Expression of NF-κB in Prostate Cancer Lymph Node Metastases

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INTRODUCTION. Nuclear factor-κB (NF-κB) is a transcription factor that transactivates genes involved in the regulation of cell growth, apoptosis, angiogenesis, and metastasis. Our aim was to assess NF-κB expression in lymph node (LN) metastases of prostate cancer.

METHODS. Immunohistochemical staining was performed using the p65 anti-NF-κB antibody. Seventy-seven paraffin-embedded LN specimens obtained from 54 prostate cancer patients were analyzed. Of the 54 patients, 32 had positive LN metastases, while 22 showed no evidence of metastasis and were considered as controls. The overall percentage of NF-κB-nuclear localization was assessed, as well as the intensity of staining.

RESULTS. Nuclear localization of NF-κB was significantly greater in the metastatic LN group compared to controls. In patients with positive-LN metastases, 84.4% showed >10% nuclear staining in tumor cells. Moreover, 64.4% of the malignant LN specimens had >10% nuclear staining in lymphocytes compared to 0% in controls. Intensity of cytoplasmic and nuclear staining was higher in the metastatic LN group than in controls (P < 0.01).

CONCLUSIONS. Nuclear localization/activation of NF-κB is up-regulated in prostate cancer LN metastasis. Such up-regulation of NF-κB activity is observed in the tumor cells as well as in the surrounding lymphocytes. Prostate 58: 308–313, 2004. © 2003 Wiley-Liss, Inc.

KEY WORDS: NF-κB; lymph nodes; prostate; human

INTRODUCTION

The majority of prostate cancer deaths are due to metastases that are resistant to therapy [1]. The exact molecular events associated with carcinogenesis, progression, and metastasis in prostate cancer are still unclear. However, it is well documented that pathogenesis of cancer metastasis comprise a series of sequential and selective steps that include tumor cell proliferation, angiogenesis, invasion, survival, and growth in distant organs [2].

Nuclear factor-κB (NF-κB) is an inducible dimeric transcription factor that belongs to the Rel/NF-κB family [3,4]. Classical NF-κB transcription factor is formed by the p50 and RelA (p65) proteins [5–8]. NF-κB activation involves its release from its inhibitor, IκB, and its subsequent translocation from the cytoplasm to the nucleus, where it binds to cognate sequences in the promoter region of multiple genes [3–8]. Regulation of gene expression by NF-κB is controlled mainly by the inhibitory IκB proteins, which include IκBa [8]. Upon stimulation, IκBa is rapidly phosphorylated and degraded via the ubiquitin-proteasome pathway, permitting activation and nuclear importing of NF-κB [8]. Numerous in vivo and in vitro
It has been recently reported that inhibition of NF-κB are essential for angiogenesis, as well as tumor IV collagenases (MMP-2 and MMP-9) [13,14]. These factors are essential for angiogenesis, as well as tumor cell motility, invasiveness, and/or metastasis [13–15]. It has been recently reported that inhibition of NF-κB activity in PC-3 cells was associated with decreased expression of VEGF, IL-8, and MMP-9 [16]. Interestingly, inhibition NF-κB activity resulted in decreased angiogenesis, invasion, and metastasis of human prostate cancer cells grown the prostate of nude mice [17]. We previously reported that nuclear localization of NF-κB in prostate cancer tissues can be used in correlation with Gleason score as predictors for poor outcome, and that NF-κB activation correlated with bone metastases, and hence poor prognosis [18].

These data suggest that NF-κB expression might have an important role in the development of metastatic prostate disease. However, to the best of our knowledge, local NF-κB expression in prostate cancer lymph node (LN) metastasis has not been well documented. Our objective was to identify NF-κB expression in LN of patients with metastatic prostate disease.

**MATERIALS AND METHODS**

**Tissues**

LN specimens were obtained from 54 patients with localized prostate cancer, who had undergone radical prostatectomy. No patients received hormonal treatment prior to surgery. Of the 54 patients, 32 had evidence of prostate cancer LN metastasis as confirmed by PSA staining, while 22 had no evidence of metastasis and were considered as controls. From the 32 metastatic prostate cancer patients, 55 LN specimens were evaluated, of which 45 were positive for metastasis, while ten were negative and were evaluated as internal controls. In addition, 32 LN specimens from the 22 patients with no evidence of metastasis were studied. One to two LN specimens were evaluated for each of the 54 patients.

**Immunohistochemistry**

Tissue sections were immunostained for NF-κB using the biotin–streptavidin immunoperoxidase method as previously described [18]. Briefly, paraffin-embedded LN sections (4 μm) were initially deparafinized with toluene and rehydrated through graded ethanol. All steps were performed at room temperature. Following each step, sections were washed with 0.01 M phosphate buffered saline (PBS solution) for 10 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide/50% methanol for 15 min. Tissue sections were incubated with a protein blocking serum-free reagent (Dako Diagnostics, Inc., Ont., Canada) for 15 min to block non-specific binding. An antigen retrieval technique was applied for NF-κB by boiling slides for 10 min at 95°C in a 0.01 M sodium citrate buffer (pH 6.0). NF-κB expression was studied using monoclonal NF-κB p65 (F-6) antibody (Santa Cruz Biotech, Santa Cruz, CA) that recognizes the amino-terminal sequences of the p65 subunit. The NF-κB primary antibody was applied at a concentration of 1:50 in PBS and was incubated for 60 min at room temperature. Immune complexes were revealed using a biotin-conjugated broad spectrum secondary antibody (20 min), then streptavidin-peroxidase conjugate for 20 min (Dako Diagnostics, Inc.), followed by chromogen (0.06% 3,3'-diaminobenzidine tetrahydrochloride, 0.01% hydrogen peroxide in PBS).

Sections were counter stained with Mayer’s haematoxylin, dehydrated, and then mounted. Negative controls were included by omitting the primary antibody, while positive controls were included using prostate tissue sections.

**Quantification**

LN sections were evaluated using light microscopy. NF-κB localization within the cell cytoplasm and/or nucleus was identified. Nuclear localization of NF-κB was categorized as positive or negative, as well as an overall proportion of cells (<1%, 1–10%, and >10%) with positive nuclear staining in the studied field at 100× magnification [18]. For the positive-metastatic LN group, tumor cells and the surrounding lymphocytes were evaluated separately. A minimum of five different areas for each specimen were evaluated, and the mean was assessed. The intensity of NF-κB staining was evaluated semi-quantitatively on a scale ranging from 1 to 4+. Statistical analysis was performed using the χ²-test.

**RESULTS**

The mean age of prostate cancer patients with LN metastases was 64.2 ± 4.7 years as compared to 65.1 ± 4.3 years in the control group (P = 0.6). The mean Gleason score was similar in both groups (Gleason 8–10; P = 0.9), while the mean preoperative prostate specific antigen (PSA) was 18.3 ± 7.8 μg/L in the metastatic prostate cancer patients compared to 17.6 ± 7.1 μg/L in controls (P = 0.8). NF-κB staining was identified in the cytoplasm of lymphocytes in both groups, as well as in the malignant epithelial cells of the tumor-positive LN group. In contrast, nuclear localization of NF-κB was variably expressed (Fig. 1).
Table I shows NF-κB-nuclear localization in patients with positive LN metastases, where 27/32 (84.4%) of patients had >10% nuclear staining in tumor tissues as compared to 9.3 and 6.3% with <1% and 1–10% nuclear staining, respectively (P = 0.001; Fig. 1A,B). Lymphocytic-nuclear localization of NF-κB of >10% was seen in 68.7% of patients with positive-LN metastases and 0% of controls (P = 0.0001, Table II, Fig. 1C,D). The negative LN specimens from patients with metastatic prostate cancer (internal controls; n = 10) showed similar lymphocytic NF-κB-nuclear localization as controls (P = 0.8), which was significantly different from the positive-LN specimens from the same patients (Table II).

The intensity of NF-κB staining in the LN specimens of metastatic prostate cancer patients and controls is shown in Table III. While 68.8% of patients with positive-LN metastases had 3 to 4+ intensity of staining in both lymphocytes and malignant cells, only 9.1% of the control group showed strong staining in the lymphocytes. It is noteworthy to mention that, in >90% of the metastatic LN specimens up-regulation of NF-κB activation and expression was observed simultaneously in lymphocytes and malignant cells. In all examined specimens, nuclear localization of NF-κB was persistently observed in lymphocytes and/or malignant cells in the vicinity of lymphatic or vascular channels.

**DISCUSSION**

In the present study, we report for the first time NF-κB expression in prostate cancer LN metastasis. Our data shows that NF-κB nuclear localization/activation is up-regulated in tumor cells, as well as in the surrounding lymphocytes of metastatic LN of prostate cancer patients. It is well documented that Gleason score is an important prognostic marker for prostate cancer metastasis [19,20]. In addition, NF-κB has been reported to regulate the expression of PSA [21], another important marker for prostate cancer progression. Hence, the control group included in this study was selected to match the positive-LN group with respect to age, preoperative PSA, and Gleason score. Furthermore, to minimize the effect of known and/or unknown coexisting variables that may be responsible for increased NF-κB activity, LN with no evidence of malignancy, as confirmed by PSA staining, from patients with LN metastases were evaluated separately as internal controls. The internal control group showed similar NF-κB behavior as controls, which was significantly different from the positive-metastases LN group. Based on these observations, we hypothesize that the increased expression of NF-κB and its subcellular localization in metastatic LN is directly

<table>
<thead>
<tr>
<th>Percentage of NF-κB nuclear localization</th>
<th>Patients (n = 32)</th>
<th>Specimens^a (n = 45)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1%</td>
<td>3 (9.3%)</td>
<td>5 (11.1%)</td>
</tr>
<tr>
<td>1–10%</td>
<td>2 (6.3%)</td>
<td>8 (17.8%)</td>
</tr>
<tr>
<td>&gt;10%</td>
<td>27 (84.4%)</td>
<td>32 (71.1%)</td>
</tr>
</tbody>
</table>

^aOne to two specimens were evaluated for each patient.
related to the process of malignant cell progression and ability to metastasize. This might in part explain our observation of persistent nuclear-NF-κB expression in malignant cells and lymphocytes in the vicinity of lymphatic and vascular channels.

NF-κB has been implicated in the development, activation, and function of B and T lymphocytes [22]. NF-κB importance for B and T cell function was proven by the fact that lymphocytes lacking either one of the two major inducible NF-κB transcription activators, C-Rel and RelA, have B and T cell proliferation defects in response to certain stimuli [23–25]. Hence, we observed diffuse cytoplasmic expression of NF-κB in all lymphocytes of the metastasis-positive LN group and controls. However, significant up-regulation of NF-κB nuclear expression was observed in the metastatic LN group especially in the lymphocytes surrounding malignant cells. It is noteworthy to mention that none of our positive-LN specimens had histological features suggestive of hyperplasia and/or an inflammatory reaction that might be responsible for the lymphocytic activation of NF-κB.

Several studies have previously suggested that NF-κB activation is associated with oncogenesis in mammalian systems [26–36]. Amplification and over expression of genes coding for RelA/NF-κB factors have been found in leukemia [26], lymphoma [27], pancreatic adenocarcinoma [28], hepatocellular carcinoma [29], thyroid C-cell tumor [30], and breast cancer [31]. Furthermore, constitutive activation of NF-κB has been reported to be a common characteristic of many cell lines from hematopoietic and solid tumors [5,28,29]. The inactivation of NF-κB in carcinoma cell lines by different approaches has been shown to dramatically reduce their ability to form colonies in agar, as well as to grow in vivo and in vitro [32,33]. NF-κB has been also shown to have a key role in cell protection against diverse apoptotic stimuli including chemotherapeutic drugs and γ-irradiation through activation of the anti-apoptotic gene program in cells [5].

With respect to prostate cancer, NF-κB activation has been detected in a large number of prostate cancer cell lines, such as the androgen-resistant PC-3 and DU145 cell lines [12,34,35]. The tumor suppressor PTEN which inhibit NF-κB activation, has been implicated in prostate cancer [36]. In addition, it has recently been shown that bcl-2 gene expression induced by tumor necrotic factor (TNF)-α in LNCAP cells is mediated by NFκB binding to specific sites in the bcl-2 P2 promoter [37]. A possible role for NF-κB in regulating apoptosis in androgen-independent prostate cancer cells has also been suggested by the fact that inhibition of NF-κB activity is necessary, although not sufficient, for the induction of apoptosis [38]. The IL-6 gene, a target of NF-κB, has been shown to be overexpressed in localized prostate cancer and is thought to promote prostate cancer cell growth [39]. Furthermore inhibition of NF-κB activity in PC-3 cells was reported to be associated with decreased expression of various potent angiogenic factors, such as VEGF, IL-8, and MMP-9 [16]. Recently, it has been reported that inhibition of NF-κB activation by dehydroxymethylpoxyquinomicin (DHMEQ) results in suppression of hormone-refractory prostate cancer cells growth in nude mice [40].

### TABLE II. NF-κB-Nuclear Localization in the Lymphocytes of Patients With Prostate Cancer LN Metastases and Controls

<table>
<thead>
<tr>
<th>NF-κB nuclear staining</th>
<th>Metastatic patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patients (n = 32)</td>
<td>Specimens (a) (n = 45)</td>
</tr>
<tr>
<td>&lt;1%</td>
<td>3 (9.4%)</td>
<td>6 (13.3%)</td>
</tr>
<tr>
<td>1–10%</td>
<td>7 (21.9%)</td>
<td>10 (22.2%)</td>
</tr>
<tr>
<td>&gt;10%</td>
<td>22 (68.7%)</td>
<td>29 (64.4%)</td>
</tr>
<tr>
<td>P value a</td>
<td>0.005</td>
<td>0.003</td>
</tr>
</tbody>
</table>

*aSignificance between <1 and >10% NF-κB localization in patients and specimens.

### TABLE III. Intensity of NF-κB Staining in Patients With Positive-Prostate Cancer LN Metastases and Controls

<table>
<thead>
<tr>
<th>Intensity of NF-κB staining</th>
<th>Patients with LN metastases (n = 32)</th>
<th>Controls (n = 22)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1+</td>
<td>3 (9.4%)</td>
<td>15 (68.2%)</td>
<td>0.005</td>
</tr>
<tr>
<td>2+</td>
<td>7 (21.9%)</td>
<td>5 (22.7%)</td>
<td>0.8</td>
</tr>
<tr>
<td>3–4+</td>
<td>22 (68.8%)</td>
<td>2 (9.1%)</td>
<td>0.001</td>
</tr>
<tr>
<td>P value a</td>
<td>0.001</td>
<td>0.002</td>
<td></td>
</tr>
</tbody>
</table>

*aSignificance between 1+ and 3–4+ intensity of NF-κB staining in both lymphocytes and malignant cells of metastatic patients and lymphocytes of controls.
reported that NF-κB-nuclear localization/activity in primary prostate cancer tissues correlates with poor patient outcome and bone metastasis [18]. The present study is in agreement with all these observations, as we were able to identify increased nuclear localization of NF-κB in prostate cancer LN-metastases. These results support and expand the possible role played by NF-κB, and its activators, in the development of metastatic prostate disease. However, further studies are essential to confirm such a hypothesis, as well as to correlate metastatic NF-κB expression to disease progression, and its possible implication as a predictor of poor prognosis. In addition, it would be interesting to determine whether our novel observation on NF-κB activation can correlate to clinical parameters such as time to recurrence and survival. Moreover, studies aiming at revealing the exact mechanisms by which the activation of NF-κB contributes to metastasis are essential. Finally, this study in conjunction with previously published reports [16–18] support the notion that targeting and/or blocking NF-κB signaling in positive-malignant cells may be a valid therapeutic strategy in the management of prostate cancer.

CONCLUSIONS

We report for the first time NF-κB expression in prostate cancer LN metastases. Nuclear localization/activation of NF-κB is up-regulated in metastatic LN. Such observation suggests, and further supports, the possible role played by NF-κB in the development of metastatic prostate disease.

REFERENCES


