Human Cancer Biology

Methylation Induced Gene Silencing of HtrA3 in Smoking-Related Lung Cancer

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Abstract

Purpose: Some 85% of lung cancers are smoking related. Here, we investigate the role of serine protease HtrA3 in smoking-related lung cancer.

Experimental Design: We assess HtrA3 methylation and its corresponding expression in the human bronchial cell line BEAS-2B following cigarette smoke carcinogen treatment, in lung cancer cell lines and in primary lung tumors from light, moderate, and heavy smokers. We also show the effects of HtrA3 downregulation on MTT reduction and clonogenic survival with etoposide and cisplatin treatment and the corresponding effects of HtrA3 re-expression during treatment.

Results: We show for the first time that HtrA3 expression is reduced or completely lost in over 50% of lung cancer cell lines and primary lung tumors from heavy smokers. Treatment of HtrA3-deficient cell lines with 5-aza-2′-deoxycytidine resulted in a dose-dependent increase in HtrA3 transcription. Further, sequence analysis of bisulfite-modified DNA from lung cancer cell lines and from primary lung tumors showed an increased frequency of methylation within the first exon of HtrA3 with a corresponding loss of HtrA3 expression, particularly in tumors from smokers. In BEAS-2B, treatment with the cigarette smoke carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane resulted in HtrA3 downregulation with a corresponding increase in methylation. Additional studies indicate resistance to etoposide and cisplatin cytotoxicity as a functional consequence of HtrA3 loss. Finally, immunohistochemical analysis of primary lung tumors revealed a strong correlation between low HtrA3 expression and heavy smoking history.

Conclusions: Collectively, these results suggest that cigarette smoke–induced methylation of HtrA3 could contribute to the etiology of chemoresistant disease in smoking-related lung cancer. Clin Cancer Res; 16(2): 398–409. ©2010 AACR.

Lung cancer is the leading cause of cancer-related deaths in the United States and worldwide (1, 2). Standard first line treatment for small-cell lung cancer (SCLC) includes platinum-based chemotherapy, generally with the DNA and protein cross-linking agent cis-platinum-(II)-diamine dichloride (cisplatin) or carboplatin plus etoposide, a topoisomerase II inhibitor, with or without radiation (3–6). Treatment of non–small cell lung carcinoma may include surgical resection, radiotherapy, and/or platinum-based chemotherapy (7–10). Despite a promising initial response to therapy, however, some 86% of lung cancer patients still die within 5 years of diagnosis, many from chemoresistant disease (2, 11–14). This grim prognosis underscores the need to characterize not only those factors involved in lung cancer etiology and progression but also those that compromise treatment by promoting chemoresistance.

Smoking increases the risk of all histologic types of lung cancer but is most strongly linked with small-cell lung cancer and squamous cell carcinoma (15, 16). Among carcinogens that have been isolated from cigarette smoke, the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane (NNK) is widely accepted as a major causative agent for lung cancer in smokers (17–19). NNK metabolites can interact directly with DNA, forming methyl adducts primarily in target tissues most affected by NNK carcinogenicity (17, 18). Further, of adenocarcinomas induced by NNK in rats, 94% showed gene silencing through promoter hypermethylation within pin1 (20, 21). In humans, pin1 silencing is associated with promoter hypermethylation in non–small cell lung cancers (22).

HtrA3 was first characterized as an upregulated factor during mouse uterus placentation (23). More recently, HtrA3 has been identified as a possible target for cigarette smoke–induced changes in normal human bronchial epithelial cells, showing dramatic transcriptional downregulation within 2 hours of condensed cigarette smoke exposure in contrast to the steady HtrA1 and
Translational Relevance

Smoking-induced methylation changes are associated with lung cancer development. This is the first report linking HtrA3 gene silencing to cigarette smoke–mediated methylation with high statistical significance (P < 0.05) both in vitro and in vivo. We have identified five specific CpG sites within the exon 1 of HtrA3 that are unmethylated following 5-aza-2′-deoxycytidine treatment with corresponding increase in HtrA3 expression. These sites become methylated following prolonged treatment with the cigarette-smoke carcinogen 4-(methylamino)-1-(3-pyridyl)-1-butanone with corresponding loss of HtrA3 expression. Including HtrA3 methylation index with other commonly methylated genes such as p16 and others in oral cavity may lead to the identification of patients with the highest risk of developing lung cancer. We have also shown resistance to etoposide- and cisplatin-induced cytotoxicity as a functional consequence of HtrA3 downregulation that is rescued by HtrA3 re-expression in vitro. Thus, our study provides a rationale for exploring treatment strategies that include chemotherapy combined with demethylating agents.

HtrA2 expression observed over the same time period (24). Long and short HtrA3 splice variants that differ in the COOH-terminal PDZ domain, which is lacking in the short isoform, and in the last seven COOH-terminal residues of the short form have been cloned (23). Of the four known human High-temperature requirement A (HtrA) family members, HtrA3 shares the greatest sequence homology and amino acid identity with HtrA1 (25). We previously reported that HtrA1 is downregulated in the short isoform, and in the last seven COOH-terminal residues of the short form have been cloned (23). Of the four known human High-temperature requirement A (HtrA) family members, HtrA3 shares the greatest sequence homology and amino acid identity with HtrA1 (25). We previously reported that HtrA1 is downregulated, in part, through promoter hypermethylation in ovarian cancer (26).

In the present study, we have shown that HtrA3 is epigenetically silenced through cigarette smoke–induced methylation. Additionally, we provide preliminary evidence that clearly implicates HtrA3 loss as a factor in promoting resistance to etoposide and cisplatin cytotoxicity. More importantly, using patient-derived lung tumor samples, we show a statistically significant correlation between heavy smoking history and low HtrA3 expression. Collectively, these results suggest that cigarette smoke–induced methylation of HtrA3 could contribute to the etiology of chemoresistant disease in smoking-related lung cancer.

Materials and Methods

Cell culture. Cell lines were cultured according to the American Type Culture Collection guidelines.

RNA extraction, semiquantitative reverse transcription-PCR, and real-time PCR. RNA extraction, cDNA synthesis, and reverse transcription-PCRs (RT-PCR) were done as previously described (19). HtrA3 RT-PCR primers and control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers are listed in Supplementary Table S1. Real-time duplex PCR was carried out as previously described (16) with HtrA1, HtrA2, and HtrA3 primers obtained from SABiosciences.

Anti-HtrA3 monoclonal antibody. The anti-HtrA3 monoclonal antibody was developed at the Mayo Antibody Core Facility Rochester. It is characterized in Supplementary Fig. S1. The antibody was raised against a polypeptide corresponding to amino acids 144 to 453 of HtrA3 in BALB/c mice. Supernatants from mouse hybridoma cultures were tested over several weeks until optimal HtrA3 recognition with minimal cross-reactivity to purified HtrA1 was obtained by ELISA and Western with culture medium as a negative control. Anti-HtrA3 monoclonal antibodies were purified from supernatants of these hybridomas using Immunopure Immobilized Protein A Plus IgG Purification kit (Pierce). Purified antibodies were tested for HtrA3 specificity by Western analysis using purified protein and lysates from confirmed HtrA3-expressing cell lines and from cell lines lacking the HtrA3 expression.

Immunoblotting. Immunoblotting was done as previously described (16). HtrA2 (V-17) antibody was obtained from Santa Cruz Biotechnology (dilution of 1:200 in TBST +5% bovine serum albumin).

Densitometric analysis. Immunoblots were analyzed by the Bio-Rad Quantity One 4.40 software. Arbitrary intensity values were normalized to β-actin loading controls and expressed as fold change over controls.

Treatment of cells with 5-aza-2′-deoxycytidine, trichostatin-A, and LBH589. Treatments were done as previously described (16). LBH589 was purchased from Novartis Pharmaceuticals and was dissolved in DMSO before use.

Genomic DNA extraction and sequencing of bisulfite-modified DNA. These procedures were done as previously described (19). Primers used for methylation analysis are given in Supplementary Table S1.

NNK treatment. NNK (ABCR GmbH & Co.) was prepared in DMSO to a final concentration of 25 mmol/L. BEAS-2B cells were seeded at 1 × 10⁵ cells in 35-mm tissue culture dishes. Either NNK or DMSO was added to the culture medium after 24 h in culture. Culture medium was changed every 48 h.

Short hairpin RNA-mediated downregulation of HtrA3 in BEAS-2B. Lentiviral supernatants were generated by transfecting short hairpin RNA (shRNA) transfer vectors for nontarget control, or HtrA3 shRNA targeting either the 3′ untranslated region (UTR) or the open reading frame into 293T in combination with both VSV-G and gag/pol vectors (Sigma). Cells were seeded at 5 × 10⁴ in 100-mm tissue culture dishes. A total of 12 μg of DNA was included in the transfection mixture with 6 μg transfer vector and 3 μg each of VSV-G and gag/pol vectors. Transfections were carried out 24 h after seeding using Lipofectamine LTX (Invitrogen). Transfection medium was replaced with 5 mL culture medium after overnight incubation. Supernatants containing lentiviral particles for transduction were collected 48 h after

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transfection. Transduction was carried out using 2 mL supernatant on BEAS-2B and EKVX 24 h after seeding at 1.5 × 10^3 in six-well plates. Efficiency of knockdown was determined by immunoblot after 48 h of transduction. We selected stable batch clones in BEAS-2B using 0.5 μg/mL puromycin after 2 d of transduction.

**Rescue of shRNA downregulation.** Rescue experiments were done as previously described (19). The full-length HtrA3 open reading frame was obtained from the Invitrogen Full-Length Mammalian Gene Collection and subcloned into pcDNA3.1 (+)myc-His A (Invitrogen). Inactive mutants containing an S305A point mutation within the conserved trypsin H-S-D catalytic triad were generated by site-directed mutagenesis using the QuickChange Site Directed Mutagenesis Kit (Stratagene). Primers are given in Supplementary Table S1. A polyclonal antibody raised against a peptide corresponding to amino acids 250 to 300 within the trypsin domain was obtained from Abcam against a peptide corresponding to amino acids 250 to 300 within the trypsin domain was obtained from Abcam (dilution of 1:200 in TBST+5% bovine serum albumin) and was used to detect PDZ2-deleted variants.

**Chemotherapeutic drug treatment.** Treatment was carried out as previously described (21). Etoposide and cisplatin were purchased from Sigma-Aldrich and were dissolved in DMSO before use.

**MTT survival assays.** MTT assays were carried out as previously described (19).

**Clonogenic survival assays.** Clonogenic assays were carried out as previously described (22).

**TMA construction and digital imaging for TMA analysis.** Tissue microarray (TMA) construction and digital imaging system were done as previously described (18). Levels of HtrA3 expression were calculated and compared by two observers in a double blinded fashion and categorized as low (weak staining, 0), moderate (1+), or high (intense staining, 2+).

**Immunohistochemistry.** Immunohistochemistry for HtrA3 in normal lung and lung tumors was done using the HtrA3 monoclonal antibody as previously described (12).

**Statistics.** Two-tailed Student’s t test, ANOVA, and two-sided χ² analyses were done using Prism 3.0 (GraphPad Software). P < 0.05 and α = 0.05 were considered statistically significant.

### Results

**HtrA3 is expressed in the human bronchial cell line BEAS-2B and is downregulated in lung cancer cell lines.** Expression analysis of the human bronchial cell line BEAS-2B and 23 lung cancer cell lines by RT-PCR revealed that HtrA3 was expressed in BEAS-2B but was transcriptionally downregulated in 10 of 22 lung cancer cell lines (Fig. 1A, top). Immunoblot analysis using an HtrA3 monoclonal antibody (Supplementary Fig. S1) revealed that HtrA3 was expressed in BEAS-2B but was lost in over half (13 of 23) of lung cancer cell lines analyzed including adenocarcinoma lines H122, H1648, and H2122, which each showed mRNA expression (Fig. 1A, bottom). Collectively, these data suggest that HtrA3 is expressed in the normal lung but may be specifically lost in lung cancer.

**HtrA3 expression is regulated through methylation in the first exon.** As epigenetic modification has been implicated in regulating HtrA1 expression in ovarian cancer (26, 27), we assayed lung cancer cell lines lacking the HtrA3 expression for aberrant methylation and histone deacetylation. Treatment of H157, A549, CL1.0, and H460 with increasing concentrations of the methyltransferase inhibitor 5-aza-2′-deoxycytidine (5-aza-dC) resulted in a dose-dependent increase in HtrA3 transcription (Fig. 1B). Treatment with the histone deacetylase inhibitor trichostatin-A did not result in HtrA3 reexpression and no synergistic effects on HtrA3 expression were observed in combination with 5-aza-dC, indicating that HtrA3 expression is primarily under methylation control (Fig. 1C). However, although real-time PCR analysis showed that the novel histone deacetylase inhibitor LBH589 did not induce HtrA3 re-expression in A549 or H157, a synergistic effect on HtrA3 induction was observed in A549 when 5-aza-dC and LBH589 were combined (Supplementary Fig. S2A-B). Further, HtrA3 expression was induced by LBH589 in H358 and a synergistic effect on HtrA3 expression was again observed in combination with 5-aza-dC, indicating that HtrA3 expression in lung cancer cell lines may also be regulated by histone deacetylation in a manner that is cell type specific (Supplementary Fig. S2C).

To identify the target regions of HtrA3 methylation, we analyzed the methylation status of the HtrA3 promoter and of the first exon in BEAS-2B and in nine lung cancer cell lines, and observed differential methylation within the first exon, but not within the promoter, between cell lines with and without HtrA3 expression (Supplementary Fig. S3A; Table 1A). Sequence analysis revealed that 78% of lung cancer cell lines analyzed (7 of 9) showed an increase in methylation frequency in the first exon with corresponding a loss of HtrA3 expression (Table 1A). In contrast, cell lines with HtrA3 expression were consistently unmethylated at 19 of 23 CpG sites. These sites were consistently methylated in cell lines with low HtrA3 expression.

We next induced HtrA3 expression in H157 and A549 using 5.0 μmol/L 5-aza-dC and determined the methylation status of CpG sites within this region by direct sequencing following sodium bisulfite treatment (Fig. 1D). Sites 4, 7, 8, 11, 17, 20, and 21 within the first exon became unmethylated following 5-aza-dC treatment in H157 and A549 with corresponding increase in HtrA3 mRNA and protein expression (Supplementary Fig. S3A; Table 1A). These sites were unmethylated in cell lines with HtrA3 expression but were methylated in cell lines without HtrA3 expression (Table 1A).

To determine if HtrA3 was methylated in primary tumors from lung cancer patients with a history of smoking, we next assessed HtrA3 methylation and expression in primary lung tumors from heavy smokers (>40 pack-years) and from light smokers (<10 pack-years). Our analysis revealed an increase in the frequency of HtrA3 methylation in 69% (11 of 16) of tumors from heavy smokers with...
corresponding loss of HtrA3 expression when compared with tumors from light smokers (Supplementary Fig. S3B; Table 1B). Collectively, these data support CpG island methylation within the first exon of HtrA3 as a major mechanism of regulating HtrA3 expression in lung cancer, particularly related to smoking.

The cigarette smoke carcinogen NNK regulates HtrA3 expression through exon 1 methylation. Cigarette smoke is a known etiologic factor for lung cancer that exerts its effects, at least in part, through promoter hypermethylation in crucial tumor suppressor genes (22). We therefore investigated whether the cigarette smoke–derived carcinogen NNK regulates HtrA3 expression through methylation in vitro. BEAS-2B human bronchial cells were treated with increasing concentrations of NNK and were assayed for mRNA and protein expression. We observed a 2-fold loss of HtrA3 protein expression within 24 hours of 500 μmol/L NNK treatment compared with HtrA3 expression at treatment initiation and a more rapid 2-fold loss within 2 hours of treatment when the dose was doubled (Fig. 2A). In contrast, HtrA3 expression was unsuppressed in the untreated cell population. Real-time PCR further revealed significant downregulation of HtrA3 transcription within 2 hours of 1.0 mmol/L NNK treatment (Fig. 2B). In contrast, HtrA1 and HtrA2 mRNA and protein expression were induced by 1.0 mmol/L NNK treatment (Fig. 2B). In contrast, HtrA1 and HtrA2 mRNA and protein expression were induced by 1.0 mmol/L NNK treatment (Fig. 2B). In contrast, HtrA1 and HtrA2 mRNA and protein expression were induced by 1.0 mmol/L NNK treatment (Fig. 2B). In contrast, HtrA1 and HtrA2 mRNA and protein expression were induced by 1.0 mmol/L NNK treatment (Fig. 2B). In contrast, HtrA1 and HtrA2 mRNA and protein expression were induced by 1.0 mmol/L NNK treatment (Fig. 2B).

As chronic smokers are subject to long-term, low-dose cigarette smoke exposure, we assessed the effect of prolonged low-dose NNK treatment on HtrA3 expression in BEAS-2B. We observed a steady decline in HtrA3 protein...
**Table 1.** *HtrA3* methylation analysis in the human bronchial cell line BEAS-2B, lung cancer cell lines, and primary lung tumors

<table>
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**NOTE:** ○, unmethylated; ●, methylated; θ, hemimethylated. Data represent three independent trials.

*Abbreviations: ADC, adenocarcinoma; SCC, squamous cell carcinoma; BAC, bronchioalveolar carcinoma.*

*Pack-years are the number of packs of cigarettes smoked each day per year of active smoking.*

†*HtrA3 expression was determined by RT-PCR and immunoblot (Fig. 1A and B). Expression was characterized as either present (+) or absent (–).*

‡*HtrA3 expression was determined by real-time PCR. Expression was characterized as either high (+) or low (–).*
Fig. 2. HtrA3 expression is regulated by NNK in BEAS-2B. A, immunoblot showing HtrA3, HtrA1, and HtrA2 expression with NNK or DMSO treatment, and β-actin loading control. Densitometric analysis showing fold changes in intensity normalized by β-actin loading controls is represented graphically below blots. B, real-time PCR showing HtrA3, HtrA1, and HtrA2 transcription with 1.0 mmol/L NNK treatment. C, immunoblot showing HtrA3, HtrA1, and HtrA2 expression with low-dose 10 μmol/L NNK treatment over 15 d and β-actin loading control. D, real-time PCR showing HtrA3, HtrA1, and HtrA2 transcription with low-dose 10 μmol/L NNK treatment over 15 d.
and mRNA expression, with an 8-fold decrease in protein expression over 15 days (Fig. 2C-D). In contrast, both HtrA1 and HtrA2 were induced over the same time period. Interestingly, we also observed HtrA3 protein re-expression 72 hours after an initial 24-hour treatment with 500 μmol/L NNK, suggesting that NNK-mediated downregulation of HtrA3 expression was reversible (Supplementary Fig. S4A). Further, we found that NNK treatment slowed BEAS-2B cell proliferation when measured by mitochondrial reduction of the tetrazolium salt MTT (Supplementary Fig. S4B).

In addressing whether NNK-mediated HtrA3 suppression occurred through methylation, bisulfite-modified DNA from NNK-treated samples was analyzed by direct sequencing. We observed an increase in methylation frequency in the first exon of HtrA3 with corresponding loss of HtrA3 expression with both 500 μmol/L NNK treatment over 24 hours and 10 μmol/L treatment over several days when compared with untreated controls (Table 2A-B). Collectively, these data suggest that HtrA3 may be a specific target for cigarette smoke carcinogen-mediated regulation through methylation in smoking-related lung cancer.

**Loss of HtrA3 expression attenuates etoposide and cisplatin cytotoxicity.** Downregulation of human HtrA family members has been implicated in resistance to cisplatin and paclitaxel in ovarian and gastric cancers and in renal cells (28, 29). We therefore investigated whether the loss of HtrA3 expression could result in chemoresistance in vitro. We observed a 2- to 3-fold decrease in HtrA3 protein expression in pooled BEAS-2B clones stably transduced with shRNAs directed at either the open reading frame or the 3'UTR compared with endogenous HtrA3 expression in a clonal line stably transduced with a nontargeted shRNA control (Fig. 3A). MTT assay (Fig. 3B, top) and clonogenic survival assay (Fig. 3B, bottom) showed that endogenous HtrA3 expression sensitized cells to both etoposide and cisplatin treatment compared with clonal lines in which HtrA3 was stably downregulated (P < 0.05).

We next subcloned the long HtrA3 isoform (full length) and a shortened variant lacking the PDZ domain (PDZ

### Table 2. HtrA3 methylation analysis following NNK treatment in the human bronchial cell line BEAS-2B

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#### A. Methylation analysis of Cpg sites 1-25 in the first exon of HtrA3 with corresponding HtrA3 expression following 500 μmol/L NNK treatment in BEAS-2B

#### B. Methylation analysis of Cpg sites 1-25 in the first exon of HtrA3 with corresponding HtrA3 expression following 10 μmol/L NNK treatment in BEAS-2B

**NOTE:** Data represent three independent trials.

*HtrA3 expression was determined by real-time PCR (Fig. 2A and C). Expression was characterized as high (2+), moderate (1+), or low (0).
deleted) as well as catalytically inactive forms of each. We transiently re-expressed each form in a clonal cell line in which HtrA3 was stably suppressed by a 3′UTR-targeted shRNA (Fig. 3C). Re-expression of the functional full-length and PDZ-deleted HtrA3 variants in BEAS-2B significantly attenuated proliferation as determined by MTT assays with either etoposide or cisplatin treatment compared with the re-expression of protease inactive variants or expression of vector control \((P < 0.05; \text{Fig. 3D})\). Moreover, re-expression of functional variants significantly decreased clonogenic survival with etoposide treatment compared with the re-expression of protease-inactive variants or vector control \((P < 0.05)\). Taken together, these data implicate HtrA3 as a key factor in modulating sensitivity to etoposide and cisplatin in smoking-related lung cancer that mediates this function, at least in part, through its catalytic activity.

**Smoking history is correlated with HtrA3 expression in smoking-related lung cancer.** As HtrA3 expression is regulated by cigarette smoke in vitro, we next addressed whether HtrA3 expression in primary lung tumors is strongly correlated with smoking history in vivo. HtrA3 expression was determined by immunohistochemistry in normal lung tissue and on a TMA containing 70 primary lung tumors from patients identified as light (<10 pack-years, 0), moderate (between 10 and 40 pack-years, 1), and heavy (>40 pack-years, 2) smokers. Levels of HtrA3 expression were calculated and compared by two separate observers in a double-blinded fashion and were categorized as low (weak staining, 0), moderate (1+), or high (intense staining, 2+; Fig. 4A-B). Supplementary Table S2 shows the relevant patient and tumor characteristics. Statistical analyses were done by V. S. and D. B. Light and moderate smoking groups were combined for statistical analysis. As given in Fig. 4C, 100% (35 of 35) of tumors from heavy smokers showed either low or moderate HtrA3 expression with 66% (23 of 35) of tumors showing markedly low HtrA3 expression. Likewise, 91% (32 of 35) of tumors from light and moderate smokers combined showed moderate to high levels of HtrA3 expression with 49% (17 of 35) showing markedly high expression. Results given in Fig. 4C show a statistically significant difference between the low- and high-staining groups \((P < 0.0001)\). The odds ratio for light smoking history in the cohort with low levels of HtrA3 expression was 0.05 (95% confidence interval, 0.0221-0.2313). No significant correlations were noted between HtrA3 expression and patient gender or tumor grade, stage, or histology. These results clearly support a strong correlation between heavy smoking history and low HtrA3 expression in primary lung tumors.

**Discussion**

Gene silencing through aberrant promoter and first exon hypermethylation has been widely implicated in a variety of pathologic processes including cancer induction, progression, and resistance to chemotherapery. Here, we have characterized a novel mechanism for HtrA3 regulation through cigarette smoke–mediated first-exon methyl-
Fig. 3. HtrA3 modulates cytotoxicity to etoposide and cisplatin in BEAS-2B. A, immunoblot showing normal HtrA3 expression and stable HtrA3 downregulation. Densitometric analysis showing fold changes in intensity normalized by β-actin loading controls is given below blot. B, top, MTT reduction assays showing greater reduction in cell proliferation with HtrA3 expression than with HtrA3 downregulation during etoposide or cisplatin treatment; bottom, clonogenic survival assays showing greater clonogenic survival with HtrA3 downregulation than expression following etoposide or cisplatin treatment. Points, mean independent trials done in triplicate; bars, SEM. P values were calculated using unpaired two-tailed Student’s t test for two groups, and ANOVA. C, top, domain structural schematics of catalytically functional and protease-inactive full-length and PDZ-deleted HtrA3 variants that are COOH-terminally myc and poly-histidine tagged. The functional PDZ-deleted variant is untagged. Protease inactive mutants contain a serine to alanine point mutation at position S305 within the conserved serine protease H-D-S catalytic triad; bottom, immunoblot showing transient HtrA3 re-expression and vector control in the BEAS-2B 3’UTR clonal cell line 2 d after transfection. PDZ-deleted variants were detected using a polyclonal antibody. D, left, MTT reduction assays showing a decrease in cell proliferation with re-expression of functional HtrA3 variants during etoposide or cisplatin treatment; right, clonogenic survival assay showing decreased clonogenic survival with re-expression of functional HtrA3 variants following etoposide or cisplatin treatment. Points, mean of independent trials done in triplicate; bars, SEM. P values were calculated using unpaired two-tailed Student’s t test for two groups, and ANOVA.
the HtrA1 promoter region. Initial induction of HtrA3 protein expression observed within 4 hours of 500 μmol/L NNK treatment followed by HtrA3 suppression within 24 hours (Fig. 2A) could suggest that NNK-induced methylation of the HtrA3 XRE both attenuates the HtrA3 stress response and contributes to HtrA3 silencing.

Comparison of Tables 1 and 2 reveals that in some instances, overall levels of methylation in A549 and H157 after treatment with 5-aza-dC, which induced HtrA3 expression, were similar to levels of methylation following NNK treatment, which resulted in HtrA3 suppression. A more detailed analysis identified CpG sites 4, 6, 7, 8, 11, 17, 20, and 21 as sites demethylated in both A540 and H157 within 48 hours of 5-aza-dC treatment. Sites that become methylated with NNK treatment seem to differ with dose and the duration of treatment. With 500 μmol/L NNK treatment, CpG sites 4, 6, 7, 8, 11, and 19 were fully methylated, whereas site 20 was hemimethylated within 24 to 48 hours of treatment. In contrast, with 10 μmol/L NNK treatment, CpG sites 4, 6, 8, 11, 20, and 21 were fully methylated, whereas sites 7 and 17 were hemimethylated within 15 days of treatment. Based on these data, minimal CpG sites within the first exon of HtrA3 involved in the regulation of HtrA3 expression seem to be sites 4, 6, 8, 11, and 20.

The identification of HtrA3 as a novel target for suppression through cigarette smoke–mediated methylation and the evidence of its role in modulating sensitivity to etoposide and cisplatin in vitro may contribute to studies of both biomarkers of chemoresistance, particularly related to smoking, and targeted epigenetic therapies in lung cancer. CpG sites 4, 6, 8, 11, and 20 could eventually serve as screening sites with strong predictive value with regard to treatment outcome that could alter the target approach to lung cancer treatment from a general to an individual treatment strategy. Further, as HtrA3 is downregulated in lung tumors by epigenetic mechanisms, it represents a strong potential target for epigenetic modulation (39). Chemotherapeutic strategies for cancer treatment have included 5-aza-dC in the treatment of myelodysplastic syndrome, chronic myelogenous leukemia, and acute myelogenous leukemia (40). Future clinical trials involving the evaluation of epigenetic therapies should eventually include HtrA3 as a therapeutic marker to assess the biological response of epigenetic therapies and should evaluate its prognostic potential to predict tumor response to combined epigenetic and conventional chemotherapy. Until then, the analysis of HtrA3 expression and function still warrants further investigation in confirmatory studies and perhaps even in different tumor types.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest exist.

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Methylation Induced Gene Silencing of HtrA3 in Smoking-Related Lung Cancer

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