Lack of PTEN Expression in Non-Small Cell Lung Cancer Could Be Related to Promoter Methylation

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ABSTRACT

Purpose: The PTEN gene at chromosome 10q23.3 is a tumor-suppressor gene that is inactivated in several types of human tumors. Although mutation and homozygous deletion are the most common mechanisms of PTEN inactivation, promoter methylation and translational modification can also account for PTEN silencing. The aim of this study was to investigate the expression of PTEN protein in primary non-small cell lung cancer (NSCLC) samples and to investigate the promoter methylation status of the gene in a panel of NSCLC cell lines as well as primary tumors.

Experimental Design: We analyzed PTEN expression by immunohistochemistry in tissue samples from 125 patients with early-stage NSCLC. We also evaluated PTEN promoter methylation status by methylation-specific PCR in 20 microdissected PTEN-negative primary tumors from among the last specimens as well as in a panel of 16 NSCLC cell lines. Western and Northern blotting were performed in the same panel of NSCLC cell lines.

Results: Thirty (24%) of the 125 specimens showed a lack of staining for PTEN. PTEN methylation was detected in 7 (35%) of the 20 PTEN-negative NSCLC samples and in none of the 10 PTEN-positive NSCLC samples that were microdissected. Furthermore, PTEN methylation was observed in 11 (69%) of the 16 NSCLC cell lines tested. PTEN mRNA expression was increased in the NCI-H1299 cell line by in vitro treatment with the demethylating agent 5-aza-2’-deoxycytidine. PTEN methylation was well correlated with PTEN expression in NSCLC cell lines by Western and Northern blot (P = 0.025).

Conclusions: Although genetic alterations of the PTEN gene are rare in NSCLC, loss of PTEN protein is not an uncommon event in early-stage NSCLC. Lack of PTEN expression may be partially explained by promoter methylation.

INTRODUCTION

Lung cancer is the most common cause of cancer death worldwide, accounting for more deaths than those caused by prostate, breast, and colorectal cancers combined (1). The prognosis for patients with lung cancer is strongly correlated with disease stage at the time of diagnosis: patients with clinical stage I disease have a 5-year survival rate of about 60%, whereas in patients with clinical stage II-IV disease the 5-year survival rate ranges from 40% to less than 5% (2). Improving the survival rate of patients with this disease requires a better understanding of tumor biology and the subsequent development of novel therapeutic strategies.

PTEN/MMAC1/TEP1, located at 10q23.3, is a tumor-suppressor gene that encodes a cytoplasmic protein that has a protein tyrosine phosphatase domain and a domain extensively homologous to the cytoskeletal proteins tensin and auxilin (3). Germ-line mutations of PTEN are found in patients with Cowden syndrome, a familial syndrome associated with a predisposition for multiple benign hamartomas and malignant breast, skin, and thyroid neoplasms (3). Somatic mutation or deletion of PTEN has been reported in a variety of tumor types, including glioblastoma, melanoma, breast, prostate, renal, and endometrial carcinomas (4–9). Genetic analysis of PTEN in NSCLC cancers has demonstrated alterations in PTEN in 8–16% of the examined NSCLC cell lines suggesting that PTEN is frequently targeted in NSCLC tumorigenesis (10–13). Because alternative mechanisms such as promoter hypermethylation, alternative splicing of pre-mRNA, and posttranslational modification, may also inactivate gene function, the actual frequency of PTEN abnormalities in NSCLC may be underestimated. To date, few analyses of PTEN protein expression in NSCLC have been performed, and little is known about epigenetic or posttranslational mechanisms that could participate in PTEN inactivation.

The abbreviations used are: NSCLC, non-small cell lung cancer; PTEN, phosphatase and tensin homologue deleted on chromosome ten; TSA, trichostatin A; MSP, methylation-specific PCR; PIP-3, phosphati- dylinositol-3,4,5-triphosphate; HBE, human bronchial epithelial (cells); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PI3K, phosphoinositol-3-kinase; PKB, protein kinase B.

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To determine PTEN expression in patients with NSCLC and to ascertain whether promoter methylation accounted in part for the loss of PTEN, we examined immunohistochemically the expression pattern of PTEN in 125 patients with early-stage NSCLC and evaluated promoter methylation status in 20 microdissected tumor samples as well as in a panel of 16 NSCLC cell lines.

MATERIALS AND METHODS

Clinical Samples and Immunohistochemical Staining for PTEN Protein. Surgical specimens were obtained from a total of 125 patients with early stages of lung cancer who underwent a surgical resection at the Department of Thoracic and Cardiovascular Surgery at The University of Texas M. D. Anderson Cancer Center. Paraffin-embedded, 4-μm-thick tissue sections from 125 primary tumors were stained for the PTEN protein using a primary rabbit polyclonal anti-PTEN antibody (Zymed Laboratories, San Francisco, CA), as reported previously (14). All of the sections were deparaffinized by using a series of xylene baths and then rehydrated using a graded alcohol series. To retrieve the antigenicity, the tissue sections were microwaved in 10 mM citrate buffer (pH 6.0) once for 2 min. The sections were then immersed in methanol containing 0.3% hydrogen peroxide for 20 min to block endogenous peroxidase activity and then incubated in 2.5% blocking serum to reduce nonspecific binding. Sections were incubated overnight at 4°C with primary anti-PTEN antiserum (1:50). The sections were then processed using standard avidin-biotin immunohistochemical techniques according to the manufacturer’s recommendations (Vector Laboratories, Burlingame, CA). Diaminobenzidine was used as a chromogen, and commercial hematoxylin was used for counterstaining. Adjacent normal-appearing epithelium within the tissue sections served as a positive internal control.

Representative areas of each tissue section were selected, and cells were counted in at least four fields (at ×200). On the basis of the results of the immunohistochemical staining, specimens were classified into three groups, as reported previously (6): increased or equal staining intensity compared with the corresponding normal tissue (+ +), decreased staining intensity (+), and absence of staining (−). All of the slides were evaluated and scored independently by two investigators (J. I. L. and J.-C. S.) who were blinded to the clinical information pertaining to the subjects.

Cell Lines and Culture Conditions. Normal HBE cells were grown from bronchial epithelium that was harvested from fresh surgical specimens obtained from patients undergoing lobectomy procedures. The mucosal layer was sterilely stripped from bronchial specimens, cut into small pieces, and placed on a plastic tissue culture plate containing a thin layer of medium. For each experiment, normal HBE cells from a single patient were used. Normal HBE cells were grown in keratinocyte serum-free medium (Life Technologies, Inc. Grand Island, NY) on standard plastic ware (Falcon; Becton-Dickinson, Bedford, MA) at 37°C in a 5% CO2 atmosphere. The NSCLC cell lines NCI-H1792, -H1299, -H661, -H596, -H591, -H460, -H441, -H358, -H322, -H292, -H226, and -H157, A549, Calu-6, Calu-1, and SK-MES-1 were routinely maintained in RPMI 1640 supplemented with 10% FCS. The NSCLC cell lines were obtained from the American Type Culture Collection (Manassas, VA). 5-Aza-2′deoxycytidined was added to the RPMI 1640 containing 2% serum.

Tissue Microdissection and Genomic DNA Extraction. Sections (4 μm thick) from formalin-fixed and paraffin-embedded tissue blocks were obtained. Tissue microdissection was performed manually under a stereomicroscope using a 25-gauge needle. Dissected tissues were digested in 200 μl of digestion buffer containing 50 mM Tris–HCl (pH 8.0), 1% SDS, and proteinase K (0.5 mg/ml) at 42°C for 36 h. The digested products were purified by extraction with phenol-chloroform twice. DNA was then precipitated by the ethanol precipitation method in the presence of glycogen (Roche Molecular Biochemicals, Indianapolis, IN) and recovered in distilled water.

MSP. Sample DNA (at least 100 ng) from the microdissected tumor specimens and from NSCLC cell lines, mixed with 1 μg of salmon sperm (Life Technologies, Inc., Gaithersburg, MD), were submitted to chemical modification following the protocol by Herman et al. (15). Briefly, DNA was denatured with 2 M NaOH, followed by treatment with 10 mM hydroquinone and 3 M sodium bisulfite (Sigma Chemical Co., St. Louis, MO). After purification in a Wizard SV Plus kit column (Promega, Madison, WI), the DNA was treated with 3 M NaOH and precipitated with three volumes of 100% ethanol, a one-third volume of 10 μl H2OAc, and 2 μl of glycerol at −20°C. The precipitated DNA was washed with 70% ethanol and dissolved in distilled water. PCR was conducted with primers that were specific for either the methylated or the unmethylated versions of the PTEN gene: PTENM forward, 5′-gctttggggattttttgtc-3′, and PTENM reverse, 5′-aaccctttcctactggg-3′, for the methylated sequence; PTENU forward, 5′-TTAGTTTTGGGATTTTTTTTTGT-3′, and PTENU reverse, 5′-CCTACCATTCTACACCA-3′, for the unmethylated sequence. Primers location is shown in Fig. 1. The 12.5-μl total reaction volume contained 25 ng of modified DNA, 3% DMSO, all four deoxynucleoside triphosphates (each at 200 μM), 1.5 mM MgCl2, 0.4 μM PCR primers, and 0.625 units of HotStar Taq DNA polymerase (Qiagen, Valencia, CA). Water was substituted for DNA as a negative control, and NCI-H460 cell line

Fig. 1  Map of the PTEN promoter area. Vertical bars on top, CpG sites. Gray box on the bottom, exon 1; arrow, putative transcription start site. Horizontal lines, the areas of homology to the PTEN pseudogene, the location of the primers used in Salveson et al. (MSP-old; Ref. 28), and the location of the primers used in the current study (MSP). The area shown is 3454 bases long.
DNA, treated with SsSI Methylase (New England Biolabs, Beverly, MA), was used as a positive control. DNA was amplified by an initial cycle at 95°C for 15 min as required for enzyme activation, followed by 40 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min, and ending with a 5-min extension at 72°C in a thermocycler (Applied Biosystems, Foster City, CA). PCR products were separated on 2% agarose gels and visualized after staining with ethidium bromide.

**Western Blot Analysis.** Whole-cell lysates were prepared in lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 0.2 mM EGTA, 1% NP40, 10% fetal bovine serum]. Lysates were incubated for 20 min on ice, followed by centrifugation at 13,000 × g for 10 min. The supernatants were collected, and protein concentrations were determined with a BCA protein assay kit (Bio-Rad, Hercules, CA). Cell lysates were electrophoresed using SDS-PAGE and then transferred onto a BA-S-83-reinforced nitrocellulose membrane (Schleicher and Schuell, Inc., Keene, NH). Membranes were immunoblotted overnight at 4°C with a rabbit polyclonal antibody against human PTEN and a goat antibody against β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in Tris-buffered saline containing 5% nonfat dry milk. Antibody binding was detected using the ECL kit (Amersham, Inc., Arlington Heights, IL) according to the manufacturer’s directions.

5-Aza-2′-deoxycytidine Treatment and Northern Blot Analysis.** NCI-H1299 cells were transferred onto a 100-mm dish and, 1 day later, 0.001, 0.01, 0.1, 1, or 5 µM 5-aza-2′-deoxycytidine or 0.001, 0.01, 0.1, 1, or 5 µM TSA were added in RPMI 1640 containing 2% FCS. Cells were changed to a new medium containing 5-aza-2′-deoxycytidine or TSA every day. After 6 days of treatment, the cells were lysed in 4.0 M guanidinium isothiocyanate, and total cellular RNA was extracted. RNA was subjected to electrophoresis (20 µg/lane) on a 1% agarose gel containing 2% formaldehyde, transferred to a Zeta-Probe membrane (Bio-Rad Laboratories), and hybridized to a [γ-³²P]dCTP-labeled PTEN or GAPDH probe as a control.

**Statistical Analysis.** Fisher’s exact test or the χ² test was used to analyze the association between two categorical variables. All of the tests were two-sided. P < 0.05 was considered to be statistically significant.

**RESULTS**

The usual pattern of positive staining for PTEN in NSCLC was cytoplasmic and not nuclear (Fig. 2). Even when tumor cells were negative for PTEN staining, normal bronchial epithelial cells in the section were positive and were used as an internal positive control of the staining for PTEN. PTEN staining among the tumor specimens was either negative, diffusely weak, strong, or of a heterogeneous pattern of variable intensity. Among the heterogeneously stained specimens, staining was prominent in the well-differentiated areas (Fig. 2D). According to our scoring criteria, loss of PTEN expression (−) was noted in 30 (24%) of the 125 NSCLC specimens. Weak expression (+) was seen in 57 (46%) of the tumors, and strongly positive expression (++) was seen in 38 (30%) of the tumors. The frequency of PTEN expression did not differ significantly by tumor-node-metastasis stage, sex, smoking status, age, race, and histological subtype between the group with PTEN positive staining (+ or ++) and the group with PTEN negative staining (−); Table 1). Survival times were similar for patients with PTEN-negative tumors and with PTEN-positive tumors (P = 0.88, log rank test; data not shown).

To understand the mechanism explaining loss of PTEN expression in 24% of 125 NSCLC patients, we manually microdissected 20 randomly selected PTEN-negative slides along with 10 PTEN-positive slides. MSP was performed on genomic

![Fig. 2 Immunohistochemical staining patterns of PTEN in early-stage NSCLC. A, an adenocarcinoma with most cancer cells expressing PTEN in the cytoplasm. B, an adenocarcinoma tumor negative for PTEN expression. C, a squamous cell carcinoma tumor with diffuse and strong PTEN expression. D, a squamous cell carcinoma tumor with diffusely weak staining. The staining is prominent in the well-differentiated areas. (All panels, ×400).](image-url)
DNA extracted from those samples (Fig. 3). No methylation was found in the 10 PTEN-positive samples, whereas a methylated band was observed in 7 (35%) of the PTEN-negative samples. To further explore PTEN promoter methylation status, we evaluated a panel of 16 NSCLC cell lines. Overall, 11 (69%) of the 16 NSCLC cell lines displayed a methylated band (NCI-H1792, -H1299, -H661, -H441, -H358, -H322, -H292, -H157, Calu-6, Calu-1, and SK-MES-1). The following cell lines only displayed an unmethylated band: NCI-H226, -H460, -H591, -H596, and A549. The NSCLC cell line NCI-H1299 displayed a clear methylated band and had very low levels of PTEN mRNA, making it a suitable candidate for treatment with the demethylating agent 5-aza-2'-deoxycytidine. A net increase of PTEN mRNA was seen after treatment of NCI-H1299 cells with 5-aza-2'-deoxycytidine (Fig. 4) but not with TSA (data not shown), confirming the role of the 5' region CpG methylation in regulating PTEN expression.

We further evaluated the correlation between PTEN promoter methylation status and PTEN expression by Western blot analysis in the same panel of 16 NSCLC cell lines. PTEN expression by Western blot in the NSCLC cell lines was either absent, weak, or strong (Fig. 5). Eleven cell lines displayed a methylated band: of these 11, 5 had a weak PTEN band, and 5 had no PTEN band detectable by Western blotting. Among the five cell lines without a methylated band by MSP, four had a strong PTEN band by Western blotting. The presence of a methylated band by MSP was statistically correlated with weak or absent PTEN expression by Western blotting (P = 0.025, Fisher’s exact test; Fig. 6). Northern blotting further confirmed a low mRNA message in the NSCLC cell lines that had a methylated band and a weak or absent PTEN band by Western blot (Fig. 6).

### DISCUSSION

The PTEN gene product dephosphorylates tyrosine and serine/threonine residues and exhibits phosphatase activities with both protein and lipid substrates (16, 17). The major

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<td>5-yr overall survival rate (95% CI)</td>
<td>52.3% (44.2–61.9%)</td>
<td>53% (43.8–64.2%)</td>
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* P was calculated comparing smoking versus nonsmoking patients.
substrate of PTEN is PIP-3, a product of PI3K (18). The loss of PTEN function increases the concentration of PIP-3, which in turn leads to Akt hyperactivation, which suggests that the tumor-suppressor function of PTEN is exerted through the negative regulation of the PI3K/Akt cell survival pathway (3, 19, 20). The loss of PTEN expression results in increased Akt activity and continued cell survival and cell proliferation. In studied glioma, breast, and prostate cancer cell lines, PTEN has been shown to mediate G1 cell cycle arrest and/or apoptosis through the suppression of the PI3K/Akt pathway (21). PTEN, therefore, seems to play an important role in modulating cell cycle progression and/or apoptosis. Although the protein phosphatase activity of PTEN is not considered to be as important as its lipid phosphatase activity for tumor suppression, the PTEN function as protein phosphatase has been implicated in the inhibition of cell migration and invasion via dephosphorylation of focal adhesion kinase (FAK), a molecule critical in the regulation of integrin signaling (22–24) and also in the inhibition of cell cycle progression (24).

Recently, frequent genetic alterations and loss of expression of the PTEN gene have been found in several malignant neoplasms (4–9). The studies of PTEN in NSCLC have focused exclusively on searching for mutations or deletions of the gene, with little emphasis on abnormalities at the protein level (10–13). In one study of NSCLC cell lines, homozygous deletions of PTEN were reported in 2 (8%) of 25 cell lines tested (10). Two subsequent studies, however, failed to demonstrate mutation of PTEN in the NSCLC samples studied, neither in cell lines nor in primary tumors (11, 13). Nevertheless, Forgacs et al. (12) described point mutations in 3 (16.7%) of 18 NSCLC cell lines analyzed (12). Using immunohistochemical analysis of 125 NSCLC patients, we showed that the rate of PTEN inactivation at the protein level is more frequent (24%) than that identified at the genetic level (0–16.7%). Interestingly, one previous study (25) that screened a wide variety of human neoplastic tissues, also reported PTEN protein loss in 25% of the NSCLC samples tested. We did not find any significant correlation between the loss of PTEN and clinicopathological characteristics in our NSCLC patients. Furthermore, no association was found between PTEN expression and survival. This is in contrast to the results of a recent report in which patients with breast cancer who lacked PTEN expression in their tumor had a shorter survival time (26). Tissue specificity or technical differences (e.g., different antibody used for immunohistochemical evaluation) may account for these different results.

The loss of PTEN expression in 24% of 125 patients with early-stage NSCLC suggests that abrogation of PTEN function may occur through multiple mechanisms. Loss of PTEN expression may be explained by decreased protein synthesis, elevated protein degradation or turnover, or other posttranslational modifications. Another possible mechanism is the epigenetic inactivation of the gene through hypermethylation of the promoter region (27, 28). Indeed, inactivation of other tumor-suppressor genes by methylation has been previously reported in patients with NSCLC (29, 30). We found promoter methylation in 7 (35%) of 20 PTEN-negative microdissected tumors, whereas none of the 10 PTEN-positive microdissected tumors displayed a methylated band. In the 16 NSCLC cell lines, we found methylation in 69% of the cell lines tested. The higher percentage of methylation found in the lung cancer cell lines compared with primary tumors might be explained by additional changes acquired in culture or by the fact that the tumor cell lines were derived from more aggressive and more advanced tumors (stage III and IV). Furthermore DNA, quality and preservation in the sections from formalin-fixed and paraffin-embedded tissue blocks might not always be optimal. This could partially explain why only 35% of the PTEN-negative sections displayed a methylated band. Additional mechanisms (e.g., posttranslational modifications) could also account for the lack of PTEN expression in some of the primary tumor samples.
tested. It is noteworthy that our primer design for PTEN MSP differed from the one previously reported by Salvesen et al. (28). Indeed, through BLAST search, we have found extensive homology between the PTEN DNA region explored by Salvesen et al. (28) and the chromosome 9 duplication of the T-cell receptor β gene (AF029308), as well as with the highly conserved processed PTEN pseudogene (31). Therefore, we have designed primers to explore a CpG island, with no homology, located ∼1.9 kb upstream from the region evaluated by Salvesen et al. (28). Fig. 1 represents the PTEN promoter area with the different CpG islands. It is not clear to date, which CpG island in the very large PTEN upstream regulatory region is best related to the gene expression. An additional fact that supports the importance of PTEN methylation in inactivating this gene is the efficacy of the demethylating agent 5-aza-2’-deoxycytidine in increasing PTEN mRNA in NCI-H1299 cell line. It is noteworthy that TSA treatment had no effect on PTEN expression, thus highlighting the specificity of the demethylating effect of 5-aza-2’-deoxycytidine. Taken together, these findings suggest that the methylation of PTEN may be an important mechanism for silencing this gene in NSCLC.

Our findings are in line with similar findings reported in endometrial cancer (28) and suspected in ovarian carcinomas and melanoma (16, 32). In fact, the loss of PTEN function in endometrial, breast, prostate, ovarian, and melanocytic tumors is more frequent than can be adequately explained by structural genomic changes alone (33). We believe that reports on PTEN methylation are few because proving an epigenetic mechanism of PTEN silencing is technically challenging. Indeed, one has to take into account the large size (>250 kb) of the PTEN upstream regulatory region, the existence of a highly conserved processed pseudogene with homology maintained up to 1 kb upstream from the translational start site, and technical challenges in linking epigenetic events with expression level (31, 33).

The elucidation of the mechanism that mediates the loss of PTEN expression has important clinical implications. The role of PI3K/Akt/PKB in apoptosis and survival, as well as the effects of dysregulation of the PI3K/Akt/PKB pathway in the pathogenesis of a large fraction of human cancer, has been identified (34–37). Because demethylating agents are under clinical evaluation, our finding may provide an advantage in therapeutic strategies, especially in the treatment of NSCLC, in which constitutive activation of Akt/PKB occurs at a high frequency (34).

In summary, we have demonstrated that the loss of PTEN is not a rare event in NSCLC, although genetic alterations of the PTEN gene are rare in this setting. The lack of PTEN expression may be partially explained by promotor methylation. We found methylation of PTEN in 35% of the primary tumors and in 69% of the NSCLC cell lines tested. Moreover, we were able to show that methylation of this gene is reversible with 5-aza-2’-deoxycytidine. Our findings of a frequent acquired tumor-related epigenetic alteration favor the candidacy of PTEN as a tumor suppressor gene also subject to methylation in addition to point mutations and homozygous deletions.

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