Neutral Endopeptidase Protein Expression and Prognosis in Localized Prostate Cancer

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ABSTRACT

Purpose: Neutral endopeptidase (NEP) is a cell-surface peptidase that inactivates neuropeptide growth factors implicated in prostate cancer progression. The clinical significance of decreased NEP expression observed in prostate cancer is unclear. We investigated whether decreased NEP expression in localized prostate cancers is associated with prostate-specific antigen (PSA) relapse after radical prostatectomy.

Experimental Design: NEP expression patterns were examined by immunohistochemistry in 223 men, who underwent radical prostatectomy between 1990 and 2000 at the Veterans Administration Medical Center (New York, NY) with available representative tissues and adequate follow up. We also examined whether hypermethylation of the NEP promoter contributes to down-regulation of NEP protein expression in a subset of patients that showed decreased NEP expression (n = 22).

Results: Three patterns of NEP expression were observed: (a) membranous expression similar to benign prostate epithelium (n = 82; 37%); (b) complete loss of NEP expression in prostate cancer compared with adjacent benign prostate glands (n = 105; 47%); and (c) heterogeneous NEP expression (n = 36; 16%). In a multivariate analysis, complete loss of NEP expression was associated with PSA relapse after controlling for grade, stage, pretreatment PSA, and race simultaneously (hazard ratio, 1.99; 95% confidence interval, 1.13–3.52; two-sided $\chi^2 P = 0.017$). In addition, DNA hypermethylation of the NEP promoter was frequently (73%) identified in a subset of 22 cases that showed decreased NEP expression.

Conclusion: Our data suggest that decreased NEP expression might contribute to progression of localized prostate cancer after surgery. Data also suggest that methylation is an important mechanism of NEP protein silencing. Larger prospective studies are required for confirmation.

INTRODUCTION

Neuropeptides such as neurotensin, bombesin, and endothelin-1 bind to G-protein coupled receptors and initiate signaling pathways that stimulate cell growth (1). Several studies demonstrated that alterations in neutral endopeptidase 24.11 (NEP; CD10), a cell-surface metallopeptidase that inactivates neuropeptides, contribute to the development and progression of prostate cancer (2, 3). We showed previously that NEP loss in cultured prostate cancer cells results in stimulation of cell proliferation and migration (4) and ligand-independent activation of insulin-like growth factor-1 receptor leading to Akt phosphorylation (5).

Studies of NEP expression in tissues suggested that NEP loss might contribute to prostate cancer clinical progression. A microarray gene expression profile analysis of 50 normal and neoplastic prostate specimens found decreased NEP expression in primary and metastatic prostate cancer relative to normal prostate epithelium (6). In addition, lack of NEP expression was observed in tumor cells of metastatic prostate cancer tissue specimens from patients with androgen-independent prostate cancer (7). However, the prognostic significance of NEP loss in primary prostate cancer is unclear.

In this study, we investigated the clinical relevance of NEP expression in a well-characterized cohort of homogeneously treated prostate cancer patients with adequate clinical follow up and used the time to prostate-specific antigen (PSA) relapse as the unified response variable. In addition, we examined a subset of cases for evidence of NEP promoter methylation and correlated the results with NEP expression to determine the mechanism for the lack of NEP expression in prostate cancer tissues. This was based on our preclinical data, which revealed that hypermethylation of the NEP promoter is associated with loss of expression of NEP protein in prostate cancer cell lines (8).

MATERIALS AND METHODS

Patient Characteristics and Tissues. Patients were identified through review of the Department of Urology database at the Veterans Administration Medical Center/New York University School of Medicine. This prospective database enrolled patients with prostate cancer from 1990 to the present, documenting patient demographics including race, stage, and grade of the primary tumor. After Institutional Review Board approval and activation of the protocol, we retrospectively reviewed all of the relevant clinical information. Tumor grade,
stage, pretreatment PSA values, pathological stage, PSA recurrence, and survival data were entered into a database. Representative H&E-stained tissue sections were examined to evaluate the histopathological characteristics of each case. Of the 261 patients whose tumors were resected at the Veterans Administration Medical Center, representative tissue blocks of formalin-fixed, paraffin-embedded primary tumors were obtained from 223 patients. The high retrieval rate (85.4%) of tissues provided confidence in the results as it minimized the chance of selection bias, which can be a major issue in conducting a retrospective analysis. Patient selection was based solely on the availability of both adequate clinical follow-up and the availability of repre-

Fig. 1 Photomicrographs of neutral endopeptidase (NEP) expression analyzed by immunohistochemistry of benign and malignant prostate. Normal NEP expression: A, low power magnification (×10) of benign hyperplastic prostatic glands demonstrating predominantly membranous localization of NEP and (B) high power magnification (×40) of a single hyperplastic prostatic gland showing predominantly membranous staining with NEP antibody. Positive NEP expression: C, power low magnification (×4) and (D) high power magnification (×40) of poorly differentiated prostatic adenocarcinoma (Gleason score 9) showing solid sheets intermixed with acinar carcinoma cells. The NEP stains positively at the apical membrane of the cancerous glandular epithelium (note the arrow). Negative NEP expression: E, low power magnification (×4) and (F) higher power magnification (×40) of prostatic adenocarcinoma (Gleason grade 8) with loss of membranous staining compared with internal positive control benign prostatic gland. Heterogeneous NEP expression: G, low power magnification (×10) and (H) higher power magnification (×40) of prostatic adenocarcinoma showing focal NEP expression.
NEP Protein Expression in Prostate Cancer

Table 1  Results of multivariate cox regression analysis relating NEP<sup>a</sup> loss of expression and other factors to time to PSA relapse

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard ratio</th>
<th>95% confidence interval</th>
<th>Hazard ratio limits</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline PSA</td>
<td>2.280</td>
<td>1.399 3.716</td>
<td>0.0009</td>
<td></td>
</tr>
<tr>
<td>Tumor size</td>
<td>1.891</td>
<td>1.080 3.311</td>
<td>0.0257</td>
<td></td>
</tr>
<tr>
<td>Gleason</td>
<td>2.450</td>
<td>1.371 4.379</td>
<td>0.0025</td>
<td></td>
</tr>
<tr>
<td>Race</td>
<td>2.697</td>
<td>1.068 6.814</td>
<td>0.0358</td>
<td></td>
</tr>
<tr>
<td>NEP</td>
<td>1.993</td>
<td>1.129 3.516</td>
<td>0.0173</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>NEP, neutral endopeptidase; PSA, prostate-specific antigen.

Methylation Assay. We previously used methylation-specific PCR to show that hypermethylation of the NEP promoter is associated with loss of expression of NEP protein in prostate cancer cell lines (8). Therefore, we examined 22 randomly chosen cases that showed lack of NEP expression for evidence of NEP promoter methylation. Because the methylation assay requires the complete exhaustion of the tissue block to isolate enough DNA, we had to limit our study to a relatively small subset of patients to preserve the precious tissue resources. We also studied, as a control, 6 cases that showed normal NEP expression and correlated the results with NEP expression.

Statistical Analyses. The response variable, time to PSA relapse, was defined as the time from radical prostatectomy to the time of the first detectable (non-zero) PSA measurement. To confirm PSA relapse, three consecutive increases of PSA were required; however, the time of relapse was defined as the time of the first detectable PSA measurement (12–14). All of the patients were hormone naïve at the time of PSA relapse. Eighteen patients were excluded from the time to PSA relapse analysis because three consecutive follow-up PSA measurements (n = 5) were missing, or post-surgery PSA never went down to zero (n = 13). The F and χ<sup>2</sup> tests were used to explore associations between NEP expression patterns and age, race, baseline PSA, Gleason score, and tumor stage. The Cox proportional hazards model was used to assess the relationship between NEP expression patterns and time to PSA relapse controlling for baseline PSA, Gleason score, tumor stage, and race. All of the P values were two sided. All of the statistical analyses were done using SAS Release 8.2 (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Complete membranous staining was detected in the luminal surface of benign prostatic glands, which was considered an internal control for each tissue section (Fig. 1, A and B). We identified three different expression patterns in neoplastic prostate: pattern 1, positive staining similar to benign prostatic glands (n = 82, 37%; Fig. 1, C and D); pattern 2, negative NEP

Table 2  Correlation between different NEP<sup>a</sup> patterns of expression and baseline characteristics

<table>
<thead>
<tr>
<th></th>
<th>NEP negative (n = 105)</th>
<th>NEP heterogeneous (n = 36)</th>
<th>NEP positive (n = 82)</th>
<th>All (n = 223)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>#</td>
<td>%</td>
<td>#</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2</td>
<td>67</td>
<td>46.5%</td>
<td>27</td>
<td>18.8%</td>
<td>50</td>
</tr>
<tr>
<td>≥3</td>
<td>38</td>
<td>48.1%</td>
<td>9</td>
<td>11.4%</td>
<td>32</td>
</tr>
<tr>
<td>Gleason</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;7</td>
<td>56</td>
<td>45.9%</td>
<td>22</td>
<td>18.0%</td>
<td>44</td>
</tr>
<tr>
<td>≥7</td>
<td>49</td>
<td>48.5%</td>
<td>14</td>
<td>13.9%</td>
<td>38</td>
</tr>
<tr>
<td>Baseline PSA&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;5</td>
<td>14</td>
<td>53.9%</td>
<td>1</td>
<td>3.8%</td>
<td>11</td>
</tr>
<tr>
<td>5–10</td>
<td>44</td>
<td>54.3%</td>
<td>9</td>
<td>11.1%</td>
<td>28</td>
</tr>
<tr>
<td>≥10</td>
<td>37</td>
<td>41.6%</td>
<td>20</td>
<td>22.5%</td>
<td>32</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>African-American</td>
<td>66</td>
<td>42.0%</td>
<td>31</td>
<td>19.8%</td>
<td>60</td>
</tr>
<tr>
<td>Caucasian</td>
<td>39</td>
<td>59.1%</td>
<td>5</td>
<td>7.6%</td>
<td>22</td>
</tr>
</tbody>
</table>

<sup>a</sup>NEP, neutral endopeptidase; PSA, prostate-specific antigen.

<sup>b</sup>196 patients available.

<sup>c</sup>χ<sup>2</sup> test.
Although there was no significant association of NEP with time to PSA relapse in univariate analysis, we adjusted for known prognostic factors and found that the hazard ratio for patients with complete loss of expression of NEP was 1.99. In a univariate analysis, the association of a new tumor marker with disease outcome may be confounded by other prognostic factors, e.g., an association between the new marker and disease outcome may be observed (and statistically significant) simply because the new marker associates with one or more of the prognostic factors identified previously. In a multivariate analysis, this association between the new marker and disease outcome may no longer be present (or weakened), indicating that the new marker is no better in predicting disease outcome than the previously known prognostic factors. Conversely, an association between a new marker and disease outcome may not be apparent (or may be weak and not statistically significant) in a univariate analysis due to negative confounding. If the association becomes significant in a multivariate analysis, it indicates that the new marker is associated with disease outcome independently of the previously known prognostic factors. Therefore, the new marker contributes information regarding prognosis beyond the information contributed by known prognostic factors (16). The result of the multivariate analysis indicates that the risk of PSA relapse was ~2-fold greater in men who had tumors with complete loss of expression of NEP than in men with other patterns of NEP expression and that this increase in risk was independent of other known risk factors. Although this result needs to be replicated in other studies, as is customary, before its clinical relevance may be fully assessed, the strength of the association (doubling of the hazard rate, small P value) in multivariate analysis indicates the strong clinical relevance of NEP loss.

We observed a significant association between NEP expression and race (African-American versus Caucasian); the proportion of subjects with normal NEP expression was similar in the two race groups (33% in Caucasians versus 38% in African-Americans). However, the proportion of patients with heterogeneous expression was greater in African-Americans (8% in Caucasians versus 20% in African-Americans). The clinical relevance of this result is not clear, because the heterogeneous pattern is the least common (16.1%; Table 2).

DNA hypermethylation of the NEP promoter was identified frequently in a subset of cases that showed loss of NEP expression. We found that methylation was detected in the NEP CpG island in 16 of 22 (72.7%) cases with decreased NEP expression. No methylation was detected in 6 prostate cancer cases that showed a NEP protein expression pattern similar to that of benign prostate glands (Fig. 2). This suggests that methylation is an important mechanism of NEP protein silencing.

We report an independent association between negative NEP expression and PSA relapse after prostatectomy in our cohort. Our data are in concordance with two recently reported studies in prostate cancer (17, 18), showing that diminished NEP expression is a common and early event in prostate cancer and does not associate with prostate cancer grade and stage at the time of diagnosis. These studies did not detect an association between NEP expression and clinical relapse. However, both studies retrospectively included a significant proportion of patients from 1984 to 1990, the pre-PSA era, when evaluation of
recurrence was based on developing gross metastatic disease from imaging studies. In contrast, we used the unified response variable of time to PSA relapse. Furthermore, one study included patients who received neoadjuvant hormone treatment, which is known to influence time to PSA relapse after surgery (14) and to affect NEP expression (7), whereas our cohort was homogenous, hormone-naïve patients at the time of surgery and did not receive hormone ablation before their PSA relapse. Although African-American patients represented the majority in our cohort compared with the other two studies in which Caucasian patients were the majority, it is unlikely that the distinct cohort contributed to differences in correlation with PSA outcome because the association between NEP expression and PSA relapse in our study persisted after controlling for race (Table 1B).

In conclusion, our data corroborate our preclinical studies on the ability of NEP to regulate cellular pathways and imply that loss of NEP by prostate cancer cells contributes to neuropeptide-mediated progression of localized prostate cancer. Larger prospective studies are required for confirmation.

ACKNOWLEDGMENTS

We thank Drs. Jonathan Melamed and Rosemary Wieczorek from New York University School of Medicine and Dr. Satish Tickoo from Weill Medical College of Cornell University for assistance with the pathology review.

REFERENCES

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