopathy [151].

Tumor necrosis factor alpha (TNF-α) is a pleiotropic cytokine that regulates inflammatory responses and contributes to some autoimmune diseases [152]. A greater level of secreted TNF-α in the blood was observed among T1DM patients with no link to age, disease duration and ethnicity [153]; the elevated level of this cytokine is attributed to the presence of rs1800629A allele, which may influence the synthesis and transcription factor binding capacity [154].

No significant difference was observed in the rs1800629 genotype in our study, but the presence of rs1800629G:361525A haplotype played a role in protecting against T1DM disease. Both rs361525 and rs1800629 are promotor region SNPs not involved in direct protein coding but may affect the quantity of proteins. Specific SNPs in the TNF-α gene were found to affect susceptibility to many diseases [155].

The rs1800629A allele was observed at significantly high frequency in Australian and United Kingdom T1DM patients [151]. Another Saudi study also demonstrated a significant level of this SNP, but the authors did not correct their results using the Bonferroni test [156].

Strong linkage disequilibrium (LD) was demonstrated between the TNF gene and HLA [157] (the telomeric HLA class III harbours many genes, including TNF) [158]. Furthermore, the association of HLA with T1DM is demonstrated globally, including in Saudi Arabia [159] [160]. Our investigation for HLA class II in this study confirmed that DRB1*03 and DRB1*04 alleles are risk factors for T1DM while DRB1*07, DRB1*13 and DRB1*15 are protective. Moreover, stratification of specific HLA alleles with rs18006629 and rs361525 genotypes have shown DRB1*07 in the presence of rs18006629A/G is a twelve times risk factor for T1DM than those having DRB1*07 and rs18006629G/G.

It is worth noting that individuals positive for DRB1*07 and DRB1*03/04 were observed at a higher significant frequency in T1DM patients despite DRB1*07 being considered a protective factor. Individuals positive for DRB1*07, DRB1*03/*04 and rs1800629A/G showed no significant difference between patients and control, while those positive for DRB1*07 and rs18006629 A/G were only found in the T1DM group. Accordingly, we observed the predominant immunological influence of DRB1*03/*04 alleles over DRB1*07 when inherited in one person, and the compromise situation was observed when DRB1*07, DRB1*03/04 and rs1800629A/G inherited together. However, the association with T1DM could be due to an HLA-DRB1-TNF-alpha-HLA-B haplotype association[161] [162], and the effect could be due to HLA-B, not TNF. The 26-08-07-03-02 haplotype, the second most frequent haplotype observed in **Publication 1**, showed the link between the HLA-B and HLA-DRB1 alleles.

This study is part of a broader set of recent literature looking for a link between TNF gene polymorphisms and T1DM in different populations. Szabo *et al*. investigated the role of adiponectin and TNF in susceptibility to T1DM in Romanian children, but no associations were detected in their study [163]. Keles *et al*. demonstrated a significantly increased of TNF-α level in T1DM patients with gingivitis [164]. Lin *et al*., in 2021, identified a strong link between TNF and naive B cell/naive CD4+ T cell in T1DM patients and concluded that TNF may play a vital regulatory role in T1DM development [165].

Publication 6; The presence of rs 2070874C/C genotype at Interleukin-4 is linked to a high risk of type 1 diabetes mellitus

Significance and Background

Usually, self-reactive T cells are suppressed to avoid autoimmune reactions; instead, HLA genetically susceptible individuals can break the peripheral tolerance and harm B cells in the islet of Langerhans. Releasing pro- and anti-inflammatory cytokines is crucial in the immune mechanism to initiate the destruction of B cells. In this study, we genotyped five different SNPs within IL-4, IL4R and IL-10 genes and HLA- DRB1, DRA1, and DQB1 loci in T1DM and healthy control. The purpose was to identify possible significant differences between the two groups.

HLA-DRB1, -DA1, and DQB1 molecules present self-peptide through TCRs to T cells, and then the cytokines regulate the immune reactions to determine whether they are expanded or suppressed [166], [167]. Interleukin-4 (IL-4) and interleukin 10 (IL-10) are anti-inflammatory cytokines that down-regulate the inflammatory response. The IL-4 gene is on chromosome 5, and the IL-10 gene is on chromosome 1. Blood levels for both cytokines were observed at low concentrations at the time of T1DM diagnosis or in non-obese diabetic patients [168]. Additionally, IL-4 genetic overexpression or administration prevents insulitis and minimizes T1DM incidence [169]. The combination of IL-4 with the IL-4R involves the PI3K and JAK/STAT pathways in the immune reaction process. It initiates B cell viability and increases the expression of IL-4R on their surface [170].

IL-10 is an anti-inflammatory cytokine that can suppress Th1-producing cytokines, including IFN-γ and TNFα [171]. IL-10 has a short half-life and a short range of activity, so multiple cells that secrete this cytokine can respond quickly at various needed sites [172]. A variety of SNPs in non-coding sequences on both the Il-10 and IL-4 genes may affect levels of these cytokines [168]. For instance, the rs2070874 SNP located at the untranslated region of the IL-4 gene regulates the level for this cytokine secretion in blood. Furthermore, the rs1800871 and rs1800872 SNPs present within the promoter region for this gene were found to regulate the messenger RNA and gene expression for the IL-10 cytokine [173].

Our hypothesis in this study assumed that self-peptides are attached to beta cell in the pancreas. The combined reaction was bound to autoimmune cell clones, exacerbated by pro-inflammatory cytokines such as TNF-α or missed the influential roles of anti-inflammatory cytokines such as IL-4 and IL-10. Accordingly, we genotyped rs1800896, rs1800871, rs1800972, rs2070874 and rs1801275 SNPs located within IL-4, IL4R and IL-10 genes and the HLA- DRB1, DRA1, and DQB1 loci in T1DM and healthy control. The purpose was to identify possible significant differences between cases and controls and to determine if there was any link between the onset of T1DM above or below the age of 13 among the patients.

Results

DRB1*03 and DRB1*04 were observed at higher significant frequencies among T1DM patients while DRB1*07, DRB1*13 and DRB*15 were significantly higher among the healthy controls. For DQ, DQA1*03, DQA1*05 and DQB1*02 were found to play a role in T1DM susceptibility, whereas DQA1*04, DQA1*06 and DQB1*06 alleles were protective.

Stratification of DRB1*03/04 or DRB1*07/13 with rs2070874 C/C genotype (these genotypes have shown significant differences between patients and control) as indicated in Table 9 of **Publication 6**, no meaningful associated withstood post statistical calculation. However, a grouping of patients based on the age of onset (≤13 or >13 years) demonstrated a significant increase of rs2070874 C/C genotype in patients who were ≤13 compared with those >13 of age. Stratification of specific HLA-DRB.DQA1. DQB1 haplotypes with rs2070874 C/C have shown 03.05.02/04.03.03 in the presence of rs2070874 C/C was in significant among early onset of T1DM compared with late ones (≤13 years).

Commentary and Impact

Low IL-4 production was associated with T1DM in NOD mice and patients with T1DM, and the administration of IL-4 was observed to prevent autoimmune disease in animal experiments [174]. IL-4 mRNA expression in peripheral blood mononuclear cells was almost double in T1DM males compared to females [175]. Others have shown no

association between T1DM and the IL-4 gene using the affected sibpair (ASP) and transmission/disequilibrium test (TDT) [176]; however, they did not consider the age of disease onset.

Early onset of T1DM was more significant in younger twin patients because of non-HLA genes that control B and T cell activity [177]. HLA class II presents exogenous peptides to T-helper cells to initiate immune response or produce β-cell self-peptides to induce tolerance. Accordingly, our data demonstrated specific HLA haplotypes (03.05.02/04.03.03) are strongly associated with T1DM, while others (07.02.02/11.05.03) were observed as protective factors against T1DM.

The standard immune mechanism depends on the balance between pro- and anti-inflammatory cytokines; an imbalance between these groups of cytokines leads to an immune defect. Elevated levels of pro-inflammatory or low anti-inflammatory cytokines induce autoimmune disease [178]. Our findings in **Publication 5** indicated a high TNF producer gene or the current one that provides evidence of a contribution from an IL-4 gene variant associated with low IL-4 production is consistent with the statement of imbalance cytokine in T1DM development.

Multiple genes linked to aggressive early-onset T1DM in young individuals under seven years, such as the GLIS3 gene, were detected. It is a crucial transcriptional factor in the Islet β-Cell, and its presence in a deletion form in mice and humans induces neonatal diabetes [179]. In a recent GWAS meta-analysis study, multiple genes were found to be associated with the risk of T1DM, including PTPN22, HLA-DQB1, IL2RA, RNLS, INS, IKZF4-RPS26-ERBB3, and SH2B3. Moreover, PTPN22, HLA-DQB1, INS, and ERBB3 were found to be associated with the onset of T1DM [180]. Also, a significant association with IL-10 in T1DM patients under seven years of age was detected, which disagreed with our findings [181]. Our cases and controls differed in age range, which might have introduced bias to the study. We will consider using age-matched controls in future studies. However, the current study only focused on candidate SNPs in IL-10, and the selection of additional SNPs in this gene for study may have resulted in an association being found.

In conclusion, the presence of high-risk HLA alleles for T1DM with rs2070874 C/C genotypes was significantly increased in early-onset T1DM patients; this is commensurate with the statement that low IL-4 secretion can enhance autoimmune destruction of beta cells.

Recently, some further studies looking for a link between IL-4 gene polymorphisms and T1DM in different populations were conducted. An in vitro study of the effect of IL-4 in the T1DM model was conducted by Pfeifer *et al*. in 2022; they observed that IL-4 failed to preserve beta cell endocrine function [182]. In another study by Preisser *et al*., investigating the role of recombinant Lactococcus lactis that carries an IL-4 coding vector in the T1DM nod mice model, they demonstrated some protection against T1DM because it attenuates the insulitis process [183]. The association between IL-4 polymorphisms and T1DM was also shown in a recent study (2023) by Haider *et al*. on the Kuwaiti population [184].

Analysis of P values for allele frequencies for the 17 SNPs that were studied in Publication 5 and 6 with correction for multiple testing

In Publication 5, I studied the role of SNPs present within the pro-inflammatory cytokines and how these SNPs may exacerbate the autoimmune reaction against pancreatic cells and lead to T1DM. In Publication 6, I focused on the role of anti-inflammatory cytokines in controlling the immune response and preventing T1DM development. Allele frequencies and the genotype models for rs16944, rs3212227, rs1800629 and rs361525 SNPs have shown significant differences between T1DM patients and controls when data for each publication was analyzed separately using the Bonferroni correction test. Publication 6 was an extension of publication 5 using the same samples for both studies to investigate a new set of SNPs further. In the two original publications, the Bonferroni correction test was used separately for each publication. However, for this thesis, I now include all the SNPs (17) investigated in the two publications based on how pro- and anti-inflammatory cytokines could modulate the human immune system and lead to T1DM. When I applied the Bonferroni correction test, only the allele frequency of the rs2070874 SNP demonstrated a significant difference between the patients and control (corrected p values < 0.002941), as shown in Table 5. I also applied the false discovery rate (FDR) analysis using the method

of Benjamini-Hochberg to see if that could make any other SNPs potentially significant and to minimize the possibility of false positive association, as the studied SNPs have not been functionally proven. As shown in Table 5, when Q=0.05, only rs2070874 and rs361525 were significant. The FDR results suggest that the most significant SNPs in Publications 5 and 6 (ie rs2070874 and rs361525 in IL4 and TNF-alpha respectively) remain significant as predictors of T1DM in a more extended analysis.

Table 5

Allele frequencies for the 17 SNPs that were studied in Publications 5 and 6 with correction for multiple testing.

No	SNP ID	Gene	Alleles	P value	Bonferroni Corrected P value	FDR corrected P value (Q=0.05)
1	***rs2070874***	**IL-4**	C T	C0.0013*	0.0221	0.002941
2	**rs361525**	**TNF-alpha**	G A	0.007*	0.119	0.008824
3	**rs16944**	**IL-1B**	A G	0.03	0.51	0.014706
4	***rs1800871***	**IL-10**	G A	0.033	0.561	0.020588
5	***rs1801275***	**IL-4R**	A G	0.08	1.36	0.026471
6	**rs315952**	**IL-1RN**	T C	0.18	3.06	0.032353
7	**rs1143634**	**IL-1B**	G A	0.19	3.23	0.038235
8	**rs2069762**	**IL-2**	C A	0.2	3.4	0.044118
9	**rs2234650**	**IL-R1**	C T	0.22	3.74	0.05
10	**rs3212227**	**IL-12B**	T G	0.3	5.1	0.005882
11	***rs1800872***	**IL-10**	G T	0.3	5.1	0.011765
12	**rs4986791**	**TLR-4**	C T	0.4	6.8	0.017647
13	**rs1800587**	**IL-1A**	G A	0.42	7.15	0.023529
14	**rs3804099**	**TLR-2**	T C	0.5	8.5	0.029412
15	**rs4986790**	**TLR-4**	A G	0.5	8.5	0.035294
16	**rs1800629**	**TNF-alpha**	G A	0.6	10.2	0.041176
17	***rs1800896***	**IL-10**	T C	0.64	10.88	0.047059

SNPs in italic bold for publication 5; SNPs in bold for publication 6; C indicates the p value is significant after correction; values in rows 1, 2, 3 and 4 of column "Bonferroni Corrected P value" represent the threshold for the Bonferroni test. *Significant using FDR correction.

Copies of publications for Chapter 3

Publication No: 4

My contribution to this study included obtaining the IRB approval, which was under my name. I contributed equally with a senior author in the research design and sample selection. I generated the bulk of laboratory work and analyzed the raw data, drafted the manuscript, communicated with the other co-authors for review, acted as the corresponding author and responded to all reviewers' comments throughout the publication.

Human Immunology 77 (2016) 110–114

Contents lists available at ScienceDirect

journal homepage: www.elsevier.com/locate/humimm

Rapid Communication

Investigation of activating and inhibitory killer cell immunoglobulin-like receptors and their putative ligands in type 1 diabetes (T1D)

Awad E. Osman [a,*], Nezar Eltayeb-ELSheikh [a], Mohamed Mubasher [a], Hanan Al Harthi [a], Sahar Alharbi [a], Muaawia A. Hamza [a], Gehad ElGhazali [b]

[a] King Fahad Medical City, Riyadh 11525, Saudi Arabia
[b] Mafraq Hospital and Sheikh Khalifa Medical City, Abu Dhabi, United Arab Emirates

ARTICLE INFO

Article history:
Received 31 December 2014
Revised 24 May 2015
Accepted 31 October 2015
Available online 2 November 2015

Keywords:
Killer immunoglobulin-like receptor
HLA class I ligands
Saudi population
Type 1 diabetes

ABSTRACT

Genetic and environmental factors play important roles in predisposing an individual to the development of type 1 diabetes (T1D). Several studies have investigated the role of killer cell immunoglobulin-like receptors (KIRs) and their HLA-class I ligands in susceptibility to T1D development, but only some of these studies have demonstrated an association. KIRs and their corresponding HLA class I ligands were investigated in Saudi patients with T1D compared with healthy controls. No significant differences in KIR gene distribution were observed between T1D patients and healthy controls. However, the homozygous C1/C1 ligand was considered a risk factor in predisposing individuals to T1D, whereas C2/C2 and HLA-Bw4 were considered protective factors against T1D. KIR2DL2/2DS2-C1C1 and KIR2DL3-C1C1 were significantly associated with T1D, and KIR2DS1-C2C2 and KIR2DL1-C2C2 were significantly less frequent in T1D patients. Stratification of KIR-HLA class I ligands in terms of the absence/presence of specific genotypes has different indications for susceptibility to T1D.

1. Introduction

Type 1 diabetes (T1D) is a multifactorial and polygenetic T cell-mediated autoimmune disease that leads to pancreatic B-cell destruction [1]. Natural killer (NK) cells are implicated in the development of T1D and might be important regulators and inducers of autoimmune diseases. NK cells, which are considered part of the innate immune system and can act against target cells after direct contact, can also kill islet cells [2,3]. NK cell activity is governed by a delicate balance between activating and inhibitory signals that arise from corresponding killer cell immunoglobulin-like receptors (KIRs), which are expressed on the cell surface and modulate NK and T cell functions by interacting with HLA class I-specific ligands on target cells [4]. Healthy human cells express HLA class I ligands, which interact with inhibitory KIRs to avoid spontaneous cell destruction, and failure of NK cells to recognize a suitable HLA ligand will activate these cells to kill their target [5,6]. KIR HLA class I-specific ligands include HLA-C group 1 (C1), HLA-C group 2 (C2) and HLA-Bw4 (Bw4) motifs that span residues 77–83 of the α1 domain; HLA-C is the most important ligand for NK cell regulation [7]. The different chromosomal locations of the KIR (residing on chromosome 19q13.4) and HLA (residing on chromosome 6p21) genes indicate the independent segregation of their existence within an individual, which is important for maintaining functional interactions and the immune response [2,8].

The presence of HLA molecules/genes is an important risk factor for susceptibility to T1D development [4], and certain combinations of HLA-KIR genotypes have been linked to susceptibility to autoimmune diseases. Further, several population studies have found associations between KIR/HLA class I ligands and T1D [1,3,4,9,10]. To our knowledge, no previous study on the role of KIRs and HLA class I ligands in T1D in the Saudi population has been published. Thus, this study aimed to investigate the distribution of certain KIRs and their corresponding HLA class I ligands in T1D patients compared with healthy controls in a Saudi population.

2. Materials and methods

KIRs and HLA class 1 alleles were defined in 106 unrelated Saudi individuals with T1D (39 individuals diagnosed prior to 5 years of age and 67 individuals diagnosed between 5 and 14 years of age); T1D diagnosis was based on WHO criteria (technical report series

* Corresponding author.
E-mail address: awadelsid@yahoo.com (A.E. Osman).

http://dx.doi.org/10.1016/j.humimm.2015.10.019
0198-8859/

727). In addition, 148 individuals who met the inclusion criteria of being healthy and unrelated [11] were randomly drawn from a pool of healthy donors at the Stem Cell Transplant Program of King Fahad Medical City (KFMC), Saudi Arabia, for inclusion as controls. The study was approved by the Hospital Institutional Review Board, and informed consent was obtained from the participants or their guardians prior to the time of blood collection. Blood samples were collected in containers containing EDTA, and genomic DNA was extracted using a MagNA pure compact instrument (Roche Diagnostics Ltd. Rotkreuz). DNA was measured using a NANODROP® 2000c spectrophotometer. KIR genes (KIR2DL1, KIR3DL2, KIR2DL2, KIR2DL3, KIR3DL1, KIR2DS1, KIR2DS2, KIR3DS1, KIR2DL5, KIR2DP1, KIR2DS3, KIR2DS4, KIR2DS5, KIR2DL4, KIR3DL3 and KIR3DP1) were identified using a sequence-specific oligonucleotide probe (SSOP) method that utilized Luminex® technology, and HLA-A, HLA-B and HLA-C were genotyped using the Luminex method (One Lambda, Canoga Park, CA, USA). The details of the two procedures can be obtained from https://www.onelambda.com. The frequencies of individuals positive for each KIR gene were counted based on http://www.allele-frequencies.net. Individuals carrying one or more of the KIR2DL2, KIR2DL5, KIR3DS1, KIR2DS1, KIR2DS2, KIR2DS3 and KIR2DS5 genes were grouped as Bx haplotypes (*x* can be either an A or B haplotype), and individuals who did not carry any of these genes were grouped as A haplotypes. In addition, haplotype group ID profiles were obtained [12]. Bx genotypes were classified into four subsets based on the presence or absence of centromeric (C) and telomeric (T) clusters: C4T4 (presence of both C and T), C4Tx (presence of C and absence of T), CxT4 (absence of C and presence of T) and CxTx (absence of both) [13].

The frequency distribution of the C1, C2, A*03/11 and Bw4 ligands was also assessed. According to Mehers et al., C1 was assigned if an individual had a C*01, *03, *07, *08, *12 and *14 allele lineage, whereas C2 was assigned if an individual had a C*02, *04, *05, *06, *15, *17 or *18 allele lineage [14]. HLA-C alleles not possible to group into C1 or C2 (subtyped classifications were needed for HLA-C) were removed. Additionally, Bw4 was defined based on the HLA-allele database (http://hla.alleles.org), and only the Bw4 motif that presents within HLA-B was considered.

Using the SAS program [15], the frequency distributions for the KIR genes and their ligands were derived using a direct counting method, and Fisher's exact test and logistic regression methodology were applied. To determine significant differences between patients and healthy controls, the log of the odds (logit) for the presence of each KIR gene and its ligand was modeled as a function of each group, and the overall significance level was set at 0.05. Bonferroni corrections were applied whenever necessary to address multiple comparisons, and significant results are presented as corrected *p* values.

3. Results

The KIR gene frequency distribution results among Saudi patients with T1D and healthy controls are shown in Table 1. All tested KIR genes were represented in the patient and control samples, and no significant differences were observed in KIR gene frequencies between T1D patients compared with healthy controls. KIR haplotype variations, which depend on the presence of KIR genes within an individual, were calculated, and no differences were observed for the A and Bx haplotype groups between the patients (20.8% and 79.2%, respectively) and controls (18.1% and 81.9%, respectively). Forty-six different KIR genotype groups were observed in the patients and controls, as shown in Fig. 1. For the HLA class I ligands for the KIR genes, a significantly higher frequency of homozygous C1C1 ligands was observed in patients with T1D (45% vs 15.9%, $p = 0.0002$, corrected $p = 0.0008$), whereas the frequency of homozygous C2C2 ligands was higher in healthy controls (36.4% vs 14%, $p = 0.0002$, corrected $p = 0.0008$).

Table 1
Frequency of KIR genes in children with type T1D compared with healthy controls.

Gene	All type 1 diabetes $n = 106$ (%)	Control $n = 148$ (%)
KIR2DL1	103 (97.2)	145 (98)
KIR2DL2	74 (69.8)	101 (68.2)
KIR2DL3	92 (86.8)	123 (83.1)
KIR3DL1	104 (98.1)	140 (94.6)
KIR3DL2	106 (100)	148 (100)
KIR2DS1	37 (34.9)	65 (43.9)
KIR2DS2	76 (71.7)	108 (73)
KIR3DS1	34 (32.1)	53 (35.8)
KIR2DL5	61 (57.5)	96 (64.9)
KIR2DP1	103 (97.2)	145 (98)
KIR2DS3	44 (41.5)	64 (43.2)
KIR2DS4	96 (90.5)	137 (92.6)
KIR2DS5	45 (42.5)	65 (43.9)
KIR2DL4	106 (100)	148 (100)
KIR3DL3	106 (100)	148 (100)
KIR3DP1	106 (100)	148 (100)

The Bw4 motif, which is the specific ligand for KIR3DS1 and KIR3DL1, was also found at a higher frequency in healthy controls (63.5% vs 41.5%, $p = 0.001$, corrected $p = 0.008$), and no difference was observed in the frequency of A*03/11 (ligand for KIR3DL2) between the patients and controls (18.9% vs 17.9%, $p = 0.84$).

An assessment the effect of KIR-HLA ligand genetic variation on susceptibility for T1D development is presented in Table 2. KIR2DL2-C1C1 and KIR2DS2-C1C1 combinations were present more frequently in T1D patients than controls (43.7% vs 19.4%, $p = 0.0008$, corrected $p = 0.0032$), and KIR2DL3-C1C1 was also present at a higher frequency in T1D patients than controls (46% vs 14.1%, $p = 0.0001$, corrected $p = 0.0004$). However, KIR2DS1-C1C1 had a higher frequency in controls (41.3%), and only one individual in each group of T1D patients was positive for KIR2DS1-C1C1.

Further, investigations were conducted on KIR-HLA ligands that exhibited significant differences between T1D patients and healthy controls. The presence of certain combinations, including KIR2DL2/3-C1C1, KIR3DL1-Bw4, KIR2DL1-C2C2, KIR2DL1 C2C2 + KIR3DL1-Bw4 or KIR2DL2/3-C1C1 + KIR3DL1-Bw4, was evaluated in each individual. As displayed in Table 3, higher frequencies with significant values were observed in T1D patients who exhibited KIR2DL2/3-C1C1 in the absence of KIR3DL1-Bw4 and KIR2DL1-C2C2 (p value = 0.0015, corrected p value = 0.0075) and KIR2DL2/3-C1C1 + 3DL1-Bw4 in the absence of 2DL1-C2C2 (p value = 0.001, corrected p value = 0.005), whereas 3DL1-Bw4 in the absence of 2DL2/3-C1C1 and 2DL1-C2C2 was significantly increased in healthy controls.

4. Discussion

Our results comparing T1D patients and controls in terms of KIRs genes frequencies are in agreement with Chinese and Basque populations [4,16]; however, Mehers et al. reported only a significant difference in the KIR2DL3 genes in a British population [14]. Significant differences were detected in HLA class 1 ligands. Accordingly, the homozygous C1C1 was considered a predisposing risk factor for T1D development, whereas the significantly increased frequencies of C2C2 and HLA-Bw4 in controls indicated these ligands might play a protective role against T1D in the Saudi population. Notably, the HLA-Bw4 motif in the HLA-B locus had a better effect on NK cell inhibition than the Bw4 motif in HLA-A [2]. No significant differences were observed in the heterozygous

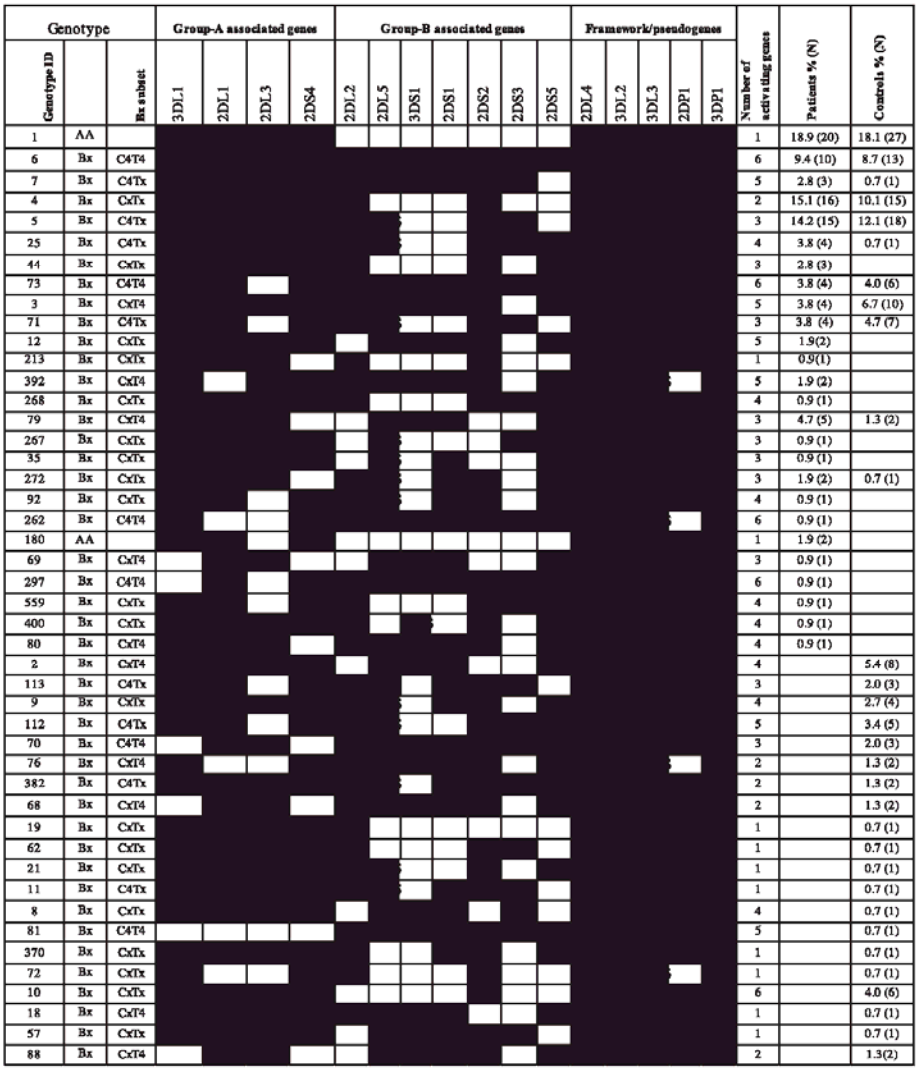

Fig. 1. Comparison of KIR gene diversity between patients and controls. This figure includes the genotype IDs, A and Bx haplotype-associated groups, framework/pseudogenes and the activating KIR gene frequencies. Black areas indicate positive genes, and blank areas indicate negative genes.

C1C2 ligand between patients and controls, but Zhi et al. (2011) reported a significant association between heterozygous C1C2 and T1D in a Chinese Han population [4].

The significantly increased frequency of KIR2DL2/-S2 with the C1C1 ligand in T1D patients is in agreement with a Latvian study in which an increased frequency of KIR2DL2 and KIR2DS2 with its C1 ligand was observed in patients compared with controls [17]. Additionally, a Dutch study found a significantly increased association of KIR2DS2 with its putative ligand in T1D patients [3].

An analysis of the gene content variation in the KIR region from a global set of populations by Single et al. demonstrated that KIR2DL2 and KIR2DS2 are in strong linkage disequilibrium [18]. Despite the predominant distribution of C1 found here and the consequent limitation of C2 through its receptors, KIR2DL1-C2C2 was observed at a relatively high frequency in controls compared with T1D patients (36.4% vs 14%, $p = 0.0002$, corrected = 0.0008). Yawata et al. demonstrated that individuals positive for KIR2DL1-C2 or KIR3DL1-Bw4 pairs had higher numbers of NK cells expressing these gene combinations [19]. However, Bw4 may coincide with HLA-C1 because many HLA-C and HLA-B haplotypes, such as C*07 (part of the C1 ligand) and B*51 (separate from Bw4), are in linkage disequilibrium [20].

Table 2
Frequency of KIR genes in the presence of HLA-C1 and HLA-C2 in children with T1D compared with healthy controls.

HLA-C genotype	T1D patients	Controls	p value of T1D vs controls
KIR2DL2/-S2 (C1-ligand)	n = 71 (%)	n = 98 (%)	
C1C1	31 (43.7)	19 (19.4)	0.0008
C1C2	30 (42.3)	44 (44.9)	0.73
C2C2**	10 (14)	–	–
KIR2DL3 (C1-ligand)	n = 87 (%)	n = 114 (%)	
C1C1	40 (46)	16 (14.1)	0.0001
C1C2	36 (41.4)	56 (49.1)	0.28
C2C2**	11 (12.6)	42 (36.8)	
KIR2DS1 (C2-ligand)	n = 34 (%)	n = 58 (%)	
C1C1*	20 (60.6)	7 (12.1)	
C1C2	12 (36.4)	27 (46.6)	0.35
C2C2	2 (6)	24 (41.3)	0.002
KIR2DL1 (C2-ligand)	n = 100 (%)	n = 132 (%)	
C1C1*	45 (45)	21 (1539)	
C1C2	41 (41)	63 (47.7)	0.31
C2C2	14 (14)	48 (36.4)	0.0002

* C1C1 is not a ligand for KIR2DS1 or KIR2DL1.
** C2C2 is not a ligand for 2DL2/-S2 or KIR2DL3.

Further analysis on KIR-HLA ligands that exhibited significant difference between patients and controls indicated that individuals with KIR2DL2/3-C1C1 in the absence of KIR3DL1-Bw4 and KIR2DL1-C2C2 or KIR2DL2/3-C1C1 + 3DL1-Bw4 in the absence of 2DL1-C2C2 genotypes were at risk for T1D. However, the significantly higher frequency of KIR2DL1-C2C2 + KIR3DL1-Bw4 in the absence of KIR2DL2/3-C1C1 detected in healthy controls suggests a protective role of approximately fivefold in individuals with this genotype than those lacking this combination. Therefore, fewer inhibitory KIRs with their corresponding ligands could be a possible cause of T1D development. Finally, the discrepant results obtained in different population studies may be due to variations in the KIR/HLA gene distribution in various ethnic groups and/or the substructuring effect or stratification of populations. One potential limitation of our study is that HLA-class II genes were not typed; thus, verifying whether the association between T1D and HLA-class I genes resulted from the linkage disequilibrium between HLA class I and HLA class II (diabetes high-risk HLA) was not possible. However, Van der Silk et al. suggested no linkage between HLA class I and II in patients and controls in a diabetes high-risk HLA match study [21]. In contrast, Jobim et al. found that the presence of the KIR2DL2 gene together with HLA class II alleles was more frequent in T1D patients than controls [10].

Table 3
Frequency distribution of KIR-HLA class I ligands exhibiting significant differences between T1D patients and healthy controls: 2DL2/3-C1C1 (A), 3DL1-Bw4 (B), and 2DL1-C2C2 (C).

KIR ligand	T1D patients (n = 75)	Controls (n = 113)	p value	Corrected p value
Positive for A, negative for B and C	24 (32%)	14 (12.4%)	0.0015	0.0075
Positive for B, negative for A and C	17 (22.6)	45 (39.8)	0.0154	0.077
Positive for C, negative for A and B	8 (10.7)	17 (15%)	0.39	
Positive for B and C, negative for A	5 (6.7)	31 (27.4%)	0.001	0.005
Positive for A and B, negative for C	21 (28%)	6 (5.3%)	0.001	0.005

In conclusion, we found no association between KIR gene frequencies and T1D. However, the homozygous C1/C1 ligand was a risk factor for T1D development. Various combinations of KIR genes and HLA class 1 ligands either had a role of protection against or led to susceptibility to T1D. Genetic studies using a large sample size and including HLA class II and functional assays are recommended to better clarify the roles of KIRs and HLA class I ligands.

Acknowledgments

This study was fully supported by King Fahad Medical City, Riyadh, Saudi Arabia. The authors thank Professor Derek Middleton for critical comments on the manuscript. We would also like to thank Dr. Abdulwahid Al-Dehaimi for his support, and appreciation is extended to the patients and BMT donors who participated in this study.

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Publication No: 5

My contribution to this study included obtaining the IRB approval, which was under my name. I contributed equally to the research design and sample selection, the supervision of the laboratory work, and the interpretation of the data. I drafted the manuscript, communicated with the other co-authors for review, and acted as the corresponding author.

Received: 11 January 2020 | Revised: 3 February 2021 | Accepted: 22 February 2021

DOI: 10.1111/iji.12535

ORIGINAL ARTICLE

INTERNATIONAL JOURNAL OF IMMUNOGENETICS WILEY

Association of single-nucleotide polymorphisms in tumour necrosis factor and human leukocyte antigens genes with type 1 diabetes

Awad Elsid Osman[1] | Imad Brema[2] | Alaa AlQurashi[3] | Abdullah Al-Jurayyan[1] | Benjamin Bradley[4] | Muaawia Ahmed Hamza[3]

[1]Pathology and Clinical Laboratory Management Department, King Fahad Medical City, Riyadh, Saudi Arabia

[2]Obesity, Endocrine and Metabolism Center, King Fahad Medical City, Riyadh, Saudi Arabia

[3]Research Center, King Fahad Medical City, Riyadh, Saudi Arabia

[4]National Centre for Biomedical Engineering Science, National University of Ireland, Galway, Ireland

Correspondence
Awad Elsid Osman, Pathology and Clinical Laboratory Management Department, King Fahad Medical City, Riyadh, Saudi Arabia.
Emails: awadelsid@yahoo.com; aeosman@kfmc.med.sa

Funding information
King Fahad Medical City, Grant/Award Number: 017-034

Abstract

Type 1 diabetes (T1D) is an autoimmune disease characterized by progressive destruction of insulin-producing pancreatic beta cells. This multifactorial disease has a strong genetic component associated with the human leukocyte antigens (HLA) and non-HLA regions. In this study, we compared frequencies of HLA-DRB1 alleles and single-nucleotide polymorphisms (SNPs) associated the genes coding for: toll-like receptors (TLRs), tumour necrosis factor (TNF), interleukin-1 (IL-1), interleukin-1 receptor type 1 (IL-1R1), interleukin-1 receptor antagonist (IL-1RN), interleukin-2 (IL-2) and interleukin-12B (IL-12B), between T1D patients and healthy controls. The aim was to identify frequency differences and linkage between these genetic markers in T1D patients and healthy controls. Twelve SNPs were investigated as follows: rs16944 (IL-1B), rs1143634 (IL-1B), rs1800587 (IL-1A), rs2069762 (IL-2), rs3212227 (IL-12B), rs2234650 (IL-1R1), rs315952 (IL-1RN), rs3804099 (TLR2), rs4986790 (TLR4), rs4986791 (TLR4), rs1800629 (TNF) and rs361525 (TNF). TaqMan genotype assay method was used for SNPs genotyping. HLA-DRB1* genes were typed by Sequence Specific Oligonucleotide Probe (SSOP). SPSS and SNPStats programs were used for the statistical analysis. Significant differences between T1D and control groups were found for the dominant model of rs361525 and rs1800629A:rs361525G genotypes for TNF. Increased frequencies of DRB1*03 and DRB1*04 and decreased frequencies of DRB1*07, DRB1*11 and DRB1*13 and DRB1*15 were observed in T1D patients compared with controls. However, the genotype, DRB1*07 with rs1800629A/G was associated with T1D. We have confirmed that DRB1*03 and DRB1*04 are associated with increased risk and DRB1*07, DRB1*11 and DRB1*13 and DRB1*15 with decreased risk of T1D. Also, the dominant model of rs361525A, and the rs1800629G:361525A genotype were associated with increased risk. The simultaneous presence of DRB1*07 and rs1800629A/G genotypes in 23 out of 27 DRB1*07 positive T1D patients implied that islet cell peptide processing may have been biased towards autoimmunity by upregulation of TNF associated intronic SNPs.

KEYWORDS

IL-1R, Saudi population, single-nucleotide polymorphisms, tumour necrosis factor, type 1 diabetes

1 | INTRODUCTION

Type 1 diabetes (T1D) is an incurable autoimmune disease characterized by permanent destruction of insulin-producing pancreatic beta cells: the islets of Langerhans. Pathogenesis is characterized by a chronic inflammatory responses involving autoimmune T lymphocytes and macrophages infiltrating and destroying the islets, eventually causing hyperglycaemia through insufficient secretion of insulin (Mandrup-Poulsen et al., 1994; Thomas et al., 2013). Strong genetic susceptibility to T1D is supplemented by non-genetic factors, as evidenced by monozygotic twin concordance rates ranging from 13% to 65% (Kaprio et al., 1992, Redondo et al., 2008; Törn et al., 2015).

Susceptibility genes for T1D are grouped into three categories, namely, immune function, insulin expression and islet cell function; but the highest risk elements are genes involved in autoimmune responses (Noble & Erlich, 2012). Foremost among these are the human leukocytes antigen (HLA) genes on the short arm of chromosome 6, encoding cell surface proteins involved in the initial stages of peptide processing (Kaprio et al., 1992; Ovsyannikova et al., 2014; Risch, 1987). However, other genetic markers, particularly those associated with regulation of pro and anti-inflammatory cytokines may also play a role.

Both self and non-self peptides bind to HLA molecules on the external cell membrane of antigen presenting cells (APCs). APCs then offer these peptides to selected T-cell clones via T-cell receptors (TCR) (Mogensen, 2009; Purcell et al., 2016). Depending on availability, pro- and anti-inflammatory cytokines induce expansion and/or suppression of the selected clones.

We hypothesised that in T1D the 'self' peptides associated with pancreatic islet cells are targeted by autoimmune clones normally directed down tolerogenic pathways, but instead are directed down immune pathways where they become amplified into self-destructive clones under the influence of pro-inflammatory cytokines.

The pro-inflammatory cytokines interleukin-1 (IL-1) and tumour necrosis factor (TNF) have been demonstrated in the islets cells of patients with T1D (Clark et al., 2017; Van Belle et al., 2011). Furthermore, toll-like receptors (TLRs), a group of proteins fundamental in innate immune responses involved in the pathogenesis of various autoimmune diseases (Kawai & Akira, 2010; Lien & Zipris, 2009), also play a crucial role in T1D (Sun et al., 2014; Xie et al., 2018). Multiple research studies have investigated associations between single-nucleotide polymorphisms (SNPs) and the susceptibility to developing T1D with variable outcomes (Allam et al., 2018; Hollegaard & Bidwell, 2006; Javor et al., 2010).

In this study, we investigated both HLA-DRB1 genes, and SNPs associated with cytokines that regulate autoimmune responses for their association with T1D, namely, toll-like receptors (TLR-2, TLR-4), tumour necrosis factor (TNF), interleukin-1 (IL-1), interleukin-1 receptor 1 (IL-1R1), interleukin-1 receptor antagonist (IL-1RN), interleukin-2 (IL-2) and interleukin 12 (IL-12). Our aim was to identify significant differences between T1D and healthy controls and possible linkages between SNPs and HLA-DRB1.

2 | MATERIALS AND METHODS

This case–control cohort study involved 150 patients diagnosed with T1D and 178 healthy normal controls with no history of T1D. The sample size calculation was based on the Saudi descriptive epidemiology study by Robert et al. that compared patients and control data using the Fisher's exact test at a level of 5% and power 80. Patients were diagnosed according to the American Diabetes Association criteria (Standards of Medical Care in Diabetes, 2019). Diagnosis was confirmed by the presence of one or more of the following: glutamic acid decarboxylase (GAD), islet-cell antibodies (ICA) or insulin autoantibodies (IAA). The healthy unrelated control group was selected randomly from the list of potential bone marrow transplant (BMT) donors in our hospital who had no history of diabetes or other autoimmune diseases.

The study was conducted according to the guidelines of the Helsinki Declaration on Human Experimentation and approved by the Institutional Review Board (IRB) at King Fahad Medical City (KFMC). An exemption for obtaining informed consent from patients was approved by the IRB as we used the remaining clinical samples previously collected from patients and the archived DNA of potential BMT donors. Peripheral blood samples were collected into EDTA tubes between January 2016 and December 2017 for T1D patients and from February 2015 to July 2017 for controls. The integrity of blood samples was checked for quality purposes prior to storage at −20°C until the time of DNA extraction. All procedures adhered to the rules and regulations of the Saudi Arabia government, the KFMC/IRB policies and procedures, and the IHC Good Clinical Practice guidelines.

A MagNa Pure Compact instrument (Roche Diagnostics GmbH; Roche Applied Science) was utilized to extract genomic DNA according to manufacturer's instructions, and a DNA concentration of 20 ng/μl with 260/280 nm value of between 1.6 and 2.0 was accepted for both quantity and the quality.

Twelve SNPs, which included: rs16944 (IL-1B), rs1143634 (IL-1B), rs1800587 (IL-1A), rs2069762 (IL-2), rs3212227 (IL-12B), rs2234650 (IL-1R1), rs315952 (IL-1RN), rs3804099 (TLR2), rs4986790 (TLR4), rs4986791 (TLR4), rs1800629 (TNF −308) (TNF) and rs361525 (TNF −238) (TNF) were investigated (Table 1).

Sequence-specific forward and reverse primers with two TaqMan® MGB probes and its dyes (VIC™ and FAM™) were used to detect alleles 1 and 2 for each SNP. Genotype procedures were carried out according to manufacturer's instructions (Applied Biosystems).

HLA-DRB1 genotyping was performed by sequence-specific oligonucleotide probe (SSOP) utilizing a Luminex-based method, The Lab Type kit (One Lambda, San Diego, USA) according to manufacturer's instructions (http://www.onelambda.com). The HLA

TABLE 1 List of IL1A, IL1B, IL1R1, IL12B, IL2, TLRs, IL1RN and TNF genes and the selected single-nucleotide polymorphisms (SNPs)

SNP ID	Reference	Gene Name	Location	Alleles	GMAF[a]	Codon change	Amino acid change
rs16944	(Allam et al., 2018)	Interleukin 1 beta (IL1B)	Chr.2 (Intron)	A > G	0.4651	TTC, TTT	F105F
rs1143634	(Mahmoudi et al., 2011)	Interleukin 1 beta (IL1B)	Chr.2 (coding region	G > A	0.1455		
rs1800587	(Javor et al., 2010)	Interleukin 1 alpha (IL1A)	Chr.2 (UTR 5)	G > A	0.253		
rs2069762	(Kim et al., 2012)	Interleukin 2 (IL2)	Chr.4 (Intron)	A > C	0.247		
rs3212227	(Morahan et al., 2009)	Interleukin 12B (IL12B)	Chr.5 (UTR 3)	T > G	0.3384		
rs2234650	(Mahmoudi et al., 2011)	Interleukin 1 receptor type 1 (IL1R1)	Chr.2 (Intron)	C > T	0.4109		
rs315952	(Mahmoudi et al., 2011)	Interleukin 1 receptor antagonist (IL1RN)	Chr.2 (coding region)	T > C	0.3907		
rs3804099	(Jaen et al., 2009)	Toll-like receptor 2 (TLR2)	Chr.4 (coding region)	T > C	0.4307	AAC, AAT	N199N
rs4986790	(Jaen et al., 2009)	Toll-like receptor 4 (TLR4)	Chr.9 (coding region)	A > G	0.04408		
rs4986791	(Rudofsky et al., 2004)	Toll-like receptor 4 (TLR4)	Chr.9 (coding region)	C > T	0.02984		
rs1800629	(Boraska et al., 2009)	Tumour necrosis factor (TNF)	Chr.6 (Intron)	G > A	0.0955		
rs361525	(Boraska et al., 2009)	Tumour necrosis factor (TNF)	Chr.6 (Intron)	G > A	0.05051		

Abbreviation: GMAF, global minor allele frequency.
[a]https://www.snpedia.com/index.php/SNPedia

genotyping procedure at our laboratory is continuously monitored for quality assurance by the College of American Pathologists (CAP).

2.1 | Statistical analysis

The Hardy–Weinberg equilibrium (HWE) for the expected and observed distribution of allele frequencies for the control data was analysed by implementing the χ^2 distribution (degree of freedom = 1) (Rodriguez et al., 2009).

The frequencies for alleles and genotype models for each SNP, that include the codominant, dominant and recessive alleles were derived by an algorithm based on the direct counting method using SNPStats software (https://www.snpstats.net/start.htm). The dominance phenomenon is defined as the relationship between the two alleles located at one gene, in which the effect of the first allele in the phenotype masks the contribution of the second allele—the first allele is known as the dominant allele, and the second as the recessive allele ('New Oxford American dictionary', 2013). Codominance is defined as a form of dominance in which two alleles (heterozygous) located at one gene are fully expressed, and the resulting phenotype in the offspring is neither dominant nor recessive (Carr et al., 2003).

The D′ statistic and p value tests were used to analyse the linkage disequilibrium (LD) between the SNPs. The D′ LD measurement is from 0 (no LD) to 1 (strong LD), and the p value was set at 5% (Brown et al., 2006). Fisher's exact test and the log of the odds (logit) for the presence of each SNP was calculated to determine the significant differences at an overall level of $p < .05$. HLA allele frequency analysis and stratification of significant results were conducted implementing SPSS software version 22.0. The Bonferroni correction test was applied whenever it was necessary to address multiple comparisons, and results are shown as corrected p ($^c p$) values.

3 | RESULTS

For this study, we recruited 150 patients with T1D of whom 60.7% were female, aged 3–30 years (median17 years), and 178 healthy controls of whom 51.1% were female, aged 1–79 (median 29 years), detailed in Table 2. The conformation for HWE was observed in nine SNPs with expected statistical values ($p > .05$); however, there were 3 SNPs, rs16944, rs4986790 and rs1143634, that did not conform with HWE ($p < .05$). The LD values for rs1800629 and rs361525 were high (D′ = 0.9919, and $p < .011$). No other significant values for LD were identified.

3.1 | Single-nucleotide polymorphisms

The allele frequencies and genotype models for SNPs were tested between the patients and controls for possible differences as shown in Table 3. Four SNPs (rs16944, rs3212227, rs1800629 and rs361525) had significant differences between the patients and controls in terms of alleles or genotype model frequencies. Two of these values (rs16944, rs3212227) did not reach a significant level after Bonferroni correction. However, significant difference after correction was observed for the rs361525 dominant model ($^{c}p < .024$). Thus, individuals positive for the rs361525A/G or -A/A genotypes had a more than fivefold protective effect against T1D than those who were positive for rs361525G-G genotype (risk factor = 5.3).

Although rs16944 had differed in some values, these differences were not satisfied after correction for the G allele, the codominant model and the recessive model. Similar observations were made with recessive models of rs3212227 and rs1800629.

Interestingly, the rs1800629G:361525A haplotype was higher after correction among the control group than in the patient group ($^{c}p < .032$) and was considered to be a protective factor against T1D (Table 4).

3.2 | HLA-DRB1

Twelve different alleles of HLA-DRB1 were analysed, of which DRB1*03 ($^{c}p < .001$) and DRB1*04 ($^{c}p < .002$) had higher frequencies among T1D patients compared with healthy controls. In contrast, DRB1*07 ($^{c}p < .0001$), DRB1*11 ($^{c}p < .001$), DRB1*13 ($^{c}p < .0004$) and DRB1*15 ($^{c}p < .0002$) had lower frequencies in T1D patients. Of the remaining alleles DRB1*01, *08, *09, *10, *14 and *16 were not significantly different between T1D and Controls (Table 5).

TABLE 2 Details of demographic and clinical data for type 1 diabetes (T1D) patients and healthy control

	T1D patients $N = 150$ (%)	Healthy control $N = 178$ (%)
Females	91 (60.7)	91 (51.1)
Male	59 (39.3)	87 (48.9)
Age range (years)	3–30	1–79
Age median (years)	17	29
Onset of T1D		
Female	9.7 years	
Male	8.6 years	
Duration of T1D		
Female	9.7 years	
Male	9.8 years	
Nephropathy		
Female	7 (7.7)	
Male	7 (11.9)	

When T1D patients were stratified according to presence or absence of DRB1*03, DRB1*04, DRB1*07 and DRB1*13 (homozygous or heterozygous), and association with the two TNF SNPs, rs1800629 and rs361525 analysed, a significant increase in the DRB1*07 and rs1800629A/G genotype was observed compared to the DRB1*07 in the absence of rs1800629G/G genotype ($^{c}p < .0001$) as shown in Table 6. We did not attempt to stratify DRB1*11 due to its low frequency (1.7%) among T1D patients. Stratification of DRB1*07 in presence of DRB1*03/04 with or without rs180629A/G genotypes was shown in Table 7, higher significant frequency of individuals positive for DRB1*07 and DRB1*03/04 genotype was detected among T1D patients ($^{c}p = .018$) when compared to healthy control. Also, DRB1*07, DRB1*03/04 and rs1800629A/G was found at higher significant frequency in patients ($^{c}p = .0003$). No other significant differences were observed.

4 | DISCUSSION

In this study, we compared the frequencies of twelve SNPs associated with genes coding for TNF-α, IL1, IL1R, IL2, IL12B and TLR in unrelated T1D patients of Saudi origin with a healthy control group. Furthermore, we compared frequencies of HLA-DRB1 alleles and identified possible linkages with these SNPs.

A deviation from HWE was observed in three SNPs (rs16944, rs4986790 and rs1143634) and was possibly attributed to the high consanguinity among the Saudi population (El-Hazmi et al., 1995), or to the Wahlund effect caused by a subpopulation structure (Garnier-Géré & Chikhi, 2013).

Multiple differences in SNP allele and genotype models were identified, but these did not reach significance after applying the Bonferroni correction test, except for the rs361525 where individuals having genotypes A/G or A/A appeared to be protected against T1D compared with those having the G/G genotype. This observation was consistent with a South Croatian study (Boraska et al., 2009) and a Caucasian study (Noble et al., 2006) who investigated the rs361525 (G-238A) SNP in T1D. In contrast, no associations were observed with this SNP and T1D patients in a cohort study by Kaidonis et al. (2016).

The rs1800629 SNP showed no significant associations with T1D after applying the Bonferroni correction test, but a significant association was detected with the rs1800629G:361525A haplotype, and considered a protective factor against T1D (Table 4). A similar observation of was made in a South Croatia study (Boraska et al., 2009). Moreover, a higher frequency of the rs1800629A allele was found among Australian and United Kingdom T1D patients compared with healthy controls (Kaidonis et al., 2016). By contrast, a study of Saudi patients by Allam et al claimed that the TNF-α −308A was a risk factor for T1D; however, the authors did not implement the Bonferroni correction test for multiple comparisons to adjust their results (Allam et al., 2018).

Rs1800629 and rs361525 SNPs are present within the TNF gene, previously known as TNF- α or TNFA, located on the short arm

TABLE 3 Association between IL1A, IL1B, IL1R1, IL12B, IL2, TLRs, IL1RN and TNF genes single-nucleotide polymorphisms (SNPs) and the susceptibility to type 1 diabetes (T1D)

SNP ID	Genotype	T1D patients $n = 150$ (%)	Healthy control ($n = 178$)	SNPs model	OR (95% CI)	*p*-Value	Corrected *p*-value
rs16944 (IL1B)	A	192 (64)	191 (56)	Alleles	1.4 (1.1, 2.0)	.03	.36
	G[a]	108 (36)	153 (44)				
	A/A	62 (41.3)	60 (34.9)	Codominant			
	G/A	68 (45.3)	71 (41.3)		1.1 (1.1,4.0)	.7	
	G/G	20 (13.3)	41 (23.8)		2.2 (1.1, 4.0)	.022	.264
	A/A versus G/A + G/G			Dominant	1.3 (0.8, 2.1)	.23	
	A/A + G/A versus G/G			Recessive	2.0 (1.1, 3.7)	.015	.18
rs1143634 (IL1B)	G	180 (60)	208 (65)	Alleles	0.9 (0.6, 1.1)	.19	
	A[a]	120 (40)	112 (35)				
	G/G	59 (39.3)	74 (46.2)	Codominant			
	G/A	62 (41.3)	60 (37.5)		0.8 (0.5, 1.3)	.5	
	A/A	29 (19.3)	26 (16.2)		0.7 (0.4,1.3)		
	G/G versus G/A + A/A			Dominant	0.8 (0.5, 1.12)	.2	
	G/G + G/A versus A/A			Recessive	0.8 (0.5, 1.5)	.5	
rs1800587 (IL1A)	G	166 (55)	201 (58)	Alleles	0.9 (0.7, 1.2)	.42	
	A[a]	134 (45)	143 (42)				
	G/G	48 (32)	62 (36)	Codominant			
	A/G	70 (46.7)	77 (44.8)		0.8 (0.43–1.5)	.7	
	A/A	32 (21.3)	33 (19.2)		0.7 (0.38,1.3)		
	G/G versus G/A + A/A			Dominant	0.8 (0.5, 1.3)	.4	
	G/G + G/A versus A/A			Recessive	0.9 (0.5, 1.5)	.6	
rs2234650 (IL1R1)	C	152 (51)	193 (56)	Alleles	0.8 (0.6, 1.1)	.22	
	T[a]	146 (49)	153 (44)				
	C/C	43 (28.9)	58 (33.5)	Codominant			
	C/T	66 (44.3)	77 (44.5)		0.9 (0.5, 1.5)	.5	
	T/T	40 (26.9)	38 (22)		0.7 (0.4, 1.3)		
	C/C versus C/T + T/T			Dominant	0.8 (0.5, 1.3)	.4	
	C/C + C/T versus T/T			Recessive	0.8 (0.5, 1.3)	.3	
rs3212227 (IL12B)	T	223 (74)	242 (70)	Alleles	1.2 (0.9,1.7)	.3	
	G[a]	77 (26)	102 (30)				
	T/T	80 (53.3)	89 (51.7)	Codominant			
	G/T	63 (42)	64 (37.2)		0.9 (0.6, 1.5)	.09	
	G/G	7 (4.7)	19 (11.1)		2.5 (1.0, 6.1)		
	T/T versus G/T + G/G			Dominant	1.1 (0.7, 1.7)	.78	
	T/T + G/T versus G/G			Recessive	2.5 (1.0, 6.2)	.032	
rs2069762 (IL2)	C[a]	171 (57)	184 (62)	Alleleles	0.8 (0.6, 1.1)	.2	
	A	129 (43)	114 (38)				
	C/C	52 (34.7)	58 (39.9)	Codominant			
	C/A	67 (44.7)	68 (45.6)		0.7 (0.3, 1.3)	.47	
	A/A	31 (20.7)	23 (15.4)		0.9 (0.6, 1.5)		
	C/C versus C/A + A/A			Dominant	0.82 (0.50–1.36)	.4	
	C/C + C/A versus A/A			Recessive	0. 7 (0.4, 1.3)	.2	

(Continues)

TABLE 3 (Continued)

SNP ID	Genotype	T1D patients $n = 150$ (%)	Healthy control ($n = 178$)	SNPs model	OR (95% CI)	p-Value	Corrected p-value
rs3804099 (TLR2)	T	150 (5)	182 (53)	Alleles	0.9 (0.7, 1.2)	.5	
	C[a]	150 (5)	162 (47)				
	T/T	42 (28)	50 (29)	Codominant			
	C/T	66 (44)	82 (47.7)		1.1 (0.6, 1.8)	.6	
	C/C	42 (28)	40 (23.3)		0.8 (0.4, 1.5)		
	T/T versus C/T + C/C			Dominant	0.9 (0.58–1.5)	.8	
	T/T + C/T versus C/C			Recessive	0.8 (0.5–1.3)	.3	
rs4986790 (TLR4)	A	285 (96)	281 (96)	Alleles	1.3 (0.6, 2.8)	.5	
	G[a]	13 (4)	17 (4)				
	A/A	136 (91.3)	137 (92)	Codominant	0.5 (0.2, 1.4)	.012	
	A/G	13 (8.7)	7 (4.7)				.144
	G/G		5 (3.4)				
	A/A versus G/A + G/G			Dominant	0.9 (0.4, 2.1)	.8	
	A/A + G/A versus G/G			Recessive		.008	.096
rs4986791 (TLR4)	C	289 (96)	325 (95)	Alleles	1.4 (0.6, 3.0)	.4	
	T[a]	11 (4)	17 (5)				
	C/C	140 (93.3)	156 (91.2)	Codominant			
	C/T	9 (6)	13 (7.6)		1.3 (0.5, 3.1)	.8	
	T/T	1 (0.7)	2 (1.2)		1.8 (0.2, 22)		
	C/C versus C/T + T/T			Dominant	1.4 (0.6, 3.1)	.5	
	C/C + C/T versus T/T			Recessive	1.8 (0.2, 1.9)	.7	
rs315952 (IL1RN)	T	239 (80)	256 (75)	Alleles	1.3 (0.9, 1.9)	.18	
	C[a]	61 (20)	85 (25)				
	T/T	97 (64.7)	98 (57)	Codominant			
	C/T	45 (30)	63 (36.6)		1.4 (0.9, 2.2)	.4	
	C/C	8 (5.3)	11 (6.4)		1.4 (0.5, 3.5)		
	T/T versus C/T + C/C			Dominant	1.4 (0.9, 2.2)	.2	
	T/T + C/T versus C/C			Recessive	1.2 (0.5, 3.1)	.7	
rs1800629 (TNF)	G	234 (78)	264 (76)	Alleles	1. 1 (0.8, 1.6)	.6	
	A[a]	66 (22)	82 (24)				
	G/G	87 (58)	105 (61.7)	Codominant			
	A/G	60 (40)	54 (31.2)		0.8 (1.1, 13)	.018	.216
	A/A	3 (2)	14 (8.1)		4.3 (1.2, 15)		
	G/G versus G/A + A/A			Dominant	0.76 (0.5, 1.2)	.6	
	G/G + G/A versus A/A			Recessive	4.45 (1.2, 16.1)	.01	
rs361525 (TNF)	G	297 (99)	328 (94)	Alleles	5.4 (1.6, 18)	.007	.084
	A[a]	3 (1)	18 (6)				
	G/G	147 (98)	156 (90.2)	Codominant	5.0 (1.4, 17)	.0075	
	A/G	3 (2)	16 (9.2)				.09
	A/A		1 (0.6)				
	G/G versus G/A + A/A			Dominant	5.3 (1.5, 18)	.002[c]	.024
	G/G + G/A versus A/A			Recessive		.26	

Note: [c]p Value statistically significant after correction.

[a]Minor allele frequency (MAF).

TABLE 4 association of rs1800629:rs361525 haplotypes ($n = 323$) with the susceptibility to type 1 diabetes (T1D)

rs1800629	rs361525	Patients *n* (%)	Control *n* (%)	OR (95%)	*p* Value	Corrected *p*-value
G	G	116 (77)	127 (71)	1		
A	G	33 (22)	42 (23.3)	1.2 (0.8, 1.7)	.39	
G	A	1 (1)	9 (5.2)	5.4 (1.6, 18)	.008[c]	.032
A	A	0	1 (0.5)			

Note: [c]*p* Value statistically significant after correction.

TABLE 5 Frequency distribution and association of HLA-DRB1 allele lineages in type 1 diabetes (T1D) patients and healthy control

	T1D		Control				
DRB1	$n - 144$[a]	AF%	$n - 142$[a]	AF%	OR (95% CI)	*p* Value	Corrected *p* value
*03	122	42.4	48	16.8	10.8 (6.1, 19.2)	<.0001	<.0012
*04	88	30.6	52	18.3	2.7 (1.7, 4.4)	.0002	.0024
*07	31	10.7	63	22.2	0.3 (0.2, 0.6)	.0001	<.012
*11	5	1.7	23	8	0.2 (0.1, 0.5)	.001	.012
*13	16	5.6	40	14.1	0.3 (0.2, 0.6)	.0004	.0048
*15	3	1	26	9.2	0.1 (0.03, 0.3)	.0002	.0024
*01	7	2.4	11	3.9	0.6 (0.2, 1.6)	.31	
*08	2	0.7	5	1.8	0.4 (0.07, 2)	.3	
*09	1	0.4	1	0.4	0.98 (0.06, 15.9)	.99	
*10	4	1.4	3	1.1	1.3 (0.3, 6)	.7	
*14	1	0.4	4	1.4	0.2 (0.02, 2.2)	.21	
*16	8	2.7	8	2.8	0.98 (0.3, 2.7)	.98	

Note: [c]*p* Value statistically significant after correction.

[a]Indicates number of samples genotyped.

TABLE 6 Association of rs361525 and rs1800629 SNPs in type 1 diabetes (T1D) patients positive for DRB1*03, −04, −07 and −13 and T1D patients negative for these allele lineages

	rs361525					rs1800629			
HLA allele linage	A/A *n* (%)	A/G *n* (%)	G/G *n* (%)	OR (95% CI); *p* Value		A/A *n* (%)	A/G *n* (%)	G/G *n* (%)	OR (95% CI); *p* Value
Positive DRB1*03 ($n = 96$)	0	2 (2.1)	94 (97.9)	0.9 (0.1, 11.1); 0.9	Positive DRB1*03 ($n = 95$)	1 (1.1)	43 (45.3)	51 (53.6)	1.3 (0.7, 3.0); .3
Negative DRB1*03 ($n = 47$)	0	1 (2.1)	46 (97.9)		Negative DRB1*03 ($n = 46$)	2 (4.2)	17 (35.4)	29 (60.4)	
Positive DRB1*04 ($n = 72$)	0	3 (4.1)	69 (95.9)		Positive DRB1*04 ($n = 72$)	1 (1.4)	30 (41.6)	41 (57)	1.0 (0.5, 2.0); .9
Negative DRB1*04 ($n = 72$)	0	0	72 (100)		Negative DRB1*04 ($n = 72$)	2 (2.8)	29 (40.3)	41 (56.9)	
Positive DRB1*07 ($n = 30$)	0	0	30 (100)		Positive DRB1*07 ($n = 27$)		23 (76.7)	4 (13.3)	12.5 (4.0, 38.5); <.0001[c]
Negative DRB1*07 ($n = 114$)	0	3 (2.6)	111 (97.4)		Negative DRB1*07 ($n = 114$)	0	36 (31.6)	78 (68.4)	
Positive DRB1*13 ($n = 17$)	0	0	17 (100)		Positive DRB1*13 ($n = 17$)	0	8 (47.1)	9 (52.9)	1.3 (0.5, 3.5); .6
Negative DRB1*13 ($n = 127$)	0	3 (2.4)	124 (97.6)		Negative DRB1*13 ($n = 127$)	3 (2.4)	51 (40.2)	73 (57.4)	

Note: [c]*p* Value statistically significant after correction.

TABLE 7 association of rs1800629A/G in type 1 diabetes (T1D) patients positive for DRB1*07 and DRB1*03/04 compared with healthy control

Genotype model	T1D	CON	OR (95% CI)	*p* Value	Corrected *p* value
Positive DRB1*07, positive DRB1*03 /*04	24/30 (80%)	25/52 (48.1%)	4.3 (1.5, 12.3)	.006	.018
Positive DRB1*07, positive DRB1*03 /*04, positive rs1800629A/G	22/30 (73.3%)	16/25 (64%)	1.5 (0.5, 4.9)	.5	
Positive DRB1*07, rs18006629 A/G	23/27 (85.2%)	25/52 (48.1%)	11.9 (3.7, 38.3)	.0001	.0003

of chromosome 6 (p21.3) between HLA class I and class II, spanning three kilobases and containing four exons (Nedwin et al., 1985). The TNF gene is in strong LD with HLA, and the DQ and DR genes are considered as a major determinant for T1D.

A short sequence of about 200 base pairs within the untranslated region of the lymphotoxin gene, (TNF-β), was found in strong LD with various HLA haplotypes. In 1995 Monos et al. investigated microsatellites within this untranslated region and demonstrated strong LD between TNFa2b1 and DR4-B62 and between TNFa6b5 and DR4-B44 among insulin dependent diabetics (Monos et al., 1995).

Multiple SNPs identified within the TNF-α locus affect the rate of transcription and protein production in many diseases (Qidwai & Khan, 2011). Elevation of serum TNF-α was demonstrated among T1D patients regardless of age, disease duration and ethnicity (Qiao et al., 2017). Additionally, a biallelic substitution of G allele with A in rs1800629A (TNF-308A),(namely TNF2) was associated with increased TNF-α levels and was thought to affect the transcription factor binding and synthesis (Mira et al., 1999).

Association of HLA with T1D has been studied worldwide and in Saudi Arabia (El Wafai et al., 2011; Eltayeb-Elsheikh et al., 2020); however, to the best of our knowledge, no study has combined the HLA genes with the SNPs we genotyped in this study. Higher frequencies of DRB1*03 and DRB1*04 were observed among T1D patients with odd ratios 2.7 and 10.8, respectively, and are a strong risk factor; whereas, DRB1*07, DRB1*13 and DRB1*15 had lower frequencies with odd ratios 0.3, 0.3 and 0.1, respectively, and acted as protective factors against T1D. Our results confirm data of Nezar et al. in a recent Saudi study (Eltayeb-Elsheikh et al., 2020).

Stratification of DRB1*03, DRB1*04, DRB1*07 and DRB1*13 with rs1800629 and rs361525 SNPs showed a higher frequency of rs1800629A/G in DRB1*07 positive patients (odds ratio 12.5). Thus individuals positive for rs1800629A/G and DRB1*07 appeared highly susceptible to T1D compared with those negative for DRB1*07 and positive for rs1800629. It is worth noting that DRB1*07 was found at lower frequency among T1D patients when compared to healthy controls (10.7% versus 22.2% respectively) and the DRB1*07 genotype (homozygous or heterozygous) showed a lower frequency in T1D compared to DRB1*03 and DRB1*04. However, when we stratified the patients, 85.2% (23/27) were positive for both DBR1*07 and rs1800629A/G, suggesting this phenotype confers a high risk not withstanding DRB1*07's apparent protective role. Higher significant frequency of individuals DRB1*07 *positive* and DRB1*03/04 *positive* genotype was found in T1D patients when compared to healthy controls (80% versus 48.1%), although the presence of DRB1*07 allele was higher in control group. This result indicated the predominant role of DRB1*03/04 over DRB1*07 as risk factor when inherited together. In contrast, the presence of DRB1*07 *positive*, DRB1*03/04 *positive* and rs1800629A/G *positive* showed no significant variations between T1D patients and healthy control ($p = .5$). Also, we found no link between DRB1*07 and rs1800629A/G genotypes in healthy control and demonstrated similar frequencies between DRB1*07 *positive*, rs1800629A/G *positive* (48.1%) and DRB1*07 *negative*, rs1800629 *positive* (51.9%). Accordingly, the simultaneous presence of DRB1*07 *positive* and rs1800629A/G *positive* genotype was suggested as a risk factor independent from the DRB1*03/04 group in this study.

The DR3/ DR4 haplotype group is known to confer the highest susceptibility risk for T1D (Noble & Valdes, 2011). Furthermore, raised TNF-α expression levels were found to be associated with the susceptibility for autoimmune diseases (Kroeger et al., 1997). Torn et al. demonstrated that DR3-DQ2/DR4-DQ8 haplotypes and the TNF microsatellites TNFa2/2 (or TNF-α-308 A/A) were identified as risk factors for T1D in Swedish population (Törn et al., 2006).

In contrast, no LD were found between the TNF-α gene −308G/A and the Lymphotoxin-α gene +249A/G haplotypes and the predisposing HLA DRB1:DQB1 haplotypes in Bahraini T1D patients (Stayoussef et al., 2008). This contradiction of the impact of TNF and HLA may be attributed to variations in the surrounding environmental factors.

In summary, we have shown that the dominant models for TNF SNPs, namely, rs361525 and rs1800629G:361525A haplotype, increase the risk of developing T1D in this Saudi population. Whereas the DRB1*03 and DRB1*04 allele increase the risk, DRB1*07, DRB1*11, DRB1*13 and DRB1*15 protect against T1D. However, the genotype DRB1*07 with rs1800629A/G increases the risk of T1D. We speculate that 'Self' peptides derived from pancreatic islets are efficiently processed by the DRB1*07 surface receptor and are normally directed along a tolerogenic pathway, but in the presence of TNF genotype associated promoter genes linked to the SNPs rs1800629 A/G, peptide processing is redirected along a pro-inflammatory pathway triggering autoimmunity, eventually leading to T1D.

Future studies using sequence-based typing methods including HLA-DQ genotyping, supplemented by vitro and in vivo TNF-α functional studies, may clarify our findings.

ACKNOWLEDGEMENTS

This study is fully supported by King Fahad Medical City, Riyadh, Saudi Arabia. The authors would like to thank the patients and BMT donors who participated in this study.

AUTHOR CONTRIBUTIONS

AO and MH design the study. IB provide clinical information, A AlQurashi, A Al-Jurayyan, acquired information. AO and A Al-Jurayyan supervised laboratory work. AO and BB interpreted the data. AO drafted the manuscript. AO, BB and IB revised the manuscript. BB supervised the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Awad Elsid Osman https://orcid.org/0000-0003-1945-4363

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How to cite this article: Osman AE, Brema I, AlQurashi A, Al-Jurayyan A, Bradley B, Hamza MA. Association of single-nucleotide polymorphisms in tumour necrosis factor and human leukocyte antigens genes with type 1 diabetes. *Int J Immunogenet*. 2021;00:1–10. https://doi.org/10.1111/iji.12535

Publication No: 6

My contribution to this study included obtaining the IRB approval, which was under my name. I equally contributed to the research design and laboratory work supervision and interpreted the raw data. I drafted the manuscript, communicated with the other co-authors for review, and acted as the corresponding author.

Original Research Article

Single nucleotide polymorphism rs 2070874 at Interleukin-4 is associated with increased risk of type 1 diabetes mellitus independently of human leukocyte antigens

International Journal of Immunopathology and Pharmacology
Volume 36: 1–10
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DOI: 10.1177/03946320221090330
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Awad E Osman[1], Imad Brema[2], Alaa AlQurashi[3], Abdullah Al-Jurayyan[1], Benjamin Bradley[4] and Muaawia A Hamza[3]

Abstract

Introduction: Type 1 diabetes mellitus (T1DM) is characterized by autoimmune destruction of insulin-producing pancreatic beta (β-) cells. Previous studies suggested an imbalance between and pro- and anti-inflammatory cytokines exacerbates T1DM development.

Objectives: We aimed to test the hypothesis that patients with T1DM carry a higher frequency of regulatory genes associated with low levels of the anti-inflammatory cytokines interleukin-4 (IL-4), its receptor (IL-4R), and interleukin-10 (IL-10).

Methods: Accordingly, we compared frequencies of five different single nucleotide polymorphisms (SNPs) in T1DM patients and healthy controls who had been typed for HLA-DRB1, HLA-DQA1, and HLA-DQB1 genes.

Results: The frequencies of rs2070874 (IL-4) alleles C and T differed between T1DM patients and controls ($^c p = 0.0065$), as did their codominant ($^c p = 0.026$) and recessive ($^c p = 0.015$) models. Increased frequencies were observed in T1DM patients for HLA alleles: DRB1*03 (pc < 0.0013), DRB1*04 ($^c p = 0.0169$), DQA1*03 ($^c p = 0.0222$), DQA1*05 ($^c p < 0.0006$), DQB1*02 ($^c p = 0.0005$), and DQB1*06 ($^c p < 0.0005$). And lower frequencies were observed for: DRB1*07 ($^c p = 0.0078$), DRB1*11 ($^c p = 0.0013$), DRB1*13 ($^c p < 0.0364$), DRB1*15 ($^c p < 0.0013$), DQA1*01 ($^c p < 0.0006$), and DQA1*02 ($^c p = 0.0348$). Certain DRB1: DQA1: DQB1 haplotypes showed greater frequencies, including, 03:05:02 ($p < 0.0001$) and 04:03:03 ($p = 0.0017$), whereas others showed lower frequencies, including 07:02:02 ($p = 0.0032$), 11:05:03 ($p = 0.0007$), and 15:01:06 ($p = 0.0002$). Stratification for the above HLA haplotypes with rs2070874 C/C exhibited no significant differences between T1DM patients overall and controls. However, when stratified for the vulnerable HLA haplotype (03:05:02/04:03:03), young patients in whom T1DM began at ≤13 years had a higher frequency of the SNP (rs2070874 C/C); a gene associated with low IL-4 production ($p < 0.024$).

Conclusion: This study suggests that possession of the rs2070874 C/C genotype, which is associated with low production of IL-4, increases the risk of T1DM in young individuals carrying vulnerable HLA alleles/haplotypes.

[1]Pathology and Clinical Laboratory Management Department, King Fahad Medical City, Riyadh, Saudi Arabia
[2]Obesity, Endocrine and Metabolism Center, King Fahad Medical City, Riyadh, Saudi Arabia
[3]Research Center, King Fahad Medical City, Riyadh, Saudi Arabia
[4]National Centre for Biomedical Engineering Science, National University of Ireland, Galway, Ireland

Corresponding author:
Awad E Osman, Pathology and Clinical Laboratory Management Department, King Fahad Medical City, Riyadh 11525, Saudi Arabia.
Email: awadelsid@yahoo.com

Keywords
Type 1 diabetes mellitus (T1DM), single nucleotide polymorphisms (SNPs), Interleukin-10, Interleukin-4, and Interleukin-4 receptor (IL-10, IL-4, IL-4R)

Date received: 7 July 2022; accepted: 9 March 2022

Introduction

Type 1 diabetes mellitus (T1DM) is a chronic disease characterized by autoimmune destruction of insulin-producing pancreatic beta cells (β-cells), leading to insulin deficiency.[1] Between 5% and 10% of all diabetes patients worldwide have T1DM, and the incidence is estimated to be approximately 3% annually with significant geographical variations[2]; the highest incidence being in Finland (40.2/100,000),[3] and the lowest in China (0.1/100,000).[4] In 2017, Saudi Arabia was ranked eighth worldwide regarding the numbers of TIDM patients and fourth in terms of the incidence rate of the disease, which was estimated to be (33.5 per 100,000 individuals) of TIDM.[5]

T1DM occurs more frequently in genetically susceptible individuals who are subject to various environmental and epigenetic factors, as indicated by the concordance rate of T1DM in monozygotic twins, ranging between 13% and 65%.[6,7] Importantly, in twins of patients with early onset of T1DM (<24 years) the probability of progression to T1DM was 38% compared to late-onset patients (>24 years) where the probability was 6% (Redondo et al. 2001)[6]; suggesting an inverse association between the burden of genetic risk and age of onset. This is accounted for by multiple genes, including those associated with peptide processing and regulation of inflammatory reactions being implicated in pathogenesis.

Self-reactive T cells are normally heavily suppressed to prevent autoimmunity. However, in T1DM, genetic predisposition carried by alleles within the human leukocyte antigen (HLA) region leads to a breakdown in peripheral tolerance and the progressive destruction of insulin-producing β-cells.[8] In addition, genetic regulation of expression, function, and production of both pro-and anti-inflammatory cytokines plays a role in triggering the inflammatory β-cell destruction.[9-11] While the HLA-DRB1/DQA1/DQB1 genes on chromosome six encode cell surface molecules that determine which self-peptides are presented via T-cell receptors (TCRs), cytokines regulate the expansion or suppression of T-cell clones.[12,13] The interleukin-4 (IL-4) gene on chromosome five and the interleukin 10 (IL-10) gene on chromosome one encode cytokines that down-regulate inflammatory responses. However, decreased levels have been observed in both newly diagnosed T1DM patients and non-obese diabetic (NOD) mice thereby facilitating insulitis.[14,15] Conversely, genetic overexpression of IL-4 prevents insulitis and decreases the incidence of diabetes.[16] Notwithstanding these findings, others have claimed to have refuted a role for IL-4 regulator genes (Riemsnider et al. 2000).[17] IL-10 also generates feedback regulation of autoimmunity by binding to the IL-10 receptor (IL-10R) expressed on the surface of many immune cells.[15]

Multiple research studies, including our own, have investigated associations between single nucleotide polymorphisms (SNPs) linked to regulatory or structural regions of cytokine genes and T1DM.[18-21] Depending on their location within the genome, SNPs are flags highlighting possible differences in the expression, function, and/or production of cytokines within T1DM phenotypes. For example, the IL-4 SNP rs2070874 is located in the 5′-untranslated region of exon one of the IL-4 gene and, as such, could regulate the levels of cytokine production. In addition, rs1800871 and rs1800872 are located in the promoter region of the IL-10 gene, influencing messenger RNA transcription and expression.[22]

In this study, we hypothesized that in T1DM "Self-peptides" associated with β-cell are targeted by autoimmune T-cell clones, whose evolution is facilitated by an excess of pro-inflammatory cytokines (e.g., TNF-α) and/or a dearth of anti-inflammatory cytokines (e.g., IL-4 or IL-10); both scenarios facilitating β-cell destruction. Based on this hypothesis, we studied SNPs located within the IL-10 (rs1800896, rs1800871, and rs1800972), IL-4 (rs2070874), and IL-4R (rs1801275) genes in T1DM patients and controls typed for HLA - DRB1, DRA1, and DQB1 loci. Our objective was to identify significant differences in gene frequencies between patients, with age of onset above and below 13, and controls.

Materials and methods

This case-controlled cohort study of 371 individuals included 180 patients diagnosed with T1DM and 191 healthy controls. The sample size calculation was performed based on a descriptive epidemiological study on the Saudi population by Robert et al. utilizing Fisher's exact test at a level of 5% and power of 80. Patients were diagnosed according to the American Diabetes Association criteria.[23] We included individuals of Saudi origin (patients and controls) and only patients who were less than 30 years of age at the time of blood collection. Exclusion criteria

included non-Saudi patients, patients with type 2 diabetes, young patients with maturity-onset diabetes, patients with other forms of secondary diabetes, and immunocompromised patients. The healthy unrelated control group was selected randomly from the list of bone marrow transplant (BMT) donors of King Fahad Medical City (KFMC) Hospital who had no history of diabetes or other autoimmune diseases.

Guidelines of the Helsinki Declaration on Human Experimentation were implemented, and the Institutional Review Board (IRB) at KFMC approved the study. An exemption from obtaining informed consent from patients was also approved by the IRB because we used clinical samples collected from patients for hemoglobin A1c or archived DNA of BMT donors. Peripheral blood sample were collected into EDTA tubes between January 2016 and December 2019 for T1DM patients and from February 2015 to July 2020 for healthy controls. Blood samples integrity was assessed for quality purposes at the time of collection. All procedures adhered to rules and regulations of the Saudi government, the KFMC/IRB policies and procedures, and the IHC Good Clinical Practice guidelines.

A MagNa Pure Compact instrument (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) was used to extract genomic DNA according to the manufacturer's instructions, and a minimum of 20 ng/µl of DNA with 260/280 nm values between 1.6 and 2.0 was set as the standard for performing the genotype assays.

Five SNPs, including rs2070874 (IL-4), rs1800896 (IL-10), rs1800871 (IL-10), rs1800872 (IL-10), and rs1801275 (IL-4R), were investigated (Table 1). A PCR-based assay, which included sequence-specific forward and reverse primers with two TaqMan® MGB probes and dyes (VIC™ and FAM™), was used for genotyping procedures according to the manufacturer's instructions (Applied Biosystems, Foster City, CA).

For HLA genotyping, we used a sequence-specific oligonucleotide probe (SSOP) utilizing a Luminex-based method (One Lambda, San Diego, USA) to detect the HLA-DRB1, HLA-DQA1, and HLA-DQB1 genes according to the manufacturer's instructions (http://www.onelambda.com). The HLA genotyping procedure performed in our laboratory is continuously monitored for quality assurance by the College of American Pathologists (CAP).

Statistical analysis

Hardy-Weinberg equilibrium (HWE) for expected and observed allele frequencies for control data was assessed based on the χ^2 distribution (degree of freedom = 1).[24] For each SNP allele, allele frequencies and genotype models (codominant, dominant, and recessive) were derived by an algorithm based on the direct counting method utilizing SNPStats software (https://www.snpstats.net/start.htm).

Dominance is defined as the relationship between the two alleles of one gene, where the effect of the first allele (dominant) in the phenotype masks the contribution of the second allele (recessive).[25]

Codominance is defined as a form of dominance in which two different heterozygous alleles of one gene are fully expressed, and the offspring's phenotype is neither dominant nor recessive.[26]

D′ statistic and p-value tests were implemented to analyze the linkage disequilibrium (LD) between the SNPs. The D′ LD measurement ranged from 0 (no LD) to 1 (strong LD), and significant differences were indicated by a p-value less than 5%.[27] Fisher's exact test and the log of the odds (logit) for the presence of each SNP were used to specify the significant differences between patients and controls at an overall level of $p < 0.05$. HLA alleles, haplotype frequencies, and stratification with SNPs were conducted using SPSS software version 22.0. The Bonferroni correction test was used whenever it was necessary to correct multiple comparisons, and the results are presented as corrected p (^{c}p) values.

Results

Of 180 patients with T1DM, 90 (50%) were females (aged 3–28) and 90 (50%) were males (aged 16–30). The age of onset of T1DM was below 13 years in 142 (78.9%) and above 13 in 34 (18.1%) patients. In four males date of onset was unavailable. Of 191 healthy controls, 95 were female aged 1–76 and 96 males aged 1–79 (Table 2).

Single nucleotide polymorphisms

Conformation to HWE was observed for the three SNPs, rs1800871, rs1800872, and rs1801275 with expected statistical values ($p > 0.05$); however, two SNPs, rs2070874 and rs1800896, did not conform to HWE ($p < 0.05$). Among the five SNPs tested for possible LD, only rs1800896 and rs1800872 showed significant statistical values ($D' = 0.9993$ and $p < 0.001$) and were considered to exhibit high LD. No other significant values for LD were detected.

The allele frequencies and genotype models for SNPs in patients and controls are shown in Table 3. Only rs2070874 SNP (IL4) showed significant differences in terms of alleles ($^c p < 0.0065$), and codominant ($^c p < 0.026$) and recessive ($^c p < 0.0105$) genotype model frequencies furthermore these differences remained significant after Bonferroni correction.

The allele frequency for rs1800871 (IL-10) exhibited significant variation that did not remain after Bonferroni correction. No other differences were found, and interaction analysis for these SNPs revealed no difference between males and females.

Table 1. Selected SNPs for IL-10, IL-4, and IL-4R genes (https://www.snpedia.com).

SNP ID	Reference	Gene name	Chromosome location	Alleles	GMAF*
rs2070874	[18]	Interleukin-4 (IL-4)	5q31.1 (5'UTR)	C>T	0.4279
rs1800896	[27]	Interleukin-10 (IL-10)	1q32.1 (intergenic/intragenic)	A>G	0.3026
rs1800871	[27]	Interleukin-10 (IL-10)	1q32.1 (intron)	G>A	0.4086
rs1800872	[27]	Interleukin-10 (IL-10)	1q32.1 (intron)	A>C	0.4091
rs1801275	[18]	Interleukin-4 receptor (IL-4R)	16p12.1 (intron)	A>G	0.3453

*Global Minor Allele Frequency (GMAF) is frequency of the minor allele as reported in a default global population.

Table 2. Demographic and clinical data for T1DM patients and Controls.

		T1DM patients (no. = 180)			
	Sex	All patients (no. = 180)	Onset <13 years (no. = 142)	Onset ≥13 years (no. = 34)	Controls (no. = 191)
Totals	Females	90	75	15	95
	Male	90	67*	19*	96
Age range (yrs.)	Female	3–28	1–12	13–27	1–76
	Male	16–30	0.8–12	13–32	1–79
Age median (yrs.)	Female	15.2	9.0	15.0	25
	Male	22.7	8.0	15.0	25
Onset of T1DM (mean yrs.)	Female	9.7	8.0	16.3	
	Male	8.6	7.0	17.2	
Duration of T1DM (mean yrs.)	Female	9.7	9.9	8.7	
	Male	8.9	8.7	8.4	

*In four cases date of onset was unavailable.

HLA-DRB1

Thirteen alleles for DRB1 were detected (Table 4), and DRB1*03 (^{c}p = 0.0013) and DRB1*04 (^{c}p = 0.0169) were observed at higher frequencies in patients than in controls, representing a total of 249 (71.5%) out of 348 DRB1 alleles among T1DM patients. In contrast, DRB1*07 (^{c}p = 0.0078), DRB1*11 (^{c}p = 0.0013), DRB1*13 (^{c}p = 0.0364), and DRB*15 (^{c}p = 0.0013) were at greater frequencies in healthy controls with a total of 200 (52.3%) out of 382 alleles among the control samples. None of the remaining alleles differed between T1DM and controls.

HLA-DQA1

Six alleles for DQA1 were observed (Table 5). DQA1*03 (^{c}p = 0.0222) and DQA1*05 (^{c}p = 0.0006) were present at higher frequencies in T1DM patients, whereas DQA1*01 (^{c}p = 0.0006) and DQA1*02 (^{c}p = 0.0348) were found at higher frequencies in healthy controls. DQA1*04 and DQA1*06 did not differ between the two groups.

HLA-DQB1

Five alleles for DQB1 were detected (Table 6). DQB1*02 was increased in frequency in T1DM patients compared with healthy controls (^{c}p = 0.0005), whereas DQB1*06 was found more frequently in controls (^{c}p = 0.0005). HLA-DQB1*05 did not reach significance after the Bonferroni correction, whereas DQB1*03 and DQB1*04 did not show any differences.

HLA-DRB1:DQA1: DQB1 haplotypes

Fifty-one different haplotypes for DRB1:DQA1: DQB1 were observed (Table 7), but only five exhibited frequencies greater than 5% and differed between patients and controls. Specifically, 03:05:02 ($p < 0.0001$) and 04:03:03 ($p = 0.0017$) exhibited greater frequencies in patients compared with controls; whereas 07:02:02 ($p = 0.0032$), 11:05:03 ($p = 0.0007$) and 15:01:06 ($p = 0.0002$) were higher in healthy controls.

Stratification by SNP and HLA-DRB1 genotype

According to previously published studies,[28-31] we classified T1DM subjects and controls according to genotype models based on the presence or absence of DRB1*03/04 or DRB1*07/13, and presence or absence of rs2070874 C/C. We report that after controlling for HLA the

Table 3. Association between SNPs and susceptibility to T1DM.

SNP ID	SNP model	Genotype	T1DM (total alleles = 360)	Controls (total alleles = 382)	OR (95% CI)	p-value	Corrected [c]p value
rs2070874 (IL-4)	Alleles	C	308 (85.6%)	291 (79%)	1.8 (1.3, 2.7)	0.0013	0.0065
		T*	52 (14.4%)	91 (21%)			
	Codominant	C/C	131 (72.8%)	116 (60.7%)	1.0	1.0	
		C/T	46 (25.6%)	59 (30.9%)	1.4 (0.9, 2.3)	0.1	
		T/T	3 (1.7%)	16 (8.4)%	6.0 (1.7, 21.2)	0.0052	0.026
	Dominant	C/C versus C/T+T/T			1.7 (1.7, 21.5)	0.23	
	Recessive	C/C+C/T versus T/T			5.4 (1.5, 18)	0.0021	0.015
rs1800896 (IL-10)	Alleles	T	215 (60%)	240 (62%)	0.9 (0.7, 1.2)	0.64	
		C*	145 (40%)	144 (38%)			
	Codominant	T/T	65 (36.1%)	81 (43.2%)	1.0	1.0	
		T/C	85 (47.2%)	75 (39.9%)	0.7 (0.5, 1.1)	0.13	
		C/C	30 (16.7%)	32 (17%)	0.9 (0.5,1.8)	0.8	
	Dominant	T/T versus T/C+C/C			0.8 (0.5, 1.6)	0.6	
	Recessive	T/T+T/C versus C/C			1.1 (0.6, 1.7)	0.9	
rs1800871 (IL-10)	Alleles	G	290 (81%)	156 (82%)	1.5 (1.1, 2.1)	0.033	0.165
		A*	70 (19%)	34 (18%)			
	Codominant	G/G	110 (61.1%)	102 (54.3%)	1.0	1.0	
		G/A	70 (38.9%)	73 (38.8%)	1.2 (0.7,1.7)	0.5	
		A/A	0%	13 (6.9%)	1.0	1.0	
	Dominant	G/G versus A/G+G/G			1.3 (0.9, 2.0)	0.18	
	Recessive	G/G+A/G versus A/A			1.0	1.0	
rs1800872 (IL-10)	Alleles	G	264 (73%)	267 (70%)	1.2 (0.9, 1.6)	0.3	
		T*	96 (27%)	115 (30%)			
	Codominant	G/G	97 (53%)	90 (48.1%)	1.0	1.0	
		T/G	70 (38.9%)	82 (43.9%)	1.3 (0.8, 1.9)	0.2	
		T/T	13 (7.2%)	15 (8%)	1.2 (0.6, 2.8)	0.4	
	Dominant	G/G versus T/G+T/T			1.3 (0.8, 1.9)	0.3	
	Recessive	G/G+T/G versus T/T			1.1 (0.5, 2.4)	0.8	
rs1801275 (IL-4R)	Alleles	A	286 (79%)	274 (74%)	1.4 (0.9,1.9)	0.08	
		G*	74 (21%)	96 (26%)			
	Codominant	A/A	114 (63.3%)	100 (55.2%)	1.0	1.0	
		A/G	58 (32.2%)	66 (36.5%)	1.3 (0.8, 2.0)	0.2	
		G/G	8 (4.4%)	15 (8.3%)	2.1 (0.8, 5.2)	0.1	
	Dominant	A/A versus A/G+G/G			1.4 (0.9, 2.4)	0.12	
	Recessive	A/A+A/G versus G/G			1.9 (0.8, 4.7)	0.13	

*The second most common allele occurring in the population.

rs2070874 C/C association with T1DM risk was no longer significant (Table 8).

However, when we divided patients into two groups, based on the age of onset of T1DM,[32] we demonstrated a significant increase in the frequency of rs2070874 C/C in patients with onset age ≤13 years ($p \leq 0.0027$), and no increase in patients with onset >13 years (Table 9).

Stratification by SNP and HLA-DRB: DQA1: DQB1 haplotype

We classified T1DM subjects and controls according to genotype models based on the presence or absence of HLA-DRB: DQA1:DQB1 haplotypes, and the presence or absence of the rs2070874 C/C genes and found no significant differences between cohorts overall ($p = 0.09$). See Table 10 for details.

However, further stratification based on the date of onset of T1DM showed patients with early-onset disease (≤13 years) had a greater frequency of the genotype, 03:05:02/04:03:03; rs2070874 C/C, ($p \leq 0.024$). There was no difference between the groups for the presumed protective genotypes, 07: 02:02/ 11: 05:03 who carried rs2070874 C/C (Table 11).

Discussion

Our findings indicate genes associated with low production of IL-4 facilitate the early onset of T1DM in individuals

Table 4. Frequency distribution and association of HLA-DRB1 alleles in T1DM and controls.

DRB1	T1DM (n = 348)	Controls (n = 382)	OR (95%CI)	p Value	^{c}p value
*01	8 (2.3)	14 (3.7)	0.5 (0.2–1.3)	0.1624	
*03	142 (40.8)	56 (14.7)	4.0 (2.8–5.7)	0.0001	0.0013
*04	107 (30.7)	78 (20.4)	1.7 (1.2–2.4)	0.0013	0.0169
*07	38 (10.9)	78 (20.4)	0.5 (0.3–0.7)	0.0006	0.0078
*08	2 (0.6)	5 (1.3)	n/a	n/a	n/a
*09	2 (0.6)	2 (0.5)	n/a	n/a	n/a
*10	6 (1.7)	10 (2.6)	n/a	n/a	n/a
*11	5 (1.4)	34 (8.9)	0.2 (0.06–0.4)	0.0001	0.0013
*12	n/a	1 (0.3)	n/a	n/a	n/a
*13	24 (6.9)	53 (13.9)	0.5 (0.3–0.8)	0.0028	0.0364
*14	1 (0.3)	5 (1.3)	n/a	n/a	n/a
*15	4 (1.2)	36 (9.4)	0.1 (0.04 0.3)	<0.0001	<0.0013
*16	9 (2.6)	10 (2.6)	n/a	n/a	n/a

Table 5. Frequency distribution and association of HLA-DQA1 alleles in T1DM and controls.

DQA1	T1DM (n = 358)	Controls (n = 370)	OR (95%CI)	p Value	^{c}p value
*01	58 (16.2)	132 (35.7)	0.3 (0.2 0.5)	0.0001	0.0006
*02	46 (12.8)	76 (20.5)	0.6 (0.4–0.8)	0.0058	0.0348
*03	106 (29.6)	75 (20.3)	1.7 (1.2–2.3)	0.0037	0.0222
*04	2 (0.6)	2 (0.5)	n/a	n/a	n/a
*05	146 (40.8)	84 (22.7)	2.3 (1.7 3.2)	0.0001	0.0006
*06	0	1 (0.03)	n/a	n/a	n/a

Table 6. Frequency distribution and association of HLA-DQB1 alleles in T1DM and controls.

DQB1	T1DM (n = 358)	Controls (n = 382)	OR (95%CI)	p Value	^{c}p value
*02	183 (51.1)	127 (33.3)	2.1 (1.6–2.8)	<0.0001	<0.0005
*03	111 (31)	110 (28.8)	0.9 (0.7–1.2)	0.5	n/a
*04	4 (1.1)	7 (1.8)	n/a	n/a	n/a
*05	31 (8.7)	54 (14.1)	0.6 (0.4 0.9)	0.0268	n/a
*06	29 (8.1)	84 (22.0)	0.3 (0.2–0.5)	<0.0001	<0.0005

Table 7. Frequency distribution and association of DRB1: DQA1: DQB1 haplotypes in T1DM and controls.

Haplotype	Case (n = 344)	CON (n = 342)	OR (95%CI)	p Value
3.05.02	134 (40.2)	46 (13.5)	4.1 (2.8114–5.9967)	<0.0001
4.03.03	94 (28.3)	59 (17.3)	1.8 (1.2491–2.6040)	0.0017
7.02.02	32 (9.6)	58 (17)	0.5 (0.3157–0.7932)	0.0032
1.01.05	7 (2.1)	15 (4.4)		
13.01.06	22 (6.6)	35 (10.2)	0.6 (0.3438–1.0447)	0.0709
10.01.05	6 (1.8)	10 (2.9)		
15.01.05	9 (2.7)	2 (0.6)		
11.05.03	3 (0.9)	23 (6.7)	0.1 (0.0363–0.4103)	0.0007
15.01.06	3 (0.9)	28 (8.2)	0.1 (0.0299–0.3298)	0.0002
4.03.02	6 (1.8)	4 (1.2)		
Other	17 (5.1)	63 (18.3)		

Table 8. Stratification rs2070874 C/C genotype with HLA- DRB1.

Genotype models[1]					
DRB1*03/04	DRB1*07/13	Rs2070874 C/C	T1DM no. (%)	Controls no. (%)	OR (95%CI); p value
+	-	+	86 (72.9%)	44 (62.9%)	1.2 (0.7, 1.9); 0.5
+	-	-	32 (27.1%)	26 (37.1%)	
-	+	+	8 (61.5%)	38 (63.3%)	0.9 (0.3, 3.2); 0.9
-	+	-	5 (38.5%)	22 (36.7%)	
Totals			131	130	

[1]DRB1*03 or DRB1*04 with DRB1*07 or DRB1*13 individuals were excluded.

Table 9. Stratification of rs2070874 C/C genotypes with HLA - DRB1 by age of onset of T1DM.

	Genotype models[1]					
Age of onset. (yrs.)	DRB1*03/04	DRB1*07/13	rs2070874 C/C	T1DM no. (%)	Controls. No. (%)	OR (95%CI); p value
>13	+	-	+	**77 (78.6%)**	44 (62.9%)	**2.2 (1.1, 4.3); 0.027**
	+	-	-	21 (21.4%)	26 (37.1%)	
	-	+	+	6 (66.7%)	38 (63.3%)	1.2 (0.3, 5.1); 1.2
	-	+	-	3 (33.3)	22 (36.7%)	
Totals				107	130	
≤13	+	-	+	9 (45%)	44 (62.9%)	0.5 (0.2,1.3); 0.2
	+	-	-	11 (55%)	26 (37.1%)	
	-	+	+	2 (50%)	38 (63.3%)	0.6 (0.1, 4.4); 0.6
	-	+	-	2 (50%)	22 (36.7%)	
Totals				24	130	

[1]DRB1*03 or DRB1*04 with DRB1*07 or DRB1*13 individuals were excluded.

Table 10. Stratification of rs2070874 C/C genotype with HLA - DRB1: DQA1; DQB1 haplotypes.

Genotype model (DRB1: DQA1: DQB1 and SNP)[1]					
03:05:02/04:03:03	07:02:02/11:05:03	rs2070874 C/C	T1DM. No. (%)	Controls. No. (%)	OR (95%CI); p value
+	-	+	98 (74.8%)	43 (63.2%)	1.7 (0.9, 3.2); 0.09
+	-	-	33 (25.2%)	25 (36.8%)	
-	+	+	7 (58.3%)	31 (67.4%)	0.7 (0.2, 2.5); 0.6
-	+	-	5 (41.7%)	15 (32.6%)	
Totals			143	114	

[1]DRB103:05:02 or 04:03:03 with 07:02:02 or 11:05:03 DRB1 individuals were excluded.

with vulnerable HLA genotypes. The association between HLA and T1DM has been extensively studied, but the involvement of IL-4 has been unclear; and whereas low IL-4 was implicated in NOD mice (Shoda et al. 2004)[33] and patients with T1DM (Hagar and Zohreh 2016),[34] others claimed to have disproved a role for IL-4 regulatory genes (Reimsnider et al. 2000).[17] But no account was taken of the age of onset of T1DM in their analysis. Our data are consistent with and add to, the observation that early-onset T1DM is associated with a higher twin-concordance rate in younger patients due to an increased burden of non-HLA genes controlling B and T-cell development (Inshaw et al. 2020, Redondo et al., 2001and 2020).[35,36]

The well-established role of HLA Class II molecules is to process peptides external to the cell surface and present them to T-helper cells to generate an immune response, or to T-regulator cells to generate tolerance. In this context, our data imply the HLA haplotype most strongly associated with T1DM (03:05:02/04:03:03) presents a unique array of β-cell derived self-peptides to T-helper cells triggering autoimmunity. Conversely, the HLA haplotype most strongly associated with protection against T1DM (07:02:02/11:05:03) produces β-cell self-peptides triggering tolerance. Thus

Table 11. Stratification of rs2070874 C/C with HLA - DRB1: DQA1; DQB1 haplotypes, according to age of onset of T1DM.

	Genotype models[1]					
Age of onset (yrs.)	03:05:02/04:03:03	07:02:02/11:05:03	rs2070874 C/C	T1DM no.(%)	Controls. No. (%)	OR (95%CI); p value
≤13	+	-	+	83 (79%)	43 (63.2%)	2.2 (1.1, 4.3); 0.024
	+	-	-	22 (21%)	25 (36.8%)	
	-	+	+	5 (71.4%)	31 (67.4%)	1.2 (0.2, 6.7); 0.8
	-	+	-	2 (28.6%)	15 (32.6%)	
Totals				112	114	
>13	+	-	+	15 (55.6%)	43 (63.2%)	0.7 (0.3, 1.8); 0.7
	+	-	-	12 (44.4%)	25 (36.8%)	
	-	+	+	1 (25%)	31 (67.4%)	0.2 (0.02, 1.7); 0.12
	-	+	-	3 (75%)	15 (32.6%)	
Totals				31	114	

[1]DRB103:05:02 or 04:03:03 with 07:02:02 or 11:05:03 DRB1 individuals were excluded.

immunity, or tolerance, to β-cell peptides is dictated initially by the HLA haplotype, and subsequent evolution of responses is directed by pro or anti-inflammatory cytokines.

Within the context of the simplistic paradigm whereby autoimmune diseases are facilitated by an imbalance between anti-inflammatory (IL-4, IL-10, IL-13) and pro-inflammatory cytokines (TNF- α, IFN- γ) low levels of the former and high levels of the latter facilitate T1DM onset (Moudgil and Chouby 2011)[39]. Both our former finding (Osman et al. 2021),[37] that T1DM was associated with high TNF producer genes (rs361525), and current findings of low IL-4 producer genes are consistent with this model.

As demonstrated by next-generation genotyping array technology, several other genes are associated with aggressive early-onset T1DM in children under 7 years.[36] These include genes expressed in β-cells (GLIS3) and others affecting immune function via B-cell, T-cell, and Thymus development (IL2-RA, IL-10, IKZF3, THEMIS, CTSH). In Inshaw et al.'s study SNPs detecting IL-10 differed from those in ours and may account for our negative finding of an association between T1DM and IL-10. Furthermore, no SNPs linked to IL-4 and IL-4R, in particular the rs2070874, were used in their study. Hence, our results are concordant with their observation that the number of susceptibility genes is inversely correlated with the age of onset of T1DM.

Selection of controls in our study deserves comment in so far as bone marrow volunteer donors are selected for health and absence of autoimmune diseases; a feature that may have increased the differences between them and patients. Furthermore, they were not strictly matched for age range; the T1DM patients being 3–30 and controls ranging from 1 to 79 years. These differences may have introduced artefacts into the comparison between the groups and be addressed in future studies with larger numbers designed to take account of the entire age range of disease onset. Such studies will allow cumulative effects of susceptibility genes to be compared between the young and elderly. The ultimate application of this work is to develop early prophylactic screening of vulnerable individuals allowing intervention designed to arrest disease progression.

Conclusions

In this study, young T1DM patients (age of onset ≤13 years old) carrying vulnerable HLA genes had a higher frequency of the IL-4 "low producer" linked SNP rs2070874 C/C, consistent with the view that low levels of anti-inflammatory cytokines facilitate autoimmunity to β-cells.

Acknowledgements

This study is fully supported by King Fahad Medical City, Riyadh, Saudi Arabia. The authors would like to thank the patients and BMT donors who participated in this study.

Author's Contribution

AO and MH designed the study. IB provided clinical information, and A AlQurashi and A Al-Jurayyan acquired information. AO and A Al-Jurayyan supervised laboratory work. AO and BB interpreted the data. AO drafted the manuscript. AO, BB, and IB revised the manuscript. BB supervised the manuscript and critically added comments.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

This study was completely funded by King Fahad Medical City.

Ethics approval

Ethical approval for this study was obtained from Institutional Review Board (IRB) at King Fahad Medical City, Riyadh, Saudi Arabia (IRB Log No: 16 054)

Informed consent

Informed consent was not sought for the present study because we used leftover clinical blood specimens in this project and approval from our IRB was obtained.

ORCID iD

Awad E Osman https://orcid.org/0000-0003-1945-4363

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Chapter 4. Conclusion

In conclusion, I have characterized HLA, KIRs and STRs genes in the Saudi population to pave the way for understanding their distributions among this population. Allele frequencies were determined and compared to other national and international studies, and as a result, I have detected similarities and variations. LD was detected between specific markers and haplotypes for HLA loci and KIRs genes. I have implemented PCA analysis in specific HLA alleles or MDS plots in STRs markers on data from Arab-related ethnic groups and other unrelated populations; the clustering of Saudis was closer to Arabs than other unrelated populations. However, PCA on the KIR data has shown clustering of the Arab populations near the middle of the plot between Africans and Asians, supporting the dispersion out of Africa theory (**Publication 2**).

Various informative results were detected in the studies I have conducted on T1DM; in the study of KIRs and their corresponding HLA ligand, there was no significant difference between patients and controls for all 16 KIRs gene frequencies but, the differences were detected in C1C1, C2C2 and Bw4 ligands. Also, KIR2DL2-C1C1, KIR2DS2-C1C1, KIR2DL3-C1C1 and KIR2DS1-C1C1 significantly differed between the two groups. In studies of SNPs within selected pro- and anti-inflammatory cytokines in conjunction with HLA in T1DM, I confirmed that HLA DRB1*03 and DRB1*04 are risk alleles for T1DM, and that DRB1*07, DRB1*11 and DRB1*13 are protective against T1DM. The dominant model for rs361525 (TNF) and rs1800629G.361525A haplotype also increases the risk. In contrast, individuals positive for both DRB1*07 and rs1800629A/G were found to be at risk of T1DM. On the other hand, the carriers of the high-risk HLA genes in the presence of rs2070874 C/C (IL-4) genotype showed increased risk of T1DM which is consistent with the notion that low levels of this anti-inflammatory cytokines induce autoimmunity to beta cells in children under 13 years old.

The presented publications are one-centre-based studies, the sample sizes were not large enough, and we utilized low-resolution methods for HLA and KIRs genotyping, which were considered limitations for these research works. Also, there may not have been enough correction for multiple testing when a number of different genes are genotyped, as in **Publications 5 and 6**. However, further analysis which has not been published but is included in the commentary suggests that the findings for rs361525 (TNF-alpha) and

rs2070874 (IL4) as T1DM risk factors in these publications are still significant when a more global correction is performed. Further genetic studies in larger samples and high-resolution methods for KIRs and HLA genotypes are recommended to better understand the interrelationship between the different ethnic groups and sort out complex ambiguities in specific alleles. These should be used in genome-wide approaches instead of further candidate gene studies. Also, in vitro and in vivo functional assays are recommended for evaluating the practical significance of particular genes and their associations with diseases. However, the work has had a direct impact on the day-to-day management of patients in the clinic.

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