Chapter IV:

Influence of higher affinity binding allele 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

4.1 INTRODUCTION:

Natural Killer (NK) cells are important effector lymphocytes that are specialized in the recognition and cytolysis of virus-infected and tumor cells (1). Various subpopulations of NK cells are determined by the density and presence of CD16 (FcgRIII) and CD56 (N-CAM) surface molecules. The CD56 (CD56dim) subsets constitute 90% of peripheral blood NK cells, express killer Ig-like receptors (KIR), and perforin, and are highly cytotoxic. On the other hand, the CD56bright subset exhibits immune-regulatory functions through the secretion of diverse cytokines. The CD16 marker-expressing cells are involved in Ab-dependent cellular cytotoxicity (ADCC) (2). Direct recognition of target cells through inhibitory and activating receptors determines the activation of NK cell-mediated cytotoxicity against susceptible cells and requires a finely regulated mechanism of control(3). The functional activation of human NK cells is regulated by an array of receptors that includes a polygenic and polymorphic family of cell surface glycoproteins known as the Killer Cell Ig-like Receptors (KIR) (4).The Human KIR multigene family consists of as many as 14 expressed KIRgenes [KIR2DL1–5, KIR3DL1–3, KIR2DS1–5, KIR3DS1] along with 2 pseudogenes (5). Vast polymorphism of *KIRs* has been reported globally, which includes allelic polymorphisms, gene content, and copy number variations. The role of Copy Number Variations (CNVs) as risk factors for cancer is an intriguing area of study due to the structural dynamism and genomic instability that characterize the cancer cells (6). The KIR genes are arranged in a close head-to-tail orientation and have high sequence similarity. This often leads to copy number variation by sequential non-allelic homologous recombination (NAHR) or unidirectional alignment. Besides, unfamiliar KIR haplotypes with abnormal gene content or fusion genes have also been identified (7). Copy numbers leading to susceptibility to some diseases have already been reported in diseases like HIV where CNV of *3DL1S1* was found to influence HIV control. (8)

The inhibitory KIRs signal through an immune-receptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic domain. Inhibitory KIRs engage with the MHC-I (HLA-A, -B, and –C allotypes) followed by phosphorylation of the ITIMs and subsequent binding of Src homology region 2-containing protein tyrosine phosphatase-2 (SHP-2) to the N-terminal domain of the ITIM. The recruitment of

SHP-2 leads to the suppression of the activating receptor signals (9, 10). Every HLA-C allotype forms a ligand for KIR2DL and these interactions modulate the NK cell responses (11). However, KIR-HLA-C interactions are dependent upon the residue at position 80 in the α1 domain where Lysine residue represents the HLA-C2 ligand and the HLA-C1 ligand is defined by the presence of asparagine at that position(11). The inhibitory receptor KIR2DL1 binds HLA-C allotypes bearing a C2 epitope and is reported to deliver the strongest inhibitory signal (12). Epistatic interactions between *KIR2DL1* and *HLA-C* have been linked with the pathogenesis of some human infections such as autoimmune diseases, pregnancy disorders, HIV/AIDS, and transplantation outcomes. However, some of the strongest associations of *KIR2DL1* and *HLA-C2* are reported with certain types of cancers (13).

Nonetheless, apart from the direct cytotoxic mechanisms,the mechanism of action of Natural Killer (NK) cells are also dependent on production of immune-regulatory cytokines. Recent evidences from various investigators has shown that NK cells, similar to the adaptive immune cells display memory and produces cytokines which plays a pivotal role in the maturation, activation and survival of NK cells(14-16). Interleukins and interferons are known for positively regulating NK cell function. Depending on the context and the need, cytokines have the ability to enhance or supress the NK cells function either independently or in cooperation with other cytokines(17, 18). On the contrary,when induced in the tumor microenvironment, some cytokines like TGFβ, IL-10 and IL-6 have the ability to modulate or suppress the NK cell activity both directly and indirectly regulating other immunosuppressive cells in the TME and by antagonizing the stimulatory cytokines. This in turn dampens the antitumor response of the NK cells resulting in tumor aggression and metastasis(19-21). Therefore, understanding of the NK cell functioning and response towards cytokines in context of tumors and tumor microenvironment may help us in aiding novel immunotherapeutic interventions for a better and enhanced NK cell response in cancer patients.

4.2. Materials and methods

4.2.1. Study site, study design, and participants

The present study was designed as a hospital-based case-control study distributed as 204 HNSCC and 225 healthy participants from the state of Assam, North-East India. Clinical and histopathologically confirmed HNSCC cases were recruited from two hospitals in Northeast India- Gauhati Medical College and Hospital (GMCH)and North East Cancer Hospital and Research Institute (NECHRI). The study was ethically approved vide letter No **IEC/17/03/002** and the participants of the study were informed about the study before the collection of the specimens. A detailed information sheet was distributed among the patients and their attendants. All the participants voluntarily consented to participate in the study. Clinically and histopathologically confirmed cases of HNSCC (post operated and biopsy samples) were included in the study after obtaining written informed consent from the participants. Clinically and histopathologically confirmed adjacent normal tissues were also collected for the conduct of the study. The following were the exclusion and inclusion criteria of the study-

Inclusion Criteria

I. Clinically and histopathologically confirmed cases of HNSCC
II. Patients below the age limit of 86 years
III. Both male and female patients selected

Exclusion Criteria

I. Pediatric age group patients with head and neck cancer
II. Metastatic neck diseases from other parts of the body
III. Patients with Cancer of esophagus and thyroid
IV. Other vulnerable subjects (like terminally ill/seriously ill)

The information about gender, ethnicity, clinical history, demographic and other characteristics were collected and recorded in the form of proformas by the research staff of NECHRI. American Joint Committee on Cancer's TNM staging protocol was used as a reference for the staging and diagnosis of HNSCC tumors. Tumor stages were grouped from early clinical stages (I-II) to advanced stages (III-IV). Tumors

were also graded histologically as – well-differentiated, moderately differentiated and poorly differentiated as given by the World Health Organization (WHO).

4.2.2 Variations in Copy Number of KIR2DL1/S1 and disease association

TaqMan Copy Number Assay for *KIR2DL1* and *KIR2DS1* genes was tested in a cohort comprising 70 participants each from HNSCC patients and healthy controls as per the manufacturer's protocol(Applied Biosystems, California, USA). Briefly, the CN assays (FAM dye-labelled MGB probe) were run together with the RNase P H1 RNA reference assay (VIC dye-labelled TAMRA probe), 20 ng DNA, and TaqMan Genotyping Master-mix in a 14 µl PCR (2 replicates per sample). The PCR reactions were run on the Applied Biosystems Quant-Studio 3 system, using Relative Quantification (RQ) settings for 10 minutes at 95° C followed by 40 cycles: 95° C for 15 seconds and 60° C for 60 seconds. Real-time PCR results were analysed by SDS v2.3 software using an auto baseline and manual C_t threshold of 0.2. Results export files were opened in Copy Caller software for sample copy number analysis by relative quantification (RQ) using a comparative CT method (ΔΔ CT). The HPV status of the samples was extracted from a part of the work that has been already published (22).

4.2.3 Gene expression assay:

For this phase of the study, peripheral blood sample (0.5 mL) was collected by phlebotomy from the confirmed cancer patients and healthy controls by trained personal. Blood was then transferred in RNAlater (1.4 mL) and kept in 4° C overnight and finally stored at -80° C until used. Total RNA was isolated from blood stored in RNAlater using Ribo-Pure Blood Kit (Ambion Inc., Austin, Texas, USA) as described by the manufacturer. Yield (2-4 µg from 0.5 mL) and purity (1.9-2.1) of the extracted RNA were checked using NanoVue Plus Spectrophotometer (GE Healthcare). Around 500 nanogram of total RNA was reverse transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) in a thermal cycler (Applied Biosystems, Foster City, CA, USA) using random hexamers for initiating cDNA synthesis. Using these single-stranded cDNA products, gene expression analysis was performed using SYBR Green based assay (SYBR Select Master Mix, Applied Biosystems, CA, USA) forNK cell-associated cytokines (IL-2, IL-1b, IL-12b,

IL-10, IFN-gamma, IL-15, IL-18, TNF-α, and TGF-β) and for SHP-2 Phosphatase on an Applied Biosystems Quant-Studio 3real-time PCR (RT-PCR) system. Expression levels were normalized using GAPDH as the endogenous control and healthy controls were used as calibrators. Melt curve analysis was performed to confirm the presence of specific amplification product. The relative fold change (2-ΔΔCt) for each gene was calculated based on the following formula:

ΔCt of cancer patient = Target gene Ct mean – Endogenous control Ct mean

Δ Ct of healthy control = Target gene Ct mean – Endogenous control Ct mean

ΔΔ Ct = Δ Ct of cancer patient – Δ Ct of healthy controls

Table 3: Reverse transcription - PCR master mix composition

Reverse transcription-PCR master mix (20 µL reaction volume)	
Component	**Volume/Reaction (µL)**
10X Reverse Transcription Buffer	2
25X dNTP Mix (100 mM)	0.8
10X RT Random Primers	2
Reverse Transcriptase	1
RNase Inhibitor	1
RNA	10 (0.3 to 0.5 µg)
H_20	Adjusting volume

Table 4: PCR program for cDNA synthesis from total RNA

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

Table 5: Sequences of primers

Gene name	Primers (Forward and reverse)
IL-1	CCTGCCCCAATCCCTTTATT GCCCCCAATTCTCTTTTTGAG
IL10	GTGATGCCCCAAGCTGAGA CCCCCAGGGAGTTCACATG
TGFβ1	CTATTCAAGACCACCCACCTTCTG CTCCCGGCAAAAGGTAGGA
IL12	TTAAAAAGTTGGAGGAATTGGAACAG TGAAGGTGCGGTCCCATAA
IL15	GACCAAGTTTATCTGTGTGACACCAT GTAGCGCACTCCGAGGTCAA
IL18	CTATTCAAGACCACCCACCTTCTG CTCCCGGCAAAAGGTAGGA
TNF-α	GTGATGCCCCAAGCTGAGA CCCCCAGGGAGTTCACATG
IFN-γ	TTAAAAAGTTGGAGGAATTGGAACAG TGAAGGTGCGGTCCCATAA

en based RT- PCR master mix composition using RT2qPCR Primer Assay

RT- PCR master mix (10 µL reaction volume)	
Composition	**Concentration/Reaction**
2X SYBR Green PCR master mix	1X
10X RT2 qPCR Primer Assay	1X
cDNA	100 ng
H_20	Adjusting volume

Table7:SYBR Green based RT- PCR master mix composition usingdesigned primer

RT- PCR master mix (10 µL reaction volume)	
Composition	**Concentration/ reaction**
2XSYBR Green PCR master mix	1X
Forward primer	0.4 mM
Reverse primer	0.4 mM
cDNA Primer mix	100 ng
Water	Adjusting volume

Table 8: PCR program for SYBR Green based RT-PCR assay

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

4.2.4 Protein expression study by Immunohistochemistry:

KIR2DL1 and HLA-C protein expression were determined by immunohistochemistry (IHC) on formalin-fixed, paraffin-embedded (FEPE) tumor tissues using monoclonal KIR2DL1(ThermoFisher Scientific) and HLA-C primary antibody raised in mouse (Sigma-Aldrich, Merck, Germany). Additionally, VEGF, Ki67, p16, Pan-cytokeratin, and CD45 (Sigma-Aldrich, Merck, Germany) markers were also analyzed in 18 patients. All IHC results were independently checked by pathologists from the respective medical institutes.

Tissues showing 50% or more staining of all the proteins were considered as positive for all the three proteins. Staining intensity was calculated and samples were graded from 1+ to 4+ based on the staining intensity. Histo score (H score) was also calculated as described previously (23).We defined positivity as an H score greater than 200 for tumor cells.

H score = 1 × (% of 1 + cells) + 2 × (% of 2 + cells) + 3 × (% of 3 + cells), where staining was determined and scored according to four categories: 0 for 'without any

staining', 1 + for 'light staining visible only at high magnification', 2 + for 'intermediate staining' and 3 + for 'dark staining, visible even at low magnification.

4.2.5. Statistical analysis of data

Participants with incomplete data or incomplete investigations were excluded from the analysis. Statistical analysis of the data was performed using XLSTAT Biomed 2018.7 and 2015 versions.Student's t-test was used for comparison between the mean values. The odds ratios (OR) with 95% confidence interval (CI) were calculated to estimate the relative risk. A p-value < 0.05 was considered statistically significant.

4.3Results:

4.3.1Patient characteristics:

The clinical characteristics of the HNSCC patients and control participants are summarized in Table 1. The mean age of the HNSCC patients was 55 years and the male-to-female ratio was 3:1. The 225 healthy participants were not related to HNSCC patients. 148 patients were followed up for 2 years after treatment and 77 patients responded to treatment. The other 71 patients mainly detected in the advanced stages of cancer reported complications within 2 years of treatment. (Table 1). Of the total patients (68.8 %) were diagnosed in the advanced tumor stage.

Age group	**HNSCC Patients**	**Controls**
<45	22 (10.8)	120(53.3)
45-54	69(34.1)	55(24.4)
55-64	67(33.1)	35(15.5)
≥65	39(19.37)	11(4.88)
Gender	**HNSCC Patients**	**Controls**
Male	150(74.2)	132(58.6)
Female	52(25.7)	93(41.33)
Histological Grade	**HNSCC Patients**	
Well differentiation	153(75.7)	

Moderate differentiation	34(16.8)	
Poor differentiation	6(2.9)	
Unknown	9	
Treatment	**HNSCC Patients**	
Radiotherapy	38(18.8)	
Surgery	29(14.3)	
Chemotherapy	10(4.9)	
C+R	72(35.6)	
S+R	23(11.3)	
S+C	3(1.4)	
S+C+R	12(5.9)	
No Treatment	17(8.4)	
Response to Treatment ()	Patients(148)	
Responded	77 (52.02)	
Not-Responded	71 (47.97)	
Clinical Tumor Stage	**HNSCC Patients**	
I-II	55(27.2)	
III-IV	139(68.8)	
Blank	8(3.9)	

Table 9: Demographic and clinical characteristics of patients

Description: The mean age of the HNSCC patients was 55 years and the male-to-female ratio was 3:1. The 225 healthy participants were not related to HNSCC patients. The patients were diagnosed mainly in the advanced tumor stage (68.8 %). Histologically, 75.7% of the malignancies were classified as well-differentiated while 16.8 % were moderately differentiated and 2.9% were poorly differentiated HNSCC.

4.3.2 Positive association of KIR2DL1*003-HLA-C2 genotype with HNSCC increases the odds of the disease at a younger age:

KIR2DL1*003-HLA-C2 genotype 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). In contrast, the frequency of the KIR2DL1*003-HLA-C1genotype was higher in healthy controls and was negatively associated with HNSCC. It may be pertinent to mention here that the *KIR2DS1* gene is the activating homolog of *KIR2DL1.* Therefore, we examined the possible association of KIR2DS1-HLA-C1 and KIR2DS1-HLA-C2 in HNSCC patients and healthy controls and observed that KIR2DS1-HLA-C2 was negatively associated with HNSCC, however, the association was not statistically significant.

In the present study, the median age of the HNSCC patients was 55 years, and therefore the patients with age < 55 years were considered as younger age groups and patients with age ≥ 55 years were grouped under the older age group patients. 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 yrs. Notably, the odds of the disease at a younger age with the KIR2DL1*003+HLAC2+ genotype increased by 2.0 folds (p=0.0008 OR=2.0, 95% CI 1.157–2.363).

4. 3.4*KIR2DL1*003* was the most frequent allele among the studied alleles:

Among the five *KIR2DL1* alleles studied, the KIR2DL1*003 allele was seen at the highest frequency of 79% in all the 429 participants in the study (Figure 1). This was also comparable when stratified between HNSCC patients and healthy controls with frequencies of 76.2% and 81% respectively (Figure 11). Additionally, the frequency of *KIR2DS1,* the activating homolog of the *KIR2DL1* gene was found to be 45% in HNSCC patients as compared to 59.1 % in healthy controls. This was suggestive that the occurrence of the gene/allele alone cannot be considered a risk factor for HNSCC.

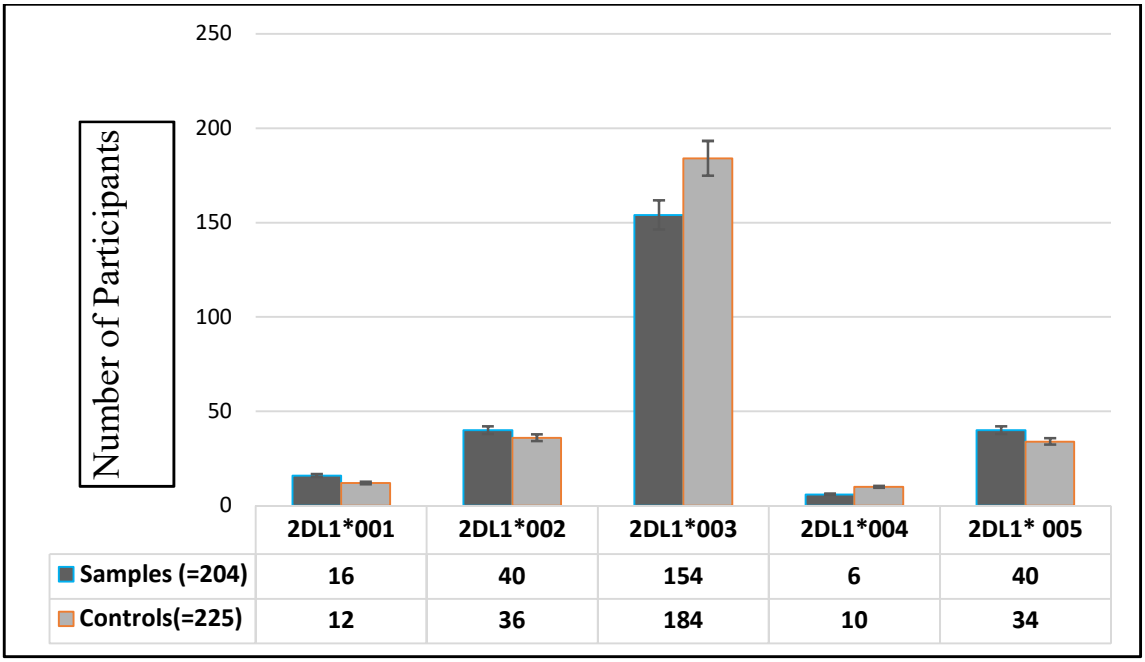


Figure 11: Distribution of *KIR2DL1 alleles* in HNSCC patients and Healthy controls

Interestingly, the comparison of HLA-C ligands between HNSCC patients and healthy controls showed that HLA-C2C2 and HLA-C1C2 genotypes were significantly higher in HNSCC patients ($p<0.0001$). On the contrary, the HLA-C1C1 genotype was lower and negatively associated with HNSCC ($p<0.0001$).

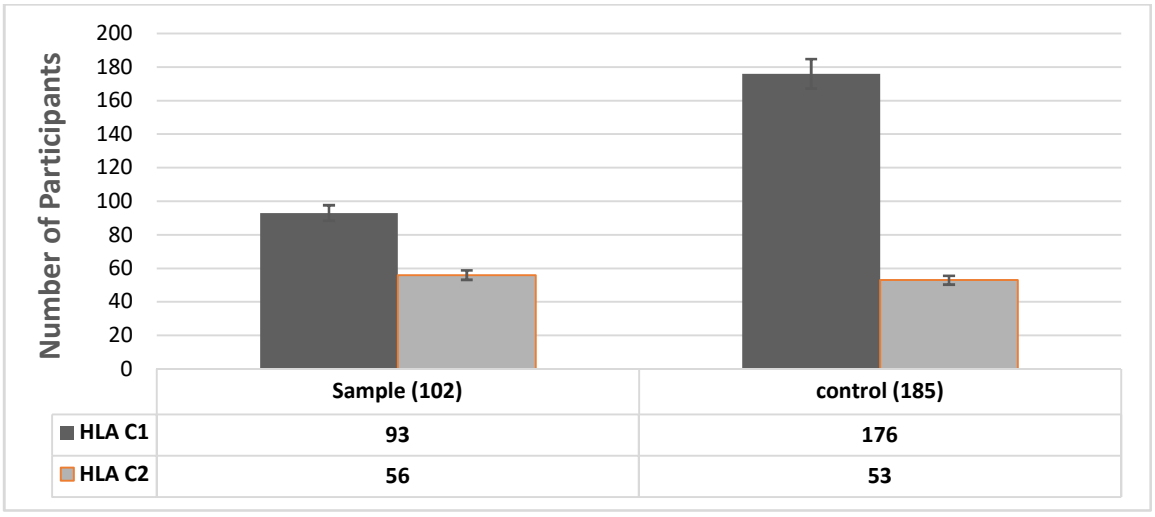


Figure 12: Distribution of *HLA-C1 and HLA-C2* in HNSCC patients and Healthy controls

	Samples	Control
CICI	45	128
CIC2	48	48
C2C2	8	5

Table 10: Comparison of HLA-C ligands between HNSCC patients and healthy controls.

In addition, the occurrence of HLA-C*04:01 and HLA-C*07:01:01, cognate ligands for KIR2DL1 were seen to be higher (25% and 28.5% respectively) in the cancer patients based on sequence alignment with reference sequences from the National Centre for Biotechnology Information (NCBI) database (24).

4.3.5Higher Copy Number of KIR2DL1 in KIR2DL1*003+HLA-C2+ genotype HNSCC patients:

The copy Number for the *KIR2DL1* gene was found to be higher for both cancer and control samples but was not associated with HNSCC (p = 0.385). Moreover, it was seen that healthy participants tended to have a higher copy number of the gene as compared to patients. Interestingly, when patients were stratified based on the KIR2DL1-HLA-C compound genotype, the patients with KIR2DL1*003-HLA-C2 had a higher copy number of the *KIR2DL1* gene as compared to healthy controls **(p< 0.0001)**.Similarly, healthy participants with the KIR2DL1*003-HLA-C1 genotype were seen to have a higher copy number of the *KIR2DL1* gene **(p < 0.0001)**. The copy number variation for the *KIR2DS1* gene was also checked but no significant difference in the copy number variation between patients and healthy control was seen (p=0.252).

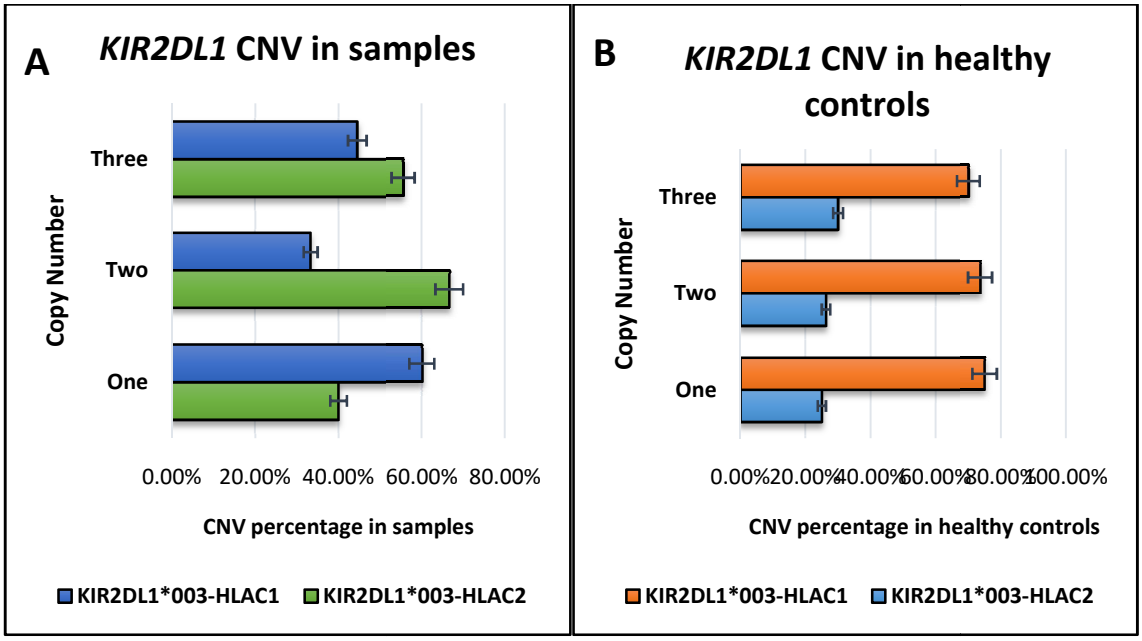
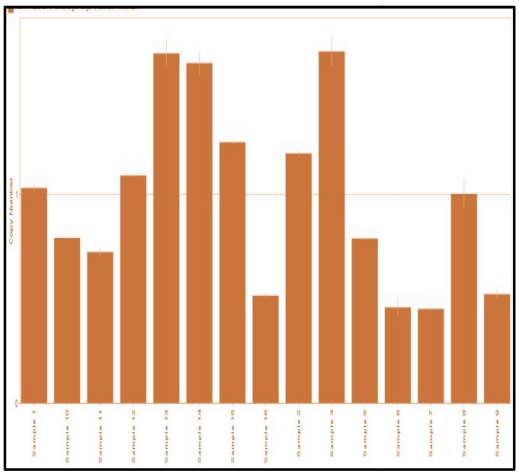
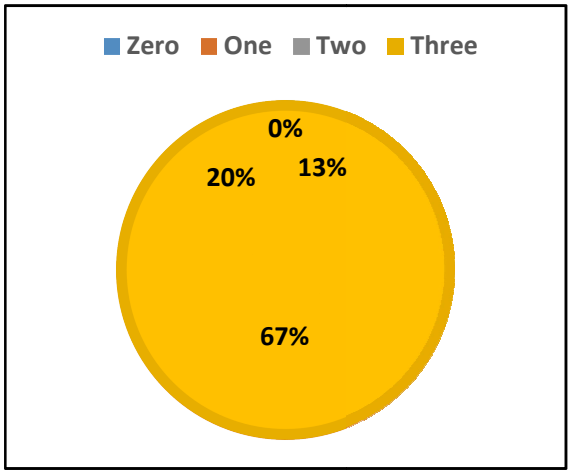


Figure 13: Comparison of *KIR2DL1* copy number variation in (A) HNSCC patients (B) Healthy controls

Further, we analysed the variation in copy numbers for both HPV positive and HPV negative HNSCC samples. Interestingly, the copy number for the *KIR2DL1* gene in HPV-positive patients ranged from 0-2 and approx. 80% of the individuals had less than 2 copies of the gene. However, the *KIR2DL1* copy number variation ranged from 0 to 3 in the case of HPV-negative patients. Similarly, the Copy number for the *KIR2DS1* gene for HPV positive and HPV negative HNSCC patients were also checked and no significant difference between the copy numbers with the status of HPV and HNSCC patients was found.

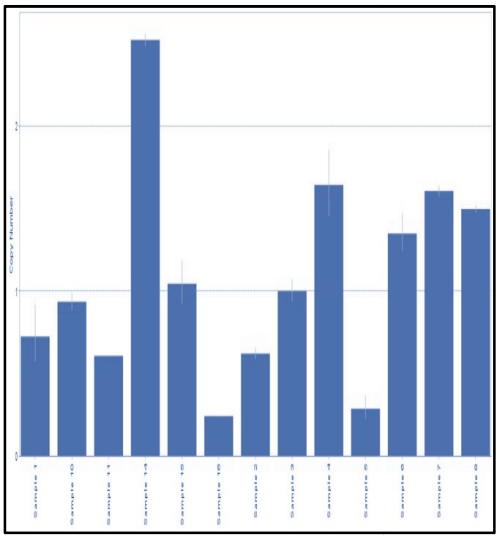


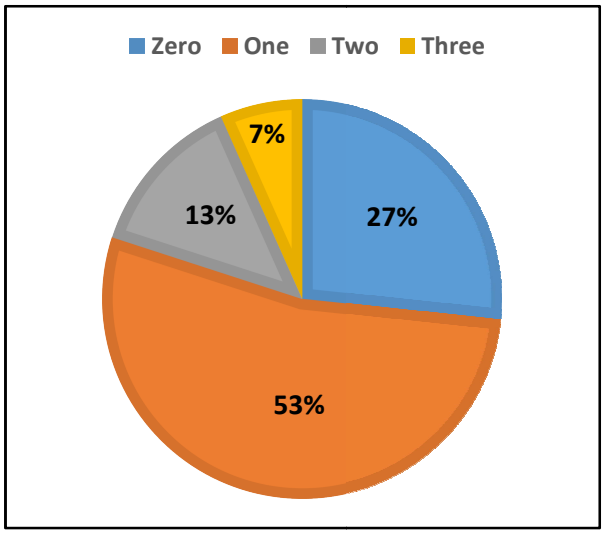


(A) (B)

Figure 14: (A)Copy Number Variation of KIR2DL1 in HPV Positive HNSCC samples (N=15)

(B)Percentage of individuals with KIR2DL1 CNV in HPV positive HNSCC Patients

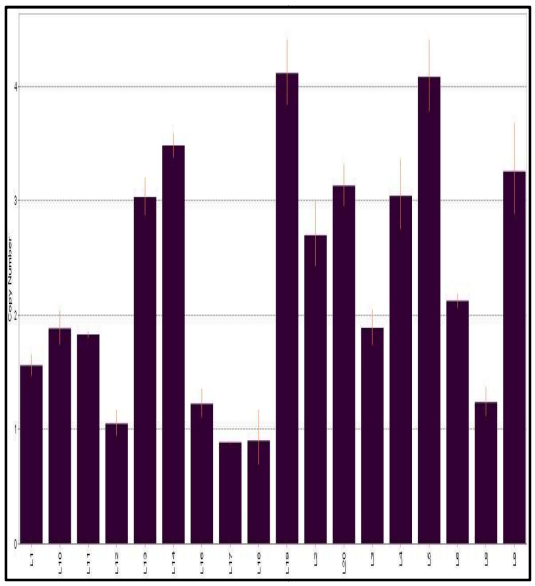


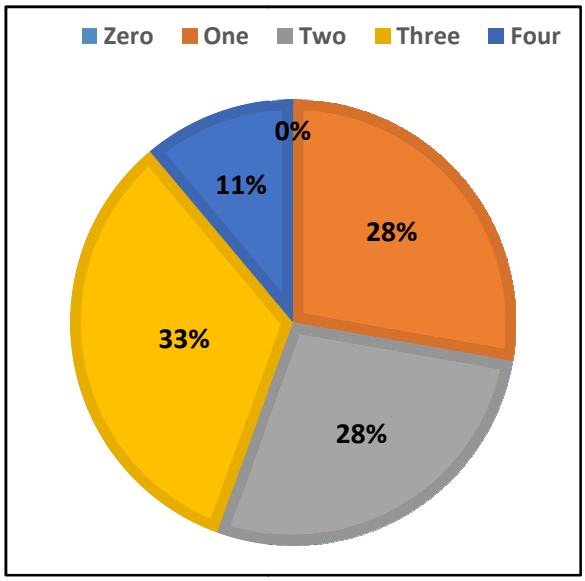


(A) (B)

Figure 15: (A)Copy Number Variation of KIR2DS1 in HPV Positive HNSCC samples (N=15)

(B)Percentage of individuals with KIR2DS1 CNV in HPV positive HNSCC Patients

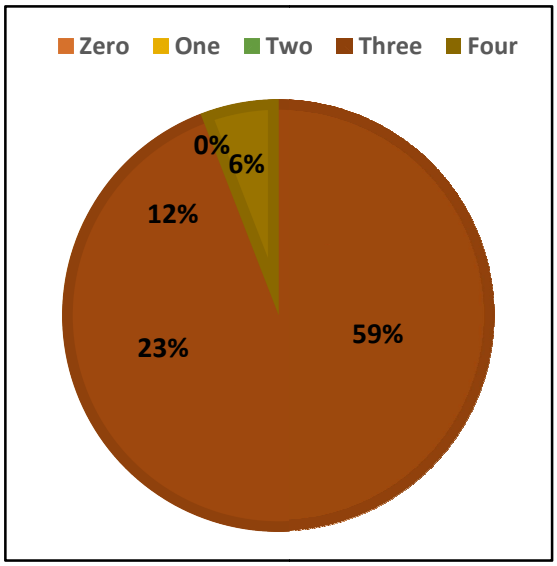




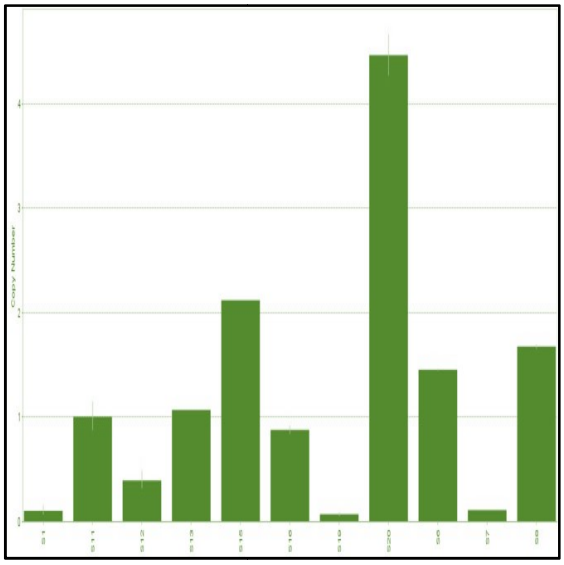
(A) (B)

Figure 16: (A)Copy Number Variation of KIR2DL1 in HPV Negative HNSCC samples (N=15)

(B)Percentage of individuals with KIR2DL1 CNV in HPV Negative HNSCC Patients



(A)



(B)

Figure 17: (A)Copy Number Variation of KIR2DS1 in HPV negative HNSCC samples (N=15)
(B)Percentage of individuals with KIR2DS1 CNV in HPV negative HNSCC Patients

4.3.6 SHP-2 expression and NK activation status:

It is worth mentioningthat the recruitment of SHP-2 leads to the suppression of the activating receptor signals. A higher activation threshold in patients with KIR2DL1*003-HLA-C2 genotype was further evident from our SHP-2 Phosphatase expression study. HNSCC patients with a compound genotype of KIR2DL1*003-HLA-C1-C2 heterozygote had a 4.6-fold higher expression of SHP2 phosphatase. On the other hand, patients of KIR2DL1*003-HLA-C2-C2 homozygote had an upregulated phosphatase activity of 5.2 fold. Interestingly, patients with the KIR2DL1*003-HLA-C1-C1 genotype had higher activity of SHP-2 of only 1.48 fold.

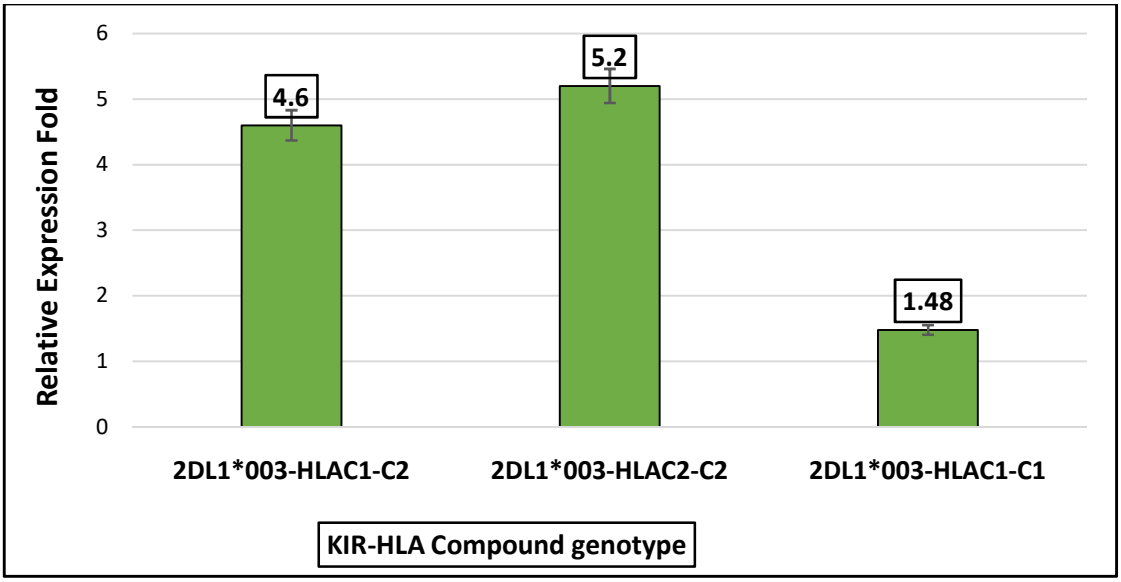


Fig 18: Expression of SHP-2 Phosphatase in HNSCC patients with different KIR-HLA compound genotype (N=50)

4.3.7: Cytokine expression in HNSCC regulates NK cell activation.

We examined the expression of cytokines that are known to either activate the NK cells or are secreted by the activated NK cells. The transcript expressions of the pro-inflammatory cytokines – IL-1β, IL-2, IL-12β, IL-15, IL-18, IFN-γ, and TNF-α and anti-inflammatory cytokines – IL-10 and TGF-β of HNSCC patients are summarized in Fig. 19.

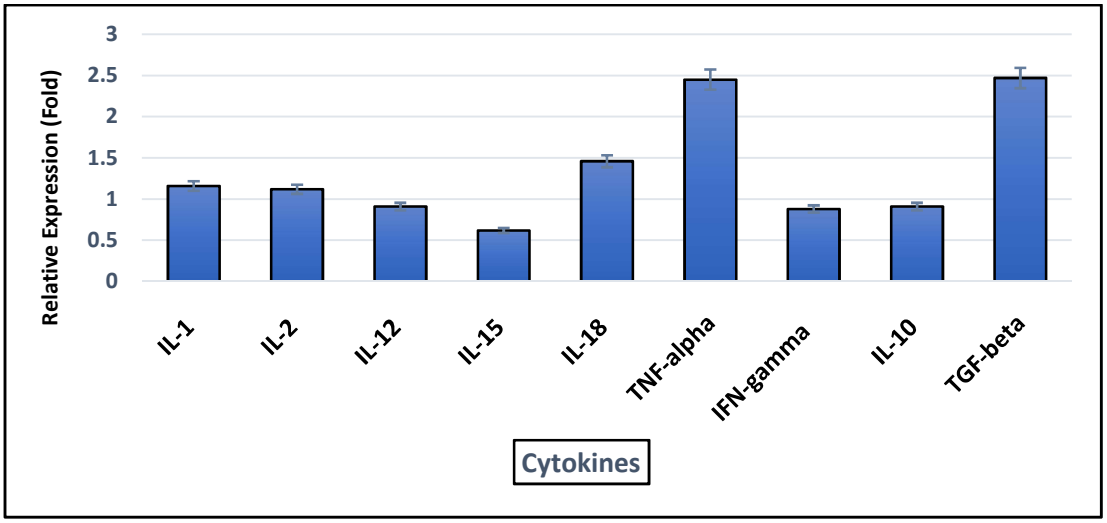


Fig 19. Expression of pro- and anti-inflammatory cytokines in HNSCC patients relative to healthy controls.

The expressions of the key pro-inflammatory cytokines having a role in NK cell activation – TNF-α (2.45-fold) along with IL-18 (1.46-fold) and anti-inflammatory cytokine– TGF-β (2.47-fold) was found higher in HNSCC patients. Also, the transcript expression of IFN-γ, a key cytokine of NK cells and a master regulator of immune responses along with IL-12 was found to be downregulated in patients. The above observations suggested an immunosuppressive microenvironment in HNSCC patients favouring the proliferation of suppressive immune cells.

4.3.8: High CNV of KIR2DL1 gene in patients with a history of cancer relapse:

Ki67 expression was higher in stage III-IV patients whereas expression of VEGF was seen in patients from all tumor stages (p=008). Notably, 22% of patients having

overexpression of both Ki67 and VEGF markers together, showed relapse within 2 years of follow up suggesting poor prognosis as well as aggressive proliferation and angiogenesis ($p< 0.05$). Interestingly these patients also had a higher copy number for the *KIR2DL1* gene. Besides, lower expression of PanCK was seen in patients with poor histological differentiation, whereas PanCK expression was higher in patients with advanced clinical stage and recurrence.

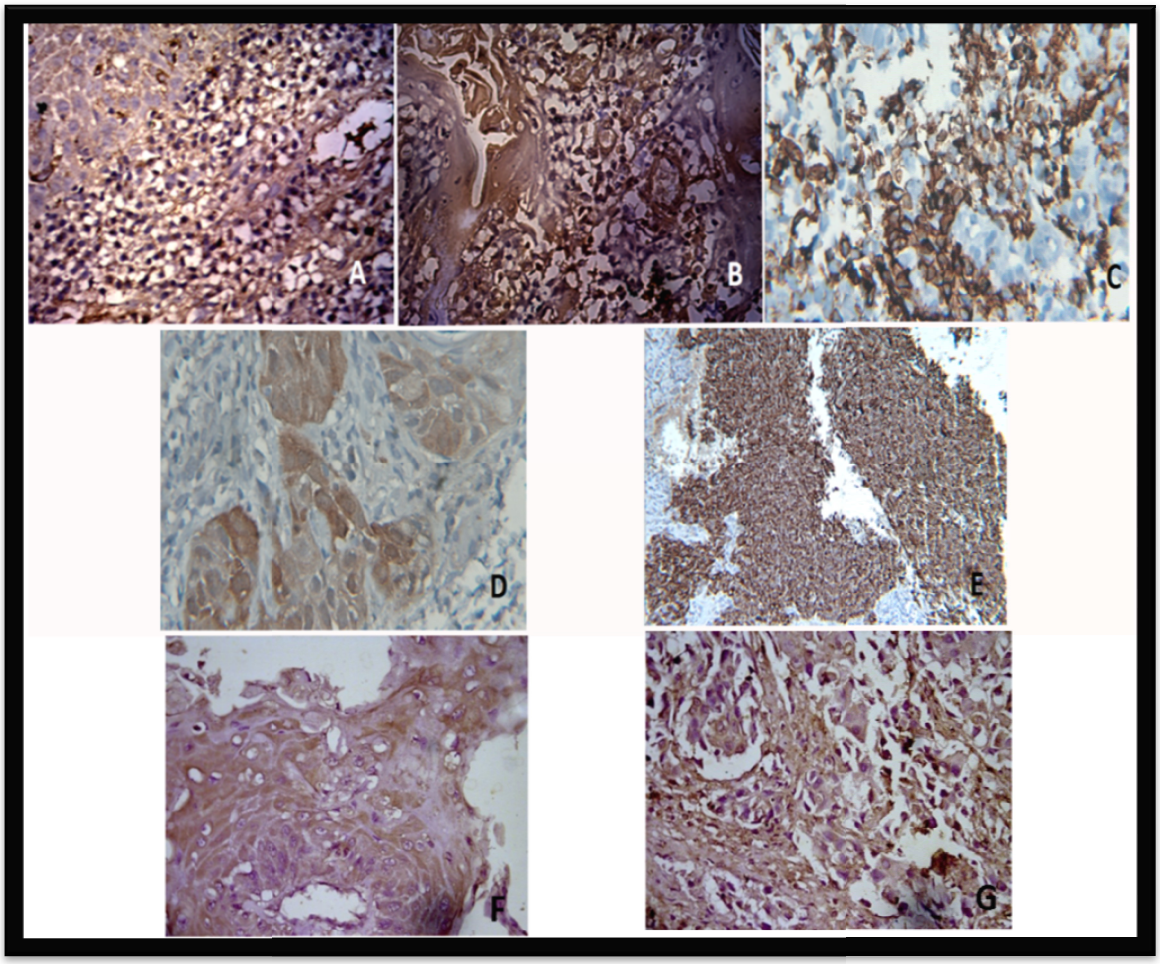


Figure 20: Expression of proliferation and differentiation markers and KIR2DL1 and HLA-C in HNSCC tumor tissue. Images were captured in Axio Vert.A1 inverted microscope (Carl Zeiss, Oberkochen, Germany) at 40X magnification (A) Immunohistochemical staining of VEGF showing the positivity of VEGF in HNSCC tumor cells counter stained by haematoxylin (B) Immunohistochemical staining of Ki67 showing the positivity of Ki67 in HNSCC tumor cells counter stained by haematoxylin (C) Immunohistochemical staining of CD45 showing the positivity of CD45 in HNSCC tumor cells counter stained by haematoxylin (D)

Immunohistochemical staining of p16 showing the positivity of p16 in HNSCC tumor cells counter stained by haematoxylin (E) Immunohistochemical staining of PanCK showing the positivity of PanCK in HNSCC tumor cells counter stained by haematoxylin (F) Immunohistochemical staining of KIR2DL1 showing the positivity of KIR2DL1 in HNSCC tumor cells counter stained by haematoxylin (G) Immunohistochemical staining of HLA-C showing the positivity of HLA-C in HNSCC tumor cells counter stained by haematoxylin.

4.4Discussion:

Natural killer (NK) cells are the principal innate lymphocytes mediating anti-tumor and anti-viral responses and have shown great promise in NK-based immunotherapy (25). However, data by several investigators shows tumor resident NK cells to have lower cytolytic ability. One of the factors that modulate NK cell functions in human immunity and reproduction is the multiple interactions between KIRs of Natural Killer cells and HLA-I ligands on the surface of cells(26). Knowledge of the extensive polymorphism of KIR genes and its binding affinity with its cognate HLA ligand are fundamental in understanding the role of Natural Killer cells in human diseases, transplantation, and reproductive health (27). The *KIR2DL1* gene,in particular, exhibits a higher affinity for the HLA-C2 epitope among the inhibitory KIRs (28). We have earlier reported the KIR2DL1+-HLA-C2+ genotype to bea heritable risk factor in OSCC predisposing to OSCC at a younger age (29).To gain a better understanding of the above findings, we next examined the possible association of alleles of *KIR2DL1* and its cognate ligand HLA-C with HNSCC in the study population(28). The higher frequency of the KIR2DL1*003 allele along with a stronger binding affinityto its cognate ligand HLA-C2 in HNSCC patients and its positive association with disease suggested a stronger inhibitory signal and thereby an increased activation threshold of NK cells. Notably, the KIR2DL1*003-HLA-C1 genotype was negatively associated with HNSCC suggesting its protective role in the population perhaps due to a lower NK activation threshold.

Src homology region 2-containing protein tyrosine phosphatase-2 (SHP-2) is known to be an inhibitor of human NK cells upon recruitment to the KIRs. Several research groups have demonstrated that SHP-2 binding to KIR-ITIM correlated to a stronger

inhibitory response from NK cells (30). Our study confirms and extends the findings to show that SHP-2 recruitment leads to inhibition of activating KIR signals. The present study demonstrated that KIR2DL1*003-HLA-C2-C2 homozygote HNSCC patients had an upregulated phosphatase activity of 5.2 folds resulting in a higher NK activation threshold.

Copy number variation (CNV) is known to contribute to many human disorders, particularly cancer (31, 32). During the process of NK cell education, interactions between inhibitory KIRs and their cognate HLA class I ligands set the activation threshold for NK cells. Therefore, the expression of multiple copies of an inhibitory KIR could lead to a better NK cell education, in turn strengthening antiviral response and immunity (33). The question that we asked here is "Does the variations in the copy number of the KIR2DL1 gene contribute to disease association?"In the present study, the copy number for the *KIR2DL1* gene was found to be higher for both cancer and control samples and was not associated with HNSCC suggesting that a higher copy number of the gene alone was not associated with the disease. However, HNSCC patients with the combined genotype of KIR2DL1*003-HLA-C2 had a higher copy number of the *KIR2DL1* gene which is again suggestive of a higher NK activation threshold in those patients.A higher Copy number is reported to confer antiviral immunity as reported earlier (30). In addition, expression of angiogenesis and cell proliferation markers, VEGF and Ki67 along with higher *KIR2DL1*copy numbers were seen in patients with a history of cancer relapse. This observation also suggested a feasible approach to combine the two markers for establishing a molecular grading model for the prognosis and identification of individuals at risk. Nonetheless, the identification of more sensitive biomarkers remains the need of the hour (34-36).

Activated NK cells are known to produce a wide array of cytokines that includes IL-1β, IL-2, IL-12, IL-15, IL-18, and IFN-γ (37). Therefore, we wanted to find out "Whether the cytokine expression data was consistent with NK cell inhibition?" Upregulation of TGF-β along with the downregulation of IFN-γ and IL-12 in our study was indeed suggestive of a potential inhibition of NK cells. Similar findings were also noted by Bittencourt et.al 2018 and these authors attributed it to TGF-β mediated inhibition of glycolysis which in turn results in impaired production of IFN-

γ and IL-12 eventually resulting in a decreased cytolytic effect of the NK cells (38). IFN-γ is a potent NK effector cytokine that is known to play a crucial role in the antiviral and antitumor activity of NK cells and is induced by the cytokine IL-12 produced by dendritic cells, macrophages, and neutrophils (3). TGF-β may also potentially signal through an alternative pathway that represses the mTORC1 signalling as reported by Viel et.al. paving a way for more studies on the potential pathways influenced by the cancer cells to mask the immune response (39).

In conclusion, the data obtained from the study will contribute to our understanding of the allelic diversity of *KIR2DL1* and *HLA-C* in the population of Assam, North-East India. To the best of our knowledge, this is the first study from the region. KIR2DL1*003 was the predominant *KIR2DL1* allele in the population and the KIR2DL1*003-HLA-C2 genotype was positively associated with HNSCCand the odds of this genotype in HNSCC patients were nearly 2 times. Our findings also suggest that the binding affinity of KIR2DL1 with HLA-C was dependent on the allele of *KIR2DL1* present. A higher copy number of *KIR2DL1*along with its cognate ligand HLA-C2 appears to compromise the NK activation for an anti-tumor response which was also reflected by the positive association of CNV with tumor proliferation, angiogenesis, and advanced tumor stage. Our data suggested that the higher affinity binding alleles together with higher CNV of inhibitory *KIR2DL1* compromised NK-mediated immune surveillance in HNSCC and opens up the possibility of the use of KIR-HLA allotype as prognostic markers

REFERENCES:

1. Huhn O, Chazara O, Ivarsson MA, Retiere C, Venkatesan TC, Norman PJ, et al. High-resolution genetic and phenotypic analysis of KIR2DL1 alleles and their association with pre-eclampsia. The Journal of Immunology. 2018;201(9):2593-601.

2. Béziat V, Duffy D, Quoc SN, Le Garff-Tavernier M, Decocq J, Combadière B, et al. CD56brightCD16+ NK cells: a functional intermediate stage of NK cell differentiation. The Journal of Immunology. 2011;186(12):6753-61.

3. Paul S, Lal G. The molecular mechanism of natural killer cells function and its importance in cancer immunotherapy. Frontiers in immunology. 2017;8:1124.

4. Thomas R, Yamada E, Alter G, Martin MP, Bashirova AA, Norman PJ, et al. Novel KIR3DL1 alleles and their expression levels on NK cells: convergent evolution of KIR3DL1 phenotype variation? The Journal of Immunology. 2008;180(10):6743-50.

5. Uhrberg M, Valiante NM, Shum BP, Shilling HG, Lienert-Weidenbach K, Corliss B, et al. Human diversity in killer cell inhibitory receptor genes. Immunity. 1997;7(6):753-63.

6. Traherne JA, Martin M, Ward R, Ohashi M, Pellett F, Gladman D, et al. Mechanisms of copy number variation and hybrid gene formation in the KIR immune gene complex. Human molecular genetics. 2009;19(5):737-51.

7. Norman PJ, Abi-Rached L, Gendzekhadze K, Hammond JA, Moesta AK, Sharma D, et al. Meiotic recombination generates rich diversity in NK cell receptor genes, alleles, and haplotypes. Genome research. 2009;19(5):757-69.

8. Jiang W, Johnson C, Jayaraman J, Simecek N, Noble J, Moffatt MF, et al. Copy number variation leads to considerable diversity for B but not A haplotypes of the human KIR genes encoding NK cell receptors. Genome research. 2012;22(10):1845-54.

9. Purdy AK, Campbell KS. SHP-2 expression negatively regulates NK cell function. The Journal of Immunology. 2009;183(11):7234-43.

10. Dhillion S, Das A, Saxena RK. Activating and inhibitory receptors on Natural Killer cells. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES (INDIA). 1999;3(69 (B)):229-43.

11. Hilton HG, Guethlein LA, Goyos A, Nemat-Gorgani N, Bushnell DA, Norman PJ, et al. Polymorphic HLA-C receptors balance the functional characteristics of KIR haplotypes. The Journal of Immunology. 2015;195(7):3160-70.

12. Norman PJ, Hollenbach JA, Nemat-Gorgani N, Guethlein LA, Hilton HG, Pando MJ, et al. Co-evolution of human leukocyte antigen (HLA) class I ligands with killer-cell immunoglobulin-like receptors (KIR) in a genetically diverse population of sub-Saharan Africans. PLoS genetics. 2013;9(10):e1003938.

13. Parham P. MHC class I molecules and KIRs in human history, health and survival. Nature Reviews Immunology. 2005;5(3):201.

14. Zwirner NW, Domaica CIJB. Cytokine regulation of natural killer cell effector functions. 2010;36(4):274-88.

15. Lanier LLJAroi. NK cell recognition. 2005;23(1):225-74.

16. Ohteki T, Ho S, Suzuki H, Mak TW, Ohashi PSJTJoI. Role for IL-15/IL-15 receptor beta-chain in natural killer 1.1+ T cell receptor-alpha beta+ cell development. 1997;159(12):5931-5.

17. Konjević GM, Vuletić AM, Martinović KMM, Larsen AK, Jurišić VBJC. The role of cytokines in the regulation of NK cells in the tumor environment. 2019;117:30-40.

18. Min-Oo G, Kamimura Y, Hendricks DW, Nabekura T, Lanier LLJTii. Natural killer cells: walking three paths down memory lane. 2013;34(6):251-8.

19. Stojanovic A, Correia MP, Cerwenka AJCM. Shaping of NK cell responses by the tumor microenvironment. 2013;6(2):135-46.

20. Meazza R, Azzarone B, Orengo AM, Ferrini SJJoB, Biotechnology. Role of common-gamma chain cytokines in NK cell development and function: perspectives for immunotherapy. 2011;2011.

21. Vignali DA, Kuchroo VKJNi. IL-12 family cytokines: immunological playmakers. 2012;13(8):722-8.

22. Sarmah N, Baruah MN, Baruah S. Immune Modulation in HLA-G Expressing Head and Neck Squamous Cell Carcinoma in Relation to Human Papilloma Virus Positivity: A Study From Northeast India. Frontiers in oncology. 2019;9:58.

23. Pirker R, Pereira JR, Von Pawel J, Krzakowski M, Ramlau R, Park K, et al. EGFR expression as a predictor of survival for first-line chemotherapy plus

cetuximab in patients with advanced non-small-cell lung cancer: analysis of data from the phase 3 FLEX study. The lancet oncology. 2012;13(1):33-42.

24. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. Journal of molecular biology. 1990;215(3):403-10.

25. Abel AM, Yang C, Thakar MS, Malarkannan S. Natural killer cells: development, maturation, and clinical utilization. Frontiers in immunology. 2018;9:1869.

26. Hilton HG, Norman PJ, Nemat-Gorgani N, Goyos A, Hollenbach JA, Henn BM, et al. Loss and gain of natural killer cell receptor function in an African hunter-gatherer population. PLoS genetics. 2015;11(8):e1005439.

27. Nicholas RE, Sandstrom K, Anderson JL, Smith WR, Wetzel M, Banerjee P, et al. KIR3DL05 and KIR3DS02 Recognition of a Nonclassical MHC Class I Molecule in the Rhesus Macaque Implicated in Pregnancy Success. 2022;13.

28. Parham P, Norman PJ, Abi-Rached L, Guethlein LA. Human-specific evolution of killer cell immunoglobulin-like receptor recognition of major histocompatibility complex class I molecules. Philosophical Transactions of the Royal Society B: Biological Sciences. 2012;367(1590):800-11.

29. Dutta A, Saikia N, Phookan J, Baruah MN, Baruah S. Association of killer cell immunoglobulin-like receptor gene 2DL1 and its HLA-C2 ligand with family history of cancer in oral squamous cell carcinoma. Immunogenetics. 2014;66(7-8):439-48.

30. Yusa S-i, Campbell KSJTJoI. Src homology region 2-containing protein tyrosine phosphatase-2 (SHP-2) can play a direct role in the inhibitory function of killer cell Ig-like receptors in human NK cells. 2003;170(9):4539-47.

31. Hull RM, Cruz C, Jack CV, Houseley J. Environmental change drives accelerated adaptation through stimulated copy number variation. PLoS biology. 2017;15(6):e2001333.

32. Craddock N, Hurles ME, Cardin N, Pearson RD, Plagnol V, Robson S, et al. Genome-wide association study of CNVs in 16,000 cases of eight common diseases and 3,000 shared controls. Nature. 2010;464(7289):713.

33. Höglund P, Brodin P. Current perspectives of natural killer cell education by MHC class I molecules. Nature reviews immunology. 2010;10(10):724.

34. Boonkitticharoen V, Kulapaditharom B, Leopairut J, Kraiphibul P, Larbcharoensub N, Cheewaruangroj W, et al. Vascular endothelial growth factor A

and proliferation marker in prediction of lymph node metastasis in oral and pharyngeal squamous cell carcinoma. Archives of Otolaryngology–Head & Neck Surgery. 2008;134(12):1305-11.

35. Basilio-de-Oliveira RP, Pannain VLN. Prognostic angiogenic markers (endoglin, VEGF, CD31) and tumor cell proliferation (Ki67) for gastrointestinal stromal tumors. World journal of gastroenterology: WJG. 2015;21(22):6924.

36. Chen J-X, Deng N, Chen X, Chen L-W, Qiu S-P, Li X-F, et al. A novel molecular grading model: combination of Ki67 and VEGF in predicting tumor recurrence and progression in non-invasive urothelial bladder cancer. Asian Pacific Journal of Cancer Prevention. 2012;13(5):2229-34.

37. Zitvogel L, Kroemer G. Cytokines reinstate NK cell–mediated cancer immunosurveillance. The Journal of clinical investigation. 2014;124(11):4687-9.

38. Zaiatz-Bittencourt V, Finlay DK, Gardiner CM. Canonical TGF-β signaling pathway represses human NK cell metabolism. The Journal of Immunology. 2018;200(12):3934-41.

39. Viel S, Marçais A, Guimaraes FS-F, Loftus R, Rabilloud J, Grau M, et al. TGF-β inhibits the activation and functions of NK cells by repressing the mTOR pathway. Sci Signal. 2016;9(415):ra19-ra.