Prognostic value of nuclear factor κ B expression in patients with advanced cervical cancer undergoing radiation therapy followed by hysterectomy

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ABSTRACT

Aims The nuclear factor κ B (NF-κB) family comprises transcription factors that promote the development and progression of cancer. The NF-κB pathway is induced by radiation therapy and may be related to tumour radioresistance. The aim of this study was to evaluate the expression of NF-κB as a predictor of the response to radiotherapy and its value as a prognostic marker.

Methods A retrospective analysis was performed in a series of 32 individuals with stage IB2 and IIB cervical cancer who underwent radiotherapy, followed by radical hysterectomy, from January 1992 to June 2001. NF-κB-p65 and NF-κB-p50 expression was examined by immunohistochemistry in biopsies from all patients before radiotherapy and in 12 patients with residual tumours after radiotherapy.

Results 16 (50%) patients had residual disease after radical hysterectomy. The median follow-up time was 73.5 months, and the 5-year overall survival was 66.5%. Before radiotherapy, cytoplasmic expression of NF-κB-p65 and NF-κB-p50 was noted in 91% and 97% of cases, respectively, versus 59% of cases with nuclear expression of these subunits. Cytoplasmic expression of NF-κB-p65 and NF-κB-p50 in the residual tumours after radiotherapy was observed in 50% of cases; 75% of cases with residual tumours had nuclear expression of NF-κB-p50 versus none with NF-κB-p65. NF-κB-p65 and NF-κB-p50 did not correlate with the risk of residual tumours after radiotherapy or recurrence or death.

Conclusions These data suggest that NF-κB does not predict the response to radiotherapy and does not correlate with poor outcomes in advanced cervical cancer.

Cervical cancer is the third most common cancer among women worldwide, with approximately 500 000 new cases per year and more than 250 000 deaths.[1]

The causal relationship between human papilloma virus (HPV) and invasive cervical cancer is supported by epidemiological and molecular data.[2–4] Approximately 40 types of HPV infect the genital tract, 12 of which are considered carcinogens.[2 5]

High-risk HPV types encode two oncoproteins, E6 and E7, both of which mediate human epithelial cell transformation and immortalisation. They stimulate cell proliferation after interfering with regulatory proteins, including the p53 and pRb suppressor tumour genes. In addition, E6 and E7 promote transformation, the suppression of apoptosis and cancer development.[3 6]

Patients with locally advanced disease (stage IB2–IVA) are treated with definitive concurrent cisplatin-based chemoradiation therapy and can achieve a 5-year overall survival rate of 66%.[7] However, 44% of such patients experience a recurrence, and 35% of recurrent tumours after radiation therapy occur exclusively in the pelvis.[7]

Molecular profiling of the tumour and biomarker studies have attempted to predict the patients who are more likely to experience a recurrence after standard therapy and develop individualised targeted therapies that improve survival rates.

Nuclear factor κ B (NF-κB) is a family of transcription factors, comprising five subunits: p50 (NF-κB1), p52 (NF-κB2), c-Rel, RelB and p65 (RelA). The classic, or canonical, NF-κB is the chief heterodimer, consisting of p50 and p65. The NF-κB complex is rendered inactive in the cytoplasm by the inhibitor of κ B (IκB). The classic NF-κB pathway is activated by the phosphorylation of IκB by the IκB kinase complex, resulting in its ubiquitination and proteosome-mediated degradation. NF-κB is released and translocates to the nucleus, where it binds to κB-responsive elements in NF-κB target genes.[8 9]

Constitutive NF-κB activation has been detected in several human malignancies, as evidenced by the increased nuclear localisation of NF-κB subunits, such as p65 and p50.[10 11] Furthermore, accumulating evidence suggests that NF-κB promotes the development and progression of cancer.[12] Increased NF-κB signalling can block apoptosis, increase proliferation, and induce epithelial mesenchymal-transition[12 13] by activating its target genes. NF-κB is also activated by radiation therapy and might be related to tumour radioresistance.[14 15]

Previous data have shown that HPV oncoproteins also correlate to the NF-κB pathway. HPV-16 E5 mediates cervical carcinogenesis partly by the upregulation of COX-2 expression through NF-κB and AP-1.[16] In cervical keratinocytes, HPV-16 E6 and E7 also enhance the expression of genes regulated by NF-κB and AP-1, and stimulate the

secretion of specific proinflammatory and immunoregulatory cytokines. Furthermore, HPV-16 E6 induces NF-κB DNA binding sites.[17]

The value of NF-κB as a prognostic biomarker has only recently been explored. In several cancers, including breast, prostate, skin, lung and pancreatic cancer, NF-κB expression has been linked to poor clinical outcomes.[18]

Few studies[8 18] have evaluated the prognostic significance of NF-κB expression in human cervical cancer, and none has examined it in response to radiation therapy.

Our aim was to evaluate the immunohistochemical expression of NF-κB retrospectively as a predictor of the response to radiotherapy in a cohort of patients with advanced cervical cancer who received radiotherapy, followed by hysterectomy, and determine its value as a prognostic marker.

MATERIALS AND METHODS

Patient characteristics

This retrospective analysis included a historical series of 32 individuals with cervical cancer who were admitted to the Department of Gynecologic Oncology, AC Camargo Cancer Hospital, from January 1992 to June 2001. The Institutional Review Board approved the study. All patients had squamous cell carcinomas (SCC) and underwent radiotherapy, followed by class II radical hysterectomy. Biopsies from all 32 patients taken before radiotherapy were suitable for analysis. Of the 16 patients with residual tumours, 12 had paraffin-embedded tissues that were suitable for analysis.

Thirty-one (97%) patients had stage IIB tumours, and one patient had stage IB2 disease. All patients received preoperative treatment with external beam radiotherapy (EBRT) and high-dose vaginal brachytherapy. Treatment with EBRT was delivered with 4 or 6-mV linear accelerators. Fractionation was 1.8 Gy per day five times per week. The median dose of EBRT was 45 Gy (range 29–45 Gy).

High-dose vaginal brachytherapy treatment was delivered with Fletcher after-loading applicators with an iridium-192 source. The median dose of brachytherapy to point A was 12 Gy (range 10–15 Gy). The median interval between radiotherapy and hysterectomy was 81.5 days (range 45–154 days).

The clinical features that were analysed were age and time between radiotherapy and surgery. The pathology data included histological type, grade, pathological response to radiotherapy and immunohistochemical expression of NF-κB-p65 and NF-κB-p50.

Immunohistochemical staining

Briefly, formalin fixed, paraffin-embedded tissues were deparaffinised and prepared in successive passages through xylol and ethanol and subjected to antigenic recovery in a pressure cooker using citrate buffer. Once the sections were prepared, they were blocked with 3% hydrogen peroxide in methanol and incubated overnight with the antibody. The reactions were always accompanied by a positive control and two negative controls—one that lacked the primary antibody and another that lacked the secondary antibody. All slides were analysed by light microscopy.

Rabbit polyclonal anti-human NF-κB-p65 and rabbit polyclonal anti-human NF-κB-p50 were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). The primary antibody for NF-κB generated granular or diffuse cytoplasmic and/or nuclear staining. Nuclear and cytoplasmic immunostaining was evaluated semiquantitatively.

Cytoplasmic staining was scored 0 when there was no stain or weak staining in fewer than 10% of tumour cells; 1+ with weak immunostaining in more than 10% of tumour cells; 2+ with moderate staining in more than 10% of tumour cells; and 3+ with strong staining in more than 10% of tumour cells.[19] Tissues that received a score of 3+ were considered positive for cytosolic expression.

Nuclear staining was scored as follows: 0 if there was no stain or weak staining in fewer than 30% of tumour cells; 1+ with weak staining in more than 30% of tumour cells; 2+ with moderate staining in more than 30% of tumour cells; and 3+ with strong staining.[20] Samples were considered positive for nuclear expression if they received a score of 2+ or 3+.

Figures 1 and 2 show examples of NF-κB p65 and p50 expression by immunohistochemistry.

Statistical analysis

The database was set up using SPSS, version 16.0 for Mac. Follow-up time was considered the interval from the date of surgery to the last date for which information was available. The association between immunostaining and other variables was analysed by the χ^2 test. The survival curves were estimated using the Kaplan–Meier method, and curves were compared by log-rank test. For all tests, an α error up to 5% ($p<0.05$) was considered significant.

RESULTS

The median age was 45 years (range 22–67 years). All patients had SCC, and six (17.6%) patients had grade three tumours.

Twenty-four (75%) patients underwent pelvic lymphadenectomy in addition to radical hysterectomy. Four (16.7%) patients had lymph node metastasis, with a median of 8.5 resected lymph nodes (range 2–28).

After radical hysterectomy, 16 patients (50%) had pathological residual disease. With regard to lymph node metastasis, one patient had lymph node involvement without residual disease.

The median follow-up time was 73.5 months (range 8–151 months). Eight patients (25%) relapsed (four local recurrence and four distant metastases), and all died from the disease. Three (9.4%) died from other causes. The 5-year overall survival rate was 66.5% (figure 3), and the 5-year disease-free survival rate was 73.7%.

Of the primary tumours, we found a higher prevalence of NF-κB cytoplasmic than nuclear expression, whereas 29 (91%)

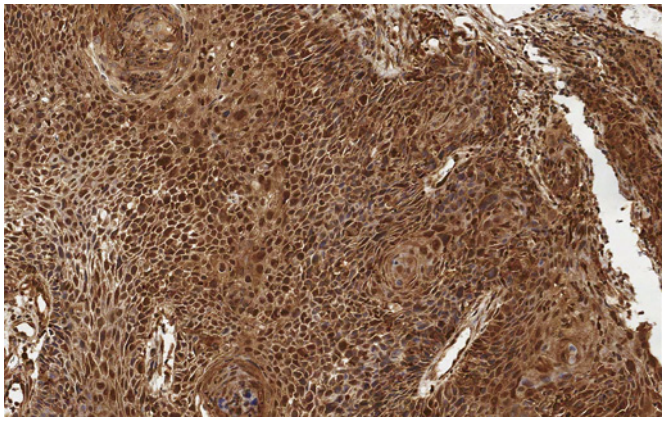

Figure 1 Microphotograph of nuclear factor κ B p65 immunohistochemical staining with '3+' cytoplasmic expression (20×).

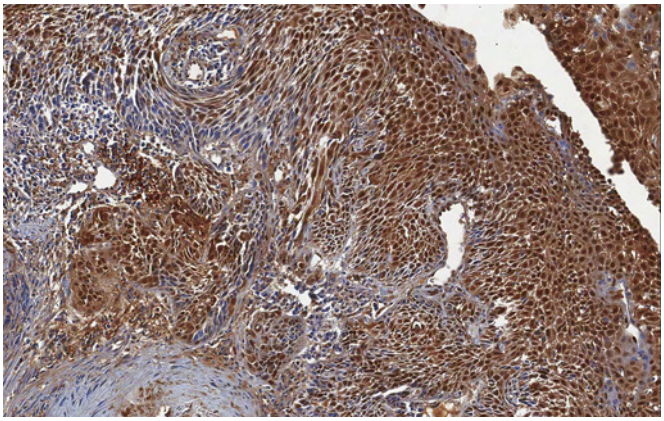

Figure 2 Microphotograph of nuclear factor κ B p50 immunohistochemical staining with '3+' cytoplasmic expression (20×).

and 19 (59%) cases were positive for cytoplasmic and nuclear NF-κB-p65, respectively, versus 31 (97%) and 19 (59%) cases positive for NF-κB-p50, respectively (table 1).

Twelve patients with residual disease after radiotherapy were analysed. We noted a lower cytoplasmic expression for NF-κB-p65 and NF-κB-p50 when compared with the primary tumours, as positive expression of NF-κB-p65 or NF-κB-p50 was found in six (50%) cases. Interestingly, we did not observe nuclear NF-κB-p65 expression in these cases. On the other hand, for NF-κB-p50 we found a higher nuclear expression when compared with the primary tumours before radiotherapy, as nine (75%) cases had positive nuclear expression (table 1).

There was no association between age, lymph node metastasis, or histological grade with NF-κB-p65 or NF-κB-p50 expression.

Regarding residual disease after radiotherapy, no clinical-pathological variable correlated significantly with the risk of residual disease (table 2).

Nuclear NF-κB-p65 expression in the primary tumour had no impact on disease-free (p=0.97) or overall survival rates (p=0.90), nor did nuclear NF-κB-p50 expression (p=0.53 and p=0.28, respectively). Furthermore, the presence of residual disease after radiotherapy was not associated with the risk of recurrence or death.

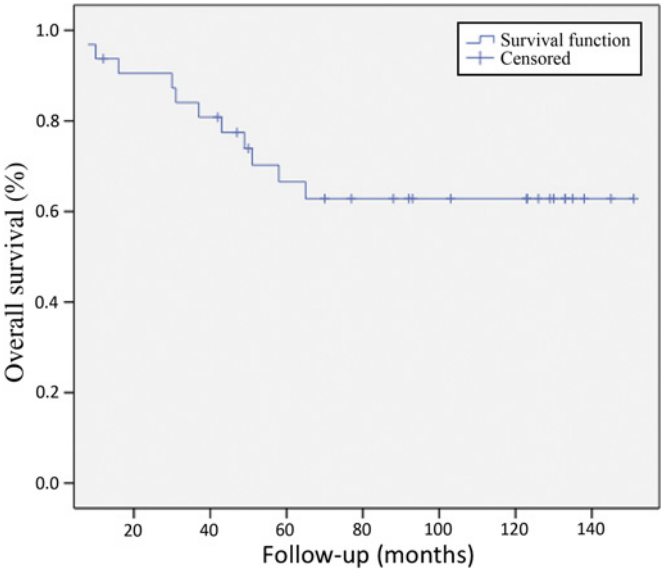

Figure 3 Overall survival curve for patients with cervical cancer submitted to radiotherapy followed by radical hysterectomy.

Table 1 Immunohistochemical positive expression of NF-κB-p65 and NF-κB-p50 for the 32 patients submitted to radiotherapy followed by radical hysterectomy and for the 12 patients with residual disease

Variable	All patients, n=32 (%)	Residual disease, n=12 (%)
NF-κB-p65 (cytoplasm)	29 (91)	6 (50)
NF-κB-p65 (nucleus)	19 (59)	0 (0)
NF-κB-p50 (cytoplasm)	31 (97)	6 (50)
NF-κB-p50 (nucleus)	19 (59)	9 (75)

NF-κB, nuclear factor κ B.

DISCUSSION

Clinical-pathological factors, such as stage, size, histological type and lymph node involvement, are the only prognostic factors for advanced cervical cancer that is treated by radiotherapy.[21] Therefore, the molecular events and mechanisms of resistance to radiotherapy must be recognised.

Several markers have been studied regarding the response to radiotherapy and have been linked to apoptosis, angiogenesis and tumour progression.[21]

NF-κB regulates important signalling pathways, immune responses, inflammation and proliferation. In carcinogenesis, NF-κB is anti-apoptotic.[22] Radiation therapy aims to eliminate tumour cells by inducing apoptosis and, conversely, the NF-κB pathway may be activated by radiotherapy. Nevertheless, the inhibition of NF-κB favours tumour susceptibility to pro-apoptotic inducers, such as chemotherapy and radiotherapy.[14]

Loercher *et al*[23] suggested that the NF-κB pathway is intrinsically activated in SCC in vitro. Yet few studies have examined NF-κB expression in the various stages of cervical intra-epithelial neoplasia and cervical cancer, noting a progressive increase in immunohistochemical NF-κB expression during the progression of cervical intra-epithelial neoplasia to cancer.[8 21 23 24]

Nair *et al*[22] studied the immunohistochemical expression of NF-κB in 38 cervical SCC. In low-grade SCC, nuclear NF-κB-p50 and NF-κB-p65 were expressed in 64% and 75% of cases, respectively, versus 81% and 92% of high-grade SCC, respectively. The authors proposed the nuclear translocation of NF-κB through the canonical pathway.

Table 2 Association between clinical-pathological variables and the presence of residual disease after radiotherapy

Variable	Category	Residual disease (no of patients)		
		Absence	Presence	p Value
NF-κB-p65 (nucleus)	Negative	5	8	0.2
	Positive	11	8	
NF-κB-p50 (nucleus)	Negative	0	1	1.0
	Positive	16	15	
Lymph node metastasis	Absence	10	10	0.5
	Presence		1	3
Histological grade	Grade 2	14	12	0.6
	Grade 3	2	4	
Age, years	<45	7	10	0.2
	≥45	9	6	
Interval between radiotherapy and radical hysterectomy, days	≤80	7	8	0.7
	>80	9	8	

NF-κB, nuclear factor κ B.

Ramdass *et al*[24] published a series of 114 cervical SCC, 97% and 95% of which expressed cytoplasmic NF-κB-p50 and NF-κB-p65, respectively, compared with 86% and 59% of cases with nuclear NF-κB-p50 and NF-κB-p65 expression. Branca *et al*[25] examined only p65 levels, observing cytoplasmic and nuclear NF-κB expression in 39% and 8.8%, respectively, of 150 cervical cancer cases.

In our study, we observed higher expression of NF-κB. As our series included only advanced cervical cancers, we may speculate that more advanced stages have higher expression of NF-κB. However, this statement still needs further confirmation.

Li *et al*[8] described a gradual increase in nuclear immunohistochemical expression of NF-κB and nuclear translocation by western blot. In 79 cervical cancer cases, nuclear p65 and p50 expression correlated with higher grade, larger tumours and stromal invasion. NF-κB-p65 expression was also linked to lymph node metastasis and had a negative impact on survival. The authors suggested that NF-κB activation in cervical cancer correlates with tumour progression, aggressive behaviour and worse prognosis.

Only Garg *et al*[18] have analysed the prognostic value of NF-κB expression in locally advanced tumours (stages IB2—IIIB) in those who have undergone definitive treatment with chemoradiation. Sixteen patients had biopsies of tumour samples performed before and 48 h after the start of radiotherapy, three (19%) and five (31%) of which, respectively, were positive for nuclear NF-κB, although the subunits that were detected were not cited. Despite the small sample, positive NF-κB expression had a negative impact on locoregional recurrence and the risk of death from cancer. Yet their correlation with prognosis might be overstated, because they did not attain this result by Kaplan—Meier analysis or log-rank test. This finding is in contrast to our results, as we did not note a correlation between NF-κB expression and the risk of recurrence or death.

Patients with residual disease after radiation therapy had lower levels of p65 and p50 compared with the primary tumour and increased nuclear NF-κB-p50 expression (75%).

NF-κB expression in the cytoplasm or nucleus before radiotherapy could not predict which patients had residual disease after radiotherapy. However, radiation might induce the translocation of NF-κB into the nucleus, as evidenced by lower cytoplasmic expression and higher nuclear expression of NF-κB-p50 in patients with residual disease after radiation. For this subgroup, the NF-κB pathway might stimulate the production of anti-apoptotic factors that impair the response to radiation. However, it is not possible to determine whether translocation occurred immediately before cell death in patients who experienced complete responses from our data.

Notably, no patient with a residual tumour expressed nuclear NF-κB-p65. Prusty *et al*[26] reported high p50 and low p65 expression in the nucleus, proposing that preferential homodimerisation of p50/p50 occurs in cervical cancer. Our findings may corroborate this hypothesis.

Our series is the first to analyse NF-κB expression in advanced tumours in patients who have undergone radiotherapy followed by surgery. However, some limitations should be recognised, such as its retrospective setting and the small sample. On the other hand, we used a valuable model to evaluate new markers and the response to radiation, obtaining tumour samples from a cohort before and after radiation therapy.

In conclusion, NF-κB is highly expressed in advanced cervical cancer, and NF-κB does not predict the response to radiotherapy or outcome. Future studies should reproduce our findings and evaluate the value of the addition of chemotherapy.

Take-home messages

- The NF-κB pathway is induced by radiation therapy and may be related to tumour radioresistance.
- Few studies have evaluated the prognostic significance of NF-κB expression in human cervical cancer, and none has examined NF-κB in response to radiation therapy.
- NF-κB is highly expressed in advanced cervical cancer. However, NF-κB does not predict the response to radiotherapy or the outcome.

A greater understanding of the predictive factors of radiation therapy will allow clinicians to identify patients who are at higher risk of recurrence and more likely to benefit from new treatment regimens.

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Competing interests None.

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