Killer cell immunoglobulin-like receptors mediated self tolerance and activation of natural killer cell in oral squamous cell carcinoma

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

Natural killer (NK) cells are the key lymphocytes in solid tumours as these are not human leukocyte antigen (HLA) restricted unlike cytotoxic T-cells. Its activity is regulated by the germline encoded killer cell immunoglobulin-like receptors (KIRs) on interacting with its cognate HLA class I ligand. These two independent loci – KIR and HLA class I segregate independently to produce diversity in terms of number and type of KIR-HLA genotype. Recent studies have explained how these varied KIR-HLA interactions can influence susceptibility/resistance to a variety of diseases, including cancer by influencing NK cell reactivity. The present study aims to investigate KIR mediated self-tolerance and NK cell activation in oral squamous cell carcinoma (OSCC). Polymerase chain reaction (PCR)-sequence specific priming (SSP) approach was used to type 16 KIR genes in individuals and SSP-real-time PCR was used for HLA class I ligand genotyping. NK cell activation in OSCC was assessed in context of – 1) NK cell cytotoxicity and 2) expression of NK cell receptors (NKp46 and KIRs) and NK cell associated cytokines (pro-inflammatory cytokines – IL-1β, IL-2, IL-12β, IL-15, IL-18, IL-21, IFN-γ and TNF-α, and anti-inflammatory cytokines – IL-10 and TGF-β). Real-time quantitative reverse transcriptase-PCR was used for the expression study. In the first phase of the study, we examined the diversity of KIR locus in the three populations – Kachari (n=108), Ahom (n=104) and Adivasi (n=101) of Assam, Northeast India. Our KIR data revealed that the KIR complex of the Mundari-speaking Adivasi was distinct from the two Mongoloid populations – Kachari and Ahom of the region. In the next phase of the study, we conducted a case-control study to evaluate KIR/HLA immunogenetic background and NK cell activation status in OSCC patients of the Mongoloid populations. We found two KIR-HLA genotypes – KIR2DL1^{+}-HLA-C2^{+} and KIR2DL3^{+}-HLA-C^{+} to be associated with OSCC. It was noted that presence of KIR2DL3^{+}-HLA-C^{+} genotype was protective in OSCC, particularly in patients with family history of cancer (FHC), while KIR2DL1^{+}-HLA-C2^{+}

genotype appeared to be a heritable risk factor in OSCC, predisposing to cancer at younger age (<45 and 45-54 years). In addition, RNA transcript abundance of the inhibitory KIR2DL1 in peripheral blood of FHC patients, particularly of younger age, supports early onset of disease in FHC patients. Based on the binding and functional studies done by earlier investigators, it can be interpreted that the presence of strong inhibitory KIR2DL1$^+$-HLA-C2$^+$ genotype in OSCC may be deleterious in cancer due to decreased NK cell response to tumour. On the contrary, weaker inhibitory KIR2DL3$^+$-HLA-C$^+$ genotype in OSCC may be anticipated to be protective in oral cancer, possibly because it lowers NK cell activation threshold leading to anti-tumour response. Further, our case-control study on NK cell activation suggested that tumours of OSCC employ different strategies at tissue and systemic level to reduce NK cell activation. In the peripheral circulation, the possible mechanisms involved are: quantitative deficiency of NK cell number and reduced cytotoxicity, and qualitative NK impairments caused by – 1) decreased expression of NK activating receptor NKp46, 2) increased expression of NK suppressive cytokines – IL-10 and TGF-β and 3) induction of FOXP3$^+$CTLA4$^+$ suppressor cells. On the other hand, in the tumour tissue, escape of NK immune surveillance appeared to be modulated by upregulation of TGF-β and IL-10 together with downregulation of NK cell-activating cytokines (IL-2, IL-12β, IL-15, IL-18, IL-21 and IFN-γ) and NK receptors (NKp46 and KIRs). Moreover, our study supported the earlier contention that TNF-α and IL-1β expression levels may be used as markers of malignant transformation in oral leukoplakia. In conclusion, the study indicated immunogenetic modulation of NK cell in oral cancer and provided a potential biomarker (KIR2DL1$^+$-HLA-C2$^+$ with family history of cancer) for stratifying populations at risk for OSCC. In addition, the study provided an insight into the negative regulation of NK cell in tumour tissue and peripheral blood of OSCC patients. Thus, the present study has given useful leads to understand KIR mediated self-tolerance and NK cell activation in OSCC, which can be exploited

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The results embodied in the thesis have not been submitted to any other University or Institution for award of any degree or diploma.

Date: 07th November 2014

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CERTIFICATE OF THE EXTERNAL EXAMINAR AND ODEC

This is to certify that the thesis entitled "Killer cell immunoglobulin-like receptors mediated self tolerance and activation of natural killer cell in oral squamous cell carcinoma" submitted by Mr. Anupam Dutta to Tezpur University in the Department of Molecular Biology and Biotechnology under the School of Sciences in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy in Molecular Biology and Biotechnology has been examined by us and found to be satisfactory.

The Oral Defence Evaluation Committee (ODEC) recommends for the award of the degree of Doctor of Philosophy.

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LIST OF ABBREVIATIONS

AMCH	Assam medical college and hospital
C4	Centromeric 4 KIR gene cluster
CF	Carrier frequency
CI	Confidence interval
C_T	Cycle threshold
CTLA4	Cytotoxic T-lymphocyte-associated antigen 4
Cx	Centromeric 4 KIR gene cluster absent
FHC	Family history of cancer
FOXP3	Forkhead box p3
GMCH	Gauhati medical college and hospital
HLA class I	Human leukocyte antigen class I
HUGO	Human Genome Organization
KIR	Killer cell immunoglobulin-like receptor
NCR	Natural cytotoxicity receptor
NECHRI	North east cancer hospital and research institute
NK cell	Natural killer cell
NKT cell	Natural killer T cell
OR	Odds Ratio
OSCC	Oral squamous cell carcinomas
PB-NK cell	Peripheral blood NK cell
PCR-SBT	PCR-sequence based typing
PCR-SSP	Polymerase chain reaction-sequence specific priming
qRT-PCR	Real-time quantitative reverse transcriptase PCR
RR	Relative Risk
T4	Telomeric 4 KIR gene cluster
TLR	Toll-like receptor
TNM	Tumor, Nodes, Metastasis
Treg cell	Regulatory T cell
TUEC	Tezpur university ethical committee
Tx	Telomeric 4 KIR gene cluster absent
WU Blast tool	Washington university blast tool

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Chapter I

INTRODUCTION

Oral cancer ranks amongst the three most common cancers in India: second most common malignancy diagnosed in men and the fourth most common in women [1, 2]. The World Health Organization has regarded oral cancer as a major public health challenge in India [3, 4]. Approximately, 70,000 new cases and more than 48,000 oral cancer-related deaths occur yearly [3, 5]. Of all the newly diagnosed cases of oral malignancy, 90-95% belongs to oral squamous cell carcinomas (OSCC) [3, 6]. It arises from dysplastic squamous epithelium of the lips, buccal mucosa, gums, front two-thirds of the tongue, floor of the mouth below the tongue and hard palate.

In general, in cancers including oral cancer, alteration in host immunity triggered or enhanced by the genetic variation of immune response genes is a significant step in the multistep process of malignant transformation of self-cell [7, 8]. Further, once the normal cells are transformed into cancerous cells, tumour progression could be linked to a failure of the immune system since the host immune system functions as an extrinsic tumour suppressor by eliminating tumour cells or preventing their outgrowth. This dynamic interaction of tumour and immune system is composed of three distinct phases: elimination, equilibrium and escape [7]. In the elimination phase, also known as cancer immunosurveillance, innate and adaptive immune cells and molecules recognize altered cells and destroy them, resulting in a return to normal physiological tissue. Nevertheless, if host antitumor immunity is unable to eliminate altered cells, surviving tumour variants may enter into the equilibrium phase, where cells and molecules of adaptive immunity prevent tumour outgrowth. Eventually these tumour variants may acquire further mutations that result in the evasion of tumour cell recognition and killing, and progress to clinically detectable malignancies in the escape phase.

Molecular epidemiological studies suggest that polymorphisms in two independent loci of immune response genes – killer cell immunoglobulin-like receptors (KIRs) and human leukocyte antigen (HLA) class I are associated with cancer development [9-14]. KIRs, also referred to as CD158, are a group of

polygenic and polymorphic family of receptors [15] expressed mainly in natural killer cells (NK cells) [16]. They are described as the key regulators for development, tolerance and activation of NK cells [17, 18]. Other than NK cells, natural killer T (NKT) cells, a minor lymphocyte population of T cells also express KIRs [19, 20]. KIRs are encoded by a family of tightly clustered fourteen genes and two pseudogenes (2DP1 and 3DP1) on leukocyte receptor complex at chromosome 19q13.4 [21]. Of the 14 KIR genes, KIR3DL1-3 and KIR2DL1-5 are described as inhibitory KIRs, while KIR3DS1 and KIR2DS1-5 are activating KIRs [21, 22]. Interestingly, KIR2DL4 has also features of activating receptors. The KIR gene complex exhibits extensive diversity for variability in its three components: haplotypic gene content, allelic polymorphism and the combination of maternal and paternal haplotypes [23]. The combined effects of these three components are such that unrelated individuals and populations differ in KIR genotype and KIR gene frequencies respectively [23]. Several studies have verified the KIR diversity in different populations [23-27] and analyzed the genetic relationships among populations from different geographical areas [28, 29]. It has been hypothesized that genetic backgrounds, migration history and length of exposure to pathogens could have bearing on the KIR profile of different populations. However, KIR variability in the Northeast Indians remains poorly studied.

Distinguishing the KIR locus has been the evolution of two distinct groups of haplotypes (A and B) and genotypes (AA and Bx, where x can be A or B) [21, 22, 30, 31]. KIR genotype-AA is homozygous for the A-haplotype and has only genes of the group-A KIR haplotype (3DL3, 2DL3, 2DL1, 2DP1, 3DP1, 2DL4, 3DL1, 2DS4, and 3DL2). In contrast, KIR genotype-BB is homozygous for group B haplotype and has genes of the group-B KIR haplotype (2DS1, 2DS2, 2DS3, 2DS5, 2DL2, 2DL5, and 3DS1) and lacked any of the four A-haplotype associated genes (2DL1, 2DL3, 3DL1, and 2DS4). The AB genotype has all four A-haplotype associated genes (2DL1, 2DL3, 3DL1, and 2DS4), as well as one or more B-haplotype specific genes. Both A and B haplotypes are present in all populations, but at different relative frequencies [32]. Further, KIR

genotypes can also be classified based on linkage disequilibrium as centromeric and telomeric gene clusters [30, 33]. The centromeric half of the KIR gene complex comprises of KIR 2DS2, 2DL2, 2DS3, 2DL5 genes while telomeric half of the complex comprises of KIR 3DS1, 2DL5, 2DS1, 2DS5 genes.

The multigene KIRs interact with their polymorphic HLA -A, -B and -C ligands to diversify and individualise the human immune system [34]. Only a fraction of the HLA -A, -B and -C variants interact with KIRs and these all carry one of four mutually exclusive epitopes (A3/11, Bw4, C1 and C2) [32]. HLA ligands for the inhibitory KIRs are well documented; however, ligands for all the activating KIRs have not been determined [34-37]. Of the inhibitory KIRs, KIR2DL1 recognizes HLA-C2 epitope whereas KIR2DL2 and KIR2DL3 exhibit higher affinity for HLA-C1 epitope [34]. HLA-C1 and HLA-C2 epitopes are characterized by asparagine and lysine respectively at position 80 [34]. HLA-A and HLA-B allotypes having the HLA-Bw4 epitopes are recognized by KIR3DL1. The HLA-Bw4 epitopes are classified based on isoleucine or threonine at position 80 [38]. Further, HLA-B Bw4 80 threonine is dimorphic at position 81, expressing either alanine or leucine. KIR3DL1 binds to Bw4 80 isoleucine variants with higher affinity than those carrying Bw4 80 threonine [38]. All the HLA-A alleles expressing the Bw4 epitope bear isoleucine at position 80 [38].

The two independent loci – KIR and HLA class I segregate independently to produce diversity in terms of number and type of KIR-HLA genotype. Recent studies have explained how these varied KIR-HLA interactions can influence susceptibility/resistance to a variety of diseases, including cancer by influencing NK cell reactivity [39-43]. The diversity in KIR-HLA interactions are the major source of immunogenetic modulation of NK cell biology [32, 44]. This modulation occurs both during the NK cell response and during NK cell development in response to – 1) perturbations in expression of cognate KIR and HLA class I ligands and 2) strength of KIR-HLA interaction

[32]. On NK cell development, the KIR interacts with self-HLA class I ligand to produce functionally responsive mature NK cells through the process called ''education'' [35, 45-48]. Depending on the strength of KIR-HLA interaction, the activation thresholds of these responsive cells are finely tuned such that each NK cell ensures self-tolerance and at the same time shows useful reactivity against potential threats [34, 49].

NK cells are the key immune lymphocytes in solid tumours [50]. They participate in anti-tumour immunity via their ability to – 1) lyse nonself and/ or altered cells without prior sensitization unlike cytotoxic T-cells, and 2) produce cytokines that stimulate and guide the response of other agents of innate and adaptive immune system. Apart from peripheral blood, NK cell subsets can be found to reside in certain tissues, these subsets represent tissue-resident NK cells [51]. The basis for tissue-resident NK cells is not entirely clear. However, recent studies describe these tissue-resident NK cells under steady-state conditions to show minimal tendencies to recirculate [51]. They express markers that are similar to immature conventional NK cells and undergo further maturation on stimulation by inflammatory conditions in the tissue of residence [51].

There is substantial evidence to support the role of lower NK cell effector functions in rapid progression of oral carcinoma [52, 53]. Broadly, NK cell activation is impeded by inhibitory networks promoted by the tumour cells, tumour cell derived factors and immunosuppressive cells (immature dendritic cells, tumour-associated macrophages, myeloid derived suppressor cells and regulatory T cells) [54]. The outcome is an impairment of NK cell function at multiple levels including expression of activating receptors and effector molecules such as cytokines [54].

Apart from activating forms of KIRs (KIR2DS and KIR3DS), the other major lysis receptors in NK cell are the natural cytotoxicity receptors (NCRs), FcγRIIIA (CD16) and NKG2D (CD314). Among these activating receptors, NCRs are the most prominent [55] and include three immunoglobulin-like

proteins: NKp46 (NCR1; CD335), NKp44 (NCR2; CD336) and NKp30 (NCR3; CD337). Although NKp30 and NKp44 have similar cellular functions, NKp46 is structurally distinct from the other two molecules and is located in a different region of the genome [55, 56].

NKp46 is stably expressed on both resting and activated NK cell, and is considered as a unique NK cell marker [55, 56]. Recent studies have demonstrated NKp46 to be critically involved in influenza [57, 58], tumourogenesis [59] and diabetes [60, 61]. NKp46 was described to be the key receptor in controlling the spread of various primary tumours in melanoma, lymphoma and carcinoma [62]. Further, experiments in an NKp46-knockout mouse model raised the possibility that the receptor is essential for controlling both cancer metastasis and influenza infection [57, 62].

Although the ligand(s) for NKp46 remain elusive [62, 63], several previous studies had suggested that the ligands for NKp46 are probably numerous and that the engagement of each may diversify NKp46 functions [62]. The influenza hemagglutinin is a lectin, and its interaction with NKp46 causes direct killing of the infected cells [58, 64], while the other ligands such as the ligand found on 3-methylcholanthrene (MCA)-induced sarcoma, cause indirect killing by cytokine secretion [65].

NK cell activity is not only regulated by its germline encoded 'receptors-ligand' interaction but also by a correct cytokine microenvironment [66]. The prominent cytokines that are related to NK cell include the pro-inflammatory cytokines – Interleukin-1 (IL-1), IL-2, IL-12, IL-15, IL-18, IL-21, interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α), and anti-inflammatory cytokines – IL-10 and transforming growth factor-beta (TGF-β). Of these cytokines, IFN-γ, TNF-α, IL-10 and IL-1 are effector molecules of NK cell [67, 68], while IL-2, IL-12, IL-15, IL-18 and IL-21 are NK cell-activating cytokines [66, 69]. TGF-β and IL-10 are described as negative factors for NK cell activation [70-77]. The cytokines can influence NK cell effector functions either directly or by inducing other immune cells in the

environment. The altered profiles of these cytokines have been implicated in pathogenesis of many diseases including cancer [66, 78-82]. However, the nature of the relationship among these NK cell related cytokines, NK cells and tumour remains to be explored to full extent.

The present study aimed to investigate the role of NK cells in oral cancer, particularly KIR mediated self-tolerance and NK cell activation in OSCC. Accordingly, three objectives were defined – 1) to investigate the polymorphism and expression of KIR genes in relation to OSCC, 2) to verify the cognate HLA-KIR recognition in OSCC and 3) to assess NK cell activation in OSCC. In the first part of the study, we examined the diversity of the KIR loci in three populations – Kachari, Ahom and Adivasi of Assam, Northeast India. Our results demonstrated that the KIR complex of the Mundari-speaking Adivasi was distinct from the two Mongoloid populations – Kachari and Ahom of the region. In the next phase of the work, association of KIR genes and their HLA class I ligands with OSCC was evaluated in the two Mongoloid populations of the region. In this case-control study, two KIR-HLA genotypes – KIR2DL1^{+}-HLA-C2^{+} and KIR2DL3^{+}-HLA-C^{+} were found to be associated with OSCC. The presence of KIR2DL3^{+}-HLA-C^{+} genotype was noted as protective in OSCC, while KIR2DL1^{+}-HLA-C2^{+} genotype appeared to be a heritable risk factor in OSCC. In the last part of the study, NK cell activation was assessed in the OSCC patients in context of – 1) NK cell cytotoxicity and 2) expression of NK receptors and key cytokines related to NK cell. Our data revealed that NK cells were negatively regulated in tumour tissue and peripheral blood of OSCC patients. In conclusion, our study indicated immunogenetic modulation of NK cell in OSCC via diversified interactions of KIR with its HLA class I ligands. In addition, tumours of OSCC employ different strategies at tissue and systemic level to reduce NK cell activation. Further, investigations on KIR complex and HLA class I ligands in the two Mongoloid populations of the region demonstrated their genetic affinity with Oriental populations of East Asia.

Chapter II

REVIEW OF LITERATURE

2.1. THE IMMUNE SYSTEM

Immune system protects human body against invading pathogens and stressed cells such as infected or tumour cells. This immune system is an interactive network of organs, tissues, cells and cell products that integrates to produce effective means to distinguish healthy normal cells from infected and dysfunctional cells and eliminates the nonself recognized as a potential threat by the host. Fundamentally, the immune system is sub-divided into two categories - innate immune system and adaptive immune system. Both innate and adaptive arms of the immune system have defined functions by employing its distinct set of effector mechanisms, which are not mutually exclusive (Figure 1). The innate immune system is the first line of defence which mediates the early protection against infections in a generic way while the adaptive immune system mediates antigen-specific response and takes days or longer to provide defence against infection.

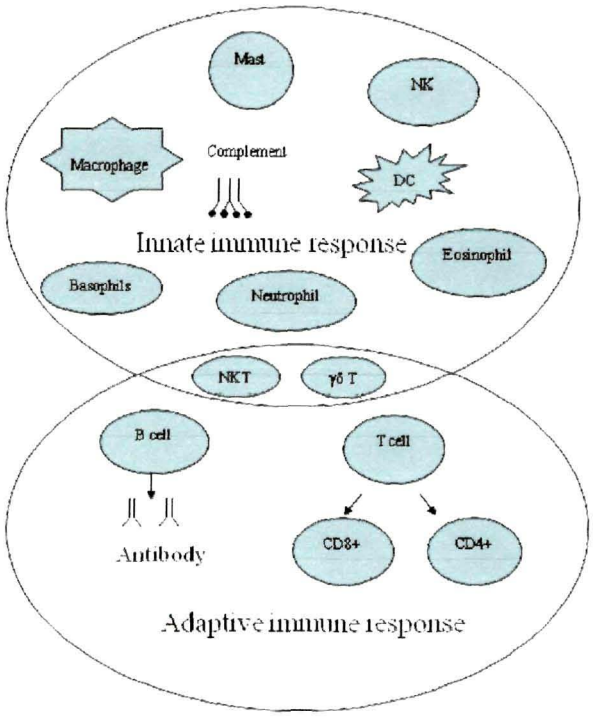

Figure 1. Cells of innate and adaptive immune system [83]

2.1.1. Innate immunity

The elements of the innate immune system include anatomical barriers, soluble molecules and cellular components, summarized in Table 1. Although the innate immune system is non-specific, it has effective recognition systems to discriminate self versus non-self and self versus altered self. Its importance can be understood from the fact that even when the adaptive immune system is intact, defect in any innate immune component, can result in increased susceptibility to disease.

Innate immunity	**Components**
Anatomical barriers	Skin and mucous membranes
Cells	Classical: Phagocytes (Dendritic cells, Macrophages and Neutrophils), Natural killer cells, Mast cells, Eosinophils and Basophils Non-Classical: Natural killer T cells and γδ T cells
Soluble molecules	Complement, Acute phase proteins, Cytokines and Chemokines

Table 1. Elements of innate immune system

2.2. NATURAL KILLER CELLS

Recent work over the last few years have now classified NK cells as the member of a family of hematopoietic effector cells termed innate lymphoid cells (ILCs). Currently, ILCs can be broadly named as ILC1, ILC2 and ILC3 based on the on their phenotype, function, and transcriptional regulation [84]. NK cells belongs to the Group 1 ILCs (ILC1).

NK cells are the key innate immune lymphocytes in solid tumours [50] as these are not HLA restricted unlike cytotoxic T-cells. They circulate throughout the human body and undergo proliferation and maturation in both lymphoid and non-lymphoid tissues (Figure 2) [55, 85]. In peripheral blood, NK cells represent 2-18% of the lymphocytes while in liver it represent up to 50% of the resident lymphocytes [55].

NK cells show phenotypic and functional heterogeneity [85-88]. Based on the differential expression of classical marker – CD56, the human mature NK cells are classified into two distinct subgroups: $CD56^{dim}$ and $CD56^{bright}$ NK cells [86]. These two subgroups differ in their phenotype, functional potential and preferential locations [87]. Most circulating NK cells are $CD56^{dim}$ while the majority of NK cells in peripheral lymphoid tissues are $CD56^{bright}$ [88]. $CD56^{dim}$ NK cells express high amounts of perforin and granzymes and are able to recognize and kill target cells like transformed cells [87]. They also produce cytokines following target cell recognition and killing. In contrast, $CD56^{bright}$ NK cells express very low amounts of cytolytic granules and display poor cytotoxicity [87]. These $CD56^{bright}$ NK cells are commonly termed as the immunomodulatory subset [87] which can extensively proliferate and produce high levels of cytokines in response to stimulation by pro-inflammatory cytokines from other immune cell source.

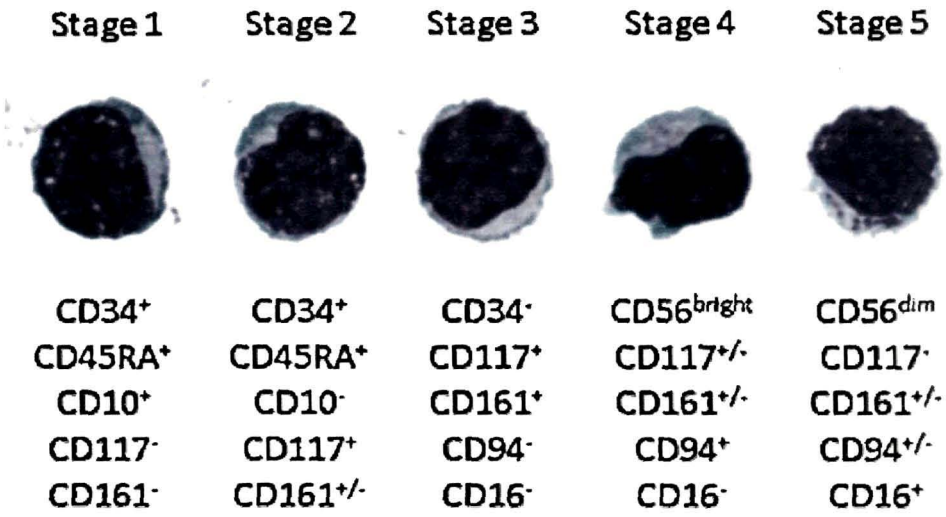

Figure 2. Morphology and immunophenotypes of the stages of human NK cell maturation in secondary lymphoid tissues [85]

2.2.1. NK cell receptors

NK cells express many different receptors that can either trigger activation or mediate inhibition of their effector functions (Figure 3) [69]. Receptors that mediate activation belong to three main families: 1) NK cell activating receptors that recognize specific ligands on the cell surface of target cells, 2) cytokine receptors, and 3) Toll-like receptors (TLRs; some of which are located in phagolysosomes and endosomes but not on the cell surface) [69]. On the other hand, receptors that negatively regulate NK cell functions are: 1) NK cell inhibitory receptors that recognize mainly MHC class I products on the cell surface of target cells and 2) the TGF-β receptor [69].

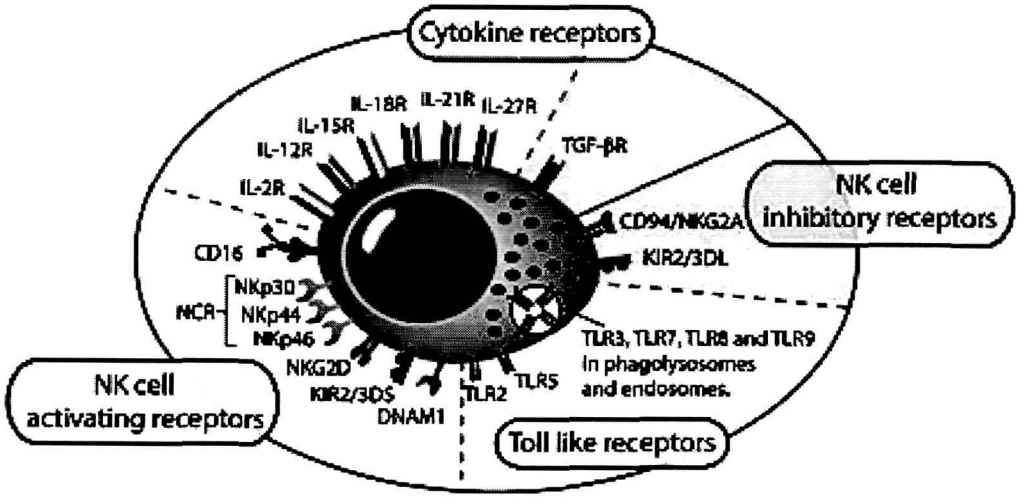

Figure 3. Diagrammatic representation of the categories of receptors that regulate NK cell functions [69]

Further, the NK cell activating and inhibitory receptors that recognize ligands and HLA class I products respectively on the cell surface of target cells are structurally differentiated into two distinct molecules: C-type-lectin-like-molecules and the immunoglobulin (Ig)-like molecules. Both the classes consist of activating and inhibitory molecules, summarized in Table 2.

NK Receptor Family	Molecular Nature	Genetic Complex
Killer immunoglobulin-like receptor	Ig-superfamily	Leukocyte receptor complex (19q13.4)
Immunoglobulin-like transcript / Leukocyte Ig-like receptor	Ig-superfamily	Leukocyte receptor complex
CD94/NKG2 -A, -B, -C, -D, -E, -F and -H (Killer cell lectin-like receptor)	C-type lectin-like	Natural Killer Gene Complex (12p12.3 - 13.2)
Natural cytotoxicity receptor	Ig-superfamily	NKp46: Leukocyte-receptor complex; NKp44 and NKp30: chromosome 6.

Table 2. NK cell receptor families (from online human gene database - www.genecards.org)

2.2.2. Killer cell immunoglobulin-like receptors

Killer cell immunoglobulin-like receptors (KIRs) are a group of polygenic and polymorphic family of receptors [15] expressed mainly in NK cells [16]. Other than NK cells, natural killer T (NKT) cells, a subset of T cells also expresses KIR [19, 20].

The KIR multigene family consists of fourteen KIR genes and two pseudogenes [22]. The Human Genome Organization (HUGO) nomenclature system is routinely followed to describe KIR genes. In this nomenclature system, genes are grouped according to whether they have two domains (2D) or three extracellular domains (3D) in addition to whether they have a short (S) or long (L) cytoplasmic tail (Figure 4) [22]. Of the 14 KIR genes, KIR3DL1-3 and KIR2DL1-5 are described as inhibitory KIRs, while KIR3DS1 and KIR2DS1-5 are activating KIRs. KIRs 2DP1 and 3DP1 represent the two pseudogenes [21, 22].

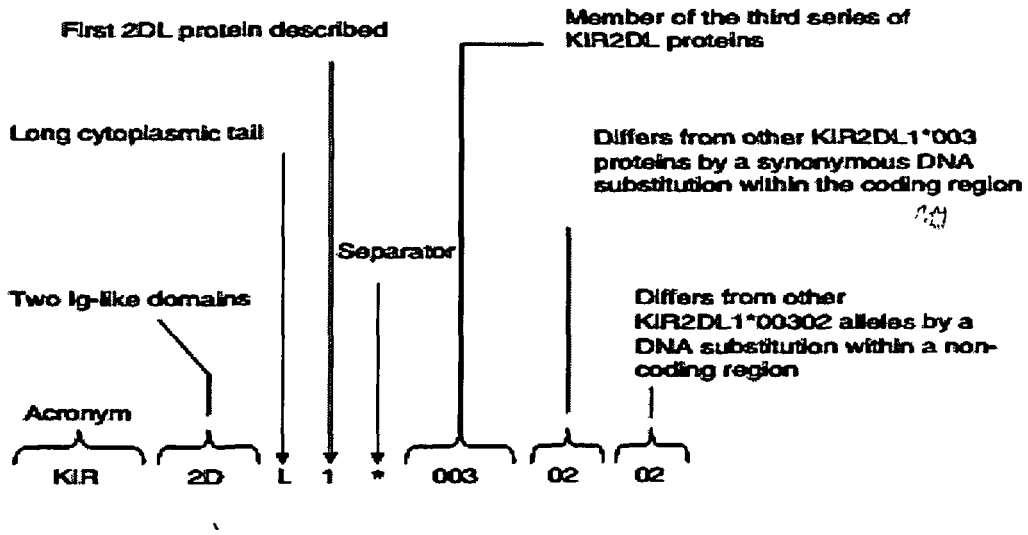

Figure 4. Nomenclature of KIR genes and alleles based on Human Genome Organization (HUGO) nomenclature system [22]

The inhibitory and activating KIRs contain immunoreceptor tyrosine-based motifs (ITIM/ITAM) to deliver inhibition/activation signals respectively to NK cells [89]. ITIM phosphorylation results in association with SHP (Src-

homology domain-bearing tyrosine phosphatase), which specifically inhibits the proteins involved in the intracellular activation cascade [21, 90]. On the other hand, ligation of activating KIR leads to phosphorylation of DAP12 (ITAM-bearing adaptor molecule), recruitment of Zeta-chain-associated protein kinase 70/Spleen tyrosine kinase and the induction of an intracellular signalling cascade [21, 91, 92].

The KIR gene cluster spans a region of approximately 150 kb within the leukocyte receptor complex at chromosome 19q13.4 [21]. The genes are organised in head to tail fashion and each gene is roughly 10-16 kb in length [21]. KIRs with three immunoglobulin domains (KIR3DL1-2 and KIR3DS1) are organized in nine exons, except KIR3DL3 where exon 6 is absent (Figure 5) [15, 93]. In KIR2DL1-3 and KIR2DS1-5, exon 3 is a pseudo exon, while in KIR2DL4-5 exon 4 is completely missing [21]. Between the two KIR pseudogenes, KIR2DP1 contains two pseudo exons (Exons 3 and 4) and KIR3DP1 is severely truncated [21].

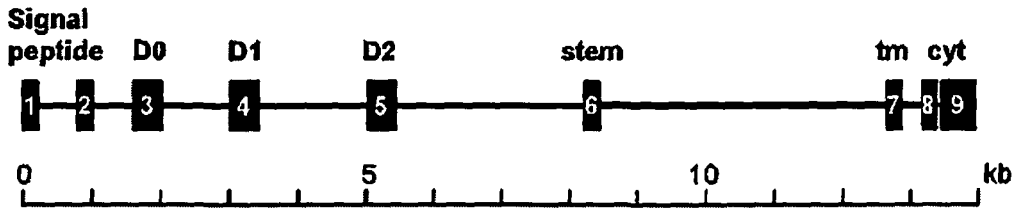

Figure 5. Organization of the KIR3DL1 gene. Exons (boxes) and introns (lines) are represented approximately to scale [15, 93]. *Exons 1 and 2 encode signal peptide plus the first two amino acids of the mature polypeptide; Exons 3, 4, and 5 encode Ig-like domain D0, D1, and D2 respectively; Exon 6 encodes linker that connects the D2 domain to the transmembrane region encoded by exon 7; Exons 8 and 9 encode the cytoplasmic tail.*

2.2.3. KIR haplotypes and genotypes

KIR gene complex exhibits variability in the quantity and quality of genes [21, 93] (Figure 6). The extensive diversity is sum of three components:

haplotypic gene content, allelic polymorphism, and the combination of maternal and paternal haplotypes [23]. The combined effects of these three components are such that unrelated individuals usually differ in KIR genotype and ethnic populations have widely differing KIR genotype frequencies [23]. Despite considerable variations in gene number and content as well as allelic polymorphism, two distinct forms of haplotypes (A and B) and genotypes (AA and Bx, where x can be A or B) can be distinguished based on KIR gene content [21, 22, 30, 31]. KIR genotype – AA is homozygous for the A-haplotype and has only genes of the group-A KIR haplotype (3DL3, 2DL3, 2DL1, 2DP1, 3DP1, 2DL4, 3DL1, 2DS4, and 3DL2). In contrast, KIR genotype – BB is homozygous for group B haplotype and has genes of the group-B KIR haplotype (2DS1, 2DS2, 2DS3, 2DS5, 2DL2, 2DL5, and 3DS1) and lacked any of the four A-haplotype associated genes (2DL1, 2DL3, 3DL1, and 2DS4). The AB genotypes have all four A-haplotype associated genes (2DL1, 2DL3, 3DL1, and 2DS4), as well as one or more B-haplotype specific genes. Further, KIR genotypes can also be classified based on linkage disequilibrium as centromeric and telomeric gene clusters [30, 33]. The centromeric half of the KIR gene complex comprises of KIR 2DS2, 2DL2, 2DS3, 2DL5 genes while telomeric half of the complex comprises of KIR 3DS1, 2DL5, 2DS1, 2DS5 genes.

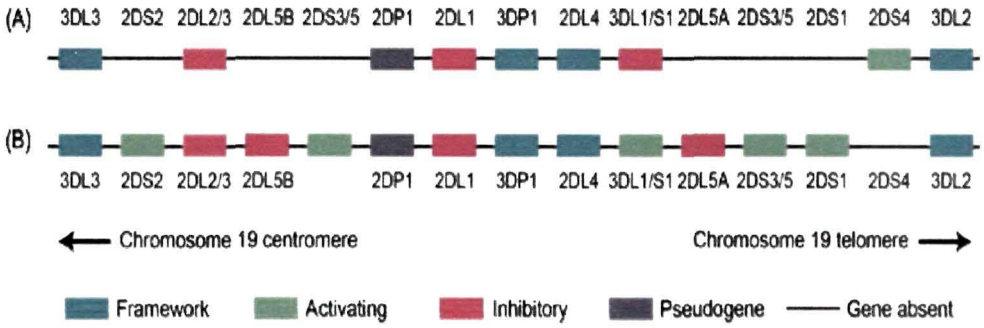

Figure 6. A map of human KIR gene locus on chromosome 19q. *The two haplotypes - A (top) and B (bottom) are illustrated. Adapted from http://www.ebi.ac.uk/ipd/kir/sequenced_haplotypes.html*

Studies on KIR gene complex in different populations provide valuable information about the history and geographic distribution of human genes [94]. Several studies have verified the KIR diversity in different populations [23-27] and analyzed the genetic relationships among populations from different geographical areas [28, 29]. It has been hypothesized that genetic backgrounds, migration history and length of exposure to pathogens could have bearing on the KIR profile of different populations.

2.2.4. KIR ligands

Crystallographic study of three-dimensional structure of KIR and its ligand had revealed that KIR interacts with the upper face of the HLA class I molecule, which comprises tops of the α1 and α2 domains and the peptide bound between them [32]. Its recognition is restricted to residues 7 and 8 of the bound peptide [32]. Further, its recognition is also restricted to four epitopes of HLA-A, -B, and -C (A3/11, Bw4, C1 and C2) which are determined by polymorphisms within residues 79-83 of the α1 helix.

HLA ligands for the inhibitory KIRs are well documented. However, ligands for all the activating KIRs have not been determined (Table 3) [34-37]. Of the inhibitory KIRs, KIR2DL1 recognizes HLA-C2 epitope whereas KIR2DL2 and KIR2DL3 exhibit higher affinity for HLA-C1 epitope. HLA-C1 and HLA-C2 epitopes are characterized by asparagine and lysine respectively at position 80. HLA-A and HLA-B allotypes having the HLA-Bw4 epitopes are recognized by KIR3DL1. The HLA-Bw4 epitopes are classified based on isoleucine or threonine at position 80 [38]. Further, HLA-B Bw4 80 threonine is dimorphic at position 81, expressing either alanine or leucine. KIR3DL1 binds to Bw4 80 isoleucine variants with higher affinity than those carrying Bw4 80 threonine. All the HLA-A alleles expressing the Bw4 epitope bear isoleucine at position 80 [38].

The inhibitory KIRs specific for the HLA -C1 (KIR2DL2/3), -C2 (KIR2DL1) and -Bw4 (KIR3DL1) epitopes are all highly polymorphic [32]. The

amino acid differences that distinguish the KIR variants can affect the avidity and/or specificity of the ligand-binding site, the level of cell-surface expression, the frequency of cellular expression, and the capacity for signal transduction [32, 95].

The combinations of KIR and its HLA class I ligand produce diversity in terms of number and type of KIR-HLA genotype to influence NK cell reactivity [40]. This receptor-ligand interaction therefore becomes an attractive target for disease association studies [39]. Such studies help to identify populations at higher risk of disease and identify molecular markers associated with susceptibility/resistance to disease. Previous studies have reported association of KIR [96-98] and KIR-HLA receptor-ligand combinations [41-43] with several diseases including cancer.

Inhibitory KIRs	Ligand(s)	Effect of receptor/ligand interaction
2DL1	C2 epitope	Inhibition
2DL2/2DL3	C1/C2 epitope; few HLA-B (C1 epitope)	Inhibition
2DL4	HLA-G	Inhibition/IFNγ induction
2DL5	Unknown	Inhibition
3DL1	HLA-B and HLA-A ($Bw4^+$)	Inhibition
3DL2	HLA-A3, -A11	Inhibition
3DL3	Unknown	Unknown
Activating KIRs	Ligand(s)	Effect of receptor/ligand interaction
2DS1	C2 epitope (weaker than 2DL1)	Activation (cytokine/cytotoxicity)
2DS2	HLA-C1 (weak), HLA-A*11:01	Activation
2DS3	Unknown	Unknown
2DS4	HLA-A11; some C1 and C2 HLA-C	Activation
2DS5	Unknown	Unknown
3DS1	Unknown	Unknown

Table 3. KIRs and their ligands [34-37]. *The ligands for some of the KIRs are unknown*

2.2.5. NKp46 receptor

NKp46 (NCR1, CD335) is the key NK activating receptor belonging to the natural cytotoxicity receptors (NCRs) family [99]. Although the other two NCRs – NKp30 (NCR3) and NKp44 (NCR2) have similar cellular functions, NKp46 is structurally distinct from the other two molecules and is located in a different region of the genome [55, 56]. It is more stably expressed on all resting and activated human NK cells [55, 56]. This group of NK cell receptors is defined as NCR because of their ability to strongly activate NK cell cytolytic activity [36, 100].

The structure of NKp46 consists of two extracellular C2-type Ig-like domains, a trans-membrane region, and a short cytoplasmic tail [101]. The receptor acts by complexing with intracellular signalling molecules, such as CD3ζ and FcεRIγ, which contain immunoreceptor tyrosine-based activation motifs that initiate signal-transduction cascades resulting in NK cell activation [102].

The surface expression of NKp46 correlates with NK cytolytic activity [103]. Recent studies have demonstrated NKp46 to be critically involved in protection against influenza [57, 58], tumourogenesis [59], and diabetes [60, 61]. In melanoma, lymphoma and carcinoma NKp46 was described to be the key receptor in controlling the spread of various primary tumours [62]. Further, experiments in an NKp46-knockout mouse model raised the possibility that the receptor is essential for controlling both cancer metastasis and immunity to influenza [57, 62].

Although the ligand(s) of NKp46 remain elusive (Table 4) [62, 63], several studies have suggested that the ligands for NKp46 are probably numerous and that the engagement of each may diversify NKp46 functions [62]. The influenza hemagglutinin is a lectin, and its interaction with NKp46 causes direct killing of the infected cells [58, 64], while the other ligands, such as the ligand found on 3-methylcholanthrene (MCA)-induced sarcoma, cause indirect killing by

cytokine secretion [65]. Finally, recently it was demonstrated that upon human cytomegalovirus infection of dendritic cells, an unknown ligand of NKp46 is downregulated to prevent NK cell-mediated lysis of the infected dendritic cells [104-107].

The expression of the NK activating receptors can be manipulated for the control of tumour formation [62]. Manipulation of NKp46 expression might be particularly important since there is no tumour-beneficial activity with regard to NKp46. Therefore, earlier investigators have suggested that enhancing NKp46 activity either through the elevation of its expression or by cytokine activation might be beneficial for the treatment of tumour metastasis [62].

Activating NK receptor	Ligand(s)	Ligand(s) expression on nontransformed cells	Ligand(s) expression on tumor cells
NKp46	Unknown[a]	Pancreatic β-cells, stellate cells (liver)	Highly expressed on a variety of tumor cell lines
	HSPG[b]	All cells	Upregulated/modified in tumor cells
NKp30	BAT3[c]/BAG6[d]	Monocyte-derived DC in response to stress/activation (released in exosomes)	Upregulated in tumor cells (released in exosomes): Raji (Burkitt lymphoma), 293T (human embryonic Kidney transformed cells)
	B7-H6	Not expressed (at steady state). Expressed on monocytes and neutrophils in response to TLR ligands or proinflammatory cytokines	Highly expressed in carcinomas, leukemias, lymphomas, melanomas
	HSPG[b]	All cells	Upregulated/modified in tumor cells
NKp44	MLL5[e] isoform	Not expressed	Highly expressed on several tumor cells of hematopoietic and nonhematopoietic origin: erythroleukemia; B-lymphoma; T-cell leukemia; cervical carcinoma; melanoma; kidney carcinoma; bladder carcinoma;
	HSPG[b]	All cells	Upregulated/modified in tumor cells

Table 4. NCR receptor-ligand pairs involved in NK cell-mediated cell killing [62, 63]

a) The expression of the ligand has been suggested by its reactivity with human recombinant NKp46-Fc chimera and/or by mAb-mediated blocking of the

receptor in functional assays. b) HSPG: heparan sulfate proteoglycans. c) BAT3: HLA-B-associated Transcript 3. d) BAG6: BCL-2-associated athanogene 6. e) MLL5: Mixed-lineage leukemia-5.

2.2.6. NK cell cytolysis

Activated NK cells can kill target cells by a variety of mechanisms, one being the direct release of cytoplasmic granules (containing membrane-disrupting proteins called perforin and a family of serine protease called granzymes), which induce apoptosis in the target cell by activating caspases. This is probably the main pathway used by NK cells to eliminate virally infected or malignant cells [108]. These granules in human NK cells also contain another membrane-damaging protein called granulysin that kills bacteria, fungi and mycobacteria [109].

Death receptor-mediated apoptosis is another mechanism by which NK cells can kill target cells. NK cells express Fas ligand (FasL) and TNF-related apoptosis inducing ligand (TRAIL) upon activation [110, 111]. When these ligands are bound by their receptors expressed on target cells, apoptosis is induced. NK cells may further mediate antibody-dependent cellular cytotoxicity (ADCC) through binding of CD16 (FcγRIII) to the Fc portion of IgG antibodies [112].

2.2.7. NK cell and cytokines

Cytokines are soluble low-molecular-weight proteins (~10-30 kDa) which mediate cellular interactions between immune cells [113]. The biological functions of these small soluble protein include induction of the inflammatory response; cellular proliferation, differentiation and activation; development of cellular and humoral responses and regulation of haematopoiesis. They most often exert their effects locally in an autocrine/paracrine fashion on target cells expressing the corresponding receptor [113].

NK cells are potent source of IFN-γ, but depending on the nature of the stimuli they can also produce TNF-α, IL-1, IL-10, IL-13, IL-5 and granulocyte macrophage-colony stimulating factor (GM-CSF) [67, 68, 113]. IFN-γ functions as an activator of effector cells of the immune system. It can regulate the Th1 subset (of helper T cells) response, activate dendritic cells and macrophages and has anti-proliferative effects on virally infected and malignant transformed cells [18]. On the other hand, NK cells are targeted by several cytokines such as 1) TGF-β and IL-10 to inhibit their activity [70-77], and 2) IL-2, IL-12, IL-15, IL-18, IL-21, and IFN-α/β to promote their development and effector functions (Table 5) [66, 69]. The cytokines influence the activation state of NK cells by augmenting or diminishing the threshold required for triggering NK cell activity [66].

Cytokine	Cytotoxicity	Cytokine production	Proliferation	Survival	Receptor expression
IL-2	Increase	Increase	Increase	Increase	Increase (NKG2D; NCR and KIR)
IL-12	Increase	Increase	Increase	No change	Increase (NKp46); Decrease (CD16) and No change (KIR)
IL-15	Increase	Increase	Increase	Increase	Increase (NKp46)
IL-18	No change	No change	No change	Increase	
IL-21	Increase	Increase	Increase	Increase	Increase (NKp46 and NKG2D); No change (NKp44; KIRDL1 and KIRDL2/3)
IFN- α / β	Increase	Increase		No change	

Table 5. Summary of human NK cell responses to different cytokines [69]

2.2.8. NK stimulating cytokines

2.2.8.1. Interleukin-2 and Interleukin-15

IL-2 is primarily produced by activated T cells, while activated dendritic cells and macrophages produce IL-15 [113]. Both IL-2 and IL-15 have positive functional effects on NK cells to enhance its antitumor responses [66, 114-117]. The signal through the IL-2/15 receptor stimulates NK cell development and homeostasis, induces proliferation, costimulates cytokine production and enhances cytotoxic effector mechanisms [66, 114-117]. The downstream signalling of IL-2/15 receptors has been extensively characterized which includes activation of Janus kinase (JAK) 1/3 and Signal transducer and activator of transcription (STAT) 3/5, the Phosphatidylinositide 3-kinases (PI3K) pathway, the Mitogen-activated protein kinases (MAPK) pathway, and ultimately Nuclear factor-kappa B (NF-κB) [66].

IL-2 has been extensively used in cancer clinical trials [115, 116], however response of the patients was unexpectedly low. It has been suggested that this may be due to simultaneous induction of regulatory T cells (Treg cells), which can limit NK cell responses [66]. IL-15 has also entered clinical trials and has promised to modulate NK cells in the absence of Treg induction [66]. Recently, these cytokines are being used in ex vivo activation and/or expansion of NK cells for adoptive immunotherapy [66].

2.2.8.2. Interleukin-12

IL-12 was originally identified as "NK cell stimulatory factor (NKSF)" based on its ability to enhance NK cell cytotoxicity [66, 118]. Upon encounter with pathogens, activated dendritic cells and macrophages release IL-12. IL-12 receptor transduces signal through activation of Janus family kinases (JAK2 and Tyrosine kinase 2) and STAT family members (STAT3, STAT 4, and STAT5), which triggers cytokine secretion and cytotoxicity by NK cells [66, 69, 118-120]. It has been observed that IL-12 is also involved in NK cell proliferation

and upregulation of NK cell receptor such as NKp46, without affecting the expression of the inhibitory KIR3DL1 receptor, thus facilitating target cell recognition [69, 121].

Notably, the IL-12 receptor is expressed on resting NK cells to facilitate rapid immune responses without prior activation [66, 120]. However, recent evidence provided by in vitro and in vivo studies of synergism between IL-12 and other activating stimuli suggests that IL-12 likely acts on NK cells in concert with other cytokines, such as IL-2 and IL-18, or with receptor-based interactions from pathogenic cells [66, 120-122].

2.2.8.3. Interleukin-18

IL-18 is also secreted by activated phagocytes [66]. Unlike other cytokines that transduce signals through the JAK-STAT pathway, the IL-18 receptor primarily transduces signals through the adapters - MyD 88 and TNF receptor associated factor 6 to activate MAPK and NF-kB, although minor activation of STAT3 has been reported [66]. In NK cells, IL-18 has traditionally been described as a costimulatory cytokine that functions synergistically with IL-12 and IL-15 [66, 123, 124]. However, it is involved in stimulation of the crosstalk of NK cells with dendritic cells [125], and protection of NK cells against self-destruction through inhibition of caspase 3 and upregulation of the prosurvival molecules Cellular inhibitor of apoptosis 2 and TRAF1 [69, 126].

2.2.8.4. Interleukin-21

The IL-21 receptor is predominantly expressed on T, B, and NK cells and binds to IL-21 produced by dendritic cells and T cells [66, 127]. It signals through activation of JAK1/JAK3 and STAT1, STAT3, and STAT5. STAT3 has been shown to be the dominant transducer following receptor engagement [66, 128]. Minor engagement of the PI3K and MAPK pathways by STAT-independent activation has also been reported [66, 128]. This cytokine stimulates

NK cell survival, cytotoxicity, and IFN-γ secretion [69, 129-132]. It also exhibits proliferative effect on human NK cells without promoting IFN-γ secretion [69, 133]. Further, it also regulates the expression of several NK cell receptors such as activating NKG2D receptor in human cells [69, 134].

2.2.8.5. Interferon-alpha / beta

IFN-α / β are produced during viral infections mainly by plasmacytoid dendritic cells [69]. IFN- α transduce signals through JAK1 and different STATs (STAT1/STAT2/STAT4). Activation of STAT1 and STAT2 regulates IFN- α - mediated cytotoxicity, whereas activation of STAT4 promotes IFN-γ secretion [69, 135]. In addition, IFN- α stimulates NK cell proliferation [136] and NK cell-mediated cytotoxicity due to an upregulation of perforin and FasL (CD95) ligand [69, 135, 137-140]. Notably, endogenous type I IFN plays an important role in the homeostasis of NK cells [69, 141]. However, it has been shown that type I IFNs can inhibit IFN-γ secretion by NK cells, suggesting that it's signalling can occur through different intracellular signalling pathways and may be critical for the regulation of the intensity of the immune responses to viral infections [69, 142].

2.2.9. NK inhibiting cytokines

2.2.9.1. Transforming growth factor-β

TGF-β is a member of the TGF-β superfamily that consists of members such as TGF-β 1, TGF-β 2 and TGF-β 3, bone morphogenetic protein, inhibins, and activins [143, 144]. The TGF-β superfamily signals through the TGF-β receptors – TGF-βRI and TGF-βRII which dimerize and function as a serine-threonine kinase upon ligand binding, resulting in the phosphorylation of Smad2, followed by a signal cascade [143, 144]. TGF-β is an immunosuppressive cytokine that affects many immune cell types including NK cells [70-77]. It controls the homeostasis of NK cells and suppresses their cytokine production and cytolytic activity [145, 146].

TGF-β inhibits IFN-γ production by NK cells stimulated with IL-2 [145, 147, 148]. It has been shown that stimulation of TGF-β-resistant NK cells produces higher IFN-γ production, which promotes Th1 subset differentiation [145, 146]. In another study, TGF-β was suggested to be a negative regulator of NK cell production and proliferation in $CD11c^{dnR}$ (transgenic mice expressing a dominant negative form of TGF-β receptor type II under the control of the promoter of CD11c) mouse model [149].

The inhibition of NK cell cytolytic function by TGF-β was demonstrated nearly two decades ago. In a recent study, microRNA-183 has been described as a key factor in TGF-β – mediated immunosuppression [150]. The study revealed that TGF-β induces microRNA -183 to repress DAP12 transcription/translation, which is critical for surface NK receptor stabilization and downstream signal transduction. The same study reported that reduced levels DAP12 were accompanied by loss of surface expression of activating killer Ig-like receptor 2DS4 and NKp44. Also, the reduced DAP12 expression in NK cells was associated with lung cancer. In HBV-infected patients, it was reported that TGF-β downregulates expression of NKG2D- and 2B4- activating receptors on NK cells together with DAP10 and SAP (Signalling lymphocytic activation molecule-associated protein), the intracellular adaptor proteins of NKG2D and 2B4 respectively, to impair NK cell-mediated cytotoxicity and IFN-γ production [151]. Thus, TGF-β can influence NK cytolytic functions by regulating the expression of NK activating receptors.

2.2.9.2. Interleukin-10

IL-10 is produced by Th0 and Th2 subsets of helper T cells, $CD5^+$ B cells, thymocytes, ketatinocytes, and macrophages that regulates the function and/or development of both lymphoid and myeloid cells [152-157]. The functionally active IL-10 receptors are composed of two distinct subunits (IL-10 receptor α and IL-10 receptor β) belonging to the class II cytokine receptor family [152, 158]. The engagement of the IL-10 receptor activates the JAK-STAT

signalling pathway [152]. Specifically, IL-10 effects the activation of JAK1 (associated with the IL-10 receptor α chain) and Tyk2 (associated with the IL-10 receptor α chain) and induces the activation of STAT1, STAT3, and, in some cells, STAT5 [152, 159-163].

IL-10 is a cytokine with potent immunoregulatory functions [164]. It is known to reduce the secretion of IFN-γ by NK cells and to reduce NK activity in cancer patients. In gastric cancer, decreased cytotoxic function of NK cell is reported to be accompanied by increased serum levels of IL-10 [165]. Further, it has been described that IL-10 and TGF-β can induce regulatory T cell that suppresses the functions of effector immune cells [166].

2.2.10. NK cell education

In a process called education, also known as licensing, NK cell activation threshold is tuned and is educated on self-cell (Figure 7) [167]. The interaction of constitutively expressed self-HLA class I molecules with specific receptors on NK cell surface plays a major role in fine-tuning NK cell responsiveness. However, these NK cell tuning exhibits plasticity in steady state conditions, meaning that it can be re-set if the HLA class I environment changes [49]. Among the NK cell receptors (NKR), the HLA class I specific KIR are vital in NK cell education [36]. Recent studies have explained how KIR diversity resulting in varied KIR-HLA class I ligand interaction can influence susceptibility to a variety of diseases, including cancer by determining NK cell reactivity [39].

The concepts of arming and disarming NK cells are not mutually exclusive [49]. Many researchers now favour the tuning model, according to which, the responsiveness of each NK cell is quantitatively adjusted depending on net signalling received from the receptors to ensure self-tolerance while at the same time ensuring useful reactivity against potential threats [49]. Ultimately, these receptor-ligand interactions manifest benefits directly as NK specific

recognition or indirectly via effects on their environment and other immune cells [167].

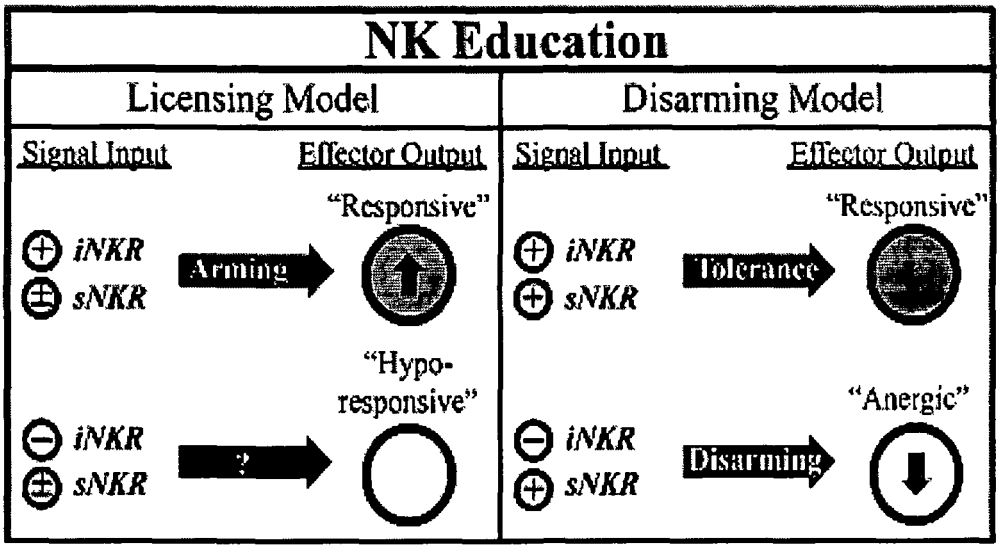

Figure 7. NK cell education [167]. *The balance of signals from inhibitory NKR (iNKR) and activating NKR (aNKR) determines NK cells reactivity. NKs that do not receive inhibitory signals are generally less responsive to sNKR stimulation (in terms of cytokine production and cytotoxicity) than NK that receive iNKR input. However, whether this occurs via a licensing mechanism, disarming mechanism or both is still not fully worked out.*

2.3. NATURAL KILLER T CELLS

Natural killer T (NKT) cells are the subsets of T cells that exhibit phenotypic and functional characteristics of both T cells and NK cells [168].They represent only 0.01–2% of human peripheral blood mononuclear cells [169], but are equipped to link between innate and adaptive immunity [170].

NKT cells are a heterogeneous population which can be further subdivided into three groups- the invariant or type I NKT cells, non-invariant or type II NKT cells and NKT-like cells [171]. The NKT-like cells are one of the critical effectors in the tumour immunosurveillance [172-174], while the type I

NKT cell relies on activation of NK and $CD8^+$ T cells to kill cancer cells [169]. In contrast the type II NKT cells suppress anti-tumour immunity responses [169].

The role of KIR on these NKT cells is less clear, with some studies suggesting that they may inhibit T cell receptor-mediated activation of NKT cells while other reports have suggested that they may function to facilitate activation of NKT cells [175, 176]. Moreover, it is not known whether HLA class I molecules have a KIR-dependent educational effect on human NKT cells function as it has on NK cells [177].

2.4. ORAL SQUAMOUS CELL CARCINOMA

Oral cancer arises from dysplastic squamous epithelium of the lips, buccal mucosa, gums, front two-thirds of the tongue, floor of the mouth below the tongue and hard palate (Figure 8). Among these different sites, the risk of cancerous transformation is greater in the thin non-keratinized epithelia (viz. floor of the mouth and ventral surface of the tongue) than in thick keratinized epithelia (viz. hard palate and the gingiva) [178, 179]. During the earlier form of the disease called carcinoma in situ, the cancer cells are present only in the epithelium, but in the invasive squamous cell carcinoma, the cancer cells grow into the deeper layers of the oral cavity [180]. Presently, of all newly diagnosed cases of oral malignancy, 90–95% belongs to oral squamous cell carcinomas (OSCC) [3, 6].

The origin of the precursor cell for OSCC is uncertain [178]. As in other cancers, they might have arisen from a tissue-specific stem cell or its progenitor cell, which has acquired epigenetic and/or genetic alterations [178]. However, it is necessary that these precursor cells possess the capacity for self-renewal, and hence are capable of initiating and sustaining growth of a cancer. Accordingly, OSCC might comprise of a heterogeneous population of cancer stem cells, cancerous transit-amplifying cells and keratinocytes at different stages of transformation [178, 181-183]. Thus, the overall growth of OSCC is brought about by the multiplication of cells with a cancer stem cell phenotype

and by the uncontrolled proliferation of monoclonal cancerous transit-amplifying cells [178, 182].

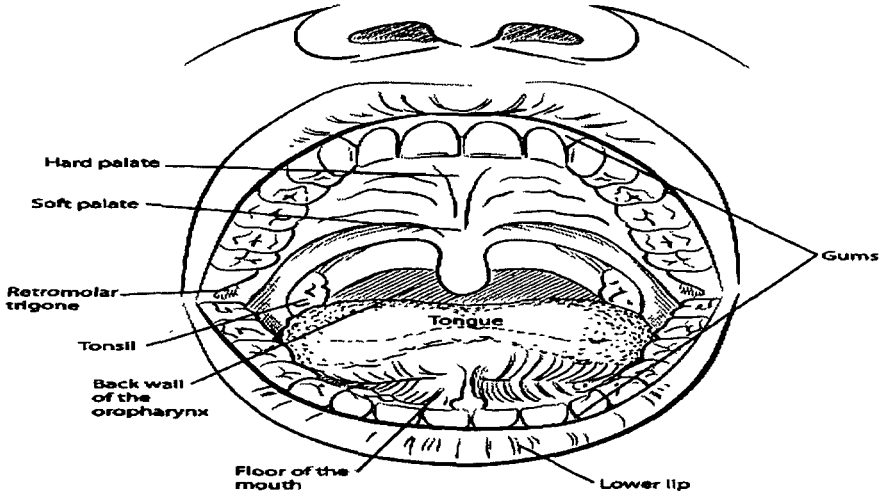

Figure 8. Anatomical sub-sites of human mouth [180]. *It includes the lips, the inside lining of the lips and cheeks (buccal mucosa), the teeth, the gums, the front two-thirds of the tongue, the floor of the mouth below the tongue, and the bony roof of the mouth (hard palate)*

The lip and oral cavity cancer represents the eleventh most common type of cancer in the world [1, 2]. Estimated incidence, mortality and 5-year prevalence of lip and oral cavity cancer, as estimated by GLOBOCAN 2012 [1, 2], for the whole world, East Asia, South East Asia and for India, are summarised in Table 6. It was observed that the global burden of lip and oral cavity cancer has decreased as previously GLOBOCAN 2008 ranked lip and oral cavity cancer as the sixth most common cancer in the world [3].

India has been regarded as the global epicentre of oral cancer because the frequency of oral cancer is high in its large population [3]. It ranks as the third most common cancers in India: second most common malignancy diagnosed in men and the fourth most common in women (Figure 9) [1, 2]. Over five people die from oral cancer every hour every day in India [3]. As cancer registration is not compulsory in India, the actual burden it imposes in terms of

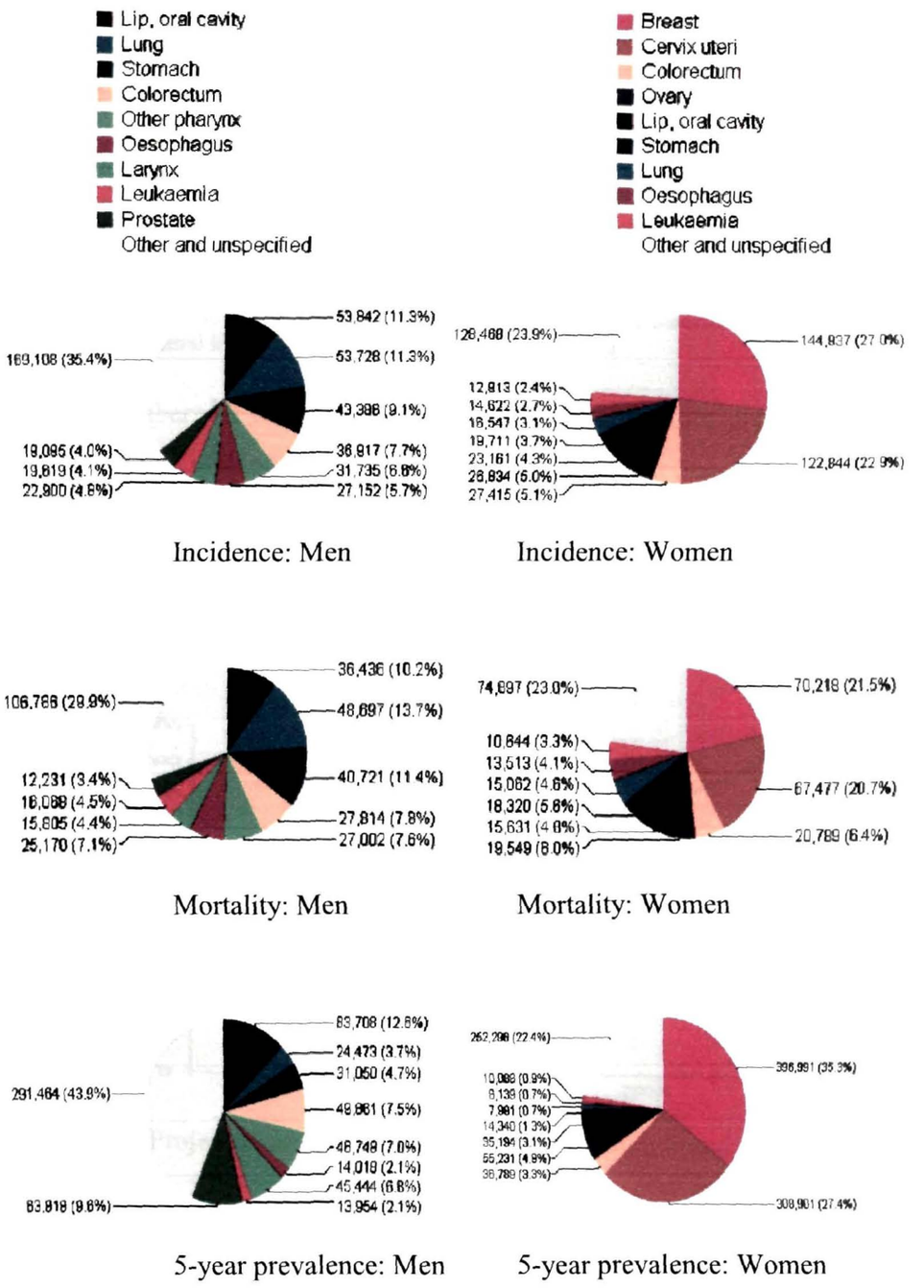

Figure 9. Incidence, mortality and 5-year prevalence rates for the top ten cancers in India. *Data was derived from GLOBOCAN 2012 (http://globocan.iarc.fr/)* [1, 2]

- A lump or thickening in the cheek
- A white or red patch on the gums, tongue, tonsil, or lining of the mouth
- A sore throat or a feeling that something is caught in the throat that doesn't go away

• Trouble chewing or swallowing

• Trouble moving the jaw or tongue

• Numbness of the tongue or other area of the mouth

• Swelling of the jaw that causes dentures to fit poorly or become uncomfortable

• Loosening of the teeth or pain around the teeth or jaw

• Voice changes

• A lump or mass in the neck

• Weight loss

• Constant bad breath

2.4.2. TNM Staging

Histopathologically, OSCC can be categorized into three degrees of differentiation (the degree of differentiation may vary from one part of the tumour to another [186]):

1) Well differentiated disease (shows greater than 75% keratinization)
2) moderately differentiated disease (characterized by 25% to 75% keratinisation) and
3) poorly differentiated disease (demonstrates less than 25% keratinisation)

Cancers of oral cavity like other cancers are staged based on the results of physical and endoscopy exams, biopsies and imaging tests (like CT scan, MRI and chest x-ray). TNM staging system of the American Joint Committee on Cancer (AJCC) is a standard way to describe the extent of oral cavity cancer, where T-describes the size of the tumour, N-describes the extent of spread to nearby lymph nodes and M-indicates whether the cancer has spread to other organs of the body. The numbers 0 through 4 after letters T, N and M indicate increasing disease severity. The T, N and M information is combined by a process called stage grouping to assign an overall stage of – 0, I, II, III or IV (Table 7) [180].

Tumour Stages	Description
Stage 0	
(Tis, N0, M0)	Cancer only in the epithelium
Stage I	
(T1, N0, M0)	Tumour is 2 cm across or smaller (T1) and no spread to nearby structures, lymph nodes (N0), or distant sites (M0)
Stage II	
(T2, N0, M0)	Tumour is larger than 2 cm across but smaller than 4 cm (T2) and has not spread to nearby structures. Also N0 and M0
Stage III	
T3, N0, M0	Tumour is larger than 4 cm across (T3), but it hasn't grown into nearby structures or spread to the lymph nodes (N0) or distant sites (M0)
T1 to T3, N1, M0	Tumour in any size and hasn't grown into nearby structures (T1 to T3). It has spread to one lymph node on the same side of the head or neck, which is no larger than 3 cm across (N1). The cancer hasn't spread to distant sites (M0)
Stage IVA	
T4a, N0 or N1, M0	The tumour is growing into nearby structures (T4a). It can be of any size. It has either not spread to the lymph nodes (N0) or has spread to one lymph node on the same side of the head or neck, which is no larger than 3 cm across (N1). The cancer hasn't spread to distant sites (M0)
T1 to T4a, N2, M0	The tumour in any size and may or may not grow into nearby structures (T1 to T4a). It has not spread to distant sites (M0). It has spread to one of the following: • One lymph node one the same side of the head and neck that is between 3 and 6 cm across (N2a) • One lymph node on the opposite side of the head and neck that is no more than 6 cm across (N2b) • 2 or more lymph nodes, all of which are no more than 6 cm across. The lymph nodes can be on any side of the neck (N2c)
Stage IVB	
T4b, any N, M0	Tumour growing into deeper areas and/or tissues (T4b). It may (or may not) have spread to lymph nodes (any N). It has not spread to distant sites (M0)
Any T, N3, M0	The tumour in any size and it may or may not have grown into other structures (any T). It has spread to one or more lymph nodes larger than 6 cm across (N3), but it hasn't spread to distant sites (M0)
Stage IVC	
Any T, Any N, M1	The tumour in any size, and it may or may not have spread to lymph nodes. It has spread to distant sites, most commonly the lungs

Table 7. TNM staging system of the American Joint Committee on Cancer (AJCC) [180]

2.4.3. Treatment

The main treatment options for diagnosed oral cancer patients are – Surgery, Radiation therapy, Chemotherapy, Targeted therapy and Palliative treatment. These options can be used either alone or in combination, depending on the stage and location of the tumour. In general, surgery is the first treatment for cancers of the oral cavity, and may be followed by radiation or combined chemotherapy and radiation [180].

2.4.4. Risk factors for oral cancer

In Indian populations, tobacco in all its forms, betel quid and alcohol are the established risk factors for oral cancer [3, 180]. The other contributory or predisposing factors include – viral infections (Human Papilloma Virus, Epstein - Barr virus, Herpes Simplex Virus type 1 and Cytomegalovirus), radiation, dietary deficiencies, hormones, physical irritants and age [3, 180].

The modulation of host immunity is also a significant step in the multistep process of malignant transformation of self-cell [7, 8]. The alterations in host immunity may be triggered or enhanced by factors such as genetic variability of genes that control the immune response [7, 8]. Further, once the normal cells are transformed into cancerous cells, tumour progression could be linked to a failure of the overall immune system since the host immune system functions as an extrinsic tumour suppressor by eliminating tumour cells or preventing their outgrowth.

2.5. TUMOURS AND IMMUNE CELLS

Burnet and Thomas in the year 1950 proposed the concept of immune surveillance of cancer [187]. This physiological function would have the ability to recognize tumour cells as altered cells and to destroy them before they develop into detectable tumours [187, 188]. Tumour acquires different mechanisms to escape from immune response. Some of the mechanisms adopted by the tumours are – 1) defect of expression of antigens on the tumour cell surface, 2)

loss or reduction of the expression of HLA class 1 molecules, 3) loss of expression of costimulatory molecules, 4) production of immunosuppressive molecules such as TGF-β, IL-6 and IL-10, 5) the resistance to apoptosis and/or 6) expression of Fas ligand that leads to the death of tumour-infiltrating lymphocytes [187, 189-192].

Histopathological analyses of human tumours have provided evidence that immune cells are located in the core of the tumour, in the invasive margin or in the adjacent tertiary lymphoid structures (TLS) (Figure 11) [193]. These immune infiltrates are heterogeneous between tumour types, and are very diverse from patient to patient [193]. The roles of different subtypes of immune cells in antitumor immunity and tumour-promoting inflammation are summarized in Table 8 [194].

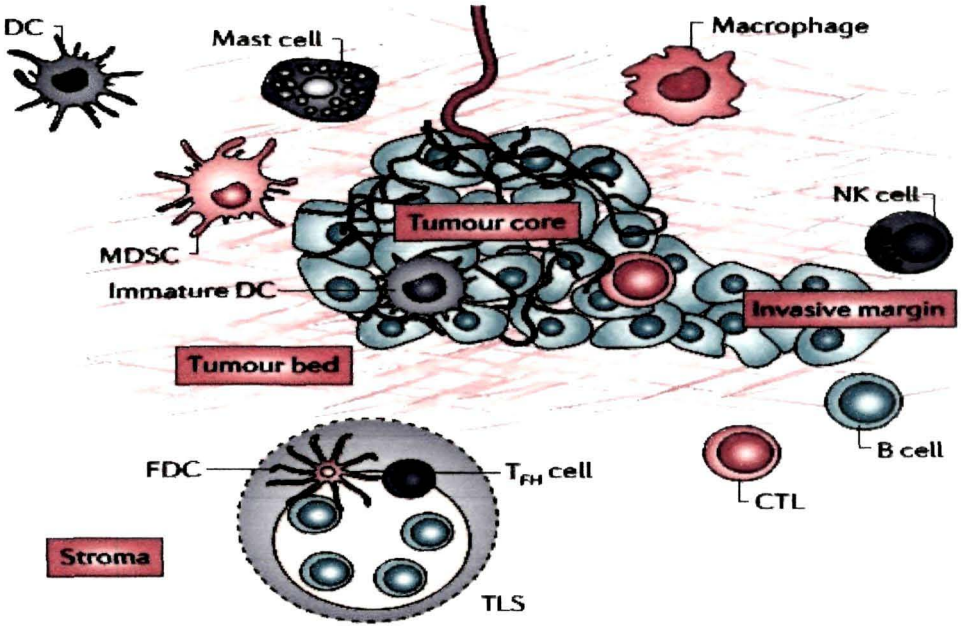

Figure 11. Tumour anatomy showing the features of the immune contexture [193]

Cell Types	Antitumor	Tumor-Promoting
Macrophages, dendritic cells, myeloid-derived suppressor cells	Antigen presentation; production of cytokines (IL-12 and type I IFN)	Immunosuppression; production of cytokines, chemokines, proteases, growth factors, and angiogenic factors
Mast cells		Production of cytokines
B cells	Production of tumor-specific antibodies?	Production of cytokines and antibodies; activation of mast cells; immunosuppression
$CD8^+$ T cells	Direct lysis of cancer cells; production of cytotoxic cytokines	Production of cytokines?
$CD4^+$ Th2 cells		Education of macrophages; production of cytokines; B cell activation
$CD4^+$ Th1 cells	Help to cytotoxic T lymphocytes (CTLs) in tumor rejection; production of cytokines (IFNγ)	Production of cytokines
$CD4^+$ Th17 cells	Activation of CTLs	Production of cytokines
$CD4^+$ Treg cells	Suppression of inflammation (cytokines and other suppressive mechanisms)	Immunosuppression; production of cytokines
Natural killer cells	Direct cytotoxicity toward cancer cells; production of cytotoxic cytokines	
Natural killer T cells	Direct cytotoxicity toward cancer cells; production of cytotoxic cytokines	
Neutrophils	Direct cytotoxicity; regulation of CTL responses	Production of cytokines, proteases, and ROS

Table 8. Roles of different subtypes of immune cells in tumours [194]

2.5.1. NK cells and tumours

NK cells are the primary innate immune cells that are capable of killing non-HLA expressing cancer cells, by releasing cytotoxic proteins such as perforin and granzyme which cause apoptosis in tumour cells [195]. Besides their direct cytolytic effect against tumour cells, NK cells also shape the adaptive arm of the immune response [63, 196-200]. Several previous studies in solid tumours, such as head and neck, lung, gastric and colorectal tumours have shown evidence that a high NK cell infiltration correlates with a better prognosis and improved survival probability [201-205]. However, NK cell effector functions can be evaded by the tumour microenvironment. Broadly, the tumour cells, tumour cell derived factors and tumour-induced aberrant immune cells (i.e. tolerogenic or suppressive macrophages, dendritic cells and T cells) can

interfere with NK-cell activation pathways or the complex receptor array that regulate NK-cell activation to hinder its antitumor activity (Figure 12) [63, 206].

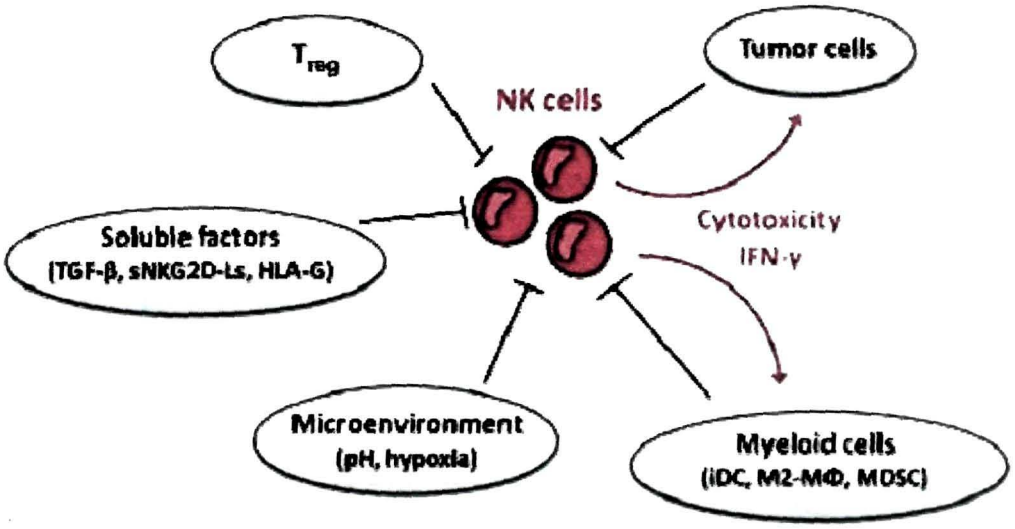

Figure 12. Factors that suppress NK cell effector function in the tumour microenvironment [206]

The tumour microenvironment can favour tumour escape from NK-cell-mediated cytotoxicity by two main mechanisms: 1) Suppression of the effector NK cell function, and 2) evasion through the selection/editing of poorly immunogenic tumour cells [7, 63]. The interactions of various immune cell types with NK cells can potentially improve its antitumor immune responses [63]. The dendritic cells and macrophages promote NK cell activation, whereas neutrophils can favour the terminal differentiation of fully functional NK cells [63, 196, 207-210]. Conversely, NK cells boost the maturation and activation of dendritic cells, macrophages and T cells [63, 196, 207-211]. However, at the tumour site, most immune cells may be altered, as a result of which their interaction with infiltrating NK cells may be ineffective or even lead to NK cell suppression. The evidence of NK cell suppression was demonstrated by tumour-associated immune cells like T regulatory cells, tolerogenic dendritic cells, tumour associated macrophages and myeloid derived suppressor cells, which modulate NK cell function by producing various cytokines. The Treg cells from patients with gastrointestinal sarcoma have been shown to inhibit

NK cells through membrane-bound TGF-β [63, 212, 213]. Further, recent data suggested that both tumour associated macrophages and myeloid derived suppressor cells could modulate NK cell function in patients with hepatocellular carcinoma [63, 214, 215]. The chronic exposure of tumours to NK cells may favour the selection or the induction of tumour cells with altered expression of ligands for either inhibitory or activating NK cell receptors [63]. Accordingly, in a study, the melanoma cells were reported to have increased expression of both classical and non-classical HLA class I molecules to become resistant to NK-cell-mediated lysis [63, 216]. In another study, it was shown that tumours which originated in NKp46-deficient mice displayed a higher expression of the NKp46 ligand as compared with the levels detectable in tumours from wild type mice [63, 65].

In solid and hematologic malignancies, NK cell-based immunotherapy is a promising strategy to enhance or trigger host immune response, particularly when used in combination with chemotherapy, radiation or monoclonal antibody [217]. Earlier studies have reported that antitumor activity of NK cell can be increased by certain antitumor drugs [63]. For example, NK-cell-mediated ADCC is increased by lenalidomide [218]. NKG2D-ligand expression can be upregulated on melanoma cells by dacarbazine [219]. The tyrosine kinases inhibitors – Imatinib and Sorafenib enhance the effector function of NK cells by promoting dendritic cell-mediated NK cell activation, and by modulating macrophage polarization, respectively [215, 220]. Further, NK cell-based immunotherapy can be combined with radiation therapy as irradiation-induced tissue injury increases the expression of NK-activating ligands (e.g., NKG2D ligands) on malignant cells, thereby rendering tumours more susceptible to NK cell cytotoxic activity [217, 221]. Thus, these initial results allow the scientific community to envisage a future scenario in which NK cell-based therapy together with chemotherapy or radiation therapy may result in favourable clinical outcomes in cancer patients.

Chapter III

MATERIALS AND METHODS

3.1. STUDY DESIGN AND PARTICIPANTS

In the first phase of the study, 313 unrelated participants were randomly selected and enrolled from three populations – Kachari (n=108), Ahom (n=104) and Adivasi (n=101) of Assam, Northeast India. Briefly, the individuals of the three ethnic groups were assigned on the basis of linguistic affinity after consultation with ethnolinguistic experts. The 16 KIR genes were typed in these individuals to examine the diversity of KIR complex in the three populations. In the next phase of the thesis, a hospital based case-control study was conducted to understand OSCC in response to – 1) KIR and its ligand diversity and 2) NK cell activation. Clinically and histopathologically confirmed cases of OSCC were recruited from E.N.T. departments of three hospitals of Northeast India - Assam Medical College and Hospital (AMCH), Gauhati Medical College and Hospital (GMCH) and North East Cancer Hospital and Research Institute (NECHRI) (Figure 13). The healthy participants were matched to cases by age (within 3 years), gender, ethnicity and geographic residence. The demographic, clinical and other characteristics of the patients and healthy control participants were recorded in questionnaires through personal interview, medical records and clinical examination by collaborating clinicians of the three respective hospitals. The tumour staging was done according to the American Joint Committee on Cancer (TNM) classification and grouped as early (clinical stages I-II) or advanced clinical stages (clinical stages III-IV) [222]. Histological grades of tumour were classified as - well differentiated, moderately differentiated or poorly differentiated as proposed by World Health Organization [223]. In formation on participant's family history of any type of cancer and relation was recorded. The family members were classified as first-degree relative (parent, sibling, or child), second-degree relative (uncle, aunt, nephew, niece, grandparent, grandchild or half-sibling) or third-degree relative (first cousin, great-grandparent or great-grandchild). Institutional ethical committee-Tezpur University Ethical Committee (TUEC), Northeast India, approved the study. All individuals

voluntarily agreed to participate and provided written informed consent. The inclusion and exclusion criterions were as follows -

Inclusion criteria:

- All newly diagnosed cases of OSCC

Exclusion criteria

- Paediatric age group patients with OSCC
- Patients with other debilitating diseases
- Vulnerable patients like terminally ill/seriously ill, mentally challenged
- Patients with carcinoma in situ, verrucous carcinoma and oro-pharynx cancer
- Metastatic diseases from other parts of the body

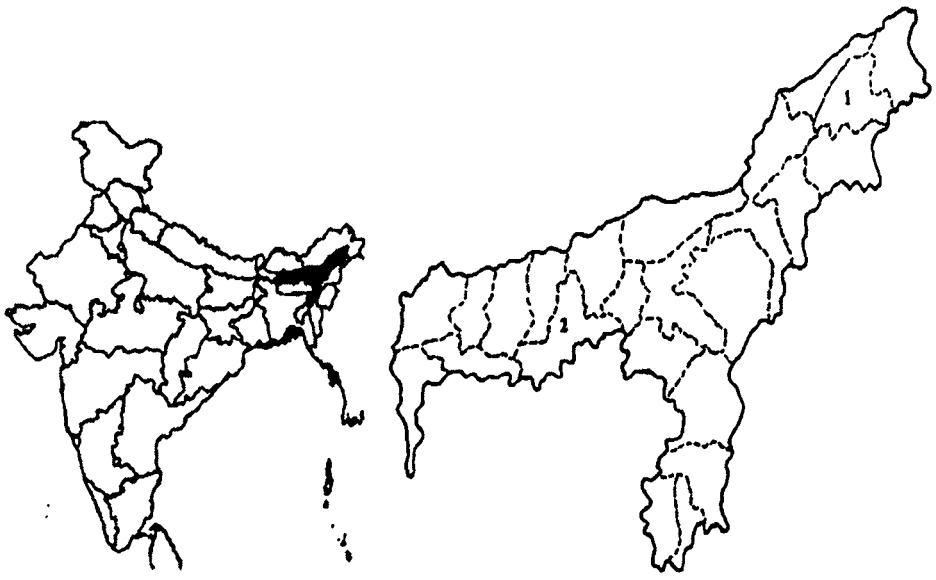

Figure 13. (a) Map of India and (b) Assam state (89° 42′ E to 96° E longitude and 24° 8′ N to 28° 2′ N latitude). *Samples were collected from Assam Medical College and Hospital (AMCH), Dibrugarh (1); Gauhati Medical College and Hospital (GMCH), Guwahati (2); and North East Cancer Hospital and Research Institute (NECHRI), Guwahati (2), as indicated in the map of Assam state*

3.2. KIR POLYMORPHISM STUDY

Briefly, peripheral blood samples (0.5 ml) were collected by venipuncture using standard operative protocol from OSCC patients and control participants by a trained staff. Genomic DNA was isolated from whole blood using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Yield (8-12 ug from 200 ul blood) and the purity (OD_{260}/OD_{280} = 1.6-1.9) of the extracted DNA were checked using NanoVue™ Plus Spectrophotometer (GE Healthcare, Little Chalfont, United Kingdom). The typing of the 16 KIR genes in individuals was done by Polymerase Chain Reaction-Sequence Specific Priming (PCR-SSP) approach [224] where the PCR product defines the presence or absence of KIR genes to evaluate KIR polymorphism on that haplotype (Table 9 and 10). The amplified products were then electrophoresed in 3% agarose gel containing ethidium bromide. DNA ladder of appropriate size was used to size the fragments and the presence of each KIR gene was determined by the presence of a band of DNA of the expected size (Table 11). The validations for PCR amplification were done according to Du et al. [225] using polyposis coli gene and the framework KIR genes as a positive control for each PCR amplification.

PCR	Segment	Temperature conditions
For KIR Set 3, 6 & 8	Initial denaturation	95°C for 3 min
	Cycle 1	7 cycles of 94°C for 15 sec, 66°C for 20 sec and 72°C for 45 sec
	Cycle 2	40 cycles of 94°C for 15 sec, 64°C for 20 sec and 72°C for 45 sec
	Final extension	72°C for 7 min
	Hold	4°C infinity
For KIR Set 4 & 7	Initial denaturation	95°C for 3 min,
	Cycle 1	7 cycles of 94°C for 15 sec, 68°C for 20 sec and 72°C for 45 sec
	Cycle 2	40 cycles of 94°C for 15 sec, 66°C for 20 sec and 72°C for 45 sec
	Final extension	72°C for 7 min
	Hold	4°C infinity
For KIR Set 2	Initial denaturation	95°C for 3 min,
	Cycle 1	7 cycles of 94°C for 15 sec, 68°C for 20 sec and 72°C for 30 sec
	Cycle 2	35 cycles of 94°C for 15 sec, 66°C for 20 sec and 72°C for 30 sec
	Final extension	72°C for 7 min
	Hold	4°C infinity
For KIR Set 1 & 5	Initial denaturation	95°C for 3 min,
	Cycle 1	7 cycles of 94°C for 15 sec, 63°C for 20 sec and 72°C for 45 sec
	Cycle 2	40 cycles of 94°C for 15 sec, 61°C for 20 sec and 72°C for 45 sec
	Final extension	72°C for 7 min
	Hold	4°C infinity

Table 9. Temperature conditions for KIR genotyping by duplex SSP-PCR [224]

Master mix (15 µl reaction volume)	
Composition	**Concentration/Reaction**
10X PCR buffer	1X
$Mgcl_2$	2 mM
dNTP's	200 µM
Forward primer for KIR2DL1	0.6 µM
Reverse primer for KIR2DL1	0.6 µM
Forward primer for KIR3DS1	0.6 µM
Reverse primer for KIR3DS1	0.6 µM
Taq polymerase	2 units
DNA	100 ng
H_20	Adjusting volume

Table 10. PCR master mix composition for KIR genotyping (Set 1) by duplex SSP-PCR [224]

KIRs	Amplicon Size (bp)	KIRs	Amplicon Size (bp)
Set I		**Set V**	
3DS1	320	3DP1	280/399
2DL1	144	2DS1	100
Set II		**Set VI**	
3DL3	202	2DL3	814
2DL2	160	2DL5	151
Set III		**Set VII**	
2DL4	243	2DS2	191
2DS3	138	3DL2	133
Set IV		**Set VIII**	
2DP1	693	3DL1	275
2DS5	153	2DS4	199/221

Table 11. PCR amplicon size of KIRs [224]. *The sixteen KIRs are grouped in eight sets in the duplex PCR-SSP genotyping platform*

3.2.1. Prediction of KIR haplotypes and genotypes

KIR haplotypes (A and B) and genotypes (AA and BX, where x can be A or B) were assigned to each individual based on KIR gene content [21, 22, 30, 31].

Individuals assigned as KIR genotype AA are homozygous for the A haplotype and had only genes of the group A KIR haplotype (3DL3, 2DL3, 2DL1, 2DP1, 3DP1, 2DL4, 3DL1, 2DS4, and 3DL2). All other individuals had one or more B haplotype specific genes (2DS1, 2DS2, 2DS3, 2DS5, 3DS1 and 2DL5) and therefore had either one (AB heterozygotes) or two (BB homozygotes) B haplotypes. Such individuals were assigned the KIR genotype Bx.

Classification of centromeric and telomeric gene clusters was done based on the linkage disequilibrium [30, 33]. The centromeric half of the KIR gene complex comprises of KIR 2DS2, 2DL2, 2DS3, 2DL5 genes while telomeric half of the complex comprises of KIR 3DS1, 2DL5, 2DS1, 2DS5 genes.

3.3. GENE EXPRESSION ANALYSIS

The expression of KIRs, NKp46 (NCR1, CD335), NK cell associated cytokines (pro-inflammatory cytokines – IL-1β, IL-2, IL-12β, IL-15, IL-18, IL-21, IFN-γ and TNF-α, and anti-inflammatory cytokines – IL-10 and TGF-β), Forkhead Box P3 (FOXP3), and Cytotoxic T-Lymphocyte-Associated Antigen 4 (CTLA4) was measured by real-time quantitative reverse transcriptase PCR (qRT-PCR).

Total RNA was isolated from RNAlater stored blood and tissue samples of OSCC patients and healthy control participants using RiboPure™ kit (Ambion Inc., Austin, Texas, USA) as described by the manufacturer. Yield (2-4 ug from 0.5 ml blood and 200-400 ug from 100 mg of tissue) and the purity (OD_{260}/OD_{280} = 1.9-2.1) of the extracted total RNA were checked using NanoVue™ Plus Spectrophotometer (GE Healthcare). One microgram of total RNA was reverse-transcribed into cDNA using high-capacity cDNA reverse transcription Kit (Applied Biosystems, Foster City, CA, USA) in a thermal cycler (Eppendorf, Hamburg, Germany) using random hexamers for initiating cDNA synthesis (Table 12a and 12b). The single-stranded cDNA products were then analyzed in real-time qRT-PCR (Table 13a and 13b).

TaqMan Gene Expression Assays specific for – six groups of KIRs (Table 14), ten cytokines, FOXP3 and CTLA4 were used to determine their expression levels in OSCC patients relative to controls. Subsequently, based on the expression level of each KIR group, real-time qRT-PCR for individual KIRs of that group was performed using SYBR Green based QuantiTect primer assays (Qiagen) (Table 15a and 15b). NKp46 gene expression was also evaluated by SYBR Green based QuantiTect primer assays.

Only samples with housekeeping genes (GAPDH and 18S rRNA) transcript levels within two standard deviations of the mean [226] were included for the expression study. Further, apart from GAPDH and 18S rRNA, NKp46 gene was used as additional housekeeping gene for cytokine and KIR expression study in the tumour [227, 228]. The real-time qRT-PCR was performed on StepOnePlus real-time PCR platform and 18S rRNA was used as the endogenous control for normalization of expression levels. The real-time amplification was recorded by the StepOne software v2.2.2 (Applied Biosystems) and the RNA transcript abundance for each gene was obtained by comparative cycle threshold (C_T) method [229]. In this method, relative expression of target gene is calculated using the formula $2^{-\Delta\Delta CT}$, which tells us the amount of target RNA transcript expressed by OSCC patients after normalizing with endogenous control relative to healthy controls.

$\Delta\Delta C_T$ value is calculated as follows –

ΔC_T of OSCC patient = Target gene (KIR) C_T mean – Endogenous control C_T mean

ΔC_T of healthy control = Target gene (KIR) C_T mean – Endogenous control C_T mean

$\Delta\Delta C_T = \Delta C_T$ of OSCC patient – ΔC_T of healthy controls

Table 12a

ReverseTranscription-PCR master mix (20 µL reaction volume)	
Component	**Volume/Reaction (µL)**
10X ReverseTranscription Buffer	2
25X dNTP Mix (100 mM)	0.8
10X RT Random Primers	2
Reverse Transcriptase	1
RNase Inhibitor	1
RNA	10 (1µg)
H_20	Adjusting volume

Table 12b

PCR program
25^0C for10 mins
37^0C for 120 mins
85^0C for 5 mins
4^0C hold

Table 12. (a) ReverseTranscription-PCR master mix composition and (b) PCR program for cDNA synthesis from total RNA

Table 13a

Real time PCR master mix (10 µL reaction volume)	
Composition	**Concentration/Reaction**
2X Taqman universal PCR master mix	1X
20X Gene Expression Assays for KIRs	1X
cDNA	100 ng
H_20	Adjusting volume

Table 13b

PCR program
95^0C for 20 mins
40 cycles 95^0C for 1 min and 60^0C for 20 mins

Table 13. (a) Taqman based real-time quantitative reverse transcriptase PCR master mix composition and (b) PCR program for gene expression study

Gene Expression Assays	KIRs
Group 1	KIR 2DL1, 2DL2 & 2DL3
Group 2	2DL5A & 2DL5B
Group 3	KIR3DL1
Group 4	KIR 3DL1 & 3DS1
Group 5	KIR 2DS2, 2DS4 & 2DS1
Group 6	KIR 2DS3 & 2DS5

Table 14. Gene Expression Assays specific for six groups of KIRs

Table 15a

Real time PCR master mix (10 µL reaction volume)	
Composition	**Concentration/Reaction**
2X SYBR green PCR master mix	1X
10X QuantiTect Primer Assay	1X
cDNA	100 ng
H_20	Adjusting volume

Table 15b

PCR program
95^0C for 15 mins
40 cycles 94^0C for 15 secs, 55^0C for 30 secs and 72^0C for 30 secs

Table 15. (a) SYBR green based real-time quantitative reverse transcriptase PCR master mix composition and (b) PCR program for gene expression study

3.4. HLA CLASS I GENOTYPING

Multiplex real-time polymerase chain reaction genotyping platform was used to determine the HLA class I ligands (Table 16a and 16b) [38]. In the first round PCR, exon no. 2 and 3 of HLA -A, -B or -C loci were amplified using locus-specific primers (Table 17a and 17b) [230]. 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 target both ligand-specific region and a non-polymorphic region of the amplicon as internal control. All

samples were run on a 96-well plate format in StepOnePlus real-time PCR system (Applied Biosystems) with the following thermo cycling program: 10 mins at 95^{0}C followed by 60 cycles of 15 secs at 95^{0}C and 1 min at 60^{0}C. The C_T 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.

PCR-Sequence Based Typing (PCR-SBT) was done in 25% of total samples to validate the results of SSP- real-time PCR described above. The respective amplicons of exon 2 and 3 of HLA -A, -B or -C loci were sequenced using sequencing primers on ABI 3100 Genetic Analyzer (Applied Biosystems) using Big-Dye Terminator v3.1 (Applied Biosystems) (Table 18a and 18b). Washington University (WU) Blast tool of IMGT/HLA database was then used to determine the respective HLA -A, -B or -C allele that constitutes any of the four epitopes – A3/A11, Bw4, C1 or C2 recognised by KIRs.

Table 16a

Master mix (10 µl reaction volume)	
Composition	**Concentration/Reaction**
2X Taqman universal PCR master mix	1X
Internal control primer	200 nm
Internal control probe	150 nm
HLA -A, -B or -C primer	200 nm
HLA -A, -B or -C probe	150 nm
DNA	5-15 ng
H_20	Adjusting volume

Table 16b

PCR program
95^{0}C for10 mins
60 cycles 95^{0}C for 15 secs and 60^{0}C for 1min

Table 16. (a) Master mix composition and (b) PCR program for SSP- real-time PCR to determine the HLA class I ligands [38]

Table 17a

Master mix (30 µl reaction volume)	
Composition	**Concentration/Reaction**
10X PCR buffer	1X
$Mgcl_2$	2 mM
dNTP's	200 µM
Forward primer	0.6 µM
Reverse primer	0.6 µM
Taq polymerase	2 units
DNA	100 ng
H_20	Adjusting volume

Table 17b

PCR program
95°C for 7 mins
45 cycles 95°C for 30 secs, 65°C for 1min and 72°C for 2 mins
72°C for 7 mins
4°C hold

Table 17. (a) Master mix composition and (b) PCR program for amplification of exon no. 2 and 3 of HLA -A, -B or -C loci [230]

Table 18a

Big-Dye v3.1 termination reaction (10 µL reaction volume)	
Composition	**Concentration/Reaction**
2.5X Reaction premix	0.25X
5X buffer	0.75X
Primer	5 pmol
DNA	10-20 ng
H_20	Adjusting volume

Table 18b

PCR program
96^0C for 2 min
30 cycle 96^0C for 30 secs, 50^0C for 1 min and 60^0C for 4 mins
4^0C hold

Table 18. (a) Master mix composition and (b) PCR program for Big-Dye v3.1 termination reaction

3.5. NK CYTOTOXICITY STUDY

Ability of freshly isolated peripheral blood NK cell to lyse HLA-negative K562 target cells was measured by fluorescence microscopy using Live/Dead cell-mediated cytotoxicity kit (Molecular Probes, Oregon, USA) (Figure 14).

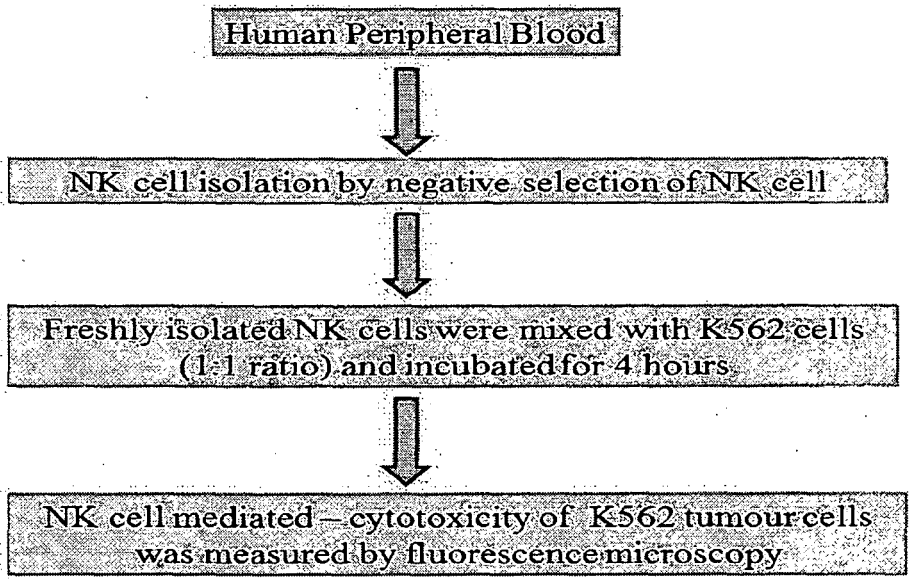

Figure 14. Steps involved in measuring NK cell-mediated cytotoxicity of K562 cells

3.5.1. NK cell isolation protocol

NK cells were isolated from peripheral blood of OSCC patients and control participants using Human NK cell Enrichment Set – DM kit (BD Biosciences, New Jersey, USA). The kit uses negative selection of NK cells from peripheral blood. It contains cocktail of monoclonal antibodies which recognizes antigens expressed on erythrocytes, platelets, and peripheral leukocytes (including NK-T cells). In the kit, streptavidin particles are covalently conjugated with magnetic nanoparticles. The NK cell isolation protocol as described in the kit is summarized below –

1. 1X BD IMag™ buffer was prepared: BD IMag™ Buffer (10X) was diluted with sterile distilled water or Phosphate Buffered Saline (PBS) supplemented with 0.5% Bovine serum albumin, 2mM Ethylenediaminetetraacetate (EDTA), and 0.1% sodium azide (1:10).
2. PBMC was prepared from anti-coagulated human blood (blood in EDTA)
3. Clumps of cells and/or debris were removed by passing the suspension through a 70-μm nylon cell strainer. Cells were counted, and resuspended in 1X BD IMag™ buffer at a concentration of 10 x 10^6 cells/ml.
4. Biotinylated human NK cell enrichment cocktail was added (5 μl per 1 x 10^6 cells).
5. Incubation was done at room temperature for 15 mins.
6. The labelled cells were washed with 10X excess volume of 1X BD IMag™ buffer.
7. Centrifugation was done at 300 g for 7 mins, and supernatant was carefully aspirated.
8. The BD IMag ™ streptavidin particles were thoroughly vortex, and 5 μl of particles were added for every 1 x 10^6 total cells.
9. Thoroughly mixed and incubation was done at room temperature for 30 mins.
10. With 1X BD IMag buffer volume was made up to 20 to 80 x 10^6 cells/ml.

11. Labelled cells were transferred to a 12 x 75 mm round-bottom test tube, maximum volume added not to exceed 1.0 ml. The tube was placed on the BD IMagnet™ (horizontal position) for 6 to 8 mins.
12. With the tube on the BD IMagnet™, the supernatant (enriched fraction that contains the NK cells) was carefully aspirated and placed in a new sterile tube.
13. The tube with positive-fraction was removed from the BD IMagnet™. 1X BD IMag™ buffer was added (same volume as in Step 10). The positive fraction was resuspended and the tube was placed back on the BD IMagnet™ for 6 to 8 mins.
14. The supernatant was aspirated and combined with the enriched fraction from Step 12 above.
15. Steps 13 and 14 were repeated. The combined enriched fraction contained NK cells with no bound antibodies or magnetic particles.
16. To increase the purity of the combined enriched fraction by another 5% or more, the tube containing the combined enriched fraction was placed on the BD IMagnet™ for another 6 to 8 mins.
17. The supernatant was aspirated and placed in a new sterile tube. The NK cells of enriched fraction were resuspended in complete medium and were ready for cytotoxicity assay.
18. The positive-fraction cells remaining in the original tube were resuspended in culture medium for other downstream applications.

3.5.1.1. Complete medium for NK Cells

RPMI 1640 medium supplemented with 10 % fetal bovine serum (filtered through 0.8-μm filter, heat treated), nonessential amino acids, sodium pyruvate, L-glutamine and antibiotic-antimycotic. HEPES not required in the complete medium.

3.5.1.2. Viability staining

Trypan blue staining method was used to distinguish viable and nonviable cells in the culture. The stained and unstained cells were counted

using a hemocytometer and the viability percentage was determined using the formula given below -

% viability = (No. of viable cells / Total no. of cells) x 100

3.5.2. K562 Cells

3.5.2.1. Cell line description

Organism - Homo sapiens (Human); Tissue – Chronic myelogenous leukemia (CML); bone marrow; Age – 53 years; Gender – female; Morphology – lymphoblast and Growth properties – suspension.

3.5.2.2. K562 complete medium

RPMI 1640 medium supplemented with 10 % fetal bovine serum (filtered through 0.8-μm filter, if not heat treated) and antibiotic-antimycotic.

The K652 cell line (Sigma-Aldrich, St. Louis, Missouri, USA) is an erythroleukemia cell line established by Lozzio and Lozzio [231]. The K562 Cell freezing and thawing protocols are summarized below –

3.5.2.3. Freezing of K562 cells

1. Cells were checked whether they are healthy and have the correct morphology.
2. The medium was changed 24 hrs before freezing the cells. Freezing is done when cells are in the logarithmic growth phase.
3. Centrifugation of the cells was done at 250 g for 10 mins, and resuspended in complete media in a culture vessel.
4. In dropwise fashion, 0.5 ml of the cell suspension (approximately 5–10 x 10^6 cells/ml) was transferred into each 1 ml freezing vial containing 0.5 ml freezing medium (K562 complete media and 20% DMSO). Vials were labelled with the name of the cell line, date and passage number.
5. Vials were stored in the –80°C freezer overnight.
6. The next day, vials were transferred to a liquid nitrogen chamber.

3.5.2.4. K562 cell thawing

1. Water bath was heated to 37°C. K562 complete medium was warmed.
2. Vial of frozen K562 cells was placed in the water bath until thawed.
3. Outside of the vial was washed with 70% ethanol.
4. Thawed cell suspension was slowly pipetted into a sterile centrifuge tube containing pre-warmed complete medium. Cells were mixed with the medium.
5. Centrifugation was done at 250 g for 10 mins. Supernatant was aspirated and the cells were resuspended in fresh medium.
6. Step 5 was repeated twice and K562 cells were transferred to an appropriate cell culture vessel.
7. K562 cells were incubated overnight under their usual growth conditions (5% CO_2, 37^0C and 95% air).
8. The next day, medium was replaced. K562 cells were ready for cytotoxicity assay.

3.5.3. Cytotoxicity Assay

In the cell-mediated cytotoxicity kit, to distinguish K562 (target) cells from NK (effector) cells, target cells were labelled with 3, 3′-dioctadecyloxacarbocyanine ($DiOC_{18}$), a green fluorescent membrane stain. The labelled target cells were then incubated with the effector cells. After incubation, membrane-impermeant nucleic acid counterstain propidium iodide was added to label any cells with compromised plasma membranes. Both live and dead effector cells / target cells were readily discriminated in the fluorescence microscope. The dead target cells had coincident green-membrane and red-nucleus staining while dead effector cells had only red-nucleus staining.

The protocols for labeling of K562 cells and cell-mediated cytotoxicity assay as described in the kit are summarized below -

3.5.3.1. Labeling of K562 cells

1. 1ml of $DiOC_{18}$ was added for labelling 5×10^5 K562 cells. To prepare 1ml of staining solution, 4 µL of staining solution was added to 1ml of complete culture medium (1:250 dilutions).

2. Culture medium from K562 cells was removed and replaced with the staining solution - $DiOC_{18}$ prepared in step 1.

3. Cells in staining solution were incubated overnight under normal culture conditions (5% CO_2, 37^0C and 95% air).

3.5.3.2. Cell-mediated cytotoxicity assay

1. K562 cells were once rinsed with PBS.
2. K562 cells were resuspended to 2×10^4 cells/ml in complete culture medium.
3. A suspension of NK cells (2×10^4 cells/ml) in complete culture medium was prepared.
4. 0.5 ml of NK cells-suspension (2×10^4 cells/ml) and 0.5 ml K562 cells – suspension (2×10^4 cells/ml) were mixed to yield 1:1 effector:target (E:T) ratio. In addition, 0.5 ml of complete culture medium (without NK cells) was mixed with 0.5 ml of the K562-cell suspension as a control to determine spontaneous cell death.
5. The above cell mixtures were incubated for a period of 4 hours.
6. Cell mixtures were concentrated by centrifugation at 250 g for 10 minutes.
7. 50 µL of propidium iodide counterstaining solution was added to 1 ml of cell mixtures. To prepare counter staining solution, 2 µL of propidium iodide was added to 1 ml of PBS (1:500 dilutions) and mixed well.
8. The cells in counterstaining solution were incubated at room temperature for 5 minutes.

9. 15 μL of cell mixture was trapped between a slide and an 18–22 mm^2 coverslip.

10. About 500 Cells were observed in the fluorescence microscope to determine the number of dead K562 cells in presence of NK cells, viable K562 cells in presence of NK cells and death K562 cells in absence of NK cells. The dead K562 cells had coincident green-membrane and red-nucleus staining while dead NK cells had only red-nucleus staining.

The NK cell-mediated cytotoxicity of K562 cells was calculated as cytotoxicity (or lysis) percentage using the following equation –

{dead cells/total cells (dead + live) in effector $^+$ cells } - {dead cells/total cells (dead + live) in effector $^-$ cells } x 100

3.6. DATA ANALYSIS AND STATISTICAL METHODS

Data analysis was done using statistical softwares - GraphPad Prism (La Jolla, CA, USA) and XLSTAT (Addinsoft, NY, USA).

The carrier frequency (CF) of each KIR/HLA gene and genotype was obtained by direct counting. CF for each gene, genotypes and KIR-HLA receptor-ligand pair were compared between the OSCC patients and healthy controls by *Chi-square test* or *Fisher's exact test*. Holm's procedure for adjustment of the p-values for multiple comparisons was applied when necessary. Odds Ratio (OR) / Relative Risk (RR) and 95% confidence interval (CI) were estimated in order to examine the magnitude and statistical significance of the association. Logistic regression analysis was done to determine the relative influences of independent variables of interest on OSCC or malignant features when applicable. The two-tailed unpaired or paired t-test was performed to compare the relative expression values of target gene or cell counts in the two groups. The results were considered significant at p values of <0.01 or <0.05.

Chapter IV

RESULTS AND DISCUSSIONS

4.1. DIVERSITY OF THE KILLER CELL IMMUNOGLOBULIN-LIKE RECEPTOR GENE COMPLEX IN THREE ETHNIC POPULATIONS OF ASSAM STATE, NORTHEAST INDIA

4.1.1. Results

4.1.1.1. KIR genotypes and haplogroups

In the 313 unrelated individuals, the 16 KIR genes were amplified successfully by the PCR-SSP approach [224] (Figure 15). The maximum and minimum numbers of KIR genes seen in an individual were 16 and 7 respectively. The KIR genotypes of the Adivasi were rich in KIR gene content as compared to Ahom and Kachari. In the three populations, 145 distinct KIR genotypes were detected (Table 19). Of the 145 genotypes, 82 genotypes were recorded in allelefrequencies website [232] and 63 genotypes were novel. The frequency of known genotypes from allelefrequencies website [232] was higher in Kachari (86/108) as compared to Ahom (70/104) and Adivasi (69/101). Conversely, the proportion of novel genotypes was highest in Adivasi and least in Kachari. The Bx-haplogroup individuals were preponderate in the three-study population like any other Indian population. The Adivasi had the higher share of Bx-haplogroup compared to Kachari and Ahom.

The presence of centromeric (KIR 2DS2, 2DL2, 2DS3, 2DL5) and telomeric (KIR 3DS1, 2DL5, 2DS1, 2DS5) KIR gene clusters were analyzed in the three populations. Like other native Indian population, the Adivasi had higher C4Tx genotype (four centromeric genes present; four telomeric genes absent) as compared to Kachari and Ahom. The CxTx genotype (four centromeric and telomeric genes absent) was more prevalent in three ethnic populations despite having higher proportion of B haplotypes.

Figure 15a.

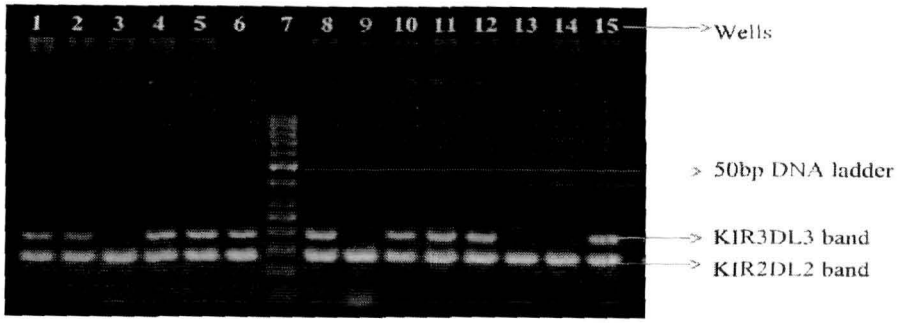

Figure 15b.

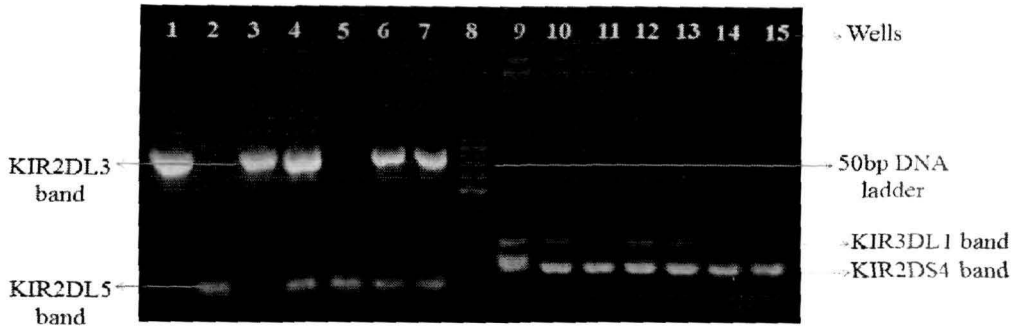

Figure 15c.

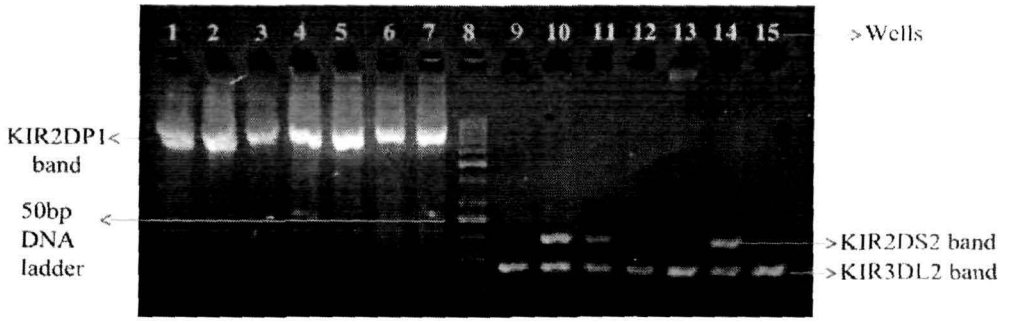

Figure 15. Agarose gels showing amplified KIR bands in 14 individuals – (a) Presence of KIR3DL3 (202bp) & KIR2DL2 (160bp); (b) Presence of KIR2DL3 (814bp) & KIR2DL5 (151bp) and 3DL1 (275bp) & 2DS4 (221bp); c) Presence of KIR2DP1 (693bp) & KIR2DS5 (153bp) and KIR2DS2 (191bp) & KIR3DL2 (133bp). *In all the individuals, both the genes were not present*

Sl. No.	2DL1	2DL2	2DL3	2DL5	3DL1	2DS1	2DS2	2DS3	2DS4	2DS4 - full	2DS4 - del	2DS5	3DS1	2DP1	Profile	Genotype ID	Kachari GF (%)	Ahom GF (%)	Adivasi GF (%)
1	1	0	1	0	1	0	0	0	1	1	0	0	0	1	AA	1	7.41	7.69	
2	1	1	1	1	1	1	1	0	1	0	1	1	1	1	Bx	3	0.93	0.96	
3	1	1	1	0	1	0	1	0	1	1	0	0	0	1	Bx	4	0.93	2.88	
4	1	1	1	1	1	0	1	1	1	1	0	0	0	1	Bx	5		2.88	4.95
5	1	1	1	1	1	1	1	1	1	0	1	1	1	1	Bx	6	0.93	0.96	
6	1	1	1	1	1	1	1	0	1	1	0	1	0	1	Bx	9	0.93		2.97
7	1	0	1	0	1	0	1	0	1	1	0	0	0	1	Bx	10		0.96	
8	1	1	1	1	1	1	1	1	1	1	0	0	0	1	Bx	11			0.99
9	1	0	1	1	1	1	1	0	1	1	0	1	1	1	Bx	12	0.93		
10	1	1	1	1	1	0	1	1	1	1	0	0	1	1	Bx	13			1.98
11	1	0	1	0	1	0	0	0	1	0	1	0	1	1	Bx	14	3.7	2.88	
12	1	0	1	0	1	1	0	0	1	1	0	0	0	1	Bx	15	5.56	4.81	
13	1	0	1	0	1	1	0	0	1	1	0	0	1	1	Bx	16	1.85	0.96	
14	1	1	1	1	1	1	0	0	1	1	0	1	1	1	Bx	18		0 96	
15	1	1	1	0	1	0	0	0	1	0	1	0	0	1	Bx	19	1.85	3.85	
16	1	1	1	1	1	0	0	0	1	1	0	1	0	1	Bx	20		2.88	0.99
17	1	1	1	1	1	0	1	0	1	1	0	1	0	1	Bx	21			1.98
18	1	1	1	1	1	0	1	1	1	1	0	1	1	1	Bx	22			4.95
19	1	0	1	0	1	0	0	0	1	1	0	1	0	1	Bx	23	1.85	1 92	2.97
20	1	1	1	1	1	0	1	1	1	1	0	1	0	1	Bx	25			6.93
21	1	0	1	1	1	0	0	1	1	1	0	0	0	1	Bx	30		1.92	
22	1	0	1	1	1	0	0	0	1	0	1	1	0	1	Bx	32	2.78	0.96	0.99
23	1	0	1	1	1	1	0	0	1	1	0	0	1	1	Bx	33	2.78	0.96	
24	1	0	1	1	1	0	1	1	1	1	0	0	0	1	Bx	36			1.98
25	1	0	1	1	1	1	0	1	1	1	0	0	0	1	Bx	37		0.96	
26	1	1	1	1	1	0	0	0	1	0	1	0	0	1	Bx	38	0.93	4.81	
27	1	1	1	0	1	0	1	1	1	0	1	0	1	1	Bx	41	0.93		
28	1	0	1	0	1	0	1	0	1	1	0	0	1	1	Bx	43		0.96	
29	1	1	1	0	1	0	1	0	1	1	0	1	0	1	Bx	44		0.96	0.99
30	1	1	1	1	1	1	0	0	1	1	0	1	0	1	Bx	46		0.96	1.98
31	1	1	1	1	1	1	0	1	1	1	0	1	0	1	Bx	50		0.96	
32	1	1	1	1	1	0	0	1	1	0	1	0	0	1	Bx	51		0.96	
33	1	1	1	0	1	0	1	1	1	0	1	0	0	1	Bx	62	0.93		1.98
34	1	1	1	0	1	1	1	0	1	0	1	1	1	1	Bx	63		0.96	
35	1	1	1	1	1	1	0	1	1	1	0	0	1	1	Bx	64		0.96	

Table 19. KIR locus profile (12 non-ubiquitous KIR genes) of the three populations. *Presence and absence of each KIR gene is indicated by 1 and 0 respectively. The capital letter 'N' represents novel genotype. The genotype frequency percentage (GF %) of each population is defined as – number of individuals carrying the genotype divided by the total number of individuals studied in a population. Genotypes (AA and Bx) were grouped based on KIR gene content* [21, 22, 30, 31]

Table 19 continued

Sl. No.	2DL1	2DL2	2DL3	2DL5	3DL1	2DS1	2DS2	2DS3	2DS4	2DS4 - full	2DS4 - del	2DS5	3DS1	2DP1	Profile	Genotype ID	Kachari GF (%)	Ahom GF (%)	Adivasi GF (%)
36	1	1	1	1	0	1	1	0	0	0	0	1	1	1	Bx	68	1.85		
37	1	1	0	1	1	0	1	1	1	1	0	0	0	1	Bx	71			2.97
38	1	1	1	1	0	1	1	1	1	0	1	1	1	1	Bx	87	0.93		
39	1	1	0	0	1	0	1	0	1	1	0	0	0	1	Bx	89		0.96	
40	1	1	0	1	1	1	1	1	1	0	1	1	0	1	Bx	91			3.96
41	1	1	0	1	1	0	1	1	1	1	0	0	1	1	Bx	94			1.98
42	1	1	0	1	1	0	1	1	1	0	1	1	0	1	Bx	112			0.99
43	1	1	0	1	1	1	1	1	1	0	1	0	0	1	Bx	113		0.96	0.99
44	1	0	1	1	0	1	0	0	1	1	0	1	1	1	Bx	154	0.93		
45	1	0	1	1	0	0	0	0	1	1	0	1	1	1	Bx	155	0.93	0.96	
46	1	0	1	1	0	1	1	1	0	0	0	1	1	1	Bx	166	0.93		
47	0	0	1	1	1	1	0	0	1	1	0	1	0	1	Bx	170			0.99
48	1	1	0	0	1	0	1	0	1	0	1	0	0	0	Bx	172			0.99
49	1	1	0	1	1	0	0	0	1	1	0	1	0	1	Bx	175			0.99
50	1	0	1	0	1	0	1	1	1	0	1	0	0	1	Bx	191			2.97
51	1	1	1	0	1	0	0	0	1	0	1	1	0	1	Bx	193		0.96	
52	1	0	1	1	1	0	0	0	1	1	0	0	0	1	Bx	200	10.2	1.92	
53	1	0	1	0	1	1	0	0	1	1	0	1	1	1	Bx	202		1.92	
54	0	1	0	1	1	1	1	1	1	1	0	0	1	0	Bx	204		0.96	
55	1	0	1	0	1	1	0	1	1	0	1	0	0	1	Bx	205		1.92	
56	1	0	1	0	1	1	0	0	0	0	0	0	0	1	Bx	207			0.99
57	1	1	1	0	1	1	1	1	1	1	0	0	1	1	Bx	233	0.93		
58	1	0	1	0	1	0	0	1	1	0	1	0	0	1	Bx	260	1.85		0.99
59	1	0	1	0	1	0	0	1	1	1	0	0	1	1	Bx	264	0.93	1.92	
60	1	1	1	0	1	0	1	1	1	1	0	1	0	1	Bx	268			0.99
61	1	1	1	0	1	1	1	1	1	1	0	1	0	1	Bx	269	0.93		
62	1	1	1	1	1	0	0	1	1	0	1	1	0	1	Bx	270			0.99
63	1	1	1	0	1	0	0	1	1	0	1	0	0	1	Bx	275	1.85	1.92	
64	1	0	1	0	0	0	1	1	1	0	1	1	0	1	Bx	312	0.93		
65	1	1	0	0	1	0	1	1	1	1	0	0	0	1	Bx	317			0.99
66	1	1	1	0	1	1	1	1	1	1	0	1	1	1	Bx	319	0.93		
67	0	1	1	1	1	0	1	0	1	1	0	1	0	1	Bx	323			0.99
68	1	0	1	1	1	1	0	0	1	1	0	0	0	1	Bx	326	4.63		
69	1	1	1	0	1	1	1	0	1	1	0	0	1	1	Bx	328	1.85		
70	1	1	1	0	1	0	0	0	1	1	0	0	1	1	Bx	336	0.93		
71	1	1	1	1	1	1	0	1	1	1	0	0	0	1	Bx	339			1.98
72	0	0	1	1	1	1	0	0	1	1	0	1	1	1	Bx	359		0.96	
73	1	0	1	0	1	1	0	1	1	1	0	0	1	1	Bx	372	0.93		
74	1	1	1	1	1	1	1	0	1	1	0	0	1	1	Bx	381		0.96	
75	1	1	1	1	1	1	1	1	1	1	0	1	0	1	Bx	382	0.93		

Sl. No.	2DL1	2DL2	2DL3	2DL5	3DL1	2DS1	2DS2	2DS3	2DS4	2DS4 - full	2DS4 - del	2DS5	3DS1	2DP1	Profile	Genotype ID	Kachari GF (%)	Ahom GF (%)	Adivasi GF (%)
76	1	1	1	1	1	0	0	0	1	0	1	0	1	1	Bx	383	0.93		
77	1	1	0	0	1	1	1	0	1	1	0	0	0	1	Bx	388			0.99
78	1	1	0	1	1	1	1	1	1	1	0	1	0	0	Bx	396	0.93		
79	1	1	0	1	1	0	1	1	1	1	0	1	1	1	Bx	401			1.98
80	1	0	1	1	1	0	1	1	1	1	0	1	0	1	Bx	426	0.93		0.99
81	1	0	1	1	1	0	0	0	1	1	0	0	1	1	Bx	433	7.41	0.96	
82	1	0	1	0	1	1	0	0	1	1	0	1	0	1	Bx	439	0.93		
83	1	1	0	1	1	0	0	0	1	1	0	0	0	1	Bx	N 1			0.99
84	1	1	0	1	1	0	0	1	1	0	1	0	0	1	Bx	N 2			0.99
85	1	1	1	1	1	0	1	0	0	0	0	1	1	1	Bx	N 3	0.93		
86	1	0	1	1	1	0	1	1	0	0	0	1	1	1	Bx	N 4		0.96	
87	1	0	0	0	1	1	0	0	1	1	0	1	0	1	Bx	N 5			0.99
88	1	0	0	0	1	0	0	0	1	1	0	1	0	0	Bx	N 6			0.99
89	1	1	0	0	1	1	1	1	1	1	0	1	0	1	Bx	N 7			2.97
90	1	0	1	0	1	0	0	0	0	0	0	0	1	1	Bx	N 8	0.93		
91	1	0	1	0	0	0	0	0	0	0	0	1	0	1	Bx	N 9	0.93		
92	0	0	1	0	0	0	1	0	0	0	0	0	1	1	Bx	N 10		0.96	
93	1	0	1	1	1	0	0	0	0	0	0	0	0	1	Bx	N 11	0.93		
94	1	0	1	1	1	0	1	0	0	0	0	0	0	0	Bx	N 12		0.96	
95	1	0	1	0	1	1	1	0	1	1	0	0	1	1	Bx	N 13		1.92	
96	1	0	1	0	1	1	1	0	1	1	0	0	0	1	Bx	N 14		1.92	
97	1	0	1	1	1	1	0	0	1	1	0	1	1	0	Bx	N 15	0.93		
98	1	1	0	1	0	0	0	1	1	0	1	1	0	1	Bx	N 16			0.99
99	1	1	1	0	1	1	1	1	0	0	0	0	0	1	Bx	N 17			0.99
100	1	0	1	1	1	0	1	0	1	1	0	0	0	1	Bx	N 18		2.88	
101	0	1	0	1	0	0	1	1	1	1	0	1	0	1	Bx	N 19			0.99
102	1	1	0	0	1	1	1	1	1	0	1	0	0	1	Bx	N 20			0.99
103	0	0	0	0	1	0	0	0	1	1	0	0	0	1	AA	N 21			0.99
104	1	0	1	0	1	0	1	0	1	0	1	1	0	1	Bx	N 22			0.99
105	0	0	0	1	0	0	1	0	1	1	0	1	0	1	Bx	N 23	0.93		
106	1	1	0	0	1	0	0	1	1	1	0	1	0	1	Bx	N 24			0.99
107	1	1	0	0	1	0	1	0	1	0	1	1	0	1	Bx	N 25			0.99
108	1	1	0	1	1	0	0	0	0	0	0	1	0	1	Bx	N 26			0.99
109	1	0	1	1	0	0	1	0	0	0	0	1	0	0	Bx	N 27		0.96	
110	0	0	1	1	0	1	1	1	1	0	1	1	1	1	Bx	N 28	0.93		
111	1	1	1	0	1	0	0	1	1	1	0	1	0	1	Bx	N 29	0.93		
112	1	1	0	0	1	0	1	1	1	1	0	1	0	1	Bx	N 30	0.93		0.99
113	1	0	1	1	1	1	0	0	1	1	0	1	0	1	Bx	N 31	0.93		
114	0	0	1	1	1	1	0	0	1	1	0	0	1	1	Bx	N 32	0.93		
115	1	0	0	1	1	0	0	1	1	0	1	1	0	1	Bx	N 33			0.99

Sl. No.	2DL1	2DL2	2DL3	2DL5	3DL1	2DS1	2DS2	2DS3	2DS4	2DS4 - full	2DS4 - del	2DS5	3DS1	2DP1	Profile	Genotype ID	Kachari GF (%)	Ahom GF (%)	Adivasi GF (%)
116	1	0	1	0	1	1	1	1	1	0	1	1	1	1	Bx	N 34		0.96	
117	1	0	1	1	1	1	1	1	1	1	0	0	0	1	Bx	N 35		1.92	
118	1	1	1	0	1	1	0	0	1	1	0	0	0	1	Bx	N 36	1.85	2.88	
119	1	1	1	1	1	0	0	0	0	0	0	1	0	1	Bx	N 37		0.96	
120	1	0	1	1	1	0	1	1	1	1	0	0	1	1	Bx	N 38			2.97
121	1	1	1	1	1	0	1	0	1	1	0	0	1	1	Bx	N 39	0 93	0.96	
122	1	0	1	1	1	0	1	1	1	1	0	1	1	1	Bx	N 40			1.98
123	1	0	1	1	1	1	0	0	1	1	0	1	1	1	Bx	N 41		2.88	0 99
124	1	1	1	0	1	0	1	1	1	0	1	1	1	1	Bx	N 42			0.99
125	1	0	1	0	1	0	0	1	1	0	1	1	0	1	Bx	N 43		0.96	0.99
126	1	1	1	0	1	1	0	1	1	1	0	0	0	1	Bx	N 44		0.96	
127	1	0	1	0	1	0	1	0	1	1	0	1	1	1	Bx	N 45	0 93	0.96	
128	1	1	0	1	1	0	0	1	1	0	1	1	1	1	Bx	N 46			1.98
129	1	0	1	1	1	1	1	0	1	0	1	0	1	1	Bx	N 47	1.85	1.92	
130	1	1	0	1	1	1	0	0	1	1	0	0	0	1	Bx	N 48	0.93		
131	1	0	0	1	1	0	1	1	1	0	1	1	0	1	Bx	N 49			0.99
132	0	1	0	1	1	0	1	1	1	1	0	1	1	1	Bx	N 50			0.99
133	1	1	1	0	1	1	0	1	1	0	1	1	0	1	Bx	N 51		0.96	
134	1	0	1	0	1	0	1	1	1	0	1	1	0	1	Bx	N 52			0.99
135	1	0	1	1	1	0	1	0	0	0	0	1	0	1	Bx	N 53		0.96	
136	1	0	0	1	1	0	1	0	0	0	0	0	0	1	Bx	N 54		0.96	
137	1	0	0	1	1	0	0	0	1	1	0	0	1	1	Bx	N 55		0.96	
138	1	0	0	1	1	1	1	0	1	1	0	0	0	1	Bx	N 56		0.96	
139	1	1	0	0	1	1	0	0	1	1	0	1	0	1	Bx	N 57	0.93		
140	1	1	1	0	1	0	0	0	1	1	0	1	1	0	Bx	N 58		0.96	
141	1	0	1	0	1	1	1	1	1	1	0	0	0	1	Bx	N 59	0.93		
142	1	0	1	0	1	0	1	1	1	1	0	0	1	1	Bx	N 60	0.93		
143	1	0	1	0	0	0	0	1	1	0	1	0	1	1	Bx	N 61		0.96	
144	1	0	1	0	1	1	1	0	1	0	1	1	0	1	Bx	N 62	0.93		
145	1	0	0	1	1	0	1	1	1	1	0	1	1	1	Bx	N 63			0.99

4.1.1.2. KIR carrier frequencies

The ubiquitous KIR genes – 2DL4, 3DL2, 3DL3 and 3DP1 were present in all individuals. The distributions of carrier frequency of 12 non-ubiquitous KIR genes in the three ethnic populations are given in the Table 20.

The pairwise analysis of the populations revealed that the two Mongoloid populations – Kachari and Ahom had comparable KIR carrier frequencies. The Adivasi differed markedly from these Mongoloid populations

in having higher KIR 2DL2, 2DS2, 2DS3 and 2DS5, but lower 2DL3 (p value < 0.0001).

Considering 3DL1/3DS1 as two genes, their frequencies were examined in the three populations (Table 21). The distribution of KIR3DL1$^-$/3DS1$^+$ genotype was higher in the two Mongoloid populations, while KIR3DL1 homozygosity was higher in Adivasi, though it was not significant. Further, zygosity of KIRs 2DL2 and its allele 2DL3 was also estimated. In Adivasi, KIR2DL2 homozygosity was higher and 2DL3 homozygosity was lower as compared to Kachari and Ahom (p value < 0.0001).

	Kachari (Ka)	Ahom (Ah)	Adivasi (Ad)			
	n=108	n=104	n= 101	p values		
KIR	CF %	CF %	CF %	Ka-Ah	Ka-Ad	Ah-Ad
2DL1	97.2	97.1	95	1	1	1
2DL2	28.7	39.4	72.3	0.297	**< 0.0001**	**<0.0001**
2DL3	95.4	95.2	61.4	1	**< 0.0001**	**<0.0001**
2DL5	51.8	48.1	69.3	1	**0.029**	**0.006**
3DL1	90.7	96.1	98	0.32	0.054	1
2DS1	42.6	39.4	24.7	1	**0.019**	0.072
2DS2	28.7	34.6	73.3	1	**< 0.0001**	**< 0.0001**
2DS3	21.3	25	72.3	1	**< 0.0001**	**< 0.0001**
2DS4	93.5	93.3	97	1	0.683	0.615
2DS4 Full	62.9	59.6	57.4	0.769	0.49	0.693
2DS4 Del	30.5	33.6	39.6	0.665	0.479	0.786
2DS5	30.5	28.8	63.4	1	**< 0.0001**	**< 0.0001**
3DS1	41.7	33.6	21.8	0.685	**0.006**	0.171
2DPI	98.1	96.1	98	1	1	1

Table 20. Comparisons of KIR carrier frequency (CF) percentage of 12 non-framework KIR genes in the three ethnic populations. *KIR 2DS4 alleles were divided into two groups – 2DS4 Full and 2DS4 Del based on a 22-bp deletion from the exon 5. Only significant p-values (<0.05) are displayed in bold*

Genotype	Kachari (Ka) n=108	Ahom (Ah) n=104	Adivasi (Ad) n= 101	p values Ka-Ah	Ka-Ad	Ah-Ad
$3DL1^+/3DS1^+$	35	31	22	0.605	0.079	0.211
$3DL1^+/3DS1^-$	56	65	76	0.31	0.2	0.19
$3DL1^-/3DS1^+$	7	3	0	0.203	**0.008**	0.085
$2DL2^+/2DL3^+$	25	38	40	0.071	**0.032**	0.723
$2DL2^+/2DL3^-$	4	2	33	0.414	**< 0.0001**	**< 0.0001**
$2DL2^-/2DL3^+$	71	58	22	0.174	**< 0.0001**	**< 0.0001**

Table 21. Frequency (%) of KIR 3DL1/3DS1 and 2DL2/2DL3 zygosity between the three populations. *Only significant p-values (<0.05) are displayed in bold*

4.1.1.3. Genetic distances and Phylogenetic tree

The three study populations were compared with 43 world populations in respect of their KIR gene frequencies to understand their genetic relationship. KIR data of 43 populations were collected from the allelefrequencies website (Table 22) [232]. Neighbor-joining dendrogram was constructed based on Nei's standard genetic distances using KIR frequencies of 11 KIR genes excluding the framework genes and KIR2DPI (Figure 16).

Seven groups were seen in the neighbor-joining dendrogram. The Kachari mapped in the first group of the dendrogram was close to the China Eastern Mainland Han, China Jiangsu Province Han, Japan population 3 and South Korea populations. In the second group, the Ahoms were mapped between an Oriental population (Bangkok2) and two Polynesian populations (Samoa and Tokelau). The Adivasi was mapped in the fifth group with the African populations – Gabon, Senegal, South Africa San and South Africa Xhosa. The neighbor-joining dendrogram also inferred that among the three study populations, Adivasi was more close to other Indian populations – India North Hindu, India Mumbai Maharashtrian, India Tamil Nadu Kanikar, India Tamil Nadu Mollukurumba and India Tamil Nadu Paravar.

Sl. No.	Name as in Dendrogram	Population (n)	Ethnic origin
1	Kachari	Kachari (108)	Austro Asiatic
2	Ahom	Ahom (104)	Tibeto-Burman
3	Adivasi	Adivasi (101)	Indo-European
4	Chiriguano	Argentina Chiriguano (54)	Amerindian
5	Wichi	Argentina Chaco Wichi (82)	Amerindian
6	Amazon	Brazil Amazon (40)	Amerindian
7	Huichol	Mexico Huichol (73)	Amerindian
8	Purepecha	Mexico Purepecha (53)	Amerindian
9	Tarahumara	Mexico Tarahumara (65)	Amerindian
10	Bari	Venezuela Bari (80)	Amerindian
11	Warao	Venezuela Warao (89)	Amerindian
12	Yucpa	Venezuela Yucpa (61)	Amerindian
13	IranArab	Iran Khuzestan Arab (76)	Arab
14	Palestine	Jordan Palestine (105)	Arab
15	SaudiArab	Saudi Arab (162)	Arab
16	InMM	India Mumbai Maharashtrian (139)	Asian
17	InNH	India North Hindu (72)	Asian
18	InTNK	India Tamil Nadu Kanikar (35)	Asian
19	InTNM	India Tamil Nadu Mollukurumba (41)	Asian
20	InTNP	India Tamil Nadu Paravar (77)	Asian
21	IndoJava	Indonesia Java	Austronesian
22	Timor	East Timor (50)	Austronesian
23	Gabon	Gabon (54)	Black
24	Ghana	Ghana (41)	Black
25	Sengal	Senegal (118)	Black
26	SASan	South Africa San (91)	Black
27	SAXhosa	South Africa Xhosa (50)	Black
28	BAires	Argentina Buenos Aires (365)	Caucasoid
29	BrazBHCua	Brazil Belo Horizonte Caucasians (90)	Caucasoid
30	England5	England population 5 (584)	Caucasoid
31	FranSE2	France SE pop 2 (38)	Caucasoid
32	SpBasque	Spain Basque (71)	Caucasoid
33	Turkey	Turkey (154)	Caucasoid
34	Iran	Iran (200)	Caucasoid
35	USACau	USA Caucasian (255)	Caucasoid

Table 22. Populations analysed in the neighbor-joining phylogenetic tree [232]

Table 22 continued

Sl. No.	Name as in Dendrogram	Population (n)	Ethnic origin
36	Portugal	Portugal (65)	Caucasoid
37	PGJaya	Papua New Guinea Irian Jaya (50)	Melanesians
38	SoloIsland	Soloman Islands(40)	Melanesians
39	ChEMHan	China Eastern Mainland Han (106)	Oriental
40	ChJPHan	China Jiangsu Province Han (150)	Oriental
41	Japan3	Japan population 3 (132)	Oriental
42	Skorea	South Korea(154)	Oriental
43	Bangkok2	Thailand Bangkok population 2 (100)	Oriental
44	Cook Is	Cook Islands (48)	Polynesian
45	Samoa	Samoa (50)	Polynesian
46	Tokelau	Tokelau (47)	Polynesian

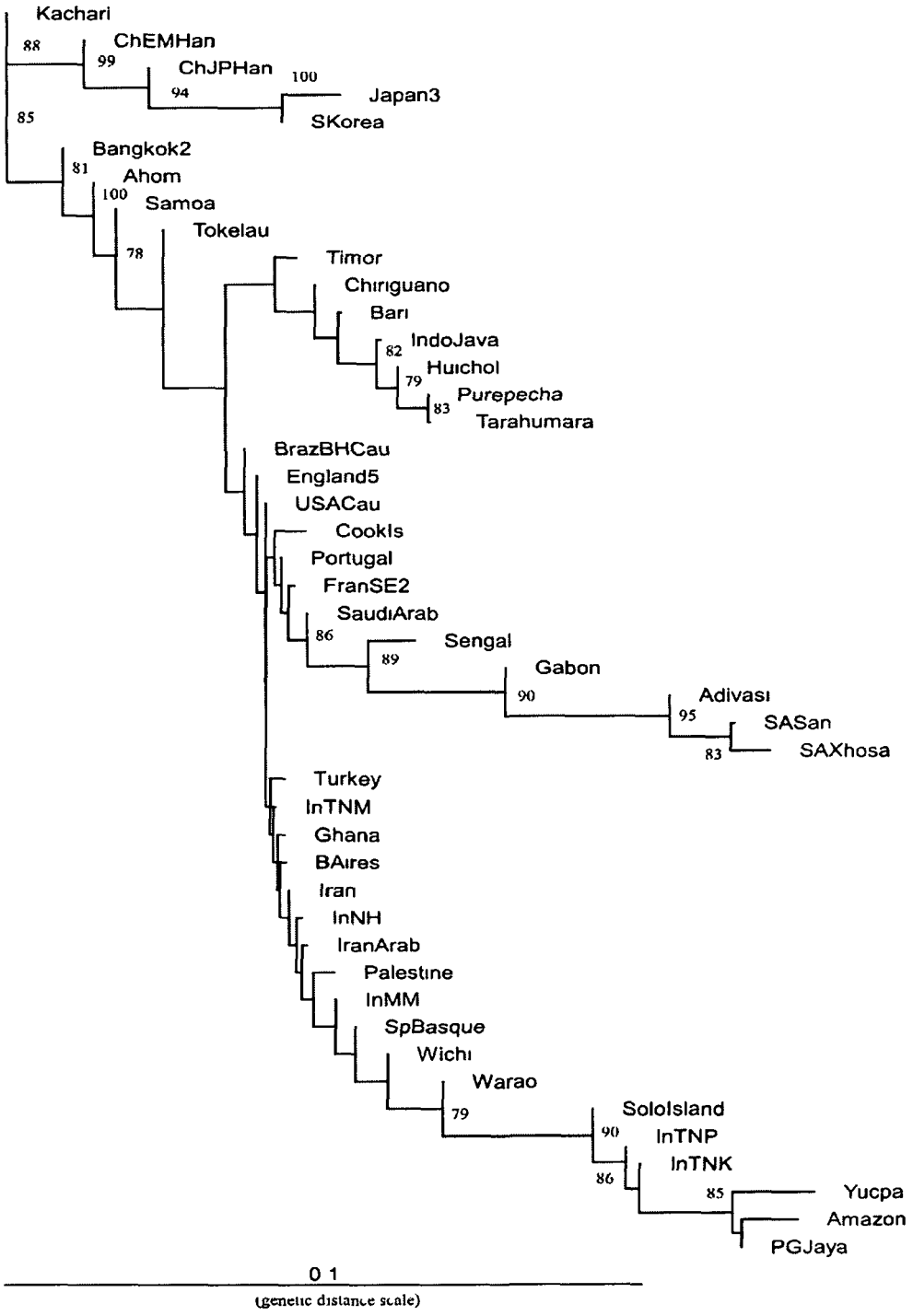

Figure 16. Neighbor-joining tree. *The phylogenetic tree was generated based on Nei's genetic distances using KIR gene frequencies of our study population with 43 world populations. Bootstraps were calculated with 100 iterations, and confidence values above 75 % are depicted*

4.1.2. Discussion

We had explored the diversity of the KIR complex in the Mundari-speaking Adivasi and two Mongoloid populations – Kachari and Ahom of Assam, Northeast India. Our data revealed that the frequencies of KIR genes in Kachari and Ahoms were comparable, while that of Mundari-speaking Adivasi was distinct from the two Mongoloid populations. Moreover, the neighbor-joining dendrogram that was constructed based on KIR gene frequencies mapped the two Mongoloid populations closely and clustering with East and Southeast Asian populations. The Kachari was clustered with Chinese populations while the Ahom in another clade was clustered with Thailand Bangkok and Polynesian populations. Interestingly, the mapping of the two Mongoloid populations in two clades of the dendrogram is in consistent with their migration histories. Historically, the Kachari intruded Northeast India through several waves of migration from Southern China while the Ahom migrated mainly from Thailand and Southeast Asia [233-235]. The mapping of Ahom with the Polynesian supports the current postulations on colonization of the Pacific region and intermixing of Austronesians, Polynesians and Melanesians population with mainland Asian [236].

The genetic studies describing mitochondrial-DNA and Y-chromosome revealed that Mundari-speaking Adivasi belongs to the Austro-Asiatic linguistic group and are reported to be distinct from Mongoloid populations of Northeast India [237]. In accordance, our data also showed that Mundari-speaking Adivasi was distinct from the two Mongoloid populations. The Adivasi had predominant Bx-haplogroup and centromeric KIR gene cluster as reported in native Indians. However, unlike other Indian populations, KIR3DS1 gene frequency was lower in Adivasi, which was comparable to the African population. This may be speculated to be reflection of their ancient origin or persistent selection probably maintained by their isolation from racial admixtures due to endogamous tradition of this tribe. Maternally inherited mitochondrial-DNA studies strongly support the view that Austro-Asiatic

Mundari speakers are the earliest inhabitants of India with highest frequencies of the ancient sub-haplogroup – M2 [238]. Earlier studies also show similarities between Mundari and African populations in having human-specific – Alu elements in the nuclear genome, supporting their ancient origin [239]. In the neighbor-joining dendrogram of the present study, clustering of Adivasi with African populations further supports genetic affinity of Adivasi and their distinctiveness from the two Mongoloid populations. Importantly, our KIR data revealed the migration history of the three populations, which is in parallel to the findings based on mitochondrial-DNA and Y-chromosome [240-242].

In summary, our results demonstrated that the KIR locus of the Mundari-speaking Adivasi was distinct from the two Mongoloid populations – Kachari and Ahom of Assam, Northeast India. The mapping of Kachari and Ahom with East and Southeast Asian populations, and Adivasi with African populations in neighbor-joining dendrogram was consistent with their migration history. Overall, our data supported the concept that KIR diversity may be used to understand genetic affinity and migration history of the populations. However, high resolution typing of KIR genes in extensive sampling would further reveal the relationship of the three populations.

4.2. KILLER CELL IMMUNOGLOBULIN-LIKE RECEPTOR / HUMAN LEUKOCYTE ANTIGEN IMMUNOGENETIC BACKGROUND IN ORAL SQUAMOUS CELL CARCINOMA PATIENTS OF ASSAM, NORTHEAST INDIA

4.2.1. Results

4.2.1.1. Patient characteristics

In this case-control study, the association of KIR genes and their HLA class I ligands with OSCC was evaluated in the two Mongoloid populations – Kachari and Ahom of Assam, Northeast India. The demographic and clinical characteristics of the OSCC patients (n=169) and control participants (n=177) are given in the Table 23. The median age of the OSCC patients was 55 years (inter-quartile range 49-60 years) and the male to female ratio for OSCC was 3.6:1. Of the 169 OSCC patients, 92 patients (54.4%) had family history of cancer (FHC), of which 68 cases (73.9%) were reported in their first-degree relatives and 24 cases (26.1%) in their second-degree relatives. Interestingly, 79/92 OSCC patients with FHC reported paternal history of cancer. The median age of the OSCC patients with FHC and no FHC were 55 years (inter-quartile range 47-61 years) and 56 years (inter-quartile range 52-60 years) respectively. The 177 healthy participants were not related to the OSCC patients and had no FHC.

Buccal mucosa was seen to be the most prevalent sub-site of OSCC (119/169 cases). The patients were diagnosed mainly in advance tumour stage (95.3%) and our data based on questionnaire indicated that poor socioeconomic conditions of the patients and inadequate access to healthcare were related to diagnosis of patients in advance tumour clinical stage. Histologically, 82.2% of the malignancies were classified as well differentiated, while 15.4% were moderately differentiated and 2.4% were poorly differentiated OSCC. The majority of the moderately and poorly differentiated OSCC occurred in the tongue sub-site with no nodal involvement.

Age (Years)	OSCC patients (169)	OSCC with FHC (92)	OSCC with no FHC (77)	Controls (177)
<45	7 (4.1)	5 (5.4)	2 (2.5)	8 (4.5)
45-54	69 (40.8)	40 (43.5)	29 (37.7)	63 (35.6)
55-64	73 (43.2)	38 (41.3)	35 (45.5)	86 (48.6)
≥65	20 (11.8)	9 (9.8)	11 (14.3)	20 (11.3)
Sex				
Male	132 (78.1)	70 (76.1)	62 (80.5)	138 (78)
Female	37 (21.9)	22 (23.9)	15 (19.5)	39 (22)
TNM Stage				
T2	8 (4.7)	4 (4.3)	4 (5.2)	
T3	75 (44.4)	45 (48.9)	30 (39)	
T4	86 (50.9)	43 (46.7)	43 (55.8)	
N0	43 (25.4)	24 (26.1)	19 (24.7)	
>N0	126 (74.6)	68 (73.9)	58 (75.3)	
M0	169 (100)	92 (100)	77 (100)	
Clinical tumour stage				
I-II	8 (4.7)	4 (4.3)	4 (5.2)	
III-IV	161 (95.3)	88 (95.7)	73 (94.8)	
Histological grade				
Well differentiation	139 (82.2)	75 (81.5)	64 (83.1)	
Moderate differentiation	26 (15.4)	14 (15.2)	12 (15.6)	
Poor differentiation	4 (2.4)	3 (3.3)	1 (1.3)	
Treatment				
Surgery	83 (49.1)	41 (44.6)	42 (54.5)	
Surgery + Radiotherapy	59 (34.9)	34 (37)	25 (32.5)	
No treatment	27 (16)	17 (18.5)	10 (13)	
Sub-site				
Buccal mucosa	119 (70.4)	62 (67.4)	57 (74)	
Floor of mouth	28 (16.6)	17 (18.5)	11 (14.3)	
Tongue	17 (10.1)	9 (9.8)	8 (10.4)	
Lip	5 (3)	4 (4.3)	1 (1.3)	

Table 23. Demographic and clinical characteristics (count; frequency %) of 169 OSCC patients and 177 healthy controls. *OSCC patients were classified into – 92 OSCC patients with family history of cancer (FHC) and 77 patients with no FHC*

4.2.1.2. KIR genotypes and haplogroups

In 169 OSCC patients, 59 distinct KIR genotypes were detected, of which 36 genotypes were recorded in allelefrequencies website [232] and 23 genotypes were novel. Similarly, in 177 healthy control participants, we found 52 distinct genotypes, out of which 35 were recorded in allelefrequencies website and 17 were novel genotypes. However, 38 KIR genotypes (27 genotypes known; 11 genotypes novel) were shared between the cancer and control group. The Bx-haplogroups were preponderate in individuals with 93.5% OSCC patients and 96% healthy participants. In oral cancer and healthy control group, we also analyzed the centromeric (KIR- 2DS2, 2DL2, 2DS3, 2DL5) and telomeric (KIR -3DS1, 2DL5, 2DS1, 2DS5) KIR gene. The CxTx genotype (four centromeric and telomeric genes absent) was more prevalent in the two groups (82%). However, we did not observed any significant difference in distribution of CxTx genotype between the two groups.

4.2.1.3. KIR carrier frequencies

The ubiquitous KIR genes – 2DL4, 3DL2, 3DL3 and 3DP1 were present in all 346 individuals typed. The 12 non-ubiquitous KIR genes in 169 OSCC patients and 177 healthy participants are given in the Table 25. Inhibitory KIR2DL3 frequency was lower in OSCC patients and negatively associated with OSCC ($p = 0.0001$, $pc = 0.0014$; OR= 0.193, 95% CI: 0.077 - 0.482). The total number of stimulatory and/or inhibitory KIR was comparable in OSCC patients and controls suggesting that the number of activating or inhibitory KIR genes in a genotype was not related to disease. Further, the frequencies of KIR genes were comparable between the patients with FHC and no FHC (Table 25).

Sl. No.	2DL1	2DL2	2DL3	2DL5	3DL1	2DS1	2DS2	2DS3	2DS4	2DS4 Full	2DS4 Del	2DS5	3DS1	2DPI	Profile	Genotype ID
1	1	0	0	0	1	0	0	0	1	1	0	0	0	1	AA	180
2	1	1	0	1	1	1	1	1	1	1	0	1	0	0	Bx	396
3	1	0	1	1	1	1	1	0	1	1	0	1	1	1	Bx	12
4	1	0	1	0	1	1	0	1	1	0	1	0	1	1	Bx	372
5	1	1	0	1	1	1	1	0	1	1	0	1	0	1	Bx	92
6	1	0	1	1	1	0	0	0	1	0	1	1	0	1	Bx	32
7	1	1	1	0	1	1	1	1	1	1	0	0	1	1	Bx	233
8	1	0	1	0	0	0	0	0	1	1	0	0	0	1	AA	156
9	1	0	1	0	1	0	0	0	1	1	0	0	0	1	AA	1
10	1	0	1	0	1	1	0	1	1	1	0	0	0	1	Bx	205
11	1	0	1	0	1	0	0	1	1	1	0	0	1	1	Bx	264
12	1	1	0	1	1	1	1	0	1	1	0	1	1	1	Bx	118
13	1	1	1	0	1	0	1	1	1	0	1	0	1	1	Bx	41
14	1	0	1	0	1	1	0	0	1	1	0	1	1	1	Bx	202
15	0	0	1	1	1	1	0	0	1	1	0	1	1	1	Bx	359
16	1	1	1	0	1	0	0	0	1	0	1	0	1	1	Bx	336
17	1	0	1	0	1	1	0	0	1	1	0	1	0	1	Bx	439
18	0	1	1	1	1	0	1	0	1	1	0	1	0	1	Bx	323
19	1	1	1	0	1	0	0	0	1	0	1	0	0	1	Bx	19
20	1	0	1	1	1	0	0	0	1	1	0	0	0	1	Bx	200
21	1	1	1	0	1	0	1	0	1	0	1	0	0	1	Bx	4
22	1	0	1	1	1	1	0	1	1	1	0	0	0	1	Bx	37
23	1	0	1	1	1	1	0	0	1	0	1	0	0	1	Bx	326
24	1	1	1	0	1	0	0	1	1	1	0	0	0	1	Bx	275
25	1	1	1	0	1	0	1	1	1	1	0	0	0	1	Bx	62
26	1	0	1	1	1	0	0	0	1	1	0	0	1	1	Bx	433
27	0	1	0	1	1	1	1	0	1	1	0	0	0	0	Bx	423
28	1	0	1	1	0	0	0	0	1	1	0	1	1	1	Bx	155
29	1	0	1	1	1	1	0	0	1	1	0	0	1	1	Bx	33
30	1	1	1	0	1	0	1	0	1	1	0	1	0	1	Bx	44
31	1	0	1	1	1	0	1	1	1	1	0	1	0	1	Bx	426
32	1	0	1	0	1	1	0	0	1	1	0	0	0	1	Bx	15
33	1	1	0	1	1	0	1	1	1	1	0	0	0	1	Bx	71
34	1	1	1	1	1	1	1	1	1	1	0	0	0	1	Bx	11
35	1	1	1	1	1	1	0	1	1	1	0	1	0	1	Bx	50
36	1	0	1	0	1	0	0	0	1	1	0	0	1	1	Bx	14
37	1	1	1	1	0	1	1	0	0	0	0	1	1	1	Bx	68

Table 24. KIR locus profile of OSCC and control participants. *In the 346 individuals typed, 111 distinct genotypes were observed that differed from each other by the presence (indicated by 1) or absence (indicated by 0) of 12 KIR genes*

Table 24 continued

Sl. No.	2DL1	2DL2	2DL3	2DL5	3DL1	2DS1	2DS2	2DS3	2DS4	2DS4 Full	2DS4 Del	2DS5	3DS1	2DPI	Profile	Genotype ID
38	1	0	1	1	1	0	0	1	1	0	1	0	0	1	Bx	30
39	1	0	1	0	1	1	0	0	1	0	1	0	1	1	Bx	16
40	1	1	1	0	1	0	0	0	1	1	0	1	0	1	Bx	193
41	1	1	1	1	1	0	0	0	1	1	0	0	1	1	Bx	383
42	1	1	0	1	1	0	1	1	1	0	1	1	1	1	Bx	401
43	1	1	0	1	1	1	1	1	1	1	0	0	0	1	Bx	113
44	1	1	1	0	1	1	1	0	1	1	0	1	1	1	Bx	63
45	1	0	1	0	1	0	0	0	1	0	1	1	0	1	Bx	23
46	1	1	0	1	1	0	1	0	1	1	0	1	0	1	Bx	228
47	1	1	1	1	1	1	0	1	1	0	1	0	1	1	Bx	64
48	1	1	1	1	1	1	1	0	1	1	0	0	1	1	Bx	381
49	1	0	1	0	1	0	1	0	1	1	0	0	0	1	Bx	10
50	1	0	1	1	1	1	0	0	1	0	1	1	1	1	Bx	2
51	1	0	1	1	0	1	0	0	1	0	1	1	1	1	Bx	154
52	1	1	1	1	1	1	1	1	1	1	0	1	0	1	Bx	382
53	1	0	1	0	1	0	0	1	1	0	1	0	0	1	Bx	260
54	1	1	1	0	1	1	1	0	1	1	0	0	1	1	Bx	328
55	1	0	1	0	1	0	1	0	1	0	1	0	1	1	Bx	43
56	1	0	0	1	1	0	0	0	1	1	0	1	0	1	Bx	238
57	1	0	1	0	1	1	0	0	0	0	0	0	0	1	Bx	207
58	1	0	1	1	0	1	1	1	0	0	0	1	1	1	Bx	166
59	1	1	1	0	1	1	1	1	1	0	1	1	0	1	Bx	269
60	1	1	1	1	1	0	1	1	1	1	0	0	0	1	Bx	5
61	1	1	1	1	1	1	1	0	1	1	0	1	1	1	Bx	3
62	1	1	0	1	0	1	1	1	0	0	0	0	1	1	Bx	190
63	1	1	1	1	1	0	0	0	1	1	0	0	0	1	Bx	38
64	1	1	0	1	1	1	1	1	1	1	0	0	1	1	Bx	90
65	1	0	1	1	1	0	0	0	1	1	0	1	1	1	Bx	17
66	1	0	1	1	1	0	1	1	1	1	0	0	0	1	Bx	36
67	1	1	1	1	0	1	1	1	1	1	0	1	1	1	Bx	87
68	0	0	1	1	1	0	0	0	1	1	0	0	0	1	Bx	287
69	1	1	1	1	1	1	0	1	1	0	1	0	0	1	Bx	339
70	1	1	0	1	1	0	1	0	1	1	0	0	0	1	Bx	176
71	0	0	1	1	1	1	0	0	1	0	1	1	0	1	Bx	170
72	1	0	0	1	1	1	0	0	1	1	0	0	0	1	Bx	New 1
73	1	0	0	1	0	0	0	0	0	0	0	0	1	1	Bx	New 2
74	1	0	1	1	1	1	0	0	1	0	1	1	0	1	Bx	New 3

Sl. No.	2DL1	2DL2	2DL3	2DL5	3DL1	2DS1	2DS2	2DS3	2DS4	2DS4 Full	2DS4 Del	2DS5	3DS1	2DPI	Profile	Genotype ID
75	1	0	0	1	1	0	1	0	0	0	0	0	0	1	Bx	New 4
76	1	0	0	0	1	0	0	0	0	0	0	0	1	0	Bx	New 5
77	1	0	1	1	1	1	0	0	1	1	0	1	1	1	Bx	New 6
78	1	0	0	1	1	0	1	0	0	0	0	0	1	1	Bx	New 7
79	1	0	1	1	1	1	1	0	1	1	0	0	0	0	Bx	New 8
80	1	0	0	1	1	0	0	0	1	1	0	0	0	1	Bx	New 9
81	1	0	1	1	1	1	0	1	1	1	0	0	0	0	Bx	New 10
82	1	0	1	1	1	0	0	0	0	0	0	0	1	1	Bx	New 11
83	1	0	0	1	0	0	0	0	1	1	0	1	1	1	Bx	New 12
84	1	0	1	0	1	1	1	1	1	0	1	0	0	1	Bx	New 13
85	1	0	0	0	1	0	0	0	0	0	0	0	1	1	Bx	New 14
86	1	0	1	1	0	1	1	0	0	0	0	0	1	1	Bx	New 15
87	1	0	0	0	1	1	0	0	1	0	1	0	1	1	Bx	New 16
88	1	1	1	0	1	1	0	0	1	0	1	0	0	1	Bx	New 17
89	1	0	1	1	1	1	1	0	1	1	0	0	1	1	Bx	New 18
90	1	0	0	1	0	0	0	0	0	0	0	0	1	1	Bx	New 19
91	1	1	1	0	1	1	0	0	1	1	0	0	0	1	Bx	New 20
92	1	0	1	1	1	1	1	0	0	0	0	0	1	1	Bx	New 21
93	1	0	0	1	1	1	0	0	1	1	0	0	1	1	Bx	New 22
94	1	0	1	1	1	0	1	1	1	0	1	0	0	1	Bx	New 23
95	1	0	0	1	1	0	0	0	1	0	1	0	0	1	Bx	New 24
96	1	0	1	0	0	1	0	0	1	1	0	1	0	1	Bx	New 25
97	1	0	1	1	1	0	1	0	0	0	0	0	1	1	Bx	New 26
98	0	0	1	0	1	0	0	0	1	1	0	1	1	1	Bx	New 27
99	1	0	1	0	1	0	1	0	1	1	0	1	1	1	Bx	New 28
100	1	0	1	1	1	0	1	1	1	1	0	0	0	0	Bx	New 29
101	1	0	1	1	1	0	1	0	0	0	0	1	1	1	Bx	New 30
102	0	0	1	1	0	0	0	0	1	1	0	0	0	1	Bx	New 31
103	1	0	1	1	1	0	1	0	1	1	0	1	0	1	Bx	New 32
104	1	1	1	1	1	1	1	1	0	0	0	0	0	1	Bx	New 33
105	0	0	1	1	0	1	0	0	0	0	0	0	0	0	Bx	New 34
106	0	0	1	1	0	1	0	0	0	0	0	0	0	1	Bx	New 35
107	0	0	1	1	0	0	1	0	0	0	0	0	0	0	Bx	New 36
108	0	0	1	1	0	1	0	0	0	0	0	0	1	1	Bx	New 37
109	0	0	1	0	1	0	0	0	1	1	0	0	0	1	Bx	New 38
110	1	0	1	1	1	0	0	0	1	1	0	0	0	0	Bx	New 39
111	1	1	1	1	1	0	0	0	1	1	0	0	0	0	Bx	New 40

KIR Carrier frequency (%)	OSCC patients	OSCC with FHC	OSCC with no FHC	Controls
KIR2DL1	163 (96.5)	92 (100)	71 (92.2)	160 (90.4)
KIR2DL2	56 (33.1)	30 (32.6)	26 (33.7)	58 (32.8)
KIR2DL3 *	143 (84.6)	76 (82.6)	67 (87)	171 (96.6)
KIR2DL5	95 (56.2)	53 (57.6)	42 (54.5)	104 (58.8)
KIR3DL1	153 (90.5)	85 (92.4)	68 (88.3)	157 (88.7)
KIR2DS1	78 (46.2)	38 (41.3)	40 (51.9)	79 (44.6)
KIR2DS2	56 (33.1)	27 (29.3)	29 (37.7)	69 (39)
KIR2DS3	45 (26.6)	23 (25)	22 (28.6)	50 (28.2)
KIR2DS4	154 (91.1)	85 (92.4)	69 (89.6)	159 (89.8)
KIR2DS4 Full	108 (63.9)	60 (65.2)	48 (62.3)	110 (62.1)
KIR2DS4 Del	46 (27.2)	21 (22.8)	25 (32.5)	49 (27.7)
KIR2DS5	59 (34.9)	30 (32.6)	29 (37.7)	59 (33.3)
KIR3DS1	78 (46.2)	39 (42.4)	39 (50.6)	78 (44.1)
KIR2DPI	162 (95.9)	88 (95.7)	74 (96.1)	172 (97.2)

Table 25. Count (frequencies %) of 12 non-framework KIR genes. *p-values of variables <0.05 are indicated by asterisk sign. KIR2DL3 p= 0 0001, OR=0 193, 95% CI: 0.077 - 0.482 (OSCC patients vs. controls). KIR2DL3 p= 0.0002; OR= 0.1667, 95% CI: 0.06276 - 0.4426 (OSCC patients with FHC vs. controls)*

4.2.1.4. Transcript expression of KIR genes

The RNA transcript expression of KIR genes was measured in peripheral blood of 169 OSCC patients by real-time quantitative reverse transcriptase PCR (qRT-PCR) [229]. The cDNA product of the OSCC and control samples were successfully amplified for the KIRs using TaqMan Gene Expression Assays and SYBR Green based primer assays in the qRT-PCR (Figure 17 and 18).

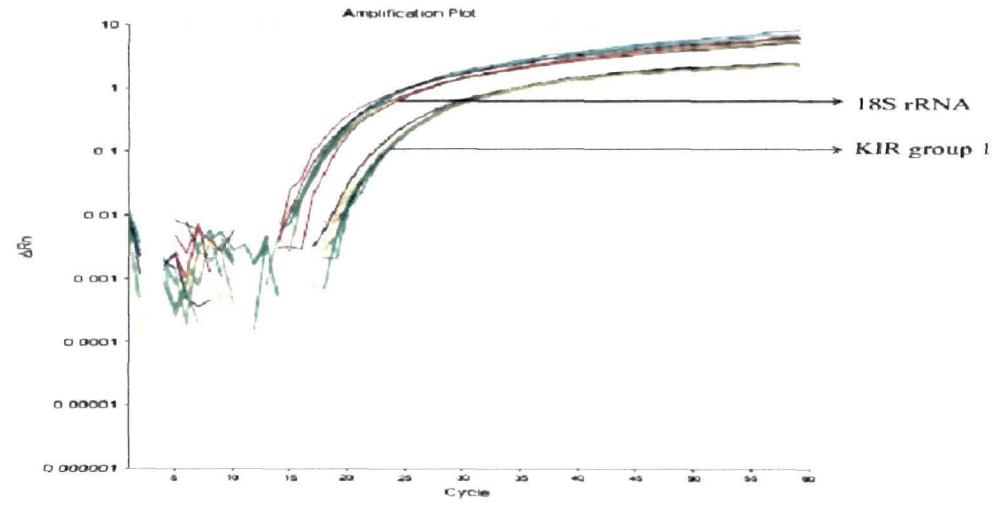

Figure 17. Amplification plot showing the variation of log (ΔRn) with PCR cycle number for KIR group 1 (KIR 2DL1, 2DL2 & 2DL3) and 18S rRNA in 3 samples in triplicates (TaqMan Gene Expression Assays). *In this graphical representation of real-time PCR data, Rn is the fluorescence of the reporter dye divided by the fluorescence of a passive reference dye. ΔRn is Rn minus the baseline* [229]

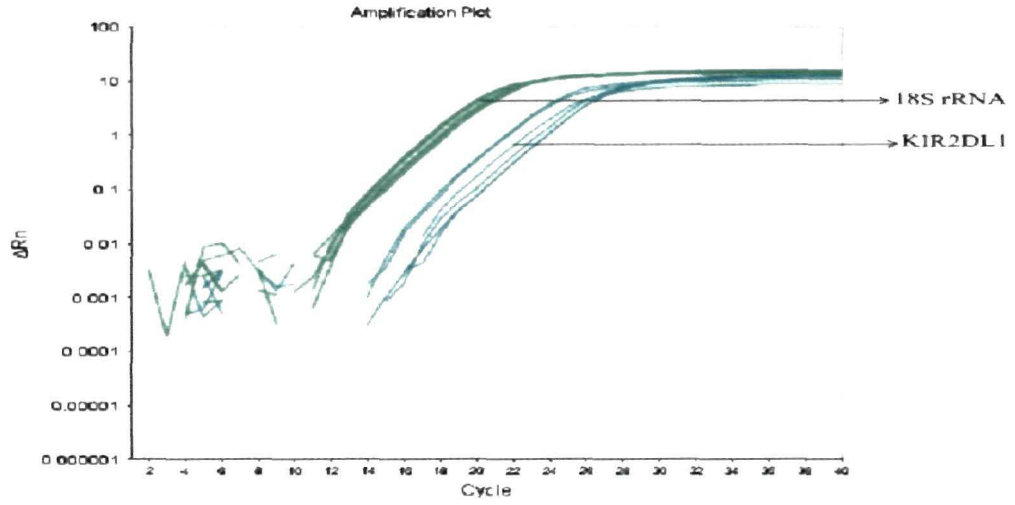

Figure 18a. Amplification plot showing the variation of log (ΔRn) with PCR cycle number for KIR2DL1 and 18S rRNA in 2 samples in triplicates (SYBR Green based primer assays)

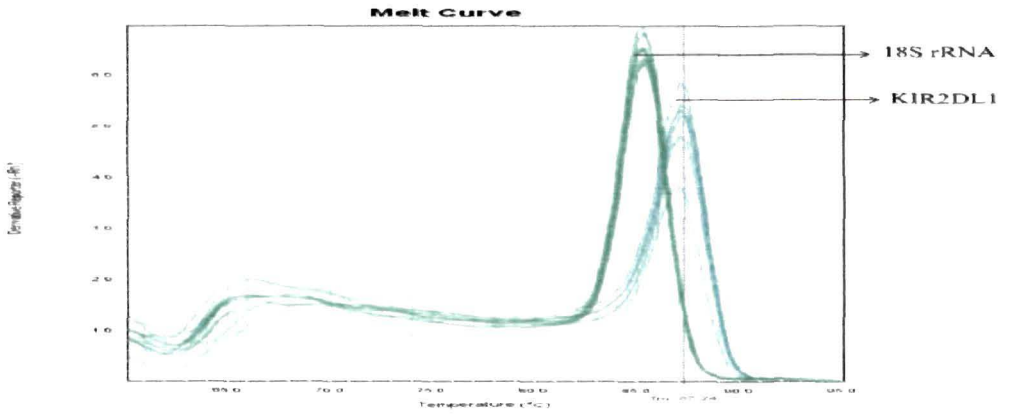

Figure 18b. Melting curves of KIR2DL1 and 18S rRNA amplicons in 2 samples in triplicates. *It was observed that KIR2DL1 and 18S rRNA were successfully amplified in the real-time PCR with no non-specific products*

Individuals containing a given KIR gene showed expression of the corresponding transcript with very few exceptions. Considering the higher transcript levels of the housekeeping genes (GAPDH and 18S rRNA) in these individual's sample there is possibility for degradation of RNA and thereby these samples were not analysed further.

Among the six KIR groups, the expression for the KIR group – 2DL1, 2DL2 & 2DL3 was 2.28-fold higher in cancer patients relative to controls (Figure 19). On classifying the OSCC patients, increased expression for the KIR group - 2DL1, 2DL2 & 2DL3 was found in patients with FHC compared to patients with no FHC (2.5-fold vs 2-fold; $p<0.001$). Further, on estimating the expression level of these three individual KIR genes, KIR2DL1 expression was seen 2.2-fold higher in OSCC patients relative to controls (Figure 20). It was noted that higher KIR2DL1 expression was in patients with FHC compared to patients with no FHC (2.3-fold vs. 1.9-fold; $p<0.001$).

Interestingly, OSCC patients with FHC of lower age groups (<45 and 45-54 years) had increased KIR2DL1 expression than higher age groups (55-64 and ≥65 years) patients ($p<0.001$) (Figure 21). Moreover, on comparing the

OSCC patients with FHC and no FHC, patients with FHC in lower age groups – <45 (2.7-fold) and 45-54 (3-fold) showed higher expression for KIR2DL1 compared to patients with no FHC ($p<0.001$).

Besides KIR 2DL1, 2DL2 & 2DL3 group, the expression of inhibitory KIR groups – KIR 2DL5 A&B, KIR 3DL1 & 3DS1 and KIR3DL1 was found to be 0.95-fold, 1.4-fold and 1.3-fold higher respectively in OSCC patients relative to controls.

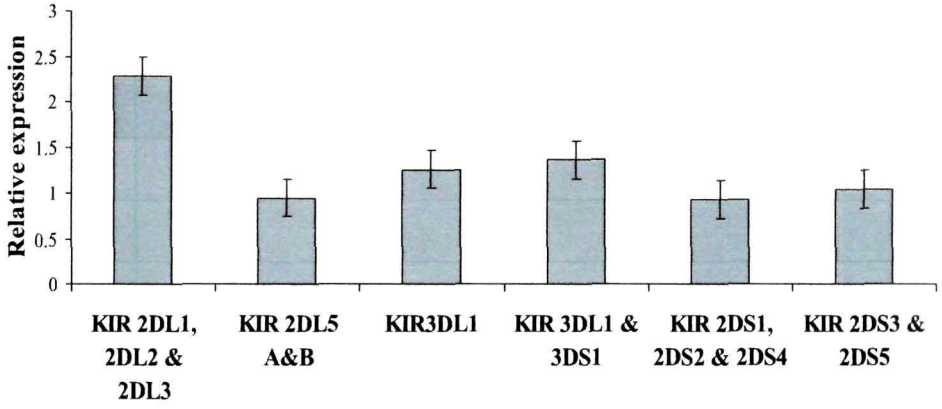

Figure 19. Mean RNA transcript expression of the six groups of KIRs – KIR 2DL1, 2DL2 &2DL3; 2DL5 A&B; KIR3DL1; KIR 3DL1&3DS1; KIR 2DS2, 2DS4, 2DS1; KIR 2DS3&2DS5 in OSCC patients relative to healthy controls. *The mean relative expression of the six groups of KIRs was calculated with the* $2^{-\Delta\Delta CT}$ *formula* [229]

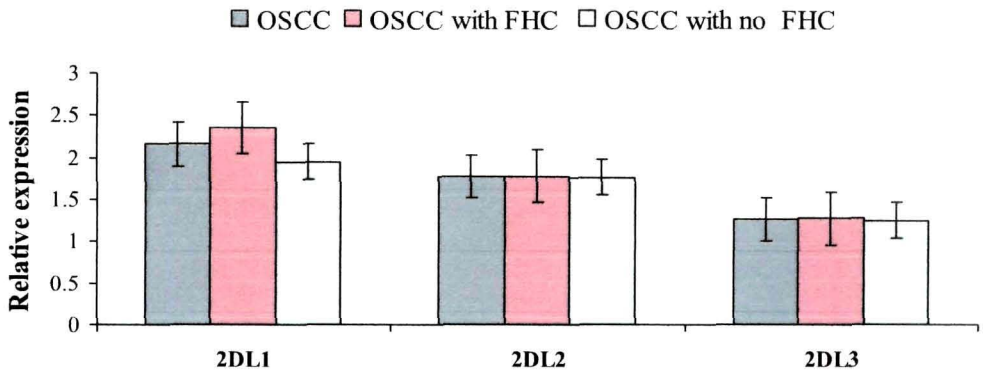

Figure 20. Relative mean expression of three KIRs – 2DL1, 2DL2 and 2DL3 in the OSCC, OSCC with FHC and OSCC with no FHC. *Among the three KIRs,*

mean expression of KIR2DL1 was higher (2.2-fold). The KIR2DL1 expression was higher in patients with FHC compared to patients with no FHC ($p<0.001$)

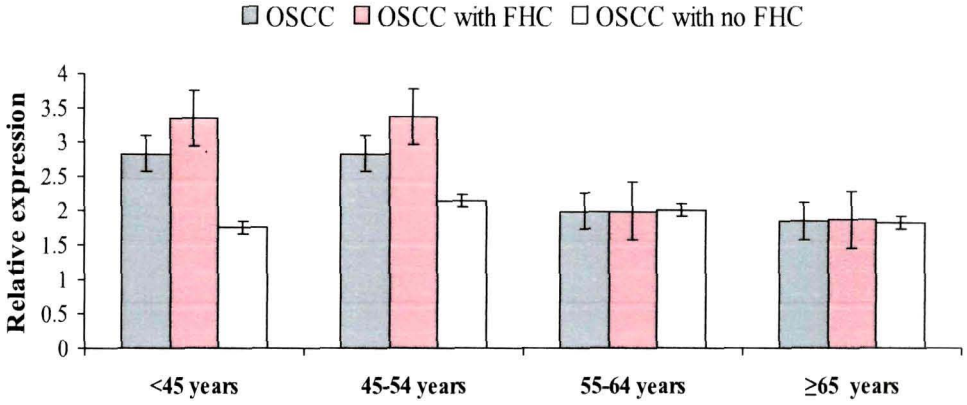

Figure 21. Relative mean expression of KIR2DL1 in four different age groups (<45 years; 45-54 years; 55-64 years; >65 years) of OSCC patients with FHC and no FHC. *In OSCC patients with FHC, KIR2DL1 expression was higher in lower age groups (<45 and 45-54 years) than in higher age groups (55-64 and ≥65 years) ($p<0.001$)*

4.2.1.5. HLA class I distribution

The presence of HLA class I ligands in 169 OSCC and 177 control samples was successfully determined by multiplex real-time PCR genotyping platform [38]. In the first round PCR, exon no. 2 and 3 of HLA -A, -B or -C loci were amplified using locus-specific primers (Figure 22) [230]. The respective PCR amplicons were then used in SSP-real-time PCR platform. C_T value of each reaction was used to determine the presence/absence of targeted HLA (Figure 23). Further, PCR-Sequence Based Typing (PCR-SBT) was done in 25% of total samples to validate the results of SSP- real-time PCR (Figure 24).

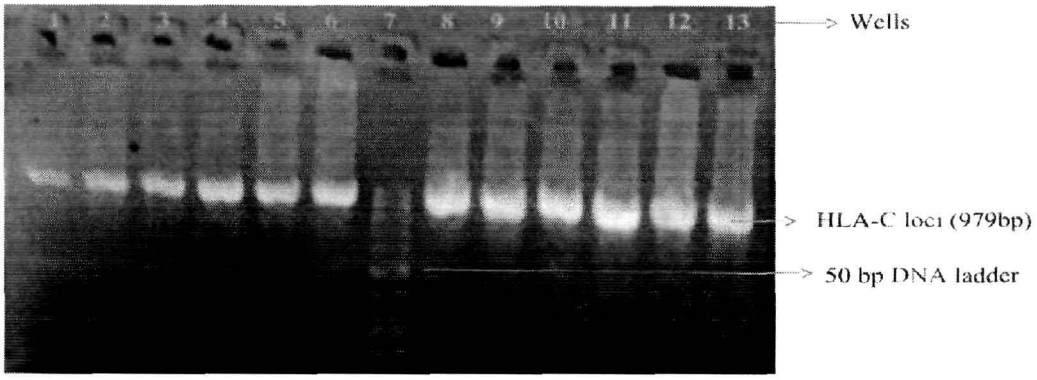

Figure 22. Agarose gel showing the DNA bands of HLA-C loci (exon 2 & 3) in 14 samples (well no. 1-6 and 8-13). *50bp DNA ladder was loaded in well no. 7*

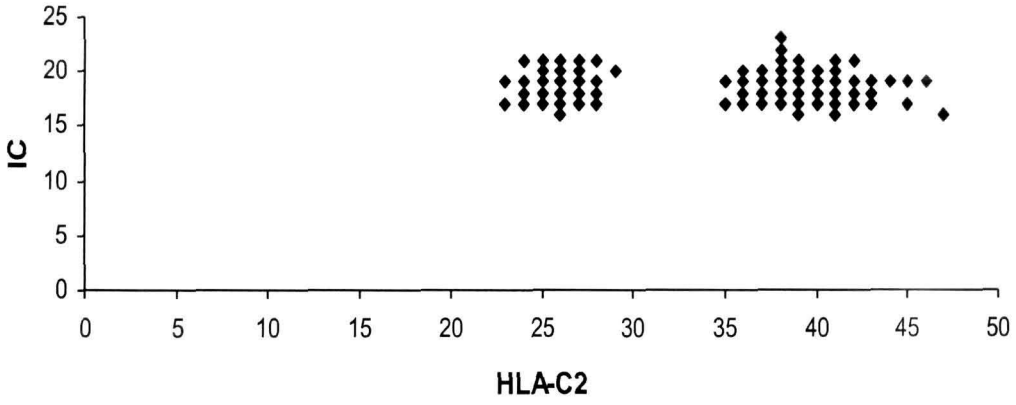

Figure 23. Distribution of C_T values of SSP-real-time PCR. The C_T values indicate the presence/absence of the KIR ligands in OSCC samples. *Samples carrying the HLA-C2 formed a distinct cluster, with similar levels of amplification for both the IC (internal control) and the specific reactions. In contrast, samples that do not have HLA-C2 did not amplify a specific product yet amplified the IC reaction comparable to carrier samples, forming a separate cluster*

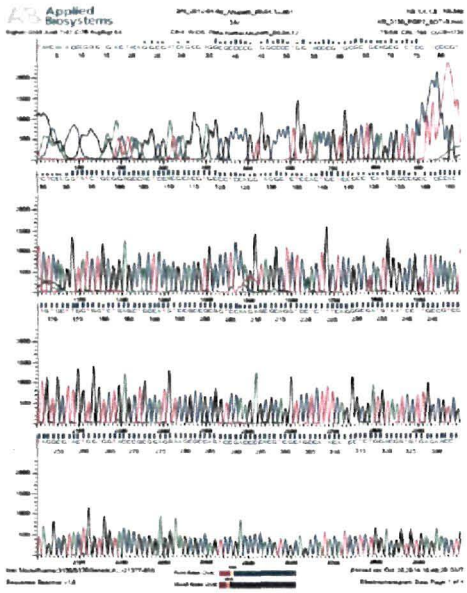

Figure 24a.

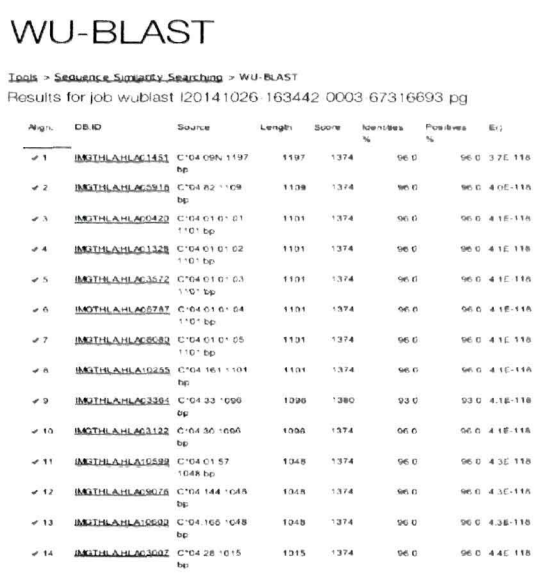

Figure 24b.

Figure 24. a) Partial sequence of the exon 2 and 3 of HLA-C locus. b) Washington University (WU) Blast tool result showing sequence similarity of the query sequence with HLA-C*04 allele of the database. *The HLA-C*04 allele form HLA-C2 epitope, which is a cognate ligand for KIR2DL1. In PCR-Sequence Based Typing approach, exon 2 and 3 of HLA -A, -B or -C loci (OSCC and control samples) were sequenced. WU Blast search was then done to determine the respective HLA -A, -B or -C allele (that constitute any of the four epitopes – A3/A11, Bw4, C1 and C2 recognised by KIRs) and validate the results of SSP- real-time PCR*

HLA-C2 and HLA-C1C2 genotype were higher and positively associated with OSCC (Table 26). The comparison of HLA ligands between OSCC patients with FHC and no FHC showed that HLA-C2 and HLA-C1C2 genotypes were significantly higher in patients with FHC ($p<0.0001$). On the contrary, the HLA-C1C1 genotype was lower and negatively associated with OSCC particularly in patients with FHC.

HLA Carrier frequency (%)	OSCC patients	OSCC with FHC	OSCC with no FHC	Controls
HLA-B C1	7 (4.1)	5 (5.4)	2 (2.6)	5 (2.8)
HLA-C C1	159 (94.1)	83 (90.2)	76 (98.7)	169 (95.5)
HLA-C1	159 (94.1)	83 (90.2)	76 (98.7)	169 (95.5)
HLA-C2 *	88 (52.1)	85 (92.4)	3 (3.9)	71 (40.1)
HLA-A Bw4	115 (68)	65 (70.7)	50 (64.9)	116 (65.5)
HLA-B Bw4 80Thr 81Ala	26 (15.4)	15 (16.3)	11 (14.3)	23 (13)
HLA-B Bw4 80Thr 81Leu	12 (7.1)	5 (5.4)	7 (9.1)	14 (7.9)
HLA-B Bw4 80Ile	69 (40.8)	39 (42.4)	30 (39)	67 (37.9)
HLA-B Bw4	94 (55.6)	53 (57.6)	41 (53.2)	92 (52)
HLA-Bw4	117 (69.2)	66 (71.7)	51 (66.2)	121 (68.4)
Genotype frequency (%)				
C1C1 *	81 (47.9)	7 (7.6)	74 (96.1)	106 (59.9)
C1C2 *	78 (46.2)	75 (81.5)	3 (3.9)	63 (35.6)
C2C2	10 (5.9)	10 (10.9)	0 (0)	8 (4.5)

Table 26. Count (frequencies %) of HLA class I ligands. *p-values of variables <0.05 are indicated by asterisk sign . HLA-C2 p< 0.0257; OR= 1 622, 95% CI: 1.059 - 2.484 (OSCC patients vs. controls). HLA-C2 p<0.0001, OR= 18.13, 95% CI: 7.925 - 41.47 (OSCC patients with FHC vs. controls) HLA-C2 p<0.0001; OR= 0.06053, 95% CI: 0.01836 - 0.1996 (OSCC patients with no FHC vs. controls). HLA-C2 p< 0.0001 (OSCC patients with FHC vs OSCC patients with no FHC). HLA-C1C1 p=0 0257; OR= 0.6165, 95% CI 0 4026 - 0.9440 (OSCC patients vs. controls). HLA-C1C1 p<0.0001; OR= 0 05516, 95% CI: 0.02411 - 0.1262 (OSCC patients with FHC vs. controls). HLA-C1C1 p<0.0001; OR= 16.52, 95% CI: 5.011 - 54.48 (OSCC patients with no FHC vs. controls). HLA-C1C1 p< 0.0001 (OSCC patients with FHC vs. OSCC patients with no FHC) HLA-C1C2 p=0.0457; OR= 1.551, 95% CI: 1.007 - 2.388 (OSCC patients vs. controls). HLA-C1C2 p<0.0001; OR= 7.983, 95% CI· 4.338 -14.69 (OSCC patients with FHC vs. controls). HLA-C1C2 p<0 0001; OR= 0.07336, 95% CI: 0.02221 - 0.2423 (OSCC patients with no FHC vs. controls). HLA-C1C2 p<0.0001 (OSCC patients with FHC vs. OSCC patients with no FHC)*

4.2.1.6. KIR2DL3$^+$-HLA-C$^+$ genotype was negatively associated with OSCC

To evaluate the association of KIR receptor and its HLA class I ligand with OSCC, the participants were stratified as +/+, +/-, -/+ and -/- for a particular combination of receptor-ligand genotype based on presence/absence of the KIR gene and its HLA ligand. Occurrence of KIR-HLA-C2$^+$ genotype was lower in OSCC patients with no FHC as only 3 patients were HLA-C2$^+$ (Table 27). Among the inhibitory KIR receptor-HLA ligand combinations, KIR2DL3$^+$-HLA-C1$^+$/C2$^+$ genotype was negatively associated with OSCC. The KIR2DL3$^+$-HLA-C1$^+$ genotype was lower in OSCC patients with FHC than in patients with no FHC. In contrary, KIR2DL3$^+$-HLA-C2$^+$ genotype was lower in OSCC patients with no FHC.

As KIR2DL3$^+$-HLA-C$^+$ genotype and KIR2DL3$^+$ were negatively associated with OSCC, logistic regression analysis was performed to determine the relative influence of these independent variables on risk to oral cancer. It suggested higher contribution of KIR2DL3$^+$ compared to KIR2DL3$^+$-HLA-C$^+$ genotype for the observed negative association with OSCC; however, it was not statistically significant.

In addition, activating KIRs – 2DS1/2/4 and 3DS1 bind with HLA-C and Bw4 ligands respectively. In this regard, the possible association of these combinations was examined between OSCC patients and controls, but no association was found with OSCC. However, 2DS1/4-HLA-C2 genotype was found negatively associated with OSCC patients with no FHC.

4.2.1.7. KIR2DL1$^+$-HLA-C2$^+$ genotype was positively associated with OSCC and family history of cancer

The odds of having oral cancer for KIR2DL1$^+$-HLA-C2$^+$ genotype was 1.7 times higher in OSCC patients (p=0.0147; 95% CI: 1.108 - 2.613) (Table 27). Interestingly, all 85 KIR2DL1$^+$-HLA-C2$^+$ genotypes occurred exclusively

in patients with FHC, showing a strong positive association of KIR2DL1$^+$-HLA-C2$^+$ genotype with FHC. Further, the association of KIR2DL1$^+$-HLA-C2$^+$ genotype with FHC was comparable between first-degree relatives and second-degree relatives of patients with FHC.

Inhibitory KIR-HLA pair	OSCC patients	OSCC with FHC	OSCC with no FHC	Controls
2DL1$^+$ - C2$^+$	**85 (50.3)**	**85 (92.4)**	0	**66 (37.3)**
2DL2$^+$ - C1$^+$	53 (31.4)	30 (32.6)	23 (29.9)	58 (32.8)
2DL2$^+$ - C2$^+$	13 (7.7)	11 (12)	2 (2.6)	17 (9.6)
2DL3$^+$ - C1$^+$	**133 (78.7)**	**66 (71.7)**	**67 (87)**	**164 (92.7)**
2DL3$^+$ - C2$^+$	**41 (24.7)**	**38 (41.3)**	**3 (3.9)**	**68 (38.4)**
3DL1$^+$ - Bw4$^+$	110 (65.1)	63 (68.5)	47 (61)	105 (59.3)
Activating KIR-HLA pair				
2DS1$^+$ - C2$^+$	20 (11.8)	**18 (19.6)**	**2(2.6)**	**30 (16.9)**
2DS2$^+$- C1$^+$	53 (31.4)	26 (28.3)	27 (35.1)	68 (38.4)
KIR2DS4 Full$^+$- C1$^+$	103 (60.9)	49 (53.3)	46 (59.7)	106 (59.9)
KIR2DS4 Full$^+$- C2$^+$	32 (18.9)	**29 (31.5)**	**3 (3.9)**	**47 (26.6)**
3DS1$^+$- Bw4$^+$	42 (24.9)	22 (23.9)	20 (26)	51 (28.8)

Table 27. Count (frequencies %) of inhibitory and activating KIR-HLA pairs. *p-values of variables <0.05 are indicated in bold. 2DL1$^+$-C2$^+$ p=0.0147; OR= 1.7, 95% CI: 1.108 - 2.613 (OSCC patients vs. controls). 2DL1$^+$-C2$^+$ p<0.0001; OR= 20.42, 95% CI: 8.915 - 46.78 (OSCC patients with FHC vs. controls). 2DL3$^+$-C1$^+$ p = 0.0002; OR= 0.2929, 95% CI: 0.149 - 0.575 (OSCC patients vs. controls). 2DL3$^+$-C1$^+$ p<0.0001; OR= 0.2012, 95% CI: 0.09749 - 0.4153 (OSCC patients with FHC vs. controls). 2DL3$^+$-C1$^+$ p= 0.0157 (OSCC patients with FHC vs. OSCC patients with no FHC). 2DL3$^+$-C2$^+$ p = 0.0046; OR = 0.5134, 95% CI: 0.3227 - 0.8168 (OSCC patients vs. controls). 2DL3$^+$-C2$^+$ p<0.0001; OR= 0.06498, 95% CI: 0.01970 - 0.2144 (OSCC patients with no FHC vs. controls). 2DL3$^+$-C2$^+$ p<0.0001 (OSCC patients with FHC vs. OSCC patients with no FHC). 2DS1$^+$-C2$^+$ p = 0.0008; OR= 0.1307, 95% CI: 0.03039 - 0.5618 (OSCC patients with no FHC vs. controls). 2DS1$^+$-C2$^+$ p =*

0.0006 (OSCC patients with FHC vs. OSCC patients with no FHC) 2DS4$^+$-C2$^+$ p<0.0001; OR= 0.1121, 95% CI: 0.03371 - 0.3730 (OSCC patients with no FHC vs. controls). 2DS4$^+$- C2$^+$ p<0.0001 (OSCC patients with FHC vs. OSCC patients with no FHC)

4.2.1.8. Association of disease onset age with KIR2DL1$^+$-HLA-C2$^+$ and family history of cancer

Considering 55 years as median age for OSCC patients, the participants of age <55 years were grouped as younger age group patients, while participants of age ≥55 years were grouped as older age group patients. It was noted that all younger age group patients with FHC were positive for KIR2DL1$^+$-HLA-C2$^+$ genotype showing significant positive association of the genotype with early onset of the disease (Table 28). The median age of these patients in younger and older age group were 47 and 61 respectively. On the other hand, in patients with no FHC, KIR2DL3$^+$-HLA-C1$^+$ genotype was lower in younger age group patients than patients in older age group (p<0.0001). Further, the socioeconomic conditions of the OSCC patients or access to healthcare had no influence on early onset of the disease in the KIR2DL1$^+$-HLA-C2$^+$ genotype positive patients with FHC.

OSCC Patients	Lower age group (<45-54)	Higher age group (≥55)
2DL1$^+$-C2$^+$		
with FHC	45 (100)	40 (85.1)
2DL3$^+$-C1$^+$		
with no FHC	21 (67.7)	46 (100)

Table 28. Count (frequency %) of 2DL1$^+$-C2$^+$ and 2DL3$^+$-C1$^+$ genotype in younger and older age groups of OSCC patients. *2DL1$^+$-C2$^+$ p=0.0124; RR= 1.175, 95% CI: 1.043 - 1.324 (younger age group OSCC patients with FHC, n=45 vs. older age group OSCC patients with FHC, n=47). 2DL3$^+$-C1$^+$ p<0.0001; RR= 0.6774, 95% CI: 0.5313 - 0.8637 (younger age group OSCC patients with no FHC, n=31 vs. older OSCC patients with no FHC, n=46)*

4.2.1.9. KIR expression in the presence of HLA class I ligand

The association of KIR2DL1, KIR2DL2 and KIR2DL3 expression in the presence of HLA class I ligands was analysed. In both OSCC and control participants, presence of HLA-C1 ligand was associated with increased expression of KIR2DL2/3, while HLA-C2 lowered the expression of KIR2DL2/3. Interestingly, the expression of KIR2DL1 was lower in controls (p=0.0028) in presence of HLA-C2 ligand, but not in OSCC patients (Tables 29-33).

	$2DL1^+HLA\text{-}C2^-$	**$2DL1^+HLA\text{-}C2^+$**
OSCC (n=169)	78 (46.2)	85 (50.3)
Controls (n=177)	94 (53.1)	66 (37.3)

Table 29. OSCC and control participants with $KIR2DL1^+$ expression when HLA-C2 ligand is present and absent in the genotype of the individual (Count; frequency %). *OSCC p= 0.4461 ($2DL1^+HLA\text{-}C2^-$ vs. $2DL1^+HLA\text{-}C2^+$); Controls p= 0.0028 ($2DL1^+HLA\text{-}C2^-$ vs. $2DL1^+HLA\text{-}C2^+$)*

	$2DL2^+HLA\text{-}C1^-$	**$2DL2^+HLA\text{-}C1^+$**
OSCC (n=169)	3 (1.8)	53 (31.4)
Controls (n=177)	0	58 (32.8)

Table 30. OSCC and control participants with $KIR2DL2^+$ expression when HLA-C1 ligand is present and absent in the genotype of the individual (Count; frequency %). *OSCC p<0.0001 ($2DL2^+HLA\text{-}C1^-$ vs. $2DL2^+HLA\text{-}C1^+$); Controls p<0.0001 ($2DL2^+HLA\text{-}C1^-$ vs. $2DL2^+HLA\text{-}C1^+$)*

	2DL2 $^{+}$HLA-C2 $^{-}$	2DL2 $^{+}$HLA-C2 $^{+}$
OSCC (n=169)	43 (25.4)	13 (7.7)
Controls (n=177)	41 (23.2)	17 (9.6)

Table 31. OSCC and control participants with KIR2DL2^{+} expression when HLA-C2 ligand is present and absent in the genotype of the individual (Count; frequency %). *OSCC $p<0.0001$ ($2DL2^{+}HLA\text{-}C2^{-}$ vs. $2DL2^{+}HLA\text{-}C2^{+}$); Controls $p = 0.0006$ ($2DL2^{+}HLA\text{-}C2^{-}$ vs. $2DL2^{+}HLA\text{-}C2^{+}$)*

	2DL3 $^{+}$HLA-C1 $^{-}$	2DL3 $^{+}$HLA-C1 $^{+}$
OSCC (n=169)	10 (5.9)	133 (78.7)
Controls (n=177)	7 (4)	164 (92.7)

Table 32. OSCC and control participants with KIR2DL3^{+} expression when HLA-C1 ligand is present and absent in the genotype of the individual (Count; frequency %). *OSCC $p<0.0001$ ($2DL3^{+}HLA\text{-}C1^{-}$ vs. $2DL3^{+}HLA\text{-}C1^{+}$); Controls $p<0.0001$ ($2DL3^{+}HLA\text{-}C1^{-}$ vs. $2DL3^{+}HLA\text{-}C1^{+}$)*

	2DL3 $^{+}$HLA-C2 $^{-}$	2DL3 $^{+}$HLA-C2 $^{+}$
OSCC (n=169)	102 (60.4)	41 (24.3)
Controls (n=177)	103 (58.2)	68 (38.4)

Table 33. OSCC and control participants with KIR2DL3^{+} expression when HLA-C2 ligand is present and absent in the genotype of the individual (Count; frequency %). *OSCC $p<0.0001$ ($2DL3^{+}HLA\text{-}C2^{-}$ vs. $2DL3^{+}HLA\text{-}C2^{+}$); Controls $p = 0.0002$ ($2DL3^{+}HLA\text{-}C2^{-}$ vs. $2DL3^{+}HLA\text{-}C2^{+}$)*

4.2.2. Discussion

The study investigated the association of two independent loci – KIR and HLA with OSCC and FHC in the two Mongoloid populations of Assam, Northeast India. Notably, 54.4% of the population had FHC, with 73.9% in their first-degree relatives and 26.1% in their second-degree relatives.

The combined inheritance of distinct KIR and HLA gene combinations has influences on immune responsiveness and it has been demonstrated that strength of KIR-HLA interactions can influence susceptibility for several human tumours, chronic inflammatory diseases and viral infections [13, 42, 243-245]. The results of the study indicated the association of two cognate KIR-HLA genotypes – KIR2DL1$^+$-HLA-C2$^+$ and KIR2DL3$^+$-HLA-C$^+$ with OSCC. The KIR2DL1$^+$-HLA-C2$^+$ genotype exhibits strongest inhibitory KIR-HLA interactions and it may be hypothesized that the presence of KIR2DL1$^+$-HLA-C2$^+$ genotype may be deleterious in cancer due to decreased NK cell response to tumour [34, 40]. In an earlier study on lung cancer, it was reported that the increased inhibitory signal decreased NK cell cytotoxic function against tumour cells that retained class I HLA expression [246]. Accordingly, considering the functional significance of KIR2DL1$^+$-HLA-C2$^+$ genotype, the strong association of KIR2DL1$^+$-HLA-C2$^+$ genotype with FHC patients in present study may be suggested as a risk factor for OSCC. Consistent with the findings of the present study, recently Campillo and his group [247] have reported association of KIR2DL1(+)/S1(-)/C2C2 genotype with susceptibility to melanoma and sentinel lymph node metastasis in Spanish Caucasian population. However, in the present study, KIR2DL1$^+$-HLA-C2$^+$ genotype was independent of KIR2DS1$^-$ and HLA-C2 homozygosity.

The present study showed that the presence of KIR2DL1$^+$-HLA-C2$^+$ genotype was strongly associated with susceptibility to OSCC in patients with FHC. In addition, higher occurrence of KIR2DL1$^+$-HLA-C2$^+$ genotype in younger age group FHC patients (<55 years) suggested that KIR2DL1$^+$-HLA-C2$^+$ genotype could predispose the FHC patients to early onset of cancer.

Further, higher RNA transcript abundance of the inhibitory KIR2DL1 in FHC patients of lower age groups (<45 and 45-54 years) supported the argument that KIR2DL1$^+$-HLA-C2$^+$ genotype was a risk factor in OSCC patients with FHC. Notably, the socioeconomic conditions and access to healthcare of the OSCC patients with FHC were comparable to non-FHC patients. In agreement with the findings of the present study, a previous study reported HLA-C1C1-KIR2DS2/2DL2 to influence the onset age of hepatocellular carcinoma in male patients with HBV infection [248]. However, in the present study, we could not analyze the association of KIR2DL1$^+$-HLA-C2$^+$ genotype in context of gender of OSCC patients with FHC, as majority of patients were male. Interestingly, we observed that FHC was predominantly from paternal lineage. This suggests the role of Y-chromosome dependent factors in cancer predisposition and needs further investigation. Overall, the findings of the present study suggest FHC to be an important factor in association studies of KIR and its HLA ligand with cancer.

Considering that KIR2DL1$^+$ frequency was only marginally higher in OSCC patients with FHC and HLA-C2$^+$ was seen predominantly in FHC patients, it could be suggested that HLA-C2$^+$ alone was the determining risk factor to OSCC. However, as HLA-C2 consistently co-occurred with KIR2DL1 in FHC patients, the independent contribution of HLA-C2$^+$ with disease could not be assessed. Further, due to presence of only three KIR2DL1$^-$-HLA-C2$^+$ genotypes in patients with no FHC and eighty-five KIR2DL1$^+$-HLA-C2$^+$ genotypes in FHC patients, logistic regression analysis was not considered.

A negative association of KIR2DL3$^+$-HLA-C$^+$ genotype was noted with OSCC suggesting its protective role. The binding and functional studies have reported that KIR2DL3 in presence of its ligand HLA-C1/C2 delivers the weakest inhibitory effect among the different inhibitory KIR-HLA-C1/C2 genotypes. In NK cell, the weaker inhibitory effect mediated by KIR2DL3$^+$-HLA-C$^+$ was hypothesized to reduce the threshold of NK activation [40].

Accordingly, it can be anticipated that the weaker inhibitory effect conferred by KIR2DL3$^+$-HLA-C$^+$ genotype may be protective in oral cancer, possibly because it lowers NK cell activation threshold leading to antitumour response.

The role of weaker inhibitory KIR-HLA genotype has been reported earlier, where KIR2DL3$^+$-HLA-C1$^+$ genotype was found to be protective in acute Hepatitis C virus infection and malignant melanoma [243, 247]. Similarly, in cerebral malaria, KIR2DL3$^+$-HLA-C1$^+$ genotype was seen to be positively associated with development of cerebral malaria in Thai population of Southeast Asia [249]. Several other studies have also negatively correlated less inhibitory KIR-HLA genotype such as KIR2DL3$^+$-HLA-C1$^+$ with virus associated-cancers like hepatocellular carcinoma where inflammatory response triggered by excess activation of NK cells was suggested to be associated with the pathogenesis of the disease [250].

In addition, the association of KIR2DL3$^+$-HLA-C$^+$ with oral cancer may also be explained in the context of 'NK licensing'. Self-recognizing KIR-HLA interactions can influence NK cell responsiveness in at least two ways: 1) during NK cell maturation where they promote the generation of functional pools of NK cells and 2) by controlling the activation threshold of the mature NK cells [40]. In the present study, KIR2DL3$^+$-HLA-C$^+$ genotype together with HLA-C1C1 homozygosity in control participants may have resulted in functionally competent mature NK cells. However, as KIR2DL3$^+$-HLA-C$^+$ genotype exhibits weaker KIR-HLA interaction, it lowers NK cell activation threshold leading to hyper-responsive NK cells. Accordingly, in the present study, higher occurrence of KIR2DL3$^+$-HLA-C$^+$ genotype in healthy participants may be protective in oral cancer but at the same time susceptible/risk factor for other diseases. Similarly, for stronger KIR2DL1$^+$-HLA-C2$^+$ genotype, the presence of KIR2DL1$^+$-HLA-C2$^+$ genotype may have resulted in hypo-responsive NK cell population in OSCC.

The association between KIR locus, HLA ligand and HLA-KIR combination with the clinical tumour stages of oral cancer could not be

determined because most oral cancer patients of the present study were detected in advanced tumour stage. On comparing the distribution of HLA class I ligands -C1 and -C2 in the present study population with reports of Parham and Moffett [251], it was observed that the distribution of these HLA class I ligands was comparable to populations of Asia. In addition, Bw4 distribution was comparable to both Asian and Melanesian population. Consistent with previous report [252], the KIR distribution was similar to Oriental populations of Asia.

In summary, the KIR and its HLA ligand genotyping data together with KIR expression data support the role of KIR-HLA interaction in oral cancer. The findings of the study suggest KIR2DL1^{+}- HLA-C2^{+} genotype as heritable risk factor in OSCC predisposing to cancer at younger age. On the other hand, the KIR2DL3^{+}-HLA-C^{+} genotype was protective in oral cancer. Since early detection of oral cancer is an important criterion to lower morbidity and death rates, this study may be useful step towards cancer surveillance and early detection of oral cancer in patients with FHC.

4.3. NEGATIVE REGULATION OF NATURAL KILLER CELL IN TUMOUR TISSUE AND PERIPHERAL BLOOD OF ORAL SQUAMOUS CELL CARCINOMA

4.3.1. Results

4.3.1.1. Patient characteristics

The characteristics of the OSCC patients and control participants are same as in 4.2.1.1.

4.3.1.2. Cytolytic activity of freshly isolated peripheral blood NK cells

Peripheral-blood NK cells (PB-NK cells) were functionally defined by their ability to exert cytolytic activity against HLA class I-negative target cell lines, including K562 [253]. In the study, the ability of freshly isolated PB-NK cells to lyse K562 tumour cells was measured in 20 OSCC patients and 10 healthy controls by fluorescence microscopy.

4.3.1.2.1. PB-NK cell count

PB-NK cells represented 6-8% of peripheral blood mononuclear cells (PBMCs). The viability percentage of freshly isolated PB-NK cells was 80-85. The average PBMC and NK cell count in OSCC and control participants is summarized in the Table 34. In OSCC patients, the PB-NK cell count was lower compared to healthy controls (p=0.0039).

	OSCC	Control
PBMCs Count (viable cells/ml blood)	$8.5 \times 10^5 \pm 1.1 \times 10^4$	$8.6 \times 10^5 \pm 1.2 \times 10^4$
NK cells Count (viable cells/ml blood)	$5.8 \times 10^4 \pm 2.8 \times 10^3$	$6.2 \times 10^4 \pm 2.3 \times 10^3$

Table 34. Average PBMC and NK cell count in peripheral blood of participants. *PB-NK cell count was lower in OSCC patients compared to healthy controls (p=0.0039)*

4.3.1.2.2. Standardization of effector:target ratio and incubation time

NK cell-mediated lysis of K562 cells was examined in three different incubation (PB-NK cells from healthy controls + K562 cells) times (4 hours, 8 hours and 12 hours) to evaluate whether there is significant rise in lysis of K562 cells with increasing incubation time (Figure 25). It was observed that lysis of K562 cells following 12 hours (70.4%) and 8 hours (69.1%) of incubation was higher than 4 hours of incubation. However, this was not statistically significant ($p>0.01$). It was thereby inferred that prolonged incubation over 8 and 12 hours do not improve the killing of target cells.

To determine the appropriate effector:target ratio (E:T ratio), the PB-NK cells from healthy controls and K562 cells were incubated for 4 hours in three different E:T ratios (E:T ratio – 0.5:1, 1:1 and 4:1) (Figure 26). Of the three E:T ratios, lowest NK cell lysis of K562 cells was observed at 0.5:1 ratio (52.8%). Further, there was no significant increase ($p>0.01$) in NK cell cytotoxicity at 4:1 E:T ratio (68.6%). This may be explained by the stringent regulation of the resting NK cells, which prevents autoimmune derived diseases [254, 255].

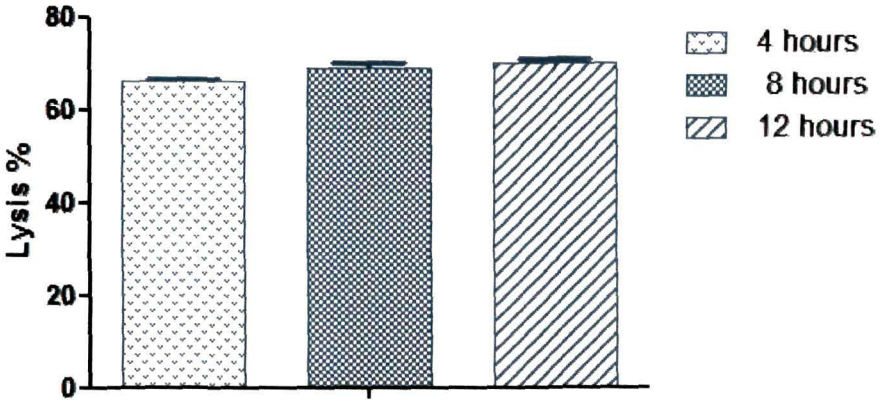

Figure 25. Lysis (%) of K562 cells by PB-NK cells in three different incubation times. *Prolonged incubation over 8 and 12 hours did not improve the killing of target cells*

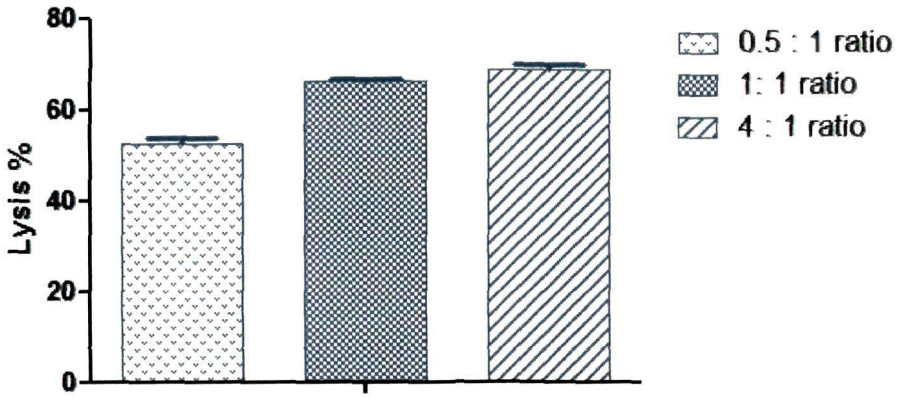

Figure 26. Lysis (%) of K562 cells by PB-NK cells in the three effector:target ratios. *Detection of lower cytotoxicity at effector:target ratio (0.5:1). No significant increase in NK cytotoxicity at 4:1 effector:target ratio (p>0.01)*

4.3.1.2.3. Decreased cytolytic activity of PB-NK cell in OSCC

Freshly isolated PB-NK cells and K562 cells were incubated for 4 hours in 1:1 E:T ratio. It was noted that, PB-NK cells of OSCC patients showed decreased lysis (%) of K562 cell compared to healthy controls (p=0.0001) (Figure 27). Further, no statistical difference in NK cell lysis (%) was observed in the two clinical stages of OSCC patients (Early stage - 52.2% vs. Advanced stage - 51.1%).

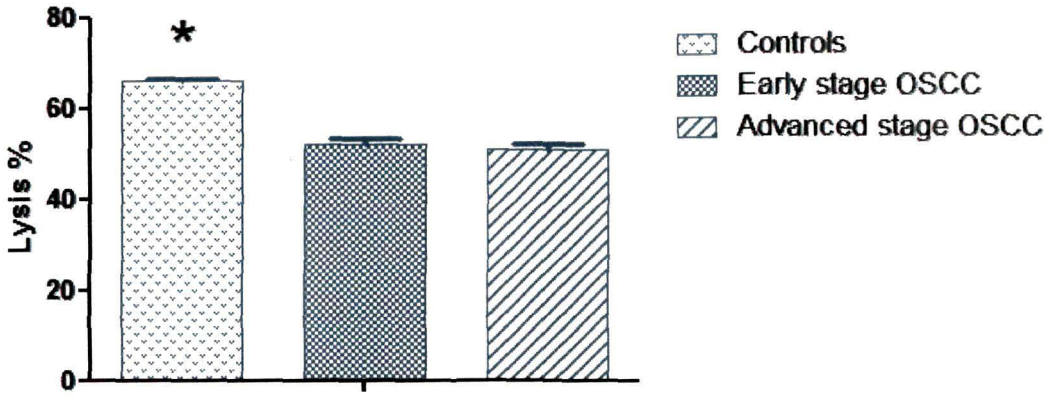

Figure 27. Lysis (%) of K562 cells by PB-NK cells isolated from peripheral blood of healthy controls, early stage and advanced stage OSCC patients. *p-*

value of <0.01 is indicated with asterisk. NK cell-mediated lysis of K562 was lower in OSCC compared to healthy controls (p=0.0001)

4.3.1.3. Transcript expression study

The RNA transcript expressions of – NKp46, NK cell associated cytokines (pro-inflammatory cytokines – IL-1β, IL-2, IL-12β, IL-15, IL-18, IL-21, IFN-γ and TNF-α, and anti-inflammatory cytokines – IL-10 and TGF-β), Forkhead Box P3 (FOXP3) and Cytotoxic T-Lymphocyte-Associated Antigen 4 (CTLA4) were measured in tumour tissue and peripheral blood of 75 OSCC patients (in triplicates) by qRT-PCR (Figure 28). Further, the transcript expressions of KIR genes in the tumour tissue were also evaluated in the 75 OSCC patients. The expression of each gene in OSCC patient was calculated relative to healthy controls [229].

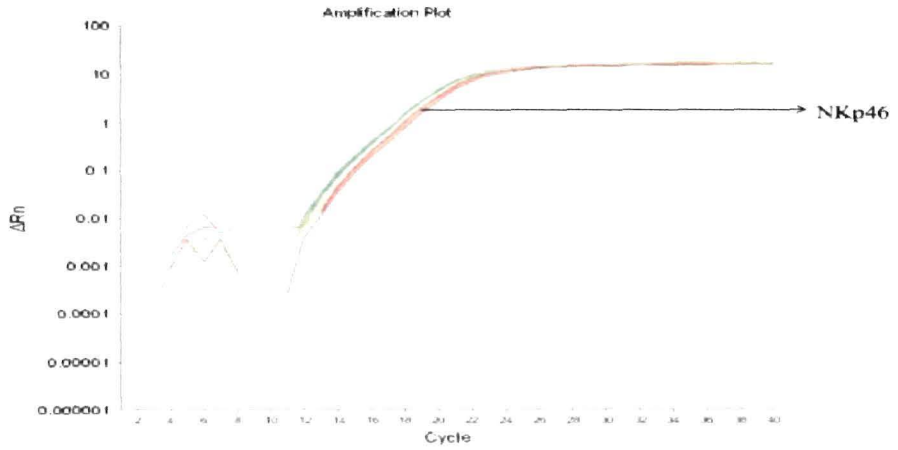

Figure 28a. Amplification plot showing the variation of log (ΔRn) with PCR cycle number for NKp46 in 3 tumour tissue samples in triplicates (SYBR Green based primer assays)

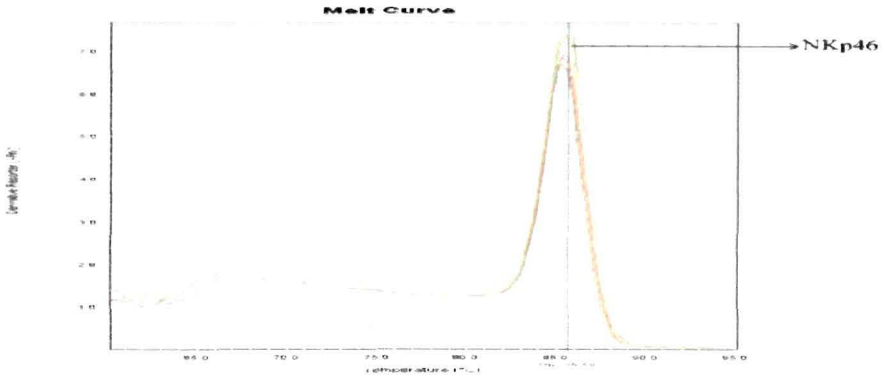

Figure 28b. Melting curves for NKp46 amplicon in 3 tumour tissue samples in triplicates. *It was observed that NKp46 was successfully amplified in the real-time PCR with no non-specific products*

4.3.1.3.1. NKp46 expression in OSCC

In both peripheral blood and tumour tissue, the transcript expression of NKp46 gene was downregulated in OSCC patients (Figure 29 and 30). However, NKp46 expression was significantly higher in the peripheral blood than in tumour tissue ($p<0.0001$). The correlation analysis revealed a strong correlation of peripheral blood-NKp46 (PB-NKp46) expression with NK cell count ($r = 0.94$, $p <0.001$) and with NK cell cytotoxicity (lysis percentage against K562 cells) ($r = 0.95$, $p <0.001$) (Figure 31).

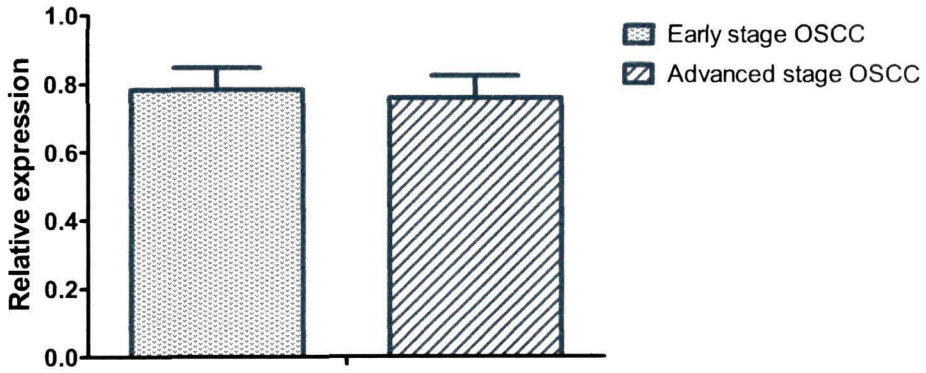

Figure 29. Mean RNA transcript expression of NKp46 gene in peripheral blood of OSCC patients relative to healthy controls. *No significant difference in the early (0.78 fold) and advanced stages (0.76 fold) of the disease ($p>0.01$)*

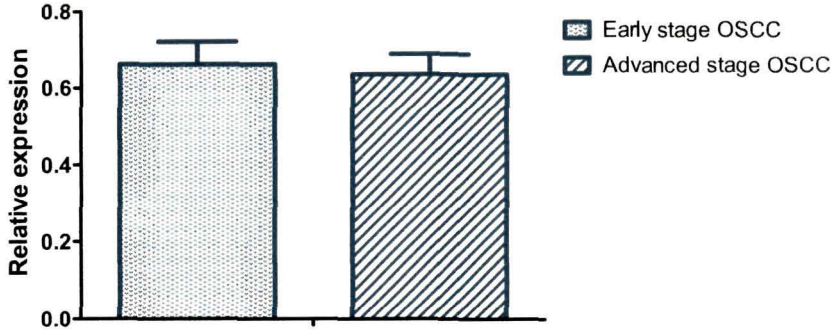

Figure 30. Mean RNA transcript expression of NKp46 gene in tumour tissue of OSCC patients relative to healthy controls. *No significant difference in the early (0.66 fold) and advanced stages (0.63 fold) of the disease (p>0.01)*

Figure 31a

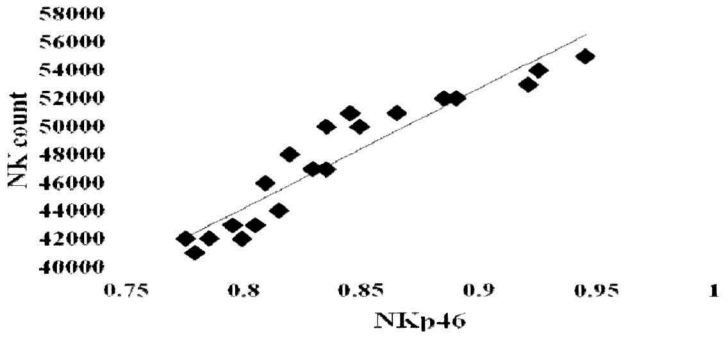

Figure 31b

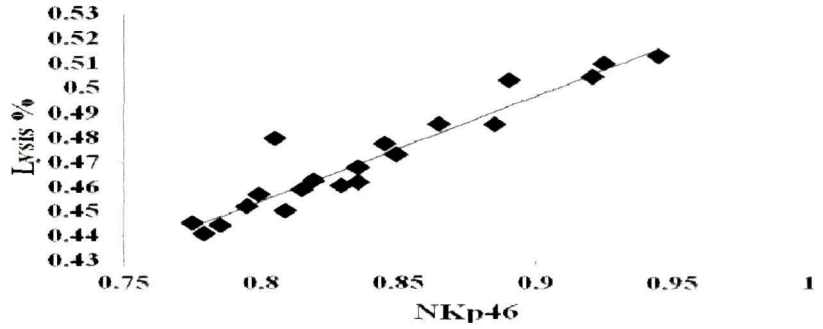

Figure 31. Correlation between transcript expression of PB-NKp46 – a) with PB-NK cell count and b) with PB-NK cell cytotoxicity (lysis percentage

against K562 cells) in OSCC patients. *Correlation analysis – NKp46 vs. NK count ($r = 0.94$, $p < 0.001$); NKp46 vs. NK cytotoxicity ($r = 0.95$, $p < 0.001$)*

4.3.1.3.2. KIR expression in OSCC

For the six KIR groups – 2DL1, 2DL2 & 2DL3; 2DL5 A & B; 3DL1 & 3DS1; 3DL1; 2DS1, 2DS2 & 2DS3; 2DS3 & 2DS5, the C_T values in triplicates could be determined in only 17, 9, 16, 17, 17 and 8 OSCC samples respectively. In these samples, the six KIR groups were downregulated relative to healthy controls (Figure 32).

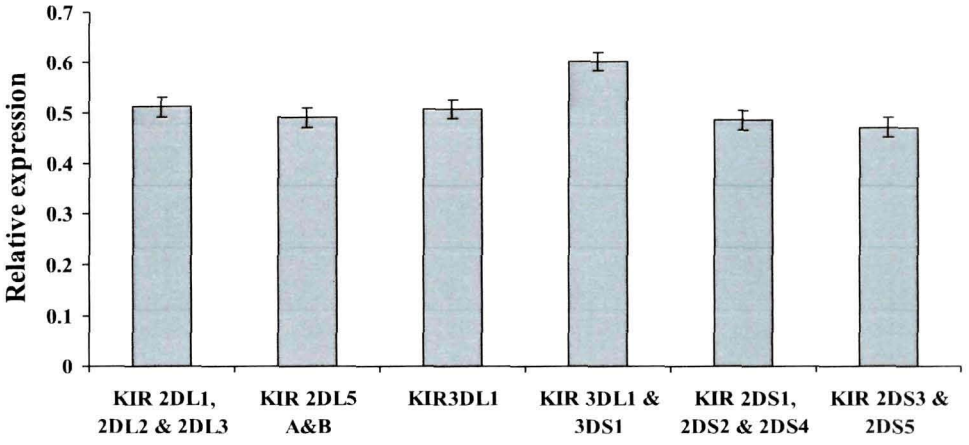

Figure 32. Mean transcript expression of six KIR groups in tumour tissue of OSCC patients relative to healthy controls

4.3.1.3.3. Cytokine expression in OSCC

The transcript expressions of the pro-inflammatory cytokines –IL-1β, IL-2, IL-12β, IL-15, IL-18, IL-21, IFN-γ and TNF-α and anti-inflammatory cytokines – IL-10 and TGF-β in the tumour tissue and peripheral blood of OSCC patients are summarized in the Figure 33. In tumour tissue, transcript abundance for the cytokines – IL-2, IL-12β, IL-15, IL-18, IL-21 which have positive functional effects on NK cells were downregulated in OSCC together with IFN-γ, the key effector and activator molecule of NK cell. However, the expressions of pro-inflammatory cytokines – TNF-α (2.01 fold) and IL-1β

(1.91 fold), and anti-inflammatory cytokines – TGF-β (3.72 fold) and IL-10 (2.25 fold) were found higher in OSCC patients. In advanced clinical stage, there was marked decrease in expression of IL-12β and IFN-γ, concomitant with increase of IL-10 and TGF-β expression (Table 35). Further, correlation analysis revealed that in OSCC patients, reduced NKp46 expression was positively correlated with diminished expression of IFN-γ, IL-12β and IL-15 in tumour tissue (Figure 32). Moreover, IL-12β and IFN-γ were found strongly correlated with the disease ($r = 0.89$, $p<0.0001$) (Figure 33).

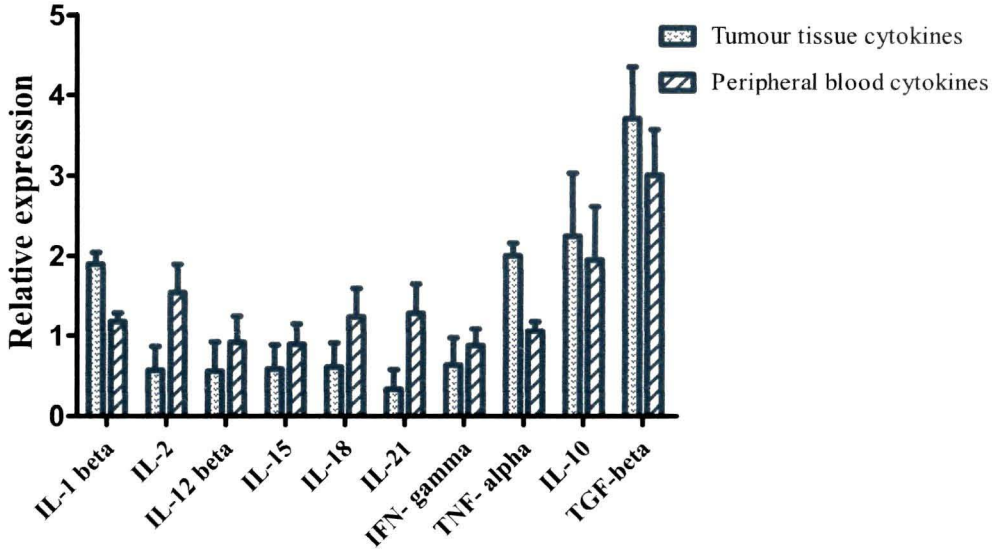

Figure 33. Mean transcript expressions of cytokines in tumour tissue and peripheral blood of OSCC patients relative to healthy controls. *The cut-off for transcript expression ≤ 0.80 and ≥ 1.5, $p<0.01$ was considered as significant (OSCC vs Healthy controls)*

In peripheral blood, a similar trend of transcript expression was observed for the two anti-inflammatory cytokines – IL-10 and TGF-β (Figure 32 and Table 35). However, unlike in tumour tissue, the expression of IL-2 was upregulated while expressions of other pro-inflammatory cytokines (IL-1β, IL-12β, IL-15, IL-18, IL-21, IFN-γ and TNF-α) were comparable with healthy controls in the peripheral blood. In addition, no significant correlation ($p>0.01$) was noted between expression of PB-NKp46 and any of the cytokines.

Cytokines	Tumour tissue (mean relative expression)		Peripheral blood (mean relative expression)	
Pro-inflammatory	**Early stage OSCC**	**Advanced stage OSCC**	**Early stage OSCC**	**Advanced stage OSCC**
IL-1β	1.887	1.931	1.16	1.22
IL-2	0.599	0.558	1.525	1.569
IL-12β	**0.676**	**0.46**	0.924	0.932
IL-15	0.625	0.565	0.882	0.923
IL-18	0.603	0.628	1.258	1.233
IL-21	0.359	0.323	1.279	1.307
IFN-γ	**0.758**	**0.523**	0.906	0.872
TNF-α	1.952	2.07	1.043	1.093
Anti-inflammatory				
IL-10	**1.958**	**2.55**	**1.7**	**2.217**
TGF-β	**3.275**	**4.158**	3.056	2.973

Table 35. Mean transcript expressions of the cytokines in the two clinical stages of the disease relative to healthy controls. *p-values for relative expression values <0.01 are indicated as bold . In tumour tissue for IL-12β, IFN-γ, IL-10 and TGF-β p<0.001 (Early stage vs. Advanced stage). In peripheral blood for IL-10 p<0.001 (Early stage vs. Advanced stage)*

The independent relative influence of the pro- and anti- inflammatory cytokines on malignant features was determined by logistic regression analysis. Interestingly, higher transcript levels of IL-10 in peripheral blood ($p<0.0001$) and TGF-β in tumour tissue ($p <0.0001$) revealed significant contributions for higher-grade tumour (T3 & T4 vs. T1 & T2), lymphatic involvement (N1, N2 & N3 vs. N0) and higher clinical stage (Advanced stage vs. early stage). In

addition, in peripheral blood IL-10 was noted to be negatively correlated with PB-NK cell count and with PB-NK cell cytotoxicity (IL-10 vs. NK cell count, r = - 0.68, p<0.001); IL-10 vs. NK cell cytotoxicity, r = -0.59, p<0.007) (Figure 34).

Figure 34a

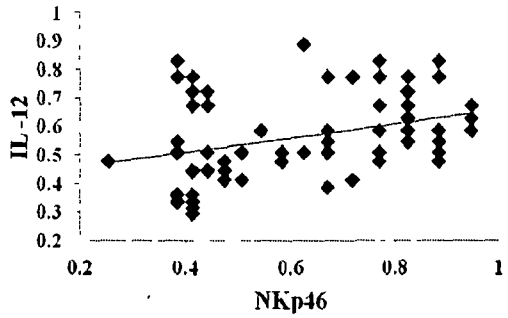

Figure 34b

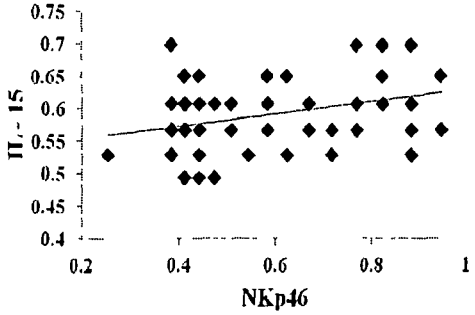

Figure 34c

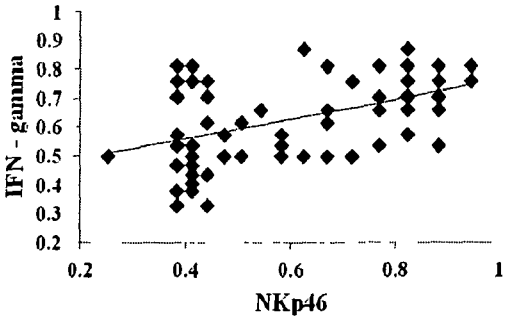

Figure 34d

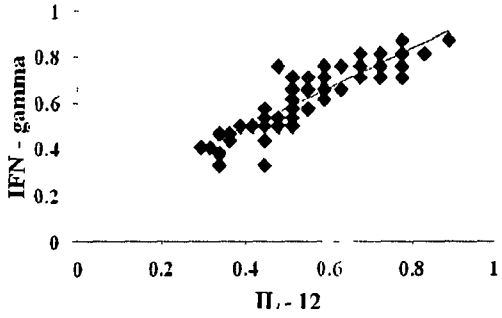

Figure 34e

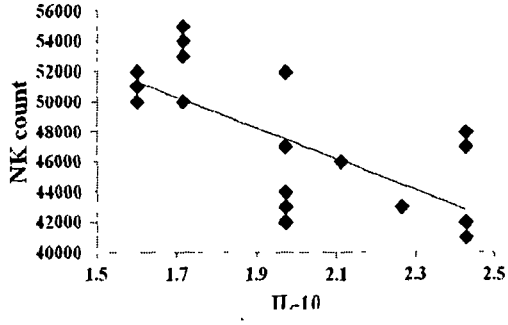

Figure 34f

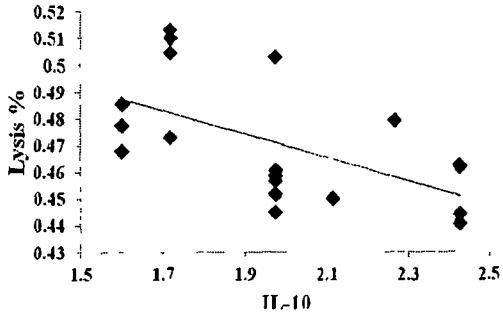

Figure 34. Correlation between transcript expression of – a) NKp46 with IL-12β, b) NKp46 with IL-15, c) NKp46 with IFN-γ in tumour tissue, d) IL-12β with IFN-γ in tumour tissue, e) Blood IL-10 with PB-NK cell count and f)

Blood IL-10 with PB-NK cell cytotoxicity (Lysis percentage against K562 cells) in OSCC patients. *Correlation analysis – NKp46 vs. IL-12β ($r = 0.35$, $p<0.002$); NKp46 vs. IL-15 ($r = 0.35$, $p<0.002$); NKp46 vs. IFN-γ ($r = 0.49$, $p<0.0001$); IL-12β vs. IFN-γ ($r = 0.89$, $p<0.0001$); IL-10 vs. NK cell count ($r = -0.68$, $p<0.001$); IL-10 vs. NK cell* cytolytic activity *($r = -0.59$, $p<0.007$)*

4.3.1.3.4. FOXP3 and CTLA4 expressions

In the study, upregulation of anti-inflammatory cytokines – TGF-β and IL-10 suggested an immunosuppressive microenvironment in OSCC. Since such microenvironment favours proliferation of suppressive immune cells, expression of FOXP3 and CTLA4 was examined in tumour tissue and peripheral blood of OSCC patients. Both FOXP3 and CTLA4 transcript expressions were found higher in tumour tissue compared to peripheral blood (Figure 35). Moreover, a significant positive correlation was found between FOXP3 and CTLA4 in both tumour tissue ($r = 0.57$, $p<0.0001$) and peripheral blood ($r = 0.68$, $p<0.0001$).

The higher transcript levels of FOXP3 and CTLA4 in tumour were not surprising since tumour cells also express FOXP3 and CTLA4. Further, upregulation of FOXP3 and CTLA4 transcripts together with TGF-β and IL-2 in the peripheral blood of OSCC suggested increased proliferation of suppressive cells like regulatory T cells (Tregs) in the circulation.

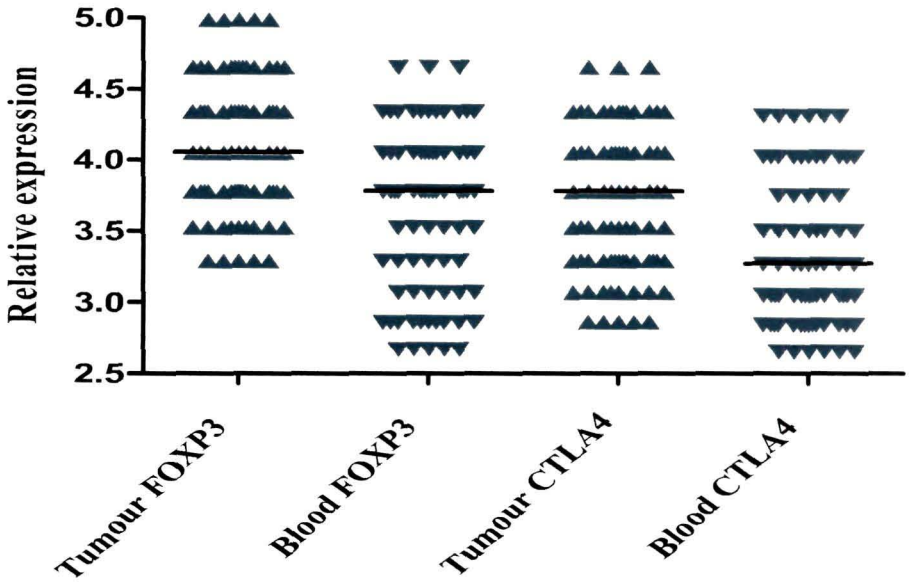

Figure 35. Transcript expressions of FOXP3 and CTLA4 gene in OSCC patients relative to healthy controls. *The horizontal bars indicate median values. Tumour – FOXP3 (4.1 fold) and CTLA4 (3.69 fold); Peripheral blood – FOXP3 (3.64 fold) and CTLA4 (3.35 fold). Paired t-test – Tumour FOXP3 vs. Blood FOXP3 ($p<0.0001$); Tumour CTLA4 vs. Blood CTLA4 ($p=0.0002$)*

4.3.2 Discussion

NK cells play important role in tumour surveillance; nevertheless, tumour develops various mechanisms to escape from NK cell immune pressure [256]. Abnormal NK cell cytolytic function has been described earlier in almost all haematological malignancies [256]. In solid tumours like head and neck cancer and breast cancer, the NK cells in the circulation were also reported to have low NK activity [257]. However, the reasons for this preferential suboptimal NK cell function are still not very clear. In this context, we examined the PB-NK cell cytotoxicity and transcript expressions of – NK cell receptors, NK cell associated cytokines, FOXP3 and CTLA4 to understand their relationship with NK cell activation in OSCC.

In the study, reduced PB-NK cell number and cytotoxicity in OSCC patients was noted. Moreover, in these patients, PB-NKp46 expression was

downregulated and strongly correlated with PB-NK cell number and cytotoxicity: supporting NKp46 as a – 1) reliable marker receptor of NK cell and 2) a major activating receptor in NK cell-mediated cytolysis in OSCC. This findings were consistent with previous studies on hepatitis C virus infection [258, 259], acute myeloid leukaemia [260, 261] and cervical cancer [103], which had also reported association of NKp46 downregulation with decreased NK cell-mediated cytolysis. Further, in melanoma, lymphoma and carcinoma, NKp46 was reported as the key receptor in NK cell-mediated lysis of tumours [62]. In leukaemia, it was suggested that insufficient activation caused by defects in NKp46 expression might represent a potent method for tumour immune evasion [256]. Accordingly, in our study it may be argued that the downregulation of NKp46 could also be an immune evasion strategy of oral tumours since downregulation of PB-NKp46 was concomitant with PB-NK cell cytotoxicity.

IL-2, IL-12β, IL-15, IL-18, IL-21 and IFN-γ have been described as the prominent soluble factors involved in differentiation, proliferation, survival and activation of NK cells [66, 67, 69]. On the other hand, the anti-inflammatory cytokines – TGF-β and IL-10 were identified as inhibitory factors for blunting NK effector functions [70-77]. In accordance, in the tumour tissue, the downregulation of NK cell activating cytokines in OSCC together with the upregulation of NK suppressive cytokines suggested the presence of immature and functionally impaired tumour resident-NK cells (TR-NK cells). Further, upregulation of TGF-β and IL-10 could also result in a 'pool of tumour infiltrating-NK cells (TI-NK cells) with lower effector functions. It was described that TGF-β could suppress NK cell by reducing the expression of NK activation receptors, IFN-γ production and cytolytic effect of NK cells [70, 71-75]. Wilson et al. [72] reported that TGF-β antagonized IL-15, which induces proliferation and gene expression associated with NK cell activation, resulting in inhibition of both NK cell activating receptor molecules and components of the cytotoxic apparatus. Further, Szkaradkiewicz and his group reported IL-10 to reduce NK activity and IFN-γ secretion in cancer patients [76].

Among the pro-inflammatory cytokines, upregulation of NK effector molecules - TNF-α and IL-1β in tumour tissue of OSCC patients but not in peripheral blood suggested the tumour-specific production of these cytokines. However, in the scenario where NK cell activity is dampened, it is unlikely that NK cells were the source of increased expression of TNF-α and IL-1β. Although the exact source of these cytokines was not determined in our study, but it is well established that oral cancer cells apart from a variety of cells including macrophages, fibroblasts and endothelial cells actively produce TNF-α and IL-1β [262-264], and that altered level of these cytokines is tightly associated with the development of OSCC [265, 266]. Considering earlier reports on increased levels of both TNF-α and IL-1β in oral cancer patients, some investigators have suggested the expression profile of TNF-α and IL-1β as a marked feature of oral cancer, which could be used for monitoring the malignant transformation of oral leukoplakia [265, 267]. The increased production of TNF-α and IL-1β in the tumour tissue but not in peripheral blood in present study supported this assumption.

In the study, the diminished NKp46 expression in the tumour tissue indicated reduced NK cell count and/or lower NK cell activation. Moreover, lower (or undetectable) levels of KIR expression hinted lower infiltration of peripheral blood NK cells to the tumour tissue or possible change in phenotype of these cells in the tumour tissue. Glasner and his group [62] had suggested that the expression of NKp46 could be enhanced by cytokine for controlling the tumour. Interestingly, in the present study, the positive correlation between expression of NKp46 in the tumour tissue with IFN-γ, IL-12β and IL-15, pointed the possible opportunity for enhancement of NKp46 expression through the elevation of these cytokines. Such manipulation of NKp46 expression might be important since recent studies have shown that tumours can successfully evade NKG2D-mediated NK cytolysis [62, 268], which is considered another potent NK activating receptor for tumour immunosurveillance.

In the peripheral blood of OSCC patients, the steady expression level of cytokines - IL-1β, IL-2, IL-12β, IL-15, IL-18, IL-21, IFN-γ and TNF-α indicated the scope for presence of functionally mature NK cell population. However, marked decrease in PB-NK cell count and cytotoxicity was observed in OSCC patients together with lower expression of NKp46. This might be explained by the increased levels of immunosuppressive cytokines - IL-10 and TGF-β in the blood. Interestingly, negative correlation of IL-10 with PB-NK cell count and with PB-NK cell cytotoxicity suggested a role of IL-10 in regulating NK cell. Modelling of data by logistic regression analysis also revealed higher level of IL-10 to be a risk factor in advanced disease.

The anti-inflammatory cytokines - TGF-β and IL-10 can recruit suppressor cells like $CD4^+$ $CD25^+$ Tregs which express intracellular and surface markers such as FOXP3 and CTLA4 respectively [269]. Recent evidences have described that for Tregs-mediated immunologic tolerance IL-2 is crucial, as it is associated with Tregs development, homeostasis and function [270]. In the present study, considering higher expression of both TGF-β and IL-10 together with IL-2 in peripheral blood, proliferation of suppressive immune cells like Tregs was examined. Subsequently, expression data of FOXP3 and CTLA4 gene indicated the induction of $FOXP3^+CTLA4^+$ suppressor cells in the circulation. Earlier studies have described that increased frequency of Treg cell number was directly correlated with cancer progression [271, 272]. In addition, the number of Treg cells was inversely correlated with the frequency and function of NK cells [273-276]. Cai et al. [277] demonstrated that in hepatocellular carcinoma patients, reduced killing capacity of PB-NK cells against K562 target cells was correlated to a high incidence of Treg cells [273]. Further, it has been described that NK cell activity can be restored with the depletion of Treg cell population [273]. Thus, based on previous investigations on NK impairment by $FOXP3^+CTLA4^+$ suppressor cells, it would appear that diminished PB-NK cell cytolysis might be related to proliferation of suppressor cells in the peripheral blood of OSCC patients. However, unlike in peripheral blood, decreased IL-2 expression in the tumour tissue suggested lower scope for induction of

$FOXP3^+CTLA4^+$ suppressor cells in the tumour, despite higher transcript levels of FOXP3 and CTLA4.

In summary, our study provided an insight into the negative regulation of NK cell in tumour tissue and peripheral blood of OSCC patients. In the peripheral circulation, the possible mechanisms involved are: qùantitative deficiency of NK cell number and reduced cytotoxicity, and qualitative NK impairments caused by - 1) decreased expression of NK activating receptor NKp46, 2) increased expression of NK suppressive cytokines – IL-10 and TGF-β and 3) induction of $FOXP3^+CTLA4^+$ suppressor cells. On the other hand, in the tumour tissue, escape of NK immune surveillance appeared to be modulated by upregulation of TGF-β and IL-10 together with downregulated of NK cell activating cytokines (IL-2, IL-12β, IL-15, IL-18, IL-21 and IFN-γ) and NK receptors (NKp46 and KIRs). However, it needs to be determined if higher expression of FOXP3 and CTLA4 gene in tumour tissue is related to the suppressor cell or if they are expressed by tumour cells as an immune evasion strategy. In addition, the present study supported the use of TNF-α and IL-1β expression levels as markers of malignant transformation of oral leukoplakia. Thus, this part of the study presented the key issues like neutralizing the immunosuppressive factors and augmenting NKp46 expression, to raise NK cell activation in oral cancer.

Chapter V

SUMMARY AND CONCLUSION

5.1. SUMMARY

KIR exhibits extensive diversity and it has been observed that populations with different ethno-history, linguistic, geographic and genetic backgrounds can differ in KIR profile [28, 29]. In this context, we examined the diversity of the KIR loci in three ethnic populations of Assam, Northeast India. Our results demonstrated that the KIR complex of the Mundari-speaking Adivasi was distinct from the two Mongoloid populations – Kachari and Ahom of the region. The neighbour-joining dendrogram that was constructed based on KIR gene frequencies mapped Kachari and Ahom with East and Southeast Asian populations, while Adivasi clustered with African populations. Interestingly, mapping of these populations with world populations was consistent with their genetic affinity and migration history as showed earlier by mitochondrial-DNA and Y-chromosome studies [240-242]. Further, our study on HLA class I loci revealed that the distribution of HLA class I epitopes – Bw4, C1 and C2 in the two Mongoloid populations – Kachari and Ahom of the region was comparable with Oriental populations of Asia. It is thus anticipated that immune response to disease in the two Mongoloid populations and Oriental populations are likely to overlap and hence provide a basis for understanding disease in context of genetic backgrounds of these populations. The similarities in clinical manifestation of systemic lupus erythematosus in the Mongoloid population of this region with that of Chinese population support our assumption (Unpublished data of our laboratory).

The germline encoded receptor-ligand (KIR-HLA) interaction is an important component of NK cell reactivity [39-43]. In the Mongoloid populations of the region, two KIR-HLA genotypes – KIR2DL1$^+$-HLA-C2$^+$ and KIR2DL3$^+$-HLA-C$^+$ were found to be association with OSCC. It was noted that presence of KIR2DL3$^+$-HLA-C$^+$ genotype was protective in OSCC, particularly in patients with family history of cancer, while KIR2DL1$^+$-HLA-C2$^+$ genotype appeared to be a risk factor in OSCC. Thus, it may be interpreted that the presence of KIR2DL1$^+$-HLA-C2$^+$ genotype and absence of KIR2DL3$^+$-

HLA-C^+ genotype in a Mongoloid individual would tend to make the person more susceptible to oral cancer probably by influencing NK cell reactivity. The two genotypes are expected to modulate NK cell biology during NK cell education and during NK cell response to tumours. In OSCC patients, during NK cell development, the interaction of KIR with self-HLA class I ligand would result in maturation of functionally responsive NK cells through the process called ''education''. However, as KIR2DL1$^+$-HLA-C2$^+$ combination exhibits strongest inhibitory KIR-HLA interactions, these functionally competent mature NK cells should have higher activation threshold. On the contrary, KIR2DL3$^+$-HLA-C^+ genotype that exhibits weaker KIR-HLA interaction should have lower NK cell activation threshold leading to antitumor response thus conferring protection in OSCC. Interestingly, in both OSCC and control participants, the presence of HLA-C1 ligand was associated with increased expression of KIR2DL2/3, while HLA-C2 lowered the expression of KIR2DL2/3. But, in case of KIR2DL1, the presence of HLA-C2 ligand lowered the expression of KIR2DL1 in control participants, but not in OSCC patients, suggesting that epigenetic regulation for KIR expression differed in case and controls. Accordingly, our data supports the presence of KIR2DL1$^+$-HLA-C2$^+$ genotype to be deleterious in cancer patients. Hence, it may be inferred that in OSCC patients, during NK cell maturation, the interaction of KIR2DL1 with its HLA-C2 ligand ensured KIR mediated self-tolerance, but compromised NK cell reactivity, thus producing a functional pool of NK cells with higher activation threshold leading to decreased response to tumour.

On understanding that KIR/HLA immunogenetic background can influence oral cancer by modulating NK cell reactivity, the study further assessed the activation status of NK cell in OSCC patients in relation to NK cell cytotoxicity and transcript expressions of – NK cell receptors and NK cell associated cytokines. It was observed that NK cell cytotoxicity in peripheral blood was lower in OSCC patients. Moreover, it was noted that the NK cells

were negatively and differentially regulated in tumour tissue and in peripheral blood. This assumption was based on our following findings –

1) NK cell activating cytokines (IL-2, IL-12β, IL-15, IL-18, IL-21 and IFN-γ) were downregulated in the tumour tissue but not in the peripheral blood. This indicates that the tumour resident-NK cells in the oral tumour tissue, if present, could be either immature or functionally downregulated.

2) NKp46 transcript abundance in the tumour tissue was lower than in the peripheral blood. NKp46 is a marker receptor of NK cell and its expression shows correlation with NK cell activation. Thus, in our study, lower NKp46 expression in the tumour tissue than in the peripheral blood tends to imply either reduced NK cell count or diminished NK activation status or both in the tumour tissue compared to peripheral circulation. Further, lower (or undetectable) levels of KIRs in the tumour tissue support lower infiltration of peripheral blood NK cell to the tumour tissue or possible change in phenotype of these cells in the tumour tissue.

3) Transcript expression of NK cell suppressive cytokines – TGF-β and IL-10 were higher in advanced clinical stage of the disease. Further, based on logistic regression analysis, TGF-β level in tumour tissue and IL-10 in peripheral blood were identified as risk factors for advanced disease.

4) The study reported that the two prominent pro-inflammatory cytokines – TNF-α and IL-1β were upregulated in the tumour tissue but not in peripheral blood. This increased expression profile of TNF-α and IL-1β supported the earlier assumption that the increased expression profile of TNF-α and IL-1β in oral cancer patients may be considered as the marked feature of oral cancer, which could be used for monitoring the malignant transformation of oral leukoplakia.

5.2. CONCLUSION

Our case-control study suggested the possible activation status of tumour resident-NK cells, peripheral blood-NK cells and tumour infiltrating-NK cells in OSCC patients (Figure 36). In the tumour tissue, the diminished levels of NK cell activating cytokines (IL-2, IL-12, IL-15, IL-18 and IL-21) and higher levels of NK cell suppressive cytokines (TGF-β and IL-10) appeared to have resulted in immature and functionally impaired tumour resident-NK cells. On the other hand, the presence of cognate KIR-HLA interaction (KIR2DL1$^+$-HLA-C2$^+$) and NK cell activating cytokines produces a functionally mature pool of NK cells in peripheral circulation of OSCC patients, although their cytotoxicity level was noted to be low. Our study hinted that diminished cytolytic activity of peripheral blood-NK cells was promoted by a strong KIR-HLA genotype, increased NK suppressive cytokines and by induction of FOXP3$^+$CTLA4$^+$ suppressor cells. Further, the functional NK cells of the peripheral blood on infiltrating the tumour tissue are yet again affected by the immunosuppressive cytokines to lower their effector functions in the tumour microenvironment. In addition, the reduced NKp46 expression together with markedly lower (or undetectable) KIR expression in tumour tissue argues for lower NK cell count in tumour tissue. The lower NK count could be for lack of recruitment of peripheral blood-NK cells to tumour tissue in absence of required chemokines, possible change in phenotype of these cells or NK cell apoptosis; however, this needs to be investigated.

In conclusion, the study provides a potential biomarker (KIR2DL1$^+$-HLA-C2$^+$ with family history of cancer) for stratifying populations at risk for OSCC. Secondly, the KIR/HLA immunogenetic background of OSCC patients, along with cytokine microenviroment will give useful leads to understand NK cell activation, which can be exploited to boost the current NK cell-based therapeutic strategies for the treatment of oral cancer.

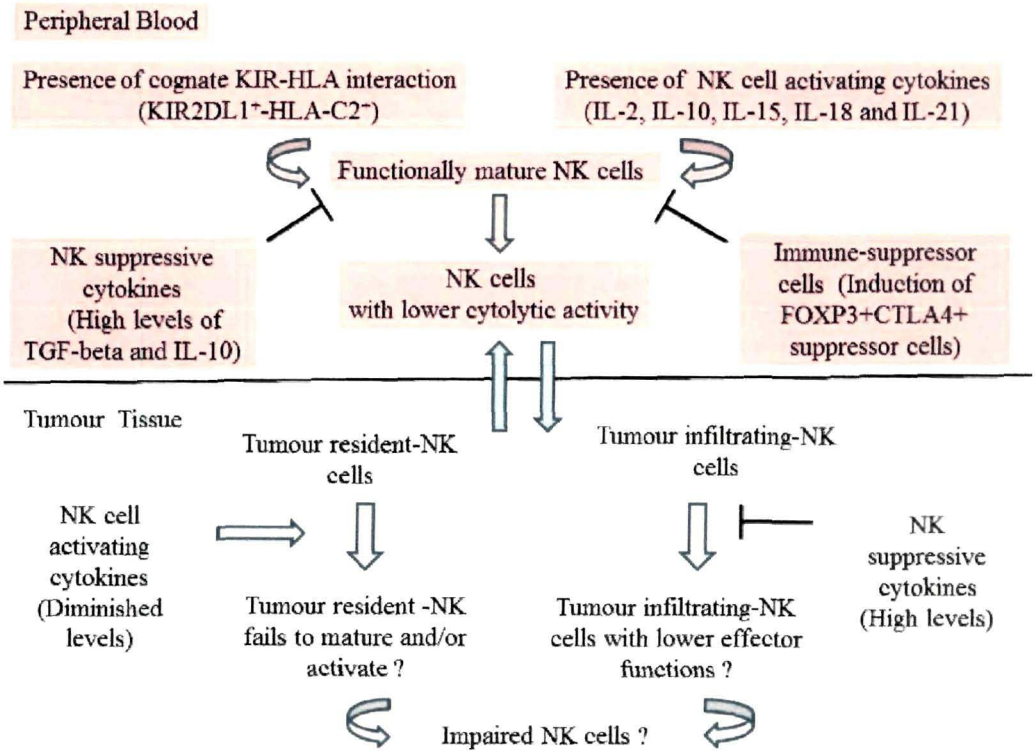

Figure 36. Summarizing the findings of the study. *In OSCC, the NK cells are negatively regulated in tumour tissue and in peripheral blood*

Chapter IV
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