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CONSTITUTIVE ACTIVATION OF NUCLEAR FACTOR -kB: PREFERNTIAL HOMODIMERIZATION OF p50 SUBUNITS IN CERVICAL CARCINOMA

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1. ABSTRACT

Infection of specific types of 'high risk' HPVs such as HPV 16 and HPV 18 has been associated with the development of cervical cancer. Deregulation of specific NF-kB members has also been implicated with the development of many cancers including cervical cancer. We have studied the expression and DNA-binding activity of NF-kB during the development of cervical cancer involving cervical precancer, cancer and control tissues with or without HPV infection. We observed constitutive activation of NF-kB to a significant level in squamous-cell carcinomas while no or negligible NF-kB binding activity was observed in normal controls or precancerous lesions. Interestingly, there is a gradual increase in binding activity of NF-kB and differential expression pattern of NF-kB family of proteins from low-grade squamous intraepithelial lesions (LSIL) to high-grade squamous intraepithelial lesion (HSIL) and to invasive squamous cell carcinoma (SCC). On further dissection of NF-kB complex, the p50 subunit which generally heterodimerizes with p65 to form an active form NF-kB appears to form a p50/p50 homodimer instead of conventional p50/p65 heterodimer. *In situ* analysis of expression of p50 and p65 subunits by immunohistochemistry in tissue sections from different grades of cervical lesions including invasive cancer also demonstrate a gradual increase in the expression and nuclear localization of p50 subunit only as the severity of lesions increases. We have observed a very high expression of nuclear p50 in HSIL and invasive cancers while the level of nuclear p65 is significantly lower or nil. We further observed that this activation is not dependent on HPV infection since both HPV positive and HPV negative tumors showed the same pattern of high binding activity and increased expression of NF-kB p50/p50 homodimer. Although nuclear translocation and localization of p65 was observed to a lesser extent in invasive tumor, p65 was not found to be involved in dimmer formation. Thus the gradual activation and expression of NF-kB as a function of severity of cervical lesions and the change in dimerization pattern in favor of p50/p50 homodimers appear to play an important role during the development of cervical carcinoma.

2. INTRODUCTION

Cervical cancer is caused by infection of specific types of high-risk human papillomaviruses (HPVs), such as HPV type 16 and HPV 18 (1-3). In India, cancer of the uterine cervix is the major cancer harboring HPV in almost 98% and more than 90% of them are exclusively infected by the HPV type 16 (4, 5). Constitutive expression of two early genes, E6 and E7 of high risk HPVs responsible for

tumorigenic transformation (6), is dependent mainly on the deregulated expression of growth factors, growth factor receptors as well as kinases associated with growth factor signaling pathways (7, 8). Being the ultimate downstream effectors of these pathways, transcription factors play a crucial role during carcinogenesis. Several host-cell transcription factors like activator protein-1 (AP-1), nuclear factor kappa B (NF-kB) etc have been shown to play a crucial role during development of cervical cancer (9). The inducible transcription factor NF-kB regulates expression of wide variety of cellular and viral genes during immune and inflammatory responses and initiation and progression of cancer (10, 11). Deregulation of specific NF-kB members has been associated with the development of many cancers. There exists at least five NF-kB subunits (p50, p65, p52, c-Rel and RelB) but the most abundant and active form is a heterodimer composed of two different proteins, p50 (NF-kB1) and p65 (RelA) (10, 12). They differ in prevalence, expression and binding activity according to physiological conditions of cells. In resting cells, except in mature B-cells, the most NF-kB/Rel dimmer remains confined to the cytoplasm in an inactive form by binding to its inhibitory components IkBs but upon stimulation by different signaling molecules including inflammation and infection, the IkBs are rapidly phosphorylated and degraded through ubiquitin-dependent proteolysis allowing the dimers free to translocate to the nucleus for further DNA binding and transactivation (10, 12, 13). NF-kB plays a central role in regulating the expression of variety of pro-inflammatory cytokines and anti-apoptotic proteins. Enhanced expression of NF-kB contribute to the immune-related infection and diseases, provide survival advantage to cancer cells by inducing proliferation, inhibiting apoptosis, promoting angiogenesis and metastasis through increasing expression of downstream genes that code for pro-inflammatory cytokines such as IL-1, IL-6 etc and inducing resistance to various chemotherapeutic drugs. Inhibition of NF-kB activity could reverse the disease / tumor progression. Thus NF-kB is a critical sensor and integration of exogenous / endogenous signals leading to activation of cellular genes serves as a suitable target that can be used for developing potential cancer drugs. Several lines of evidence also indicate involvement of NF-kB transcription factor in controlling expression of several oncogenes, tumor suppressor genes, growth factors and cell adhesion molecules that play a key role in carcinogenesis (12). Although p50/p65 NF-kB heterodimer is the most predominant, several cell-specific homo or heterodimeric NF-kB complexes have been identified (10, 12, 13). Recently, several reports indicate presence of increased expression of p50 homodimer during development of different carcinomas (14-16). Yet some reports indicate increased expression of p65 in carcinomas (17). However, the biological role of p50 homodimers is not very clear. Bcl3, a noninhibitory member of the IkB family (18) which is known to be associated with certain leukemias and lymphomas (19) also functions to stimulate transcription through interactions with the p50 or p52 NF-kB subunits (20, 21). Gene knockout study also confirms that NF-kB family of proteins plays a central role in regulating immune functions (22). Since the primary NF-kB transactivating complex is an heterodimer of p50 and p65 subunits and since other *rel* proteins (c-Rel, RelB) are largely restricted to hemopoetic and lymphoid tissues, we have investigated the role of NF-kB in the DNA binding activity and the expression profile of all its components through molecular dissection of NF-kB complex in different grades of epithelial lesions during the development of cervical carcinoma. This has also been revalidated by immunohistochemistry to demonstrate localization and expression pattern of NF-kB components *in vivo*. The role of presence or absence of high risk HPV (HR-HPV) infection has been also correlated with the DNA-binding activity and expression of NF-kB components and we demonstrate an increased expression of NF-kB p50 homodimer and constitutive activation of NF-kB as a function of severity of cervical lesions irrespective of HPV infection.

3. MATERIAL AND METHODS

3.1. Tissue specimens

We collected a total of 100 cervical tissue specimens comprising 50 invasive cancer, 25 each of LSIL (low grade squamous intraepithelial lesions) and HSIL (high grade squamous intraepithelial lesions). Thirty normal cervical tissue samples were obtained from the patients undergoing surgery for gynecological problems other than cervical cancer. The tissue samples from women diagnosed to be at different grades of cervical lesions were obtained from the patients attending the cancer clinics of Department of Obstetrics and Gynecology, Lok Nayak Hospital, Maulana Azad Medical College, New Delhi. Each biopsy specimen was bisected and one half was subjected to histopathological examination and immunohistochemical staining and the other half was immediately frozen in liquid nitrogen. Only histopathologically proven samples were considered for the study. Informed consent was taken from each patient before obtaining the biopsy specimens and the Institutional Ethical Committee approved the use of a part of human tissues obtained for routine hospital diagnosis for research investigation.

3.2. Antibodies

Affinity purified rabbit polyclonal antibodies raised against p50, p65, c-Rel and RelB components of NF-kB were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Mouse monoclonal antibody against beta-actin, which served as an internal control, was purchased from Sigma Chemicals (USA).

3.3. Immunohistochemical analysis

Immunohistochemical analysis was done as described by Welter *et al.* (25). Briefly, following deparaffinization and rehydration of the tissue sections, these were blocked in 3% bovine serum albumin for 30 minutes and incubated overnight at 37 °C with the primary antibody. Immunoreactivity was visualized using ABC Staining System Kit from Santa Cruz Biotechnologies (USA) following the manufacturer's protocol. The slides were then counterstained regressively in Mayer's hematoxylin, dipped in methanol for few seconds, cleared in xylene and mounted in Permount. To assess the specificity of staining, sections were

processed without primary or without primary and secondary antibodies as controls.

3.4. DNA extraction and PCR detection of HPV

High molecular weight genomic DNA was isolated from normal, premalignant and tumor tissue specimens of cervical tissues by standard proteinase K digestion and phenol-chloroform extraction procedure (5, 23). Detection of high risk HPV types 16 and 18 DNA were carried out by Southern blot hybridization as well as PCR, as described earlier (5, 23) using type-specific probes and oligonucleotide primers (HPV 16(1): 5'- AAG GCC AAC TAA ATG TCA C –3', HPV 16(2): 5'-CTG CTT TTA TAC TAA CCG G –3', HPV 18(1): 5'- ACC TTA ATG AAA AAC CAC GA –3', HPV 18(2): 5'- CGT CGT TTA GAG TCG TTC CTG –3'). Initially, all DNA samples were tested for the presence of any HPV type by using a pair of consensus primers located within the conserved L1 ORF of HPV genome (MY 11: 5'- GCM CAG GGW CAT AAY AAT GC –3', MY 09: 5'- CGT CCM ARR GGA WAC TGA TC –3', where M = A+C, W = A+T, Y = C+T, R = A+G). PCR was performed in a 25 µl reaction mix containing 100 ng DNA, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 125 µM of each dNTP (dATP, dCTP, dGTP and dTTP), 5 pmoles of each oligonucleotide primer and 0.5U Taq DNA polymerase (Perkin-Elmer Biosystems, Foster City, CA, USA). The temperature profile used for amplification constituted an initial denaturation at 95 °C for 5 min followed by 35 cycles with 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 4 min in the final cycle. The oligonucleotide primers were synthesized in an automated Applied Biosystems DNA synthesizer (Model 381A, Applied Biosystem Inc., Foster City, CA USA) using phosphoramidite method and purified in HPLC.

3.5. Preparation of nuclear extract

Nuclear extracts were prepared by the method of Riol *et al.* (24) with certain modifications. Frozen tissues were minced and re-suspended in ice-cold hypotonic buffer [20 mM HEPES, pH=7.6, 20% (vol/vol) Glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 0.1% Triton X-100, 100 mM PMSF, 2 µg/ml Leupeptin and 10 µg/ml A-proteinin]. After 15 min incubation on ice, lysates were centrifuged at 3000 rpm for 15 min at 40C in a microcentrifuge. The supernatant was transferred to a new tube and designated as the cytoplasmic extract. The pellet was washed once with hypotonic buffer and extracted on ice with 2.5 times the pellet size of nuclear extraction buffer [20 mM HEPES, pH=7.6, 25% (vol/vol) Glycerol, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1mM DTT, 0.1% Triton X-100, 100 mM PMSF, 2 µg/ml Leupeptin and 10 µg/ml A-proteinin]. After 1 hour, the extraction mixture was centrifuged at 14,000 rpm at 4 °C in a microcentrifuge for 15 min. The resulting supernatant was designated as the nuclear extract. The protein concentration of the extracts was determined by spectrophotometric method and the extract was stored at –70 °C deep freezer or in liquid nitrogen until use.

3.6. Electrophoretic mobility shift assay (EMSA)

For electrophoretic mobility shift assay (EMSA), the following oligonucleotides were used: a NF-kB consensus sequence (5'-AGT TGA G*GGGACTTTCC*CAGGC-3') [consensus binding sites are underlined & italicized), an Oct-1 consensus oligonucleotide 5'- TGTCGAATGCAAATCACTAGAA-3' and a Sp-1 consensus sequence 5' - ATTCGATCGGGGCGGGGCGAG-3'. The oligoprimers were synthesized in an Applied Biosystems DNA synthesizer using phosphoramitide chemistry. The above oligonucleotides were annealed and labeled with [Gamma-^{32}P] ATP (3,000 Ci/mmol, Jonaki, Hyderabad) by T4 polynucleotide kinase and gel purified in a 15% polyacrylamide gel (9). The binding reaction was performed in a 25µl 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 of bovine serum albumin per ml, 2.5 µg of poly (dI-dC) and 10 µg of nuclear extract. After 5 minute, 10,000 cpm of the [Gamma-^{32}P] ATP 5'-end labeled double stranded oligonucleotide probe was added, the incubation was continued for additional 25 min at room temperature. For monitoring NF-kB composition in supershift assays, 2 µg of polyclonal antibodies (Abs) directed against p50 and p65 (Santa Cruz Biotechnology, USA) were added and the reaction mixture was further incubated for 1 hr at 4 °C. The following Abs were used: NF-kB p50 Ab (epitope corresponding to NLS region of NF-kB p50 of human origin), NF-kB p65 Ab (epitope corresponding to amino terminus of NF-kB p65 of human origin), c-Rel Ab (epitope corresponding to amino terminal domain of NF-kB c-Rel p75 of mouse origin) and NF-kB RelB Ab (epitope corresponding to carboxy terminus of NF-kB RelB p68 of mouse origin). The DNA-protein complexes were resolved on 4.5% non-denaturing polyacrylamide gel (29:1 cross-linking ratio), dried and exposed overnight to KODAK X-Omat Films.

3.7. Western blotting

Nuclear extracts used for band shift analysis (30 µg of protein per lane) were separated in 10% polyacrylamide gel, electrotransferred to Immobilon-P membranes (Millipore Corporation, USA), and probed with polyclonal rabbit antibodies of the corresponding family members (See above for details). The incubation was carried out overnight in phosphate-buffered saline supplemented with 5% skim milk powder, 0.05% Tween 20 (Sigma Chemicals, USA), and different dilutions of respective Abs. The bands were visualized with an antirabbit immunoglobulin G antibody conjugated with horseradish peroxidase, using the Santa Cruz Luminol reagent detection kit.

4. RESULTS

The binding activity of NF-kB and the expression profile of its subunits, p50, p65, c-Rel and RelB were analyzed in all spectrums of cervical tissues comprising premalignant, malignant and normal controls. The results showed a gradual increase in the binding activity and a differential expression of NF-kB components with the increasing severity of the lesions. NF-kB components also demonstrated differential localization *in situ* through progression to invasive cancer.

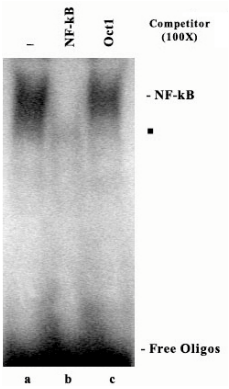

Figure 1. EMSA using nuclear extracts from invasive cervical cancer tissues, with a ^{32}P-labelled oligonucleotide harboring an NF-kB consensus sequence show a high binding activity of NF-kB. Binding specificity was evidenced by pre-incubation with a 100-fold molar addition of the homologous unlabeled oligonucleotide (lane b) in comparison with competition experiments using a heterologous consensus sequence of the Oct-1 transcription factor (lane c). The positions of the specific retarded bands are indicated. The squares mark the unspecific complexes.

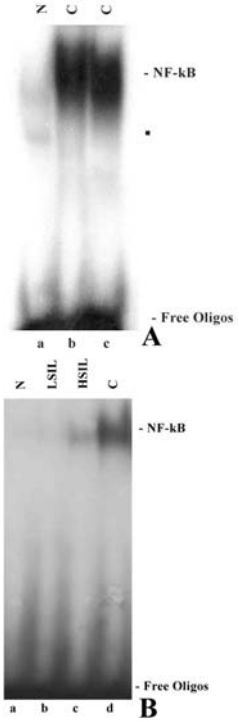

Figure 2. (A, B) Gel shift analysis using nuclear extracts from different grades of cervical biopsy tissues with ^{32}P labelled oligonucleotide probe harboring an NF-kB consensus sequence. Increasing NF-kB binding activity was observed as the severity of cervical lesions progressed from normal to invasive cancer in both figures A and B. In (A), Lane a, normal controls (N); lane b and c, invasive cancer (C). In (B), Lane a, normal controls (N); lane b, low-grade squamous intraepithelial lesions (LSIL); lane c, high-grade squamous intraepithelial lesions (HSIL) and lane d, invasive cancer (C). The positions of specific bands are indicated. The squares mark the unspecific complexes.

4.1. Enhanced DNA-binding activity of NF-kB in cervical tumors: The predominant DNA binding species is the p50/ p50 homodimer

The DNA binding affinities of NF-kB during cervical carcinogenesis were analyzed by band-shift assays using nuclear extracts from normal as well as different grades of cervical tumor tissues. ^{32}P labeled oligos harboring an NF-kB consensus sequence were used as probe. The binding specificity was confirmed when the retarded complex disappeared after competitionwith a 100 fold molar excess of a homologous but not with a heterologous probe, containing the consensus sequence for the transcription factor Oct-1 (figure 1).

We used nuclear extracts from different grades of cervical tissues e.g., LSIL and HSIL including invasive cervical cancer and normal controls and we observed a gradual increase in binding activity of NF-kB (figure 2A, B) as the lesions progressed towards severity from mild dysplasia (LSIL) to severe lesions (HSIL) to invasive cancer. While we observed an absence or a very low DNA binding activity in normal as well as in premalignant tissues, the NF-kB binding activity was found to be highly increased in malignant tissues (figure 2A, B).

But there is no difference in binding activity between control, LSIL/HSIL or cancer when Oct-1 was used as a probe, which served as a control (figure 3). To understand the role of highly elevated binding of NF-kB transcription factor in tumor tissues, complexes were further dissected to their individual components in electrophoretic mobility super shift assays using specific antibodies raised against different members of NF-kB family e.g. p50, p65, c-Rel and RelB. The supershift analysis revealed a preferential high binding of p50 instead of both p65 and p50. In almost all malignant tissues analyzed, more than 95% of the super shifted band was formed by p50 only while p65 showed no or negligible shift after incubation with the respective antibodies (figure 4A, B). Thus it appeared to be homodimerization of p50/ p50 instead of the canonical p65/ p50 heterodimer. Only in a few cases p65 involvement was observed but to a very low level. Some of the normal cervical tissues, which showed moderate to high NF-kB binding activity, harbored classical p50/p65 heterodimer (figure 4A). Although we have not observed a consistent involvement of c-Rel in nuclear NF-kB, a small number of invasive cancer samples showed presence of c-Rel as a heterodimerizing component of NF-kB (figure 4C). Presence of RelB was not detectable in either precancer or cancer tissues.

4.2. DNA binding activity of NF-kB and expression of p50 homodimer increased as a function of severity of the disease

Since inappropriate gene expression may account for absence of specific components within the NF-kB transcription complex, immunoblotting experiments were performed to analyze the level of expression of NF-kB family of proteins in cervical tissues comprising malignant, premalignant and normal controls. Interestingly enough, the analysis of expression profile of p50 and p65 proteins in nuclear extracts from different grades of cervical tissue

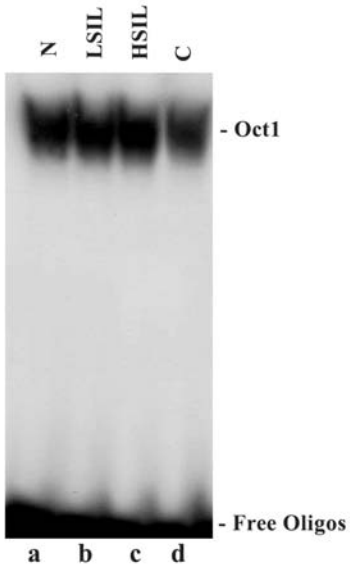

Figure 3. EMSA carried out with labeled oligos encompassing a consensus sequence of Oct-1 transcription factor showing uniform binding activity in different grades of cervical tissue extracts. Lane a, normal controls (N); lane b, low-grade squamous intraepithelial lesions (LSIL); lane c, high-grade squamous intraepithelial lesions (HSIL) and lane d, invasive cancer. The positions of the specific retarded bands are indicated.

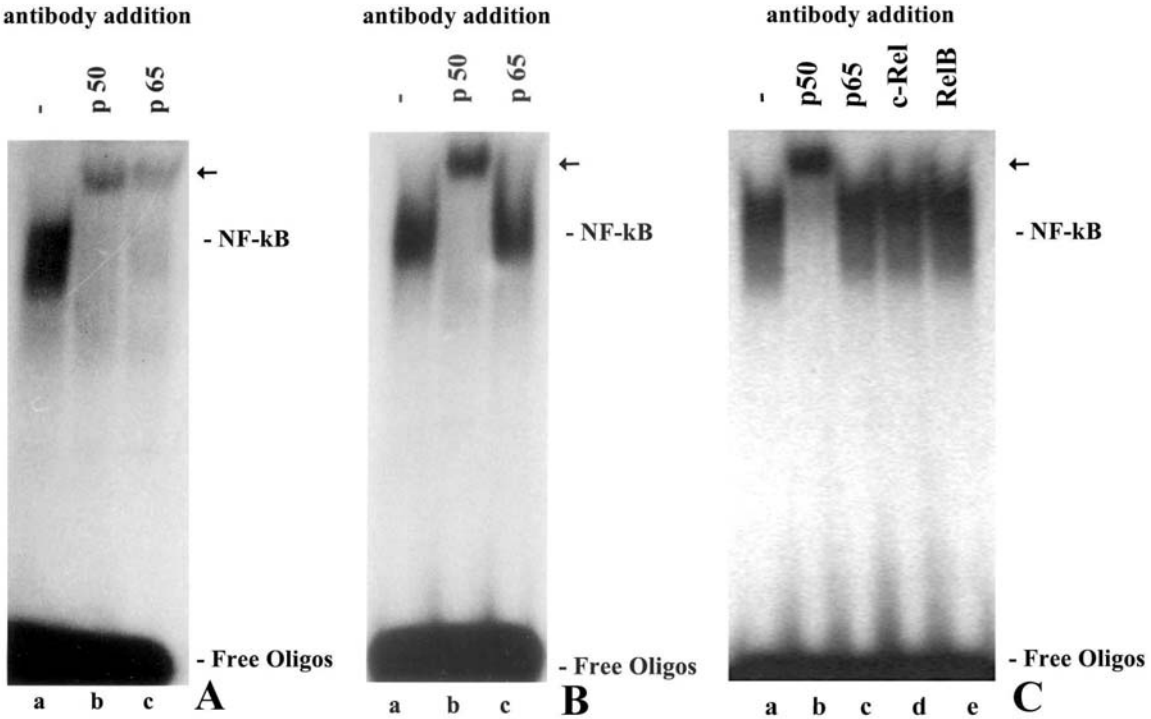

Figure 4. (A, B, C) Electromobility supershift analysis using nuclear extracts from different types of cervical tissues showing differential binding activity of NF-kB components. Nuclear extracts were incubated with specific antibodies (Abs) recognizing different members of the NF-kB family. In (A), EMSA with nuclear extract from normal cervical tissue showing presence of both p50 and p65. Lane a, without Ab; lane b, addition of p50 Ab; lane c, addition of p65 Ab. In (B), EMSA with nuclear extract from invasive cervical tissue showing exclusively high binding of p50. Lane a, without Ab; lane b, addition of p50 Ab; lane c, addition of p65 Ab. In (C), EMSA with nuclear extract from invasive cervical tissue showing binding activity of all the members of NF-kB. Lane a, without Ab; lane b, addition of p50 Ab; lane c, addition of p65 Ab; lane d, addition of c-Rel Ab; lane e, addition of RelB Ab. The position of the NF-kB specific complex is indicated. The arrowhead indicates the supershifted bands after antibody addition.. The squares mark the unspecific complexes.

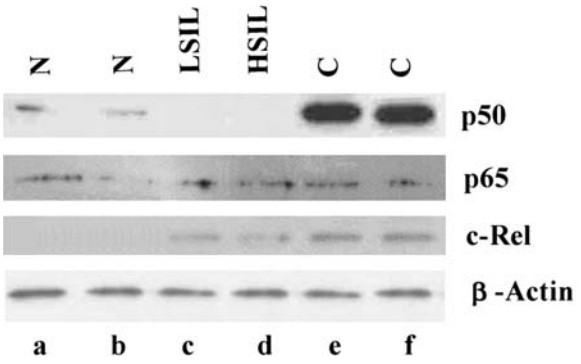

Figure 5. Immunoblotting showing differential expression pattern of different members of NF-kB family of proteins in normal, dysplasia (LSIL and HSIL) and cancerous lesions of the uterine cervix. 30µg protein each from control, dysplasia and cancer cases was separated in a 10% SDS-PAGE mini gel. After electrotransfer, the filters were consecutively incubated with different antibodies of NF-kB family (p50, p65 and c-Rel). To confirm, equal protein loading, the filters were reincubated with a monoclonal beta-actin antibody. Lanes a and b, normal controls; lane c, low-grade squamous intraepithelial lesions (LSIL); lane d, high-grade squamous intraepithelial lesions (HSIL); lanes e and f, invasive cancers.

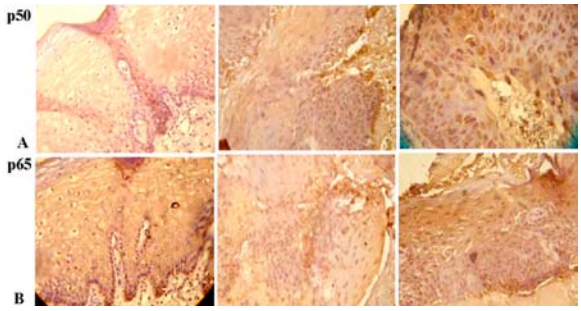

Figure 6. Immunohistochemical analysis of expression and localization of p50 and p65 in different grades of cervical lesions. Paraffin-embedded sections (4-5 µm thick) from normal, precancer and invasive cancer tissues of human cervix were immunolocalized with the antibodies specific for (A) p50 and (B) p65 of NF-kB.

specimens demonstrated gradual increase in the p50 expression as the lesion progressed to cancer. In contrast, the p65 was found to exhibit uniformly a steady state level of low to moderate expression in normal controls to premalignant to invasive cancer (figure 5). A very low or negligible expression of p50 was observed in normal and premalignant tissues, while a very high expression of p50 was observed in malignant tissues (see figure 5). Baring some minor differences between samples for the expression of NF-kB family members, the p50 expression increased as a function of severity of cervical lesions. c-Rel showed a very low expression in the nuclear extracts of normal, premalignant and malignant lesions. Differential expression pattern of all the different members of the NF-kB family in different grades of cervical tissues has been shown in table 1.

4.3. *In-situ* localization of NF-kB by immunohistochemistry: Increased nuclear translocation of p50 during progression of cervical cancer

To determine the localization of different members of NF-kB family in cervical cancer samples as compared to their normal counterparts, immuno-histochemistry was performed using antibodies raised against different members of NF-kB family on paraffin embedded tissue sections of normal as well as different grades of cervical lesions. The results showed an increase in p50, p65 and c-Rel protein levels in cervical cancer tissues when compared to that in normal as well as premalignant cervical tissues (figure 6). Although p50 staining was completely cytoplasmic in normal cervical tissues as well as LSILs, it's nuclear staining increased significantly in HSIL and invasive tumor tissues. Both cytoplasmic as well as nuclear localization of p50 was clearly visible in invasive tumor tissues (figure 6). Staining with the p65 (RelA) antibody indicated a very small increase in p65 protein level from normal to invasive cancer being predominantly cytoplasmic in all the different types (control→ precancer→ cancer) of cervical tissues. To determine whether the staining with p65 antibody was nuclear, we used an antibody specific for the activated form of p65 (p65-NLS), which specifically recognizes p65 released from IkB and we found no detectable nuclear p65 in either normal or invasive cancer tissues. c-Rel localization was mostly cytoplasmic in all types of cervical tissues. The localization and the level of expression of p50, p65 and c-Rel proteins in different grades of cervical lesions is presented in table 2 and figure 5. Although cervical cancer tissues generally showed increased expression of p50, p65 and c-Rel, but only the p50 subunits demonstrated nuclear localization and exclusive high expression (figure 5) confirming its high binding activity (figure 4) observed in nuclear extracts by band shift assay.

4.4. Constitutive activation of NF-kB and increased expression of p50 homodimer is independent of Human papillomavirus infection

In order to understand the role of viral infection on the binding activity of NF-kB, we carried out experiments in high-risk HPV positive and HPV negative cervical tumors. Since more than 90% of cervical cancer cases in India harbor high risk HPV types 16/18, all the cervical specimens (n=130) including normal cervical tissues were employed for HPV typing by PCR; first by L1 consensus primer which revealed a total HPV positivity of 10, 49 and 98 percent in normal, premalignant and malignant cervical tissues respectively. 46% of the premalignant lesions (LSIL 32%; HSIL 60%) and as high as 94% cancer cases were positive for high risk HPV types 16/18. Very interestingly, we have found that the NF-kB high binding activity has no correlation with the HPV infection, as both HPV positive as well as negative cervical tissues show high NF-kB binding activity. Thus it appears that HPV infection may be an additional mechanism but not a pre-requisite for high NF-kB DNA-binding activity.

Table 1. Quality and quantity of expression of the NF-kB proteins in normal, premalignant and malignant lesions of the uterine cervix of women

Protein	Normal (n=30)				Premalignant (n=50)				Malignant (n=50)			
	Nil	Weak	Medium	Strong	Nil	Weak	Medium	Strong	Nil	Weak	Medium	Strong
p50	08	15	07	--	21	15	14	--	--	02	18	30
p65	17	13	--	--	30	18	02	--	08	30	04	--
c-Rel	15	10	05	--	22	24	02	--	12	25	10	03

Arbitrary level of expression: Strong = ++++; Medium = ++; Weak = +; Nil = -

Table 2. Immunohistochemical localization and analysis of in-situ expression of p50, p65 and c-Rel proteins

Histology	p 50		p 65		c-Rel	
	Cytoplasmic	Nuclear	Cytoplasmic	Nuclear	Cytoplasmic	Nuclear
Normal (n=30)	22	0	15	0	12	0
LSIL (n=25)	24	01	25	0	25	0
HSIL (n=25)	12	13	14	11	23	02
Invasive cancer (n=50)	14	36	40	05	40	07

n=total number of samples analyzed.

5. DISCUSSION

We have investigated the binding activity and expression pattern of NF-kB in different grades of cervical tissues and we observed a distinct pattern of constitutive activation of NF-kB subunits, which differed significantly from that of the normal cervical cells. While normal as well as premalignant lesions showed no or, negligible amount of binding activity, a substantially increased binding activity was observed in malignant tissues. Our results further indicate that the highly increased NF-kB binding complexes contained mainly p50, which seem to be specifically activated in human cervical cancer without corresponding activation of p65 subunits. Immunohistochemical analysis of p50 in *in vivo* tissue sections revealed that while low expression of p50 was observed in normal controls or LSILs only in cytoplasm, high p50 expression was detected mainly in nuclei of tumor cells. Interestingly, c-Rel showed consistently a cytoplasmic expression. Similar observation of NF-kB activity or the expression of its components, both in HPV positive as well as HPV negative cervical tissues is indicative of the fact that it is a general phenomenon irrespective of HPV infection. The supershift analysis of NF-kB complex revealed p50/p50 homodimer since p50 was the main component (>95%) in majority of malignant lesions. The normal dimerization partner p65 was observed in some cancer cases, but to a much lesser intensity and in normal/ premalignant lesions the NF-kB complex is not discernable at all. Western blot analysis displayed consistently a very low to moderate p65 expression in nuclear extracts of normal, premalignant and malignant samples. In contrast, level of p50 was very high in most of the malignant samples and a moderate expression in the low-grade lesions. Similar high NF-kB activity in human tumors has been observed in oral and breast cancer (Das *et al*, unpublished) and in several other tumors (17, 26-29). It is often observed that variety of viral proteins can induce NF-kB activity to ensure NF-kB-dependent expression of viral genes (30). But, some viruses evolved strategies to counteract NF-kB activation. It seems that the homodimerization of p50/p50 acts as a repressor of NF-kB-dependent transcription (31) by binding to viral upstream regulatory region (URR) (32) as one potential NF-kB binding site has been identified in HPV 16- URR. Transient over-expression of RelA in HeLa cells has been shown to repress the HPV 16- URR (32). HPV transcription can be prevented by some NF-kB target proteins such as TNF-alpha and IL-1 (33, 34) and this could help the virus to interfere with NF-kB activity enabling the virus to escape from the host immune surveillance. It is also known that the HPV oncoprotein E7 obviates IKK activation in the cytoplasm and impairs TNF alpha-induced nuclear translocation of NF-kB, thus preventing NF-kB from binding to its cognate DNA. But E6 protein reduces NF-kB p65-dependent transcriptional activity within the nucleus (35). The exclusion of p65 from the NF-kB complex by forming a homodimer of p50/p50 might help in decreasing the transactivation activity of NF-kB. Evidence is there to show that HPV infection down-regulates only a subset of NF-kB dependent genes (3). The others may be controlled by additional transcription factors, which help the virus to survive in the host cell. But again these explanations seems to be confusing as high NF-kB binding activity is also observed in HPV-negative cervical tumors. Thus we may speculate that whether it is HPV-dependent or HPV independent down regulation of NF-kB, the negative control of NF-kB transactivation activity certainly helps during the process of tumorigenesis. Since p50 and p52 are not thought to be directly regulated through complexes with IkB-alpha or IkB-beta, our data suggest that a pathway is active in cervix cancer that functions to increase levels of certain forms of NF-kB and is independent of the normal inducible pathways which involve degradation of one of the IkB subunits. Similar results have also been demonstrated in breast cancer tissues (36). NF-kB regulates the susceptibility of certain cell types to apoptosis through transcriptional control of protective genes (37, 38). In addition, constitutive NF-kB activity in T-cells has also been shown to protect T-cells from apoptosis (39) and activation of T-cells by the tumor promoter factor (PMA) results in T-cell apoptosis when NF-kB translocation is inhibited (40). Consistent NF-kB activation also suppresses transformation-associated apoptosis (41). HPV infection is associated with decreased apoptosis (42) and antiapoptotic Bcl-2 protein is also strongly associated with the

development of cervical cancer (43). The antiapoptotic proteins Mcl-1 and IL-6, which are known to be regulated by NF-kB are also highly expressed in invasive cervical cancer but not in normal cervical tissues (44). Thus, NF-kB helps in suppressing apoptosis through activation of antiapoptotic proteins during development of cervical cancer. Additionally, NF-kB can promote cell cycle progression through the activation of cyclin D1 promoter activity (45, 46). Thus, the functional activation of NF-kB may induce cell proliferation and overexpression of certain cyclins, which contributes towards tumorigenesis.

Thus transcription factor NF-kB appears to play an important role during development of cervical cancer but the specific downstream effector gene(s) targets of this transcription factors that contribute to oncogenesis are poorly understood. Understanding molecular pathways involved during tumorigenesis will help in designing therapeutic approaches to fight cancer.

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Abbreviations: HPV: human papillomavirus, NF-kB: nuclear factor kappa B, EMSA: electrophoretic mobility shift assay, URR: upstream regulatory region, LSIL: low-grade squamous intraepithelial lesion, HSIL: high-grade squamous intraepithelial lesion

Key Words: Human papillomavirus, Cervical Cancer, Nuclear factor kappa B, Immunohistochemistry, p50, p65, c-Rel

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