

## EVALUATION OF SERUM TOLL-LIKE RECEPTOR 4 AND NUCLEAR FACTOR-KBP65 PROTEINS IN ORAL SQUAMOUS CELL CARCINOMA

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### ABSTRACT

**Objective:** The present study is aimed to estimate the serum toll-like receptor 4 (sTLR 4) and nuclear factor- $\kappa$ B (NF- $\kappa$ B) p65 proteins in patients of oral squamous cell carcinoma (OSCC).

**Methods:** The study was performed in prospective cases of 22 OSCC patients, 10 oral epithelial dysplasia patients, 8 control with chewing habits, and 4 control patients. The estimation of sTLR 4 and NF- $\kappa$ Bp65 proteins was done by enzyme-linked immunosorbent assay method. The Pearson correlation test was performed to find out the relationship between these two proteins.

**Results:** There was an increase in the sTLR 4 protein level in study groups OSCC, oral premalignant disorders, control with chewing habits, and control habits such as 1.31 ng/ml $\pm$ 1.06 ng/ml, 1.99 ng/ml $\pm$ 0.98 ng/ml, and 2.11 ng/ml $\pm$ 0.61 ng/ml, respectively, when comparable ( $p=0.008$ ) to control patients with 0.60 ng/ml $\pm$ 0.24 ng/ml. However, in the case of serum level NF- $\kappa$ Bp65 protein all the study groups including the control showed same values. The Pearson correlation test showed significant relationship ( $r_{\text{pearson}}=0.91$ , [ $p<0.0005$ ]) of these two proteins only in the OSCC patients.

**Conclusion:** It can be concluded that serum levels of TLR 4 are increased in OSCC patients, but there was no variation seen for the NF- $\kappa$ Bp65 protein. There is a strong interrelationship exist between the serum levels of TLR 4 and NF- $\kappa$ Bp65 proteins in the OSCC patients only.

**Keywords:** Oral squamous cell carcinoma, Oral epithelial dysplasia, Enzyme-linked immunosorbent assay, Toll-like receptor 4, Nuclear Factor- $\kappa$ Bp65.

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### INTRODUCTION

Oral cancer, malignant neoplasm ranks 3<sup>rd</sup> among the cancer types with a prevalence of 45% in India and about 90–95% of squamous cell carcinoma is the 12<sup>th</sup> most prevalent neoplasm globally [1-4]. Oral squamous cell carcinoma (OSCC) constitutes more than 90% of head and neck malignancies and ranks 6<sup>th</sup> among all other cancers which are the cause for high morbidity and mortality worldwide with an high incidence rate that varies widely by geographic location [5,6]. However, nearly 3–6% of the OSCC patients were predominantly observed in the western countries, while the rate was higher with 30% among the east including Indonesia [7]. Rapid cell division leads to clinical hypoxia causing the releases of pro-inflammatory mediators that, in turn, enlist inflammatory cells. In particular, nuclear factor- $\kappa$ B (NF- $\kappa$ B) which acts as an important intermediate between inflammation and cancer [8,9] also exerts dual action of both protumorigenic and cell survival mechanism [10,11]. Hence, it is considered as valuable therapeutic target [12]. Its activation is mediated by three molecules such as toll-like receptors (TLRs), interleukin-1 receptor, and the tumor NF receptor. TLRs, type I transmembrane protein identifies conserved microbial structures/patterns and was previously thought to play a critical role in host defense against wide range of organisms from bacteria to protozoa [13]. Recently, it is known that many cancer cells express TLRs and till now, 13 mammalian TLRs have been demonstrated, but in humans, only 11 are expressed. Recent data had demonstrated that OSCC could modulate TLRs protein expression and function in variety of immune cells [14]. Hence, TLR role in rapid cell proliferation and metastasis of oral cancer was established. The association of TLRs with NF- $\kappa$ Bp65 with different malignancies in patients with OSCC was also described [15,16]. However, the mechanism behind the interactions was not completely understood.

Many molecular markers had been proposed for the better survival of OSCC patients, but none are available in clinical benefit. Hence, early identification of ideal therapeutic targets is mandatory. Studies pertaining to the relationship of serum TLR 4 (sTLR 4) and serum NF- $\kappa$ B (sNF- $\kappa$ B) p65 for OSCC are considered to be inadequate. Hence, the objective of the study is to analyze the sTLR 4 and sNF- $\kappa$ Bp65 level in our samples using enzyme-linked immunosorbent assay (ELISA) to identify diagnostic and as well as prognostic values in our study populations.

### METHODS

#### Subject characteristics

The study was approved by the Institution Ethics Committee of (a) A.C.S Medical College, Chennai, (b) Sree Balaji Dental College and Hospital, Chennai, and (c) Government Tertiary Care Centre, Chennai. The written informed consent was obtained from all the patients ( $n=44$ ) for the prospective samples by surgical oncologist and dental surgeons, respectively. Patient details were collected based on the structured questionnaire and blood samples were obtained from patients which includes OSCC and oral premalignant disorders (OPMDs) control with chewing habits and absolute control samples.

#### Sample collection and ELISA for TLR 4 and NF- $\kappa$ B p65 proteins

Peripheral blood samples were obtained from patients under aseptic condition and collected in non-heparinized tubes. The serum was separated by centrifuging the samples at 1000 rpm for 15 min. The samples were stored at  $-80$  until further use. ELISA was performed with the help of commercially available human TLR 4 (Catalog No: E-EL-H1539, ELabsience, USA) and NF- $\kappa$ B p65 (Catalog No: E-EL-H1388), by following manufacturing instruction. Optical density was determined of each microtiter well and plate was read at 450 nm using ELISA reader.

### Statistical analysis

All statistical analyses were performed with the help of Statistical Package for the Social Sciences version 20.0 (Chicago, IL, USA). Numerical data were expressed as the mean  $\pm$  standard deviation. The comparisons of numerical data were performed by independent sample t-test, categorical variables were performed with Chi-square test or Fisher's exact test and Mann-Whitney test for continuous variables was used. The correlation between sTLR 4 and sNF- $\kappa$ Bp65 level was calculated using Pearson correlation.  $p < 0.05$  was considered to be statistically significant.

## RESULTS

### Baseline characteristics of different study group patients

The baseline clinical characteristics of the study populations are given below. Both male and female were grouped as OSCC (n=22), oral epithelial dysplasia (OED) (n=10), control with chewing habits (n=8), and absolute control patients (n=4). Overall, the mean age of the study group for each population was between 48.3 years for OSCC (ranged 33-69 years), 47.2 years for OED (ranged 32-64 years), 50 years for Control with chewing habits (ranged 33-65 years), and 49.7 years (ranged 33-80 years) for absolute control patients. When considering the gender, 81.8% of patients belong to male populations and only 18.2% of patients were categorized as female patients. When the tobacco consumption is considered 77.3% in OSCC, 90% in OED, and 100% in control with chewing habits, patients were found to be the active tobacco users against the control patients with nil tobacco consumptions. Tobacco users in the form of smoking habits are considered, OSCC 40.9%, OED 60%, and nil in control with chewing habits, and control patients were included for the study. Among the OSCC patients and OED patients, 18.2% and 60% were had the habits of alcohol consumption against the control with chewing habits and absolute control with no alcohol usage.

### Correlation of serum level expression of sTLR 4 with baseline characteristics of different study group patients

In the present investigation, sTLR 4 levels were found to be increased in OSCC, OPMD, and control with chewing habit patients when comparable to absolute control patients (Table 1) which may be correlated with their tobacco usage. In the present investigation, age group of  $>45$  showed an increased level of sTLR 4 in all the study groups when compared to the age groups of  $\leq 45$ . While considering the gender, both male and female populations exhibited almost the same levels in all the study populations. In the present investigation, it was noticed that non-smokers had a high level of sTLR 4 when compared to smokers in all the study population. Similarly, all habitual users exhibited high level of TLR 4 in OSCC patients and non-habitual users exhibited high level of TLR 4 in OED patients. The control with chewing habits and absolute control patients we could not do proper comparison as far as smoking habit, alcohol, and non-chewing habits is concerned since the cases were significantly less. The alcohol users exhibited high level of TLR 4 when comparable to non-alcoholic users (OSCC  $p=0.280$  and OED  $p=0.057$ ). Since it is a customary to do the TNM staging only for OSCC, we analyzed the correlations of the levels of the TLR 4 to the TNM staging in OSCC patients alone. It is observed that patients belonging to T4 stage have an increased level of sTLR 4 when comparable to the patients in the stage of T2.

### Correlation of serum level expression of sNF- $\kappa$ Bp65 in with baseline characteristics of different study group patients

In the present investigation, there is no significant variation in the sNF- $\kappa$ Bp65 levels was found among the study population such as OSCC, OPMD, and control with chewing habits and absolute control patients (Table 2). Similar to TLR 4 age group of  $>45$  showed an increased level of Nf $\kappa$ p65 in all study groups. While considering the gender, female population showed high level of sNF- $\kappa$ Bp65 when compared to male populations ( $p=0.013$ ) in OSCC patients and low level of sNF- $\kappa$ Bp65 in female populations when compared to male in OED cases ( $p=0.502$ ).

**Table 1: Quantification of sTLR 4 in OSCC and OED patients**

Variable	n (%)	OSCC patients sTLR 4 (ng/ml) mean $\pm$ SD	p value	n (%)	OED patients sTLR 4 (ng/ml) mean $\pm$ SD	p value
Age						
>45	12	1.45 $\pm$ 1.14 ng/ml	0.497	5	2.2 $\pm$ 0.7 ng/ml	0.545
$\leq 45$	10	1.139 $\pm$ 0.995 ng/ml		5	1.795 $\pm$ 1.20 ng/ml	
Gender						
Male	18	1.167 $\pm$ 0.916 ng/ml	0.175	8	1.9 $\pm$ 1.0 ng/ml	0.648
Female	4	1.97 $\pm$ 1.57 ng/ml		2	2.3 $\pm$ 0.5 ng/ml	
Tumor stage						
T2	6	1.248 $\pm$ 1.35 ng/ml	0.864	0	NA	NA
T4	16	1.33 $\pm$ 0.988 ng/ml		0	NA	NA
Smoking						
Yes	9	1.112 $\pm$ 1.187 ng/ml	0.473	6	1.97 $\pm$ 0.69 ng/ml	0.928
No	13	1.454 $\pm$ 0.998 ng/ml		4	2.03 $\pm$ 1.44 ng/ml	
Chewing						
Yes	17	1.443 $\pm$ 1.116 ng/ml	0.305	9	2.04 $\pm$ 1.02 ng/ml	0.651
No	5	0.8744 $\pm$ 0.815 ng/ml		1	1.54 $\pm$ 0.0 ng/ml	
Alcohol						
Yes	4	0.782 $\pm$ 0.783 ng/ml	0.280	6	2.46 $\pm$ 0.92 ng/ml	0.057
No	18	1.432 $\pm$ 1.101 ng/ml		4	1.29 $\pm$ 0.60 ng/ml	
Chewing with smokers						
Yes	7	1.36 $\pm$ 1.027 ng/ml	0.742	5	2.060 $\pm$ 0.7437 ng/ml	0.856
No	15	1.200 $\pm$ 1.22 ng/ml		5	1.93 $\pm$ 1.267 ng/ml	
Smoking with alcohol						
Yes	3	0.649 $\pm$ 0.903 ng/ml	0.255	4	2.109 $\pm$ 0.854 ng/ml	0.790
No	19	1.41 $\pm$ 1.07 ng/ml		6	1.92 $\pm$ 1.13 ng/ml	
Chewing with alcohol						
Yes	2	1.08 $\pm$ 0.700 ng/ml	0.759	5	2.65 $\pm$ 0.901 ng/ml	0.023
No	20	1.33 $\pm$ 1.106 ng/ml		5	1.34 $\pm$ 0.532 ng/ml	
All habits						
Yes	2	1.33 $\pm$ 1.10 ng/ml	0.759	7	1.86 $\pm$ 1.04 ng/ml	0.554
No	20	1.08 $\pm$ 0.700 ng/ml		3	2.298 $\pm$ 0.937 ng/ml	

sTLR 4: Serum toll-like receptor 4, OSCC: Oral squamous cell carcinoma, OED: Oral epithelial dysplasia, SD: Standard deviation

Table 2: Quantification of sNFkBp65 in OSCC and OED patients

Variable	n (%)	OSCC patients sNF-kBp65 (ng/ml) mean±SD	p value	n (%)	OED patients sNF-kBp65 (ng/ml) mean±SD	p value
Age						
>45	12	0.585±0.749 ng/ml	0.312	5	0.304±0.395 ng/ml	0.565
≤ 45	10	0.314±0.460 ng/ml		5	0.18±0.17 ng/ml	
Gender						
Male	18	0.309±0.457 ng/ml	0.013	8	0.279±0.32 ng/ml	0.502
Female	4	1.15±0.933 ng/ml		2	0.11±0.05 ng/ml	
Tumor stage						
T2	6	0.406±0.87 ng/ml	0.809	0	NA	NA
T4	16	0.483±0.557 ng/ml		0	NA	
Smoking						
Yes	9	0.290±0.503 ng/ml	0.303	6	0.33±0.3 ng/ml	0.248
No	13	0.581±0.708 ng/ml		4	0.10±0.13 ng/ml	
Chewing						
Yes	17	0.567±0.687 ng/ml	0.020	9	0.2±0.3 ng/ml	0.554
No	5	0.105±0.161 ng/ml		1	0.4±0.0 ng/ml	
Alcohol						
Yes	4	0.196±0.279 ng/ml	0.368	6	0.33±0.3 ng/ml	0.248
No	18	0.521±0.682 ng/ml		4	0.1±0.14 ng/ml	
Chewing with smokers						
Yes	7	0.364±0.551 ng/ml	0.632	5	0.320±0.384 ng/ml	0.456
No	15	0.508±0.684 ng/ml		5	0.171±0.1815 ng/ml	
Smoking with alcohol						
Yes	3	0.162±0.332 ng/ml	0.392	4	0.424±0.400 ng/ml	0.122
No	19	0.509±0.664 ng/ml		6	0.127±0.131 ng/ml	
Chewing with alcohol						
Yes	2	0.288±0.354 ng/ml	0.696	5	0.320±0.380 ng/ml	0.456
No	20	0.479±0.660 ng/ml		5	0.171±0.190 ng/ml	
All habits						
Yes	2	0.288±0.354 ng/ml	0.696	3	0.424±0.490 ng/ml	0.465
No	20	0.479±0.660 ng/ml		7	0.169±0.164 ng/ml	

sNF-kB: Serum nuclear factor-kB, OSCC: Oral squamous cell carcinoma, OED: Oral epithelial dysplasia, SD: Standard deviation

However, tobacco users had a high level of sNfkb65 (OSCC  $p=0.020$  and OED  $p=0.554$ ) when compared to non-tobacco users in the case of all study populations. Interestingly, increased sNfkb65 levels were observed in non-smokers and even in non-alcohol users when compared to smokers and alcohol users in OSCC and OED patients. The control with chewing habits and absolute control patients we could not do proper comparison as far as smoking habit, alcohol, and non-chewing habits is concerned since the cases were significantly less. Since it is a customary to do the TNM staging only for OSCC, we analyzed the correlations of the levels of the TLR 4 to the TNM staging in OSCC patients alone. It is observed that patients belonging to T4 stage have an increased level of sNF-kBp65 when comparable to the patients in the stage of T2 ( $p=0.809$ ).

#### Correlation ship between sTLR 4 and sNF-kBp65 concentrations

The Pearson correlation test showed that the TLR 4 expression in the serum of OSCC was positively correlated with NF-kBp65 expression ( $r_{\text{pearson}}=0.91$ , [ $p<0.0005$ ]) was found to be statistically significant among OSCC groups (Fig. 1). In OPMD patients, TLR 4 expression in the serum was negatively correlated with NF-kBp65 expression ( $r_{\text{pearson}}=-0.08$ , [ $p=0.81$ ]). Similarly, among the control with chewing habits patients, the sTLR 4 expression was positively correlated with NF-kBp65 expression ( $r_{\text{pearson}}=0.30$ , [ $p=0.70$ ]). Among the absolute control patients, the TLR 4 expression level in serum was found to be negatively correlated with NF-kBp65 expression ( $r_{\text{pearson}}=-0.88$ , [ $p=0.11$ ]).

#### DISCUSSION

In our present study, we have quantified the serum level of sTLR 4 and sNF-kBp65 in various study group populations. TLR 4 is considered as most important protein molecule which is involved in both non-specific and specific immune response [17,18]. TLR 4 is the important regulator of immune mechanism and is a single transmembrane non-catalytic protein which provides a microenvironment for the tumor cells to proliferate and as well to evade the immune response [19]. Recent

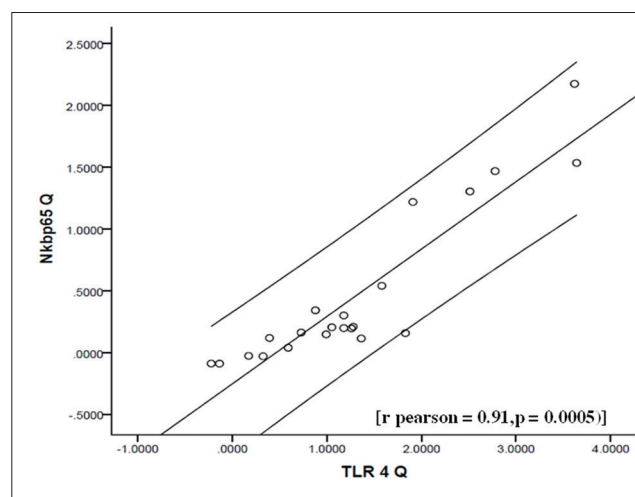


Fig. 1: Pearson correlation analysis of toll-like receptor 4 and nuclear factor-kBp65 in oral squamous cell carcinoma

studies have reported that TLRs are overexpressed in the various types of tumors. In our present investigation, significantly high TLR 4 level was observed in OSCC and control with chewing habits group of patients. Our results are comparable with studies performed by Wei et al., 2016 [20], on non-small cell lung cancer (NSCLC) patients where he showed a higher TLR 4 serum levels when compared to the healthy controls. Similarly, another study revealed from radiation pneumonia in locally advanced NSCLC exhibited higher level of TLR 4 levels in NSCLC patients when compared to healthy controls [21].

NF-kB, an important key regulator for transcription factor, is the major contributor of carcinogenesis which triggers the important

signaling pathway involved in the tumor progression such as TLR [22]. In almost all tumors such as breast, lung, and skin cancers, it is been expressed [23-25]. NF- $\kappa$ B family comprised homologous or heterologous dimers, among which the heterologous dimer of p50 and p65 is considered as most important dimer and NF- $\kappa$ B p65 plays an important nuclear transcription factor [26-28]. A study performed by Wei *et al.* [29] on gastric cardia adenocarcinoma showed the increased serum level of NF- $\kappa$ Bp65. Similar, to the above studies performed in other cancers, our study also revealed that significant association was seen between sNF- $\kappa$ Bp65 level and tobacco users. Hence, from our present study, we evaluated both levels of sTLR 4 and sNF- $\kappa$ Bp65 expression in a relatively smaller sample size. However, there might be also some possible reason for TLR 4/NF- $\kappa$ B p65 activation in various factors which is not manifested in peripheral blood samples as it is rarely investigated. Hence, further studies are needed to validate with the large number of samples to evaluate sTLR 4 and sNF- $\kappa$ Bp65 whether can be used as prognostic marker or not for OSCC patients.

## CONCLUSION

To our understanding, this is our first study to report on TLR 4 and NF- $\kappa$ Bp65 in serum samples. Although TLR 4 and NF- $\kappa$ Bp65 are considered as well-known diagnostic markers, the studies pertaining to OSCC seem to be very limited. Hence, probable targeted therapy with these above markers can help in near future for OSCC patients.

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## CONFLICTS OF INTEREST

All authors approved the final version of the manuscript for publication.

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