DEPARTMENT OF MOLECULAR BIOLOGY AND BIOTECHNOLOGY
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S.Baruah, Ph.D.
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TU/MBBT/SB/ICMR-17/038

Date: 27/06/2022

The Director-General
Indian Council of Medical Research,
V. Ramalingaswami Bhawan,
Ansari Nagar, P.Box No. 4911,
New Delhi-110029

Sub- Submission of Project closure report and other documents for the project **"Polymorphism of Toll Like and Nod ---- in Head and Neck Squamous Cell Carcinoma"**
Ref: IRIS no. 2014-2705

Dear Sir,

This is in reference to submission of closure documents for the above-mentioned project.

We are submitting the Final Report and other documents of the project. Kindly find the following documents enclosed with this letter

1. Detailed Closure Technical Report
2. Grant Utilization (UC/SE) of the project
3. Demand Draft of the unspent balance for Rs 105099/- (one lakh five thousand and ninety nine only)

We request you to kindly do the needful for the closure of the project.

Sincerely,

(Dr. S. Baruah, PI)

Prof. Shashi Baruah
Department of MBBT
Tezpur University

Format for Annual Statement of Accounts

(Period 2019- 2022)

1) Sanction Letter No. : 5/13/12/2015/NCD-III dated 14/10/2019

2. Total Project Cost : Rs 4989130

3. Sanction /Revised Project cost (if applicable) : Rs……N.A……

4. Date of Commencement of Project : 15th February 2017

5. Proposed Date of Completion : 14th August 2020

6. Statement of Expenditure : From 1st April 2019 – 31st March 2022

S. No.	Sanctioned / Heads	Funds Received (I Year)	Funds Received (II Year)	Funds Received (III Year)	Expenditure Incurred I Year (2017-18)	Expenditure Incurred II Year (2018-19)	Expenditure Incurred III Year (2019-20)	(2020-21)	BALANCE
1.	Salaries	403200	308000	476000	308000	392000	377397	NIL	
2.									
2.1.	Non-recurring (Equipments)	1200000	Nil	NIL	343400	834430.04	NIL	NIL	
2.2	Recurring	855000	810138	NIL	501052	180111	607018	418908	
3.	Travel	50000	36333	38893	36333	18709	53024		
4.	Overhead	37746	60910	12120	23591	Nil	22537	66731	
5.	Others (if any)		NA	NA	NA	NA	NA	NA	
	Total	2545946	1215381	527013	1212376	1425250	1059976	485639	105099

01/06/22

Signature of Principal Investigator with date

23/6/2022

Signature of Accounts Officer with date

Finance Officer
Tezpur University

Annexure-3a

Check list for covering note to accompany Utilization Certificate of grant for the project for the period 2019-2022

1) Title of the Project : Polymorphism of Toll like and Nod like Receptors and their role in inflammation activation in Head and Neck Squamous Cell Carcinoma

2) Name of the Institutions: **Tezpur University**

3) Principal Investigator : **Prof. S. Baruah, Dept. of MBBT, Tezpur University**

4) ICMR letter No. and date sanctioning the project: **File No 5/13/12/2015/NCD-III dated 14/10/2019**

5) Head of account as given in the original sanction letter : **Registrar, Tezpur University**

6) Amount received during the year (Please give No. & Date of ICMR's sanction letter for the amount and period) : **Rs 527013**- vide File No 5/13/12/2015/NCD-III dated 14-10-2019

7) Total amount that was available for expenditure (excluding commitments) during the year (Sl.No.6+7) : **Rs 1650713.96 (Including previous balance of Rs1123700.96 as on 31.3.2019)**

8) Actual expenditure (excluding commitments) incurred during the year: Rs **1545615/-**

9) Balance amount available at the end of the year: Rs **105098.96**

10) Amount already committed, if any: **NIL**

11) Amount to be carried forward to the next year (if applicable). Indicate the amount already committed with supporting documents.

Format for Utilization Certificate
(Annual/Final)

Certified that out of **Rs 527013** of grants-in-aid sanctioned during the year 2019-20 in favour of Registrar, Tezpur University under ICMR Letter No**5/13/12/2015/NCD-III** dated **14/10/2019** and Rs**1123700.96** on account of unspent balance of the previous year, a sum of Rs **1545615/-** has been utilized for the purpose of research for which it was sanctioned and that the balance of Rs. **105098.96/-**remaining unutilized at the end of the year has been surrendered to ICMR (vide cheque No ..... Dated.....)

Signature of Principal Investigator with date 01/06/22

Signature of Registrar/ of the Institute with date 23/6/22
Registrar
Tezpur University

Signature of Accounts Officer of the Institute with date 23/6/22
Finance Officer
Tezpur University

Format for Annual Statement of Accounts

(Period 2019- 2022)

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2.1.	Non-recurring (Equipments)	1200000	Nil	NIL	343400	834430.04	NIL	NIL	
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4.	Overhead	37746	60910	12120	23591	Nil	22537	66731	
5.	Others (if any)		NA	NA	NA	NA	NA	NA	
	Total	2545946	1215381	527013	1212376	1425250	1059976	485639	105099

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Signature of Accounts Officer with date

Finance Officer
Tezpur University

Annexure-3a

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Signature of 01/08/22
Principal Investigator
with date

Signature of Registrar/
of the Institute with date
Registrar
Tezpur University

Signature of Accounts Officer
of the Institute with date
Finance Officer
Tezpur University

Annexure-2

Format for Annual Progress Report

1. **Project title**: Polymorphisms of Toll like and Nod like receptors and their role in inflammasome activation in Head and Neck Squamous Cell Carcinoma. [IRIS Code No.-2014-2705]
2. **PI (name & address):** Dr S. Baruah, Professor, Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur-784028, Assam
3. **Co-PI (name & address**): A] Dr. Jyotirmoy Phookan, Associate Prof. Dept. of E.N.T Gauhati Medical College & Hospital B] Dr Nabajyoti Saikia, Asst.Prof. Dept of E.N.T Dibrugarh Medical College & Hospital
4. **Date of start**: 15-Feb-2017
5. **Duration**: 3 Years
6. **Objectives of the proposal:**

The objectives of the proposed investigation are:

I. To investigate the association of genetic variations (SNPs) of TLR 3, 4 and 9, NLRP3 and 6, in HNSCC.

II. Characterize macrophage sub-population based on their phenotypic and molecular markers and their activation status (based on secretion of tissue remodeling enzymes and growth factors,) in relation to tumor growth

III. NLP3 and NLP6 Inflammasome activation in relation to tumor growth.

7. **Methodology**

Introduction:

Head and Neck Carcinoma accounts for approx. 30% of all cancer cases reported in India and NE India reports a higher incidence of 33% (Bhattacharjee, A et al). Studies on molecular mechanisms that underlie development and progression of cancers, implicate inflammation to play a crucial role by providing a pro-tumorigenic environment. Inflammation besides providing a proliferation supporting environment by secreting growth promoting cytokines, chemokines and angiogenic factors, also increases mutation rates, and enhances mutagenesis in several tumor repressor genes and promotes

neo-angiogenesis and metastasis. Besides, tumors subvert and actively modulate inflammatory processes to promote tumorigenesis and facilitate evasion of immune surveillance. Further the inflammatory processes are hypothesized to alter between antitumorigenic and pro-tumorigenic responses, and the mechanisms which are responsible for this switch are not properly understood. Innate receptors like Toll like receptors (TLRs) and Nod like receptors (NLRs) are key components of inflammatory pathways and are responsible for activation of inflammasomes and initiation of inflammatory response by triggering secretion of cytokines and chemokines. SNPs within innate receptor genes may thus influence the ability to properly respond to ligands causing an altered susceptibility to disease. Aberrant signaling of inflammatory pathways are reported to be involved in the pathogenesis of inflammatory diseases and cancers.

Cancers require constant support from the microenvironment for its survival, resulting in a dynamic two-way interaction between the cancer cells and the stroma. Progression and manifestation of malignant disease are stringently dependent on interactions with the host tissue and inflammation is inevitably a key component of the process. The presence of leukocytes within tumors, observed in the 19 th century by Rudolf Virchow, provided the first indication of a possible link between inflammation and cancer 3. A role for inflammation in tumorigenesis is now generally accepted, and it has become evident that an inflammatory microenvironment is an essential component of all tumors, including some in which a direct causal relationship with inflammation is not yet proven 2. Many environmental causes of cancer and risk factors are associated with some form of chronic inflammation. Up to 20% of cancers are linked to chronic infections, 30% can be attributed to tobacco

smoking and inhaled pollutants (such as silica and asbestos), and 35% can be attributed to dietary factors (20% of cancer burden is linked to obesity). Therapy induced inflammation resulting from necrotic death of malignant cells, due to chemo and radio therapy results in the release of necrotic products and DAMPs that activate cytokine production by inflammatory cells. These cytokines activate pro survival genes in residual cancer cells, rendering them resistant to subsequent rounds of therapy. Thus, carcinogenesis and tumor progression are either stimulated or restrained by inflammatory and immune processes, respectively.

Pro-inflammatory cytokines such as IL-1β, TNF are critical and are involved in tumor pathogenesis. Inflammasomes are multi-protein complex which play a significant role in the pathway regulating IL-1β and other pro-inflammatory cytokine secretion. As their names imply, inflammasomes have a critical role in inflammatory syndromes, conditions featuring recurrent episodes of systemic inflammation 12. Inflammasome can also influence the formation, progression and therapeutic response of cancer through their contribution to tissue homeostasis, inflammation and immune responses 13. These are composed of three proteins: a nucleotide oligomerization domain (NOD)-like,

leucine-rich repeat-containing recep-tor (NLR), apoptosis associated speck-like protein containing a caspase recruitment domain (ASC), and caspase-1/ caspase-55. Each NLR differs in the makeup of its effector domain which mediates signal transduction to downstream targets leading to activation of inflammatory caspases by inflammasome. NLRs are critical for sensing DAMP signals, TLRs recognize PAMPs on the cell surface and in endosomes, and the crosstalk between TLRs and Inflammasomes executes the inflammatory response. Role of the inflammasomes in cancer is critical but contrasting, as they positively affect cell's autonomous death pathways and anticancer immunosurveillance, but they also stimulate autocrine or paracrine processes that favor carcinogenic inflammation, tumor growth, metastasis and angiogenesis. Inflammasomes have been observed to affect outcome of colorectal carcinoma and melanoma and other inflammatory diseases such as atherosclerosis, but their role in HNSCC is not well understood and information is scanty.

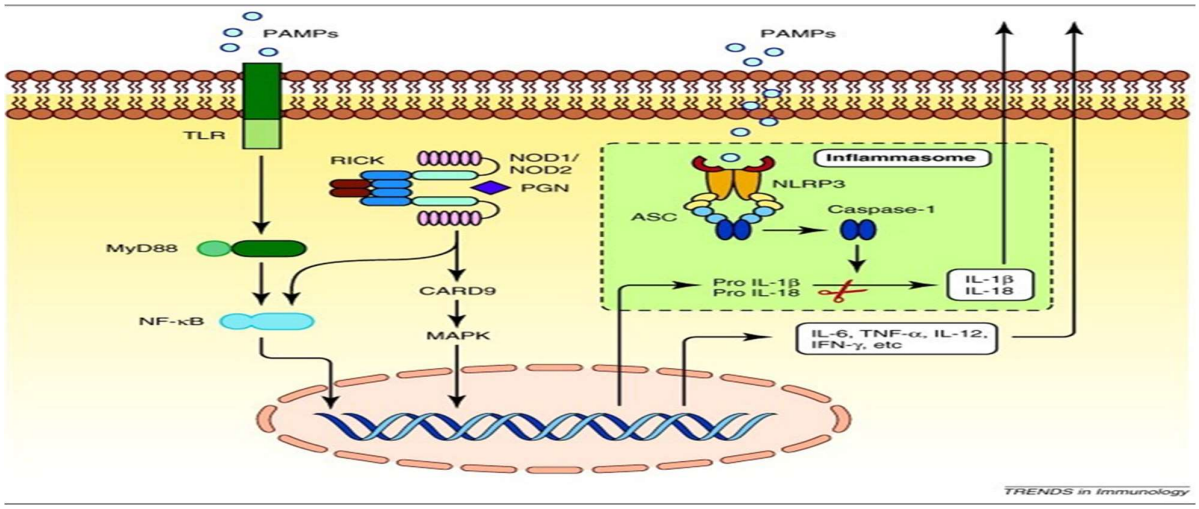

Figure: TLRs, NLRs and inflammasome signaling pathways

Image courtesy: Zaki et. al 2011

Global Genetic Diversity of the TLR Family in Human Populations:

TLRs in different worldwide populations, has shown that intracellular TLRs—principally specialized in viral recognition evolve under strong purifying selection, indicating their essential role in host survival, while the remaining TLRs display higher levels of immunological redundancy.

Large fluctuations in the overall levels of nucleotide diversity for the different TLR members Worldwide, TLR4, TLR7 and TLR9 were the least diverse, whereas TLR10 was by far the most diverse gene with a 2-fold increase of general diversity with respect to the mean values observed for the twenty noncoding regions. For all TLR family members, with the exception of TLR1, genetic

diversity was higher in Africans with respect to both Europeans and East Asians in agreement with the recent African origin of modern humans.

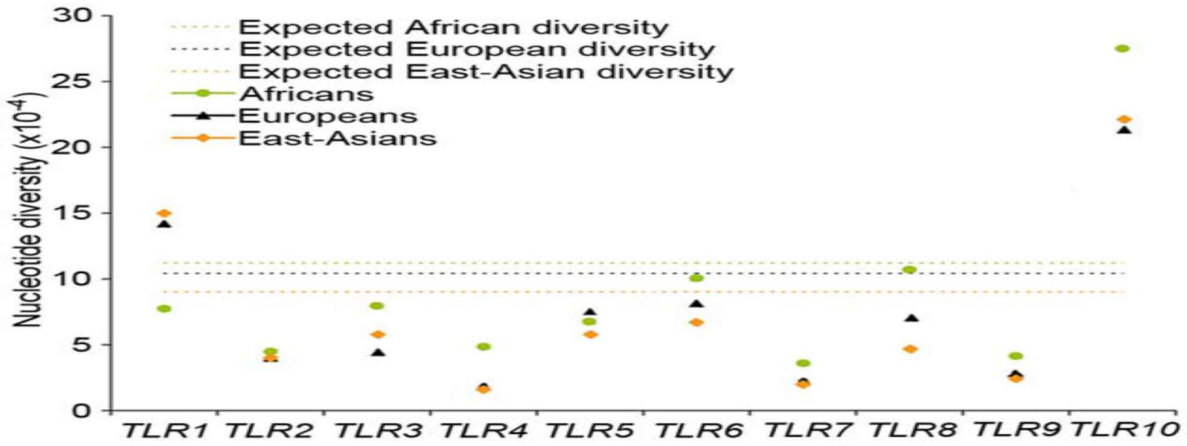

Figure 1. Global genetic diversity of the TLR family in human populations. Nucleotide diversity levels for the individual TLR genes in populations representing major ethnic groups. The expected diversity corresponds to the mean diversity levels observed for the 20 autosomal noncoding regions (‘‘neutral’’ regions) in each geographical area. doi:10.1371/journal.pgen.1000562.g001

1. Demaria,S., Pikarsky,E., et al. Cancer and Inflammation: Promise for Biological Therapy, *Journal of Immunotherapy* 33(4) 335–351, 2010.

2. Mantovani,A., et al. Cancer-related inflammation, *Nature* 454, 436-444,2008.

3. Sergei, I., et al. Immunity, Inflammation, and Cancer, *Cell* 140, 883–899, 2010.

4. Aggarwal,B.B., et al. Targeting inflammatory pathways for prevention and therapy of cancer: short-term friend, long-term foe, *Clinical Cancer Res*. 15, 425–430, 2009.

5. Dunn,J.H., et al. Inflammasomes as molecular mediators of inflammation and cancer: Potential role in melanoma, *Cancer Letters* 314, 24–33, 2012.

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7. Hanamsaga,R. Toll-like receptor (TLR) and inflammasome actions in the central nervous system, *Trends in Immunology*, 33,(7), 2012.

8. Fukata, M., et al. Toll-like receptors (TLRs) and Nod-like receptors (NLRs) in inflammatory disorders, *Seminars in Immunology* 21, 242–253, 2009

9. Wen, H., et al. A role for the NLRP3 inflammasome in metabolic diseases—did Warburg miss inflammation?, *Nature Immunology* 13(4),352-357, 2012.
10. Mejia,J.H., et al. Inflammasomes: far beyond nflammation, *Nature Immunology* 13(4),321-324, 2012.
11. Karolina,P.,Banchereau,J. Cancer immunotherapy via dendritic cells, *Nature Reviews Cancer* 12*,* 265-277, 2012.
12. Zitvogel,L., et al. Inflammasomes in carcinogenesis and anticancer immune responses, *Nature Immunology* 13(4),343-351, 2012.
13. Tschopp,J., Schroder,K. NLRP3 inflammasome activation:the convergence of multiple signalling pathways on ROS production? *Nature Reviews Immunology* 10, 211-215, 2010.
14. Ungerback,J et al. Genetic variation and alterations of genes involved in NFκB/TNFAIP3- and NLRP3-inflammasome signaling affect susceptibility and outcome of colorectal cancer *Carcinogenesis* 33,2126-2134, 2012
15. Sawian C.E, et al, Polymorphisms and expression of TLR4 and 9 in malaria in two ethnic groups of Assam, northeast India, Innate Immun. 2013;19(2):174-83.
16. Dutta A, Saikia N, Phookan J, Baruah M N, Baruah S (2014) 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.
17. Mahanta A, et al, The association of IL-8-251T/A polymorphism with complicated malaria in Karbi Anglong district of Assam, Cytokine. 2014

Materials and Methods:

Study design:

A hospital-based case-control study was conducted with 150 histopathologically confirmed cases of Head and Neck cancer patients and 150 non-cancer OPD attending participants. The study objectives were approved by the Tezpur University Ethical Committee (TUEC) (vide IEC letter No. 01/2014 dated 30/04/2014). Ethical approval has also been taken from The Institutional ethics committee, North-East Cancer Hospital and Research Institute (IEC-NECHRI) vide IEC letter no. IEC/2017/03/NP/003.

Detailed Methodology

Objective 1:

To investigate the association of genetic variations (SNPs) of TLR 4 and 9, NLRP 3 and 6, in HNSCC.

Association SNPs of TLR, NLR with HNSCC was determined using PCR Confronting Two Primer Pair technique (PCR-CTPP) technique, where the presence of SNP was determined on the basis of the electrophoretogram for the specific gene and of the SNP.

Briefly, blood samples were collected from 150 cancer patients and 150 non-cancer OPD attending participants in EDTA. Genomic DNA isolation from blood was carried out using DNA isolation kit and polymorphisms of TLR3, TLR4, TLR9, NLP3 and NLP6 were determined using PCR-CTPP method. Following amplification 10 µl of PCR products were visualized in ethidium bromide stained gels. Results obtained from the above studies were used to identify the relation of the mutations with respect to HNSCC. Based on the literature survey, the following SNPs were selected:

Gene Name	**SNP**
TLR 4	**rs4986790** (C14143T) and **rs4986791**TLR
TLR9	**rs5743836** (C-1237T) and **rs187084** (C-1486T)
NLP6	**rs141655877** (S730N)
TLR3	**rs3775291** (G1335A)
NLP3	**rs10733113** (A/G**)**

Objective 2:

Characterize macrophage sub-population based on their phenotypic and molecular markers and their activation status (based on secretion of tissue remodeling enzymes and growth factors,) in relation to tumor growth.

Macrophage being a key secretory cell for cytokine and chemokine in the tumor microenvironment we proposed to study the macrophage subpopulation based on cytokine profile and phenotypic markers and their activation status in HNSCC.

Briefly the methodology involves collection of tumor tissue (biopsy and post-operative) in RNA later and total RNA was isolated using RNA Isolation Kit followed by cDNA synthesis. Quantification of the cytokine (IL-1□□□□L-10, TGF-□, IL-6, and IL-15) and ELR positive chemokine profile of the tissue studied at mRNA level using qRT-PCR. Protein concentration of these Cytokine/Chemokine in peripheral blood and tumor tissue will be analyzed by ELISA/Multiplex bead based assays. Secretory profile of the tissue remodeling enzymes would be studied using IHC (in the phenotypic markers CD14, CD86 and iNOS for M1 macrophages) and Western Blotting in tissue extracts

Objective 3:

NLP3 and NLP6 Inflammasome activation in relation to tumor growth.

Here we are proposing to study the activation of NLRP3 and NLRP6 inflammasome in relation to tumor growth.

Collection of tumor tissue (biopsy and post-operative) will be done in RNA later and FFPE blocks for 10% of the samples. Activation of inflammasome will be studied on downstream products by detection of key signalling intermediates of NLR pathways by WB and IF. Secretion of pro-inflammatory cytokines would studied using qRT-PCR at mRNA level and by ELISA at protein level from tissue extract. Tumor growth would be assayed by markers of proliferation (Ki67) and differentiation (keratins) in FFPE tumor tissue by Immuno-Fluorescent microscopy/IHC.

Data Analysis:

Data analysis will be done for

- □Association of SNPs of TLRs and NLRs with HNSCC and with clinical features of disease.
- □Determine macrophage sub-population, identification of key cytokines and its activation status.
- □Correlation of inflammasome activation status with tumor growth.
- □Analysis of signalling pathways to identify possible immuno-intervention therapeutic targets.

8. **Interim modification of objectives/methodology (with justifications):** No

9. **Detail progress of the work carried out during the period:**

Manpower recruitment: Mr Saurav Phukan was recruited as SRF against the sanctioned position.

Instrument purchased: The following instruments were sanctioned in the project:

Sl.No.	Instrument Name	Date of Procurement
1	Gradient PCR	19-02-2018
2	UV Transilluminator	04-01-2018
3	Vertical Gel Caster and power supply	29-12-2017
4	Semi Dry transfer apparatus	06-12-2017
5	Refrigerator : Model-LG-GC-D432HLHU	20-12-2018
6	6 KVA Online UPS with Inbuilt Isolation Transformer- Orion Astra Series	05-02-2019
7	Deep Fridge	21-01-2019

Laboratory investigations:

Towards Objective 1:

To investigate the association of genetic variations (SNPs) of TLR3, 4 and 9, NLRP 3 and 6, in HNSCC.

For laboratory investigations 1.0 -2.0 ml blood were collected by qualified staff after obtaining the written informed consent of the patient.

The inclusion and exclusion criterion were as follows: -

Inclusion criteria:

All histopathologically confirmed cases of HNSCC.

Exclusion criteria

a) Pediatric age group patients with head and neck cancer.

b) Patients with other debilitating diseases.

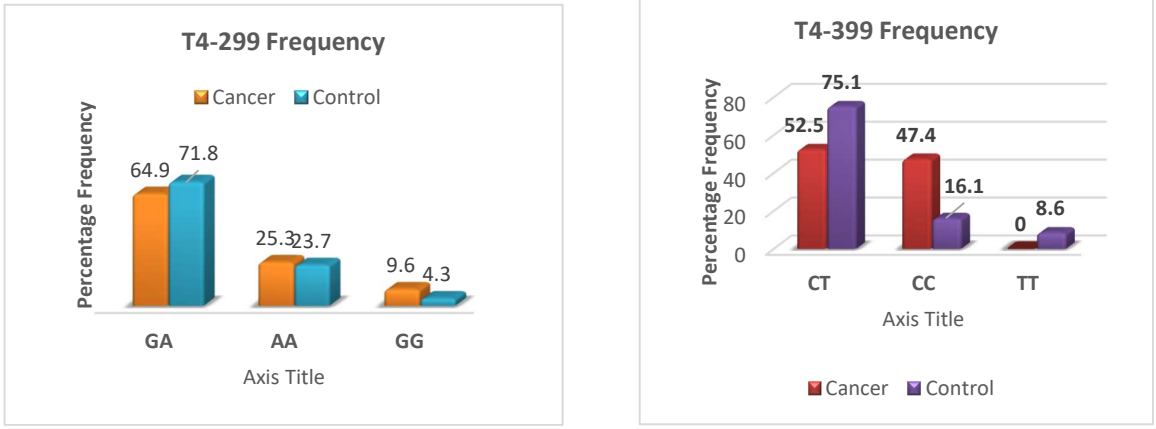
c) Metastatic neck diseases from other parts of the body, patients with cancer of esophagus, thyroid and adenocarcinomas

d) Other vulnerable participants (like terminally ill/mentally challenged)

Polymorphism Study:

Growing data about TLR/NLR polymorphisms have suggested many possible associations between the TLR/NLR SNPs and susceptibility, severity or prognosis of various cancers. The present study was aimed to investigate whether there is an association between gene polymorphisms in TLR4, TLR3 or TLR9 and NLP3/6 and to identify patients at risk for HNSCC patients from Assam. All the SNPs were typed in 300 samples which included 150 cancer and 150 control participants.

In case of TLR4-T299G, the frequency of heterozygous genotype GA as well as homozygous mutant genotype AA was found to be comparable in both cancer patients and healthy participants. In T399I polymorphism, the CT genotype was found to be higher in controls while the CC genotype was higher in cancer patients (Fig 1)

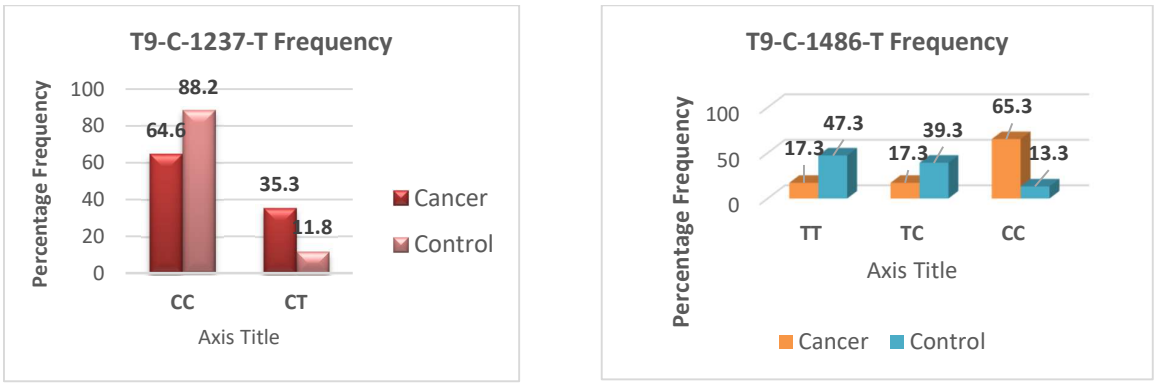


(A) (B)

Fig 1: Percentage Frequency of TLR-4 (A) T299G (B) T399I Polymorphisms in Cancer and Control

Further we studied 2 TLR-9 polymorphisms i:e TLR9 (C-1237T) and TLR-9 (C-1486-T). In TLR9 (C-1237T), the frequency of wild type homozygous CC genotype was higher in controls participants

whereas the heterozygous CT genotype was found to be higher in cancer patients. In TLR-9 (C-1486-T), the mutant homozygous genotype TT was found to be higher in control participants whereas the wild type C genotype was higher in cancer patients (Fig 2)



(A) (B)

Fig 2: Frequency of TLR-9 (A) TLR9 (C-1237T) (B) TLR-9 (C-1486-T) Polymorphisms in Cancer and Control

In case of NLRP6 (rs141655877 (S730N)) polymorphism, frequency of both the genotypes AG and GG was found to be higher in cancer patients as compared to control participants (Fig 3).

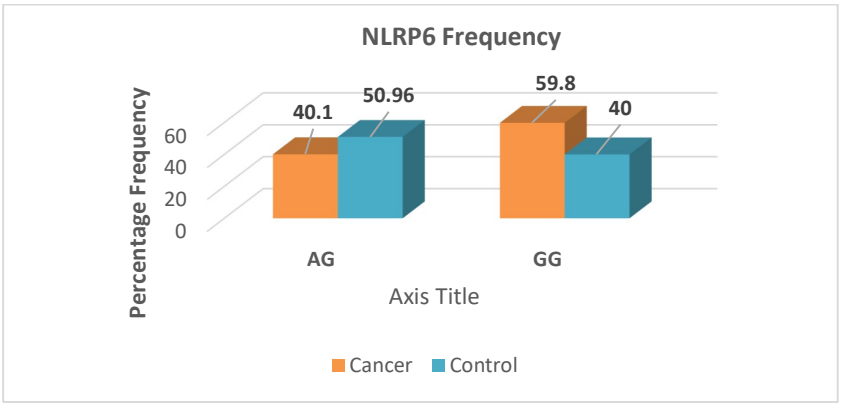


Fig 3: Percentage Frequency of NLRP6 Polymorphisms in Cancer and Control

In case of NLRP3 (rs10733113) polymorphism, the heterozygous genotype AG was found to be higher in control participants. While the wild type genotype AA was comparable between both cases and controls, the mutant genotype GG was higher in cases as compared to control participants (Fig 4).

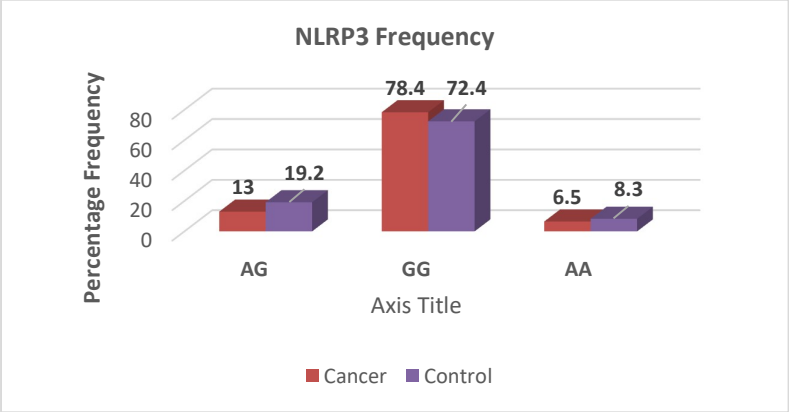


Fig 4: Percentage Frequency of NLRP6 Polymorphisms in Cancer and Control

The frequency of TLR3 L412F (G1335A) polymorphism suggested the prevalence of heterozygous GA genotype in the population. The frequency of both GG and AA genotype was found to be low and comparable between cases and controls (Fig 5).

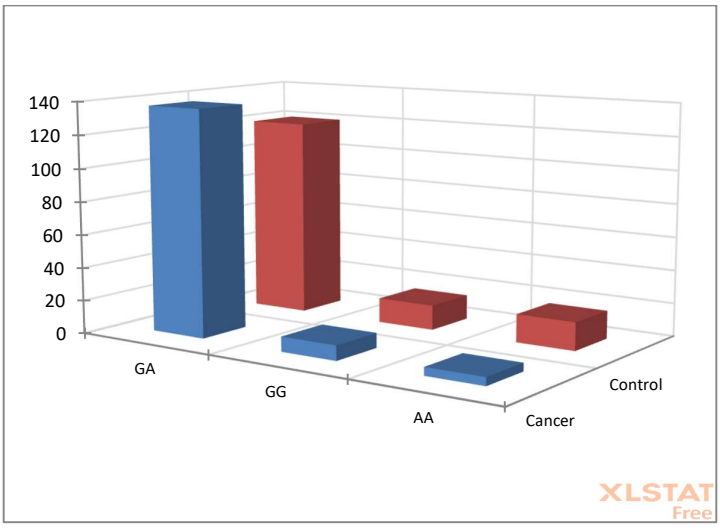


Fig 5: Frequency of TLR3 L412F (G1335A) polymorphism suggesting prevalence of GA allele in the population

Disease association study:

A comparison of frequency of wild and mutant genotype of TLR 4 T299G and TLR4 T399I (Fig 1) showed the homozygous wild type genotype in TL4-4-399 positively associated with the disease **(***p value < 0.0001)** and the presence of the ancestral genotype CC increased the odds of developing cancer **(OR: 7.830 CI=2.96-20.66).** However**,** the distribution of the genotypes of TLR-4-299 were comparable between cancer and control participants.

In TLR9 (C-1237T), the wild type allele C was found to be higher in the control group (Fig2) and was negatively associated with disease (Spearman R=-0.273). Thus the higher incidence of C allele was found to reduce the risk of developing the disease and conferred protection *(***p < 0.0001)*. However, in TLR-9 (C-1486-T), the wild allele C was found to be higher in the cancer group, and was positively associated (Spearman R=0.458, ****p* ***< 0.0001***). While the mutant allele T was higher in the control group. Therefore, **the higher incidence of the ancestral allele (C) increased the risk of developing HNSCC (OR: 6.851).**

The distribution of the alleles of NLRP6 S730N were comparable between cancer and control participants (Fig 3). Also a complete absence of the homozygous mutant genotype was noted. AG genotype was weakly associated with disease but this association did not reach statistical significance (p< 0.095). In addition, the distribution of the alleles of NLRP3 (rs10733113 A/G) were comparable between cancer and control participants (Fig 4). The mutant GG allele was weakly associated with disease but this association did not reach statistical significance **(p< 0.248**). However, the role of NLRs in tumorigenesis cannot be ruled out on the basis of lack of association of alleles with disease.

TLR3 L412F (G1335A) was also typed using PCR-CTPP approach and the data suggested the prevalence of GA genotype in the study population (Fig: 5) and was positively associated with the disease ***(*p< 0.01)***.

Logistic regression analysis of all the typed SNPs was done to model the data for predictive value of the SNPs (Fig: 6).The ancestral C allele of TLR-4-399 and the ancestral C allele of T9-C1486-T were positively associated with the diseases and increased the odds of having the disease (O.R=7.830 and 6.851 receptively for TLR-4-399 and T9-C1486-T), while TLR-3 and TLR 9 (C-1237-T) polymorphisms were found to be protective against cancer.

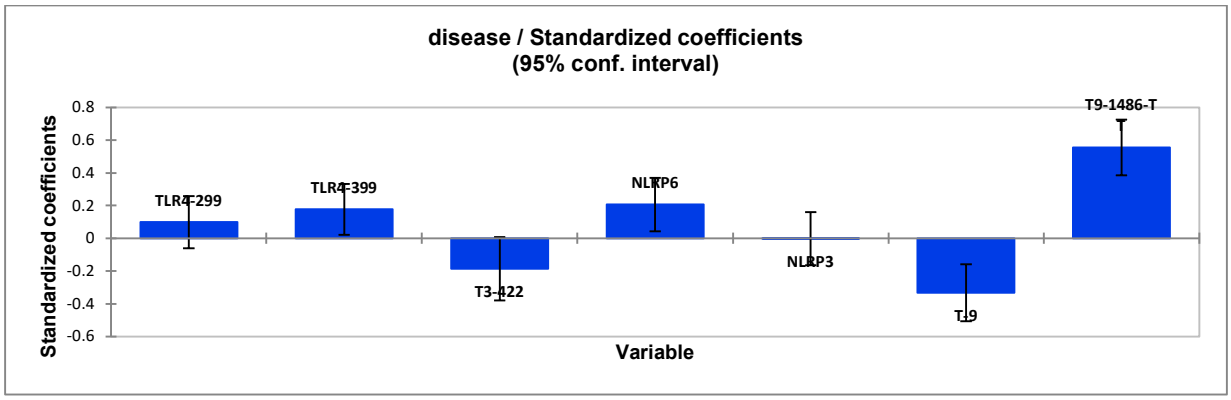


Fig 6: Logistic regression analysis of all the typed SNPs

Main findings of Objective 1

The homozygous wild type genotype in TL4-4-399 was found to be positively associated with the disease **(p = < 0.0001)** and the presence of the ancestral allele C increased the odds of developing cancer **(OR: 7.830 CI=2.96-20.66).** In contrast, the distribution of the alleles of TLR-4-299 were comparable between cancer and control participants

In TLR9 (C-1237T), the wild type allele C was found to be higher in the control group and was negatively associated with disease (Spearman R=-0.273). Thus the higher incidence of C allele was found to reduce the risk of developing the disease and conferred protection (p = **< 0.0001**). However, in TLR-9 (C-1486-T), the wild allele C was found to be higher in the cancer group, and was positively associated (Spearman R=0.458, p **= < 0.0001**). While the mutant allele T was higher in the control group. Therefore, the higher incidence of the ancestral allele (C) increased the risk of developing HNSCC **(OR: 6.851)**

The distribution of the alleles of NLRP6 S730N were comparable between cancer and control participants. Also a complete absence of the homozygous mutant allele was noted. AG genotype was weakly associated with disease but this association did not reach statistical significance (p<0.06).
In TLR3 L412F (G1335A) data suggest the prevalence of GA genotype in the study population. However, no significant association was found between the SNP and disease, thus the polymorphism demands further investigation with larger number of samples.

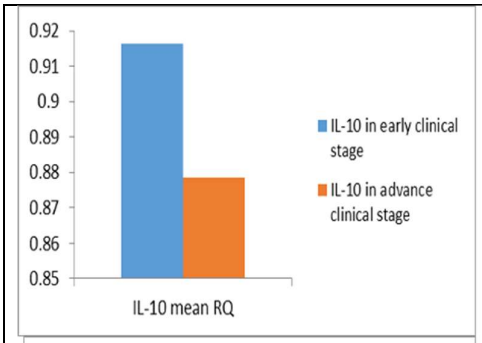
Towards Objective 2: Characterize macrophage sub-population based on their phenotypic and molecular markers and their activation status (based on secretion of tissue remodeling enzymes and growth factors,) in relation to tumor growth.

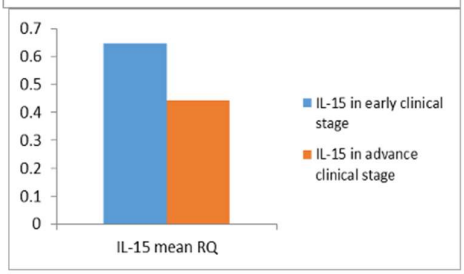
Total cellular RNA was isolated from peripheral blood of the collected samples. cDNA was generated from the isolated RNA using cDNA Reverse Transcription Kit and stored at -80°C to perform **real time qPCR** for assaying mRNA expression of the cytokines studied. The expressions were measured by comparing Ct values of the target genes with housekeeping gene (GAPDH) as reference. Fold change was calculated by determining ΔΔ Ct, using healthy control samples as calibrators. Expression of IL-10, IL-15, IL-6, IL-13, IL-1 and TGF-beta were studied in patients from both early and advanced clinical stages of cancer. The data in Fig: 7 compares the fold change in cytokine expression in early and advanced stages of cancer in comparison to calibrator samples from healthy participants.

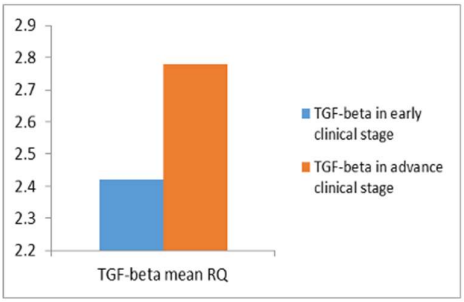
Studies have found that the activation of the TLRs signaling pathway may lead to increased secretion of proinflammatory cytokines, like IL-6, TNF-alpha, IL-1 Beta and also anti-inflammatory cytokines IL-10 and TGF-β, both of which are major immune suppressors *in vivo*.

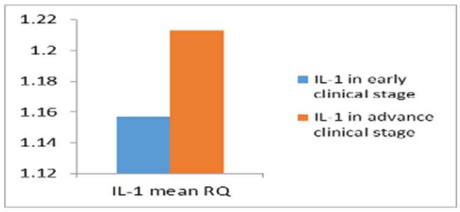
In our study, the transcript expression of cytokines IL-10 and IL-13 were found to be higher in early stages of HNSCC and downregulated in later stages, suggesting immunosuppression in early disease. However, increased levels of IL-1 and of IL-6 in later stages of disease suggest proinflammatory activation. The seemingly contradictory data suggests simultaneous activation of inflammatory environment to promote tumor growth and suppression of immunosuppressive IL-10 to suppress immune surveillance of tumor.

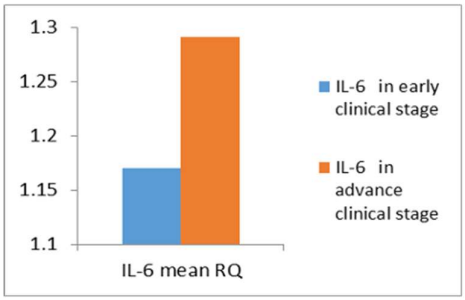
The transcript levels of cytokines IL-1, IL-6, IL-13 were not higher in comparison to healthy control and did not change appreciably with stage of disease. Levels of IL-10 and IL-13 were seen to be lower in later stages of disease, suggesting higher cytokine mediated immunosuppression in early stages of disease. However, TGF-beta levels were higher in cancer patients and were seen to increase with disease.

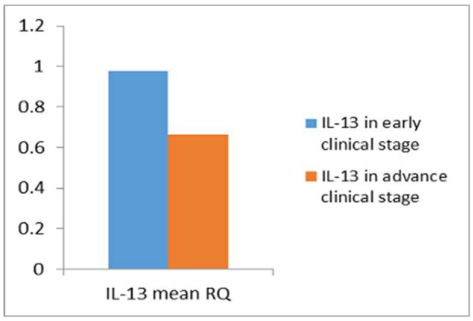

0.92
0.91
0.9
0.89
0.88
0.87
0.86
0.85
IL-10 mean RQ
IL-10 in early clinical stage
IL-10 in advance clinical stage


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
IL-15 mean RQ
IL-15 in early clinical stage
IL-15 in advance clinical stage


2.9
2.8
2.7
2.6
2.5
2.4
2.3
2.2
TGF-beta mean RQ
TGF-beta in early clinical stage
TGF-beta in advance clinical stage


1.22
1.2
1.18
1.16
1.14
1.12
IL-1 mean RQ
IL-1 in early clinical stage
IL-1 in advance clinical stage


1.3
1.25
1.2
1.15
1.1
IL-6 mean RQ
IL-6 in early clinical stage
IL-6 in advance clinical stage


1.2
1
0.8
0.6
0.4
0.2
0
IL-13 mean RQ
IL-13 in early clinical stage
IL-13 in advance clinical stage

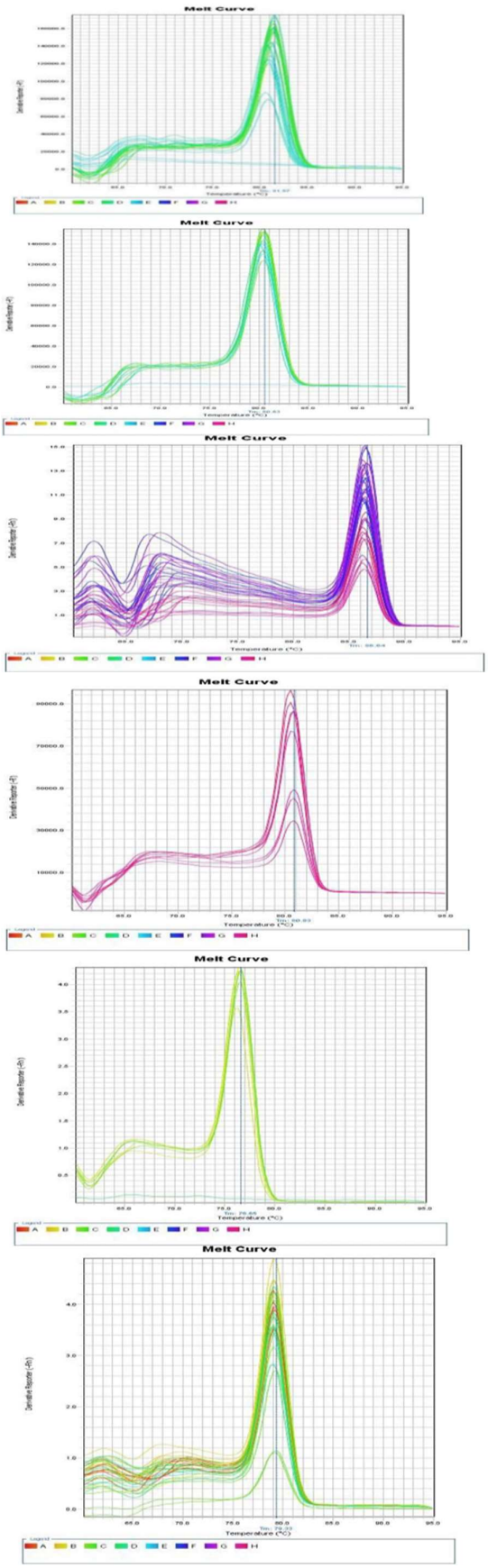

Melt Curve
Melt Curve
Melt Curve
Melt Curve
Melt Curve
Melt Curve

Fig 7: (A) mRNA expressions for cytokines performed on patients from both early and advanced clinical stages of cancer. (B) Melting curves of amplicons suggesting successful amplification in real-time PCR.

Protein expression of monocyte specific inflammatory cytokines:

In addition, BD CBA Human Inflammatory Cytokines Kit was used to quantitatively standardize the measurements of Interleukin-8 (IL-8), Interleukin-1β (IL-1β), Interleukin-6 (IL-6), Interleukin10 (IL-10), Tumor Necrosis Factor (TNF), and Interleukin-12p70 (IL-12p70) protein levels in HNSCC samples. Briefly Six bead populations with distinct fluorescence intensities was coated with capture antibodies specific for IL-8, IL-1β, IL-6, IL-10, TNF, and IL-12p70 proteins. The six bead populations were mixed together to form the BD CBA, which is resolved in a red channel (ie, FL3 or FL4) of a flow cytometer. The capture beads, PE-conjugated detection antibodies, and recombinant standards or test samples were incubated together to form sandwich complexes. Following acquisition of sample data using the flow cytometer, the sample results were generated in graphical and tabular format using the BD CBA Analysis Software or FCAP Array™ Software. High expression of IL-8, IL-1β, IL-10 and IL-6 suggested tumor migration which results in an immunosuppressive environment. The above data suggested that the cancer cells enable TLRs signals to release cytokines and chemokines in the tumor environment, which in turn binds to immune suppressive cells and further release aberrant cytokines and chemokines, ultimately leading to tumor progression.

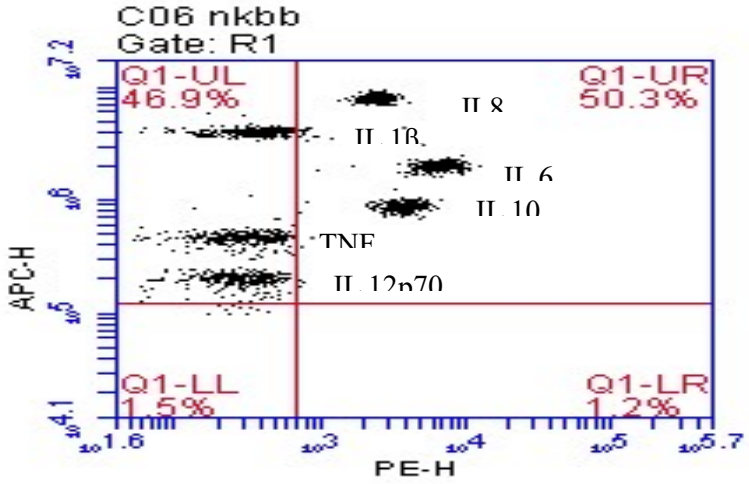


Fig 8: Detection of various cytokines using the CAB human inflammatory cytokine kit.

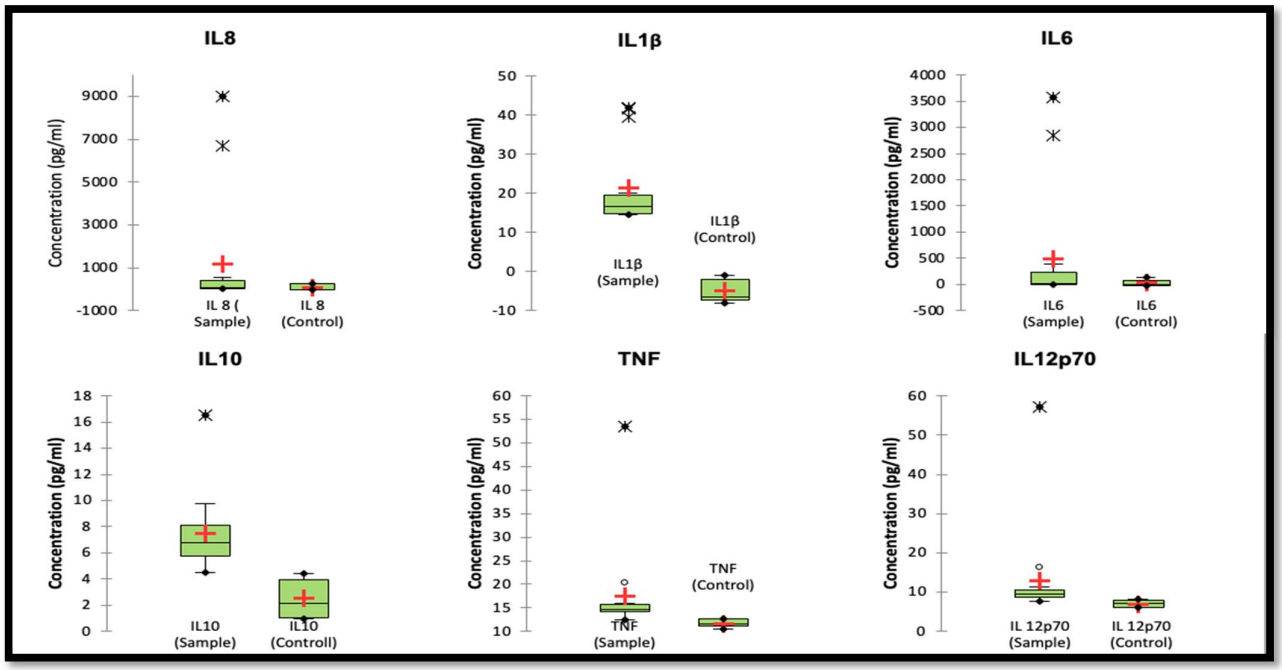


Fig 9: Quantification of cytokine proteins in tumor tissue

Levels of IL 12p70, IL-8 and TNF-alpha were seen to be comparable to healthy participants. The levels of IL-1β and IL-10 were markedly higher in tumor samples (Fig9). Correlation analysis revealed a significant positive correlation between IL-6 and IL-8 (Pearson's r =0.996, p<0.001) and between TNF and IL-1β (Pearson's r =0.970, p<0.001) indicating the synergy in the trend of expression of the pro-inflammatory cytokines as presented in Figure 14 and Figure 15 A & Fig 15B. IL-10 was positively correlated with IL-12 , but the correlation did not reach statistical significance.

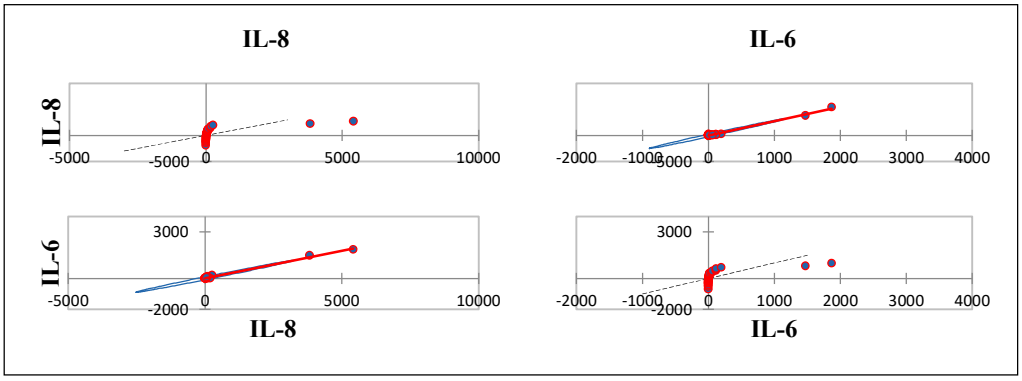


Figure 10: Correlation between the amount of IL-8 and IL-6. The quantity of IL-8 and IL-6 was positively correlated in HNSCC tumor tissues with significant p-value (P<0.001)

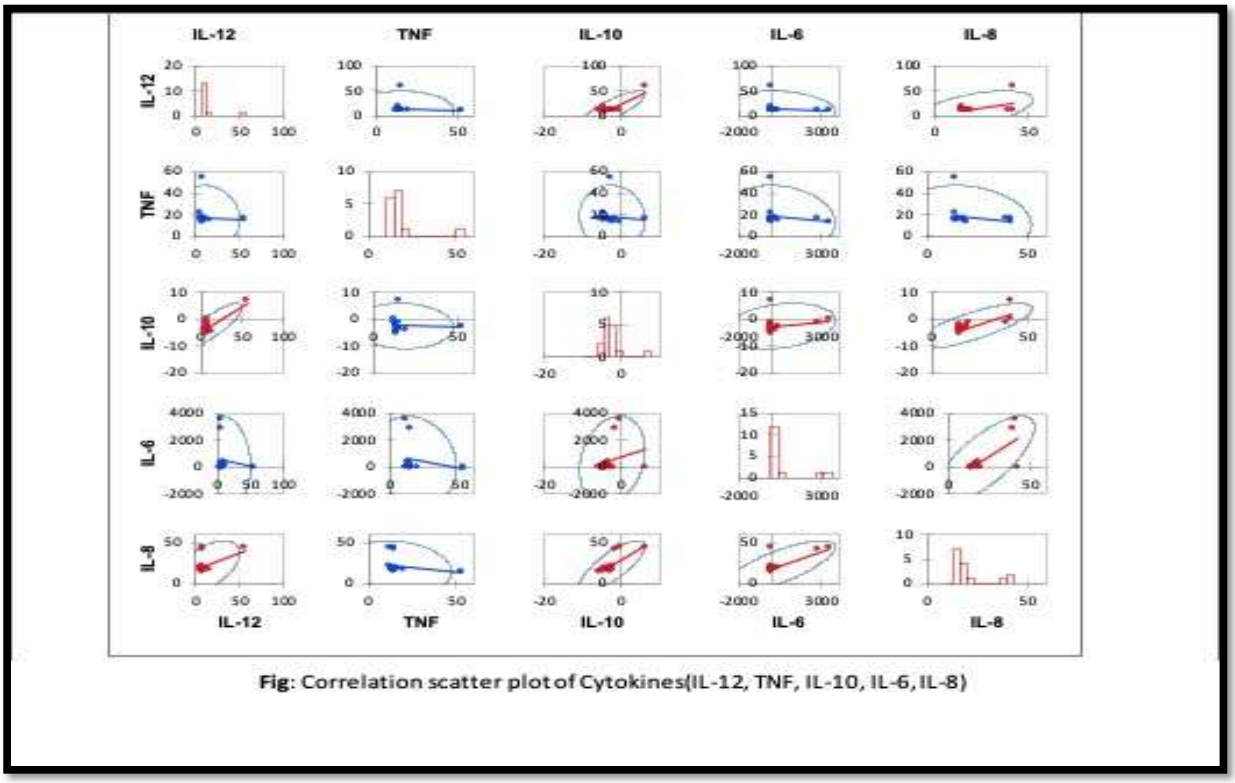


Fig 11: Correlation between cytokine levels in tumor tissue

Expression of chemokine genes, *MCSF* and *GMCSF* in the tumor tissue:

It may be noted from Figure 12 that increased expression of *CCL2*, *CCL3*, *CCL7*, *MCSF*, and *GMCSF* was observed in tumor tissue. Increased expression of *MCSF* and *GMCSF* including chemokines *CCL2* and *CCL3 i*ndicated activation and recruitment of monocytes in the tumor site.

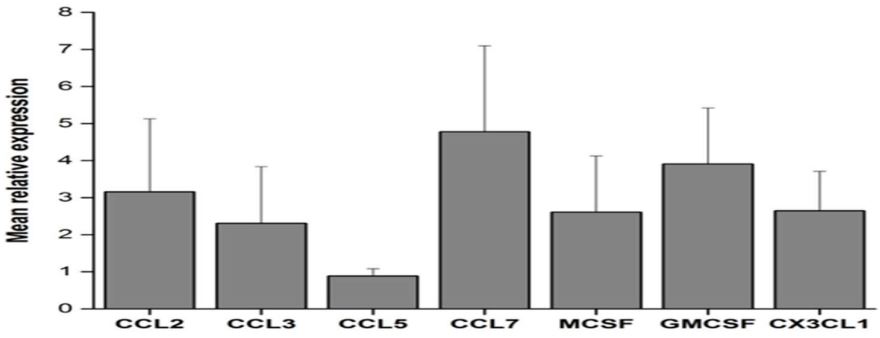
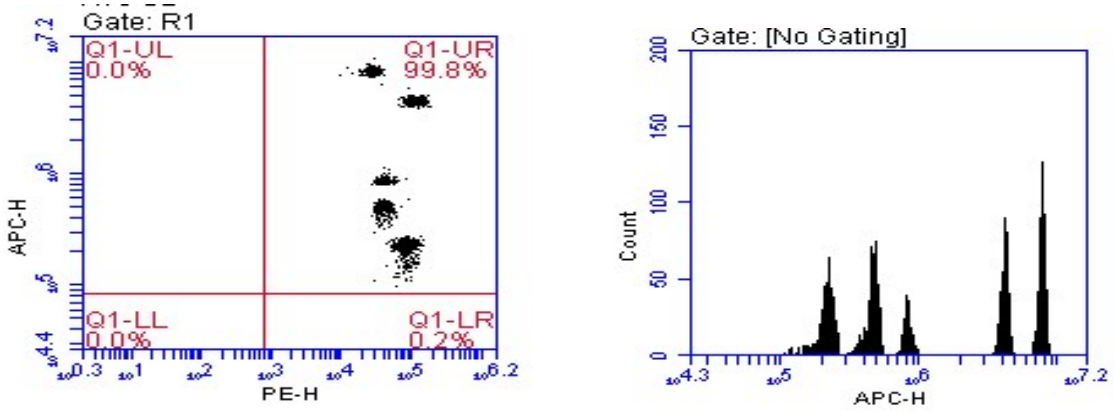


Figure 12: Transcript expression of chemokine genes, *MCSF* and *GMCSF* in tumor tissue. Values indicate fold change and error bars represent standard deviation from the mean. A $p<0.05$ was considered as statistically significant.

Protein expression of monocyte specific chemokines

The concentration of monocyte specific chemokine proteins (CCL2, CCL5, CXCL8, IP-10, and CXCL9) was determined by the CBA kit (BD Biosciences, United States) using flow cytometry as presented in Figure 17 and mean fluorescence intensity was determined. The mean fluorescence intensity of samples was compared and analyzed in relation to standard values as suggested by the manufacturer and the quantity of proteins was determined in pg/mL.



(A) (B)

Fig 13: Determination of Chemokines levels using Chemokine Bead Array Quantification of chemokine proteins in tumor tissue. Flow cytometry image showing results of bead-based capture of all the five chemokines (CCL2, CCL5, CXCL8, IP-10, and CXCL9) in an individual sample (A) and Fluorescence intensity of the proteins in that sample (B).

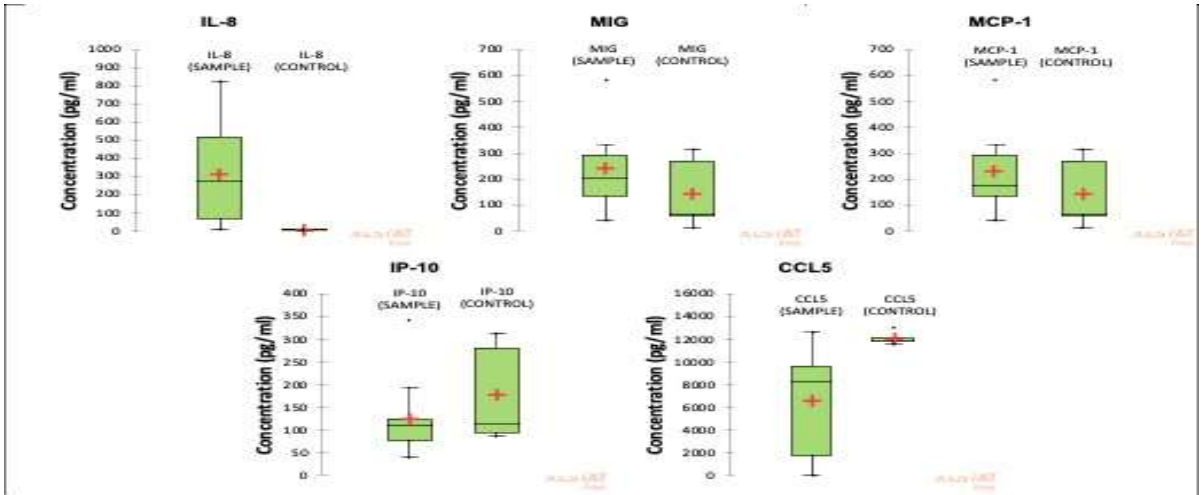


Fig: Box and whisker plots depicting levels (pg/ml) of Chemokines *(IL-8, MIG, MCP-1, IP-10, and CCL5)* in Head and Neck Squamous Cell Carcinoma samples *(n=14)* and healthy control samples *(n=5)*

Fig 14: Comparison of chemokines levels between HNSCC and normal calibrator samples

We observed high levels of all the studied chemokine proteins indicating active expression of chemokines (Figs 13 &14). The highest quantity of chemokine protein was observed for CCL5. Of note, we had low levels of *CCL5* transcript in the tissues.

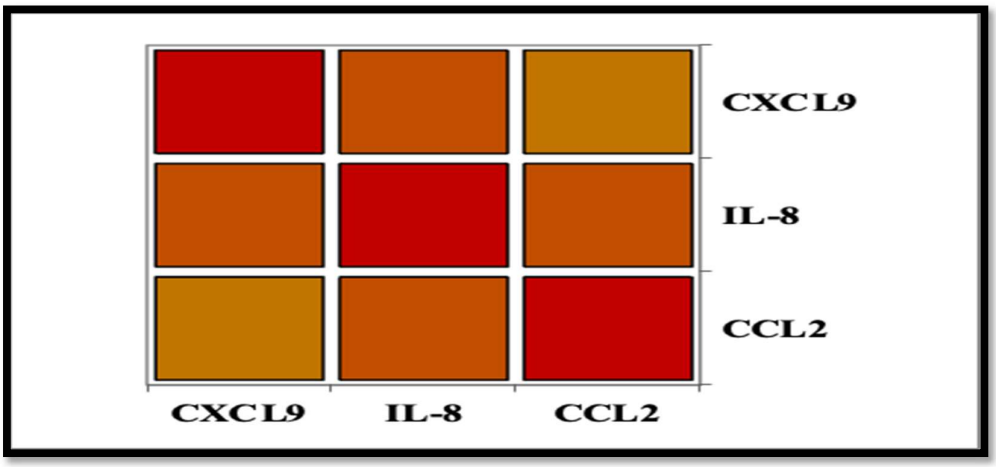


Figure 15: Correlation map of correlation analysis for CCL2, CXCL9, and CXCL8. All the proteins were positively correlated with significant p values.

As may be noted from Figure 15, significant positive correlations were observed between the quantities of CXCL9, CXCL8, and CCL2 (Pearson's r = 0.608, p=0.000 for CXCL8 and CXCL9, Pearson's r = 0.462, p=0.010 for CCL2 and CXCL9 and Pearson's r =0.758, p<0.0001 for CXCL8 and CCL2) suggesting activation of chemokines to recruit the immune cells towards the tumor site. Some studies have reported a tumor suppression role for CXCL9, -10, -11/CXCR3 axis in regulating immune cell migration, differentiation, and activation, (paracrine axis), while others report involvements of this axis in tumor growth and metastasis (autocrine axis). IL-12 and CXCL9 is also reported to promote dendritic cell licensing and $CD8^+$ T cell activation.

To further understand role of chemokines in cancer we examined expression of chemokine receptors.

Transcript expression of *CCR2*, *CCR5*, *IRF4,* and *IRF5:*

Considering higher expression of chemokines mainly CCL2 and CCL5, it was pertinent to check the expression of chemokine receptors mainly CCR2 and *CCR5* in the HNSCC tumor tissues. As presented in Figure 19, we noted increased expression of both the receptors in the tumor. However, the expression of *CCR2*, a receptor for CCL2 was much higher in comparison to *CCR5*.

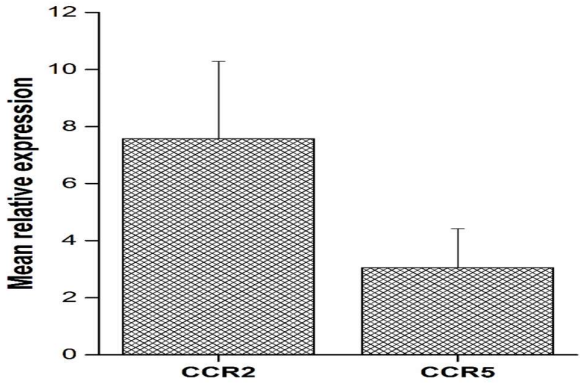


Figure 19: Transcript expression of *CCR2* and *CCR5* in tumor tissues. Values indicate fold change and error bars represent standard deviation from the mean. A $p<0.05$ was considered as statistically significant

A significant positive correlation was also observed between the expression of both the receptors indicating the similar trend of expression (Pearson's r =0.933, p=<0.0001) as presented in Figure 20. Higher expression of CCR2 together with increased CCL2 in tumor tissue indicated the activation of CCL2/CCR2 axis to regulate the monocyte recruitment to the site of the tumor.

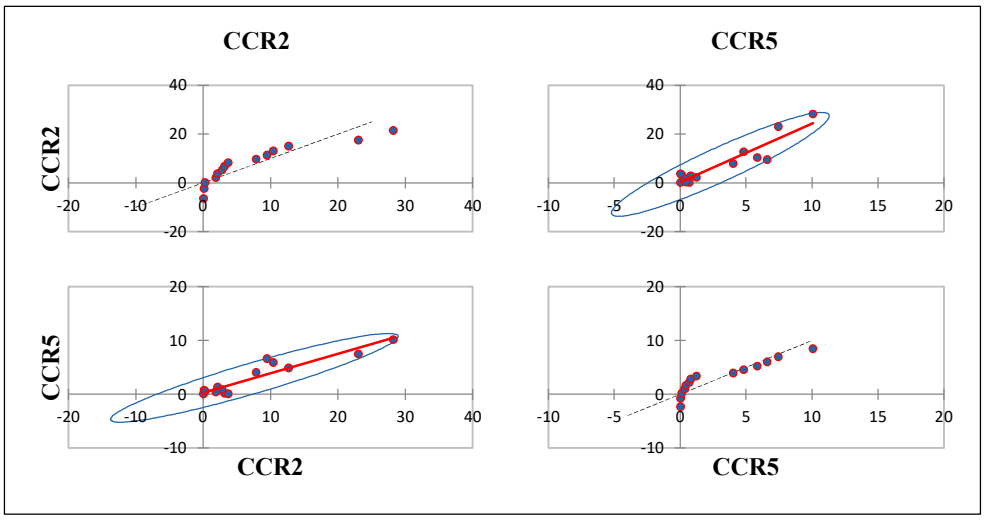
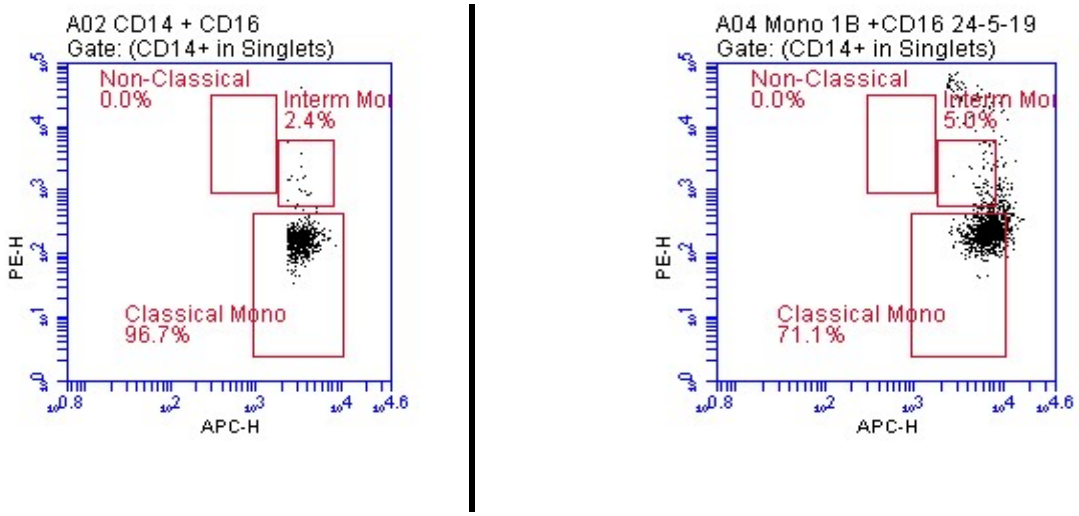


Figure 20: Correlation between the expression of *CCR2* and *CCR5* in HNSCC tissue samples. Both the genes were positively correlated with a significant p-value ($p<0.0001$).

The TLR signaling can lead macrophage polarization change, from M1 (inhibiting tumor) to M2 (promoting tumor), which might explain, at least partially, why TLR signaling promotes tumor growth. The M1/M2 polarization model has been reported in many cancer research studies in recent years. The M1 of tumor-associate macrophages (TAM) express high levels of IL-12 and IL-23, and function as inducers of Th1 responses. During tumor progression, TAM polarizes toward M2 TAM, an alternatively activated macrophage, with a tumor growth-promoting phenotype. However, this M1/M2 polarization has only been well established in vitro, not in vivo. Therefore, the role of TLR signaling in M1/M2 polarization calls for further investigation.

We used Flowcytometry to characterize macrophage sub-population in healthy and patient samples. Analysis of the samples suggests the presence of classical and intermediate monocytes in the study participants.The intermediate monocytes also known as patrolling monocytes were seen to be higher in cancer samples. Patrolling monocytes are reported to have anti-tumorogenic role by recruitment of NK cells.



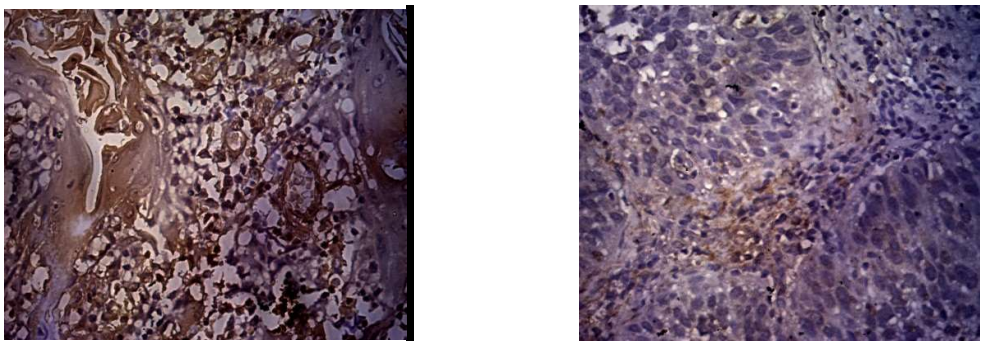
(A) **(B)**

Fig 21: Characterization monocytes subpopulation using flow-cytometer in (A) healthy control (B) HNSCC patient

Towards Objective 3:

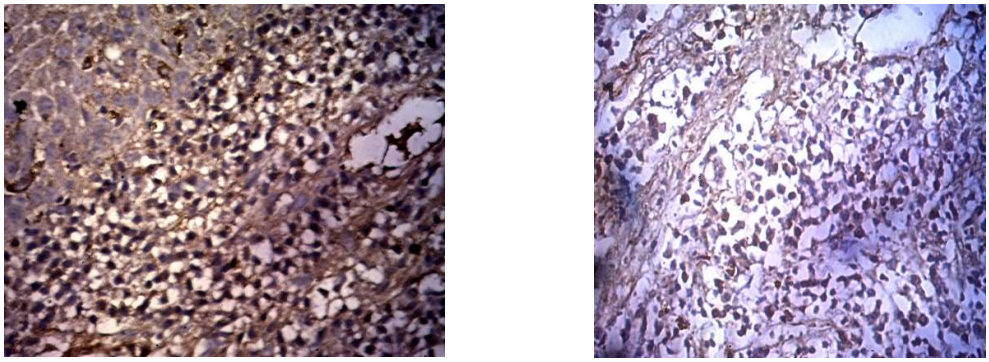
NLP3 and NLP6 Inflammasome activation in relation to tumor growth. Collection of 32 tumor tissue (biopsy and post-operative) was done in RNA later and FFPE blocks and stored in -80°C for

further processing. Immunohistochemistry for tumor growth assay by marker of proliferation (Ki67), VEGF, P16 and PanCK has been completed in those samples. -



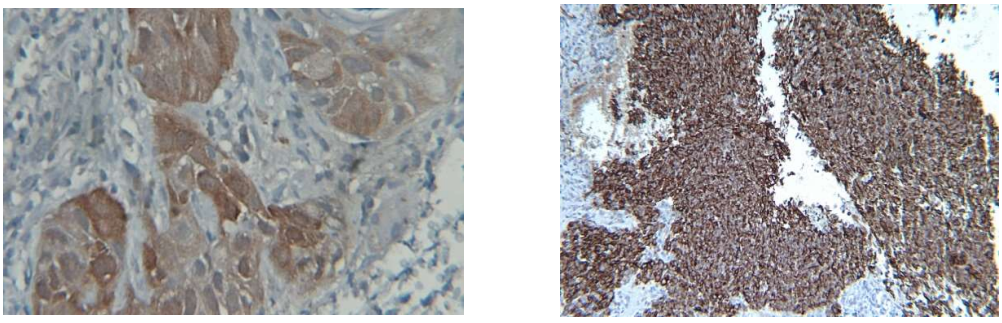
(A) **(B)**

Fig 22: IHC Staining for Ki-67 in HNSCC (40X) (A) Positive staining (B) Negative staining



(A) (B)

Fig 23: IHC Staining for VEGF in HNSCC (40X) (A) Positive staining (B) Negative staining



(A) (B)

Fig 24: Immunohistochemical staining of (A) p16 showing the positivity of p16 in HNSCC tumor cells counter stained by haematoxylin (B) Immunohistochemical staining of PanCK showing the positivity of PanCK in HNSCC tumor cells counter stained by hematoxylin

All IHC results were independently confirmed by pathologist. Staining was evaluated using the H score system. We defined Ki-67 and VEGF positivity as an H score greater than 200 for tumor cells. Ki67 expression was higher in stage III-IV patients whereas expression of VEGF was seen in patients from all tumor stages (p=008) suggesting poor prognosis as well as aggressive proliferation and angiogenesis. 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

In addition, cytokine profile in patient blood and tumor tissue was also compared (Fig 25) and a distinct difference in profile with higher IL-1 and TNF-alpha, but lower Il-18 and IL-10 transcript levels in tumor tissue. IL-1beta and IL-18 are reported to be synthesized as proIL-1beta and proIL-18that are then cleaved by caspases associated with inflammasomes for secretory IL-1beta and IL-18. Only a marginal increase in IL-1Beta transcript levels (Fig 25) and protein levels (11.81pg/mL) does not suggest inflammasome activation in tumor tissue. However, levels of IL-1 and TNF-alpha were positively correlated with tumor ($p<0.001$)

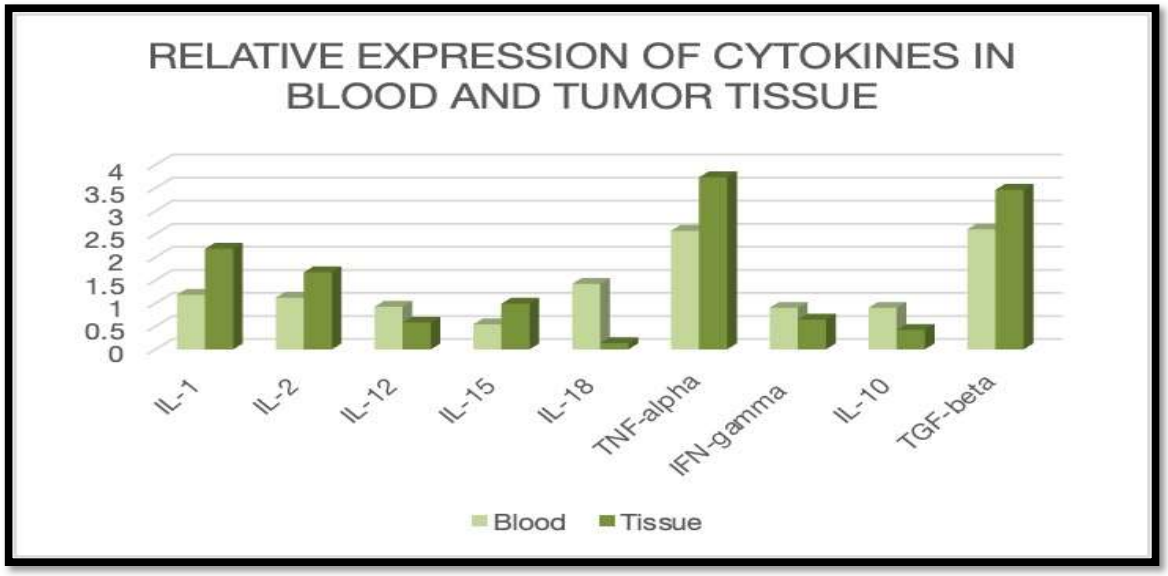


Fig -25- Relative expression of Cytokines in blood and tumor tissue of HNSCC participants

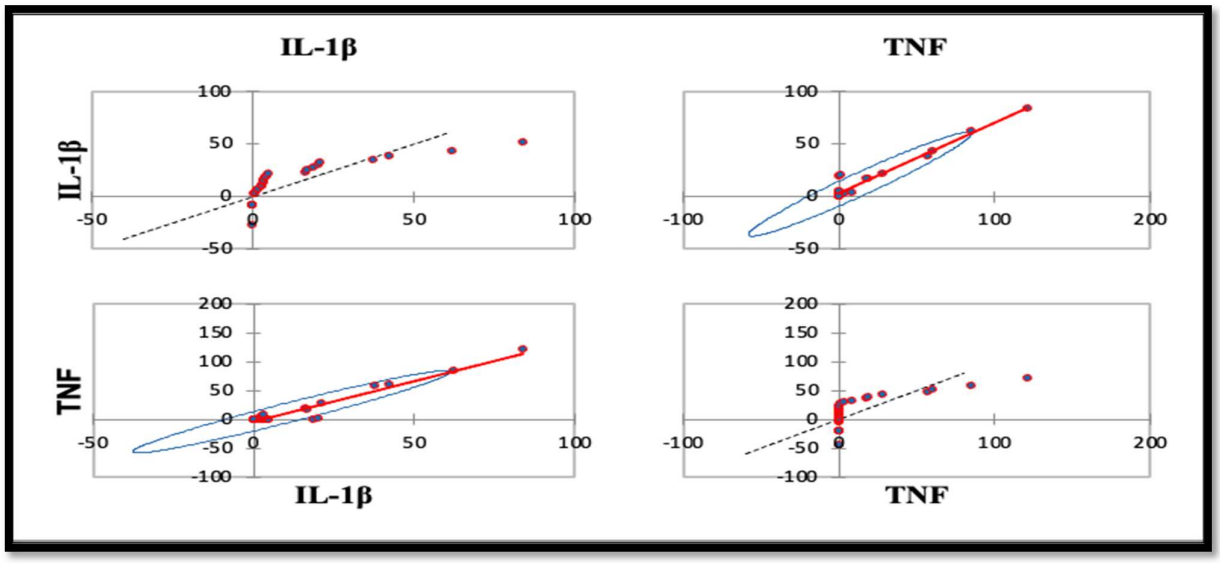


Fig 26- Correlation between the amount of IL-1β and TNF. IL-1β and TNF quantity was positively correlated in HNSCC tumor tissues with significant p-value (P<0.001).

Correlation analysis of tumor markers like tumor grade, nodes, metastasis with molecular markers showed, node status was positively correlated with levels of IFN-gamma. Further modelling of data by logistic regression showed Ki-67, a marker of proliferation was significantly (p< 0.005) related to Keratin 18 levels.

Variables	ki-67	Keratin 18	Cyclin D1	tumor grade	TGFβ	PD1	IL-10	IFNγ	Node status	Metastasis
ki-67	**1**	**0.382**	-0.084	-0.031	0.231	0.250	-0.053	0.086	0.041	0.091
Keratin 18	**0.382**	**1**	-0.132	0.018	-0.019	-0.028	-0.006	-0.044	-0.133	0.129
Cyclin D1	-0.084	-0.132	**1**	0.011	-0.067	-0.039	0.040	-0.019	-0.058	-0.100
tumor grad	-0.031	0.018	0.011	**1**	0.095	0.132	-0.007	0.062	-0.154	-0.038
TGFβ	0.231	-0.019	-0.067	0.095	**1**	**0.777**	**0.384**	0.030	0.039	-0.074
PD1	0.250	-0.028	-0.039	0.132	**0.777**	**1**	**0.464**	**0.391**	0.034	-0.090
IL-10	-0.053	-0.006	0.040	-0.007	**0.384**	**0.464**	**1**	**0.509**	0.033	0.062
IFNγ	0.086	-0.044	-0.019	0.062	0.030	**0.391**	**0.509**	**1**	**0.297**	0.172
Node statu	0.041	-0.133	-0.058	-0.154	0.039	0.034	0.033	**0.297**	**1**	0.174
Metastasis	0.091	0.129	-0.100	-0.038	-0.074	-0.090	0.062	0.172	0.174	**1**

Inflammasome Activation

Activation of inflammasomes was examined by assaying for Pycard protein, a component of inflammasome assembly. Pycard protein expression were checked by western blotting method in 22 cancer tissue and 5 non cancer tissue samples. However, positive bands were observed in only two samples. The experiment could be done only post Covid-19 lockdown, when samples had probably degraded due to power interruptions and some of the reagents had expired and hence we are not confident of the results.

Key findings from the study:

- The homozygous wild type genotype in TL4-4-399 was found to be positively associated with the disease **(p = < 0.0001)** and the presence of the ancestral allele C increased the odds of developing cancer **(OR: 7.830 CI=2.96-20.66).** In contrast, the distribution of the alleles of TLR-4-299 were comparable between cancer and control participants
- In TLR9 (C-1237T), the wild type allele C was found to be higher in the control group and was negatively associated with disease (Spearman R=-0.273). Thus the higher incidence of C allele was found to reduce the risk of developing the disease and conferred protection (p = **< 0.0001**). However, in TLR-9 (C-1486-T), the wild allele C was found to be higher in the cancer group, and was positively associated (Spearman R=0.458, p **= < 0.0001**). While the mutant allele T was higher in the control group. Therefore, the higher incidence of the ancestral allele (C) increased the risk of developing HNSCC **(OR: 6.851)**
- The distribution of the alleles of NLRP6 S730N were comparable between cancer and control participants. Also a complete absence of the homozygous mutant allele was noted. AG genotype was weakly associated with disease but this association did not reach statistical significance ($p<0.06$).
- In TLR3 L412F (G1335A) data suggest the prevalence of GA genotype in the study population. However, no significant association was found between the SNP and disease, thus the polymorphism demands further investigation with larger number of samples.
- Transcript expression of cytokines IL-10 and IL-13 were found to be higher in early stages of HNSCC and downregulated in later stages, suggesting immunosuppression in early disease.
- Increased levels of IL-1 and of IL-6 in later stages of disease suggest proinflammatory activation. Inflammation is well documented to be both protumorogenic as well as anti-tumorogenic.
- Transcript levels of TGF-beta were high in cancer samples and increased with stage of disease.
- Cytokines IL-8 and IL-6 and IL-1 and TNF-alpha were positively correlated with disease.
- High protein levels of IL-1β and IL-10 , but not of IL-8 and IL-6 was noted in tumor tissue. Oral epithelial expression of IL-1β and IL-8 was seen to be negatively correlated in OSCC by some investigators.

- Increased expression of *CCL2*, *CCL3*, *CCL7*, *MCSF*, and *GMCSF* was observed in tumor tissue. Increased expression of *MCSF* and *GMCSF* including chemokines *CCL2* and *CCL3* suggested activation and recruitment of monocytes in the tumor site.
- Chemokines CCL2, CCL5, CXCL8, IP-10, and CXCL9 were seen at concentrations ranging from 150-950 pg/mL. The receptors for chemokines CCR2 and CCR5 were also upregulated. Higher expression of CCR2 together with increased CCL2 in tumor tissue indicated the activation of CCL2/CCR2 axis to regulate the monocyte recruitment to the site of the tumor
- Intermediate Macrophage population was seen to be higher in tumor tissue. Non classical macrophage population was not observed.
- From IHC study, Ki67 expression was higher in stage III-IV patients whereas expression of VEGF was seen in patients from all tumor stages (p=008) suggesting poor prognosis as well as aggressive proliferation and angiogenesis. Besides, lower expression of PanCK was seen in patients with poor histological differentiation, whereas PanCK expression was higher in patients with advanced clinical stage.
- Correlation analysis of tumor markers like tumor grade, nodes, metastasis with molecular markers showed, node status was positively correlated with levels of IFN-gamma. Further modelling of data by logistic regression showed Ki-67, a marker of proliferation was significantly ($p< 0.005$) related to Keratin 18 levels.

Concluding Remarks:

Cancer therapeutics, by immunological intervention of the tumor microenvironment, is now considered to be stand alone or complementary to current therapeutic approaches. In the present study, the role of TLRs and NLRs in both promoting and inhibiting tumor growth and metastasis has been found which further enlightens the possible roles played by TLRs and NLRs in cancer. The present study is also a small step forward in understanding the role of genetic variations of immune system and key immune cells in providing a proliferation supporting environment in ethnically diverse populations of northeast region of India. The investigation provided a better understanding of the tumor microenvironment and the role of inflammation in cancer in context of host genetic background. However, the diverse population of NE region remain poorly studied and demands further comprehensive investigation. Besides, the specific mechanism of action is still unclear; at the same time, cancer is a multifactorial disease. Therefore, further studies may help us better understand

TLRs/NLRs and tumor immunity, and the clarification of their roles in tumorigenesis and tumor metastasis may provide new strategies and prospects for more effective cancer treatment.

Limitations of the study: It may be noted that the results of the final experiments for inflammasomes activation could only be done post lock down as research laboratories were closed. Many of the stored samples probably degraded due to long storage and power interruptions during lockdown. The purchased reagents for the experimentation too expired during the time period. Besides, the SRF on the project also left and had joined Covid Diagnostic Laboratory at TMC&H, making it difficult to pursue the work.

a. A summary sheet of not more than two pages under following heads (Title, Introduction, Rationale, Objectives, Methodology, Results, Translational Potential): **Sheet Attached**

b. **Research work which remains to be done under the project. -Nil**

c. **Applied value of the project**: Rs 49,81,130 (Rupees Forty-Nine Lakhs Eighty-One Thousand One Hundred Thirty)

d. Any publications: Manuscript under preparation. The project grant partially supported 2 doctoral work. One student has successfully defended her work and has been awarded Doctoral degree. The project has been acknowledged in her thesis.

e. Any patents applied for: No

f. If additional budget or staff is required for the remaining part of the research work, please give justifications and details: No

Date: 27 June 2022

Signature

Designation Professor

Prof. Shashi Bamal
Department of MBBT
Tezpur University

Annexure 1 (a)

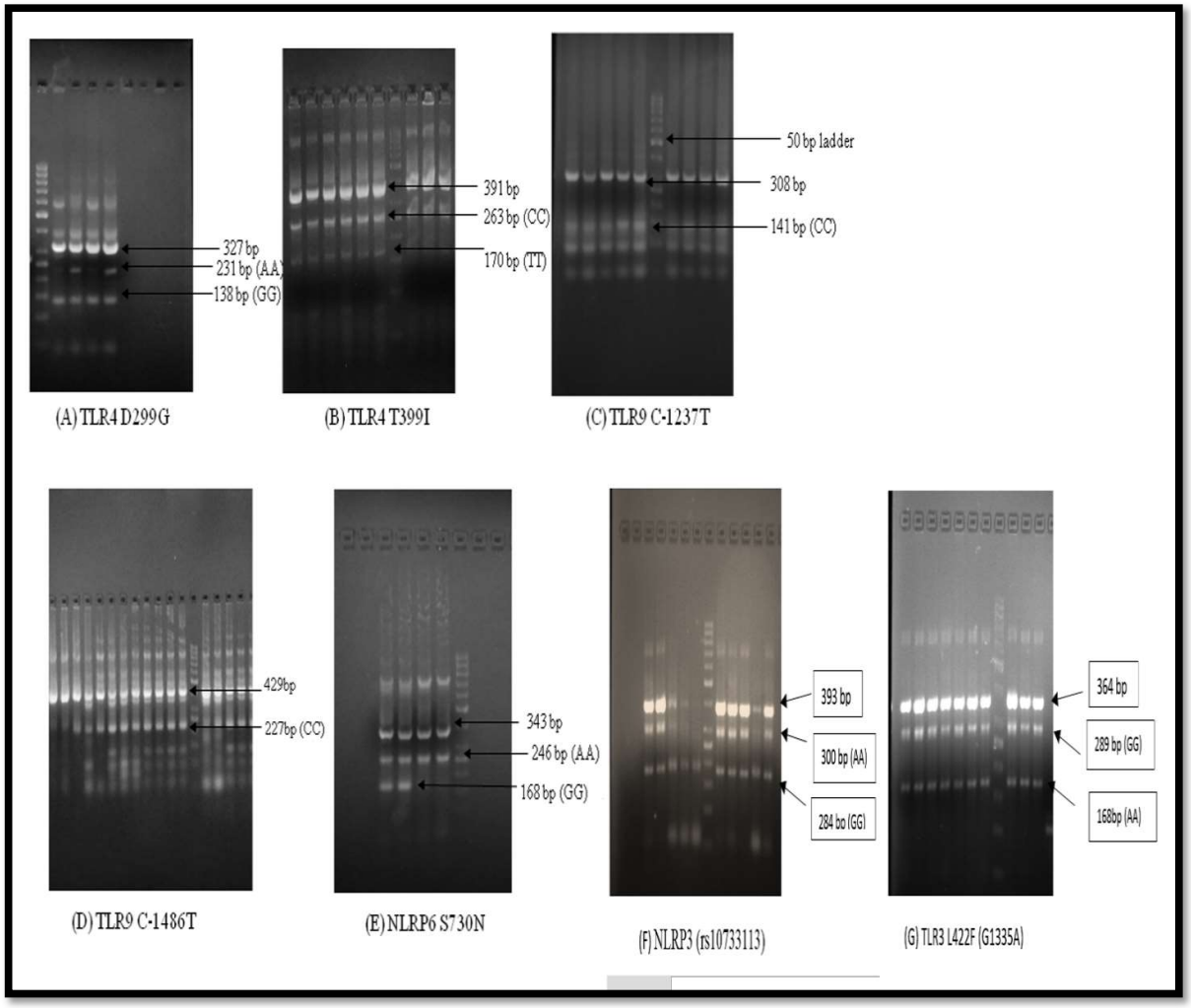


Fig: Representative Gel Electrophoresis pictures of the SNPs typed showing successful amplification