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NF-κB is constitutively activated in high-grade squamous intraepithelial lesions and squamous cell carcinomas of the human uterine cervix

Asha Nair[1], Manickam Venkatraman[1], Tessy T Maliekal[1], Balaraman Nair[2] and Devarajan Karunagaran*,[1]

[1]*Division of Cancer Biology, Rajiv Gandhi Center for Biotechnology, Thiruvananthapuram, Kerala 695 014, India;* [2]*Department of Pathology, Doctors Diagnostic and Research Center, Thiruvananthapuram, Kerala 695 011, India*

We demonstrate, for the first time, that the transcription factor NF-κB is constitutively activated during human cervical cancer progression. Immunohistochemical analysis was done using 106 paraffin-embedded cervical tissue specimens of different histological grades. In normal cervical tissue and low-grade squamous intraepithelial lesions, p50, RelA and IκB-α were mainly localized in the cytosol, whereas in high-grade lesions and squamous cell carcinomas, p50-RelA heterodimers translocated into the nucleus with a concurrent decrease in IκB-α protein. By Western blot analysis, p50 and RelA were detectable mainly in the cytosolic and nuclear extracts in normal and cancer tissues, respectively, and cytosolic IκB-α expression was detectable in normal but not in cancer cervical tissues. NF-κB DNA-binding activity increased during cervical cancer progression and the binding complex was mainly composed of the p50–RelA heterodimers as revealed by electrophoretic mobility shift assays. Semiquantitative RT-PCR analysis, however, showed increased levels of IκB-α mRNA in cancer samples presumably because of feedback regulation as a result of enhanced NF-κB DNA-binding activity and a consequent functional activation of NF-κB. Further immunohistochemical analysis with an antibody to phospho IκB-α revealed that phosphorylation occurs mainly in squamous intraepithelial lesions, suggesting that the IκB-α gets phosphorylated initially and degraded as the tumor progressed.
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Introduction

Nuclear factor-kappa B (NF-κB/Rel) is a family of transcription factors, the mammalian members of which include p50 (NF-κB1), p52 (NF-κB2), c-Rel, RelB, and RelA (p65). Heterodimers of p50 and RelA, also known as classical NF-κB, are present in the cytoplasm bound with their inhibitory proteins (IκBs), of which IκB-α is the best characterized. Inducible phosphorylation at serines 32 and 36 of IκB-α by the IκB kinase (IKK) complex is a signal for its subsequent ubiquitination and proteosome-mediated degradation leading to the translocation of NF-κB into the nucleus where it can bind to κB-responsive elements (Karin and Ben-Neriah, 2000; Baldwin, 2001; Karin *et al*., 2002). The genes encoding c-Rel, RelA, p100, p52, and Bcl-3 are located at sites of recurrent translocations and genomic rearrangements in cancer (Karin and Ben-Neriah, 2000). NF-κB controls the expression of a number of growth-promoting cytokines (Rayet and Gelinas, 1999), and the DNA-binding activity of NF-κB is induced during the G_0 to G_1 transition (Baldwin *et al*., 1991). NF-κB also activates the expression of genes important for invasion and metastasis (Newton *et al*., 1999), and IκB-α expression in tumor cell decreases the frequency of metastases (Baldwin, 2001). Mutations in the IκB-α gene have been detected in Hodgkin's lymphoma and are suggested to render NF-κB constitutively active in Hodgkin's cells, consistent with a role for IκB as a tumor suppressor (Cabannes *et al*., 1999). Inhibition of NF-κB activity potentiates cell killing of human breast cancer and fibrosarcoma cell lines by TNF-α, ionizing radiation, and daunorubicin (Beg and Baltimore, 1996; Van Antwerp *et al*., 1996; Wang *et al*., 1996). NF-κB inhibition also led to sensitization of tumors to chemotherapeutic compound CPT-11-mediated cell killing *in vivo* (Cusack *et al*., 2001). NF-κB directly causes increased expression of proteins that contribute to the survival of tumor cells such as inhibitors of apoptotic proteins (Chu *et al*., 1997; You *et al*., 1997). Results from our laboratory have shown earlier that ectopic expression of the RelA subunit of NF-κB into murine fibrosarcoma cells protects them from curcumin-induced apoptosis (Anto *et al*., 2000).

Cervical cancer is an important public health problem for adult women in developing countries, where it is the most or second-most common cancer among women. Cervical cancer causes about 250 000 deaths annually worldwide, with women in the developing countries accounting for 80% of these deaths (Schoell *et al*., 1999; Cain and Howett, 2000). Infection of the cervical epithelium with human papillomavirus (HPV) increases

*Correspondence: D Karunagaran, Division of Cancer Biology, Rajiv Gandhi Center for Biotechnology, Thiruvananthapuram, Kerala 695 014, India; E-mail: dkarunagaran@hotmail.com
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the risk of premalignant lesions and progression to cervical cancer. HPV-16, HPV-18, HPV-31, and HPV-45 are considered 'high-risk viruses' associated at a high frequency (90%) with adeno and squamous carcinoma of the cervix as well as with cervical precursor lesions (reviewed by zur Hausen, 1996). HPV E6 protein can complex p53 and promote its degradation causing chromosomal instability. HPV E7 protein binds pRB releasing E2F and inducing cell proliferation (zur Hausen, 1996). A high proportion (probably more than 50%) of HPV-related nonmalignant lesions can regress and about 10% of all carcinomas of the cervix are free of HPV genomes (zur Hausen, 1996). Hence, HPV infection is a necessary, but not sufficient, event and additional molecular alterations are needed to convert a normal cervical epithelial cell to a cancer cell, and such alterations may constitute an alternative route of carcinogenesis in HPV negative lesions (zur Hausen, 1996).

Cervical cancer progression is believed to involve progressive changes from dysplastic precursor lesions to metastatic stages of cancer. While most low-grade dysplasia regress spontaneously, about 30% of the high-grade lesions are known to take 10–15 years before cervical cancer develops (Sankaranarayanan *et al*., 2001).

Consistent with a role for NF-κB in oncogenesis and survival, aberrant constitutive activation of NF-κB/Rel is frequently found in human tumors of diverse tissue origin (Rayet and Gelinas, 1999; Karin *et al*., 2002). However, there are no reports on the activation status of NF-κB in human cervical cancer and, hence, we studied the changes in the expression of NF-κB and IκB-α proteins during cervical cancer progression using paraffin-embedded specimens of different histological grades as well as fresh tissues (normal and cancer) from human cervix. Using several techniques such as immunohistochemistry, Western blotting, semiquantitative RT-PCR and electrophoretic mobility shift assays, we provide evidences for the constitutive activation of NF-κB during cervical cancer progression.

Results

Increased nuclear translocation of p50 and RelA, and loss of IκB-α immunoreactivity during human cervical cancer progression

Using the Bethesda system for cervical cytology reporting (Nguyen and Nordqvist, 1999), we classified the cervical tissue samples as normal, low-grade squamous intraepithelial lesions (LSILs/CIN-I), high-grade squamous intraepithelial lesions (HSILs or CIN-II/CIN-III) and squamous cell carcinomas (SCCs). SCC samples were further classified into well-differentiated squamous cell carcinoma (WDSCC), which included large cell keratinizing tumors, moderately differentiated squamous cell carcinoma (MDSCC), which included large cell nonkeratinizing tumors, and poorly differentiated squamous cell carcinoma (PDSCC). These relatively well-defined stages of disease progression make cervical cancer an ideal model for investigating molecular alterations during neoplastic progression. We first analyzed the expression of NF-κB transcription factors and their classical inhibitory protein, the IκB-α, by immunohistochemistry during the sequential stages of human cervical cancer progression. Our analysis included 106 paraffin-embedded cervical tissue specimens (23 LSILs, 27 HSILs, 38 SCCs, and 18 normal). For immunohistochemical staining of p50, RelA, and IκB-α, different sections of the same sample obtained from one individual (normal/LSIL/HSIL/SCC) were used. The histologically normal cervical epithelia showed intense immunoreactivity in the cytosol of 12, 15, and 16 from a total of 18 samples for p50, RelA, and IκB-α, respectively, whereas the remaining samples showed moderate reactivity (Table 1). Similarly in LSILs, 14, 20, and 12 out of 23 total samples showed intense positivity for p50, RelA, and IκB-α, respectively, and the rest with moderate intensity, in the cytosol (Table 1), whereas the reactivity for all these molecules in the nucleus was minimum in the tissues from normal or LSIL samples (Figure 1). On the other hand, intense nuclear positivity for p50 and RelA was observed in 65 and 71 out of 100 cells, respectively, in HSILs, but IκB-α expression in the cytoplasm was low with moderate staining for seven and mild staining for 16 samples and negative staining for four samples as well (Table 1). Likewise, in WDSCC samples we found nuclear positivity for p50 and RelA in 64 and 75 out of 100 cells, respectively, and only a mild staining of IκB-α was noted in three out of nine samples and the rest were negative (Table 1). In MDSCC also 69 and 76% of cells were intensely positive in the nucleus for p50 and RelA, respectively, and again a mild staining was observed for IκB-α only in two out of seven samples while the others were negative (Table 1). In PDSCCs, the percentages were higher with 81 and 92, respectively, positive in the nucleus for p50 and RelA and, interestingly, all the samples analyzed were negative for IκB-α reactivity (Table 1). The typical intense cytosolic staining pattern for p50, RelA, and IκB-α in normal and LSIL; intense nuclear positivity for

Table 1 Analysis of immunohistochemical data on the expression of p50, RelA, and IκB-α

Histology	*p50*	*RelA*	*IκB-α*
Normal	+ + + (12)	+ + + (15)	+ + + (16)
$n=18$	+ + (6)	+ + (3)	+ + (2)
LSIL	+ + + (14)	+ + + (20)	+ + + (12)
$n=23$	+ + (9)	+ +(3)	+ + (11)
HSIL			+ + (7)
$n=27$	65%	71%	+ (16)
WDSCC $n=9$	64%	75%	+(3)
MDSCC $n=7$	69%	76%	+(2)
PDSCC $n=22$	81%	92%	–

n=total number of samples analyzed. Intensity scoring in cytosol: intense: + + +; moderate: + +; mild: +; negative: –. Nuclear positivity is shown in percentage. Figures in parentheses indicate the number of samples found positive.

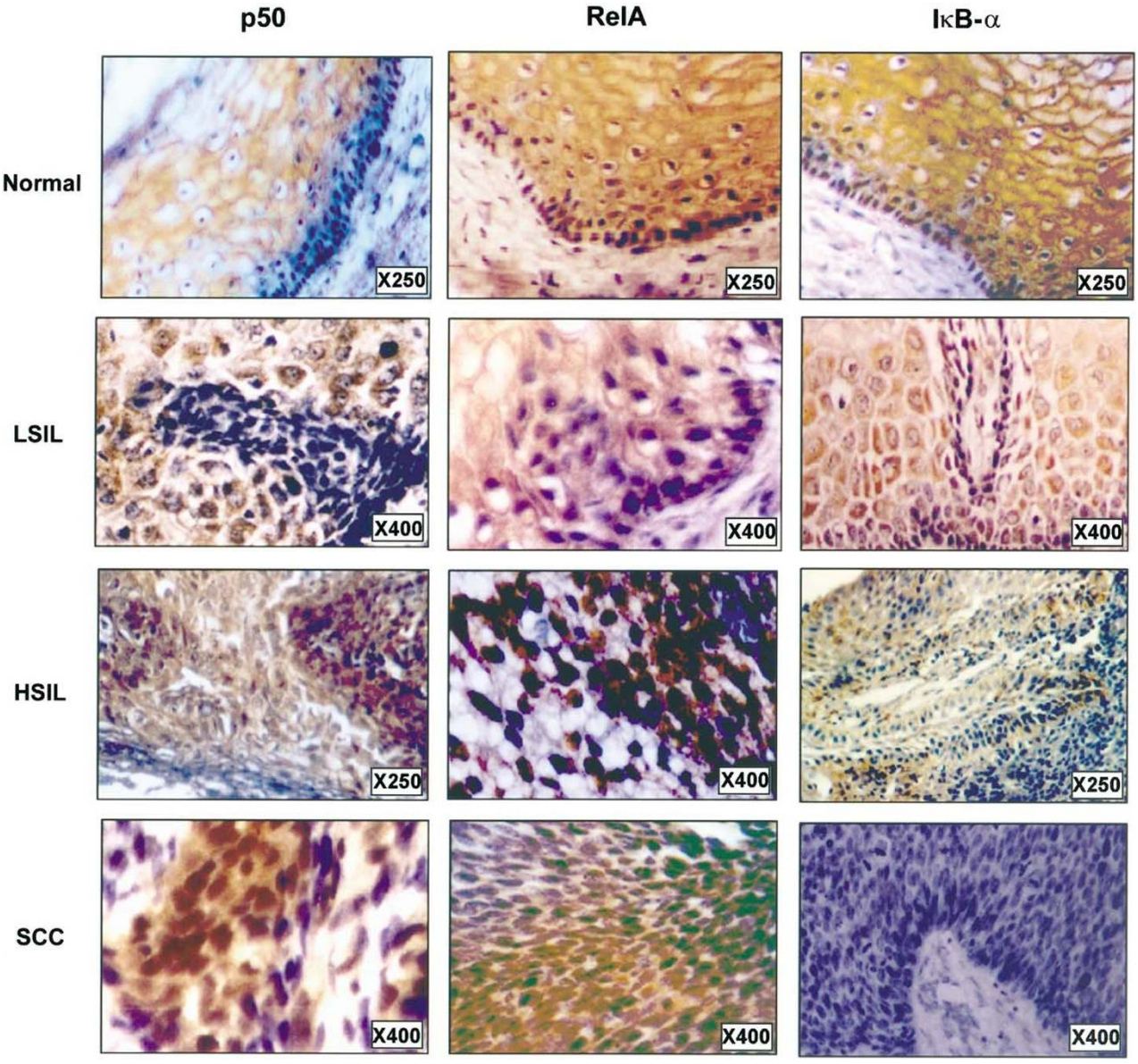

Figure 1 Expression and localization of p50, RelA, and IκB-α by immunohistochemical analysis during human cervical cancer progression. Paraffin-embedded sections (5 μm thickness) from normal, LSIL, HSIL, and SCC tissues of the human uterine cervix were immunolocalized with the antibodies specific for p50, RelA, and IκB-α as described in Materials and methods

p50 and RelA, and moderate staining for IκB-α in HSIL; intense nuclear positivity for p50 and RelA and the negative staining for IκB-α in PDSCC are shown in Figure 1. A Man–Whitney–Wilcoxon rank sum *W* test was used to determine the statistical significance between immunohistochemical expression of proteins and histological grades of the lesions. When HSILs were compared with PDSCCs, the results were highly significant for the expression of p50 and RelA ($P<0.0001$) or significant with MDSCC ($P<0.0227$) for p50. The decrease in IκB-α was highly significant for all the histological grades compared ($P<0.0001$). The paraffin-embedded specimens from normal, LSILs, HSILs, and SCCs did not differ in their immunoreactivity with antibodies for p52, c-Rel and RelB except for a mild cytosolic staining (data not shown) and, thus, we did not detect any changes in the localization or expression levels of these NF-κB proteins during cervical cancer progression. No specific immunoreactivity was observed in representative samples of tissues from normal, LSIL, HSIL, and SCC specimens when the antibody to RelA (sc-109) was omitted, or co-incubated with its competitive control peptide (sc-109 P; data not shown). To see whether the results obtained in paraffin-embedded specimens would be reflected in fresh tissues, they were analyzed for the expression levels of p50, RelA and IκB-α proteins by Western blotting using cytosolic and nuclear extracts. In

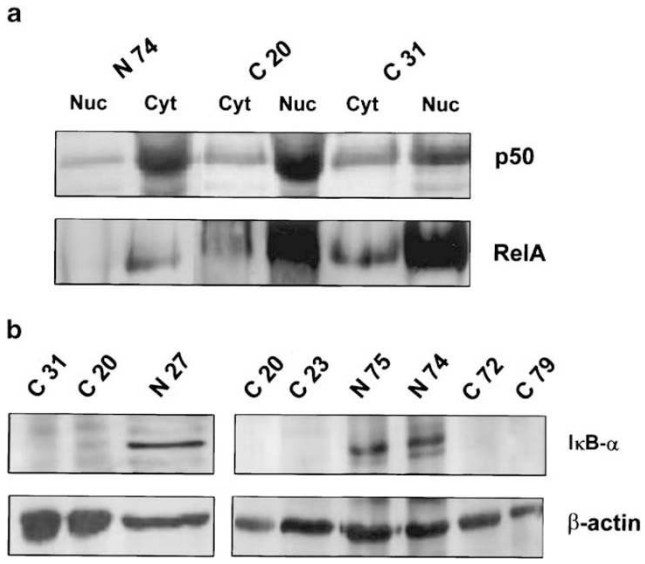

Figure 2 Western blotting of p50, RelA, and IκB-α in normal and cervical cancer tissues. Cytosolic (Cyt) and nuclear (Nuc) extracts (100 μg protein) of normal cervical tissue and SCC of uterine cervix were prepared as described in Materials and methods, and were electrophoresed by SDS-PAGE followed by Western blotting with p50 and RelA antibodies (**a**). IκB-α was similarly blotted using cytosolic extracts from normal and SCC tissues and β-actin control was used to ensure equal loading (**b**). N and C denote normal tissue and SCC samples, respectively, followed by the patient number

normal cervical tissue, p50 and RelA were detectable easily in the cytosolic extracts and in cancer tissues (SCCs), the expression of p50 and RelA increased in nuclear extracts while their expression in the cytosolic extracts was relatively less (Figure 2a). Cytosolic fractions from normal cervical tissue showed IκB-α expression, but IκB-α levels were very low or undetectable in the cytosolic fractions from the cancer tissues (SCCs) analyzed (Figure 2b). These results indicate that p50 and RelA translocate into the nucleus in cervical cancer accompanied by a decrease in the expression of IκB-α protein.

Loss of IκB-α is not caused by defective transcription

To know whether the decrease in IκB-α is caused by defective transcription, semiquantitative RT-PCR analysis was used. For this, amplification at different cycles was performed for *IκB-α* and*β2-microglobulin* control (Figure 3a and b). As shown in Figure 3a, the expression of *IκB-α* in all the cancer tissue samples analyzed was notably at a higher range than that in normal tissue samples. The variations in the expression of *β2-microglobulin* between normal and cancer samples were minimum (Figure 3b). From these experiments, a single cycle was chosen from the linear range for the expression of *β2-microglobulin* and *IκB-α* and another experiment gave similar results (Figure 3c). Thus, the decreased protein level of IκB-α in cytosol detected in HSILs and SCCs by immunohistochemical analysis (Figure 1 and Table 1) and in SCCs by Western blotting (Figure 2b) is not because of defective transcription but may be because of the rapid turnover of IκB-α.

Phosphorylation of IκB-α occurs in the squamous intraepithelial lesions but not in the advanced stages of squamous cell carcinoma

Since the rapid turnover of IκB-α requires phosphorylation at its N-terminal serine residues as a signal for ubiquitination that eventually leads to its degradation, immunoreactivity for phospho IκB-α was tested in different grades of cervical lesions using an antibody specific to phosphorylation sites at the N-terminal Ser 32/36 of IκB-α. Normal cervical tissue showed no reactivity with phospho IκB-α antibody, but LSILs showed immunoreactivity in the spinal cells and no reactivity was observed in the basal cells (Figure 4). However, in HSILs, intense staining for phospho IκB-α was observed in the differentiating basal and para basal cells (Figure 4). In SCCs, there was no immunoreactivity for phospho IκB-α in MDSCC and PDSCC samples although a mild staining was noticed in WDSCC (Figure 4). These results suggest that the phosphorylation of IκB-α occurs initially, but in the more advanced stages no phosphorylation was noticed probably because of the degradation of IκB-α.

Constitutive NF-κB DNA-binding activity during cervical cancer progression

To investigate whether the nuclear translocation of NF-κB enhanced its DNA-binding activity during tumor progression in the uterine cervix, we performed EMSA by incubating nuclear extracts from different grades of cervical lesions with a double-stranded radiolabeled oligonucleotide probe containing a high-affinity κB site. We analyzed 11 normal cervical tissues, eight LSILs, 10 HSILs and 13 SCCs. A typical EMSA result shown in Figure 5 reveals a very minimum NF-κB DNA-binding activity in normal and LSIL tissues, and enhanced activity in HSIL and SCC samples. Incubation with unlabeled probe completely abolished the specific NF-κB complexes (data not shown). We also performed supershift assays and band shifts were detectable with RelA and p50 antibodies (Figure 5), confirming that the nuclear translocation observed in HSILs and SCCs involved classical NF-κB partners and it resulted in enhanced DNA-binding activity.

Taken together, these results suggest that in normal cervical tissue and LSIL, p50, RelA and IκB-α are mainly localized in the cytosol, whereas in HSILs and SCCs, classical NF-κB translocates into the nucleus with a typical decrease in the expression of IκB-α that gets phosphorylated and presumably degraded as the tumor progressed.

Discussion

Aberrant NF-κB activity has been described in a number of tumors based on studies using cell lines

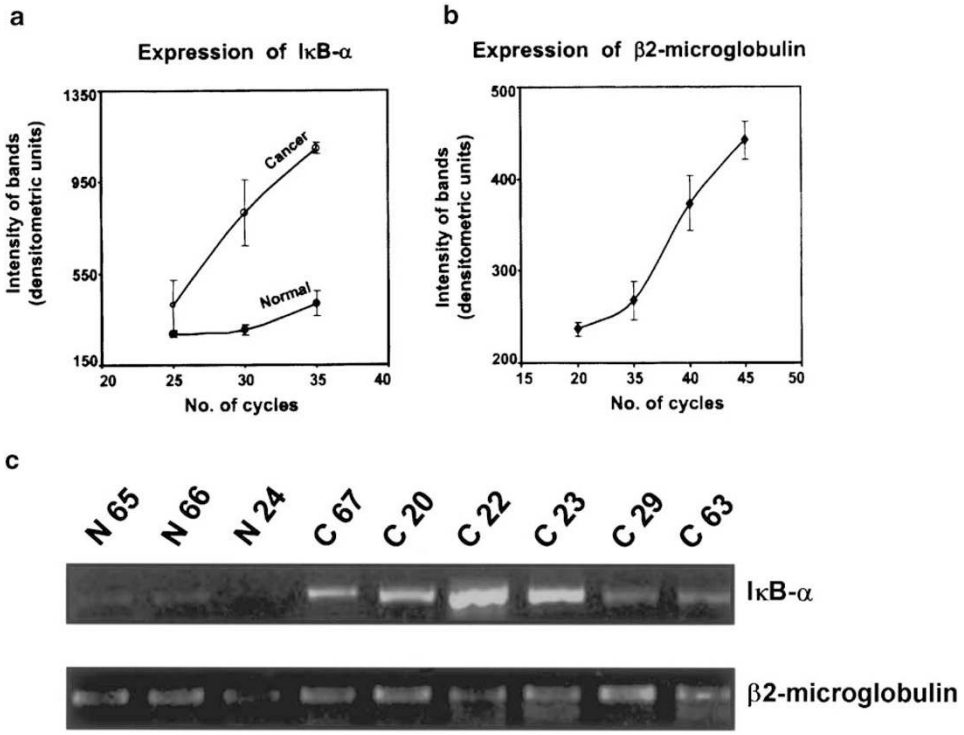

Figure 3 Semiquantitative RT-PCR of *IκB-α* mRNA in normal and cervical cancer tissues. Semiquantitative RT-PCR of *IκB-α* (25, 30, and 35 cycles) in four normal and five SCC samples were carried out as described in Materials and methods, and the bands of expected size (315 bp for *IκB-α* and 114 bp for *β2-microglobulin*) were separated by electrophoresis on 1.2% agarose gel and visualized under ultraviolet light after ethidium bromide staining. The band intensities were quantitated using Quantity One (Bio-Rad) software and plotted against the number of PCR cycles (**a**). For *β2-microglobulin* control (20, 25, 30, and 35 cycles), three normal and two SCC samples were used. Since the variations were minimum for the expression of *β2-microglobulin* between normal and cancer samples, their band intensities were quantitated together for plotting the graph (**b**). A representative band pattern of RT-PCR for *IκB-α* (30 cycles) and *β2-microglobulin* (25 cycles) of three normal and six SCC samples obtained from another independent experiment is also shown (**c**). N and C denote normal tissue and SCC samples, respectively, followed by the patient number. Bars indicate the standard deviation

(Bours *et al*., 1994; Herrmann *et al*., 1997; Nakshatri *et al*., 1997; Sovak *et al*., 1997; Visconti *et al*., 1997; Sumitomo *et al*., 1999; Dejardin *et al*., 1999; Yang and Richmond, 2001) in addition to various types of leukemia and lymphomas (Reuther *et al*., 1998; Cabannes *et al*., 1999; Mori *et al*., 1999;). While mutations and rearrangements of NF-κB/IκB family members have been identified in some hematological cancers, abnormalities in solid tumors have largely been limited to constitutive activation of NF-κB (Rayet and Gelinas, 1999). However, data on the aberrant activation of NF-κB in human tumor tissues are only available for some organs (breast, pancreas, liver, and stomach) comparing normal and cancer tissues for the expression of NF-κB/IκB proteins, but progressive stages of cancer were not studied (Sovak *et al*., 1997; Wang *et al*., 1999; Tai *et al*., 2000; Sasaki *et al*., 2001). Our investigations demonstrate, for the first time, that the transcription factor NF-κB is constitutively activated during human cervical cancer progression. In agreement with our results, nuclear translocation of RelA and NF-κB DNA-binding activity are higher in human tissues from gastric carcinoma, hepatocarcinoma, and pancreatic adenocarcinomas but not in their normal counterparts (Wang *et al*., 1999; Tai *et al*., 2000; Sasaki *et al*., 2001). Similarly, nuclear translocation of p50–RelA complex occurs in human breast tumor tissues and cell lines (Nakshatri *et al*., 1997; Sovak *et al*., 1997), but others found an accumulation of nuclear p52, Bcl-3 and c-Rel rather than RelA in human breast tumor tissues (Cogswell *et al*., 2000). Yang and Richmond (2001) demonstrated that eight melanoma cell lines exhibit enhanced nuclear localization of p50/RelA in comparison with normal human epidermal melanocytes. Constitutive activation of NF-κB also occurs during mouse skin carcinogenesis (Budunova *et al*., 1999). Our results showing a remarkable increase in *IκB-α* mRNA expression in cervical cancer is presumably the consequence of functional activation of NF-κB in cervical cancer since the activation of NF-κB is known to cause strong transcriptional upregulation of *IκB-α* as a feedback mechanism found operative in other cancers (Chiao *et al*., 1994; Wang *et al*., 1999; Cogswell *et al*., 2000). We noted that the protein levels of IκB-α are very low in HSILs and SCCs by immunohistochemistry and in SCCs by Western blotting, similar to this, cytosolic IκB-α protein expression was generally greater in nontumor tissue compared with tumor tissue in human hepatic carcinoma (Tai *et al*., 2000). In contrast, IκB-α protein was overexpressed in human pancreatic tumor tissues

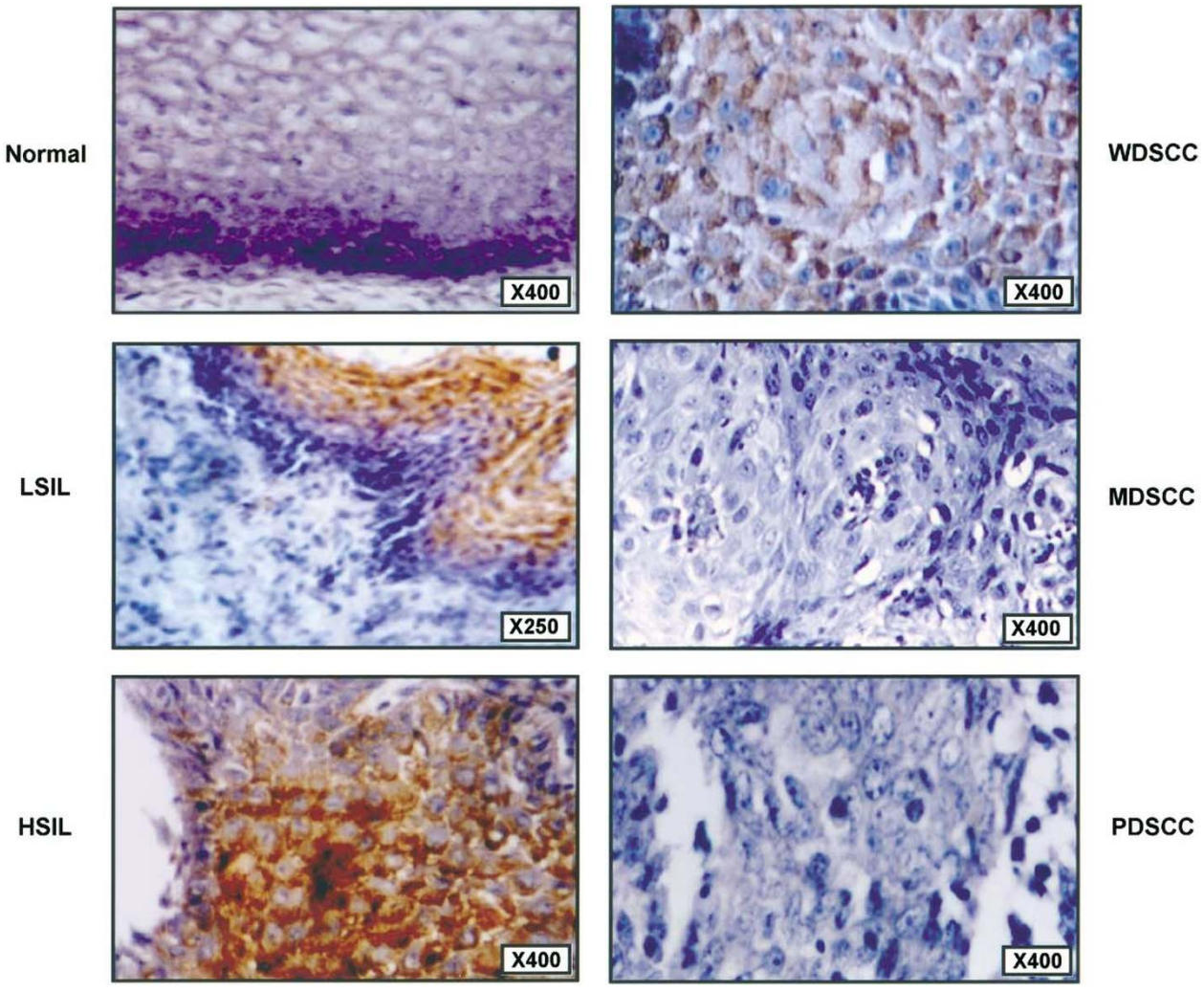

Figure 4 Phosphorylation of IκB-α, and NF-κB DNA-binding activity during tumor progression in the human uterine cervix. Paraffin-embedded sections from normal, LSIL, HSIL, and SCC tissues of the human uterine cervix were immunolocalized with an antibody that specifically recognizes the phosphorylated form of IκB-α as described in Materials and methods, and similar results were obtained in six other sections for each of the grades analyzed including the normal tissue

that also overexpress NF-κB (Wang *et al*., 1999), suggesting that IκB-α may be processed differently in different tissues. The low level of IκB-α protein with the high level of IκB-α phosphorylation at Ser 32/36 in HSILs and the decrease of phospho IκB-α expression in SCCs (more evident in MDSCCs and PDSCCs) of human cervical cancer, taken together, suggest that the IκB-α undergoes degradation in advanced stages of human cervical cancer. Constitutive phosphorylation of IκB-α has been reported in human melanoma cells as the result of the activation of IKKs (Yang and Richmond, 2001). Therefore, continuous elevated degradation of IκB-α should account for the constitutive NF-κB activity in human cervical cancer cells. The actual mechanism of constitutive activation of NF-κB in cervical and other neoplasms, however, remains to be determined. Little is known about how constitutive nuclear NF-κB activity contributes to the malignancy of these cells. There is recent evidence for the existence of alternative NF-κB activation pathways, such as that induced by UV radiation leading to the phosphorylation-independent degradation of IκB-α (Bender *et al*., 1998; Li and Karin, 1998). NF-κB may be activated by cytokines, by alterations in epidermal growth factor or receptor expression, Ras activation, or through cell damage and changes in the oxidation–reduction state (Karin *et al*., 2002). Increased immunoreactivity for N-ras was evident in the basaloid cells of malignant cervical lesions, with the maximum value of 66% found in PDSCC (Nair *et al*., 1998). A significant percentage (24.2%) of early-stage cervical cancers contain activated Ras (Grendys *et al*., 1997). Chronic inflammation and release of proinflammatory cytokines, known to induce NF-κB, might provide a selective growth advantage for abnormal cervical cells (Woodworth *et al*., 1995). Progression of cervical cancer is associated with excessive circulating levels of interleukins (IL-2, IL-6, IL-7, IL-8, and IL-10) and TNF-α (Chopra *et al*., 1998). Cytokines, IL-1 and TNF-α, stimulate proliferation of immortal and malignant cervical epithelial cells by an

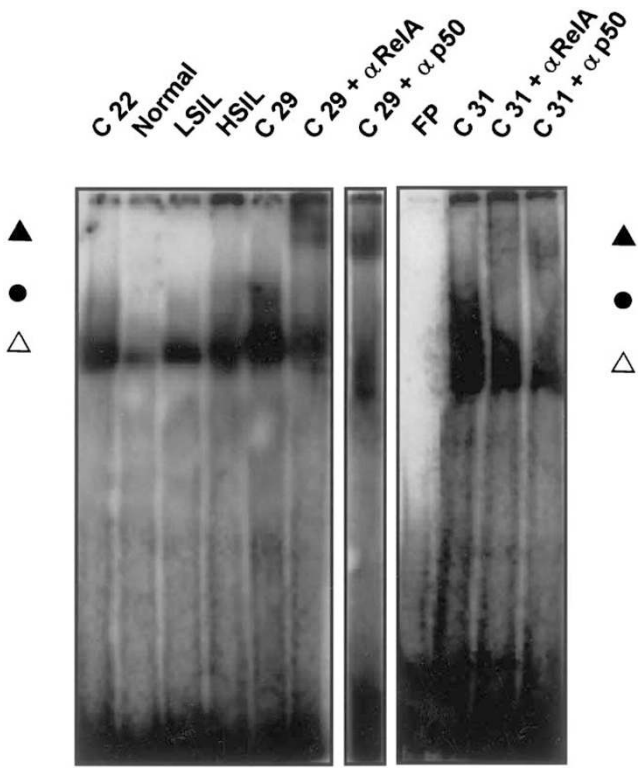

Figure 5 NF-κB DNA-binding activity during tumor progression in the human uterine cervix. Nuclear extracts were prepared from the indicated cervical lesions and normal cervical tissue, and EMSA was performed as described in Materials and methods. FP denotes free probe while C 22, C 29 and C 31 denote SCC samples followed by the patient number. Closed circles indicate the positions of active NF-κB complexes, whereas the open and closed triangles show the positions of inactive and supershifted complexes, respectively

EGF receptor-dependent pathway requiring autocrine stimulation by amphiregulin (Hazelbag *et al*., 2001). Increased levels of oxidative stress within the cell resultant from exposure to environmental carcinogens may activate expression of NF-κB and, in fact, hypoxic cervical cancers with low apoptotic index are highly aggressive (Hockel *et al*., 1999).

Dominant-negative IκB-α transfection sensitized human cervical cancer cells (ME-180) to TNF-α-induced apoptosis (Suk *et al*., 2001). HPV infection is associated with decreased apoptosis (Nair *et al*., 1999), and the presence of the antiapoptotic Bcl-2 protein is strongly associated with the development of invasive cervical disease (Pillai *et al*., 1996). Antiapoptotic protein Mcl-1 and IL-6 (known to be regulated by NF-κB) were concomitantly expressed in human cervical cancer tissues and cell lines, but not in normal cervix tissues (Wei *et al*., 2001). HPV 16 oncogenes, E6 and E7, upregulate several NF-κB-responsive genes in human cervical keratinocytes (Nees *et al*., 2001). Neoplastic progression in HPV 18/*v-fos*-transformed human skin keratinocytes, enhanced transcriptional activities of AP-1 and NF-κB, and inhibition of their activation blocked neoplastic transformation (Li *et al*., 2000). Interestingly, one potential NF-κB binding site has been identified in the HPV-16 LCR, which acts as its transcriptional repressor, and transient overexpression of RelA into HeLa cells repressed the HPV-16 LCR (Fontaine *et al*., 2000). In the light of the regulation by NF-κB of genes important in apoptosis, it is possible that the constitutive activation of NF-κB in cervical tumor cells provides a survival advantage for these tumor cells. It will be interesting to determine the changes in expression and activity of other signal transduction molecules (upstream and/or downstream of NF-κB) that lead to activation of protective pathways, or inactivation of molecules that induce apoptosis.

Our results are supportive of the notion that the nuclear expression of NF-κB might be considered as an indicator of malignant transformation. Further studies are on to understand how NF-κB activation correlates with clinical staging of cervical cancer and to examine the changes in NF-κB expression in nonsquamous cell tumors (glandular and neuroendocrine) that also develop in the human cervix. Given that p50–RelA deregulation is a characteristic common feature for cervical tumors during progression, immunohistochemical detection of these molecules in cervical tissues may be useful as a diagnostic tool. Further studies using cervical cancer cell lines may help to identify mechanisms of activation of NF-κB and shed more light on the potential use of NF-κB/IκB family members as specific targets for the therapeutic intervention of human cervical cancer.

Materials and methods

Antibodies

Polyclonal antibodies to p50 (sc-7178), RelA (sc-109), p52 (sc-298), RelB (sc-226) and IκB-α (sc-371), and a mouse monoclonal antibody to c-Rel (sc-6955) were procured from Santa Cruz Biotechnology. Monoclonal antibodies to phospho IκB-α (Ser 32/36) (9246 S) obtained from Cell Signaling Technology and β-actin (A-5441 Sigma) were also used.

Clinical samples

Fresh cervical tissues (malignant or nonmalignant) were collected from patients undergoing hysterectomy at the Sri Avittam Thirunal Hospital for women, Thiruvananthapuram. A written consent was obtained from patients and bystanders before sample collection. Samples were snap frozen in liquid nitrogen, stored at −70°C, and multiple adjacent regions of each sample were used for analysis. Paraffin-embedded specimens were obtained from the archives of the Doctors Diagnostic and Research Center.

Immunohistochemical analysis

Immunohistochemical analysis was done as described earlier (Nair *et al*., 1997). Sections were blocked with 3% bovine serum albumin for 1 h and incubated overnight at 4°C with the primary antibody. Immunoreactivity was visualized using Vectastain ABC kit (Vector Laboratories) following the manufacturer's protocol. The color development with diaminobenzidine was counterstained with Meyer's hematoxylin.

Immunoreactivities from a minimum of 500 cells/sample were assessed subjectively by at least two investigators.

Western blot analysis

Cytosolic and nuclear fractions (100 μg protein/sample) were collected using a standard protocol (Lahiri and Ge, 2000) from the fresh/frozen cervical tissue (500 mg). They were resolved on 10% SDS-PAGE and transferred to a nitrocellulose membrane (Hybond-C pure, Amersham) using a Bio-Rad Mini PROTEAN III apparatus. Specific proteins were detected with appropriate primary antibodies (Santa Cruz Biotechnology) and alkaline phosphatase-conjugated secondary antibodies (Sigma) using bromochloroindolyl phosphate/nitro blue tetrazolium substrate–dye mixture (Amersham).

Semiquantitative RT-PCR

Total RNA from tissue samples was extracted using Trizol reagent (Life Technologies Inc.) following the manufacturer's protocol and the semiquantitative RT-PCR was then carried out (Barabas *et al*., 2001). Briefly, the RNA isolated (10 μg) was reverse transcribed to cDNA in a 25 μl reaction mix containing 200 U MMLV reverse transcriptase (Life Technologies Inc.), 2 μg random hexamer (New England BioLabs), 6 U RNA guard (Amersham) and 100 μM dNTP mix at 37°C for 1 h. The enzyme was inactivated at 90°C for 4 min and quick-chilled. An aliquot of the cDNA mix (2.5 μl) was used for PCR amplification mixture (50 μl) containing 2.5 U Taq DNA polymerase (Promega) in 1 × reaction buffer, 1.5 mM $MgCl_2$, 150 μM dNTP mix, and 50 pmol of sense and antisense primers. The primer sequences used to amplify *IκB-α* and human *β2-microglobulin* were 5′-CCATGTTCCAGGCGGCCGAG-3′ (sense) and 5′-TGAGGAAGGCCAGGTCTCCC-3′ (antisense) and 5′-ACCCCCACTGAAAAAAGATGA-3′ (sense) and 5′-GCATCTTCAAACCTCCATGAT-3′ (antisense), respectively. PCR conditions for *IκB-α* and *β2-microglobulin* comprised an initial denaturation of 95°C for 3 min, and the indicated number of cycles of 95°C for 30 s, annealing for 1 min at 55°C for *IκB-α* or 65°C for *β2-microglobulin*, and 72°C for 1.5 min and a final extension for 7 min at 72°C.

NF-κB DNA-binding activity

The nuclear extracts (Lahiri and Ge, 2000) were either used immediately or stored at –70°C. Electrophoretic mobility shift assay (EMSA) was performed as described (Manna and Aggarwal, 1999) using a double-stranded ^{32}P-labeled 45-mer NF-κB oligonucleotide from the HIV terminal repeat, 5′-TTGTTACAAGGGACTTTCCGCTGGGACTTTCCAGGGAGGCGTG-3′ containing the κB site (underlined). The gel was dried and visualization of radioactive bands was carried out using Phosphor Imager (Bio-Rad Personal FX).

Abbreviations

NF-κB, nuclear factor kappa B; IκB, inhibitory kappa B; RT-PCR, reverse transcription-PCR; EMSA, electrophoretic mobility shift assay; IKK, IκB kinase; CIN, cervical intraepithelial neoplasia; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; SCC, squamous cell carcinoma; WDSCC, well-differentiated squamous cell carcinoma; MDSCC, moderately differentiated squamous cell carcinoma; PDSCC, poorly differentiated squamous cell carcinoma; HPV, human papillomavirus.

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