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Differential expression and activation of NF-κB family proteins during oral carcinogenesis: Role of high risk human papillomavirus infection

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Oral cancer is one of the most common cancers in India and south-east Asian region consisting of more than 50% of all malignant tumors. Along with many known risk factors, infection of Human Papillomavirus (HPV) has been associated with the development of oral cancer and is suggested to modulate host cell transcription. Reciprocally, cellular transcription factors, such as NF-κB and AP-1 are known to modulate the expression of viral and other genes involved in the development of cancer. In the absence of data on NF-κB in relation to HPV in oral cancer, we studied the DNA binding activity and expression pattern of NF-κB family of proteins in different stages of oral cancer and correlated with HPV infection that has been associated with better prognosis of the disease. A total of 110 fresh oral tissue biopsies were collected comprising 10 normal controls, 34 precancer and 66 oral cancer lesions prior to chemotherapy/radiotherapy. Diagnosis of HPV was done by both consensus and type-specific PCR. Electrophoretic mobility shift assays, western blots and immunohistochemical analysis were performed to assess the binding activity and expression pattern of NF-κB family of proteins (p50, p65, p52, c-Rel, RelB and Bcl-3) in oral tissue biopsies. Twenty seven percent (18/66) of the oral cancer biopsies showed the presence of HPV infection exclusively of high risk HPV type 16, which was primarily associated with the well differentiated squamous cell carcinomas (WDSCC). We observed a high constitutive activation of NF-κB with concomitant upregulated expression of all the NF-κB members in oral cancer tissues. Expression of NF-κB components gradually increased as the severity of lesion increased from precancer to invasive cancer. NF-κB p50 was found to be the major DNA binding component, which is indicative of homodimerization of p50 subunits. Interestingly, in HPV16 infected oral cancers although p50 showed high binding activity, p65 also showed a partial involvement as evidenced in supershift assay. Both by western blotting and immunohistochemistry, a differential overexpression and nuclear localization of p50, p65 and partially of Bcl-3 were observed in HPV16 positive oral cancer patients that also showed an over-expression of p21. We therefore, demonstrate a constitutive activation and differential expression of NF-κB proteins, which change as a function of severity of oral lesions during development of oral cancer. The NF-κB DNA binding is primarily due to homodimerization of p50 but infection of high risk HPV promotes participation of p65 in NF-κB complex formation, leading to heterodimerization of p50/p65. We propose that the involvement of p65 in HPV infected oral cancer may be linked to improved differentiation and better prognosis of the disease when treated.

Key words: oral cancer; carcinogenesis; human papillomavirus; high risk HPV; NF-κB; constitutive activation; Bcl-3

Oral Squamous Cell Carcinoma (OSCC) is the sixth most common cancer and accounts for approximately 5% of all malignant tumors worldwide.[1] In India and South East Asia OSCC is the most common malignancy amounting upto 50% of all malignant tumors.[2] Although most of the OSCC is attributed to smoking and alcohol consumption, a significant proportion of oral cancers have been demonstrated to contain anogenital HPV infection.[3] The high risk HPV type 16 tends to be the most predominant type detected in oral cancer.[3,4] Interestingly, HPV infected patients were found to have better overall survival than those with HPV negative tumors[5–8] and showed well-differentiated tumors.[9] However, the mechanism(s) by which HPV infection improves the overall survival/prognosis in oral cancer is not yet clearly understood.

Dysregulation of NF-κB expression and its activation is frequently observed in many cancers, such as colon,[10] breast,[11,12] pancreatic,[13] leukemia,[14] lymphoma,[15] myeloma[16] and cervical cancer.[17] NF-κB also controls the expression of several genes that regulate cell cycle (*cyclin D1*), differentiation ($p21^{Cip/Waf1}$), cell survival (*Bcl-2*, *Bcl-xL*, *cIAP*), growth factors (*VEGF*), cell adhesion (*VCAM*, *ECAM-1*) and angiogenesis (*MMPs*).[18,19] Constitutive activation of NF-κB has been observed in some head and neck cancer (HNSCC) cell lines.[20] High risk HPV type 16 has also been shown to modulate NF-κB activation and expression in different cancers.[21,22]

The NF-κB/Rel superfamily of eukaryotic proteins is a group of ubiquitously expressed, pleotropic, transcriptional regulators that bind to selective 10 base pair conserved sequence motif 5′GGGG RNN YYC 3′ (R = Pu, Y = Py, N = any base) present in the regulatory regions of the wide arrays of different genes.[23,24] There are 5 members in the mammalian Rel family that are classified into 2 categories. One group involves RelA (p65), RelB and c-Rel, which are produced in their mature forms and do not require proteolytic processing. The other category includes NF-κB1 (p105) and NF-κB2 (p100), which require processing to produce the mature p50 and p52 proteins, respectively. The most abundant NF-κB dimer is p50/RelA, which is bound to inhibitory protein IκB and retained in the cytoplasm till its nuclear translocation mediated by the degradation of IκB in response to various cellular signals. The distinct combinations of NF-κB members confer the specificity to tissue-specific and inducible gene activation[25,26]; e.g., p50/p50 dimers display high affinity binding for many distinct κB motifs but provides a strong transcriptional activation only when adopting a specific conformation induced by only a few κB motifs that have been shown to be present in a limited number of genes such as *TNFα*, *MHC Class I* and its own gene *nf-kb1*.[27,28]

In the present study we have analyzed the activation of NF-κB and alterations, if any, in the expression of different NF-κB proteins during oral carcinogenesis *in vivo* using tissue biopsies from patients with precancerous and cancerous oral lesions. We demonstrate the role of HPV infection on NF-κB trans-activation during oral carcinogenesis and show why patients with HPV 16 positive tumors seem to have a better prognosis and disease-specific survival, compared to HPV negative group.

Abbreviations: EMSA, electrophoretic mobility shift assay; HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus; NF-κB, Nuclear Factor-κB; OCL, oral cancer lesion; OSCC, Oral Squamous Cell Carcinoma; PCL, precancer lesion; WDSCC, Well-differentiated squamous cell carcinoma.

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Material and methods

Tissue specimens

A total 110 oral tissue biopsies were collected comprising 66 patients with malignant and 34 with premalignant oral lesions and 10 normal healthy controls from the Department of ENT Surgery, Lok Nayak Hospital, New Delhi. Informed consent was obtained from all the patients and control subjects. The clinical characteristics of these patients are presented in Table I. All the patients were chosen prior to any chemotherapy or radiotherapy treatment. Biopsy tissues were collected in sterile PBS. One half of the tissues were immediately frozen in −70°C and the remaining halves were sent for histopathological diagnosis in formalin solution.

TABLE I – CLINICOPATHOLOGICAL CHARACTERISTICS WITH THE SITES OF TISSUE RECOVERY OF PATIENTS AND HEALTHY CONTROLS RECRUITED FOR ORAL TISSUE BIOPSY STUDY

Characteristics	Normal	PCL	OCL
Number of biopsies	10	34	66
Mean age (years)	45.4 ± 15.5	48.2 ± 9.7	52.9 ± 10.8
Male:female ratio	4:1	7.5:1	6.3:1
Tumor Sites			
Tongue	4	14	36
Mandibular gingiva	1	4	7
Maxillary gingiva	0	3	4
Buccal mucosa	3	6	12
Palate	0	2	3
Lips	2	5	4

The numbers indicate total cases in each category. PCL: precancerous lesions consists of hyperplastic and dyplastic lesions including leukoplakia. OCL: oral cancer lesions.

DNA extraction and diagnosis of human papillomavirus infection

High molecular weight genomic DNA was isolated from normal, precancerous and cancerous oral biopsies by the standard phenol–chloroform and proteinase K digestion procedure, and PCR amplification was performed following the procedure described earlier.[29] The initial diagnosis of human papillomavirus (HPV) was performed by using a pair of consensus degenerate primers (MY09 and MY11) derived from the highly conserved L1 open reading frame (ORF) of viral genome (MY09:5′-GCM CAG GGW CAT AAY AAT GG-3′, MY11:5′-CGT CCM ARR GGA WAC TGA TC-3′where M = A/C; W = A/T; Y = C/T; R = A/G). Further typing of high risk HPV types 16 and 18 were done by type-specific primers [HPV16 – (1) 5′-AAG GCC AAC TAA TAG TCA C-3′, (2) 5′-CTG CTT TTA TAC TAA CCG G-3′; HPV18 – (1) 5′-ACC TTA ATG AAA AAC CAC GA-3′, (2) 5′-CGT CGT TTA GAG TCG TTC CTG-3′]. PCR was performed in a 25 μL reaction mixture containing 50–100 ng DNA, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$,125 μM of each dNTPs (dATP, dGTP, dCTP, dTTP), 5 pmol of oligonucleotide primers and 0.5 U Taq DNA polymerase. β-globin gene was used as internal control (1) 5′-CAA CTT CAT CCA CGT TCA CC-3′, (2) 5′-GAA GAG CCA AGG ACA GGT AC-3′). The temperature profile used for amplification constituted an initial denaturation at 95°C for 4 min followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min, which was extended for 5 min at the final cycle. The primers were synthesized in an automated DNA synthesizer (Model 381A; Applied Biosystems, Foster City, CA) using phosphoramidite chemistry and purified in high pressure liquid chromatography (HPLC; LKT Pharmacia, USA).

Preparation of protein extract

Protein extracts from all oral biopsies (precancer, cancer and control) were prepared by the method of Dignam et al.[30] with certain modification.[31] Briefly, the method involved mincing of frozen tissues and it was suspended in ice-cold buffer A [20 mM HEPES pH = 7.6, 20% (v/v) Glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, 2 μg/ml Leupeptin and 10 μg/ml Aprotinin]. The lysates were microfuged at 4,000 rpm for 10 min at 4°C after incubating them for 15 min on ice. The supernatant was transferred in a new tube and designated as cytoplasmic extracts. The pellet containing isolated nuclei was resuspended in the 2 times pellet amount of extraction buffer B [20 mM HEPES pH 7.6, 25% (v/v) Glycerol, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, 2 μg/ml Leupeptin and 10 μg/ml Aprotinin]. The extraction mixture was microfuged after 1 hr at 14,000 rpm at 4°C for 25 min. The resulting supernatant was designated as nuclear extract. The concentration of protein in the extracts was determined by standard Bradford method and the extracts were stored at −70°C freezer until use.

Electrophoretic mobility shift assay

To detect the NF-κB DNA-binding activity in the nuclear extracts, Electrophoretic mobility shift assay (EMSA) was performed as described earlier[17,31,32] using the following oligonucleotides: NF-κB consensus sequence 5′-AGT TGA *GGG GAC TTT CC*C AGGG C-3′ (consensus binding sites are underlined), Oct-1 5′-TGT CGA ATG CAA ATC ACT AGA A-3′. The oligos were synthesized in Applied Biosystems DNA synthesizer using phosphoramitide chemistry. These oligos were annealed and labeled with [γ^{32}P] ATP (3,000 Ci/mmol, Jonaki, Hyderabad, India) by T4 polynucleotide kinase and gel purified onto a 15% polyacrylamide gel. Briefly, a binding reaction of 10μg nuclear extract with [γ^{32}P] labeled oligo probe was performed in a 25 μl reaction volume containing 50% Glycerol, 60 mM HEPES pH 7.9, 20 mM Tris-HCl pH 7.9, 300 mM KCl, 5 mM EDTA, 5 mM DTT, 100 μg/ml of bovine serum albumin, 2.5 μg of poly (dI-dC) for 30 min at room temperature. The DNA–protein complexes were resolved on 4.5% nondenaturing polyacrylamide gel (29:1 cross-linking ratio), dried and exposed overnight to KODAK X-Omat films (Kodak India, India). Binding specificity was evidenced by preincubation with a 100-fold molar addition of homologous unlabelled oligonucleotide of NF-κB and heterologous consensus sequence of the Oct-1 transcription factor. For monitoring composition of NF-κB complex by supershift assay, 2 μg of polyclonal antibodies (Abs) directed against each NF-κB family member (Santa Cruz Biotechnology, Santa Cruz, CA) were added and the reaction mixture was further incubated for 1 hr at 4°C. The rabbit polyclonal antibodies against following NF-κB proteins were used: p50 (epitope corresponding to NLS region of p50 of human origin), p65 (epitope corresponding to amino terminus of p65 of human origin), p52 (epitope within a conserved domain of mouse origin), c-Rel (epitope within the amino terminal domain of human origin), RelB (epitope corresponding to carboxy terminus of mouse origin), Bcl3 (epitope corresponding to carboxy terminus of human origin). The quantitative densitometric analysis was performed using Alpha Ease FC version 4.1.0 (Alpha Innotech Corporation, IL).

SDS–Polyacrylamide gel electrophoresis and immunoblotting

Nuclear extracts (40 μg/lane) were separated in 10% polyacrylamide gel, electrotransferred to Immobilon-P, PVDF membranes (Millipore Corporation, Bedford, MA), and probed with polyclonal rabbit antibodies. The incubation was carried out overnight in PBS supplemented with 5% skimmed milk powder, 0.05% Tween 20 (Sigma, St. Louis, MO) and 1:5,000 dilution of the respective antibodies. The blots were washed, exposed to horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies for 1 hr and finally the bands were visualized by Luminol detection kit (Santa Cruz Biotech). Samples were run several times to determine the level of expression of different NF-κB proteins and every time membrane was re-probed for β-actin expression as control. The expression level of different NF-κB proteins was quantitated as described earlier[31] on an arbitrary scale where Strong= ++++; Medium = ++; Weak = +; and Nil/not detectable = −.

Immunohistochemistry

The Immunohistochemical staining was performed on 5 μm thick paraffin embedded tissue sections. Slides were deparaffinized in xylene twice for 30 min and rehydrated through gradual

addition of ethanol solution to distilled water. Heat-induced epitope retrieval was carried out by microwave treatment in the 10 mM citrate buffer (pH 6.0). Endogenous peroxidase activity was quenched by 3% H_2O_2. Nonspecific binding site was blocked using 1.5% blocking serum (ABC staining kit, Santa Cruz Biotech) diluted in Tris-buffered saline (TBS). Tissue sections were incubated with rabbit antibody diluted to 1:50 at 4°C in a humidified chamber for overnight, followed by biotinylated secondary antibody (1:200 dilution) from ABC staining kit (Santa Cruz Biotech). The peroxidase-labeled streptavidin antibody was added from the same staining kit and incubated in the similar incubation conditions. Finally 3,3′-Diaminobenzidine (DAB) was used as the chromogen in presence of hydrogen peroxide. Counterstaining of nuclei was done using Meyer's Hematoxylin. Intensity scoring of expression of assessed proteins in immunostained preparations was performed using a arbitrary semiquantitative scale: none (−); low (+); moderate (++) and high (++++).

TABLE II – HPV DIAGNOSIS AND PATHOLOGICAL GRADES OF ORAL BIOPSIES ANALYZED

Category	Grades	No. of cases	HPV positivity[1]	HPV16[2]
A	Normal/control	10	–	–
B	Precancerous	34	4 (12)	–
	Leukoplakia	4	–	–
	Erythroplakia	1	–	–
	Hyperplasia	9	1 (3)	–
	Dysplasia	20	3 (9)	–
C	Cancerous	66	18 (27)	18 (100)
	WDSCC	39	12 (18)	12 (66)
	MDSCC	18	4 (6)	4 (22)
	PDSCC	9	2 (3)	2 (11)

HPV; Human Papillomavirus, WDSCC; well-differentiated squamous cell carcinoma, MDSCC; moderately-differentiated squamous cell carcinoma, PDSCC; poorly-differentiated squamous cell carcinoma. Values in parentheses are in percentages.

[1]Percent distribution of HPV infection in different grades of pre-cancer or cancer cases with respect to total cases in each category. [2]Percent distribution of high risk HPV type 16 in different grades of cancer cases with respect to total HPV-positive cancer cases.

Results

To study the expression and activation of different members of NF-κB transcription factor during oral carcinogenesis and their modulation following HPV infection, biopsy specimens ($n = 110$) were collected from histologically proven normal ($n = 10$; negative for HPV infection), oral precancer ($n = 34$) and cancer ($n = 66$) patients displaying tumors at different sites of the oral cavity as indicated in Table I. Mean age (± SD) and Male:Female gender ratio varied from 45.4 ± 15.4 years and 4:1 in control subjects, 48.2 ± 9.7 years and 7.5:1 in precancer and 52.9 ± 10.8 years and 6.3:1 in cancer patients, respectively. The presence of HPV infection and their type specificity were determined by PCR.

Detection of HPV DNA sequences in oral tissue biopsies

To detect HPV infection in the oral biopsies, total genomic DNA was isolated and PCR amplification of L1-conserved sequence was performed using consensus primer as described earlier.[29] Type-specific primers were further used to diagnose presence of high risk HPV types 16 and 18. The prevalence of total HPV was found to be 22% (22/100) while frequency of high risk type 16 was 18%

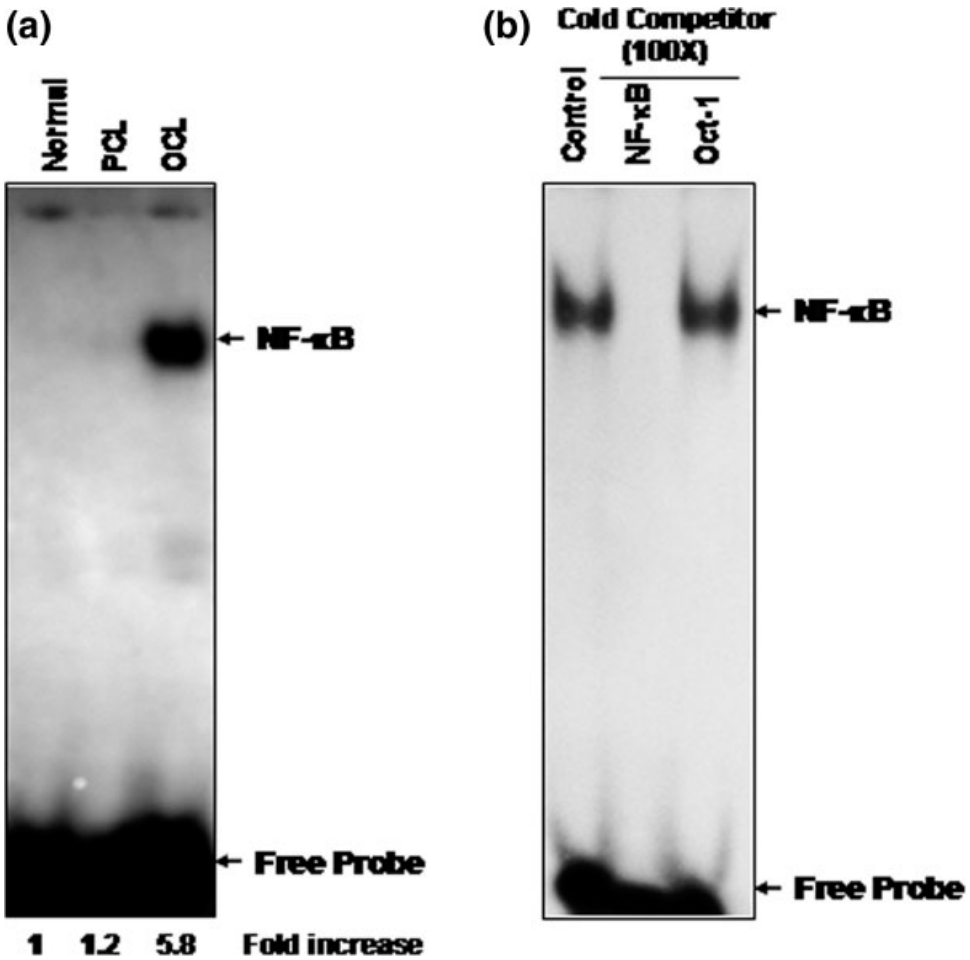

FIGURE 1 – (*a*). Constitutive NF-κB activation in malignant oral biopsies. Gel shift analysis using nuclear extracts from the different grades of oral tissue with ^{32}P-labelled NF-κB oligonucleotide probe. Oral cancer lesion (OCL) shows NF-κB binding activity. (*b*) Binding of NF-κB to DNA probe is sequence-specific. Binding specificity was evidenced using nuclear extracts (10 μg) prepared from oral cancer biopsies were incubated with unlabelled 100 M excess of specific competitor (NF-κB) probe in comparision with competition experiment using nonspecific competitor (Oct-1) and then checked for specific NF-κB binding by EMSA.

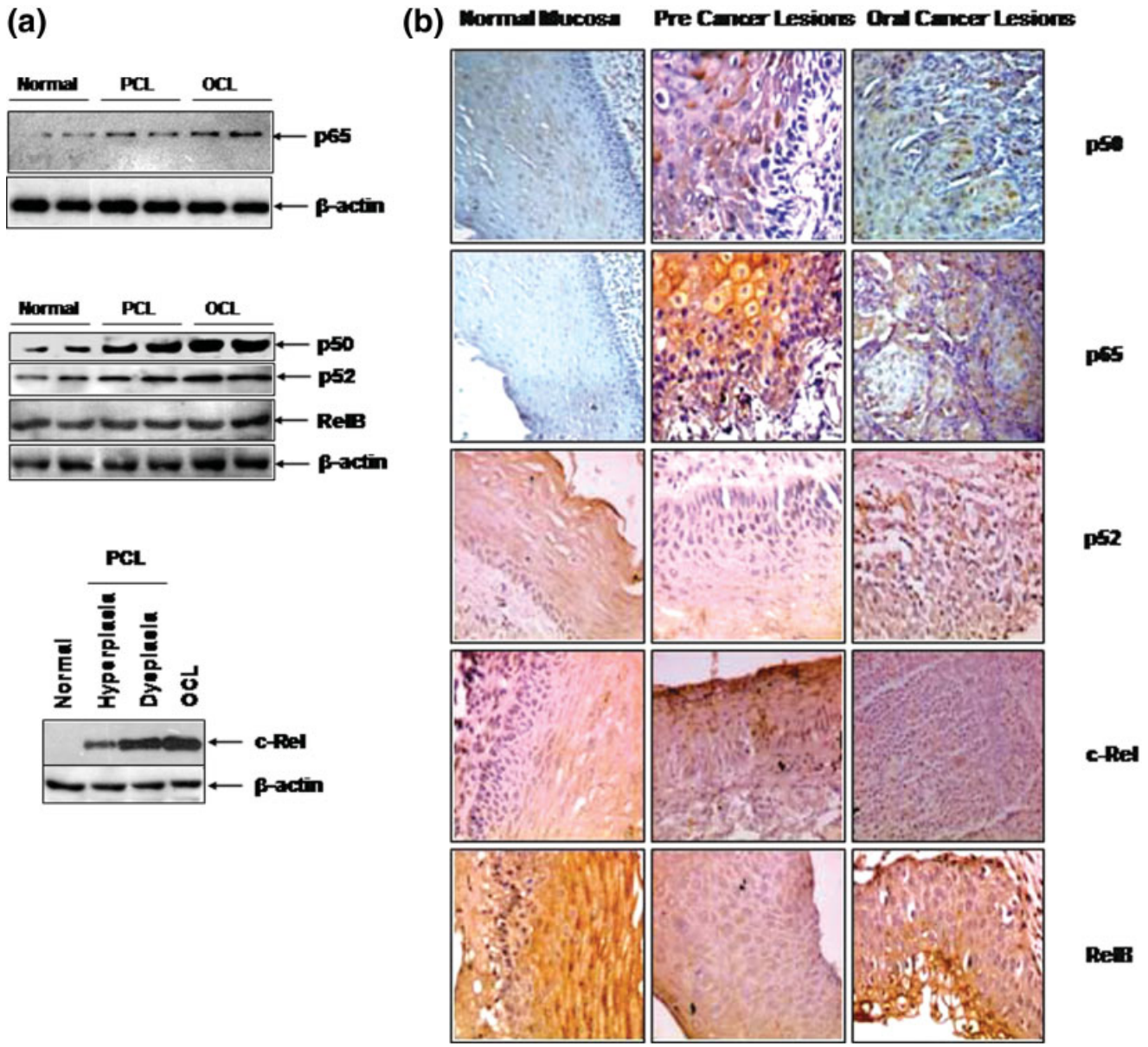

FIGURE 2 – Increased expression of p65, p50, p52, RelB and c-Rel in oral cancer lesions. (*a*) A total of 40 μg protein extracts each from normal, precancerous (PCL) or oral cancer lesions (OCL) biopsies from HPV-negative cases were separated on a 10% SDS-PAGE, electrotransferred on PVDF membrane and probed for p65, p50, p52, RelB or c-Rel expression. To confirm equal protein loading, the membranes were reprobed for β-actin expression. (*b*) Immunohistochemical analysis of normal, precancerous (PCL) and cancerous (OCL) oral tissue from HPV-negative cases for expression of different NF-κB members. Paraffin embedded (5 μm) oral tissue sections of normal mucosa, precancerous lesion and oral cancerous lesion were probed with antibodies against p50, p65, p52, c-Rel and RelB to perform immunohistochemical analysis as described in Methods (Original magnification: 200×). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(18/100) but we did not detect any HPV18 DNA sequences. The majority of cancer cases (48/66, 72.7%) were found to be negative for HPV infection. The high risk HPV type 16 was detected only in patients with oral cancer (18/66, 27.3%; Table II). Among different grades of oral cancers, majority (12/66, 18%) of the HPV infection was seen in well differentiated squamous cell carcinoma (WDSCC) followed by 6% (4/66) in moderately differentiated carcinoma (MDSCC) while the least positivity (2/66, 3%) for HPV DNA was detected in PDSCC cases. Out of 34 precancerous biopsies comprising leukoplakia, erythroplakia, and hyperplasia only 4 (11.7%) showed the presence of HPV infection (one HPV type 11 and three HPV type 6) but no high risk HPVs could be detected in any of these lesions. All the 10 normal healthy controls did not show positivity for any HPV DNA sequence.

Constitutive activation of NF-κB in oral cancer

The relative DNA binding activity of NF-κB was determined during the progression of oral cancer by electrophoretic mobility shift assay (EMSA) with the nuclear extract prepared from tissue specimens collected from biopsy of normal, precancerous (PCL) and cancerous lesions (OCL) and ^{32}P-labeled probe harboring NF-κB consensus sequence. Malignant oral tissues showed a significantly higher DNA binding activity of NF-κB while absence or very low binding was found in normal as well as in the precancerous lesions. The nuclear extracts from normal healthy controls (n = 10) showed least or no DNA binding activity for NF-κB cognate sequence (Fig. 1*a*). The binding activity of NF-κB showed a gradual increase with the increasing severity of oral lesions. The binding specificity of NF-κB to DNA was confirmed by competition assay

TABLE III – EXPRESSION OF NF-κB PROTEINS IN NORMAL ORAL MUCOSA, PRECANCEROUS AND CANCEROUS ORAL LESIONS[1]

Proteins	Normal (n = 10)				Precancerous lesions (n = 34)				Cancerous lesions (n = 66)			
	Nil	Weak	Medium	Strong	Nil	Weak	Medium	Strong	Nil	Weak	Medium	Strong
P50	–	1	8	1	–	5	20	9	–	2	24	40
P65	1	4	5	–	2	4	23	5	–	6	39	21
P52	2	3	5	–	3	5	22	4	–	7	39	20
cRel	8	2	–	–	1	6	16	11	–	2	19	45
RelB	–	2	6	2	–	2	22	10	–	2	43	21

[1]Arbitrary level of expression in immunoblotting: Strong, ++++; Medium, ++; Weak, +; Nil/not detectable, –.

using a 100 fold molar excess of cold, specific competitor probe of NF-κB and nonspecific competitor, a heterologous probe of transcription factor Oct-1. The retarded complex of DNA-protein disappeared when the nuclear extract was incubated with excess of cold oligos of NF-κB but reappeared after its incubation with nonspecific probe harboring consensus sequence for Oct-1 (Fig. 1*b*).

NF-κB family proteins show differential expression pattern during oral carcinogenesis

Western blotting and immunohistochemistry experiments were performed to analyze the level of expressions of all the NF-κB family members, e.g., p50, p65, p52, c-Rel and RelB, during progression of oral cancer. In the western blotting p50, p65 and c-Rel proteins showed a distinct gradual upregulation with the increasing severity of the lesion. p50 protein was found to be highly expressed in all malignant tissues but to a moderate level in precancerous lesions (Fig. 2*a* and Table III). A low expression of p50 protein was observed in all the normal healthy subjects. The expression pattern of p65 showed similar increasing trend with progression of lesion as of p50 but to a much lesser extent. While a minor difference was observed in the expression levels between pre-cancer and cancer for p65 protein, its level was significantly elevated when compared to those of normal healthy controls. Interestingly, c-Rel showed almost a negligible expression in normal healthy controls but a very distinct gradual increase was seen as the lesions progressed towards malignant changes (Fig. 2*a*, lower panel). RelB protein expressed almost uniformly in all grades of oral tissues from normal, premalignant to malignant lesions but certain inconsistency was also observed in some cases. The p52 protein expression also showed the similar severity-dependent trend and elevated to the maximum in cancerous tissues. The expression profile of NF-κB components was further checked *in situ* by immunohistochemistry method in paraffin embedded tissue sections. Intensity scoring of expression of NF-κB proteins in immunohistochemistry preparations was performed on an arbitrary four point scale; none (−); low (+); moderate (++) and high (++++). The results correlate very much with those obtained in western blotting analysis (Fig. 2*b* and Table IV). Expression of p50 protein was either nil or low in all the normal tissue sections examined, whereas 40% precancerous biopsies (8/20) and 70% tumors (14/20) showed a moderate to a high expression of p50. Remaining 30% of cancer cases (6/20) showed weak immunoreactivity for p50 antibody. Most of the normal oral mucosa showed nil to moderate expression for p65 protein while only 20% of cancer (5/20) and 30% of precancerous tissue showed strong positivity for p65 protein expression and that too of low to moderate scale (Fig. 2*b*). 85–90% of the cancer tissue sections showed moderate to high staining for c-Rel (18/20) and RelB (17/20) proteins. Moderate to high immunoreactivity for p52 protein was also visualized in 70% of cancerous cases (14/20) while its expression were either nil or low in normal as well as precancerous mucosa (Table IV).

TABLE IV – EXPRESSION OF NF-κB SUPERFAMILY MEMBERS IN DIFFERENT GRADES OF ORAL TISSUE BIOPSIES

NF-κB proteins	Histological grades	Level of Expression			
		− (None)	+ (Low)	++ (Moderate)	++++ (High)
p50	NM	8	2	0	0
	PCL	10	2	5	3
	OCL	2	4	6	8
p65	NM	7	2	1	0
	PCL	13	2	4	1
	OCL	12	2	3	3
p52	NM	7	2	1	0
	PCL	5	5	4	6
	OCL	1	5	7	7
c-Rel	NM	8	1	1	0
	PCL	7	3	4	6
	OCL	0	2	8	10
RelB	NM	5	2	2	1
	PCL	5	4	4	7
	OCL	0	3	8	9

Normal mucosa (NM, n = 10), precancerous Lesions (PCL, n = 20), oral cancer Lesions (OCL, n = 20). The values indicate the distribution of specimens in each category.

High risk HPV infection alters composition of NF-κB complex during DNA-binding activity of NF-κB in malignant oral tumors

To examine the effect of high risk HPV infections on the NF-κB DNA binding activity in malignant oral tumors, we compared the NF-κB activity of nuclear extracts from HPV16 positive as well as HPV negative oral tissue samples as depicted in Fig. 3*a*. There was only a minor difference in the binding activity of NF-κB between HPV positive and HPV negative oral cancers; HPV positive cancers had a bit lower binding activity. However, when the composition of NF-κB complex was further analyzed using specific antibodies raised against different members of NF-κB family, i.e., p50, p65, c-Rel, RelB, and p52, by supershift assay, we did observe a differential binding pattern of these components between HPV positive and negative oral cancer cases (Fig. 3*b*). Although, p50 was the major binding component in both HPV positive and negative oral cancer biopsies, addition of anti-p65 also showed a minor shift in only HPV positive biopsies but it is absent in HPV negative cases. Almost negligible shift was observed for other members of NF-κB family in both, HPV16 positive and negative biopsies.

Since Bcl-3 protein of IκB family is known to co-exist/co-precipitate with p50/p50 homodimer and act as a co-activator of p50/p50-mediated transcription,[33] we checked the presence of Bcl-3 in DNA-binding activity of NF-κB complex. Addition of anti-Bcl-3 antibody resulted in diminished NF-κB-DNA binding activity in both HPV negative and HPV positive malignant biopsies in the supershift assay; however, the effect was higher in the HPV negative oral cancer lesions (Fig. 3*b*, Last lanes). Despite the absence of supershifted bands which could be due to interaction of Bcl-3 antibody with DNA binding motif of p50/p50/Bcl-3 complex, the diminished binding is indicative of interaction between Bcl-3 and p50 leading to p50:p50 homodimerization during oral carcinogenesis. As the involvement of Bcl-3 is lesser in HPV positive cancer, it appears that the p50/p50 homodimer is stoichiometrically lesser in the DNA binding activity in HPV positive cancer which also contains conventional p50/p65 heterodimer.

Expression dynamics of NF-κB protein members and Bcl-3 are different in the high risk HPV infected oral cancer lesions

The expression pattern of all members of NF-κB/Rel family of proteins including Bcl-3 was comparatively analyzed in both

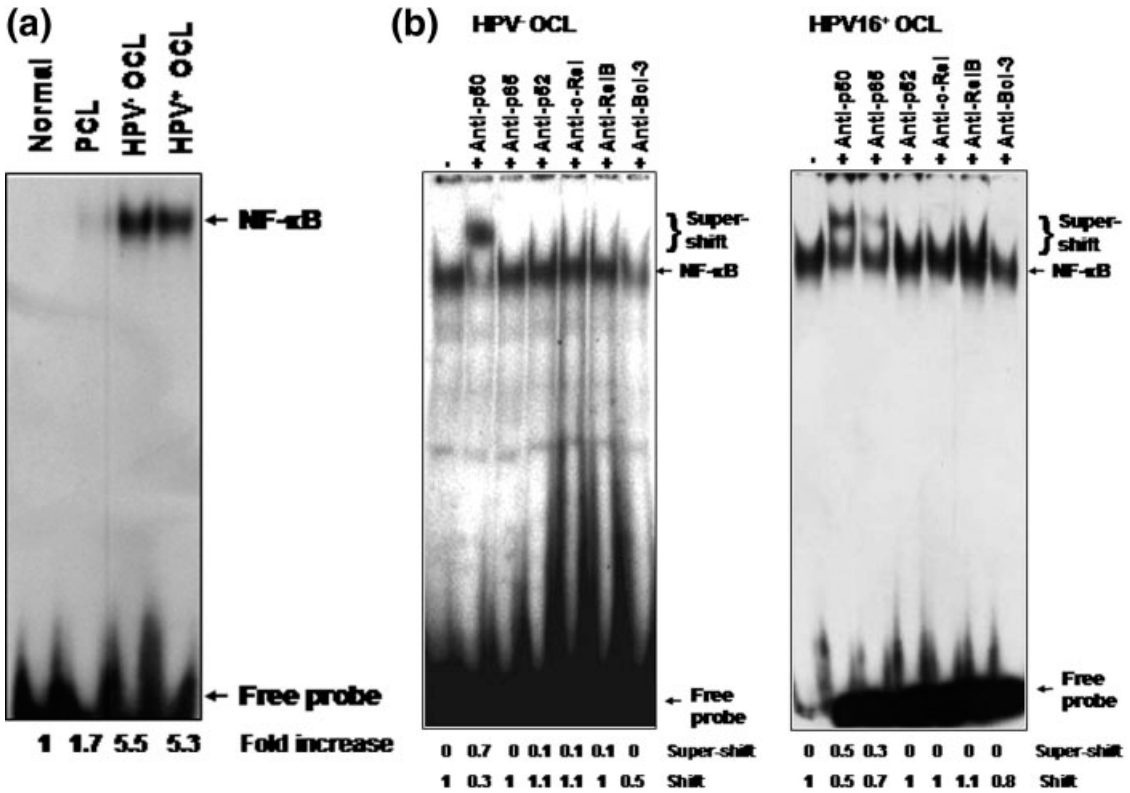

FIGURE 3 – Effect of high risk HPV type 16 infection on constitutive NF-κB activity and its composition in oral cancer lesions. (*a*) Nuclear extracts (10 μg) prepared from normal, precancerous (PCL) and cancerous (OCL) oral biopsies without HPV infection (HPV^-) or with HPV type 16 infection (HPV^+) were checked for NF-κB binding activity by EMSA as described in Methods. (*b*) High risk HPV type 16 infection results in alteration of DNA binding pattern of NF-κB members in oral cancer lesions. Nuclear extracts (10 μg) prepared from HPV^- OCL and HPV^+ OCL were incubated with specific antibodies (2 μg each) either against p50, p65, p52, c-Rel, RelB or Bcl-3 and assayed for NF-κB binding activity by EMSA as described in Methods and band intensities quantified are indicated.

HPV16 positive and HPV negative oral tumor biopsies by western blotting. The results showed an increased expression of p50 and Bcl-3 in HPV16 positive oral cancer cases as compared to that of HPV negative cancers (Fig. 4*a* and Table V). HPV 16 infected oral cancer lesions (OCL) also showed a marginal increase in the expression level of p65 protein. But there was no difference in the expression of c-Rel, RelB and p52 proteins in the HPV infected malignant tissues when compared to those were negative for HPV infection.

These results were further re-examined by immunohistochemical staining of tumor tissue sections from HPV 16 positive and HPV negative oral cancer cases with the antibodies of p50, p65 and Bcl-3 proteins. Figure 4*b* shows presence of koilocytes in HPV positive tissue sections which clearly indicate persistent HPV infection in these cancerous biopsies and they showed an increased expression of p50 as well as Bcl-3 proteins. A moderate increase in the level of p65 positivity was also observed in the HPV infected oral cancer tissue when compared to HPV negative ones. p50, p65 and Bcl-3 proteins were found to be evenly localized in the cytoplasm as well as in the nucleus of the oral cancer lesions (Fig. 4*b*).

p21WAF protein is over expressed in well differentiated squamous cell carcinoma

Since HPV16 positive oral lesions showed better differentiation, the expression of NF-κB-regulated differentiation marker, p21, was examined in different grades of oral cancer and compared between HPV negative and HPV16 positive cases (Fig. 5). Normal oral epithelium expressed very low but detectable level of p21. However, a very high expression of p21 was observed in well differentiated oral cancer. The poorly and moderately differentiated lesions showed low and moderate p21 expression respectively. Interestingly, the level of p21expression in HPV16 positive lesions was similar or slightly higher to the level observed in respective grades of HPV-negative well differentiated squamous cell carcinoma (WDSCC) and PDSCC lesions.

Discussion

In the present report, we show that a significant proportion of oral cancer patients harbor only high risk HPV types 16, which is highest in the well-differentiated squamous cell carcinomas. We observed a constitutive activation of transcription factor NF-κB which is accompanied by a generalized increase in the expression of all the members of NF-κB family proteins during development of oral cancer as revealed by western blotting and immunohistochemical analysis carried out in freshly collected oral tissue biopsies. We also report here that in oral squamous cell carcinoma, NF-κB complex is primarily of p50 protein which appears to form a p50/p50 homodimer. Although the extent of NF-κB DNA binding activity remains a bit lower in the HPV infected oral cancer tissue, a partial involvement of p65 in NF-κB complex is observed in these cases only as revealed by supershift assay of nuclear extracts as well as by immunohistochemistry. In the present investigation, we have tried to find a correlation between NF-κB DNA-binding activity, its composition and, expression and HPV infection during oral carcinogenesis.

Primary screening to detect total positivity for HPV DNA sequences in precancerous and cancerous lesions revealed 22% of oral tissue biopsies positive for HPV L1 consensus sequence with majority of infection in cancerous lesions and that too only of high risk HPV type 16 (18%). These results are in good agreement with earlier reports which showed that HPV positivity rate varies

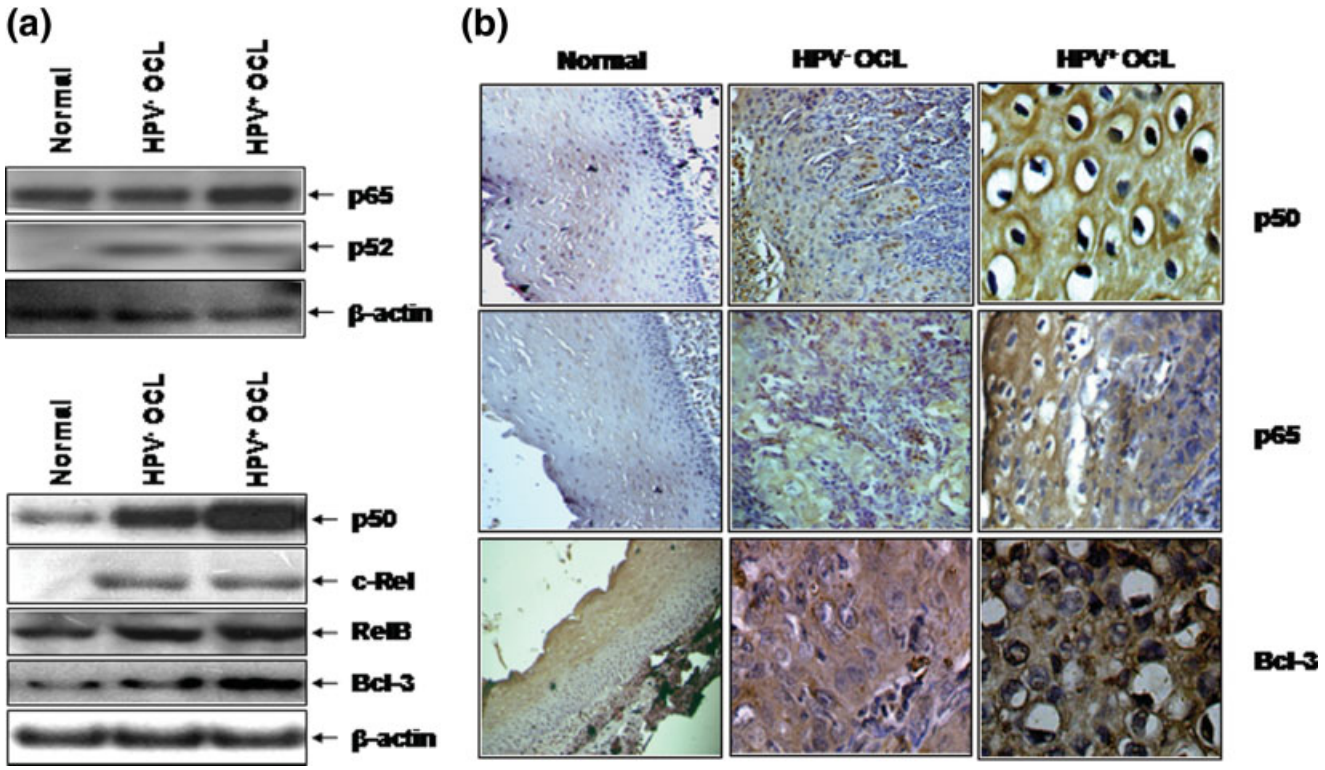

FIGURE 4 – Upregulation of p65, p50 and Bcl-3 expression in HPV16 infected oral cancer lesions. (*a*) A total of 40 μg protein extracts each from normal, HPV negative (HPV^-) oral cancer lesions (OCL) and HPV16 positive oral cancer lesion (HPV^+ OCL) biopsies was separated on a 10% SDS-PAGE, electrotransferred on PVDF membrane and probed for p65, p50, p52, RelB, Bcl-3 or c-Rel expression. To confirm equal protein loading, the membranes were re-probed for β-actin expression. (B) Comparative immunohistochemical analysis of HPV^- OCL and HPV^+ OCL for expression of canonical NF-κB members. Paraffin embedded sections of indicated tissues were probed with antibodies against p50 and p65 and Bcl-3. Immunohistochemical analysis was performed as described in Methods. (Original magnification: 200×). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE V – EXPRESSION OF NF-κB PROTEINS IN NORMAL ORAL MUCOSA AND HPV16 POSITIVE AND HPV NEGATIVE ORAL CANCEROUS LESIONS (OCL)[1]

Proteins	Normal (n = 10)				HPV-negative OCL (n = 48)				HPV16-positive OCL (n = 18)			
	Nil	Weak	Medium	Strong	Nil	Weak	Medium	Strong	Nil	Weak	Medium	Strong
P50	–	1	8	1	–	2	22	24	–	–	2	16
P65	1	4	5	–	–	4	32	12	–	2	7	9
P52	2	3	5	–	–	3	31	14	–	4	8	6
cRel	8	2	–	–	–	2	14	32	–	–	5	13
RelB	–	2	6	2	–	1	32	16	–	1	11	5
Bcl–3	2	6	2	–	–	5	31	12	–	2	12	5

[1]Arbitrary level of expression in immunoblotting: Strong, ++++; Medium, ++; Weak, +; Nil/not detectable, –.

between 10–20% in oral cancers.[4,8,34,35] These studies also found HPV16 as the predominant HPV type as observed by us in oral squamous cell carcinomas. Interestingly, in India HPV type 16 has been found to be almost exclusively prevalent in cervical squamous cells carcinoma[36] andenocarcinoma of the cervix[37] and bladder cancer[38] but not in lung cancers, which harbor only HPV18 DNA sequences.[39] However, some authors have reported higher HPV prevalence and occurrence of other high risk HPV types in oral cancer from other regions of India.[34,40–42] These variations may be due to geographical and ethnic differences or selection bias for recruiting only betel quid chewing or tobacco smoking oral cancer patients and varied PCR protocols and primers used by different investigators.

In the present study, we found a strong NF-κB DNA-binding activity contributed by p50/p50 homodimer in oral cancer, which was absent in normal mucosa. An aberrant expression of NF-κB proteins is well documented in other cancers.[43,44] There are reports indicating a constitutively activated NF-κB in epithelial cell carcinomas similar to our observations, however, the composition of NF-κB complex was strikingly different and the DNA-binding activity was predominantly due to conventional p50/p65 heterodimer.[20,45] This dissimilarity clearly shows a different composition of NF-κB compared to what we found in an *in vivo* situation where DNA-binding is primarily due to p50/p50 homodimer in fresh oral cancer biopsies. In agreement with our observation, Budunova and his colleagues[46] have also described a constitutive activation of NF-κB in a mouse skin carcinogenesis model and showed that the complex that binds κB sequence is a p50/p50 homodimer. Since composition of NF-κB regulates the transcriptional specificity,[24,26] it is quite likely that p50/p50 homodimer mediated NF-κB activity provides a better survival and proliferative advantage to the tumor cells than the canonical p50/p65 form in oral carcinogenesis. P50 homodimers transcriptionally regulate antiapoptotic Bcl-2,[47] which has been shown to overexpress in high proportions of oral cancer cells,[48,49] and to inhibit terminal differentiation of oral keratinocytes.[50] Since Bcl-2 expression is

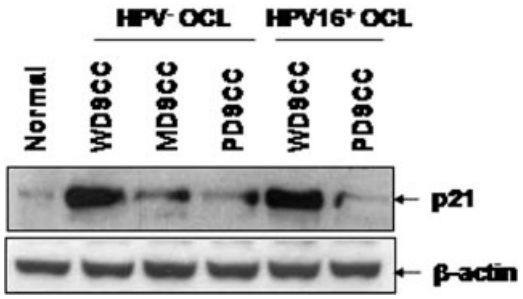

FIGURE 5 – Expression of p21 protein during oral carcinogenesis and effect of HPV infection. A total of 40 μg protein extracts each from normal, HPV negative (HPV^-) Well-Differentiated SCC, Moderately-Differentiated SCC, Poorly-Differentiated SCC oral cancer lesions and HPV16 positive Well-Differentiated SCC, Poorly-Differentiated SCC oral cancer lesion (HPV^+) biopsies were separated on a 12% SDS-PAGE, electrotransfered on PVDF membrane and probed for p21 expression. To confirm equal protein loading, the membranes were re-probed for β-actin expression.

independent of IKK/p65 activation,[51] it provides a selective survival and proliferative advantage to oral keratinocytes.

The exact mechanism by which NF-κB is constitutively activated in oral carcinoma cells is not yet fully understood but it has been suggested that autocrine expression of IL-1 and EGFR may play an important role in the activation of NF-κB.[52,53] Chromatin immunoprecipitation studies have demonstrated that p50 homodimer along with Bcl-3 binds to NF-κB consensus motifs within *egfr* promoter in nasopharyngeal carcinoma.[54] Some reports have indicated that the enhanced binding activity may be due to increased IKK and PI3 Kinase activity in oral cancer.[55,56] However, recent reports indicate that only PI3 Kinase/MEK/ERK is involved,[51,57] but IKK activity may not be required for the high binding activity of p50 homodimer.[51,58]

In our immunohistochemistry study the most intriguing observation is the presence of highly widespread koilocytes (see Fig. 4*b*) in the oral carcinoma cells. In cervical carcinoma, infection by a high-risk HPV type is an absolute requirement for malignant progression[4] whereas in oral cancer it is not. Thus, the cervical carcinoma cells are known to be the most suitable host for infection by this subset of genital HPVs than are normal oral keratinocytes. However, during progression of cervical lesions koilocytes can only be seen in HSIL and sometimes in carcinoma cells and these are certainly not as widespread as seen in oral carcinomas. This is indicative of an interesting finding that oral carcinoma cells are fully capable of supporting productive HPV infection. This needs further investigation.

Both immunoblotting and immunohistochemistry data demonstrate a differential increase in the expression of all the members of NF-κB family proteins during development of oral cancer with most remarkable increase in p50 and c-Rel expression. A significant increase of p50 expression has been shown to occur during murine oral carcinogenesis.[46] Similar increased expression of p50 has been recently observed in human cervical carcinoma.[17] Among the genes which are known to be positively regulated by p50/p50 homodimers are *TNF*α, *MHC Class I* and its own gene *NF-κB1*.[27,28,59] Thus it is very likely that oral neoplastic cells having constitutively active p50/p50 homodimers positively regulate the p50 expression and show highly increased p50 protein. Similarly, we also observed consistently a gradual increase in c-Rel expression during progression of oral lesions. c-Rel has been demonstrated to control epidermal development and homeostasis in embryonic and adult skin.[60] The overexpression of c-Rel has been implicated in hematopoietic and solid malignancies as well.[61–63] Our observation along with these data, strongly support an important role of c-Rel in oral carcinogenesis. However, our supershift assays failed to show its involvement in NF-κB DNA-binding activity. Thus, no correlation was observed between increased expressions, it's nuclear accumulation and participation in DNA-binding activity, and clinical outcome indicating that c-Rel is either not a functional target for oral carcinogenesis or it acts thorough some other mechanism yet to be identified.

In contrast to p50 and c-Rel, available literature on p65 expression indicates contradictory reports. Either no change[46] or mild[64] or even high expression of p65[55,65] in different oral cancer model systems have been reported. We find only a small proportion of cancer and precancerous lesions express moderate to high level of p65, but majority of lesions show a very low expression of p65 that too localized in the cytoplasm. Therefore, p65 even though present in the cancer cells does not take part in NF-κB complex formation in HPV negative oral cancers and hence it is unlikely to play a significant role in transcriptional regulation during oral tumorigenesis. Interestingly, our supershift assay and immunohistochemistry analysis demonstrate for the first time a partial nuclear involvement of p65 in NF-κB complex formation only in HPV16 positive oral cancer.

Activation of NF-κB is a hallmark of most viral infections. Viral infection directly or indirectly has been shown to activate canonical p50/p65 heterodimer in the NF-κB complex.[66,67] Several proteins encoded by p50/p65 target genes participate in the activation of host immune response.[23] Our study indicates a small but significant and consistent nuclear localization of p65 and its involvement in DNA-binding activity in HPV^+ oral cancer lesions indicating host's immune response to HPV infection. A functional NF-κB binding site has been detected in HPV16 LCR region which, if bound to p50/p65 heterodimer, acts as a transcriptional repressor of HPV.[19] However, as described by these authors, the affinity of this site is much lower in comparison to consensus κB promoter site. It was also observed that NF-1 binding site overlaps this NF-κB site which upregulate the LCR activity.[68,69] Thus, NF-κB p50/p65 apparently possesses a negative regulatory activity against HPV which p50/p50 homodimer does not execute due to lack of transactivation domain. However, this phenomenon was found limited only to infection by high risk HPV (HPV16) and could not be seen in infections with low risk HPVs (HPV6 and 11) which were found only in pre-cancerous lesions. This observation also gains support from a previous report which demonstrated no involvement of p65 in NF-κB complex in laryngeal papilloma infected with HPV types 6 and 11.[70]

Along with an increased expression of most of the NF-κB members, results show a small reduction in NF-κB DNA binding activity despite the presence of p65 in the nuclei of $HPV16^+$ oral cancer lesions (Fig. 3). Similarly, an enhanced expression of the functional components of NF-κB has been observed in HPV-infected tumor and showed a highly reduced DNA-binding activity.[71,72] To counter the cellular immune response through p50/p65, HPV mediate suppression of NF-κB activity by its E7 oncoprotein which supress IKK activity,[21] inhibit Type I Interferon signals and decrease the expression of IFN inducible genes.[21,71,73] On the other hand, E6 has been shown to enhance the expression of p50 and its binding to NF-κB consensus sequences[71] and it reduces transcriptional activity of p50/p65 by competitive inhibition.[21,74] Interestingly, E5 and E7 also inhibit the expression of MHC I proteins[71] which facilitate immune recognition through viral antigen presentation. In addition, HPV to escape the immune recognition requires PI3 Kinase activation,[75] which is an upstream activator of p50 homodimer.[51,57] Therefore, oral cancer cells provide appropriate environment for viral entry and persistence of HPV infection. These observations can potentially explain the productive viral infection as indicated by presence of widespread koilocytes despite presence of activated p50/p65.

Although the sample size is much less to conclude, our data do indicate a higher percentage of HPV16 positivity associated with well-differentiated SCC than the moderately or poorly-differentiated SCC which are more advanced stages of the disease and show poor prognosis. With an exception of only one report,[76] similar observations have also been made consistently in oral and other cancers which showed better overall survival of HPV-infected patients than those with HPV-negative tumors[5–8] and they showed well-differenti-

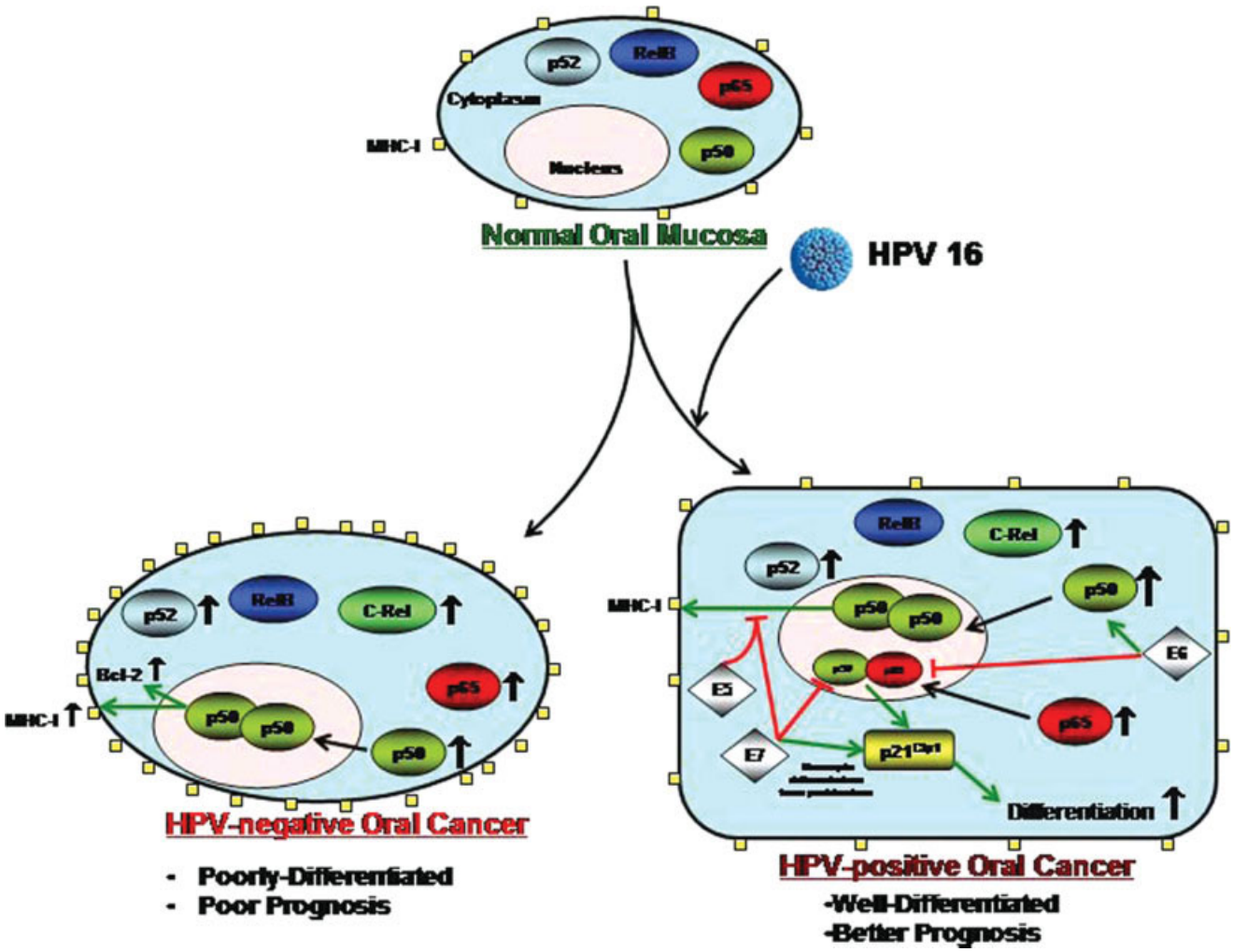

FIGURE 6 – Schematic presentation showing the role of NF-κB during progression of oral cancer lesions and its modulation by HPV16 infection. Constitutive activation NF-κB with involvement of p65 in DNA-binding activity during HPV16-infection presumably increase the expression of $p21^{Cip1}$, which may be responsible for better differentiated state in HPV infected oral lesions and thus improves prognosis and the survival of the patients. Key: □, MHC class I molecules; ◇, HPV proteins; Black arrows, nuclear translocation; Red arrows, Inhibition; Green arrows, activation; Vertical arrows, increased expression.

ated tumors.[9] We show for the first time an involvement of p65 in the constitutively active NF-κB complex generated in HPV16+ lesions. Since HPV infection positively correlates with better prognosis of patients, we hypothesize that p65 participation in NF-κB might contribute to more differentiated state of the carcinoma. However, the mechanism(s) underlying these observations has been unclear as yet. It is interesting to note that NF-κB induces the expression of $p21^{Cip/Waf1}$, a cdk inhibitor which is intimately involved in coupling growth arrest to cellular differentiation in normal epithelial cells.[77,78] In addition, patients with $p21^{Cip/Waf1}$ expression tend to have a better prognosis than those without in endometrial carcinomas.[79] However, during HPV16 infection, E7 oncoprotein has been shown to uncouple cellular differentiation and proliferation in human keratinocytes by abrogating $p21^{Cip1}$-mediated inhibition of cdk2, while differentiation mechanisms remain intact[80,81] (see Fig. 6). Our results also indicate an over-expression of p21 in well-differentiated oral squamous cell carcinomas (WDSCCs) which show better prognosis, whereas the levels of p21, which is required for normal differentiation, was found to be low in normal oral tissue. Since the protein is required only transiently, it is likely that only a subset of cells are expressing p21 in normal tissue in contrast to well-differentiated carcinomas which has constitutively active NF-κB. $p21^{Cip1}$ has been shown to be over-expressed in the HPV infected laryngeal papillomas.[70] However, we did not see any change in the expression pattern between HPV negative and HPV16 positive oral carcinomas. Further studies are being carried out at our laboratory to establish a correlation between p21 expression and HPV infection in oral cancer.

In conclusion, we show for the first time that the expression of NF-κB family of proteins along with constitutive activation NF-κB complex of comprising mainly p50/p50 homodimers increased as a function of severity of oral lesions during development of oral cancer. Infection of high risk Human Papillomavirus (HPV) promotes participation of p65 in the DNA binding activity. We propose a plausible pathway (see Fig. 6) wherein the involvement of virus-induced host's response activates p50/p65 NF-κB complex formation, which promotes differentiation of oral neoplastic cells, leading to better prognosis of the disease. Further investigations are being aimed at establishing this hypothesis and to dissect out the mechanism(s) involved in active human papillomavirus replication/transcription resulting in extensive koilocyte formation specifically in oral keratinocytes.

Acknowledgements

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