UICC global cancer control

IJC International Journal of Cancer

Transactivation and expression patterns of Jun and Fos/AP-1 super-family proteins in human oral cancer

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Transcription factor activator protein-1 (AP-1) super-family is known to modulate expression of array of genes during development of many cancers and considered as an important target for modern therapeutics. But the role of AP-1 during development of human oral cancers is still poorly understood. Because oral cancer is one of the most common cancers in India and south-east Asia, we studied the activation and expression pattern of AP-1 family of proteins and mRNA in different stages of oral carcinogenesis. Gel-shift assay, western blotting, immunohistochemistry and northern blotting have been used to assess the binding activity and expression pattern of AP-1 family (c-Jun, JunB, JunD, c-Fos, FosB, Fra-1 and Fra-2) proteins and mRNA transcripts in a total of 100 fresh oral tissue specimens comprising precancer ($n = 40$), cancer ($n = 50$) and healthy control ($n = 10$). Constitutive activation of AP-1 with concomitant upregulated expression of majority of AP-1 family of proteins and mRNA was observed in cancer cases. Interestingly, almost all precancerous cases showed JunD homodimers, whereas c-Fos/JunD was the most prevalent complex found in cancer tissues. The overexpression of EGFR mRNA, p50:p50/NF-κB homodimer formation, together with overexpression of pERK and c-Fos proteins in this study suggests an interesting cross talk between AP-1 and NF-κB pathways in oral cancers. Thus, this study demonstrates differential expression and activation of AP-1 super-family proteins in relation to severity of lesion and their crucial role in human oral carcinogenesis.

Oral squamous cell carcinoma (OSCC) is the sixth most common cancer and accounts for ~5% of all malignant tumors worldwide.[1] In India and South East Asia, it is the most common malignancy accounting to 50% of all malignant tumors. Most of the OSCC is attributed to smoking and alcohol consumption, whereas a proportion of oral cancers have been demonstrated to contain anogenital HPV infections.[2,3]

The activator protein-1 (AP-1) super-family of transcription factor is a dimeric protein complex of structurally and functionally related members of Jun, Fos, ATF (activating transcription factors) and MAF (musculoaponeurotic sarcoma) protein families. The homodimerization of Jun proteins (c-Jun, JunB and JunD) or hetrodimerization of Jun and Fos proteins (c-Fos, FosB, Fra-1 and Fra-2) generates a transcriptionally active complex interacting through basic "leucine zipper" motif. AP-1 dimers regulate downstream target gene through interaction with DNA backbone of selective 8 base pair conserved sequence 5′TGAGCTCA 3′ recognized as the TPA (12-*O*-tetradecanoyl phorbol 13-acetate) response element (TRE) of the regulatory sequences of the wide arrays of different cellular and viral genes.[4,5] In addition to tumor promoters, the DNA binding of the AP-1 complex to the TRE sequence is rapidly induced by several growth factors, cytokines and oncoproteins, implicated in the proliferation, survival, differentiation and transformation of cells.[4,6] Because DNA binding is a necessary prerequisite of transactivation, the expression of different proteins of the Jun and Fos family is crucial for the activation of downstream genes regulated by AP-1. AP-1 is also known to control the expression of several target genes that regulate cell cycle (*cyclin D1*, *p16*), differentiation (*myogenin* and *involucrin*), cell survival (*Bcl-2*, *Bcl-xL* and *FasL*), growth factors (*VEGF*), cell adhesion (*VCAM* and *ECAM-1*) and angiogenesis/invasion (*MMPs*, *uPA*, *osteopontin* and *CD44*). As each of the AP-1 family protein is differentially expressed resulting in subtly different functions and in view of heterogeneity in AP-1 complex composition, it is interesting to investigate the overall expression and transactivation pattern of AP-1 proteins in oral carcinogenesis. Dysregulated activation and aberrant expression pattern of AP-1 proteins have been observed in several human cancers including head and neck cancer[7,8] and oral cancer[9–11] mainly on cell lines and paraffin sections, but,

Key words: oral cancer, human biopsies, carcinogenesis, AP-1, constitutive activation, differential expression

Abbreviations: EMSA: electrophoretic mobility shift assay; HNSCC: head and neck SSC; OCL: oral cancer lesion; OSSC: oral SSC; PCL: precancer lesion; SCC: squamous cell carcinoma

Grant sponsor: Indian Council of Medical Research (ICMR)

DOI: 10.1002/ijc.24807

History: Received 14 Apr 2009; Accepted 23 Jul 2009; Online 3 Aug 2009

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to the best of our knowledge, this study is the first comprehensive and detailed analysis defining the role of AP-1 superfamily proteins based on fresh clinical tissue specimens from oral precancerous and cancer patients.

Therefore, in this study, we have analyzed the expression pattern and DNA binding activity of Jun and Fos members of AP-1 family proteins during human oral carcinogenesis using tissue biopsies of different histopathological grades.

Material and Methods

Tissue specimens

A total 100 fresh oral tissue biopsies were collected comprising 50 malignant, 40 premalignant and 10 normal (control) oral tissues from the Department of ENT surgery, LNJP hospital, New Delhi, after informed consent from subjects prior to any chemo/radio therapy. The clinico-epidemiological characteristics are presented in Table 1. Half portion of biopsies collected in cold 1X phosphate buffer saline (PBS) was immediately processed for molecular biological works, and the other half was sent for histo-pathological diagnosis in formalin solution.

Table 1. Clinico-pathological characteristics of tissue biopsies from subjects recruited for studies

Characteristics	NM	PCL	OCL
Number of biopsies	10	40	50
Mean age (years)	44.4 ± 12.5	46.2 ± 6.3	50.6 ± 7.6
Male: female ratio	4:1	7:1	6.5:1
Tumour sites			
Tongue	4	18	28
Mandibular gingiva	1	5	5
Maxillary gingiva	0	3	4
Buccal mucosa	3	7	7
Palate	0	2	2
Lips	2	5	4

The numbers indicate total cases in each category. Abbreviations: PCL, precancerous lesions (hyperplastic and dyplastic lesions including leukoplakia); OCL, oral cancer lesions; NM, normal mucosa.

Preparation of protein extract

Protein extracts from biopsies were prepared by the method of Dignam[12] with certain modifications.[13] Briefly, the method involved fine mincing of either fresh tissue or frozen tissue biopsies stored at −80°C, in cold 1X PBS with surgical blade in petridish on ice. The minced tissue material was later centrifuged at 4,000 rpm at 4°C to wash off 1XPBS solution. The pellet was resuspended 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, 1mM DTT, 1 mM PMSF, 2 mg/ml leupeptin and 10 mg/ml aprotinin] and incubated on ice for 10 min with frequent vortexing. Lysate was further centrifuged at 4,000 rpm for 10 min at 4°C to obtain supernatant as cytoplasmic extract. The remaining pellet containing isolated nuclei was resuspended in 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 mg/ml leupeptin and 10 mg/ml aprotinin] and centrifuged after incubation for 1 hr with repeated vortexing on ice at 14,000 rpm for 25 min at 4°C to obtain supernatant designated as nuclear extract. The concentration of protein extracts was determined by spectrophotometic method, and the extracts were stored in aliquots at −80°C till further use.

Electrophoretic mobility-shift assay

Consensus oligonucleotides of AP-1: 5′CGCTTGATGACTCAGCCGGAA-3′, Oct-1: 5′-TGTCGAATGCAAATCACTAGAA-3′ and NF-κB: 5′AGTTGAGGGGACTTTCCCAGGCC-3′ synthesized by Applied Biosystems, and annealed oligonucleotide were labeled with [γ-^{32}P] ATP (3000 Ci/mmol, Jonaki, India) with T4 polynucleotide kinase. The binding reaction and competition assays were performed to determine the specificity of DNA probes as described earlier.[14] For monitoring composition of AP-1, NF-κB and Oct-1, following antibodies of Santa Cruz Biotechnology were used: c-Jun (sc-45), JunB (sc-73), JunD (sc-74), c-Fos (sc-253), FosB (sc-48), Fra-1 (sc-605), Fra-2 (sc-171), p50 (sc-114), p65 (sc-109), p52 (sc-298), c-Rel (sc-70), RelB (sc-226) and Bcl-3 (sc-185). The quantitative densitometry analysis was performed using Alpha Ease FC version 4.1.0 (Alpha Innotech Corporation, IL).

Hybridization probes and Northern blot hybridization

Plasmid harboring cDNAs for *c-fos*, *junD* and *egfr* genes were kindly provided by Peter Angel (DKFZ, Germany), for the *fra-1* gene by M. Seiki, (Cancer Research Institute, Japan) and for β-actin by L. Kedes (Medical Center, Palo Alto, CA). Probes were labeled according to manufacturer's protocol (Bangalore Genei, India). Total RNA was extracted by TRI Reagent as per instruction manual (Sigma, USA). Northern blotting was carried out by resolving ∼15 μg of RNA on 1% agarose-MOPS-formaldehyde gel. Capillary blotted membrane was washed in 6× SSC, air dried, exposed in phosphorimager (Fujifilm FLA-5100) after prehybridization and hybridization in Perfect HYB-PLUS (Sigma) solution as suggested by manufacturer's protocol.

Quantification of signals of mRNA in Northern blots was performed by utilizing ImageJ software (Version 1.41, NIH, USA) for standard densitometric analysis. The intensity of signals from transcripts was expressed as the mean ± standard deviation (SD).

Immunoblotting

Protein extracts (50 μg/lane) were separated in 8–12% polyacrylamide gel and electrotransferred on PVDF membranes (Millipore, Bedford, MA, USA). The membrane was blocked with 5% milk and incubated overnight in PBS with 5% milk, 0.05% Tween 20 and primary antibody at 4°C. These blots

Table 2. Expression of AP-1 family proteins in normal oral mucosa and precancerous and cancerous lesions[1] as observed in western blotting

	Normal (n = 10)				Precancerous lesions (n = 40)				Cancerous lesions (n = 50)				
Proteins	Nil (–)	Weak (+)	Medium (++)	Strong (++++)	Nil (–)	Weak (+)	Medium (++)	Strong (++++)	Nil (–)	Weak (+)	Medium (++)	Strong (++++)	p value
C-Jun	–	3 (30.0)	7 (70.0)	–	2 (5.0)	4 (10.0)	28 (70.0)	6 (15.0)	–	2 (4.0)	10 (20.0)	38 (76.0)	0.36[2]
													0.03[3]
													0.13[4]
JunB	–	8 (80.0)	2 (20.0)	–	1 (2.5)	1 (2.5)	30 (75.0)	8 (20.0)	–	–	5 (10.0)	45 (90.0)	**<0.0001**[2]
													<0.0001[3]
													0.19[4]
JunD	1 (10.0)	6 (60.0)	2 (20.0)	1 (10.0)	1 (2.5)	3 (7.5)	30 (75.0)	6 (15.0)	–	–	10 (20.0)	40 (80.0)	**0.0003**[2]
													<0.0001[3]
													0.04[4]
c-Fos	6 (60.0)	4 (40.0)	–	–	–	2 (5.0)	28 (70.0)	10 (25.0)	–	–	5 (10.0)	45 (90.0)	**<0.0001**[2]
													<0.0001[3]
													0.19[4]
Fra-1	–	–	4 (40.0)	6 (60.0)	8 (20.0)	22 (55.0)	10 (25.0)	–	43 (86.0)	7 (14.0)	–	–	**<0.0001**[2]
													<0.0001[2]
													<0.0001[2]
Fra-2	2 (20.0)	3 (30.0)	4 (40.0)	1 (10.0)	2 (5.0)	9 (22.5)	25 (62.5)	4 (10.0)	2 (4.0)	5 (10.0)	18 (36.0)	25 (50.0)	0.26[2]
													0.02[3]
													0.12[4]
FosB	1 (10.0)	4 (40.0)	3 (30.0)	2 (20.0)	8 (20.0)	10 (25.0)	12 (30.0)	10 (25.0)	10 (20.0)	9 (18.0)	15 (30.0)	16 (32.0)	1.0[2]
													0.50[3]
													0.53[4]

[1]Arbitrary level of expression in immunoblotting: strong = ++++; medium = ++; weak = +; nil/not detectable. p value, probability from Fischer's exact test (using the approximation of Woolf) comparing the expression of proteins (Nil + low *vs*. moderate + strong) among. [2]precancer *versus* controls. [3]cancer *versus* controls. [4]cancer *versus* precancer. Bold type refers to statistically significant results.

Table 3. Expression of AP-1 super-family members in different grades of oral tissue biopsies as visualized by immunohistochemistry

	Normal $N = 10$ (%)				Precancerous lesions $N = 20$ (%)				Cancerous lesions $N = 20$ (%)				
Proteins	Nil (−)	Weak (+)	Medium (++)	Strong (++++)	Nil (−)	Weak (+)	Medium (++)	Strong (++++)	Nil (−)	Weak (+)	Medium (++)	Strong (++++)	*p* value
C-Jun	7 (70.0)	2 (20.0)	1 (10.0)	–	3 (15.0)	4 (20.0)	11 (55.0)	2 (10.0)	1 (5.0)	4 (20.0)	4 (20.0)	11 (55.0)	**0.006**[2]
													0.001[3]
													0.73[4]
JunB	8 (80.0)	1 (10.0)	1 (10.0)	–	2 (10.0)	2 (10.0)	11 (55.0)	5 (25.0)	–	2 (10.0)	6 (30.0)	12 (60.0)	**<0.0004**[1,2]
													<0.0001[3]
													0.66[4]
JunD	7 (70.0)	2 (20.0)	1 (10.0)	–	1 (5.0)	4 (20.0)	9 (45.0)	6 (30.0)	1 (5.0)	3 (15.0)	4 (20.0)	12 (60.0)	**0.001**[2]
													0.0004[3]
													1.0[4]
c-Fos	8 (80.0)	2 (20.0)	–	–	–	1 (5.0)	16 (80.0)	3 (15.0)	–	1 (5.0)	–	19 (95.0)	**<0.0001**[1,2]
													<0.0001[3]
													1.51[4]
Fra-1	–	1 (10.0)	2 (20.0)	7 (70.0)	2 (10.0)	2 (10.0)	10 (50.0)	6 (30.0)	15 (75.0)	4 (20.0)	1 (5.0)	–	0.64[2]
													<0.0001[2]
													<0.0001[2]
Fra-2	5 (50.0)	2 (20.0)	2 (20.0)	1 (10.0)	2 (10.0)	4 (20.0)	10 (50.0)	4 (20.0)	3 (15.0)	2 (10.0)	5 (25.0)	10 (50.0)	0.06[2]
													0.04[3]
													1.0[4]

[1]Arbitrary level of expression in immunoblotting: strong = ++++; medium = ++; weak = +; nil/not detectable. *p* value, probability from Fischer's exact test (using the approximation of Woolf) comparing the expression of proteins (nil + low *vs*. Moderate + strong) among. [2]precancer *versus* controls. [3]cancer *versus* controls. [4]cancer *versus* precancer. Bold type refers to statistically significant results.

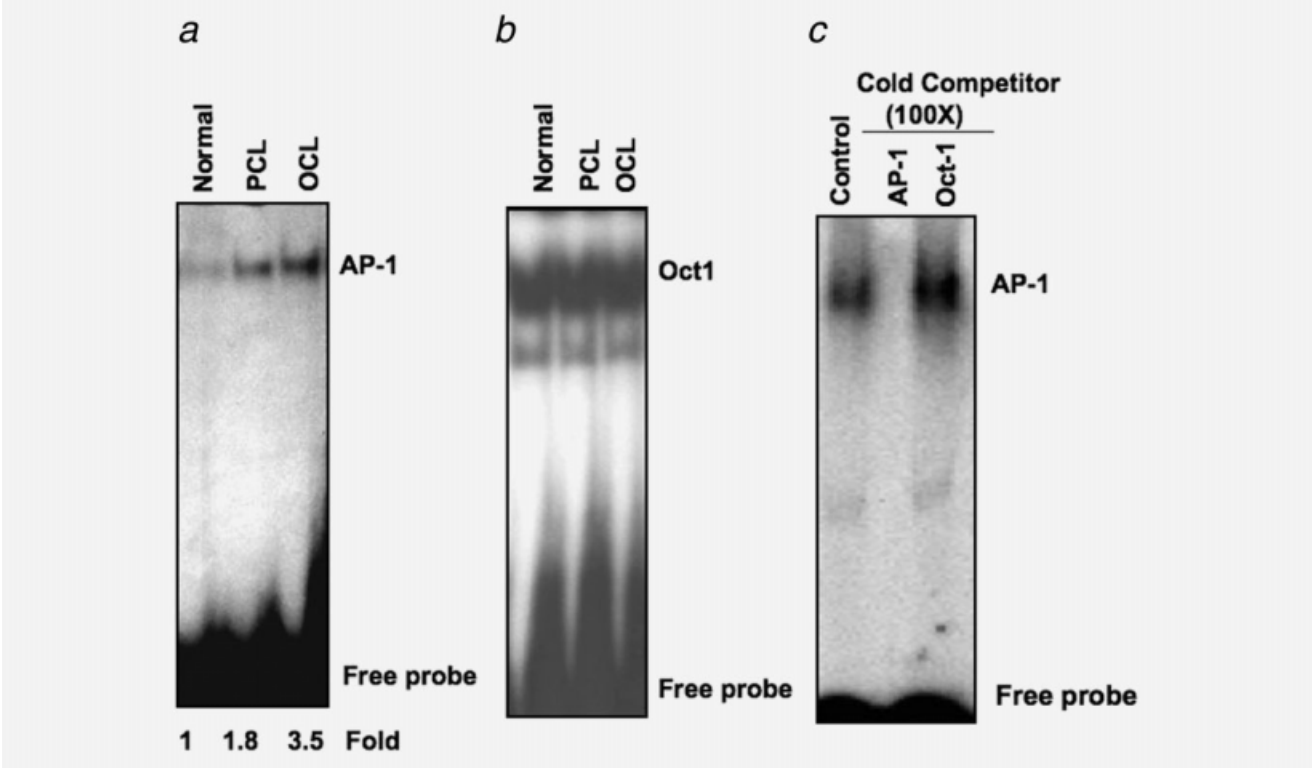

Figure 1. Constitutive AP-1 activation in malignant oral biopsies. (*a*) EMSA of the different grades of oral biopsies with [γ-^{32}P] ATP-labeled AP-1 oligonucleotide. Cancerous lesion (OCL) shows highest AP-1-binding activity. PCL, precancerous lesion. (*b*) EMSA with labeled Oct-1 probe showing uniform binding in different grades of biopsies. (*c*) Binding of AP-1 to DNA probe is sequence specific. Binding specificity was evidenced in nuclear extracts of malignant tissues incubated with unlabelled 100 molar excess of specific competitor (AP-1) probe in comparison with competition experiment using nonspecific competitor (Oct-1) and then checked for specific AP-1 binding by EMSA. Band intensities shown were quantified as described in text.

were washed, incubated with HRP-anti-rabbit IgG secondary antibodies and visualized by Luminol detection kit (Santa Cruz Biotech). Membrane was probed for β-actin expression as control. The expression level of proteins was quantitated on an arbitrary scale where strong = ++++; medium = ++; weak = + and nil/not detectable (Table 2).

Immunohistochemistry

The immunohistochemical staining was performed as following: after deparaffinization and rehydration, heat-induced epitope retrieval was done in the 10 mM citrate buffer (pH 6.0). Nonspecific binding site was blocked using 1.5% blocking serum and after incubating overnight in primary antibody, immunoreactivity was visualized according to manufacturer protocol (ABC staining kit, Santa Cruz Biotech). Intensity scoring of expression (Table 3) was performed using an arbitrary semiquantitative scale: none (−); low (+); moderate (++) and high (++++).

Statistical analysis

The data analysis was performed using the computer software Graph Pad Instat (version 4.0). Fisher's exact test (for smaller numbers on subgroup analysis) was used to compare the expression of proteins among different histopathological grades of tissue biopsies. p values (2-tailed) of <0.05 were considered statistically significant.

Results

The DNA-binding activity of transcription factor AP-1 dimers and the expression profile of its subunits c-Jun, JunB, JunD, c-Fos, FosB, Fra-1 and Fra-2 were analyzed in all spectrums of fresh oral tissues from different sites of oral cavity as indicated in Table 1. Mean age (±SD) and male: female gender ratio varied from 44.4 ± 12.5 years and 4:1 in control subjects, 46.2 ± 6.3 years and 7:1 in precancer and 50.6 ± 7.6 years and 6.5:1 in cancer cases, respectively.

Constitutive activation of AP-1 in oral cancer

The relative DNA binding activity of AP-1 complex was compared in nuclear extracts from tissue biopsies of normal, precancerous (PCL) and cancerous lesions (OCL) using [γ-^{32}P] ATP-labeled probe harboring AP-1 consensus sequence by EMSA. Malignant oral tissues showed a prominent DNA binding activity of AP-1, whereas moderate binding was also observed in all the precancerous lesions (PCLs). In contrast, nuclear extract from normal tissues did only show very weak detectable level of AP-1 activity (Fig. 1*a*).

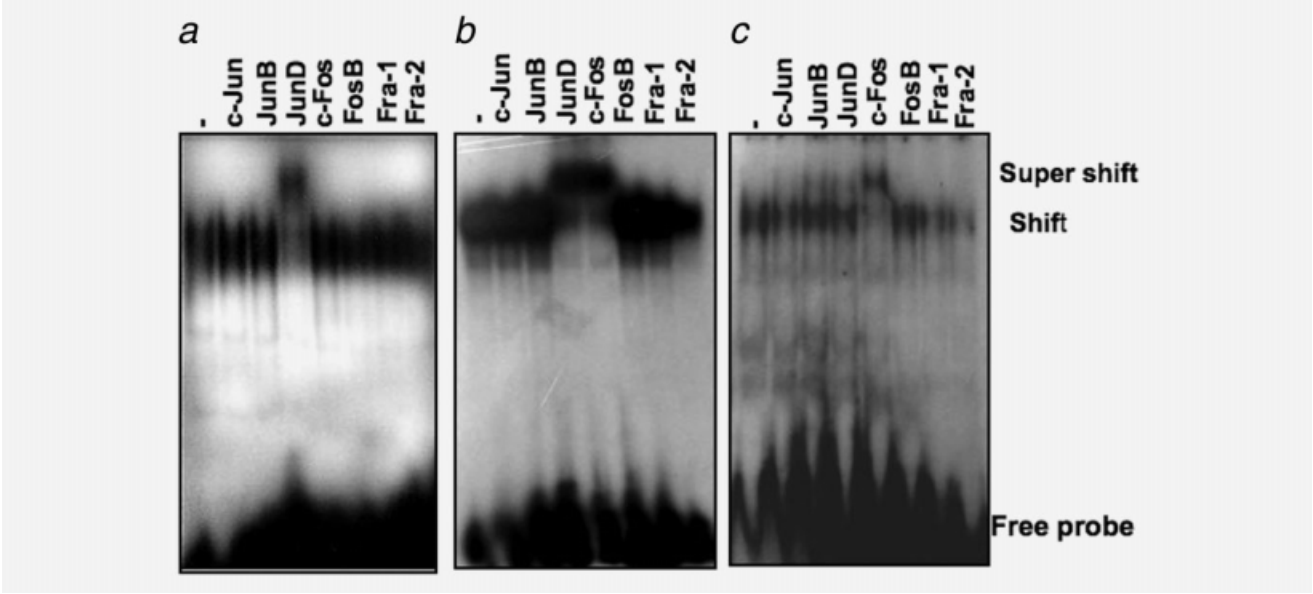

Figure 2. Alteration in composition of DNA binding AP-1 complex during neoplastic progression of oral tissue. (*a*) Precancerous lesions to (*b* and *c*) cancerous lesions. Supershift analysis using nuclear extracts (10 μg) from PCL (*a*) and OCL (*b*, *c*) with specific antibodies (2 μg each) either against Jun and Fos members.

Thus, the DNA-binding activity of AP-1 showed a gradual increase with the increasing severity of oral lesions. But no difference in the binding activity was observed between normal, PCL and oral cancer lesion (OCL) when Oct-1 was used as a probe (Fig. 1*b*), indicating that the modulation of binding activity was AP-1 specific in oral cancers. The binding specificity of AP-1 protein complex to its cognate DNA was also confirmed by competition assay using a 100-fold molar excess of cold, specific competitor probe of AP-1 and nonspecific competitor, a heterologous probe of transcription factor Oct-1 as described earlier[13] (Fig. 1*c*).

Alteration in composition of AP-1 complex during oral cancer progression

To understand the role of elevated binding of AP-1 transcription factor in premalignant (Fig. 2*a*) and malignant lesions (Figs. 2*b* and 2*c*), AP-1 complexes were further dissected to identify participating AP-1 members using elelectrophoretic mobility supershift assay. The supershift analysis in majority of the premalignant cases ($n = 37$ of 40, 92.5%) has shown the presence of JunD/JunD homodimers in the AP-1 complex. But to our surprise, no other Jun or Fos members were observed to be involved in DNA binding in any preneoplastic lesions. Interestingly, in majority of cancer cases (31 of 50; 62%) a preferential heterodimerization between c-Fos and JunD (Fig. 2*b*) instead of canonical dimerization of c-Jun with c-Fos, was observed. Among some of tumor samples ($n = 19$ of 50, 38%), JunB member has also participated in complex formation as a minor binding partner of c-Fos (Fig. 2*c*). We observed that in all malignant tissues analyzed showing c-Fos/JunB/JunD complex (Fig. 2*c*), more than 70% of supershifted band was formed by c-Fos, while only 50% of

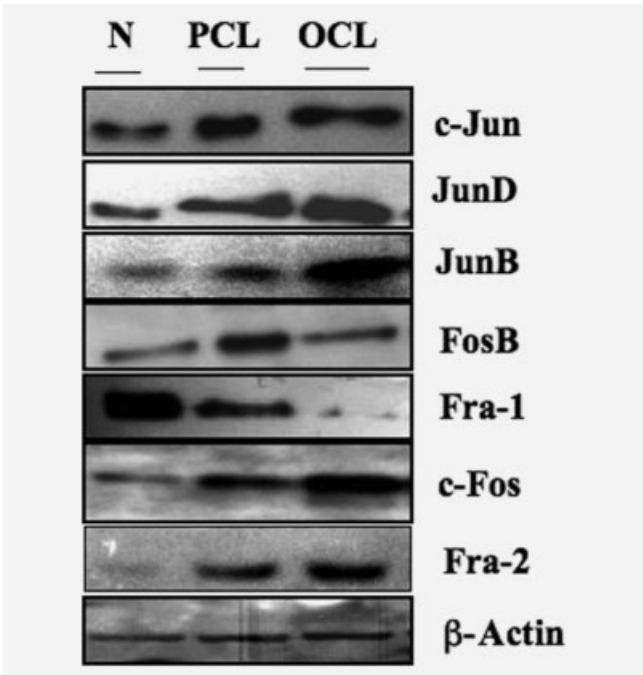

Figure 3. Immunoblotting showing differential expression pattern of AP-1 members in different spectrum of oral lesions. Increased expression of c-Jun, JunD, JunB, c-Fos and decreased expression of Fra-1 in oral cancer lesions. Fifty micrograms protein extracts each from normal, precancerous PCL and OCL cases were resolved on an 8–10% SDS–PAGE, electrotransferred on PVDF membrane and probed with antibodies described in text. Equal protein loading, confirmed by reprobing membrane with β-actin expression.

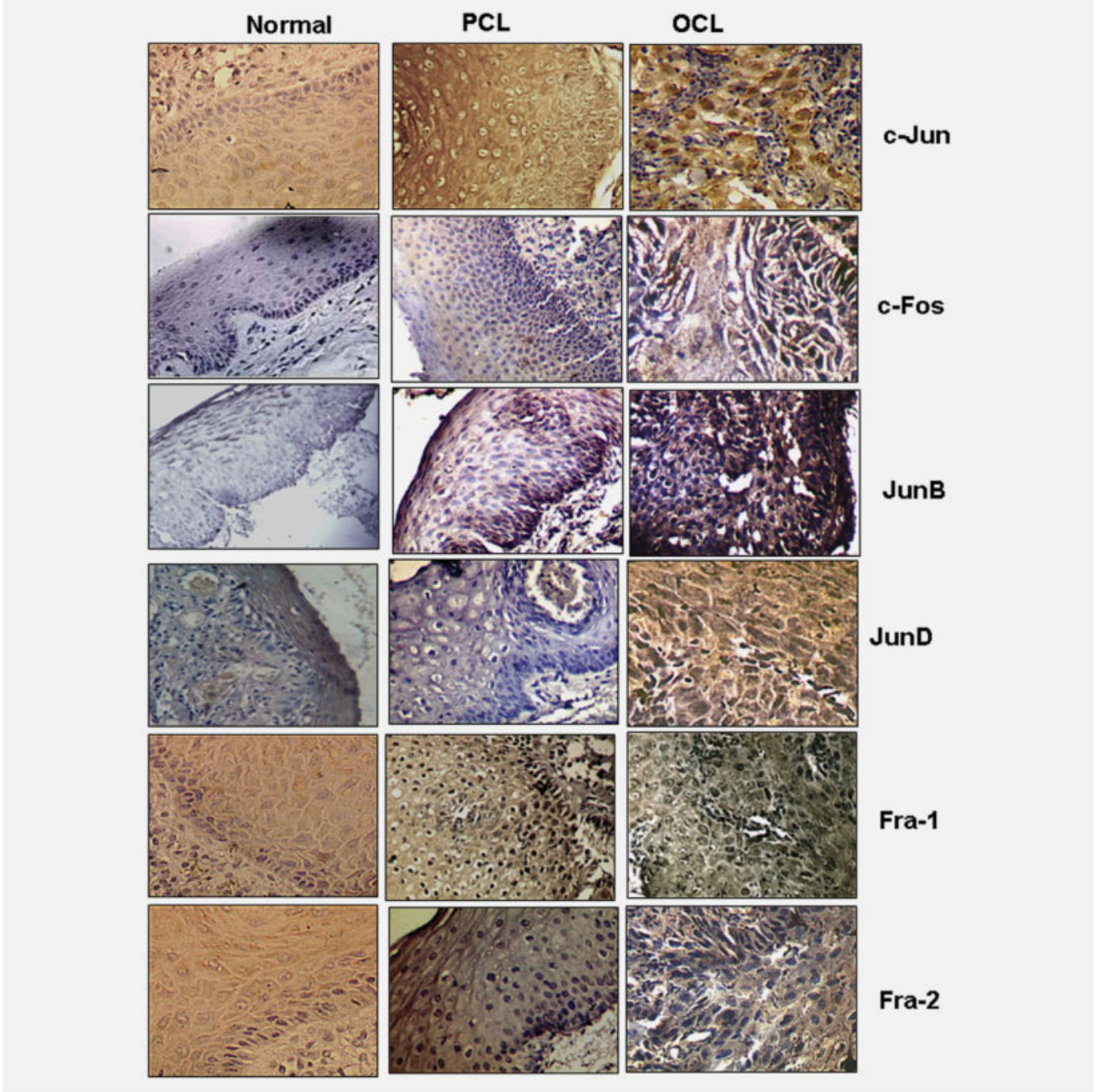

Figure 4. Representative photomicrographs of Immunohistochemical analysis of AP-1 proteins in biopsies. Paraffin embedded (5 μm) oral tissue sections of normal mucosa, precancerous lesion (PCL) and oral cancerous lesion (OCL) were probed with Jun and Fos antibodies as described in method section (Original magnification: 200×). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the supershifted band of JunD/c-Fos complex was constituted by c-Fos member only (Fig. 2*b*) indicating strongly the primary role of c-Fos protein in generation of AP-1 complex in oral cancer tissues.

AP-1 family proteins show differential expression pattern during oral carcinogenesis

Western blotting (Fig. 3 and Table 2) and immunohistochemistry experiments (Fig. 4 and Table 3) were performed to analyze the level of expression of AP-1 super-family proteins. Western-blotting analysis had shown that among AP-1 members, c-Fos, JunD and JunB proteins were found to be significantly overexpressed ($p < 0.0001$) in malignant tissues when compared with those of controls. A distinct gradual up-regulated expression was observed, as the lesions progressed towards malignant changes. Interestingly, a complete opposite trend was noticed for Fra-1 protein, which showed a very high expression in all normal oral tissues but gradually

decreased as the lesions progressed and became nil in cancer cases ($p < 0.0001$ in normal *vs.* cancer; Table 2). Although c-Jun and Fra-2 were also found to be upregulated in cancerous lesions ($p = 0.03$, c-Jun; $p = 0.02$, Fra-2), they were not elevated remarkably when compared with the high-expression levels of c-Fos, JunD and JunB in the same samples. To our surprise, JunD, the only DNA-binding constituent of AP-1 complex of all precancerous cases had not shown any remarkable overexpression when compared with the cancer cases while corroborating its overexpression (Fig. 3) with transactivation pattern (Fig. 2*b*). An inconsistent pattern of FosB protein expression was also observed in all spectrums of oral tissue biopsies from normal, premalignant to malignant lesions.

The expression profile of AP-1 protein members was further analyzed *in situ* by immunohistochemistry in paraffin-embedded sections and findings were very much in concordance with western blots. Intensity scoring of protein expression in immunohistochemistry was performed on an arbitrary 4-point scale; none (−); low (+); moderate (++) and high (++++) as described in Methods section. Results along with the statistical analyses are presented in Figure 4 and Table 3.

Expression of c-Fos protein was nil/low in 100% (10 of 10) of the normal tissue sections examined, 80% precancerous biopsies (16 of 20) showed moderate and 95% tumors (19 of 20) showed a high expression of c-Fos ($p < 0.0001$ in cancer *vs.* normal and precancer *vs.* normal). Conversely, for Fra-1, most of the cancerous oral mucosa (19 of 20) showed nil to low expression, 30% (6 of 20) of precancerous tissue showed high positivity, but 70% of normal tissue sections (7 of 10) have showed a high expression ($p < 0.0001$ in normal *vs.* cancer and precancer *vs.* cancer). About 75–90% of the malignant sections showed moderate to high immuno-staining for JunB (18 of 20; $p < 0.0001$), JunD (16 of 20; $p < 0.0004$) and c-Jun (15 of 20; $p = 0.001$) proteins, whereas their expressions were either nil or low in normal as well as precancerous mucosa. Low to high immunoreactivity against Fra-2 antibody was also observed in 85% of cancerous cases (17 of 20; $p = 0.04$) each.

Transcriptional profiling of AP-1 members (c-fos, junD and fra-1) and egfr mRNAs in oral tissue biopsies

The mRNA expression profiles from freshly collected normal, precancerous and cancerous biopsies for *c-fos*, *junD* and *fra-1* genes were further determined by northern-blot hybridization.

Densitometric analysis for mRNA transcripts in cancerous lesions revealed that *c-fos*, *junD* and *egfr* were significantly overexpressed with mean ± SD values of 3.0 ± 1.20, 2.11 ± 0.80 and 1.91 ± 0.73, respectively. On the contrary, *fra-1*expression was found to be gradually downregulated toward malignant transformation of oral lesions with mean ± SD value of 0.28 ± 0.14 (Fig. 5). Interestingly, these members (*c-fos*, *junD* and *fra-1*) have also shown similar trend of expression at their protein level as observed by western blotting (Fig. 3) and immunohistochemistry (Fig. 4), suggesting their regulation at transcriptional level during human oral carcinogenesis.

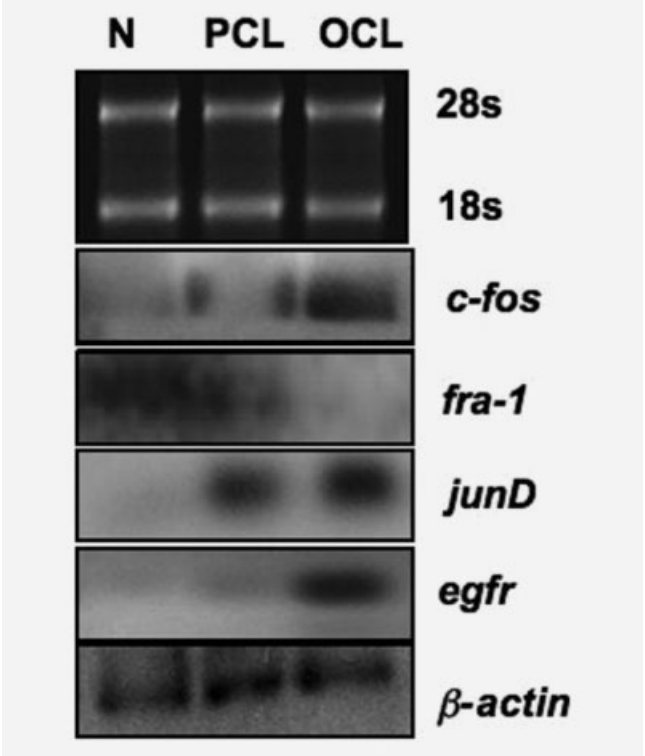

Figure 5. Northern blotting of *c-fos*, *fra-1*, *junD*, *egfr* and β-*actin* mRNA in oral biopsies. Increased expression of c-Fos, junD and egfr and decreased expression of *fra-1* mRNAs during development of cancer lesions. Reprobing filters with β-actin probes confirmed equal loading of mRNA.

c-fos upregulation, activation of NF-κB and induction of EGFR expression are correlated in oral cancer tissues

Interestingly, we have observed that oral cancer biopsies have parallel increase in *c-fos* mRNA (Fig. 5) along with overexpressed *egfr* at both mRNA and protein levels (Figs. 5 and Fig. 6*a*).

Because transcription of *egfr* gene is positively regulated by NF-κB/p50 homodimers with recruitment of Bcl-3 as a coactivator,[15] we further checked the expression and dimerization pattern of NF-κB/p50 member in oral cancer cases. As per our anticipation, upregulation of p50 (Fig. 6*a*) and Bcl-3 (Fig. 6*a*) proteins with p50/p50 homodimers (Fig. 6*b*) was found in malignant specimens. Moreover, addition of anti-Bcl-3 antibody resulted in diminished p50/p50 NF-κB DNA-binding activity in supershift assay in malignant biopsies, as visualized by densitometry analysis (Fig. 6*b*, last lane). This absence of any supershifted band with a diminished DNA binding indicates an interaction of Bcl-3 antibody with DNA-binding motif in p50/p50/Bcl-3 ternary complex present in oral cancer biopsies. To explore further the molecular mechanism involved in the induction of *c-Fos* in OSCC, we

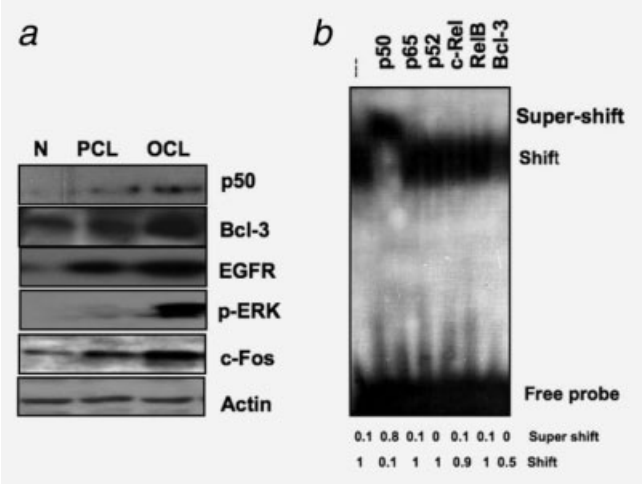

Figure 6. Upregulated c-Fos expression is correlated with NF-κB/p50:p50 homodimer mediated EGFR signals in oral cancer cells. (*a*) Immunoblotting from the same biopsies shows the elevated expression level of p50, pERK, EGFR, Bcl-3 and c-Fos proteins. (*b*) OCL category of biopsies showed the preferential homodimeration of p50/p50 in supershift assay. Nuclear extracts from OCL were incubated with specific antibodies (2 μg each) against each member for NF-κB proteins. Band intensities were quantified as described in Material and methods section of text and indicated.

checked the expression of a critical upstream regulatory MAPKinase-pERK1/2, which is affected by EGFR signals. Western-blot analysis (Fig. 6*a*) has shown that pERK expression was highest in malignant oral cases. Taken together, these data suggest that NF-κB/p50 homodimers activates EGFR expression, which is correlated with c-Fos induction *via* ERK activation in oral cancer tissues.

Discussion

High DNA-binding activity of AP-1 is reported in many epithelial tumors such as cervix, skin, breast and head and neck.[15–17] AP-1 activation is linked with alcohol consumption[9] and HPV infections in oral cancers.[18] In agreement with our data, constitutive activation of AP-1 is also reported in several different head and neck as well as oral cancer cell lines.[7–9]

Assuming that altered composition of AP-1 DNA binding complex regulates its transactivation pattern and consequently expression of downstream targets during carcinogenesis,[19] we have analyzed the composition of AP-1 complex in nuclear extracts from tissue biopsies of different grades.

We found a strong DNA-binding activity in oral cancer tissue contributed by c-Fos/JunD heterodimers in majority of cancerous tissues, whereas involvement of JunB was also noticed only in some of malignant cases. Interestingly, almost all precancerous biopsies have shown only preferential homodimerization of JunD/JunD. A very high expression of c-Fos protein was confirmed in all malignant oral tissues by western blotting and immunohistochemistry experiments, which corroborates the results of supershift assays. Presence of overexpressed c-Fos was expected in oral cancer lesions as it controls several downstream target genes such as *VEGF*, *MMPs*, *collagenase I* involved in invasiveness during development of many cancers including head and neck cancers.[10,11,20,21] We have also seen the presence of JunD with c-Fos in AP-1 complex in majority of oral cancer cases, which again corroborates our IHC and western-blotting data, suggesting gradually upregulated JunD protein during carcinogenesis. The relatively lower DNA binding of JunD containing AP-1 complex in PCL samples but highest in OCL can be explained by the fact that JunD homodimer binds its cognate DNA sequence with lower affinity and also posses lower transactivation potential.[22] Similar to our present report, it has been observed that c-Fos/JunD heterodimer together showed higher transcriptional activity than JunD homodimers.[23] Therefore, we speculate that JunD/JunD homodimer formation might prevent the precancerous cells entering into cancerous condition, but as soon as participation of c-Fos member takes place in AP-1 complex formation, the precancerous cells are pushed further in the aggressive cancerous condition. Moreover, it is also reported that JunD changes functionally from growth suppressor to growth promoter possibly by the formation of mutant form of JunD.[24] Similar to our observation of prevalent c-Fos/JunD complex, the same complex also positively regulates the expression of tyrosinase in melanomas.[25] In our supershift studies of cancerous tissues, involvement of c-Jun and JunB proteins in transcriptionally active AP-1 complex formation is found to be insignificant albeit their consistent overexpression were shown by western blotting and immunohistochemical methods. These results indicate that overexpressed JunD is the main dimerizing partner of c-Fos in the transactivated AP-1 complex. c-Jun/JunB complex in malignant cases demonstrated here are either transcriptionally inactive or have lower DNA binding capacity as reported by others too.[23] Absence of c-Jun in transcriptionally active AP-1 DNA-binding complex might be also possible due to the fact that JunB can substitute c-Jun *in vivo*.[5] In a perfect reverse correlation with upregulated *c-fos* and *junD* expression, the *fra-1* mRNA transcripts has revealed a higher expression in control specimens with a gradual downregulated trend with increasing severity of lesion as seen in many cancers.[15,26] Our observation is in sharp contrast to majority of other reports, which showed a generalized overexpressed Fra-1 in different cancers[20]; but overexpressed c-Fos in oral cancer cases found in our study is in total agreement with other investigators.[8,10,11,27]

In contrast to other reports based on cell lines[7] and tissues biopsies[28] of head and neck cancers, we observed a very distinct downregulated expression of Fra-1 protein as well as mRNA in freshly collected malignant oral tissue biopsies. Although, we understand that a more detailed studies are needed to elucidate the exact molecular mechanism of *fra-1* gene downregulation in oral cancers, some plausible pathways involved are interesting here to mention. The inhibited

positive autoregulation of *fra-1* transcription might be operating in oral cancer cells due to hampered nuclear shuttling of Fra-1 protein.[29,30] Moreover, repressed *fra-1* in oral cancer tissues infected with human Papillomaviruses cannot be ruled out as seen in 1 of the other major human epithelial cancer of uterine cervix in females.[13,15]

Lack of activation domain in Fra-2[31] can explain the absence of DNA binding in our gel-shift assays even it is upregulated in many cancers.[32,33] The low-binding activity of JunB even after its elevated expression can be attributed to its inherent lower DNA-binding activity seen in many oral cancer cell lines.[7] We expect that highly prevalent mutated p16 proteins in oral cancer cases might have altered the property of JunB from antioncogenic to prooncogenic along with its overexpression in OSCC.

In this study, we have observed overexpressed EGFR in all malignant cases as shown by others.[34–37] Moreover, many earlier reports[38,39] also support our findings of p50/p50 homodimerization with participation of Bcl-3 (Fig. 6*b*) in NF-κB complex of oral cancer biopsies. Interestingly, Thornburg *et al.*[14] have shown overexpressed *egfr* due to the presence of co-activator Bcl-3 in p50/NF-κB complex in nasopharyngeal cancers. These data together indicate that p50 homodimerization in NF-κB complex positively regulates *egfr* expression in oral cancers.

Additionally, upregulated c-Fos due to activated ERK is a well-known signaling pathway.[40] Similar to our findings of upregulated pERK1/2 including elevated c-Fos and EGFR proteins in cancer biopsies (Fig. 6*a*), ERK1/2, an upstream kinase of c-Fos, is reported to be activated in oral cancers through EGFR signals.[41,42] In fact, c-Fos is also known as an established marker of anti-EGFR therapy in many cancer cell lines including HNSCC.[8] Moreover, studies have also documented the cross-regulation of AP-1 and NF-κB[43,44] pathways through overexpressed EGFR-mediated activation by MAPKinase pathways in HNSCC cell lines.[8,14] Therefore, in light of findings of (i) p50/NF-κB activation along with *egfr* overexpression and (ii) elevated pERK and c-Fos proteins in cancer biopsies, we are tempted to speculate that in human oral cancers NF-κB and AP-1 signals might be coupled as: p50/p50 (NF-κB) → EGFR ↑ → ERK → c-Fos/AP-1 ↑. Additional functional studies and clinical correlation may provide further insights in signaling connections between AP-1 and NF-κB that may contributes toward better understanding of oral carcinogenesis.

In conclusion, this study demonstrates an important role of AP-1 as revealed by differential expression and transactivation of AP-1 super-family proteins that occur as a function of severity of lesions during progression of oral carcinogenesis.

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