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IL-16 and XRCC1 gene polymorphism along with hypoalbuminemia correlates with the risk and prognosis of NPC among the ethnic population of northeast region of India

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Abstract: *Incidence of Nasopharyngeal cancer (NPC) is high in north-eastern region of India, although it is rare in rest of country. Combined effect of environmental factors, genetic susceptibility and Epstein-Barr virus (EBV) plays an important role in NPC pathogenesis. This study investigates the association of NPC with genetic polymorphisms; XRCC1 and Interleukin 16, viral genetic variant; EBV subtype and latent membrane protein 1 (LMP1), and serum profiles; albumin and immunoglobulin. XRCC1 codon 399 and IL-16 rs11556218 gene polymorphism were determined by PCR-RFLP. PCR assay was used for typing EBV and detecting 30 bp deletion of LMP1 gene. Serum profile was measured by capillary electrophoresis. Statistical analysis was performed by GraphPad Prism. Analysis revealed that EBV type 1 was present in all NPC samples. The frequency of del-LMP1 was higher in case than controls (p = 0.002). Among NPC patients, del-LMP1 type was higher in advanced stage than in early stage (p<0.0001). Serum albumin levels were also significantly lower in NPC patients compared to controls (p<0.0001). XRCC1 Arg/Gln genotype was significantly associated with NPC as compared to Arg/Arg genotype (p=0.001). Similarly, TG and GG genotype of IL16 polymorphism was also associated with higher risk of NPC as compared to TT genotype (p=0.007). XRCC1codon 399 and IL-16 rs11556218 polymorphisms along with serum profiles can be considered as risk factors associated with NPC and might be used as molecular marker for evaluating the susceptibility of the disease.*

Keywords: *EBV, Albumin, Genetic Polymorphism, Biomarker, Nasopharyngeal carcinoma*

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I. Introduction

Nasopharyngeal carcinoma (NPC) is a highly invasive and metastatic malignant tumor that occurs in the epithelial cells lining the nasopharynx. It has high incidence in Southern China, Southeast Asia and North Africa where the average incidence reaches to 30 per 100,000 person (1, 2) whereas it is rare in most other regions of the world. Northeast region of India is a major NPC high risk-area although the rest of the country has very low incidence. The incidence rate in the states of northeast India namely Nagaland, Mizoram and Manipur are 19.4, 6 and 5 per 100,000 person/year respectively (3).

The etiology of NPC is influenced by multiple factors, including genetic, environmental, and dietary factors (4, 5). Although Epstein-Barr virus (EBV) is ubiquitous in healthy population throughout the world, NPC presents a remarkable geographic distribution. The implication of environmental cofactors has been evoked to explain this astonishing geographic distribution (6). One of the possible explanations for the geographic specific distribution of NPC may be the differences in EBV subtype in areas with high incidence of NPC. EBV is divided into two subtypes, type 1 and type 2 that are distinguished by genomic difference in a subset of latent genes that encode for the EBV nuclear antigens 2 (EBNA2), EBNA 3A, 3B and 3C (7). EBV type 1, which is predominantly detected in Asian NPC, has a greater potential to transform B lymphocyte than EBV type 2 (8). EBV genome harbours an oncogene, namely, the latent membrane protein 1 (LMP1) gene and 30-bp deletion variants has been found. The importance of LMP1 in tumorigenesis of NPC *in vivo* is supported by the finding that LMP1 was expressed in 78% NPC samples (9). Also clinical studies conducted in Taiwanese population have clearly demonstrated that LMP1 is associated with NPC development and is a tumor marker for screening NPC (10). Further studies on other population have also demonstrated higher frequency of 30-bp deletion (del-LMP1) type in NPC patients when compared with the healthy population (11, 12). However, the association between LMP1 and NPC has not been studied in Indian population yet.

Interleukin-16 (IL-16) plays a fundamental role in inflammatory diseases, as well as in the development and progression of tumors. Recent studies have shown that IL-16 rs11556218 T/G polymorphism

is significantly associated with the susceptibility to NPC among the Chinese population (13). Polymorphism of XRCC1 gene, which is involved in repairing of DNA damage, has been shown to be strongly associated with NPC development (14). In addition to the host genetic factors and viral genetic variant, malnutrition and cachexia in cancer patients are significant problems due to a variety of mechanisms involving the tumor, the host response to the tumor, and anticancer therapies (15). Malnutrition and cancer-related inflammation are crucial host-related factors that may negatively affect the outcome of cancer treatment, as they may promote tumor growth and metastasis by damaging the immune system (16, 17). Albumin (ALB) is an important serum protein that reflects the patients' nutritional status. Prior studies have demonstrated low serum ALB as an independent predictor of poor survival in several types of cancer including NPC (18, 19). Hypergammaglobulinemia is a condition that is characterized by the increased levels of a certain immunoglobulin in the blood serum (20). It is commonly observed in autoimmune diseases and chronic infections. It was proposed that hypergammaglobulinemia observed in conditions of high and persistent antigen concentrations can be caused by activated naive B cells (21). However, association of hypergammaglobulinemia and cancer particularly NPC is rarely reported. In this study, we aimed to examine the association of NPC with other host genetic polymorphisms; XRCC1 Arg399Gln, IL-16 rs11556218, viral genetic variant; EBV subtype and 30 bp deletion (del-LMP1) type, and serum profiles; albumin (ALB) and immunoglobulin status and their contribution in the development NPC in the north-eastern region of India.

II. Subjects And Methods

Study participants:

For this study, 105 NPC patients and 115 healthy volunteers whose age, sex and ethnic matched with the patients were recruited from the Regional Institute of Medical Sciences (RIMS), Manipur. The study was approved by the Institutional Human Ethics Committee, RIMS and the Institutional Human Ethics Committee, Manipur University, Manipur, India. Patient's cancer stage was defined according to the 1992 American Joint Committee on Cancer (AJCC TNM staging system). Informed consent was obtained from each participant and all the participants were requested to respond to a personal interview that elicited detailed information on potential risk factors for NPC. The characteristics of the study subjects have been reported in our previous study (5) and it is summarized in Table 1.

DNA extraction and serum collection:

Blood sample from all the participants was collected in an EDTA coated tube and the samples were divided into two parts. The first part was used to isolate blood DNA using DNA Blood Mini Isolation Kit (Qiagen, USA) according to the manufacturer's manual. The second part of the samples was simultaneously processed for subsequent separation of serum by centrifuging at 3,000 rpm for 5 minutes and separated serum was aspirated into tubes. The isolated DNA and separated serum samples were aliquoted in single use volumes to prevent freeze-thaw cycles and stored at -80°C till used.

Serology analysis:

Serum samples were analysed by capillary electrophoresis (CE) to identify abnormalities in the various separated protein bands viz: albumin, alpha 1 & 2 globulins, beta 1 & 2 globulins and gamma globulins. CE was used for the screening of protein abnormalities in serum and Sebia Capillarys (Sebia, Evry Cedex, France) is considered as the reference instrument for separation and quantification of protein fractions (22). Serum protein electrophoresis (SPEP) was carried out using a commercially available kit, the Hydra Gel Protein (E) K20 (Sebia Inc, Norcross, Ga, USA) and the separation of serum proteins was performed in alkaline buffered (pH 8.5) agarose gel. Consequently, the serum albumin levels between case and controls were also measured.

Definition of EBV types:

EBV types were determined by PCR with specific primers which yield an amplification product of 153 bp for EBV type 1 and 246 bp for EBV type 2, as described previously (7). The amplified products were examined by 2% agarose gel electrophoresis.

Genotyping of LMP1:

PCR screening for the EBV LMP 1 subtype based on exon 3, defined as wild-type (wt-LMP1) or del-LMP1, was done by using the oligonucleotide primers, LMP1-Del 2F (5'-GAGAGTCAGTCAGGCAAGC-3') and LMP1-Del 2R (5'-GACGGAAGAGGTTGAAAACA-3') as described previously (12). PCR products for the wt-LMP1 (wild type) and del-LMP1 (deleted) were detected as 233 bp and 203 bp respectively.

RFLP genotyping of XRCC1 gene:

Genotypes of XRCC1 polymorphism were determined by PCR-RFLP, using forward primers 5'-TTGTGCTTTCTCTGTGTCCA-3 and reverse 5'-TCCTCCAGCCTTTTCTGATA-'3 primers described previously (23). The reaction mixture was preheated to 95˚C for 3 min, followed by 30 cycles of denaturation at 95˚C, annealing at 57˚C, and extension at 72˚C for 1 min, and then final extension at 72˚C for 3 min in a thermal cycler (ABI Applied Biosystems, USA). 10 µL of PCR product (615 bp) was digested with 5U of *MspI* restriction enzyme (New England Biolabs, USA) at 37˚C overnight and then electrophoresed on 2% agarose gel. The *MspI* enzyme has a single recognition site on the 615-bp fragment of wild type allele. Therefore, after digestion with *Mspl,* the wild-type allele Arg/Arg resulted in two fragments, 374 and 241 bp; the heterozygous (Arg/Gln) showed fragments of 615, 374, and 241 bp; and the variant allele (Gln/Gln) showed an undigested 615-bp fragment.

Polymorphism analysis of the Interlukin-16 (IL-16) gene:

IL-16 rs11556218 polymorphism was identified by PCR-RFLP analysis with the forward primer (5′-GCTCAGGTTCACAGAGTGTTTCCATA-3′) and reverse primer (5′-TGTGACAATCACAGCTTGCCTG-3′) as described previously (24). The PCR products were digested with *NheI* restriction enzyme (New England Biolabs, USA) at 37˚C overnight to ensure complete digestion and analysed on 2% agarose gel. For rs11556218 G/T polymorphism, TT homozygote was separated as two fragments of 147 bp and 24 bp long.

Statistical analysis:

Demographic and clinical data between groups were compared using appropriate statistical tests. Data are expressed as mean ± S.D. Albumin concentration levels between case and controls and difference in percentage of normal and hypergamma population were analysed by **Student t-test** and **Fisher's exact test** respectively. Also prevalence of EBNA3C and LMP1 subtypes were analysed by **Fisher's exact test**. Genotype and allele frequencies of IL-16 and XRCC1 were compared using the **χ2 test** and **Fisher's exact test** when appropriate. Odds ratio (OR) and 95% confidence intervals (CI) were calculated to assess the relative risk conferred by a particular allele and genotype. Statistical significance was set at $p<0.05$. Analyses were performed with the use of GraphPad Prism (version 6), USA.

III. Results

Low serum albumin is associated with NPC:

The mean serum albumin level was significantly lower in NPC patients (3.47±0.7 g/dl) than controls (4.25±0.6g/dl; $p< 0.0001$) which suggested that low serum albumin levels are associated with an increased risk of NPC (Fig.1). Results also indicate that majority (78.26%) of control group had serum albumin levels within the normal range (3.5-5.0g/dl) as compared to 38.09% in NPC patients. 57.14% of NPC cases had serum albumin levels below the normal lower limit (<3.5g/dl), compared with only 18.26 % of controls suggesting that low albumin level is associated with NPC (Fig. 2).

Serologic profiles and Risk of NPC with hypergammaglobulinemia:

Serum proteins were separated by capillary electrophoresis to identify abnormalities in the various separated bands via: albumin, alpha 1 & 2 globulins, beta 1 & 2 globulins and gamma globulins. Fig. 3(A&B) represents normal and hyper gamma profiles of control and patient samples detected using CE. The percentages of normal and hyper-gamma population in control group were 67.3% and 33.1% respectively whereas in NPC patients the percentages were 12.9% and 87.1% respectively. We observe a significant (54%, $p<0.0001$) increased of hyper-gamma population in NPC compared to controls. These results strongly suggest that hypergammaglobulinemia is associated with NPC (Fig. 4).

EBV type 1 is the dominant subtype:

The frequency of EBV type 1 or type 2 infections was determined in 105 NPC samples based on the sequence variation in EBNA3C gene. Type 1 EBV was present in all 105 samples (100%) whereas type 2 EBV is not found in any of the samples (0%) as shown in Table 2. Among NPC patients, there was no significant difference in the frequency of the EBV type 1 and type 2 for sex, histological type and clinical stage.

Del-LMP1 is prevalent LMP1 subtype:

The del-LMP1 was detected in 59% (62/105) cases and in 37.3% (43/115) controls and the difference is statistically significant ($p = 0.002$) as shown in Table 3. Tumour classification were available for only 98 cases out of which, 35% (n=34) of the cases was in the early stage (stages I and II) while 65% (n=64) was in the advanced stage (stages III and IV) of disease. The frequency of the del-LMP1 in advanced stages of NPC (44/64, 68.75%) was significantly higher than in early stage (11/34, 32.3%) ($p = 0.001$). However, there was no significant difference in frequency of del-LMP1 for sex and histological type among NPC patients.

XRCC1 codon 399 polymorphism (Arg/Gln genotype) is risk factor for NPC:

Genotype and allele distribution of XRCC1 Arg399Gln codon were analysed and the frequency of genotypes and alleles are shown in Table 4. The frequencies of Arg/Arg, Arg/Gln, and Gln/Gln genotypes were 47.82, 41.73 and 10.43% in controls and 24.76, 64.76 and 10.47% in cases respectively. The results indicate that individuals with Arg/Gln genotype had increased risk of NPC development, compared to those who possess the XRCC1 Arg/ Arg genotype (OR = 2.99; 95% CI 1.59 –5.69). The risk for developing NPC for individuals with Gln/Gln genotype did not appear to differ significantly (OR = 1.94; 95% CI, 0.67–5.5). Moreover, the frequency of variant allele G was 57.14% and allele A was 42.85% in the NPC patients, and 68.69% and 31.30% in the controls. The A allele frequency was significantly higher in cancer group as compared to controls ($p<0.05$).

Polymorphism of IL-16 is risk factor for NPC:

Genotype and allele frequencies of rs11556218 T/G polymorphism are summarized in Table 5. There was significant difference in the distribution of genotype between NPC patients and healthy controls ($p<0.001$). The TG (OR =2.2; 95% CI, 1.19 –4.08 $p = 0.007$) and GG (OR =5.8; 95% CI, 1.44–33.92, $p = 0.004$) genotype variants demonstrated a significantly higher risk of NPC compared with wild type TT genotype. A significantly higher risk for NPC was observed for carriers of G allele (OR=2.32; 95% CI, 1.44 - 3.75; $p<0.001$).

IV. Discussion

EBV is associated with a wide range of human malignancies. However, contribution of EBV strains to variation in the pathogenesis of these malignancies is not clear yet. Our previous study has shown a significant association between EBV load and NPC susceptibility (5). However, the prevalence EBV type among the ethnic population of Manipur is not yet known. This study is the first investigation to explore the association between EBNA3C and LMP1 subtypes of EBV and NPC. Our study reveals that type 1 is the prevalent EBV type whereas type 2 is not detected among the population of Manipur. A study has reported that type 1 is prevalent subtype of EBV in central India whereas type 2 is found in the eastern India (25) .Our results are consistent with previous reports that the EBV type 1 is predominant among the NPC patients in Asian population but the EBV type 2 is rarely detected (26, 27) .

Analysis of LMP1 in the present study showed a significant association between the del-LMP1 subtype and NPC susceptibility ($p = 0.002$) suggesting that del-LMP1 carriers had a higher risk for developing NPC compared with wt-LMP1 carriers. Moreover, the frequency of del-LMP1 in NPC patients was associated with the clinical stage of NPC which are consistent with the other studies among Asian population (11, 12, 28).

Various types of carcinoma were investigated for XRCC1 Arg399Gln polymorphism (29-31) and all of these studies found that the 399 Gln allele was associated with higher risk of cancer. However, there is very less study conducted with XRCC1 polymorphism and NPC in this region. Our results suggest that carriers of Gln/Arg genotype of XRCC1 gene had significantly increased risk (2.99 fold) of NPC compare to Arg/Arg genotype. Association of NPC risk was also observed with the 399Gln (A) allele, compared with the Arg (G) allele (OR=1.65). Therefore our study revealed that (Gln/Arg) genotype or XRCC1 heterozygote status appears to be a strong risk factor for NPC development. Our findings are consistent with a study conducted in China which showed NPC risk in individual with defective XRCC1 gene (14). However, studies conducted in other different populations reported no such association (32-34). A similar study has reported that Gln/Gln genotype is associated with head and neck squamous cell carcinoma (35) whereas our findings suggest that Gln/Arg genotype is highly associated with NPC in the northeast region of India.

IL-16, a multifunctional cytokine, plays an important role in the development and progression of tumours. Previous studies have reported on IL-16 polymorphism and its association with the development of various cancers (13, 24, 36). In the present study, we found significant association with IL16 rs11556218 T/G polymorphism and NPC. Our study is the first in the north-eastern region which suggests that G carriers had a significantly higher risk for NPC (OR= 2.32; 1.44 - 3.75) than healthy controls. Thus, IL16 gene rs11556218 T/G polymorphism may serve as the novel genetic marker of susceptibility to NPC.

Studies conducted over the last decade or so have demonstrated association between low serum albumin (hypoalbuminemia) with a reduced quality of life, high risk of disease progression, poor response to treatment and survival in several types of cancer (37, 38). However, association of low albumin and NPC has not been reported yet. In adults, the normal ALB range is 35–50 g/L; levels < 35 g/L are termed hypoalbuminemia (18). There is slight or no hypoalbuminemia in early stages of cancer but as the disease progresses albumin levels drop significantly and serve as good indicators of prognosis of cancer (39). Serum albumin level is not only a window into the patient's nutritional status but also a useful factor for predicting patient prognosis (40). The potential advantage of serum albumin level as a pre-treatment prognostic factor in cancer patients is that it is inexpensive, reproducible and powerful (41). Our results have shown that low serum albumin levels are associated with an increased risk of NPC. Further there is also increase percentage of

hypergamma profile in NPC than controls (87.1 vs. 33.1) suggesting that hypergammaglobulinemia can be used as potential NPC specific serum biomarker which may be of great underlying significance in clinical detection and management of NPC. Therefore, we found an elevated risk for NPC associated with lower serum albumin levels and hypergammaglobulinemia. Accordingly, serum albumin level could be used in clinical trials to better define the baseline risk in NPC patients.

V. Conclusion

In summary, combination of the genetic polymorphisms of IL16 and XRCC1 along with serum profiles factors namely hypoalbuminemia and hypergammaglobulinemia can be useful for development of potential prognostic and diagnostic biomarkers for NPC among the risk population of northeast region of India. Future studies with larger study numbers of patients and longer follow-up are required to evaluate the prognostic value of these potential biomarkers.

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FIGURES:

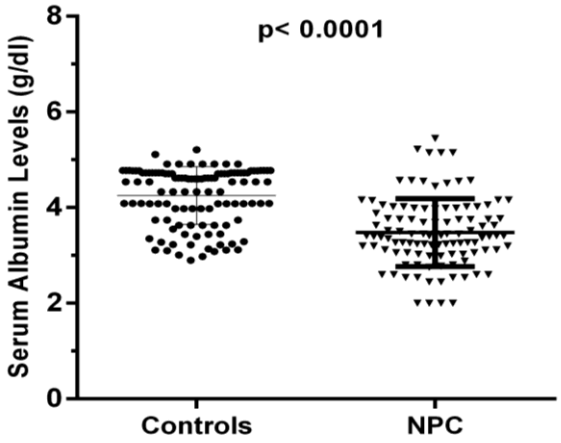

Figure 1: Scatter plot of serum albumin levels (g/dl) between healthy Controls and NPC.

Serum albumin levels were measured between NPC case and healthy controls by capillary electrophoresis. Albumin concentrations are expressed on Y-axis. The categories (Case and controls) are plotted on the X-axis. The P-values were calculated comparing between patients and control groups by Student T test. The difference was statistically significant ($P < 0.0001$).

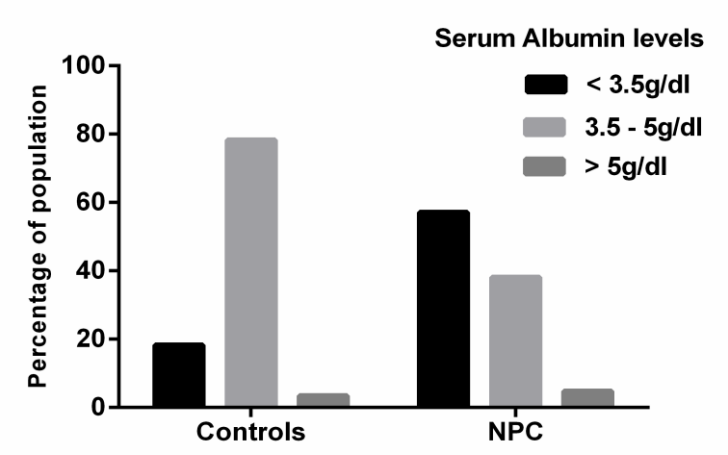

Figure 2: Range of serum albumin levels in controls and case samples.
The concentration were classified as below lower limit (<3.5g/dl), normal range (3.5-5.0g/dl) and higher limit (>5g/dl).

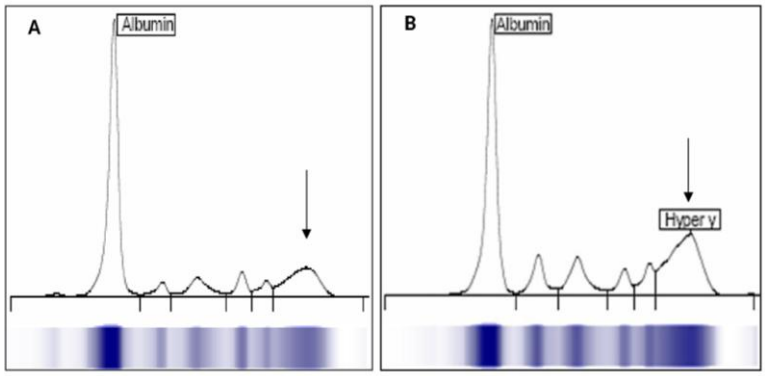

Figure 3 (A & B): Representative serum electrophoretic profile obtained by Capillary Electrophoresis identifying the various forms of gammopathies. A – Normal, B – Hypergamma

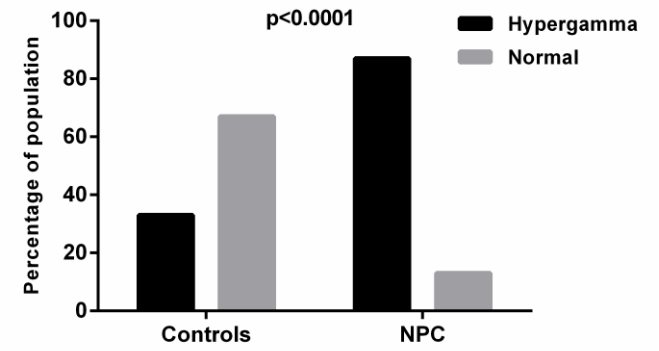

Figure 4: Analysis of normal and hyper gamma population between controls and case samples.
The percentage of normal and hyper gamma population were compared between case and controls. There was significant association in the percentage of normal and hyper gamma population between the study subjects ($p < 0.0001$) as calculated by Fishers Exact test.

TABLES:

Table 1: Characteristics of Study subjects and clinical parameter.

Variables	Case (n=105) n (%)	Controls (n=115) n (%)
Sex		
Male	72 (68.5)	71 (61.7)
Female	33 (31.4)	44 (38.2)
Age (years)		
0-30	15(14.2)	24(20.8)
30-40	21(20)	22(19.1)
40-50	25(23.8)	22(19.1)
50 and above	44(41.9)	47(40.8)
Mean Age	48.9	44
S.D.	15	10.1
Range	18-80	22-73
WHO Pathological Classification		
Type I	6 (5.7)	
Type II	16 (15.2)	
Type III	83 (79.0)	
Tumour stage		
T1 or T2	34 (34.2)	
T3 or T4	64 (65.7)	
Nodal Stage		
N0 or N1	32 (30.4)	
N2	53 (50.4)	
N3	20 (19.0)	
Overall Stage		
Early stage	34 (35)	
Advanced stage	64 (65)	

Table2. Distribution of EBV type in NPC patients.

Category	**EBNA3C**	
	EBV Type 1(%)	EBV Type 2 (%)
NPC	105 (100)	Nil
Sex		
Male	72 (68.5)	
Female	33 (31.4)	Nil
Histological type		
WHO I	6 (5.7)	
WHO II	16 (15.2)	Nil
WHO III	83 (79)	
Clinical Stage		
Stage I and II	34 (35)	
Stage III and IV	64 (65)	Nil

Table3: Distribution of LMP1 deletion subtypes in NPC patients and controls.

Category	**LMP1**	
	wt-LMP1	del-LMP1
Controls	72	43
NPC	43	62*
Sex		
Male	28	44
Female	15	18
Histological type		
WHO I	3	3
WHO II	6	10
WHO III	34	49
Clinical Stage		
Early Stage	23	11
Advanced Stage	20	44**

* P-value = 0.002 (LMP1 subtypes in between cases and controls)
** P = 0.001 (LMP1 subtypes in between Early and Advanced stage)
Frequency analysis by Chi-square test

Table 4: Distribution of XRCC1 Arg→Gln (exon10) codon 399 genotypes and allele between NPC patients and controls.

	Case n (%)	Controls n (%)	OR (95% CI)	P-value
Genotypes				
(Arg/Arg)	26 (24.76)	55 (47.82)	1	
(Arg/Gln)	68 (64.76)	48 (41.73)	2.99 (1.59–5.69)	0.001
(Gln/Gln)	11 (10.47)	12 (10.43)	1.94 (0.67–5.5)	0.164
Allele				
Arg (G)	120(57.14)	158(68.69)	1	
Gln (A)	90(42.85)	72(31.30)	1.65 (1.09–2.48)	0.012

OR = Odds Ratio, CI = Confidence Interval

Table 5: Distribution of Genotype and allele of IL-16(rs11556218) polymorphism between NPC patients and controls.

Polymorphism	Case n (%)	Controls n (%)	OR (95% CI)	P-value
Genotypes				
TT	50 (47.61)	80 (69.56)	1	
TG	44 (41.90)	32 (27.82)	2.2 (1.19–4.08)	0.007
GG	11 (10.47)	3 (2.60)	5.87 (1.44–33.92)	0.004
Allele				
T	144(68.57)	192 (83.47)	1	
G	66 (31.43)	38 (16.53)	2.32 (1.44-3.75)	< 0.001

OR = Odds Ratio, CI = Confidence Interval

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Deepak Singh Lourembam. "IL-16 and XRCC1 gene polymorphism along with hypoalbuminemia correlates with the risk and prognosis of NPC among the ethnic population of northeast region of India." IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB) , vol. 3, no. 5, 2017, pp. 45–53.