Methylation of the Neutral Endopeptidase Gene Promoter in Human Prostate Cancers


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Abstract

Neutral endopeptidase 24.11 (NEP) is a cell surface peptidase expressed by prostatic epithelial cells that cleaves and inactivates neuropeptide growth factors implicated in the growth of androgen-independent prostate cancer (PC). Decreased NEP expression in hormone-refractory metastatic PCs can result from hormonal therapies because NEP transcription is induced by androgens and down-regulated by androgen withdrawal. NEP is encoded by a gene that contains a 5′ CpG island spanning a transcriptional regulatory region. In this study, we investigate whether DNA hypermethylation of the NEP promoter accompanies decreased NEP expression in PC cell lines and whether it occurs in human PC tissues in vivo. DNA isolated from PC cell lines and from normal and neoplastic human prostate tissues was restriction-digested with a methylation-sensitive restriction endonuclease and analyzed by Southern blot using a 5′ sequence-specific NEP probe. Methylation-specific PCR was performed using PCR primers designed to discriminate between methylated and unmethylated alleles, and reverse transcription-PCR using NEP-specific primers was performed on cDNA extracted from PC cells treated with 5-aza-2′-deoxycytidine. Methylation of the NEP promoter was present in androgen-independent PC cell lines but not in androgen-dependent or small-cell derived PC cell lines and in 3 of 21 (14%) primary PCs from patients with androgen-dependent disease. Exposure of PC cells to the demethylating agent 5-aza-2′-deoxycytidine led to an increase in NEP transcripts in DU-145 and PC-3 cells. These data show that hypermethylation of the 5′ CpG NEP island is associated with a loss of NEP expression in PC. Loss of NEP expression via hypermethylation of the NEP promoter may contribute to the development of neuropeptide-stimulated PCs.

Introduction

NEP3 (CALLA and CD10) is a cell surface peptidase that inactivates neurotensin, bombesin, and endothelin-1 neuropeptide mitogens for androgen-independent PCs. We recently reported that NEP expression is decreased in androgen-independent PC cell lines in vitro and in tumor cells of metastatic biopsy specimens in vivo from patients with androgen-independent PC (1). Expression of NEP is transcriptionally activated by androgen in androgen-dependent PC cells and decreases with androgen withdrawal (1). Consequently, PC cells that survive androgen withdrawal can emerge with reduced levels of NEP. This decrease in NEP expression can contribute to the development of androgen-independent PC by allowing PC cells to use neuropeptides as an alternate source to androgen to stimulate cell proliferation.

In our previous study, we reported that diminished NEP was observed occasionally in tumor cells from patients with hormone-naïve PC (1). These data suggested that decreased NEP expression can result from other mechanisms distinct from decreased transcription of the NEP gene as a consequence of androgen withdrawal and that decreased cell surface NEP may contribute to neuroendocrine-differentiated, neuropeptide-stimulated PC in hormone-naïve patients.

A aberrant DNA methylation of promoter region CpG dinucleotides has been implicated as a cause for repressed transcription of a number of tumor suppressor or growth-related genes in PCs (2), including E-cadherin (3), CDKN2 (p16/MTS1; Ref. 4), and endothelin B receptor (5). Sequence analysis of the 5′-regulatory region of the NEP gene reveals a CpG-rich sequence (6, 7). In the current study, we examined the methylation status of the NEP promoter in human PC cell lines and in primary prostate tumors.

Materials and Methods

Cell Culture. PC cell lines were derived as described previously (8) and maintained in RPMI 1640 containing 10% fetal bovine serum. The Ichawaka cell line, which was derived

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3 The abbreviations used are: NEP, neutral endopeptidase 24.11; PC, prostate cancer; RT-PCR, reverse transcription-PCR; MSP, methylation-specific PCR.

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from a small cell carcinoma of the prostate (9), was provided by Dr. Hiroshi Okada, Kobe University School of Medicine, Japan. 5-Aza-2'-deoxycytidine was purchased from Sigma Chemical Co. (St. Louis, MO).

**Southern Blot Analysis for NEP Methylation.** Genomic DNA isolation, restriction endonuclease digestion, and Southern blot analysis were performed as described previously (5). Briefly, DNA was isolated from growing cultures of each of five human PC cell lines and from a variety of normal and neoplastic human tissues. Normal tissues were obtained at autopsy, including prostate tissues and lymph node metastases, along with matched adjacent prostate or seminal vesicle tissues, were dissected from resection specimens obtained from men treated for localized PC by radical prostatectomy at the Johns Hopkins Hospital (Baltimore, MD). Autopsy tissues were obtained from men who died of widely metastatic PC (10). For analysis of NEP tissue-specific NEP gene expression, with type 2 promoter transcripts (6). These transcripts result from alternative splicing of noncoding exons 1, 2a, or 2b to a common exon 3. These type 1 and 2 regulatory regions are believed to control splicing of noncoding exons 1, 2a, or 2b to a common exon 3.

**MSP.** Five μg of DNA were restriction-digested with KpnI (Life Technologies, Inc.) overnight at 37°C, and then bisulfite modification of 1 μg was performed using the CpGenome Modification Kit (Oncor, Inc.) according to the manufacturer’s recommendations. PCR reactions were carried out in 1× PCR buffer [16.6 mM ammonium sulfate, 67 mM Tris (pH 8.8), and 10 mM 2-mercaptoethanol], 2 mM MgCl₂, deoxynucleotide triphosphates (0.20 mM each), primers (25 pmol each per reaction), and bisulfate-modified DNA (~100 ng) in a final volume of 25 μl. Reaction mixes were prepared for multiple samples and aliquoted. A negative control consisting of a 25-μl aliquot without the addition of DNA was included in each amplification. The PCR reactions were performed using the hot start method. Reactions were hot-started at 95°C for 3 min before the addition of 1.25 units of Taq polymerase (Life Technologies, Inc.), followed by a 10-min incubation at 80°C. Amplification was performed for 33 cycles using a DNA Thermal Cycler (MJ Research Inc., Watertown, MA). A cycle profile consisted of 30 s at 95°C, 1 min at the annealing temperature listed in Table 1, and 30 s at 72°C, followed by a final 7-min extension at 72°C. Each PCR reaction (25 μl) was directly loaded onto a 2% agarose gel containing ethidium bromide and visualized directly under UV illumination. Primer pairs described in Table 1 were purchased from Integrated DNA Technologies, Inc.

**RT-PCR.** RNA extraction and generation of cDNA were performed as described previously (11, 12). PCR amplification of cDNA was performed using NEP gene-specific oligonucleotide primers [sense, NEP exon 10 (6), 5'-TTGTG-GCCAGATTGGATCGTC; antisense, NEP exon 13 (6), 5'-TTGTAGGTTGGCTGAGGCT]. β₂-Microglobulin primers [sense, 5'-TTACTCAGTCATCAGCGACAG; antisense, 5'-GTCACATGTTACACCGGA] were used as control (13). A cycle profile consisted of 30 s at 95°C, 1 min at 55°C, and 30 s at 72°C for 35 cycles, followed by a final 7-min extension at 72°C.

**Results**

The human NEP promoter contains two separate regulatory regions controlling the transcription of at least three types of NEP transcripts (6). These transcripts result from alternative splicing of noncoding exons 1, 2a, or 2b to a common exon 3. These type 1 and 2 regulatory regions are believed to control tissue-specific NEP gene expression, with type 2 promoter transcripts being more abundant in epithelial cells (7, 14). Sequence analysis of the NEP promoter reveals a CpG island in the type

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### Table 1  
**PCR primers**

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Methylation set (5’-3’; upstream/downstream)</th>
<th>Unmethylation set (5’-3’; upstream/downstream)</th>
<th>Primer position*</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alu</td>
<td>AAAATTAGTTCGGTGGTGGTTGTCG</td>
<td>TTTTTCCTAAGAAATCTCCTGTAATCG</td>
<td>−1989</td>
<td>66°C</td>
</tr>
<tr>
<td></td>
<td>AAAATTATGTTGTTGTTGTTGTTG</td>
<td>TTTTTCCTAAGAAATCTCCTGTAATCC</td>
<td>−1989</td>
<td>66°C</td>
</tr>
<tr>
<td>NEP1</td>
<td>GGGTCCGAGGAGGTAGGTAGGTAGTTC</td>
<td>CTAACCGACATCCGGAATAAACG</td>
<td>−103</td>
<td>62°C</td>
</tr>
<tr>
<td></td>
<td>GTGGTTGGGAGGGAGGTAGGTAGGTTT</td>
<td>AAACACTAAGACAACATCCAACAACCAAATA</td>
<td>−107</td>
<td>62°C</td>
</tr>
<tr>
<td>NEP2</td>
<td>GCGGATGTAAGGAGGAGGAGGAGG</td>
<td>CCACAAACTCCACACACCAAAACT</td>
<td>−196</td>
<td>62°C</td>
</tr>
<tr>
<td></td>
<td>TGGGTGATGTAATGAGGAGGAGG</td>
<td>TGGTGCCTTCACCAACACCAAATAC</td>
<td>−2</td>
<td>62°C</td>
</tr>
</tbody>
</table>

* Relative to exon 2 transcription start.
promoter region containing five Sp1 elements and juxtaposed to a region containing multiple Alu repeats (Fig. 1A). To determine whether hypermethylation of the NEP promoter occurs during aging in normal prostatic cells, DNA was isolated from normal prostate tissues from 13 men of different ages; restriction-digested with EcoRI, HindIII, and the methylation-sensitive restriction enzyme BssHII; and probed with the 1622-bp NEP-specific probe illustrated in Fig. 1A. None of the DNA derived from normal prostatic tissues showed evidence of methylation (Fig. 1B).

NEP mRNA, protein, and concomitant NEP-specific enzyme activity are present in androgen-sensitive LNCaP cells, but not in androgen-independent PC-3 and DU-145 cells, TSU-Pr1 cells (1), or PPC-1 cells (15) or in Ichawaka cells, which are derived from a small cell carcinoma of the prostate (data not shown). Southern blot analysis of DNA derived from five of

Fig. 1  A, restriction map and distribution of CpG islands in the NEP promoter. The NEP promoter contains two alternatively used promoters (shown by arrows) that splice into a common exon 3. A series of Alu repeats resides approximately 1 kb upstream from the start of exon 1. The five Sp1 sites are indicated by shaded boxes. The density of CG dinucleotides in the 5' region is shown by vertical bars. The locations of the restriction endonuclease recognition sites are indicated (R, EcoRI; H, HindIII; B, BssHII). Note that the methylation-sensitive BssHII restriction enzyme recognition site is embedded in a CpG island. The MSP products for primer pairs Alu, NEP1, and NEP2 are shown by short lines. The 1622-bp probe used for Southern hybridization is noted. B, lack of NEP promoter methylation in vivo in normal prostatic tissues obtained at autopsy from 13 men of different ages. DNA was digested with EcoRI, HindIII, and BssHII and hybridized with the 1622-bp NEP probe. Note that only a 2.0-kb band (top band) is recognized by the NEP probe, which represents complete digestion of the DNA at the EcoRI, HindIII, and BssHII restriction sites, indicating that NEP DNA is not methylated in normal prostate tissue. If the DNA were methylated at the methylation-sensitive BssHII restriction site, then the NEP probe would recognize a higher molecular weight band of approximately 2.2 kb as seen in Fig. 2 (right side) and Fig. 5.
these human PC cell lines digested with EcoRI, HindIII, and methylation-sensitive BssHII revealed a lack of digestion at the BssHII restriction site in DU-145 and PPC-1 cells, but not in LNCaP, PC-3, and TSU-Pr1 cells (Fig. 2). The Southern blot analysis only detects methylation present at the sequence recognized by BssHII. Therefore, MSP (16), which can assess several CpG sites by a single assay, was used to determine the methylation status of upstream and downstream fragments of the CpG island located in the NEP type 2 promoter as illustrated in Fig. 1A. PCR primers were designed to discriminate between methylated and unmethylated alleles after bisulfite treatment (Table 1). CpG islands are often in close proximity to Alu repeat sequences that are extensively methylated in both normal and malignant tissues (17). We therefore also synthesized PCR primers for an Alu repeat sequence upstream of the NEP promoter region as a control for bisulfite modification (see Fig. 1A). In LNCaP cells, which express NEP, CpG sites within the island were completely unmethylated [primer sets NEP1 (Fig. 3B) and NEP2 (Fig. 3C)], whereas the upstream Alu repeat was extensively methylated (Fig. 3A). In contrast, in androgen-independent PC cell lines that do not express NEP (DU-145, PC-3, TSU-Pr1, and PPC-1), extensive methylation was detected in the upstream Alu repeats, and partial methylation was detected in both regions of the CpG island (Fig. 3, B–D). Similar to other cell lines, the upstream Alu repeat in DNA derived from Ichawaka cells was methylated (data not shown); however, no methylation was detected in the NEP CpG island (Fig. 3D).

5-Aza-2′-deoxycytidine is a demethylating agent used to induce the reexpression of many methylated genes (3, 5, 18). DU-145 and PC-3 cells were treated with 0.5 μM 5-aza-2′-deoxycytidine. DU-145 and PC-3 express low levels of detectable NEP transcripts under basal conditions, but RT-PCR analysis showed that the expression of NEP transcripts appeared to increase within 24 h of incubation (Fig. 4).

To assess hypermethylation of the NEP promoter in vivo, Southern blot analysis of DNA isolated from primary PC specimens with matched control DNA prepared from normal tissues was performed. Methylation at the BssHII site of the NEP promoter was detected in 3 of 21 primary PC specimens from patients with hormone-naïve PC (representative data are shown in Fig. 5). No normal tissues or prostatic tissues exhibiting benign hyperplasia were methylated. All blots were also hybridized with a probe for the triosephosphate isomerase gene to assure complete digestion of DNA (data not shown; Ref. 5). DNA derived from these samples was not available for MSP.

**Discussion**

We have shown previously that NEP expression is decreased in most androgen-independent PCs in vivo, that the NEP gene is transcriptionally activated by androgen, and that androgen withdrawal results in a decrease in NEP transcripts and protein and enzyme activity in androgen-sensitive LNCaP cells (1). Furthermore, castration of mice implanted with xenografts of the serially transplantable androgen-dependent primary human PC CWR22 (19–21) leads to a progressive, >50% decline in tumor NEP enzyme activity over 10 days, whereas the CWR22R androgen-independent
subline that regrows lacks NEP expression.\textsuperscript{4} Taken together, these results suggest a model in which decreased NEP expression is facilitated by the elimination of androgens and consequently contributes to the development of neuropeptide-mediated, androgen-independent PC cell growth by allowing PC cells to use neuropeptides as an alternate source to androgen to stimulate cell proliferation.

In this study, we demonstrate that CpG methylation of androgen-independent PC cell lines is associated with decreased NEP protein expression, suggesting that methylation is another mechanism of NEP inactivation in PCs. LNCaP cells express abundant amounts of NEP protein and do not exhibit methylation of the NEP promoter. In contrast, androgen-independent PC cell lines PC-3, DU-145, PPC-1, and TSU-Pr1 all lack NEP protein expression and demonstrate partial methylation of the NEP promoter as determined by Southern analysis and/or MSP. The results of MSP indicate that androgen-independent cell lines possess a population of alleles containing both methylated and unmethylated alleles. The methylation pattern of neuroendocrine-derived TSU-Pr1 cells contains predominantly unmethylated alleles in NEP1 and NEP2 primer sets, whereas the small cell PC cell line Ichawaka contains no evidence of methylation, suggesting that lack of NEP expression in neuroendocrine PC may result from other factors besides promoter methylation, such as differentiation. DU-145 DNA was partially methylated as determined by both Southern analysis and MSP.

\textsuperscript{4} D. B. Agus and D. M. Nanus, unpublished observations.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{Bisulfite-modified DNA derived from PC cells was amplified with PCR amplimers that amplify methylated (M) or unmethylated (U) DNA. A, Alu sequence primers. The Alu sequence was highly methylated in DNA derived from all PC cell lines (PPC-1 and Ichawaka are not shown). B, NEP1 primers. Note the partial methylation of this region in DNA derived from androgen-independent PC cells (DU-145, PC-3, and TSU-Pr1 are illustrated; PPC-1 is not shown), but not LNCaP or Ichawaka cells (data not shown). C and D, NEP2 primers. Note the partial methylation of NEP in DNA derived from androgen-independent DU-145, PC-3, TSU-Pr1, and PPC-1 cells but not from LNCaP or Ichawaka cells. Negative control, reaction mix without DNA. ICH, Ichawaka.}
\end{figure}
and showed an increase in NEP transcripts after treatment with the demethylation agent 5-aza-2'-deoxycytidine. Expression of NEP transcripts was low in PC-3 cells and increased minimally after 5-aza-2'-deoxycytidine treatment, suggesting that other factors may also contribute to repressed NEP expression. In this regard, we have shown that NEP enzyme activity can be increased in PC-3 cells stably transfected with an androgen receptor after dihydrotestosterone treatment, but this induction is 5–10-fold less than observed in parental LNCaP cells (22).

The NEP promoter in exon 2 contains 73% C + G nucleotides and has a dense CG dinucleotide content that fulfills the criteria for CpG islands (23). Methylation of similar CpG islands in other genes can block transcription or create chromatin changes that are incompatible with transcription (24). Because NEP is developmentally regulated in both hematopoetic and epithelial progenitor cells (25), the close association of the CpG-rich region and potential Sp1-binding sites in the type 2 regulatory region suggests that methylation may play a role in Sp1 binding to the type 2 promoter and in controlling NEP transcription (7). In PC cells, NEP expression can be regulated by both androgen and methylation. Similarly, expression of the cyclin-dependent kinase inhibitor p16 (MTS1 and CDKN2) is also regulated by androgen and promoter methylation in PC cells (26), although NEP expression decreases with androgen withdrawal, whereas p16 expression decreases with androgen treatment.

In addition to cell lines, we also demonstrate methylation of the NEP promoter in DNA derived from primary androgen-dependent PCs, indicating that methylation of the NEP gene occurs in vivo. The incidence of NEP methylation in vivo may be underestimated in the current study because only one potential methylation site was examined, and DNA from these specimens was not available for MSP. However, based on the observation that only a small percentage of primary PCs exhibit neuroendocrine differentiation (27), we would predict that methylation of the NEP promoter occurs infrequently in primary PCs. A more comprehensive analysis of PC specimens using MSP is needed to determine a more accurate incidence of NEP promoter methylation in both primary and metastatic PCs.

The sequence arrangement of the CpG island in the NEP promoter region is similar to that observed in the promoter regions of a number of tumor suppressor genes, such as E-cadherin and the von Hippel-Lindau (VHL) gene (17). In these genes, the CpG island is located between densely methylated regions containing multiple Alu repeats. These methylated flanks are segregated from the CpG island by regions containing numerous Sp1 elements. There is also an Alu repeat upstream of the NEP initiation site, which is heavily methylated (Fig. IA) and separated from the NEP CpG island by multiple Sp1 sites. It has been suggested that these boundaries exist to maintain the unmethylated state in normal tissue and that they are progressively overridden in neoplasia, resulting in de novo methylation in tumor suppressor genes (17). NEP is not considered a classical tumor suppressor gene. However, overexpression of NEP in androgen-independent PC cells significantly inhibits their growth (1), and NEP-null mice develop prostatic epithelial cell hyperplasia,3 suggesting that the complete role of NEP as it relates to growth and tumorigenesis is incompletely understood.

In summary, to our knowledge, this is the first report of promoter region methylation in a cell surface peptidase. Methyla-

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tion of the NEP 5′ CpG island is associated with loss of NEP expression in PC cell lines and is also present in vivo in PC specimens. Loss of NEP expression can occur on androgen withdrawal or via hypermethylation of the NEP promoter. Both mechanisms of NEP inactivation may contribute to the development of a neuroepitope-stimulated PC cell population. Clinical studies support this model, indicating that neuroendocrine-mediated growth increases after hormone withdrawal and that neuroendocrine PCs occur de novo before hormone therapy (28). Additional studies are needed to assess the impact of NEP promoter methylation on the development of neuroendocrine differentiation and on the progression to androgen-independent PC.

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References


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