Chapter VI

SUMMARY AND CONCLUSION

6.1. Summary:

Natural killer (NK) cells are key components of the innate immune system and have potent antiviral, antitumor, and anti-metastaticactivity.Accordingly, cancer cells must overcome NK cell surveillance to form distant metastases. Researchers have shown thatcancer cells, through a dormant state, down-regulateactivating receptors to evade NK cells.However, we do not fully understand how cancer cellsescape NK cell–mediatedimmune-surveillance. We have earlier reported KIR2DL1+-HLA-C2+ genotype was found to bea heritable risk factor in OSCC predisposing to OSCC at a younger ageand tumor resident NK cells had lower cytolytic activity. The present study is a follow-up from the above findings in which we have explored further whether (a) high-affinity binding alleles of *KIR2DL1* and its copy number variation could explain the association of KIR2DL1-HLAC2 with HNSCC and (b) if NK Cell phenotype accounts for its anergy in tumor tissue.

The salient features of the study are:

1. The Phylogenetic tree based upon *KIR2DL1-HLAC2*combined genotype and *KIR2DS1* gene frequencies showed three Nodes in the neighbor-joining dendogram. Our study population was mapped in the third node consisting of South-East Asian countries. It signifies the fact that the populations of North-East India, in particular Assam, are distinct in terms of genetic makeup, evolutionary and migratory history from North Indian populations which have mainly Indo-European origins,and the South-Indian populations which have Dravidian origins.
2. 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. Additionally, the frequency of *KIR2DS1,* the activating homolog of the *KIR2DL1* gene was found to be 52.45% among the study participants.
3. We identified 9 different HLA-C alleles in the study participants based on sequence alignment with reference sequences from 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.
4. When stratified by C1 and C2 epitopes, a higher frequency of HLA C1 epitope was observed in the study participants [HLA*C1C1 (61.3%), *C1C2 (34%), *C2C2 (4.6%)].

5. The binding affinities of KIR2DL1*003/HLA-CW4 and KIR2DL1*004/HLA-CW4 were found to be higher than the already available structure of KIR2DL1/HLA-CW4. However, KIR2DL1*005 was found to have a lower binding affinity to HLA-CW4 as compared the above three structures. The above results suggest that the binding affinity of KIR2DL1 with HLA-C alters with the allele of *KIR2DL1* present.
6. 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-C1 genotype was higher in healthy controls and was negatively associated with HNSCC.
7. 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. This was also comparable when stratified between HNSCC patients and healthy controls with frequencies of 76.2% and 81% respectively. 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
8. 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).
9. 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).
10. 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).**
11. 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

12. 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.
13. 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.
14. 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.
15. CD16+CD56dim cells comprised 60.8% of the NK cell population in the tumor tissue and 57.3 % in patients' blood, suggesting the predominance of mature NK cells in tumor tissue and peripheral circulation. An intermediate stage of matured NK cells i: e CD16+CD56+ was also seen that comprised 35.4 % and 20.8% of the cells in blood and tissue extracts respectively. CD9+ decidual NK cells were seen at 0.03%-0.05% of the total NK cells in both blood and tumor tissue negating the assumption that the non-cytolytic characteristic of tumor NK cells was due to the recruitment/ predominance of decidual NK cells in the tumor microenvironment.

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.

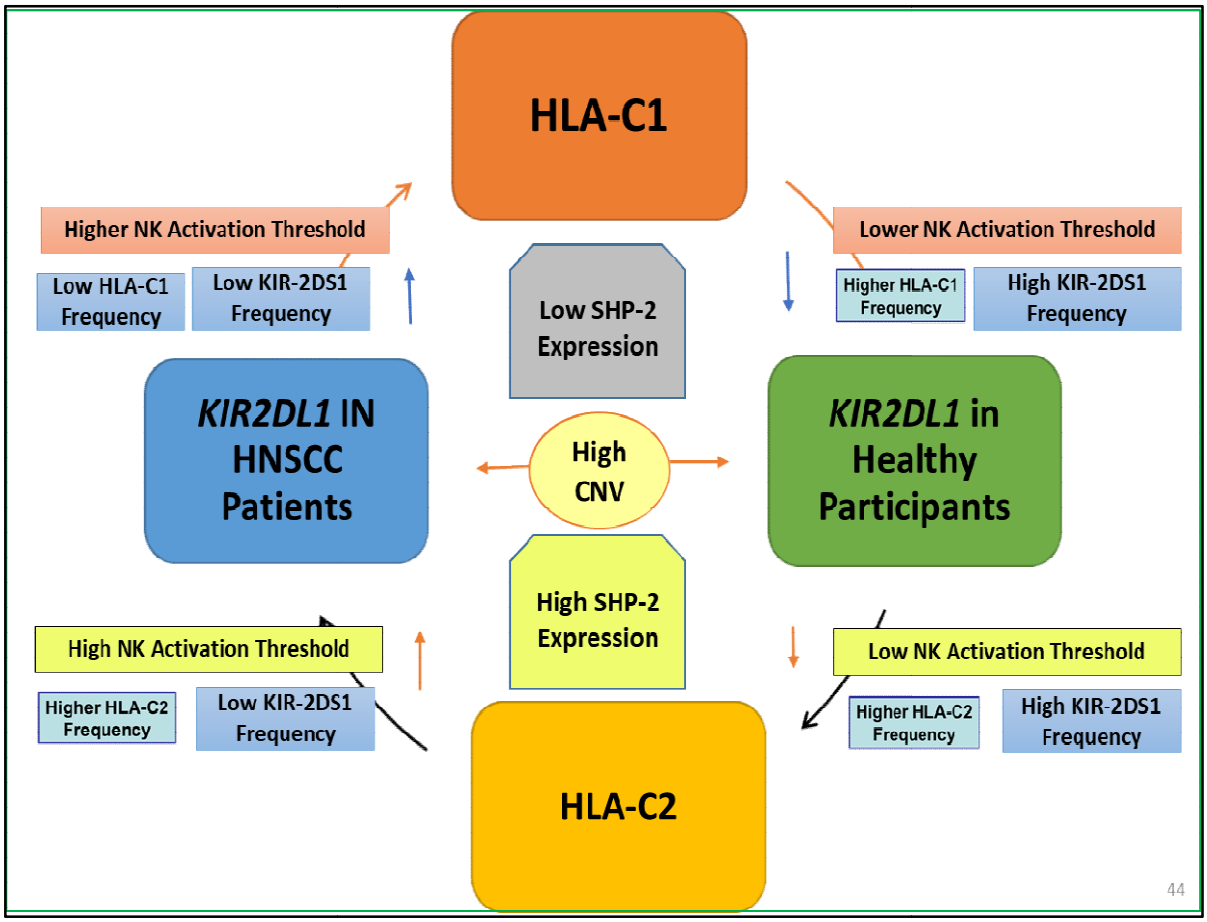

Figure 26: Hypothetical model showing the effect of KIR2DL1 Copy Number Variation and HLA-C in NK Cell activation in the study population