Chapter III

Allelic diversity of the *KIR2DL1* gene and of *HLA-C* gene in the population of Assam, North-East India.

3.1 **INTRODUCTION:**

Killer cell Ig-like receptors (KIR) which are expressed on Natural Killer cells (NK cells) are a diverse family of cell-surface glycoproteins (1)**.** The KIRs are diversified by variation in the gene as well as allelic content across individuals and populations (2) and plays an important role in modulation of human NK-cell activities. These receptors recognize the Human Leukocyte Antigen class I ligands (HLA-A, -B and -C) which are expressed on the surface of most human cells. In contrast to HLA-A and HLA-B, every HLA-C allotype forms a ligand for KIRs and it is these interactions that dominates NK cell responses. Two mutually exclusive HLA-C epitopes are defined by the residue at position 80 in the α1 domain and are recognized by different KIRs (3)**.** NK cells activity against virus-infected cells and tumor cells are directed by such interactions. The KIR-HLA interactions also induce the secretion of cytokines that activate other leukocytes and guide fetal trophoblast cells to invade the uterus during pregnancy(3) .The KIRs are found to be highly diversified by variation in the gene as well as allelic content across individuals and populations and as many as 14 expressed *KIR* genes along with 2 pseudogenes have been identified in humans so far (4)**.** Like their KIR receptors the major histocompatibility complex (MHC)/HLA is unique in that it is the most polymorphic genetic system in the human genome. Due to its high polymorphism, tight linkage among the loci and non-random association of alleles this system has become interesting from perspective of population genetics (5)**.**

The KIR-HLA genes are very good candidates for genes undergoing natural selection and coevolution in the human genome for their dual role of in immunity to infection and reproduction (6)**.** Several previous studies have showed that inhibition of NK cells by some KIR–HLA combinations are stronger than others (7, 8)**.** Weaker inhibitory interactions result in lower NK cell activation threshold and better tolerance from virus

infections, or greater susceptibility to autoimmune responses. Similarly strong inhibition by KIR–HLA interactions prevents proper NK cell activation by bringing the activation threshold higher (9). Therefore there is a strong connection between the strength of KIR-mediated inhibition and disease outcome (7) and needs to be studied across populations and of various KIR-HLA combinations both in high and low resolutions. Till date, the diversity and functional properties of the inhibitory receptor KIR2DL1 and HLA-C have been studied mainly in the European population along with studies from few of African (10, 11) and South-Asian populations. Moreover, studies involving high resolution analysis of the allelic and haplotype diversity has been very few (11, 12).

The migration of modern human across the globe as always been an area of utmost importance for modern biologist and several DNA markers has emerged as exciting targets to study human diversity as well as human migration and evolutionary relationships (5). Over the years, various markers have been identified which are helping researchers across the globe in trailing back through years and have a better understanding about the migration pattern of humans. Recently, due to their highly polymorphic nature, KIR and HLA have emerged as important DNA marker having implications in understanding human diversity, migration patterns and evolutionary relationships among populations (5). One of the most unique characteristic of the The major histocompatibility complex (MHC)/HLA is that it is only system that shows functional polymorphism. Population geneticists across the world are fascinated due to its tight linkage among the loci, non-random association of alleles and most importantly due to its high polymorphism rate(13, 14). Therefore, the HLA system has provided immense value in polymorphism studies and linking its significance in the survival and maintenance of variability within numerous world populations (15, 16). In addition,

detailed investigations about the distribution of HLA alleles in world populations have become much more important as the information would help in understanding the interplay between basic genetic origins in a population along with the effects of environmental selection and other natural phenomena like the founder effect. Interestingly, along with the HLA investigations, Immunogenetic studies on the distribution of KIR genes in various ethnic populations across the globe have shown significant differences in the distribution of both group A and B haplotypes(17) (18). For example, in the Japanese population, more than 50% of the individuals were found to have group A homozygous genotype. Whereas, among on the Australian Aborigines, group A homozygote haplotype was found in only a single individual out of 67 tested (19-21).

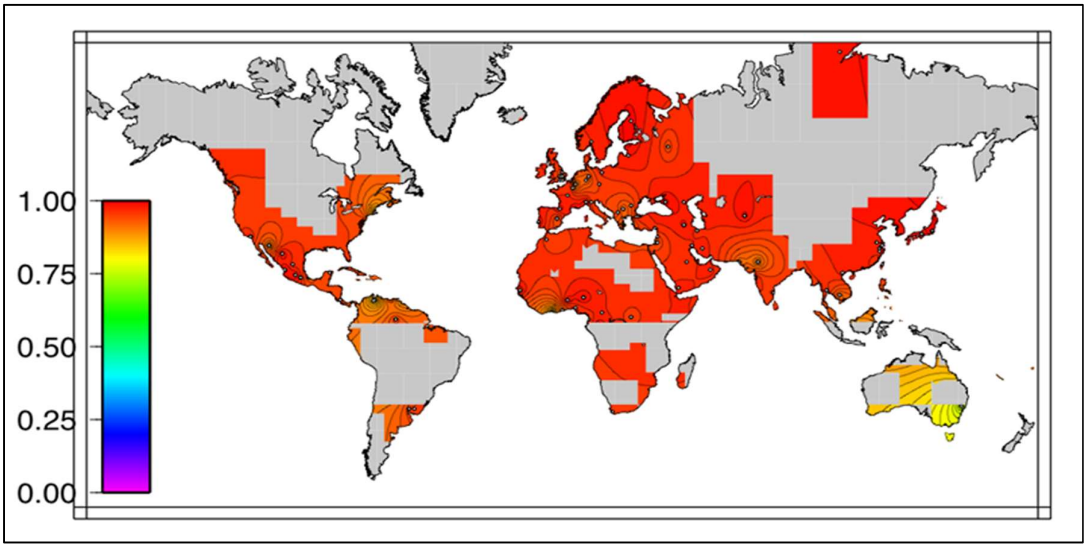


Figure 4: Distribution of KIR2DL1 in world population (Courtesy: allelefrequencies.net)

North-East India has been considered as a major corridor for modern human migration after they started dispersing out of Africa about 100000 years before present (ybp) (22).

Various group of investigators have reported that that this part of the world has witnessed at-least three waves of human migration which includes an ancient Palaeolithic migration of the modern humans, which was followed by a Neolithic migration, through the eastern horn of Fertile Crescent for the arrival of the Caucasoid population and a very dominant migration from East/Southeast Asia bringing with it the Tibeto-Burman speakers. Apart from these waves of migrations, India has also experienced colonization by races from other parts of the world which might have contributed in the genetic heterogeneity (18, 23).

Therefore, studies regarding the distribution of the KIR-HLA alleles in world populations are very important in assessing their role in conferring survival advantage to populations in varied environmental conditions.

3.2. Materials and methods

3.2.1. Study site, study design, and participants

The present study was designed as a hospital-based study of 204 HNSCC and 225 healthy participants from three linguistic affinities namely Indo-European (IE), Austro-Asiatic (AA) and Tibeto-Burman (TB) of NE India. However, the clinical status of the participants was not considered for this part of the study. Only the geographical and ethnic origin was included. The participants were recruited from two hospitals in Northeast India- Gauhati Medical College and Hospital (GMCH) and North East Cancer Hospital and Research Institute (NECHRI). The study was ethically approved vide letter No **IEC/17/03/002** and the participants of the study were informed about the study before the collection of the specimens. A detailed information sheet was distributed among the participants of the study. All the participants voluntarily consented to participate in the study. The information about gender, ethnicity, clinical

history, demographic and other characteristics were collected and recorded in the form of proformas by the research staff of NECHRI.

3.2.2. Extraction of DNA, Quality assessment of the samples

Peripheral human blood samples (0.5 ml) were collected from the participants by trained staff using a standard operative protocol. Isolation of Genomic DNA from whole blood was done using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Purity (1.8–2.0) and the yield of the extracted total RNA were checked using Nano-Vue TM plus spectrophotometer (GE Healthcare, Little Chalfont, United Kingdom)

3.2.3. PCR based detection of *KIR2DL1* Alleles:

As described by Ashouri et al. (2009), the polymerase chain reaction sequence-specific priming (PCR-SSP) approach was used for the typing of the *KIR2DL1/S1* genes in the individuals (24). The validations for PCR amplification were done using the framework KIR genes as a positive control for each PCR amplification according to Du et al. (2007) (25).For KIR subtyping, Sequence-specific primers targeting exon 4 designed to discriminate allele-specific polymorphisms were paired with *KIR2DL1* locus-specific primers targeting exons 1 and 3 as described by HG Shilling et.al (2002) (26). We typed five *KIR2DL1* alleles and their names were assigned officially by the KIR Nomenclature Committee as *001, *002, *003, *004, and *005. Additional validation of the results was done according to Le Luduec et al (2018) (27) in which six distinct ARMS PCR reactions specific for six *KIR2DL1* allele groups were done using DNA from the participants from the study. Further, Exon 8/9 of the KIR locus was amplified and sequenced in the participants according to LiHua Hou et.al (2012) (28)

3.2.4 **HLA-C Allotyping:**

HLA-C allele discrimination in 287 participants was done using multiplex real-time polymerase chain reaction (PCR) genotyping as previously described by Koehler et al. (2009)(29). In the first round PCR, exon nos. 2 and 3 of HLA-C loci were amplified using locus-specific primers (30). The respective PCR amplicons were then used as template DNA in SSP-real-time PCR platform. For typing of each HLA variant, fluorescent probes and primers targeting both ligand-specific region and a non-polymorphic region of the amplicons were used as internal control. All samples were run on a 96-well plate format in StepOne-Plus real-time PCR system (Applied Biosystems) with the following thermocycling program: 10 mins at 95 °C followed by 60 cycles of 15 s at 95 °C and 1min at 60 °C. The Cycle Threshold (CT) value of each reaction was used to determine the presence/absence of targeted HLA. For all reactions, water was used instead of genomic DNA as negative control. The validity of Multiplex real-time PCR genotyping was confirmed by randomly selecting 70 samples and sequencing exon nos. 2 and 3 of HLA-C loci using sequencing primers on ABI 3100 Genetic Analyzer (Applied Biosystems) using Big-Dye Terminator v3.1 (Applied Biosystems)

3.2.4 **KIR2DL1 molecular modelling and Protein-Protein docking**

The Protein 3D structure of KIR2DL1*003, KIR2DL1*004, and KIR2DL1*005 were predicted from the Sanger sequencing data by using I-TASSER (Iterative Threading ASSEmbly Refinement) (31). Five models each of KIR2DL1*003, *004, and *005 were computationally generated and the best-predicted structure models were selected based on confidence score (C-score). Further, the structures obtained from the I-TASSER server were docked with HLACW4 (PDB ID: 1QQD) using the PatchDock

server (32). In this case, we used the default RMSD value of 4 Å for clustering solutions. The best protein-protein docked models were chosen concerning minimum free energy and high geometric shape complementary score. The possible interacting residues across the interface of the complex and the interface were predicted by PDBsum (33). Additionally, the KIR2DL1 structure (PDB ID: 1NKR) already available in the RCSB Protein Data Bank was used as a control structure for comparison and was also docked with HLA-CW4 using the PatchDock server.

3.2.5. Genetic analyses:

For comparing the study populations with the world population with respect to their *KIR2DL1, KIR2DS1* and *HLA-C* frequencies, KIR data of 38 populations from the allele frequencies website (34) was used. The comparison was done by constructing a Neighbor-joining dendogram using the Phylogeny.fr online tool (35). Multiple sequence analysis using Clustal-Omega was done to check the level of variations among the individuals as both KIR and HLA genes are considered to be highly polymorphic. Nucleotide diversity (Pi) was calculated using DnaSP software (36) to assess the allelic diversity to an alignment of *KIR2DL1* and of *HLA-C* sequences. This measure is defined as the average number of nucleotide differences per site between two DNA sequences in all possible pairs in the sample population. Similarly, the haplotype diversity (Hp) was calculated for the both *KIR2DL1* and *HLA-C* sequences which represents the probability that two randomly sampled alleles are different. Tajima's D analysis was done using DnaSP software to study whether the genes evolved randomly ("neutrally") or were under a non-random process, including directional selection or balancing selection demographic expansion or contraction, genetic hitchhiking, or introgression.

3.2.6 Statistical analysis

For statistical analysis of the data, XLSTAT software (2015 and 2018.7 versions) was used. One sample t-test and Student's t-test were used for comparison between the mean values. A p-value < 0.05 was considered statistically significant.

3.3 **Results:**

3.3.1. Quality assessment of the DNA samples by β-globulin PCR

To determine the quality of the DNA samples, extracted DNA from HNSCC patients and Healthy controls were subjected to PCR for human β-globin gene amplification. Twelve samples that were negative for the human ß-globulin gene were excluded from the study and all other samples showing the amplicon of 110 bp were subjected to conduct of the study.

3.3.3 Genetic distances and Phylogenetic tree:

The Phylogenetic tree based upon *KIR2DL1-HLAC2* gene and *KIR2DS1* gene frequencies showed three Nodes in the neighbor-joining dendogram (Fig 4 A). Our study population was mapped in the third node consisting of South-East Asian countries and the Veracruz Mestizo population from Mexico. It signifies the fact that the populations of North-East India, in particular Assam, is distinct in terms of genetic makeup, evolutionary and migratory history from North Indian populations which have mainly Indo-European origins (37) and the South-Indian populations which have Dravidian origins (38). The results of principal component analysis also showed that our North-East Indian population clusters well with other south-east Asian populations. While the Brazilian population appears quite divergent and was located away from all

other groups, the European populations clustered in a single group signifying that the populations are less divergent as compared to the Asian or the South American populations.

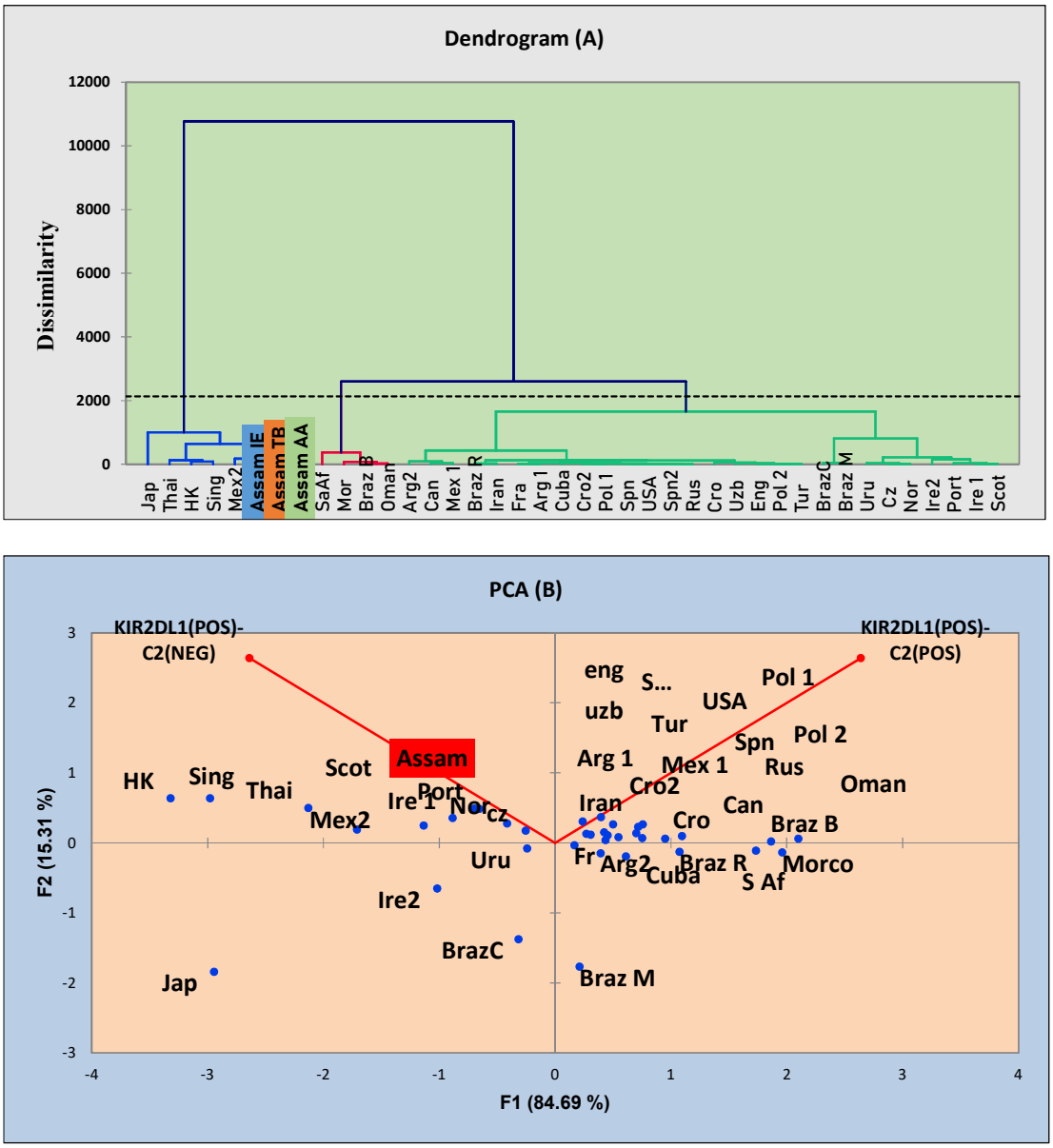


Figure 5 (A): Neighbor-Joining Dendogram of our study population with 38 world populations (B) Principal component analysis of the study population with other world populations

3.3.4 *KIR2DL1* allotyping and KIR-HLA polymorphism:

Among the five *KIR2DL1* alleles studied, the KIR2DL1*003 allele was seen at the highest frequency of 79% in all the 429 participants in the study (Figure 6). Additionally, the frequency of *KIR2DS1,* the activating homolog of the *KIR2DL1* gene was found to be 52.45% among the study participants.

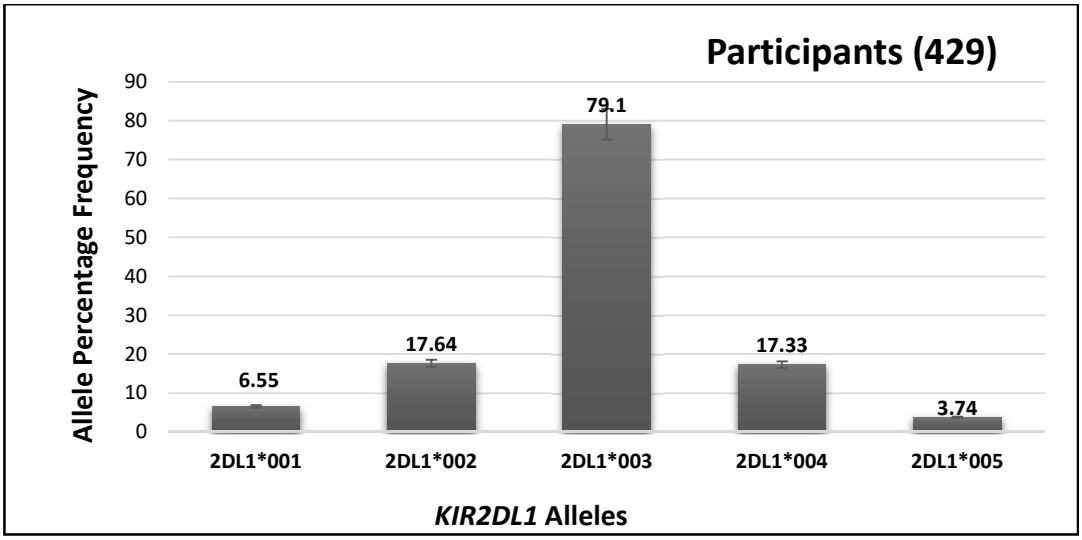


Figure 6: Frequency for *KIR2DL1* alleles in the study population

A maximum likelihood phylogenetic tree of KIR2DL1 alleles was constructed and three major clusters of alleles were observed (Figure 7), where KIR2DL1*004 formed a distinct cluster whereas KIR2DL1*001 and KIR2DL1*002 were seen in the second cluster. The third cluster consisted of KIR2DL1*003 and KIR2DL1*005 allele.

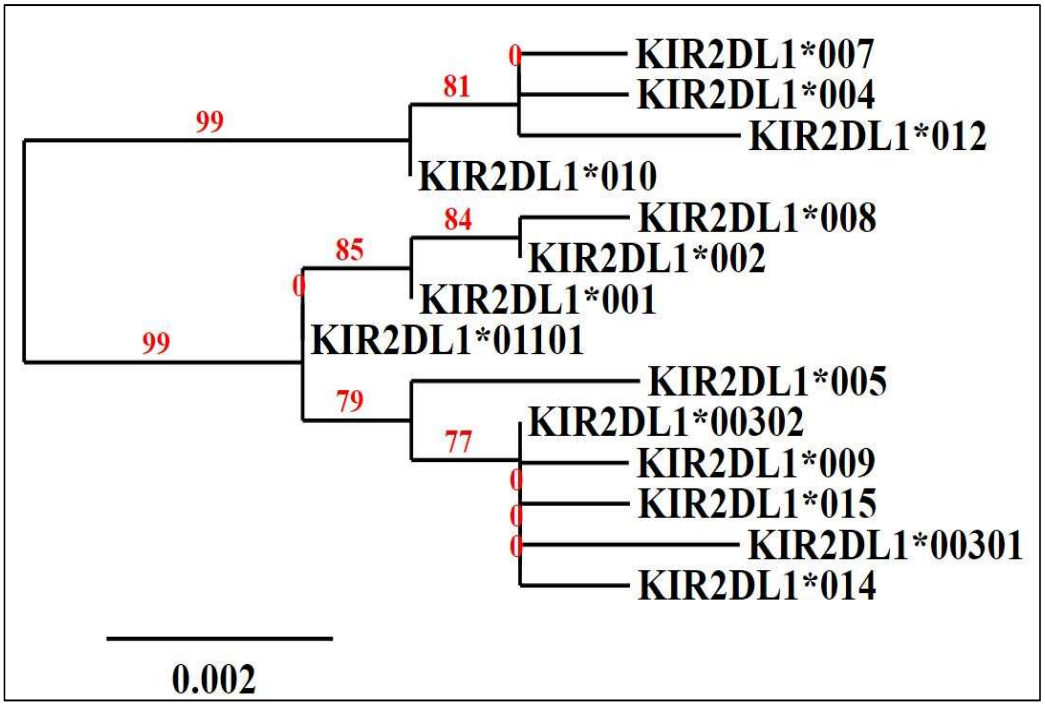


Figure 7: A maximum likelihood phylogenetic tree of most frequent KIR2DL1 alleles. Bootstraps were calculated for 100 iterations.

Further, we could identify 9 different HLA-C alleles in the study participants based on sequence alignment with reference sequences from National Center for Biotechnology Information (NCBI) database (27) . The occurrence of HLA-C*04 and HLA-C*07 were seen to be higher (28.5% and 25% respectively, Table: 2) in the study population. A phylogenetic tree of observed HLA-C alleles was constructed using a maximum likelihood technique (Fig 8). HLA-C*07:01:01 and HLA-C*07:02:01 formed a distinct cluster suggesting that the above alleles were divergent from the other reported alleles in the population. Of the 9 HLA-C alleles identified, HLA-C*16:02:01, a rare allele across populations was seen in our population, at a low frequency of 3.57 percent.

Further, when stratified by C1 and C2 epitopes, a higher frequency of HLA C1 epitope was observed in the study participants [HLA*C1C1 (61.3%), *C1C2 (34%), *C2C2 (4.6%)].

HLA-C Allele (n-70)	HLAC*07*01*01 (n-20)	HLAC*07*02*01 (n-10)	HLAC*08*01 (n-5)	HLAC*08*03*01 (n-5)	HLAC*15*05*01 (n-03)	HLAC*16*02*01 (n-3)	HLAC*01*02*01 (n-03)	HLAC*04*01 (n-18)	HLAC*14*02 (n-03)
Percentage Frequency	28.5 %	14.2 %	7.1 %	7.1 %	3.57 %	3.57 %	3.57 %	25.0 %	3.57 %

Table 1: HLA-C allele percentage frequency table from the study:

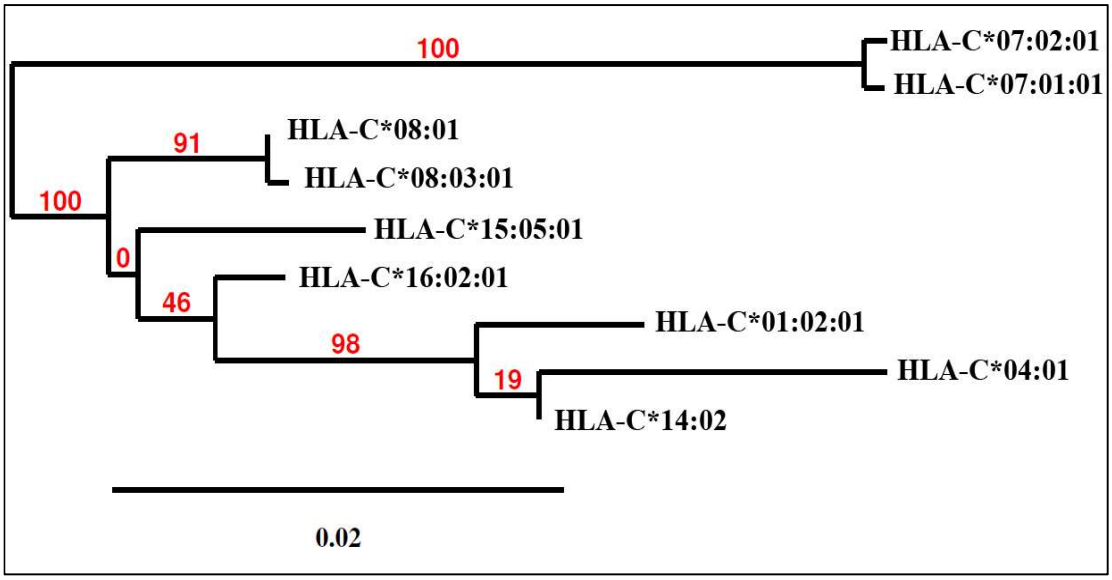


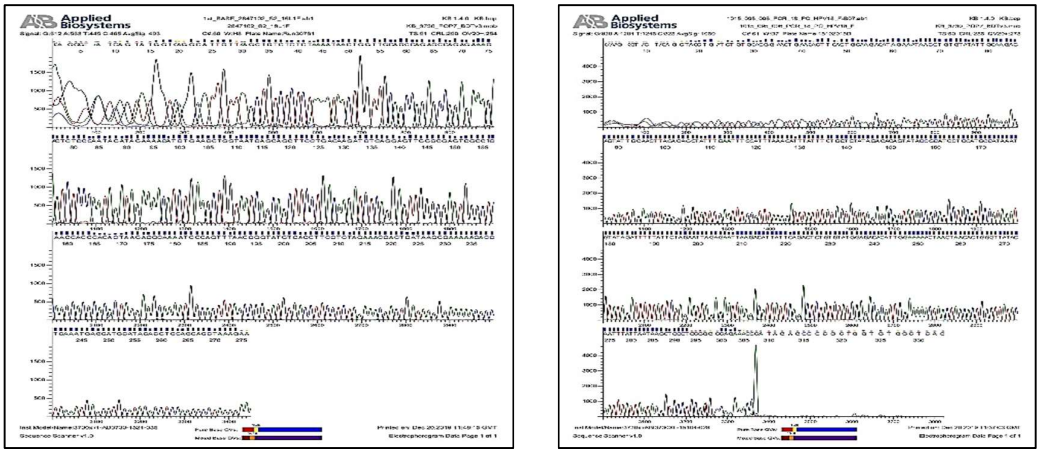
Fig 8: A maximum likelihood phylogenetic tree of HLA-C alleles in the population. Bootstraps were calculated for 100 iterations

3.4.5 KIR-HLA Nucleotide and Haplotype diversity:

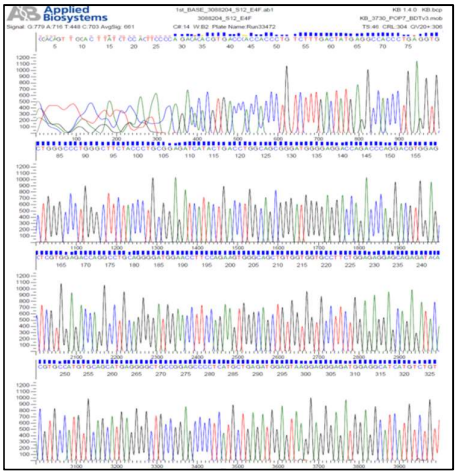
Nucleotide diversity (Pi) was calculated using DnaSP software (36) to assess the allelic diversity to an alignment of *KIR2DL1* and of *HLA-C* sequences. The nucleotide diversity of KIR2DL1 alleles (Pi: 0.00703) was found to be lower than the nucleotide diversity of *HLA-C* gene (Pi: 0.05546). Similarly, the haplotype diversity was calculated for the both *KIR2DL1* and *HLA-C* sequences. The haplotype (gene) diversity

(Hd) of *KIR2DL1* (Hd: 0.221) was found to lower than the haplotype diversity of *HLA-C* (Hd: 0.994).

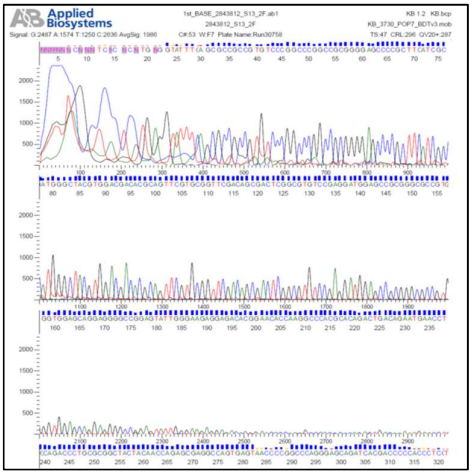
Tajima's D analysis (36) in which the D' value for both the *KIR* and the *HLA* sequences were below expected for neutrality (Tajima's D: -1.71748 and -1.53586 respectively) suggested the presence of numerous closely related variants under directional selection. The presence of rare alleles in the population together with a negative Tajima's D signifies an excess of low frequency polymorphisms relative to expectation, indicating population size expansion and/or purifying selection.



(A) (B)



(C)



(D)

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Homo sapiens natural killer-associated transcript 1 (NKAT1) mRNA, complete cds	Homo sapiens	1934	1934	100%	0.0	100.00%	1588	L41267.1
Homo sapiens natural killer cell inhibitory receptor (KIR2DL1) mRNA, KIR2DL1*002 allele, complete cds	Homo sapiens	1929	1929	100%	0.0	99.90%	1080	AY789055.1

(E)

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Homo sapiens killer cell immunoglobulin like receptor, two Ig domains and long cytoplasmic tail 1 (KIR2DL1), mRNA	Homo sapiens	1934	1934	100%	0.0	100.00%	1614	NM_014218.3
Homo sapiens KIR2DL1 mRNA, complete cds	Homo sapiens	1934	1934	100%	0.0	100.00%	1320	KJ699235.1
Homo sapiens natural killer cell inhibitory receptor (KIR2DL1) mRNA, KIR2DL1*00302 allele, complete cds	Homo sapiens	1934	1934	100%	0.0	100.00%	1157	AY789056.1

(F)

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident
Homo sapiens MHC class I antigen (HLA-C) gene, HLA-C*07var allele, complete cds	Homo sapiens	1615	1615	95%	0.0	99.55%
Homo sapiens MHC class I antigen (HLA-C) gene, HLA-C*07:01:01:01v2var allele, complete cds	Homo sapiens	1615	1615	95%	0.0	99.55%

(G)

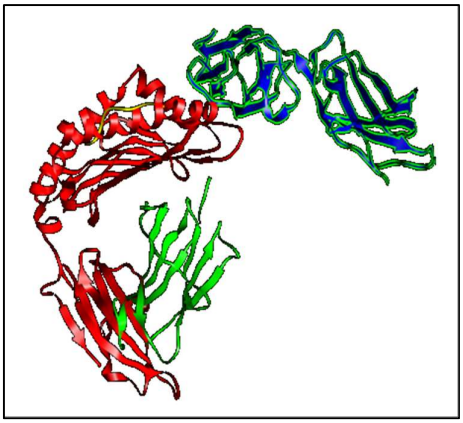
Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Homo sapiens clone PSC0248 MHC class I protein (HLA-C) gene, HLA-C*14var allele, complete cds	Homo sapiens	6185	6185	100%	0.0	100.00%	4177	MT415931.1
Homo sapiens HLA-C gene for HLA class I histocompatibility antigen C alpha chain precursor, complete cds, allele: C*14:02:01:01	Homo sapiens	6185	6185	100%	0.0	100.00%	4739	LC257770.1

(H)

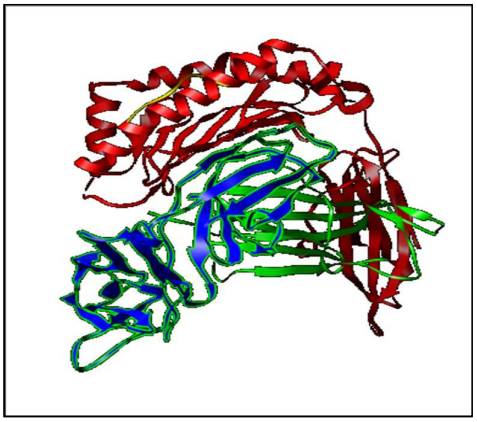
Figure 9: Representative images of chromatogram and NCBI-BLAST results for amplicons of (A) & (E) KIR2DL1*002 (B) & (F) KIR2DL1*003 (C) & (G) HLA-C*07 and (D)& (H) HLA-C*14

3.4.6 Binding affinity study of *KIR2DL1* alleles with *HLA-C.*

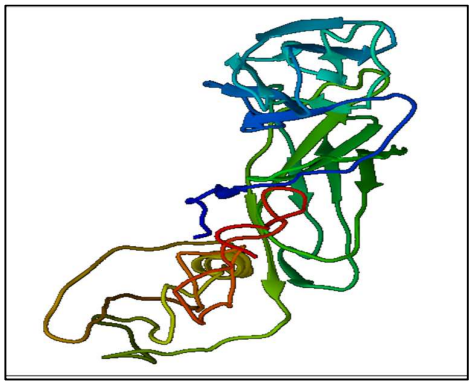
From the predicted structures obtained from the ITASSER server, the best model for KIR2DL1*003 showed a C value of -2.73 and a TM score of 0.40. The best model for KIR2DL1*004 gave a C value of -2.38 and a TM score of 0.44. Similarly, the best model for KIR2DL1*005 gave a C value of -1.14 and a TM score of 0.57. The Structures of KIR2DL1*003, KIR2DL1*004 and KIR2DL1*005 and the available structure of KIR2DL1 were then docked with HLA CW4 (PDB ID: 1QQD).



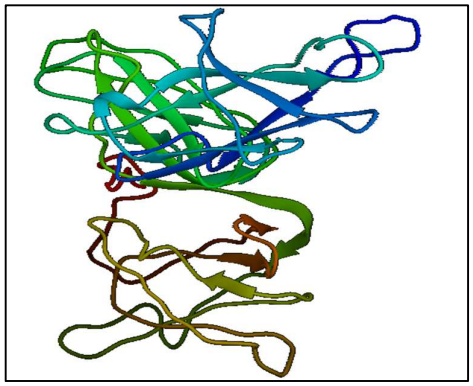
KIR2DL1*001



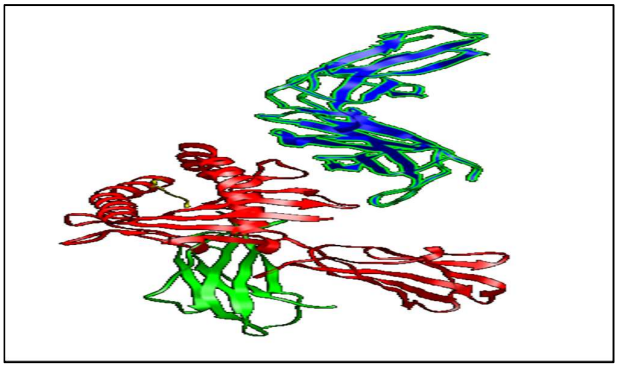
KIR2DL1*002



KIR2DL1*003



KIR2DL1*004



KIR2DL1*005

Figure 10: Representative images of predicted structures of KIR2DL1 alleles using ITASSER server.

KIR2DL1*003 interacted with HLA-CW4 with a geometric shape complimentary score of 20456. The approximate interface area of the complex was found to be 3184 A^2 with atomic contact energy of -137kcal/mol. Similarly, the complex of KIR2DL1*004-HLACW4 showed a geometric shape complimentary score of 17798. The approximate interface area of the complex was found to be 3074.30 A^2 with atomic contact energy of 473.22 kcal/mol. Further, the KIR2DL1*005-HLACW4 complex showed a geometric shape complimentary score of 17146 and the approximate interface area of the complex was 2734.10 A^2 with atomic contact energy of 240.16 Kcal/mol.

The total number of interface residues in the complex 2DL1*003-HLA-CW4 was found to be 41 while for the complex 2DL1*004-HLA-CW4, the total number of residues involved were 35 and for KIR2DL1*005, 25 residues were found to be involved (Table 2). Similarly, the interface area of each chain involved in the complex of 2DL1*003-HLA-CW4 was 1810 $A^{2,}$ for 2DL1*004-HLA-CW4, it was 1559 A^2 and for 2DL1*005-HLA-CW4, the interface area of each chain involved was 1185 $A^{\circ 2}$(Table 2). The docked complex was predicted to be stabilized by molecular interactions like hydrogen bonding and non-bonded contacts. Interestingly, the binding affinities of KIR2DL1*003/HLA-CW4 and KIR2DL1*004/HLA-CW4 were found to be higher than the already available structure of KIR2DL1/HLA-CW4. However, KIR2DL1*005 was found to have lower binding affinity to HLA-CW4 as compared the above three structures. The above results suggest that binding affinity of KIR2DL1 with HLA-C alters with the allele of *KIR2DL1* present.

Chains	No. of interface residues	Interface Area (A°)	No. of salt bridges	No. of Hydrogen Bonds	No. of Non-Bonded contacts
2DL1*003	41	1810	7	13	350
HLA-CW4	30	1910			
2DL1*005	35	1559	5	11	270
HLA-CW4	28	1693			
2DL1 (1NKR)-	32	1234	2	14	166
HLA-CW4 (1QQD)	28	1384			
2DL1*004	25	1185	5	11	159
HLA-CW4	21	1236			

Table 2. Summary of interface statistics for KIR2DL1*003-HLA-CW4, KIR2DL1*005-HLA-CW4 and KIR2DL1*004-HLA-CW4 complexes determined from PDBsum server.

3.5 Discussion:

This study attempted to investigate the allelic diversity of *KIR2DL1* and of *HLA-C* in the populations of Assam, North-East India We showed that the inhibitory *KIR2DL1* alleles cluster in distinctive phylogenetic clades that associate with specific *KIR* haplotypes. Our data showed that KIR2DL1*00302 has a stronger binding affinity for the HLA-C2 epitope which complemented previous studies from other groups (39-41). In addition, this allele

was present at a much higher frequency (79.1%) in studied populations of Assam, North-East India. The presence of the KIR2DL1*003 allele in high frequency in the population along with its strong-affinity ligand HLA-C2 suggests a higher threshold for NK cell activation, which would severely compromise NK cell activation. Interestingly it has been balanced by the presence of HLA-C1 epitope at higher frequency in the population [C1C1 (61.3%) and C1C2 (34%)]. This type of balancing act is also evident in the Japanese population where the dominant presence of KIR-A haplogroup (80%) in the population is balanced by the HLA-C1 (92%) as a dominant self-ligand for inhibitory KIR over HLA-C2 (8%)(42). It may be noted that though the presence of the combined genotype of KIR2DL1*003-HLAC2 will be effective against many autoimmune responses but the higher activation threshold is most likely to weaken the NK cell immune response against diseases like cancer. It is worthwhile to mention that we had earlier reported that increased expression of inhibitory receptors with an association of KIR2DL1-HLA-C2 genotype to be a risk factor for HNSCC patients of Tibeto-Burman origin with family history of cancer (43) which may be attributed to the strong inhibitory signal delivered by this genotype.

Further, we could identify 9 different HLA-C alleles in the study participants, of which HLA- C*16:02:01 was identified as rare allele. The presence of rare allele in the population was also evident by Tajima's D analysis in which the D' value for both the KIR and the HLA sequences were below the expected neutrality (Tajima's D: -1.71748 and -1.53586 respectively) which suggests the presence of numerous closely related variants under directional selection

North-East India is a meeting place of three distinct language families of mankind, namely the Austro-Asiatic, Tibeto-Burman and the Indo-European. Therefore, it is understandable that the admixture of genes to varied degrees between the members of diverse ancestral populations has taken place (38)**.** In the present study, the frequencies of the compound

genotype KIR2DL1-HLAC and *KIR2DS1* were mapped with South-East Asian countries. The study population also clustered with the North-American Mexican population which is not surprising owing to the fact that there is a long history of human migration between these continents(44). Interestingly, the clustering of our populations using KIR2DL1-HLAC and *KIR2DS1* genes was consistent with the clustering of our population using KIR gene frequencies (43) and it shows the genetic affinities between the study population and the other clustered South-East Asian population.

In conclusion, our study provided insight into the allelic diversity of *KIR2DL1* and *HLA-C2* in the population of Assam, North-East India in which KIR2DL1*003 was found to be the predominant *KIR2DL1* allele in the population. Further, the study demonstrated that the North-East Indian population is distinct from the rest of the Indian population and thus adds to the already varied genetic diversity of the country. Therefore, a further detailed investigation of the importance of KIR-HLA association in the population will enlighten the evolutionary as well as the disease association, particularly from the aspect of disease surveillance/ prognostic markers and identification of individuals at risk.

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