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Original Article

High Variability in HLA-DRB1*03, a Predisposing Allele in Acute Lymphoblastic Leukemia

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Abstract

Acute lymphoblastic leukemia (ALL) remains a significant health concern, particularly in children, with genetic predisposition playing a crucial role in its etiology. Among the predisposing HLA Class II alleles, –DRB1*03 has emerged as a notable candidate associated with increased susceptibility to ALL. This study aims to investigate the extent of variability within the HLA-DRB1 alleles as genetic biomarkers and its implications in ALL pathogenesis. Through methods of polymerase chain reaction–sequence-specific oligonucleotides (PCR-SSO) and sequence-based typing (SBT) analysis, our data revealed HLA-DRB1*16 as another genetic risk and HLA-DRB1*07 and HLA-DRB1*12 alleles as protective alleles in ALL patients. Further sequencing demonstrates a remarkable diversity in the HLA-DRB1*03 allele in ALL patients but none among the HLA-DRB1*16 alleles compared to normal samples. Our findings confirmed the association of HLA-DRB1 alleles with ALL and shed light on SNPs or mutations in the risk alleles. Genetic variability in HLA-DRB1 alleles is a significant factor to consider in improving outcomes in immunotherapy.

1. INTRODUCTION

Human leukocyte antigen (HLA), the counterpart of the Major Histocompatibility Complex (MHC) in humans, is critical in immune surveillance presenting diverse antigens for specific activation of the T cell community. Each HLA molecule has an invariant and variable region. The CD8/CD4 molecule on T cells binds the invariant region while the variable region which is very selective and binds with high affinity only to certain peptides of MHC class I or MHC class II amino acids, is known as the peptide binding groove (1,2). The high variability in antigen binding potential of HLA molecules makes it possible to recognize the myriad

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pathogens out there. Expression of self-peptides on MHC on the other hand, inhibits immune activation. Variability in antigen binding is due to polymorphism of the HLA genes, considered the most polymorphic set of genes seen in higher vertebrates. The HLA genes are located at the short arm of chromosome 6 (6p21.3) encoded by 3.6 Mb of genes and constitute about 0.1% of the genome (3).

HLA carries over 220 genes or loci of diverse function (1,3) and is categorized as Class I, Class II, and Class III. At least 40% of the 128 genes predicted to be expressed have immune system function (4). MHC genes and molecules are polygenic, being coded by several genes and inherited as haplotypes from paternal and maternal chromosomes. High polymorphism from the many allelic forms that exist in the population for each MHC gene makes it unlikely for two random individuals to express identical sets of genes (5). Nevertheless, universal patterns of HLA gene differences and diversity among different populations are unique to geographical regions. This is confirmed by the latest evidence of a study involving a large number of DNA sequences (n=2,062) outlined for seven HLA genes in more than 20 thousand individuals in populations worldwide. The IPD-IMGT/HLA database now contains over 35,000 alleles for HLA and is considered hyper polymorphic (6).

HLA and its genetic diversity have been associated with disease susceptibility, neutral or protective in various immunological diseases. Given the polymorphic nature of HLA antigens which influences their interactions, it is likely that these associations occur, affecting their ability to effectively present certain viruses or bacteria, either poorly or effectively. Amino acid residues and their positions in the HLA protein sequence have been linked to both susceptibility and resistance effects (7). Numerous studies have showcased notable connections between HLA class I and II molecules and vulnerability to various complex infectious diseases, spanning from viral and bacterial to parasitic infections. Though inconclusive there is an urgent need to conduct large-scale population studies to determine the true impact on susceptibility to infectious diseases (8).

Reports of genetic connections between HLA and autoimmune diseases date back to the early 1970s. Rheumatoid arthritis and type I diabetes have strong associations with HLA class II molecules. Shared epitope and self-peptides are two mechanisms proposed for diseases, respectively (9). There are even mutual HLA alleles. There is also a suggestion that infections heighten the risk of autoimmune conditions in both autoimmune and infectious diseases (7). The immune system may fail to recognize the pathogen as foreign and initiate an immune response due to its close molecular similarity to the pathogen (10).

The close association between HLA and cancers is undeniable. Descriptions of alterations in HLA expression on tumor cells predated the discovery of the role of MHC molecules in antigen presentation (11). Genetic changes acquired by tumor cells gain the ability to replicate and survive. In addition, genes of the immune system may be affected changing the proficiency of immune cells to recognize and eradicate abnormal cells. Immune escape is the principle undertaken by tumor cells to evade immunosurveillance and prevail (12,13). In a recent large population study on the susceptibility of 25 cancers and the diversity of HLA, the report was linked to a reduced risk of lung cancer, non-Hodgkin lymphoma, and Hodgkin lymphoma indicating that HLA may have protective effects against these cancers. These data were analyzed using HLA heterozygosity and HLA evolutionary divergence (HED) (11). Using an HLA-Cancer Protection/Susceptibility (P/S) estimation based on 127 alleles in Class I (A, B, C) and Class II (DPB1, DQB1, DRB1), it was reported that in 30 cancers from 14 Continental Western Europe (CWE) countries, 127 alleles common to 9 countries were broadly clustered into three different types of cancers, including digestive-skin-cervical, reproductive and endocrine systems, and brain and androgen-associated cancers. This implicated not only the risk of HLA but shared heritability in different types of cancers (14).

Abnormal expression of HLA class I molecules can occur at the genetic, epigenetic, transcriptional, or post-transcriptional level, resulting from structural alterations in the genes encoding classical HLA antigens or components of the HLA antigen processing machinery (APM) (15–17). Somatic alterations of the HLA loci rearrange the original germline configuration and reduce variability, ultimately enabling a mechanism of immune escape that promotes tumor growth and resistance to immune therapy. Somatic changes in HLA class I genes were identified in head and neck cancer, squamous cell lung cancer, stomach adenocarcinoma, and diffuse large B-cell lymphoma. Mutations occur in the invariant (exon 4) region of the α3 domain of the HLA protein, which binds the CD8 co-receptor of T cells, resulting in the loss of T cell recognition and subsequently a loss of immune reactivity. Mutations affecting peptide binding domains of the HLA molecule are also frequent affecting antigen presentation and T cell activation. The recurrence of mutations indicates that these 'hotspot' sites were positively selected (18). Copy-neutral loss in heterozygosity and focal deletions in class I HLA genes were the immune evasion mechanisms and late relapse in acute myeloid leukemia cases after allogeneic hematopoietic cell transplantation and prolonged alloreactive T cell control

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(19). Disruption of antigen presentation including down-regulation of expression favors immune escape and manifestation of cancer.

Leukemia is another cancer due to the malignant transformation of hematopoietic progenitor cells in the bone marrow. Factors that influence immune escape in leukemia are under investigation. Epidemiologic studies of acute and chronic leukemia in some populations strongly suggest HLA as a risk factor from an increase in homozygosis in certain HLA alleles frequency, which also can be inheritable basis. The review on HLA association with leukemia elucidated the significance of HLA polymorphism as genetic marker utilization in the diagnosis and pathogenesis of the disease (20). HLA association with ALL has been reported globally including among Caucasian (21–23), Iranian (24,25), Brazilian (26,27), and Chinese (28).

The high degree of allelic conservation in diverse ethnic groups suggests an important need to investigate the HLA disease association in specific populations and backgrounds to help understand the mechanism of the disease and develop possible treatments. Furthermore, the presence of somatic mutations in HLA genes is worthy of investigation in its role in promoting immune escape. In this study, pre-B acute lymphoblastic leukemia (pre-B ALL) in the Malay population was investigated for HLA-DRB1 diversity.

2. MATERIALS AND METHODS

2.1. Peripheral blood and bone marrow sample collection

Forty-two peripheral blood and excess bone marrow samples from precursor-B acute lymphoblastic leukemia (pre-B ALL) from routine tests were obtained from the Hematology Unit, Hospital Kuala Lumpur, Malaysia. The inclusion criteria for this study were of Malay ethnic group, of all ages, and both genders, and diagnosed as pre-B ALL by morphology, immunophenotyping, and cytochemistry. The diagnosis of patients was based on the reports released from the Hematology laboratory, HKL. Peripheral blood from healthy donors (n=4) representing normal controls for sequencing analysis were obtained and recruited from the students and staff from the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. Data on HLA types from healthy people (n=1445) were obtained from Institute for Medical Research Kuala Lumpur as reported by Dhaliwal, et al (2007) (29).

2.2. DNA extraction

Peripheral blood or bone marrow samples were isolated for mononuclear cells using the Ficoll-Paque PLUS solution (GE Healthcare) according to the manufacturer's protocol. DNA extraction was done according to Miller, et al. (1988) (30) following the salting out procedure. A minimum of 4.0 X 10^6 frozen mononuclear cells was recovered, washed, and pelleted before resuspension in lysis buffer containing Proteinase K (20 mg/mL) (Finnzymes, Finland, Lot No: 11161). After overnight digestion (37°C) in a rotary hybridization oven, 6M NaCl was added to precipitate protein and dissolve DNA. DNA was precipitated in absolute ice-cold ethanol (Merck, USA) and dissolved in TE buffer.

2.3. HLA-DR typing by PCR-SSO

The HLA-DR typing was performed using polymerase chain reaction-sequence specific oligonucleotides (PCR-SSO, low resolution) (LIFECODES HLA-DRB Typing Kit, Cat No: 628710-50, Gen-Probe, Inc. USA) by Luminex™ *x*MAP technology. DNA was typed using Gen-Probe according to the manufacturer's instructions with some alterations.

2.4 PCR amplification

DNA (20 ng/mL) in Master Mix (MX-DRG) was amplified with Taq Polymerase (5 U/µL GoTaq® Flexi-Promega, USA) in PCR strip tubes (Applied Biosystems, Inc. USA; ThermoScientific, USA). For negative control reactions, DNA was substituted with sterile distilled water. Samples run was performed in a PCR thermocycler (G-Storm, United Kingdom; Bio-Rad, USA) with PCR cycles; 95^{0}C for 5 min: 1 cycle; 95^{0}C for 30 s, 60^{0}C for 45 s, 72^{0}C for 45 s: 8 cycles; 95^{0}C for 30 s, 63^{0}C for 45 s, 72^{0}C for 45 s: 32 cycles and 72^{0}C for 15 min: 1 cycle. PCR product separation was performed in 2% agarose (Amresco, USA) in a gel electrophoresis apparatus (BioRad, USA) at 150 V for 45 minutes in 1X TE buffer, to confirm the presence of amplified DNA before proceeding to probe hybridization. A 100 bp DNA ladder (Promega, USA) was included in each run.

2.5. Hybridization of probes

The preparation of HLA-DRB probes and hybridization method was according to the manufacturer's procedure with some modifications. A 2.5 µL of PCR products was mixed with 7.5 µL HLA-DRB probes in 96-well PCR plates (Costar®, Fisher Scientific, USA) and hybridized on a PCR thermocycler (97^{0}C for 5 min; 47^{0}C for 30 min; 56^{0}C for 10 min and 56^{0}C hold). Streptavidin-phycoerythrin, SA-PE (Gen-Probe Inc., USA) was added when the program reached the end hold stage. Samples were analyzed using Luminex

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*x*MAP® Technology with Luminex 100 IS Software and QuickType for LifeMatch 2.6.1. software for Gen-Probe analysis.

2.6. HLA-DRB1 sequence-based typing

Targeted sequencing was performed on pre-B ALL samples with the HLA-DRB1*03 and HLA-DRB1*16 which were identified as risk alleles among Malays. Primers were designed to amplify the polymorphic exon 2 region, forward primer residing at 480 bp of intron 1 and reverse primer 50 bp of intron 2. The sense/forward primer used for HLA-DRB1*03 (DR17) (|1-RB5-2) was 5' CAG GCA CAA GGT CAG CAC TAT 3' located at intron 1 while the antisense/reverse primer was (|I2-RB28) 5' ACA CAC ACA CTC AGA TTC CCA 3', located at intron 2. The PCR product expected was approximately 760 bp for HLA-DRB1*03 (DR17) and 720 bp for HLA-DRB1*16 including the sequences of the polymorphic region of exon 2. HLA-DRB1*16 sense/forward primer was (|1RB-3) 5' AGC ACT AAG GAA GGG TTC AG 3' but shared the antisense/reverse primer as –DRB1*03 (|I2-RB28). Primers were taken from Kotsch, et al. (1998) (31) except the forward/sense primer of 1-RB5-2 which was designed with the free online Primer3 (v. 0.4.0) software. Primers were purchased from NextGene Scientific, Malaysia, and AIT Biotech, Singapore. The similarity search for DNA sequences and primers was conducted with the Basic Local Alignment Search Tool (BLAST), from the NCBI database assessable at http://blast.ncbi.nlm.nih.gov and IMGT/HLA database at http://www.ebi.ac.uk/imgt/hla for sequences of specific alleles.

2.7. PCR amplification and gel electrophoresis

The PCR mix consisted of 1X Taq Buffer, 0.16 mM $MgCl_2$, 40 mM of dNTPs, 1.0 U Taq Polymerase (all from Fermentas, Lithuania), 0.08 mM of forward and reverse primers in a 25 µL PCR reaction. PCR cycles consisted of an initial denaturation step at 94^{0}C for 2 minutes, 10 cycles of denaturation at 94^{0}C for 30 seconds, annealing at 65^{0}C for 50 seconds, and extension at 72^{0}C for 50 seconds followed by 30 cycles denaturation at 94^{0}Cfor 30 seconds, annealing temperature of 62^{0}C for 30 seconds and an extension at 72^{0}C for 30 seconds, and a final extension at 72^{0}C for 10 minutes (Eppendorf, Mastercycler ep Gradient S, Hamburg, Germany). Amplified products were viewed on 1.5% agarose gel stained with ethidium bromide (0.2 mg/mL).

2.8 Purification of PCR products and sequencing

Purification of PCR products for sequencing-based typing (SBT) was done using GeneALL®Expin ™ Combo GP DNA purification kit (Hamburg, Germany) according to the manufacturer's procedure. DNA was eluted into nuclease-free water. Sequencing of target exon 2 of HLA-DRB1 was outsourced to sequencing service providers (NextGene Scientific, Malaysia, and AIT Biotech Pte Ltd, Singapore). The chromatograms of DNA sequences of risk alleles were viewed with FinchTV 1.4.0 software and DNA alignments were done by CLC Sequence Viewer 6.0. software. DNA sequences that were aligned were checked for quality scores by Sequence Scanner v1.0 software (Applied Biosystems®, USA). All samples shown in alignment diagrams achieved a QV value of more than 20, which indicates the high quality of pure bases of DNA. The QV is a per-base estimate of the base caller accuracy and is calibrated on a scale corresponding to $QV=-10\log_{10}(Pe)$ where *Pe* is the probability of error.

3. RESULTS

3.1. HLA-DRB1 risk and protective alleles

Table 1 shows *HLA-DRB1* genotyping revealed risk *DRB1*03* and *DRB1*16* and protective *DRB1*07, and DRB1*12* alleles in pre-B ALL patients. *HLA-DRB1*12* and *DRB1*15* were the commonest alleles among Malays as reported by Dhaliwal et al. (2007) (29) Among the patients, 26 were males (61.9%) and 16 were females (38.1%). 47.6% (n=20) were adult pre-B ALL with age above 15 years old and 52.4% (n=22) were children, with age at or below 15 years old.

3.2. HLA-DRB1*03 and -DRB1*16 exon 2 sequence-based typing

PCR amplicons from seven *HLA–DRB1*03* (760 bp) and six *HLA–DRB1*16* (720 bp) pre-B ALL samples (**Figure 1A** and **1B**) were successfully sequenced and showing differential polymorphic sites. Four DNA samples from non-leukemic individuals were also included as controls. The Chromatogram of sequencing results was viewed on the FinchTV software.

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Table 1. Frequency of HLA-DRB1 alleles of Malay ethnicity in pre-B ALL patients (n=42)

HLA-DRB1 alleles	pre-B ALL (2n = 84)	Normal# (2n = 2890)	Odds ratio (95% CI)	p-value
DRB1*01	1.19	0.66		NS
DRB1*03	11.90	4.43	2.92 (1.47-5.80)	p=0.001*
DRB1*04	9.52	6.40		NS
DRB1*07	4.76	9.69	0.47 (0.17-1.28)	p=0.032
DRB1*08	1.19	1.56		NS
DRB1*09	4.76	3.56		NS
DRB1*10	3.57	2.35		NS
DRB1*11	2.38	2.80		NS
DRB1*12	17.86	26.99	0.59 (0.33-1.03)	p=0.008
DRB1*13	2.38	4.29		NS
DRB1*14	4.76	5.43		NS
DRB1*15	26.19	28.03		NS
DRB1*16	9.52	3.67	2.76 (1.30-5.87)	p=0.001*
DRB1*18	0.00	0.14		NS

Abbreviation: CI = Confidence Interval, NS = Not significant, NA = Not available, pre-B ALL = precursor B Acute Lymphoblastic Leukemia, *Indicate highly significant by statistical analysis. #(29).

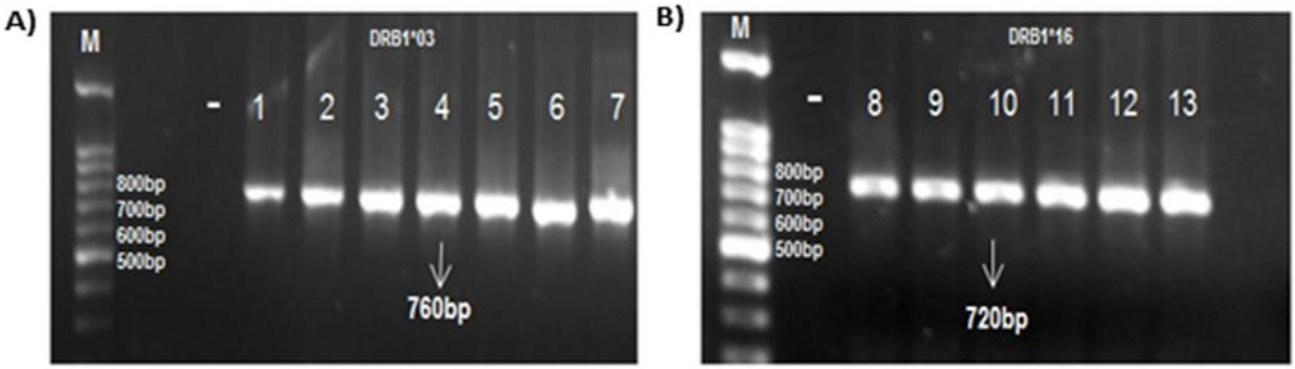

Figure 1. **PCR products of A) –DRB1*03 and B) –DRB1*16 amplification of exon 2 in pre-B ALL patients. Abbreviation: M – DNA markers.**

BLAST search outcome from the IMGT/HLA database for the targeted polymorphic exon 2 of –DRB1 genes concluded that the identified risk alleles (*-DRB1*03 and –DRB1*16*) for pre-B ALL Malay patients were *HLA-DRB1*03:01:01:01* and *DRB1*03:01:01:02* for *DRB1*03* allele and *HLA-DRB1*16:02:01* for *DRB1*16* allele.

Alignment of pre-B ALL samples (ID prefix, HKL) with healthy individuals (ID prefix, D) for *HLA-DRB1*03* and *HLA-DRB1*16* are shown in **Figure 2** and **Figure 3**, respectively. Variations in nucleotide sequences were observed among DRB1*03 pre-B ALL samples compared to the control. On the other hand, the sequencing results from *HLA–DRB1*16* alleles showed no difference between pre-B ALL patients and controls (**Figure 3** and **Figure 4**).

Unfortunately, two of seven *–DRB1*16* pre-B ALL samples had to be excluded as heterozygous chromatogram peaks were detected due to contamination from amplifying *–DRB*15* allele as these individuals also carried the DRB*15 allele. Primers that were used for –DRB1*15 and –DRB1*16 were similar, referring to **Figure 4** as reference alleles (31).

4. DISCUSSION

The discovery of the close association of human leukocyte antigens (HLA) with cancer invites closer scrutiny of this relationship. Further understanding is important to identify ways to prevent or treat this difficult disease. The HLA or major histocompatibility complex (MHC) genes have been

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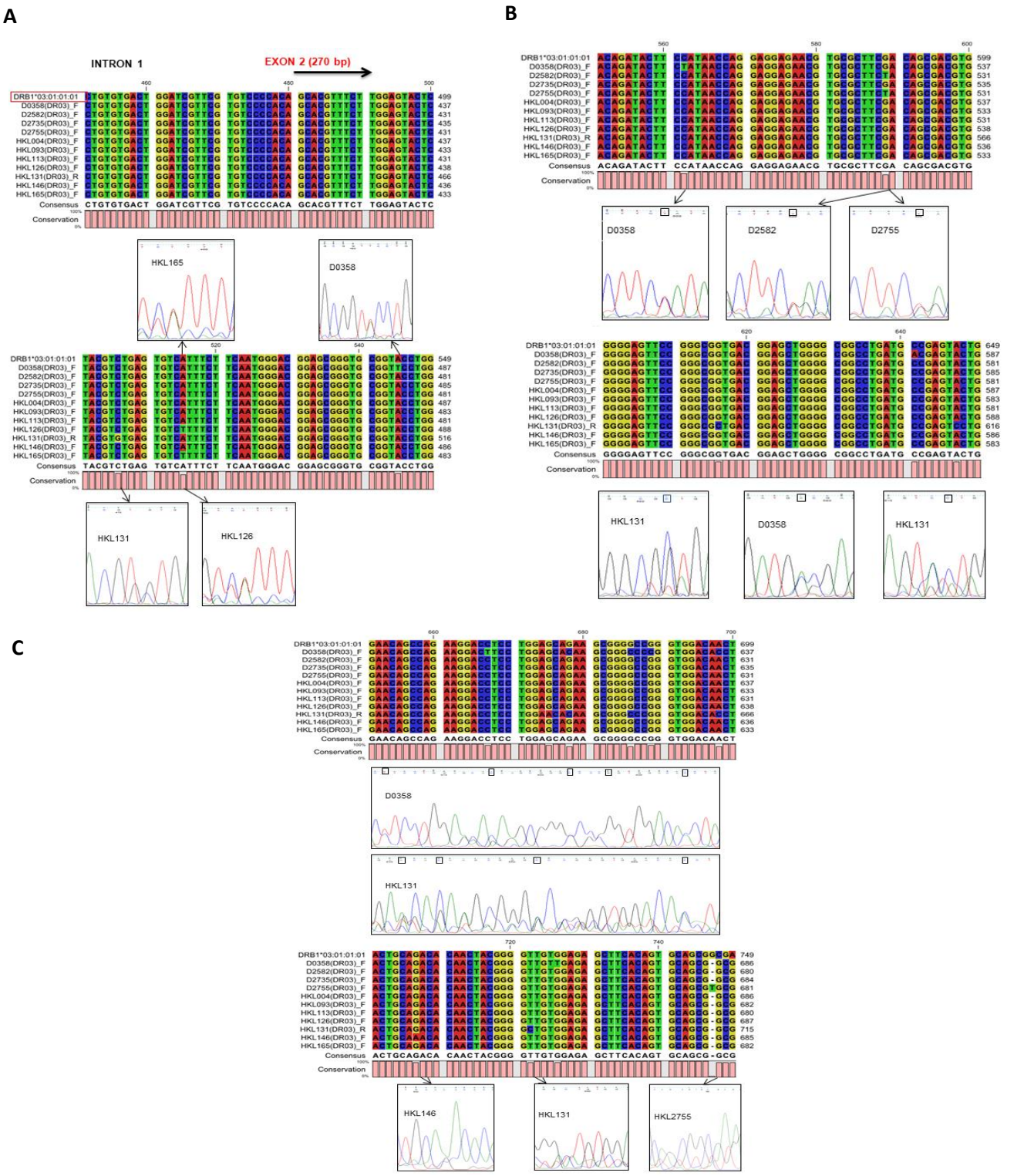

Figure 2 (A–C). Figures show the alignment of –DRB1*03 sequences of pre-B ALL samples (n=7, HKL-IDs) and normal individuals (n=4, D-IDs) of exon 2. HLA-DRB1*03:01:01:01 sequence is used as a reference in the red box. F- forward, and R- for reverse (sequencing). Boxes show examples of polymorphism sites and heterozygous (double) peaks.

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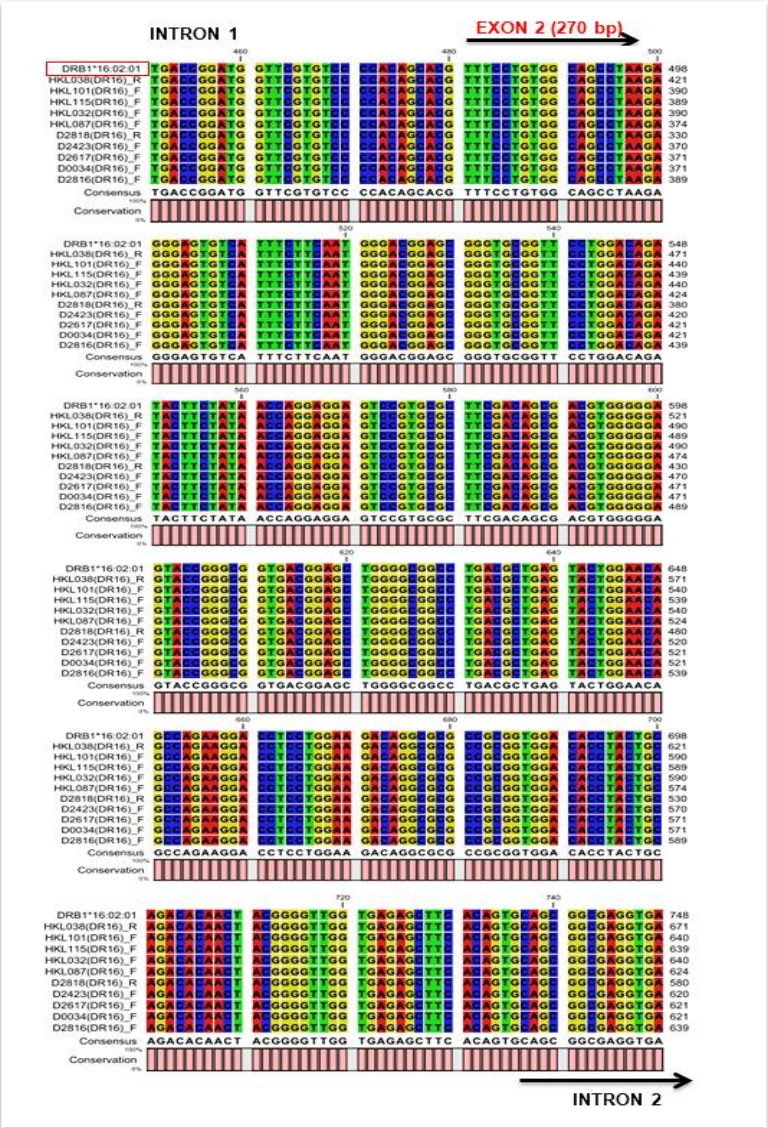

Figure 3. The figure shows the alignment of –DRB1*16 sequences of pre-B ALL samples (n=5, HKL-IDs) and healthy individuals (n=4, D-IDs) of exon 2. HLA-DRB1*16:02:01 sequence is used as a reference in the red box. F – forward, R- reverse (sequencing).

```
AA Codon                        10                  15                  20                  25
DRB1*03:01:01:01    CA CGT TTC TTG GAG TAC TCT ACG TCT GAG TGT CAT TTC TTC AAT GGG ACG GAG CGG GTG CGG TAC CTG GAC AGA
DRB1*03:01:01:02    -- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DRB1*16:02:01       -- --- --- C-- TG- C-G C-- -A- AGG --- --- --- --- --- --- --- --- --- --- --- --- -T- --- --- ---

AA Codon            30                  35                  40                  45                  50
DRB1*03:01:01:01    TAC TTC CAT AAC CAG GAG GAG AAC GTG CGC TTC GAC AGC GAC GTG GGG GAG TTC CGG GCG GTG ACG GAG CTG GGG
DRB1*03:01:01:02    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DRB1*16:02:01       --- --- T-- --- --- --- --- TC- --- --- --- --- --- --- --- --- --- -A- --- --- --- --- --- ---

AA Codon            55                  60                  65                  70                  75
DRB1*03:01:01:01    CGG CCT GAT GCC GAG TAC TGG AAC AGC CAG AAG GAC CTC CTG GAG CAG AAG CGG GGC CGG GTG GAC AAC TAC TGC
DRB1*03:01:01:02    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DRB1*16:02:01       --- --- --C --T --- --- --- --- --- --- --- --- --- --- --A G-C -G- --C -C- GC- --- --- -C- --- ---

AA Codon            80                  85                  90
DRB1*03:01:01:01    AGA CAC AAC TAC GGG GTT GTG GAG AGC TTC ACA GTG CAG CGG CGA G
DRB1*03:01:01:02    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -
DRB1*16:02:01       --- --- --- --- --- --- -GT --- --- --- --- --- --- --- --- -
```

Figure 4. The figure shows reference alleles provided by IMGT/HLA database of HLA-DRB1*03:01:01:01, DRB1*03:01:01:02, and HLA-DRB1*16:02:01 (270 bp).

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recognized for their ability to impact the vulnerability to various cancers. However, the potential influence of HLA Class II on tumor susceptibility in solid and blood cancer is rarely explored. ALL has remained a significant health concern, particularly in children and young adults, with genetic predisposition playing a crucial role in its etiology.

Many studies have reported HLA allele is associated with risk and protection in acute lymphoblastic leukemia. Although the conservation of HLA alleles within an ethnic population suggests a need to identify uniqueness between groups, identifying common alleles is just as important. Here, the risk allele *HLA-DRB1*03* was found common to that reported in ALL among Brazilians (26,27). This is likely interesting as Malays and Brazilians are not expected to have a common origin. In newer immunotherapy methods such as checkpoint inhibitors, the *HLA-A*03* allele was found associated with reduced survival (32). This emphasizes the important role of HLA in the many aspects of immunology.

Other studies found other HLA class II molecules with a positive association with ALL were *HLA-DRB1*04* (21,25) in childhood ALL, *HLA-DRB1*14* (28), *HLA-DQ5 (*0501-*0504)* (24), and *HLA-DPB1*0201* (23). None were common to *HLA-DRB1*16*, the other risk allele found among Malays here. On the other hand, the protective (*HLA-DRB1*07, HLA-DRB1*12*) alleles in pre-B ALL were not identified in other studies. The allele under-represented in ALL patients

was *HLA-DRB1*13* (25). Among male childhood, ALL of the high-risk status, allele frequencies of *HLA-DRB1*04* and *DRB4* were significantly lower while *DRB1*13* and *DRB3* were significantly lower than controls (22).

Refractory pediatric ALL (pALL) among European Americans who underwent transplantation with hematopoietic stem cells observed a greater risk of relapse in patients of homozygous predisposing genotype of which the influence of *HLA-DRB1* exceeded HLA-B which was greater than HLA-A. *HLA-DRB1*04, DRB1*14, DRB1*10,* and *DRB1*08* were predisposing while *DRB1*01* and *DRB1*03* were favourable-protective (33), in contrast to alleles reported here. Nevertheless, this emphasizes the role of HLA in the pathogenesis of pALL and a potential role for the infectious origin of pALL (33).

Loss of heterozygosity (LOH) within these HLA genes indeed can lead to false homozygosity in HLA genotyping analysis of patients as reported in a case of precursor-B acute lymphoblastic leukemia (34). In reported cases of chronic lymphoblastic leukemia (CLL), HLA homozygosity and haplotype bias were said to influence the disease progression, suggesting that CLL progression is controlled by immune surveillance in that disease (35).

The MHC also has long been known to harbor large numbers of single nucleotide polymorphisms (SNPs), MHC class I is most frequently mutated in primary and metastatic cancers while MHC class II is more restricted (36). As far as we know, this is the first study identifying mutations in acute lymphoblastic leukemia. The reason for the high number of SNPs observed in the *HLA-DRB1*03* allele and none in the *HLA-DRB1*16* is not clear. **Figure 5.** illustrates the summary of the entire study.

5. Conclusion

In conclusion, our study provides novel insights into the variability of the *HLA-DRB1*03* allele and its implications in pre-BALL predisposition. The observed diversity underscores the complexity of HLA polymorphism and its role in modulating immune responses against leukemia. Moving forward, elucidating the functional consequences of *HLA-DRB1*03* variability and its clinical relevance may pave the way for precision medicine approaches in ALL management, ultimately improving patient outcomes.

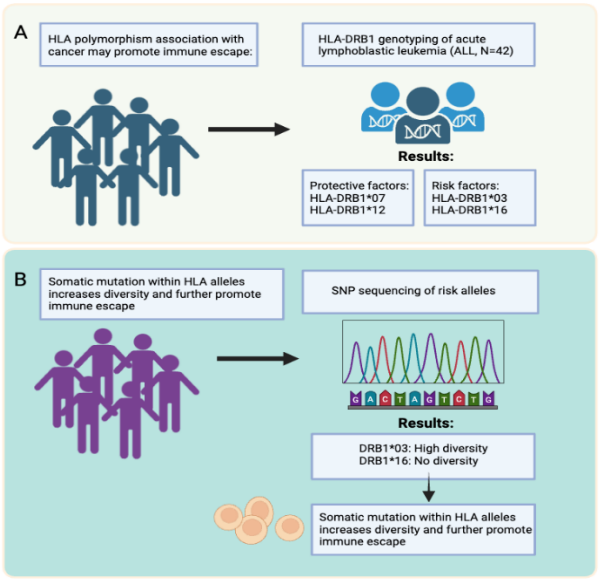

Figure 5. Graphical abstract of the overall study.

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Conflict of interest

The authors declare no conflict of interest.

Ethical statements

Approvals to conduct the study were obtained from the Medical Research Ethics Committee (MREC) of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, and the National Medical Research Register (NMRR), Ministry of Health (MOH). Samples from hospital wards were obtained after informed consent from patients or guardians, based on the Declaration of Helsinki.

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