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ORIGINAL ARTICLE

Molecular mechanism and role of NF-κB in the early diagnosis of cervical cancer

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Summary

Purpose: *The purpose of this study was to find out the activity and molecular mechanism of NF-κB subunits in cervical cancer which in turn were used as a molecular marker in diagnosing cancer progression.*

Methods: *Different cervical cancer biopsies were obtained from patients after obtaining proper written consent and approval, which were subjected to immunohistochemistry. Performed were Western blot analysis and electrophoretic mobility shift assays.*

Results: *Immunohistochemical analysis of low-grade, high-grade and squamous cell carcinoma (SCC) (stage IIIA, IIIB and IV) showed low to high nuclear expression of p52/RelB compared with p50/RelA, whereas in normal cells, c-Rel was expressed in the cytosol. p52/RelB expression was further validated by Western blot analysis. The binding ability of NF-κB to p52/RelB was increased during the progression of cervical cancer. All cervical carcinoma biopsies showed increased expression of p50/RelA and p52/RelB, but the p52/RelB NF-κB protein complex showed elevated nuclear expression and binding ability, indicating a pathway other than the classical pathway. The non-canonical NF-κB pathway also played an important role in cervical cancer progression by activating the p52/RelB NF-κB complex.*

Conclusions: *This study provides a new approach for diagnosing, and establishing an appropriate treatment against cervical cancer progression.*

Key words: *cervical cancer, NF-κB, p52/RelB, p50/RelA, c-Rel, non-canonical*

Introduction

Cancer a major health issue in humans and the high disease recurrence represents a serious problem. Many studies have reported that over 90% of cancers are associated with environmental pollutants, obesity, smoking, radiation and certain lifestyle factors [1]. However, all of these factors are linked to cancer via inflammation. There are two types of inflammation: acute and chronic. Acute inflammation is considered therapeutic, because it lasts for a short time and helps overcome infections [2]. Chronic inflammation continues for a prolonged period of time and is associated with chronic conditions, such as diabetes, cardiovascular disease and neurological disorders, as well as cancer [3].

In cancer, inflammation is governed by nuclear factor-kappa B (NF-κB) transcription factors [4], including RelA (p65), c-Rel, RelB, NF-κB1 (p50/p105) and NF-κB2 (p52/p100) [4-6]. NF-κB is found in the cytoplasm as a hetero- or homodimer in quiescent state and is translocated to the nucleus once activated [7]. Aberrant expression of these transcription factors results in cancer progression [8]. The well-characterized p50/RelA heterodimer is activated in many cancers; it also plays a vital role in

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the classic canonical pathway. In the non-canonical pathway, NF-κB undergoes activation and binds to p100, which undergoes further processing to generate p52 and liberate p52/RelB dimers. In a third pathway, NF-κB persists only as a homodimer, and its regulation remains unclear [4-6,9].

Because several studies have been conducted on NF-κB and its role in the progression of many cancers, here we focus on how NF-κB can aid in diagnosing cervical cancer. Cervical cancer an important public health issue in the developing countries and the most prevalent carcinoma in females next to breast cancer [10]. It is a major threat that accounts for approximately 80% of cervical cancer deaths in developing countries [11,12]. It affects the female reproductive system at a high rate and can result in metastasis [13]. Reports illustrated that there is a conclusive association between Human Papilloma Virus (HPV) and cervical cancer. HPV infects the cervical epithelium and increases the risk of premalignant lesions and finally progress to cervical cancer [14]. Despite advancements in the diagnosis and treatment modalities, the recurrence of cervical cancer has continued to increase [15,16]. Hence, it is important to determine the molecular mechanism and identify the biomarkers of cervical cancer progression. Several studies have illustrated the localization of homodimeric (p50/50) and heterodimeric (p50/65) NF-κB subunits in cervical cancer [17,18]. Focusing on the role of NF-κB activation, we examined the aberrant expression and localization of p52/RelB in human cervical cancer bioptic tissues. These findings open a new avenue towards prognosis in cervical cancer therapy.

Methods

Patients enrolled in the study

Both normal and malignant cervical tissue samples were collected from patients undergoing treatment in an affiliated hospital. Proper written informed consent was acquired from both the patients and/or their relatives before study entry. This study was approved by the institutional review board, ethical committee and research advisory committees. Biopsy specimens were pathologically classified following histopathological analysis. Histological analysis confirmed that the samples contained >90% tumor cells. The samples were labeled, snap frozen and stored at −70°C before subjecting to immunohistochemistry, Western blotting and DNA binding analyses.

Antibodies

Affinity-purified polyclonal antibodies raised against p50 (Cat. No. 06-886), RelA (Cat. No. SAB4502609), RelB (Cat. No. HPA040506) and c-Rel (Cat. No. PLA0217) and monoclonal antibodies against p52 (Cat. No. 05-361), IKB-β (Cat. No. MABS383) and β-actin (Cat. No. A5441) were used. All antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Immunohistochemistry

Immunohistochemical analysis was performed according to a standard protocol as described previously [19]. Cervical cancer biopsy samples were fixed in formalin and embedded in paraffin wax. Antigens were retrieved in sodium citrate buffer at 95°C for 30 min (pH 6.0), followed by blocking in 3% bovine serum albumin (BSA) for 1 h. Biopsy sections were incubated with the respective primary antibody overnight at 4°C. Immunoreactivity was observed using the ABC Staining Kit (Santa Cruz Biotechnologies, Dallas, TX, USA) according to the manufacturer's protocol. Slides were developed using appropriate chromogenic substrates and counterstained with Mayer's hematoxylin, followed by mounting and observation under a microscope. The expression of NF-κB complex proteins was calculated by dividing the positively stained cells by the total number of cells in each sample.

Western blotting

Nuclear and cytosolic extracts from cervical biopsies were collected according to a standard protocol [20]. Extracts were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins on the gel were then transferred to a polyvinylidene fluoride membrane, blocked with 5% skim milk for 1 h and incubated with the appropriate primary antibody at an appropriate dilution according to the manufacturer's instructions. After washing, the membranes were incubated with alkaline phosphate-conjugated secondary antibodies, and signals were visualized using 3,3'-diaminobenzidine (Sigma-Aldrich). Beta actin was used as a loading control for the western blotting experiments.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed by isolating nuclear extracts from cervical cancer samples as described by Lahiri and Ge [20]. Samples were subjected to EMSA according to standard protocols [21]. Nuclear extracts were incubated with a ^{32}P-labeled 45-mer double-stranded NF-κB binding site sequence from the terminal repeat of HIV1 (5'-TTCTTACAAGGGGACTTTCCGCTGGACTTTCCAGGGAGG-3'; the binding site of NF-κB is italicized). Extracts were resolved on a 7.5% polyacrylamide gel, the gel was dried, and the binding specificity was examined. A supershift assay was carried out by incubating the nuclear extracts with antibodies against p52, RelB, p50 or RelA for 30 min at room temperature. Radioactive bands were visualized on the PhosphorImager (Bio-Rad Molecular Imager FX, Bio-Rad Laboratories, Hercules, CA, USA). Mobility shift assay was performed with all the three stages of SCC (IIIA, IIIB, and IV) according to the above-mentioned protocol.

Statistics

Statistical analyses were performed using SPSS for Windows 11.0 (SPSS, Inc., Chicago, IL, USA). All experiments were performed in triplicate, and the results are

presented as the mean ± standard error of the mean. Results were analyzed by one-way analysis of variance (ANOVA), and the level of significance was set at $p<0.05$. In addition, Tukey's *post-hoc* test was used after ANOVA.

Results

Sample collection and analysis

Biopsy samples were collected from normal and cervical cancer patients. Based on the World Health Organization guidelines, samples were classified as normal, preinvasive (premalignant), squamous cell carcinoma (SCC) or malignant. Preinvasive cervical carcinoma is comprised of low-grade squamous intraepithelial lesions LSILs (Stage I) and high-grade squamous intraepithelial lesions HSILs (stage III). SCC was categorized into three stages as per FIGO guidelines namely, IIIA, IIIB and IV, respectively [22].

To analyze the expression of NF-κB complexes, a total of 118 cervical biopsy samples composed of 26 LSILs (stage I), 29 HSILs (stage III), 42 SCCs (8 samples from stage IIIA, 11 samples from stage IIIB and 23 samples from stage IV) and 21 normal samples were collected from adult females between the ages of 35 and 68 years. The samples were analyzed histologically by a pathologist and subjected to immunohistochemical, Western blot and EMSA analyses.

Nuclear translocation and expression of p52/RelB in human cervical carcinoma biopsy samples

Immunohistochemistry was performed, and the biopsies of normal and different grades of cervical cancer tissues were analyzed and compared to determine the localization of NF-κB protein complexes in cervical tissues. A total of 100 cells were counted, and the number of positively stained cells was calculated as described in methods. The immunohistochemistry results are shown in Figure 1. In the 21 normal tissue samples, cells exhibiting strong cytoplasmic expression of p52, RelB, p50, RelA and c-Rel.

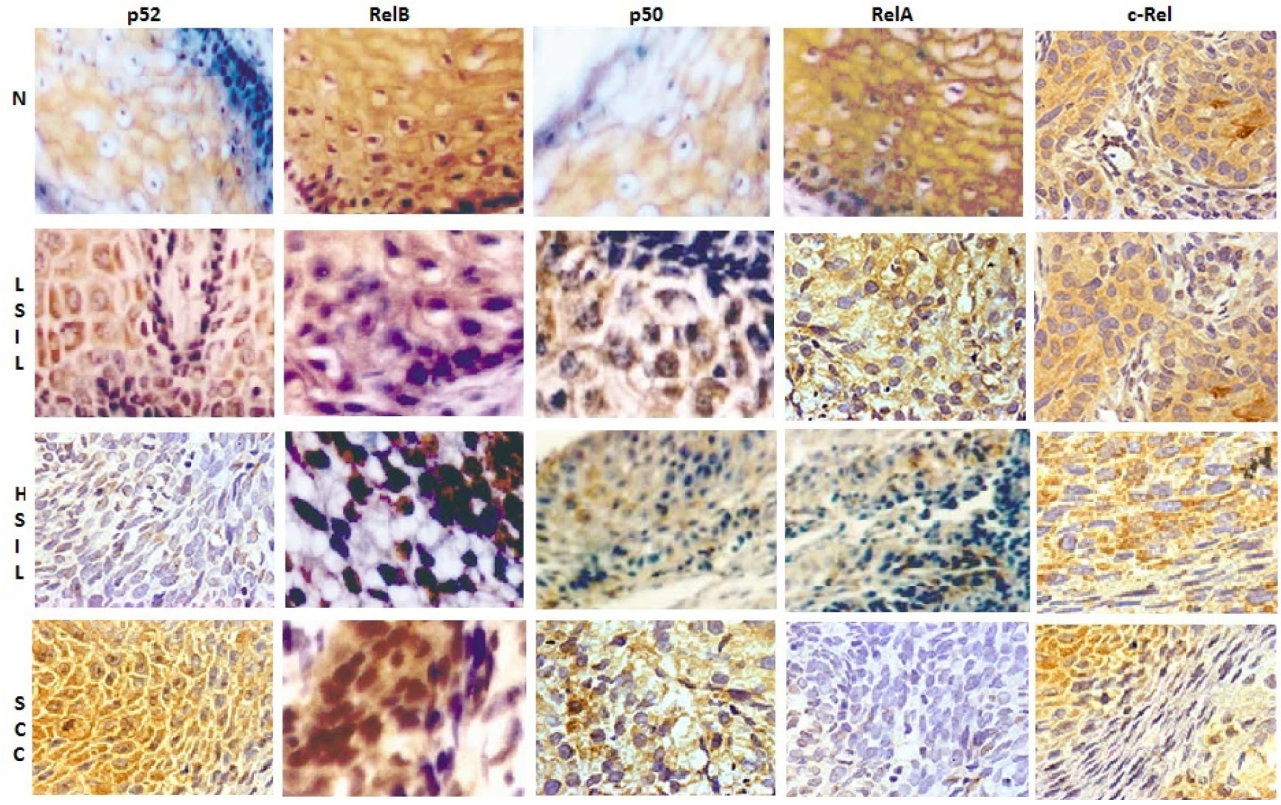

Figure 1. Immunohistochemical analysis of NF-κB in cervical cancer tissues. Nuclear expression of p52, RelB, p50, RelA and c-Rel NF-κB subunits was analyzed in cervical cancer specimens of different grades. Paraffin-embedded 4- to 5-mm-thick sections were obtained from control, premalignant (LSIL and HSIL) and malignant (SCC) human cervical tissues. Tissues were subjected to immunohistochemical analysis using specific antibodies against p52, RelB, p50, RelA and c-Rel. The cervical carcinoma biopsy samples showed increased expression of p50/RelA an p52/RelB. However, the p52/RelB NF-κB protein complex eventually showed high nuclear expression, indicating a pathway other than the classical pathway. Thus, the alternative (non-canonical) NF-κB pathway also plays an important role in cervical cancer progression by activating the p52/RelB NF-κB complex. The expression of c-Rel in the nucleus was found to be mild, but exhibited strong cytosolic expression. N: normal; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion; SCC: squamous cell carcinoma.

Interestingly, the expressions of p52, RelB, p50, RelA and c-Rel were not found in the nucleus. LSIL samples showed mild staining intensity of p52, RelB, p50, RelA and c-Rel in the nucleus compared with other cancer samples. In the LSILs, the staining intensities of p52, RelB, p50, RelA and c-Rel were noted and found to be 24, 27, 23, 22 and 14%, respectively. However, the nuclear expression of p52, RelB, p50 and RelA was very intense in both HSIL and SCC samples. Compared with p50/RelA, the expression of p52/RelB was very high in both HSIL and SCC samples.

In total, 61% and 59% cells showed nuclear expression of p50 and RelA, respectively, whereas 76% and 78% cells showed positivity for p52/RelB expression in HSIL samples. In the stage IIIA samples, nuclear p50/RelA was expressed in 64%/69% cells and nuclear p52/RelB in 77%/79% cells. Likewise, in stage IIIB and stage IV samples, the nuclear p50/RelA was expressed in 66%/72% and 79%/83% cells, respectively. In contrast, high immunoreactivity for p52/RelB in the nucleus was found in 84%/89% and 92%/96% cells of stage IIIB and stage IV samples, respectively. The expression of c-Rel in the nucleus was found to be mild, but exhibited strong cytosolic expression. These data are shown in Figure 1, and a graphical representation is provided in Figure 2.

In conclusion, all of the cervical carcinoma biopsy samples examined showed increased expression of p50/RelA and p52/RelB; however, the p50/RelB NF-κB protein complex eventually showed high nuclear expression, indicating a pathway other than the classical pathway. Thus, the alternative (non-canonical) NF-κB pathway also plays an important role in cervical cancer progression by activating the p52/RelB NF-κB complex.

Aberrant expression of p52/RelB in human cervical carcinoma tissues

The expression patterns obtained by immunohistochemistry were confirmed by Western blot analysis. Both cytosolic and nuclear extracts were collected from fresh cervical cancer tissue samples, and the expression patterns of p52, RelB, p50, RelA and c-Rel were examined and are shown in Figure 3. c-Rel expression was high in cytoplasmic extracts from normal cells, whereas in cancer specimens, the expression of p52, RelB, p50 and RelA was detected in nuclear

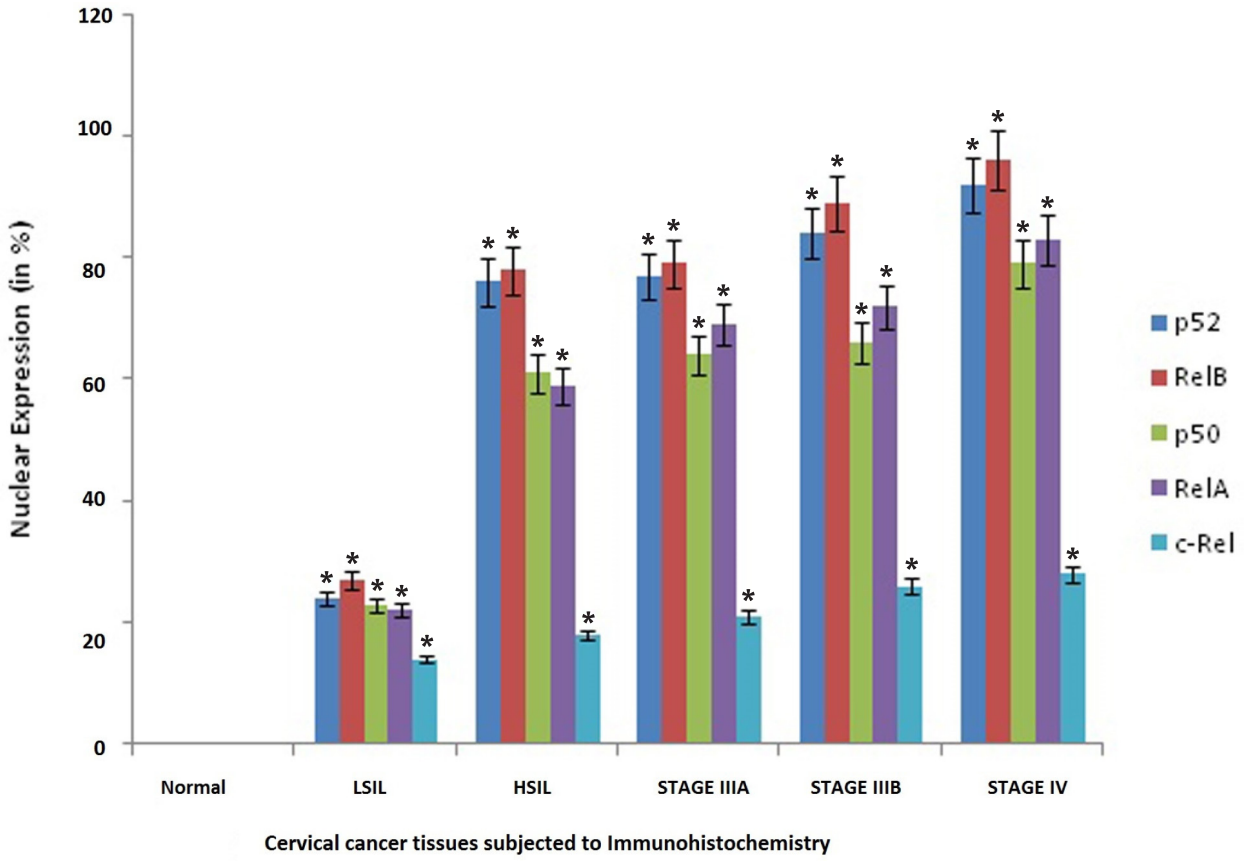

Figure 2. Nuclear expression of NF-κB in cervical cancer tissues. The graph represents the nuclear expression of p52, RelB, p50, RelA and c-Rel NF-κB subunits evaluated by immunohistochemical analysis. The graphical representation intimates that p52/RelB NF-κB protein complex showed high nuclear expression ($p<0.01$).

extracts. Compared with p50/RelA, expression of the p52/RelB NF-κB complex was elevated. This finding supports the immunohistochemistry observations, which suggest that in cervical cancer, the NF-κB p52/RelB complex is prominently activated and aberrantly expressed in the nucleus and can be considered a biomarker for cervical cancer diagnosis.

DNA binding efficiency of the NF-κB p52/RelB complex in cancer progression

The nuclear translocation of p52/RelB was observed in cancer tissues. An EMSA was conducted to determine whether this translocation improved DNA binding affinity. As described in methods, nuclear extracts were isolated from cervical cancer tissues and incubated with an oligonucleotide probe. DNA binding was apparent in all cancer tissues except for LSILs (I), but an elevated binding efficiency was observed in SCCs as shown in Figure 4.

To analyze the binding efficiencies of p52/RelB and p50/RelA, supershift assays were performed, and the results revealed enhanced DNA binding by p52/RelB compared with p50/RelA. The experiments were performed in triplicate, and preferential binding efficiency by p52/RelB to DNA was detected each time in all of the cancer samples. In addition, mobility shift assay was performed with the three stages of SCC (IIIA, IIIB, and IV) in triplicate. Similar results were observed in all the three stages as shown in the Figure 5. Mobility shift assay for low and high grade intraepithelial lesions were performed and the results are shown in the Figure 5.

In conclusion, these findings suggest that both in normal tissues and LSILs (Stage I), p52, RelB, p50, RelA and c-Rel are localized in the cytoplasm; however, in HSILs (Stage III) and SCC, the p52/RelB complex is expressed and translocated to the nucleus. This indicates that the alternative NF-κB p52/RelB pathway plays a significant role in cervical cancer progression.

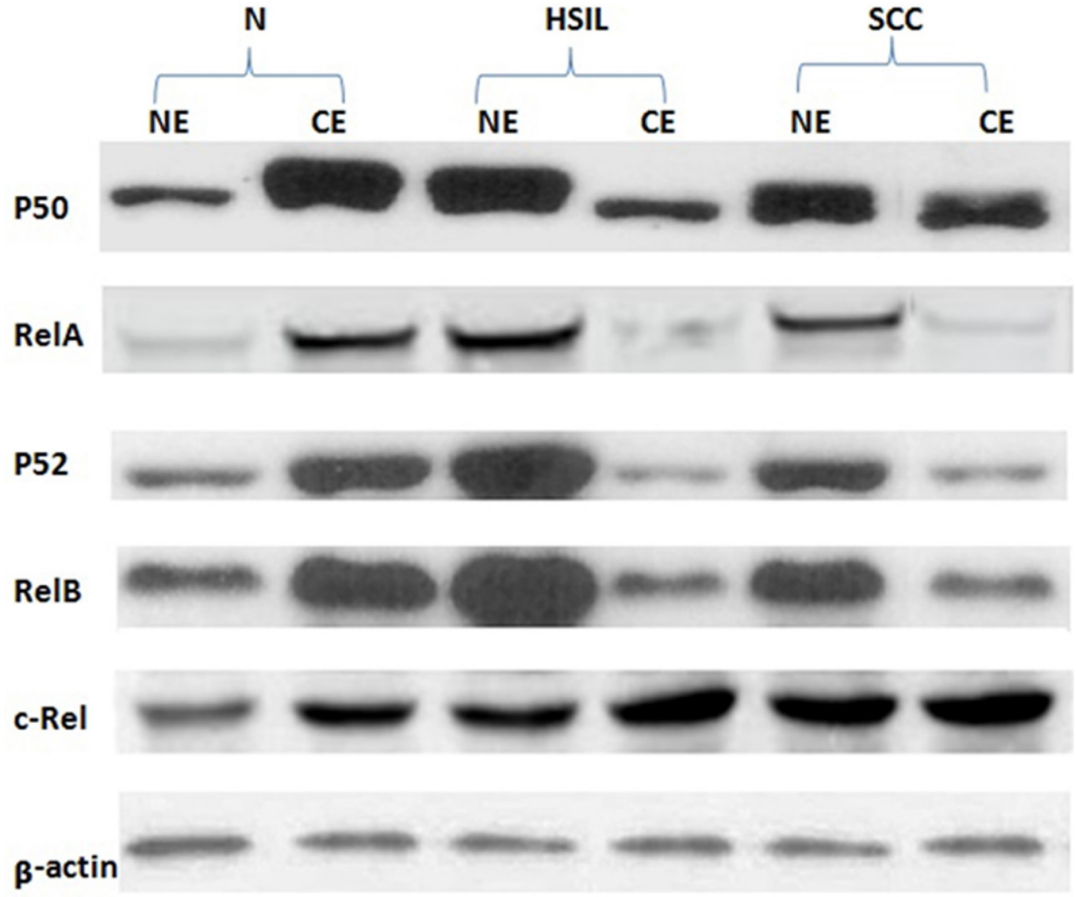

Figure 3. Western blot analysis of NF-κB in cervical cancer tissues. Nuclear and cytoplasmic extracts from normal and cancer cells were electrophoresed and immunoblotted with p50, RelA, p52, RelB or c-Rel antibodies. β-actin was used as a loading control. The expression profile of the p52/RelB NF-κB subunit was confirmed by western blot analysis, which revealed high expression in malignant tissues and moderate expression in low-grade samples. In contrast, the p50/RelA complex showed low to moderate expression. NE: nuclear extract; CE: cytoplasmic extract; N: normal; HSIL: high-grade squamous intraepithelial lesion; SCC: squamous cell carcinoma.

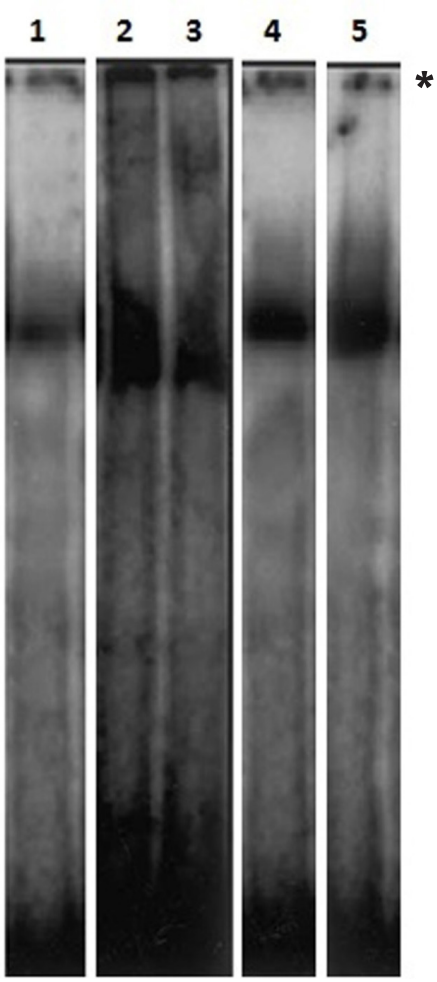

Figure 4. Electrophoretic mobility shift assay. Nuclear extracts from normal and malignant cells were prepared and incubated with specific antibodies against p52, RelB, p50 and RelA. Lane 1: nuclear extract from normal tissue; Lane 2: nuclear extract from SCC tissue incubated with the p52 antibody; Lane 3: nuclear extract from SCC tissue incubated with the pRelB antibody; Lane 4: nuclear extract from SCC tissue incubated with the p50 antibody; Lane 5: nuclear from SCC tissue incubated with the pRelA antibody. The binding efficiencies of p52/relB and p50/relA, was analyzed by means of supershift assays (*), and the results revealed enhanced DNA binding by p52/RelB compared with p50/RelA.

Discussion

In this study, the expression levels and binding affinities of NF-κB complex proteins were examined in cervical cancer tissues, and the results obtained are very interesting. NF-κB complex proteins, p52, RelB, p50, RelA and c-Rel were examined. In normal cells, NF-κB remains in a latent state and resides in the cytoplasm; however, upon activation [7,8], NF-κB undergoes degradation by means of an inhibitory protein, IkB, and the subunits are translocated to the nucleus, where they bind to specific DNA sequences and initiate transcription, followed by protein translation [23]. However, if NF-κB activation is caused by chronic inflammation, the expression of NF-κB subunits is further enhanced, which results in uncontrolled protein translation, giving rise to chronic infection, especially in cancer [24,25].

In this study, a biomarker responsible for cervical cancer was identified that may be helpful for diagnosing cancer progression. FIGO stages (stage I, III, IIIA, IIIB and IV) of cervical cancer tissues were analyzed. We observed that the normal tissues strongly expressed only cytosolic activity of NF-κB. LSIL biopsy samples showed mild nuclear expression and DNA binding affinity of NF-κB, whereas considerable nuclear expression and binding ability of NF-κB were observed in HSIL and SCC tissues. We also found enhanced expression, localization and DNA binding ability of p52/RelB NF-κB complex proteins.

Immunohistochemical analysis showed high cytoplasmic expression of c-Rel in normal tissues but vast nuclear expression of p52/RelB compared with the p50/RelA subunit in tumor tissues (Figure 1,2). The expression profile of the p52/RelB NF-κB subunit was confirmed by Western blot analysis, which revealed high expression in malignant tissues and moderate expression in low-grade samples. In contrast, the p50/RelA complex showed low to moderate expression (Figure 3). Finally, DNA binding and the supershift assay revealed that p52/RelB subunits play a major role in malignant tissues (Figure 4,5). Hence, it was confirmed that the non-canonical NF-κB pathway, which is activated by the NF-κB p52/RelB complex, plays a major role in cervical cancer progression.

Abnormal expression, rearrangement and mutation of the NF-κB complex have been evaluated in several cancer tissues, such as colon [24], pancreatic [26], breast [27] and hematological cancers [28]. Many studies on cervical cancer have reported that MALAT1 [29], EMC6 [30], integrin αVβ6 [31], survivin and FasL [32] expression can be used as markers of cervical cancer progression. Some studies on cervical cancer revealed activation of the NF-κB p50/RelA heterodimer [18] and p50/50 homodimer [17], and other studies illustrated high nuclear expression of p50/RelA in melanoma cell lines compared with normal human melanocytes [33].

In the present study, we investigated the expression of subunits of the NF-κB p52/RelB complex and showed elevated expression in HSIL and SCC tissues. Western blot analysis and DNA binding affinity assays confirmed these findings. The canonical/classical NF-κBp50/RelA pathway plays an important role in cancer progression [34,35];

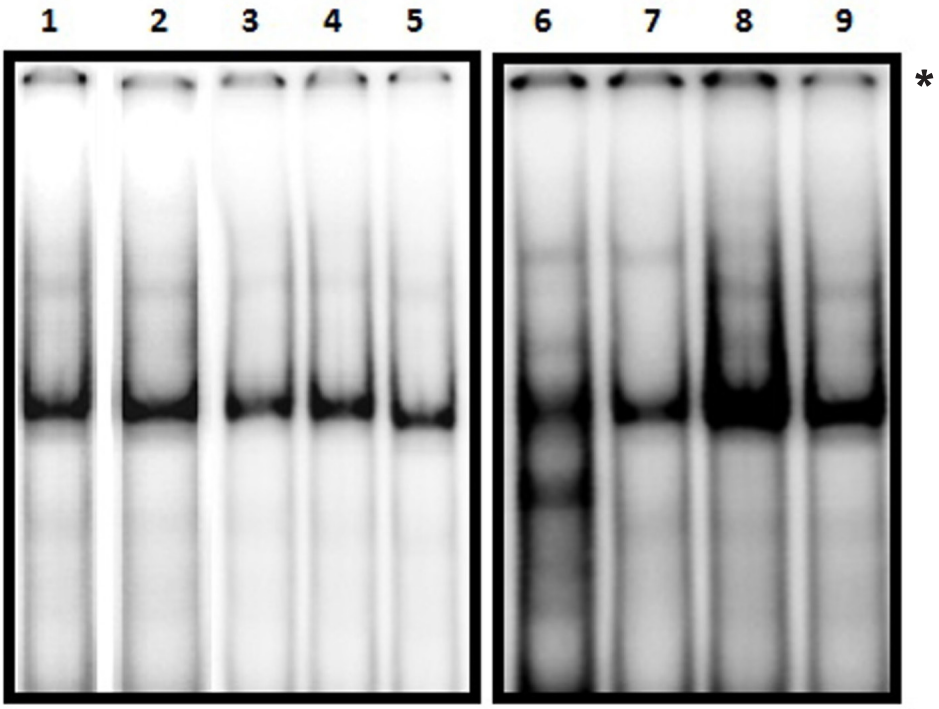

Figure 5. Electrophoretic mobility shift assay. Nuclear extracts from normal and malignant cells were prepared and incubated with specific antibodies against p52, RelB, p50 and RelA. Lane 1: nuclear extract from normal tissue; Lane 2: nuclear extract from LSIL tissue incubated with the p52 antibody; Lane 3: nuclear extract from LSIL tissue incubated with the pRelB antibody; Lane 4: nuclear extract from LSIL tissue incubated with the p50 antibody; Lane 5: nuclear from LSIL tissue incubated with the pRelA antibody; Lane 6: nuclear extract from HSIL tissue incubated with the p52 antibody Lane 7: nuclear extract from HSIL tissue incubated with the pRelB antibody Lane 8: nuclear extract from HSIL tissue incubated with the p50 antibody Lane 9: nuclear extract from HSIL tissue incubated with the pRelA antibody. * The supershift complex. Mobility shift assay for low and high grade intraepithelial lesions also showed an increased DNA binding efficiency by p52/RelB compared with p50/RelA).

however, the current study determined that, in addition to the classical pathway, the non-canonical NF-κBp52/RelB pathway is also associated with inflammation and tumor progression.

Generally after cancer treatment, the rate of recurrence is high, and this is also true for cervical cancer. One cause of recurrence is radiation. High levels of radiation activate the alternative NF-κB pathway, in which phosphorylation and independent degradation of IκB occur, causing translocation of the p52 and RelB subunits of NF-κB to the nucleus where they are aberrantly expressed. Some inflammatory agents, such as cytokines, also induce abnormal expression of NF-κB in cervical cancer [36]. Until now, there has been no reliable biomarker for diagnosing cervical cancer. Hence, it is crucial to determine the mechanism of tumor progression and identify a biomarker for cervical cancer. Consequently, this study provides a new approach for diagnosing and establishing an appropriate treatment against cervical cancer progression. Our findings suggest that high nuclear expression of the NF-κB p52/RelB complex may contribute to malignancy. The abnormal expression of these subunits detected by immunohistochemistry and Western blot analysis can be considered an essential diagnostic contrivance. Future studies are needed to investigate the activation profile of p52/RelB in cell lines, Pap smear specimens or cervicovaginal fluid sample, which will aid in the development of a new therapy for cervical cancer prevention.

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Conflict of interests

The authors declare no conflict of interests.

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