Chapter I

INTRODUCTION

1.1 Introduction:

Natural Killer cells were reported as a subset of lymphocytes that deliver a selective but spontaneous cytotoxic activity without any prior sensitization by Kiessling et al. (1975) while studying cell-mediated cytotoxicity against tumor cells. This mechanism of spontaneous cytotoxicity was termed "natural cytotoxicity" and the cells responsible for mediating this effect were named Natural Killer (NK) cells(1). NK cells are considered to be a key component of early innate immune response and constitute approximately 15% of all circulating lymphocytes (2). Despite being regarded as a part of the innate immune system, NK cells exhibit functions such as priming, education, and memory, all of which were previously considered to be characteristic of adaptive immunity (1, 3, 4).

1.2 Development of human NK cells:

The development of NK cells occurs principally in the bone marrow microenvironment. The developmental process starts with the development of Hematopoietic stem cells which develops into multipotent progenitor (MPP) cells. This is followed by the emergence of a common lymphoid progenitor from these cells, which develops primarily into a precursor NK cell, which is followed by the development of an immature NK cell, and then finally into a mature NK cell. It is to be noted that the precursor NK cells do not have the ability to differentiate into T-cells, B-cells, erythroid or myeloid cells. However, they are stimulated to form mature NK cells (5, 6).

In humans, based on the expression levels of CD56, CD34, CD94, and CD117, there are several stages of NK development in the bone marrow(2). The process of NK maturation begins with the expression of CD56 which is followed by the

simultaneous expression of CD94/NKG2A(7).Reduction in CD94/NKG2A expression and a $CD56^{low}$ phenotype correlates with the final stages of NK maturation in the bone marrow. Another important factor in the development of NK cells from precursors is the expression of interleukin 2/15 receptor beta

(CD122)(8). In humans, the precursor NK cells are characterized as $CD34^{+}$ $CD122^{+}$ $CD56^{-}$ that respond to stimulation by IL-1engage in the initiation and completion of the final maturation process(9, 10). In addition, the interaction of stromal cells with NK cells is also a crucial step that is needed for normal NK development in which the Tyro3 family of receptors on NK cells interacts with their ligands on stromal cells for the expression of NK cell receptors and functional differentiation(8, 11).

1.3 Human NK cell recognition of target cells: NK receptors

The engagement of NK cell receptors acts asa detection system which determines the cellular response and function of NK cells. There are two functionally distinct types of NK cell receptor, the NK cell inhibitory and NK cell activating receptors whose balance of positive and negative signals controls NK cell activity. In addition NK cells possess adhesion receptors which helpsin the localization of the cells to sites of injury(12, 13).

There are three major types of inhibitory receptors: killer immunoglobulin receptors (KIRs), CD94/NKG2A, Ly49 and Siglecs (14, 15). Most NK cell inhibitory receptors have Immunoreceptor tyrosine-based inhibition motifs (ITIMs) located within their cytoplasmic tails (16, 17).

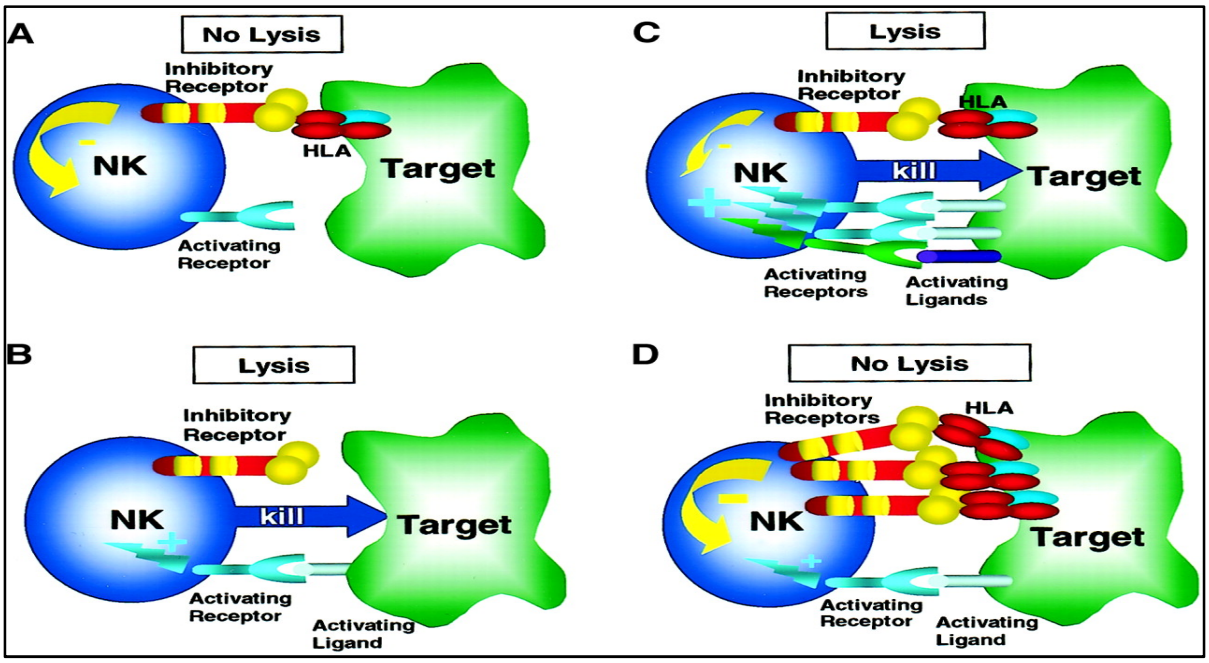

Figure 1: Recognition of target cell by NK cells. (A) inhibitory receptors engage HLA in the absence of an activating receptor/ligand interaction, a net negative signal is generated, resulting in no target cell lysis. (B) activating receptors engage their ligands on target cells in the absence of inhibitory receptor/ligand interaction, a net activation signal is generated, resulting in target cell lysis. (C) the activating receptor/ligand interactions predominate over weaker inhibitory receptor/ligand signals with the net result of NK cell activation and target cell lysis. This net result may occur when activation receptors and ligands are up-regulated, thereby amplifying the net activation signal to exceed the inhibitory signal. (D) inhibitory receptor/ligand interactions result in a net negative signal that prevents NK cell lysis of the target cell.

Most KIRs encoded in the leukocyte receptor complex (LRC) on human chromosome 19q13.4are inhibitory and their recognition of the major histocompatibility complex (MHC) suppresses the cytotoxic activity of NK cell (13, 16-18). The KIR family of genes includes twelve (12) members of which six(6) receptors are inhibitory in nature and the remaining six (6)receptors are activating, in addition to numerous allelic variants(19). The KIRs are monomeric receptors having either 2 (KIR2D) or 3 (KIR3D)immuno-globulinlike domains. Further, the receptors are subdivided into those having long (L) cytoplasmic tails (KIR2DL/KIR3DL) and those with a short (S) cytoplasmic tail (KIR2DS/KIR3DS)(20). The KIRs with a long cytoplasmic tail initiates an inhibitory signal, whereas the short cytoplasmic tail KIRs produces an activating signal. The presence of immune-receptor tyrosine-based inhibition motifs (ITIMs) in the long tail receptors results in an inhibitory signal. On the other hand, the association of adaptor proteins bearing immune-receptor tyrosine-based activating motifs (ITAMs) with that of the short tail receptors results in an activating signal(16). The KIRs have been found to be specific for a number of different MHC class I molecules, however, the predominant class I isotype is the HLA-C. The HLA-C isotype is found to be an important molecule involved in regulation of human NK cells(21-23).

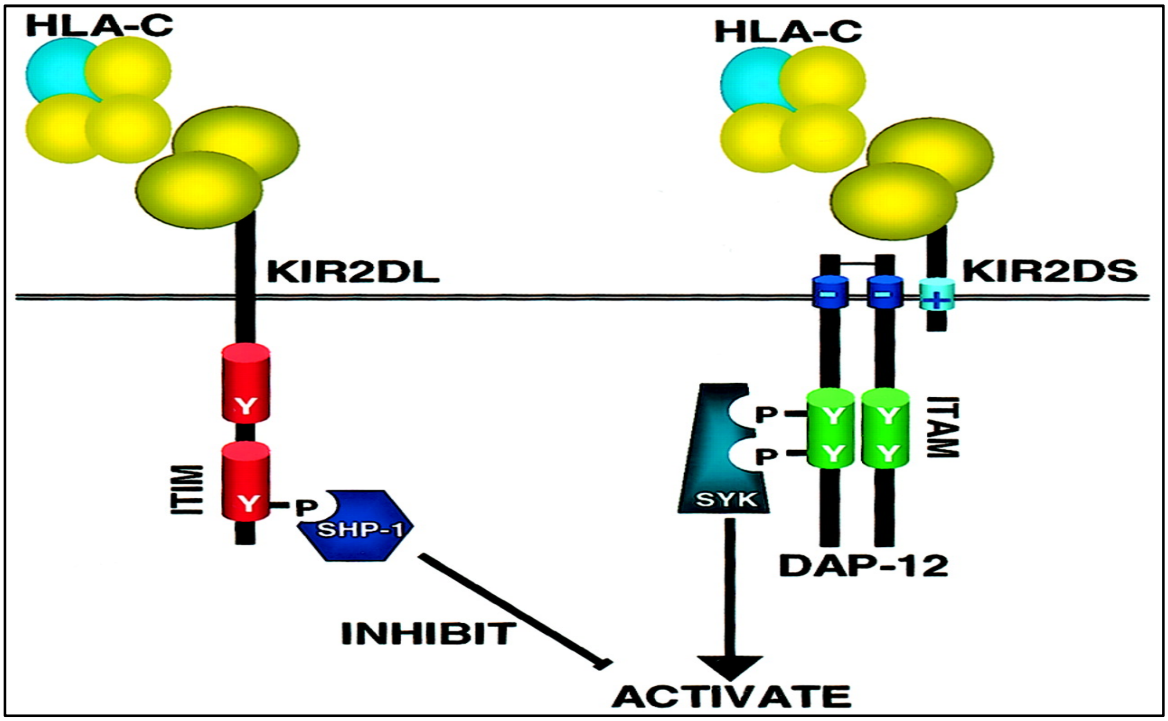

Figure 2: Operation of activating and inhibiting KIRs on NK cells

In addition, CD94/NKG2A, a family of C-type lectin receptors are expressed predominantly on the surface of NK cells. NKG2 receptors are transmembrane type II and form heterodimers afterdimerizingspecifically with CD94 molecule which is responsible for signal transduction(24, 25). Receptors of the CD94/NKG2 family bind non classical MHC class I glycoproteins which include the LILR family of genes and the CD33-related sialic acid binding Ig-like lectins (CD33rSiglecs); in particular human CD33rSiglec-7(2, 26). These receptors have inhibitory motifs (ITIMs) in their cytoplasmic domains which restricts the activation signals. CD33-related Siglecs are largely inhibitory and are expressed on human and mouse NK cells, B cells, monocytes, neutrophils, dendritic cells, basophils and eosinophils (27).NK cells also express diverse activating receptors which include CD16, NK1.1 , NKR-P1, (CD161), (KLRK1, CD314), NKG2D, NCR (NKp30, NKp44, NKp46, NKp80); and the activating isoforms of human KIRs(28-30). These molecules function as activating receptors as they have ITAM positive adaptor molecules (DAP12) and lack ITIMs (13, 31-35).

NK cells as the name suggests are known for their ability to lyse viral infected and tumor cells without any prior sensitization. This cytolytic activity is controlled by binding of the inhibitory NK receptors specifically to the human leucocyte antigen(HLA) molecules on healthy cells. In contrast, the NK cell activating receptors detects stressed cells. Whenever, theHLA class I molecules are down-regulated or lost in viral infected or on tumor cells, inhibitory signals from inhibitory receptors are lost and in turn expressing the activating signals resulting in NK cell activation. The entire process is termed as "missing-self" triggered NK activation(36). Additionally, NK cell activation receptors can detect self-molecules up regulated, a process called "stress-induced self-recognition." Once activated by the activating receptors, NK cells uses various methods to exert their cytotoxic effects which includes cytolytic granule mediated cell apoptosis and antibody dependent self-mediated cytotoxicity (ADCC). Also, when activated by cytokines or interferons NK cells promote phagocytosis by secreting interferon gamma and TNF alpha (11, 37, 38).

1.4 Natural killer cells in Cancer Biology:

NK cells were initially observed as freshly-isolated white blood cells capable of lysing certain tumor targets, such as YAC-1, a Moloney tumor virus-induced lymphoma from A/Sn mice (36)or K562, a tumor cell linederived from a patient with chronic myelogenous leukemia(1). Further, it was observed that, activation of NK cells with IL-2 or IFN enhances the cytolytic activity which results in elimination of a broad array of other tumor targets not lysed by the resting NK cells(39).

The tumor microenvironment is a highly complex phenomenon, and in recent years' immune escape has come up as an important hallmark of cancer, contributing largely to metastasis andtumor progression. Natural Killer (NK) cells,named for their ability to kill target cells autonomously serves as the principal effector innate immune cell towards elimination of cancer and are highly heterogeneous inthe tumor microenvironment(40)

Although NK cells can recognize and kill tumor cells, the mechanism followed by the cellsrepresents a process that evolved predominantly to provide antiviral immunity. Therefore, various studies to understand the mechanism of action ofNK cells in

recognition and the eventual elimination of the virus-infected cells may be directly synonymous to cancer also(41). The importance of NK cells in human for viral immunity very can be understood from a study in which a patient having a complete absence of NK cells, but with normal B and T lymphocytes, died at an early age after suffering from severe Herpesvirus infections (40, 41). Similarly, in another study, presence of NK cells in mouse have been implicated directly in host resistance to mouse cytomegalovirus (MCMV). Depletion of NK cells antibody in susceptible mouse strains was shown to beresulting in elevated levels of MCMV viral titer. On the other hand, adoptive transfer of NK cells in the hosts resulted in protection against MCMV infection(42-48).In principle, the viral-infected cells having downregulated MHC class I moleculeexpressionareeliminated by NK cells as the inhibitory MHC receptors on NK cells were no longer active.

Another NK activating receptor, NKp46, initially discovered for its contribution in NK cell killing of human tumors has been claimed to recognize and kill influenza virus–infected cells(49, 50). Recent experiments in various studies have also demonstrated the role of NK cells in Epstein-Barr virus (EBV) infections (51). CD24, an activating NK receptorthat signals by associating with signaling lymphocyte activation moleculeassociatedprotein (SAP). Interestingly, it was found that patients having X-linkedlympho-proliferative syndrome hadnon-functional SAP and that the NK cells couldnot be activated through CD244from these patients (52, 53). Intriguingly, these patients arehighly susceptible to EBV infection(54-57).Those patients were also found to be prone to development offatal infectious mononucleosis or B lymphomas, indicating a role of NK cellsin managing EBV infection. Further, the role of NK cells have also been studied extensivelyin immunity against, human herpesvirus 8(HHV8)-a virus causing Kaposi's sarcoma(58-61).Therefore, collectively,the above observationsalong with numerous other studies indicates an active participation of NK cells in immunity against viruses that can cause cancers,and evolutionarily, the NK cell immune strategies that were initially thought to have evolved primarily to fight against viral infections are equally being used by the human immune systemto control transformed cells.

1.5 NK cell mediated tumor immuno-surveillance:

MacFarland Burnet and Lewis Thomas in the 1950s proposed the concept of cancer immune-surveillance and predicted thatan immune system, if not manipulated by any internal or external factor is very much capable of recognition and the eventual elimination of the nascent transformed cells(62).Subsequent experimentations with mice deficient in perforin, RAG-2, or IFN-gammaprovided further evidences aboutthe vital roles played byNK cells and CTL in cancer immune-surveillance(63-67). Antibody depletion studies also revealed that NK cells are important in preventing spontaneous tumorsinduced by the chemical carcinogen methylcholanthrene (68). The discovery of the activating NK receptor NKG2D and its ligands on primary tumors was regarded as an important milestonethatdemonstrated a potential mechanism of immune-surveillance byNK cells (69). In another study, the expression of RAE-1 or H60 in some MHC class I–positive tumorsin syngeneicmice was shown to makethem susceptible to rejection by the NK cells. The study hypothesized that the MHC class I–mediated inhibitory signal was overcome by the NKG2D-mediated activation in responding NK cells(70). Subsequently, it was postulated that "selective upregulation of the NKG2D ligands on ''stressed'' cells may allow NK cells and T cells to shift from immune tolerance to activation, resulting in elimination of precancerous cells"(69, 71). Interestingly, in some of the studies, NK cells were found to be able to kill certain tumors that lack ligands for NKG2D which implicates the presence ofadditional ligands on tumor cells for other activating receptors and following some other mechanism for tumor-surveillance(72, 73). However, additional studies on NKG2D and various stress-induced signals are required to ascertain the role played by NKG2D and NK cells in immune-surveillance against cancer.

1.6 NK Cell Anergy and Tumor Escape Mechanisms:

The Tumor-microenvironment (TME), composed of cancer cells, endothelial cells,fibroblasts, and immune cells is considered to be a platform that provides conditions that promotetumor progression. The TME is comprised of cells of mesenchymal origin (fibroblasts, myofibroblasts, mesenchymal stem cells, adipocytes, and endothelial cells), hematopoietic origin (lymphocytes and myeloid

cells), and the extracellular matrix (ECM) (74). In order to promote tumorigenesis, the above components are often found to be hijacked by the tumors. According to Hanahan and Weinberg, the processes that triggers oncogenesis includes growth signals with self-sufficiency, continuous replicative potential, ability to inhibit apoptosis, insensitivity to anti-growth signals,increased angiogenesis, and invasion and metastasis (75).

Recent studies have showed that TME negatively regulate the processes of maturation, proliferation, and functioning of NK cells byproducing soluble modulators (76). These immunosuppressive molecules either acts either directly on NK cells or indirectly stimulates other immune cells to produce additional immunosuppressive molecules(77-79).One of the most studied cytokine produced in the TME by tumor cells, Tregs, MDSCs, and other stromal cells is TGF-β and is known to directly or indirectly impair NK cell function (80). Another mechanism by which TGF-β inhibits NK cell function is by targeting the serine and threonine kinase mTOR, a crucial signaling integrator of pro- and anti-inflammatory cytokines(81-83).Further,TGF-β is also able to dampen the CD56dim recruitment and in the other hand favoring that of CD56bright which contributes to modulation of chemokine repertoire thereby reducing the expression of those molecules that attract CD56dim NK cells(84, 85).

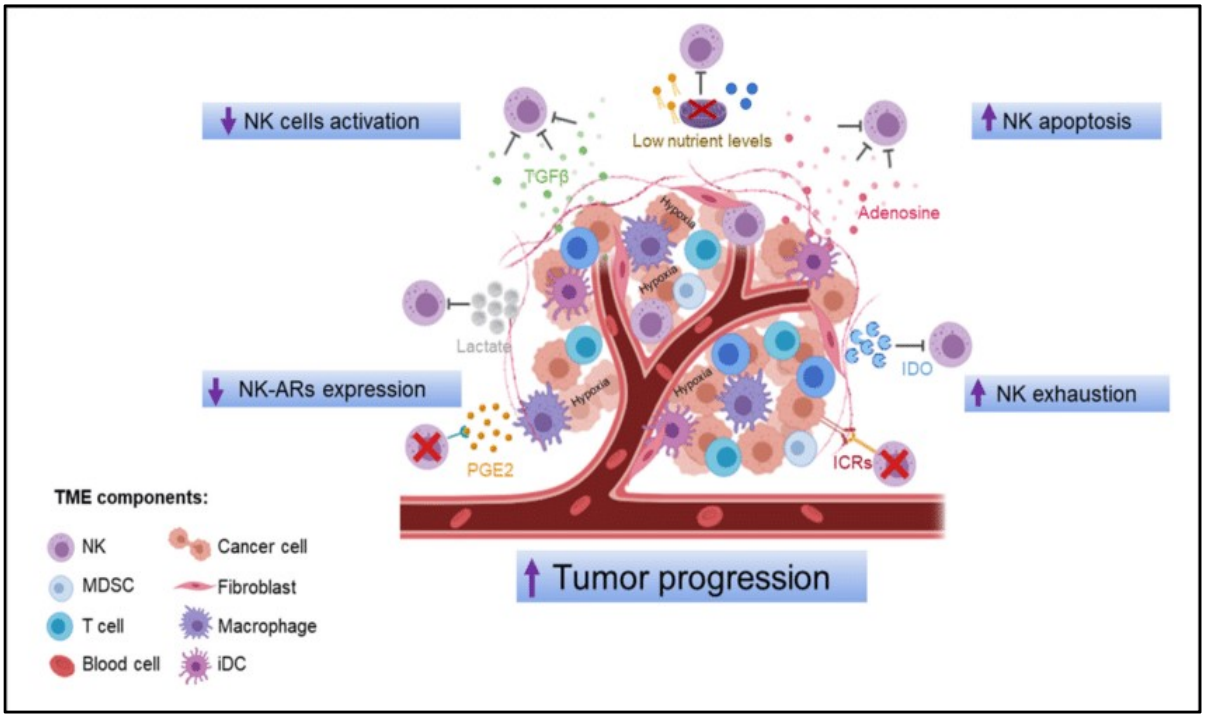

Figure 3: Mechanism of NK cell dysfunction in Tumor microenvironment

Impaired cellular metabolismis considered to be another very important factor that leads to dysfunctional NK cells in cancer. TME is known to be deprived of important nutrients, such as glucose and glutamine, which in contrast are very crucial for NK cells functioning(86). Glycolysis, an important metabolic process for normal NK cell activity, is found to be increased in activated NK cells. Upregulation of the expression of the gluconeogenesis enzyme fructose bisphosphatase 1 (FBP1) in tumor-infiltrating NK cells with the help of mechanism that's involves TGF-β in lung cancers was reported in a recent study(87). This inhibits glycolysis results in dysfunctional NK cells(88).

Other molecules found to have the potential to inhibit NK cell metabolism include two physiological products of enzymatic cholesterol oxidation, 25-hydroxycholesterol and27-hydroxycholesterol, which inhibits the activation of SREBP transcription factors, key regulators of NK cell metabolism. Another mechanism adopted by tumors to selectively suppress the cytolytic activity of NK cells is by the production of prostaglandin E2 (PGE2) in the TME (89, 90). PGE2 that are produced by tumor cells and tumor-associated macrophages are considered to be a key regulator of the NK.Studies have shown that PGE2 produced bythe thyroid

cancer cell microenvironment helps in immune-evasion by suppressing the NK cell cytotoxicity(91, 92). One of the most studied factor that results in dysfunction of tumor-infiltrating NK cells is hypoxia. Hypoxia is found to downregulate NKp46, NKp44, perforin, NKG2D, NKp30, and granzyme-B. In subsequent studies, treatment with IL-2 was seen to restore the NK cell cytotoxicity in multiple myeloma by increasing NKG2D expression(93-95).

There are extensive studies in which NK cells are proven to be vital components in tumor control that acts as brisk-acting immune effectors (96). However, one of the key mechanisms of immune evasion by tumor cells is by expressing signals that make it appear to our defense-immune cells as if it were a fetal growth (97). Recently, several reports have suggested that $CD56^{bright}CD16^{-/low}$ NK infiltrating tumors expresses CD9 and are found to be mimicking a decidual like phenotype in the tumor microenvironment in which NK cells are induced by the tumor cells to extend the conditions necessary for fetal growth. In addition, there are reports of various mechanisms that may be deployed by the cancer cells for the repression of cytotoxic NK cells andaccumulation of proangiogenic or poorly cytotoxic NK cells in the TME which includes reprogramming of NK cells,migration and decidualization of poorly cytotoxic peripheral NK cells in the TME,survival and proliferation of proangiogenic NK cells in the TME(97-99).

We have earlier reported KIR2DL1+-HLA-C2+ genotype was found to bea heritable risk factor in OSCC predisposing to OSCC at a younger age(100) and tumor resident NK cells had lower cytolytic activity. The present study is a follow-up from the above findings and accordingly the following objectives were defined:

I. To study the allelic diversity of the KIR2DL1 gene and of HLA-C gene in the population of Assam, North-East India.
II. To study the influence of higher affinity binding alleles and copy number variation of inhibitory KIR2DL1 gene in immune surveillance in head and neck squamous cell carcinoma in the population of Assam, North-East India.
III. To study the polarization of NK cells towards a decidual phenotype in the tumor microenvironment.

In summary, among the five KIR2DL1 alleles studied, the KIR2DL1*003 allele was seen at the highest frequency of 79% in all the 429 participants in the study. In addition, 9 different HLA-C allele groups were identified in the study participants based on sequence alignment with reference sequences from the National Center for Biotechnology Information (NCBI) database.The occurrence of HLA-C*04 and HLA-C*07 were seen to be higher (28.5% and 25% respectively) in the study population.Further,KIR2DL1*003 has the strongest binding affinity among the studied alleles.

The combined genotype of KIR2DL1*003-HLA-C2 was more frequent in HNSCC patients and was positively associated with HNSCC and the odds of this genotype in HNSCC patients were nearly 2 times (p=0.0152; OR=1.9, 95% CI 1.118–2.534).It was interesting to note that the combined genotype of KIR2DL1*003+HLAC2+ in the younger age group patients was positively associated with the early onset of the disease where the median age was 46 and the range 26-55 years.HLA-C2C2 and HLA-C1C2 genotypes were significantly higher in HNSCC patients as compared to the healthy controls($p<0.0001$).Patients with KIR2DL1*003-HLA-C2 had a higher copy number of the KIR2DL1 gene as compared to healthy controls ($p< 0.0001$).Patients of KIR2DL1*003-HLAC2-C2 homozygote had an upregulated phosphatase activity of 5.2 fold.Observations from cytokine expression study suggested an immunosuppressive microenvironment in HNSCC patients favoring the proliferation of suppressive immune cells.It was noteworthy that 22% of patients having overexpression of both Ki67 and VEGF markers together, showed relapse within 2 years of follow up. Tumor resident NK cells did not show decidual NK cell phenotype.In conclusion, our data suggested that higher affinity binding alleles together with higher CNV of inhibitory *KIR2DL1* compromised immune surveillance in HNSCC and opens up the possibility of the use of KIR-HLA allotyping as prognostic markers.

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