Epigenetic modulation of PTEN expression during antiandrogenic therapies in human prostate cancer

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Abstract. Although the tumor-suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is frequently mutated or deleted in a wide variety of solid tumors, some malignancies, including prostate cancer, exhibit undetectable PTEN protein without loss of PTEN gene. Aim of this study was to evaluate whether the PTEN downmodulation, observed during bicalutamide treatment, was due to epigenetic events. We analyzed the expression of PTEN in presence or absence of azacitidine or valproic acid in a panel of 50 primary cultures derived from naive (UNT, 23 ptz) and bicalutamide-based neoadjuvant hormone therapy-treated patients (NHT, 27 pts). Results showed that Western blot and PCR analyses showed that 54 and 68% of primary cultures displayed detectable amounts of PTEN protein and mRNA, respectively. Treatment with azacitidine increased the percentage of PTEN-positive cultures up to 72 and 80% for PTEN protein and mRNA determination, respectively. Treatment with valproic acid was able to increase the percentage of PTEN-positive cultures up to 80 and 74% for PTEN protein and mRNA determination, respectively. The percentage of cultures with undetectable levels of PTEN protein was significatively higher in cultures derived NHT patients respect to cultures derived from UNT men (P=0.020). Valproic acid reduced significantly the percentage of cultures PTEN-negative only at protein level and only in NHT (P=0.029) group. In conclusion, our data suggests that antiandrogenic therapy reduced PTEN expression by epigenetic mechanisms suggesting that epigenetic drugs, upmodulating PTEN expression, can reduce Akt activity and probably enhance the efficacy of antiandrogenic therapy.

Introduction

Bicalutamide monotherapy at 150 mg/day has been investigated as an alternative treatment for locally advanced prostate cancer (PC), based on its comparative benefits for quality of life issues and associated morbidity (1). However, many patients eventually progress to hormone-refractory status and require second-line therapies. Knowledge of molecular changes induced by the primary therapy may be informative in the selection and predicted success of subsequent therapies. Mutations and the loss of the tumor-suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) leads to constitutive activation of the Akt pathway, providing a mechanism whereby prostate tumor cells survive after the withdrawal of exogenous trophic factors or androgens, because activated Akt inhibits PC cell suicide (2,3). High PTEN is the most frequently mutated tumor-suppressor gene in PCa (3), where deletions, point mutations, and DNA methylation are reported to occur. Studies have shown that mice heterozygous for PTEN develop spontaneous tumors (4-8), and that conventional heterozygous Pten deletion leads to tumors in the affected tissues (4,5,9-11). In addition, conditional deletion of both alleles of Pten significantly reduces the latency of prostate intraepithelial neoplasia (PIN) lesion development and further progression to localized adenocarcinoma followed by metastasis (4-6). Therefore, the onset and progression of prostate cancer are PTEN dosage-dependent (4).

Similar to the majority of human prostate cancers, Pten null murine prostate cancer initially regress in response to androgen ablation therapy, but subsequently relapse and proliferate in the absence of androgens (5,12,13). In addition, it has been demonstrated that the overexpression of PTEN in PTEN-negative PC3 cells by transfection reduces cell proliferation and increases the pro-apoptotic effects of chemotherapeutic agents (14,15). In humans, this tumor-suppressor gene results inactivated in ~30% of primary prostate cancers and in ~60% of metastatic prostate tumors (16-20). The results showed by Bedolla and cooworkers indicate that, although PTEN by itself is not a good predictor of PSA recurrence, loss of PTEN expression, in combination with increased Gleason scores and Akt phosphorylation, is an
even better predictor of biochemical recurrence than Akt by itself (21). In addition El Sheikh and co-workers (22) demonstrated that patients negative for PTEN and AR had a poor prognosis respect to patients positive for both markers. Results from several in vitro studies using established PCA cell lines have demonstrated that PTEN and Akt are, respectively, negative and positive modulators of AR transcriptional activity (6, reviewed in ref. 23). Under experimental conditions, PTEN and AR exert opposite effects on cell growth and apoptosis.

We previously demonstrated that the in vivo androgen ablation therapy can reinforce the PI3K/Akt pathway through several mechanisms including the increased PTEN loss and EGFR/Her2 increased expression/activity (24). Activated Akt, in turn, mediates antiapoptotic signalling through the inactivation of a multitude of downstream targets involved in apoptosis regulation. Blockade of the PI3K/Akt pathway leads to apoptosis of PTEN-deficient cancer cells (24-26). Consequently, the PI3K/Akt pathway is currently a major therapeutic target (26) for treatment of cancer.

Interestingly, some malignancies exhibit undetectable PTEN protein without mutations or loss of PTEN mRNA. The cause(s) for this reduction in PTEN is unknown. To understand the mechanisms of PTEN lost we analyzed the expression levels of PTEN in a panel of 50 prostate cancer primary cultures. Our results indicated that PTEN inactivation is both a genetic (mutations or PTEN gene deletion) and an epigenetic (gene methylation and protein ubiquitination and degradation) event. The first seems to be independent to antiandrogenic therapy whereas the second is increased after antiandrogenic therapy.

**Materials and methods**

**Reagents and cell lines.** All the materials for tissue culture were purchased from Hyclone (Cramlington, NE, USA). Plasticware was obtained from Nunc (Roskilde, Denmark). Azacitidine (Vidaza®) was obtained in collaboration with Celgene Corporation (Summit, NJ, USA). Valproic acid, R1130 and 17AAG, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against PTEN, Akt and phosphor Akt were purchased from Santa Cruz (Santa Cruz, CA, USA). Anti-DNMT1, DNMT3a and DNMT3b antibodies were purchased from Biocarta (Hamburg, Germany). We used the aggressive prostate cancer model, 22rv1 (27) which was obtained from German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and grown as recommended.

**Primary tumor cultures.** The primary tumor cultures of PCa were established from specimens of consenting patients undergoing radical prostatectomy. A wedge-shaped specimen of the fresh prostate was removed within 1 h of surgery. Frozen sections of a part of this tissue were used to confirm the prostatic origin and for diagnosis. We analysed a 50-patient cohort with clinically localized tumors that were surgically treated with RP as described (24,28). Among these, 23 patients received RP as initial treatment, whereas the other 27 patients received NHT for 4 months based on casodex treatment. Tissue samples were minced and cultured in DMEM as previously described (29).

**Growth assay.** For pharmacological treatments, primary cultures and cell lines were seeded at a density of 2x10^4 cells per dish in 50 mm petri dishes. Cells were left to attach and grow in 5% FCS DMEM for 24 h. All other cells were treated with 1.0 or 10.0 μM bicalutamide or 10^{-12} M DHT. Cells trypsinized and resuspended in 20 ml saline were counted by a hemocytometer every 48 h (LabRecyclers, Gaithersburg, MD, USA) and 5 independent counts were performed for each dish. All experiments were conducted in triplicate.

**Apoptosis.** Apoptosis was quantified as the percentage of cells with hypodiploid DNA assessed using HT titer TACS assay kit, a colorimetric quantitative assay for the detection of apoptosis (Trevigen, Gaithersburg, MD).

**Preparation of cell lysates and Western blot analysis.** Cells were seeded in 24-well tissue culture plates (Costar, Corning, NY) at 20,000 cells per well. At 24 h after seeding, cells were treated with azacitidine (final concentration of 1.0 mM). After 72 h of drug exposure, cells were washed with cold PBS and immediately lysed with 1 ml lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM sodium orthovanadate, 30 mM p-nitrophenyl phosphate, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and 10 μg/ml leupeptin). Lysates were electrophoresed in SDS-PAGE, and separated proteins were transferred to nitrocellulose and probed with the appropriate antibodies using the conditions recommended by the suppliers.

**RT-PCR for PTEN expression.** Total RNA was extracted from cells with TRIzol reagent according to the manufacturer's instruction. An RT-PCR was carried out on Dnase Amp Grade (Gibco-BRL)-treated RNA using M-MuLV RT (50 units) in 100 mM Tris-HCl pH 8.3, 500 mM KCl, 5 mM MgCl2, 0.5 μM each dNTP, 1 unit of RNasin, 500 pmol of random examer primers. Two microliters of cDNA were amplified in a 50 μl reaction volume containing 0.5 μM of sense and antisense primers, 2.5 units of TaqDNA polymerase (Applied Biosystems, Milan, Italy), 200 μM each dNTP and 1.5 mM MgCl2. A co-amplification of GAPDH was performed. Primers sequences used for PCR were: PTEN 3th exon Fw 5'-ATATTTCTCTGAAAAGAGTCTGG-3', PTEN Rev 5'-TTATCGTTAGGAATCAA-3', GAPDH Fw 5'-CACCATTGGGAAAAAGGCGCGG-3' and GAPDH Rev 5'-GACGGACACATTGGGGGTAG-3'. After amplification, 20 μl of the PCR reaction mixture was analysed by 1.2% agarose gel electrophoresis and stained with ethidium bromide.

**Statistical analysis.** Statistical analysis was performed using SPSS 11.0 (SPSS, Inc., Chicago, IL) software. All P-values <0.05 were considered to indicate significance. All statistical tests were two-tailed. Continuous variables were analyzed using an unpaired Student’s t-test. Differences among categorical variables were compared with Chi-square test. When multiple comparisons were performed the Tukey test was used.
Results

PTEN expression and effects of the DNA methyltransferase inhibitor, azacitidine. We analyzed the expression levels of PTEN, both as protein and mRNA, in a panel of 50 prostate cancer primary cultures. We observed that 27/50 (54%) and 34/50 (68%) primary cultures displayed detectable levels of PTEN protein and mRNA, respectively. After treatment with 1 mM azacitidine for 48 h, although PTEN protein expression was significantly increased in all PTEN-positive cultures, 14/23 (60.7%) cultures with low levels of PTEN protein and 10/16 (62.5%) cultures with low/undetectable mRNA levels resulted once again PTEN-negative. In Fig. 1A and B we show the effects of azacitidine 1 mM in representative cultures: 14/50 (28%) and 10/50 (20%) of primary cultures were again PTEN-negative for protein and mRNA expression, respectively.

Effects of the pan histone deacetylase inhibitor, valproic acid. Considering that in addition to DNA methylation, also histone modifications are involved in gene expression we evaluated the effects of non-toxic concentration of the histone deacetyltransferase inhibitor, valproic acid (VPA, 1 mM) for 48 h. We observed that this treatment increased PTEN protein expression. In Fig. 2A we show the effects of VPA 1 mM in representative cultures. We observed that VPA increased PTEN expression (Fig. 2B). The effects of VPA were statistically significant only when we consider protein determinations (P=0.036). Further we observed that the treatment with 1 mM VPA decreased the expression and increased the degradation of DNMT1 in all cultures. No changes in DNMT3a and DNMT3b expression were observed (data not shown). In Fig. 2C we show the time-dependent effect in a representative primary culture. Since pan HDAC inhibition with VPA could increased HDAC-6/HSP90- and proteasome-dependent PTEN degradation we analyzed the effects of the proteasome inhibitor, MG132 (1.0 μM) and HSP90 inhibitor, R1130 (0.1 μM). We demonstrated that these agents was able...
to maintain elevated the basal levels of PTEN protein in primary cultures and in 22rv1 cell line. Cells were seeded in 24-well tissue culture plates at 20,000 cells per well. At 24 h after seeding, cells were treated with VPA alone or in combination with R1130 or MG132. After 72 h of drug exposure, cells were washed with cold PBS and immediately lysed with 1 ml lysis buffer. Lysates were electrophoresed in SDS-PAGE, and separated proteins transferred to nitrocellulose and probed with the appropriate antibodies using the conditions recommended by the suppliers. Each lane show the PTEN protein expression from 40 μg of protein.

Figure 3. Effects of 0.1 μM R1130 (HSP-90 inhibitor), 1.0 μM MG132 (proteasome inhibitor) alone or in combination with 1.0 mM VPA on three representative primary cultures and in 22rv1 cell line. Cells were seeded in 24-well tissue culture plates at 20,000 cells per well. At 24 h after seeding, cells were treated with VPA alone or in combination with R1130 or MG132.

Figure 4. Effects of 1.0 mM valproic acid (VPA) on mRNA and protein PTEN expression in cultures derived from untreated (UNT) or bicalutamide-treated patients in neoadjuvant regimen (NHT). (A) Summary of results showing the significant of comparisons performed by Chi-square test. (B) Densitometric analysis of gels and definition of arbitrary densitometric units as the densitometric values of PTEN compared vs. a standard extract obtained from 22rv1 cells and statistical evaluations using Tukey’s all pairs comparison analysis. d vs. a, Mean difference of 0.914815, |q| of 4.701, P=0.0068 and 95% CL ranged between 0.19526 and 1.4961. b vs. c, Mean differences of 1.34873, |q| of 2.24775, P=0.0025 and 95% CL ranged between 0.19526 and 1.4961.

Figure 5. Effects of 1.0 mM valproic acid (VPA) on mRNA and protein PTEN expression in cultures derived from untreated (UNT) or bicalutamide-treated patients in neoadjuvant regimen (NHT). (A) Summary of results showing the significativity of comparisons performed by Chi-square test. (B) Densitometric analysis of gels and definition of arbitrary densitometric units as the densitometric values of PTEN compared vs. a standard extract obtained from 22rv1 cells and statistical evaluations using Tukey’s all pairs comparison analysis. d vs. a, Mean difference of 2.144561, |q| of 11.424, P=0.0011 and 95% CL ranged between 0.24371 and 2.4775. d vs b, Mean differences of 1.543452, |q| of 7.856, P=0.0025 and 95% CL ranged between 0.19526 and 1.4961. c vs. a, Mean differences of 0.914815, |q| of 4.701, P=0.0068 and 95% CL ranged between 0.19526 and 1.4961.

PTEN expression and effects of neoadjuvant hormone bicalutamide-based therapy. Grouping prostate cancer cell primary cultures in those derived from naive prostate cancer patients (23 pts) and those derived from neoadjuvant hormone therapy (NHT)-treated patients (27 pts) we found that PTEN protein loss (Fig. 4A) were higher in the later group (17/27 (63%) vs. 6/23 (26.1%), P=0.020). Parallel 5/23 (21.7%) cultures from untreated patient group resulted negative in RT-PCR determination whereas 11/27 (40.7%) cultures derived from NHT patient group were negative for mRNA. Thus we performed a densitometric analysis and defined arbitrary densitometric units as the densitometric values of PTEN compared vs. a standard extract obtained from 22rv1...
cells. However, the densitometric amount of PTEN measured in untreated (UNT) patient group was not statistically higher (P=0.0616) when compared to NHT group (Fig. 4B). After treatment with 1 mM azacitidine (Fig. 1), although PTEN expression was significantly increased in all PTEN-positive cultures, 4/6 (66.7%) and 2/5 (40%) cultures with low levels of PTEN protein and mRNA resulted once again PTEN-negative in UNT group whereas 12/17 (70.6%) and 8/11 (72.7%) cultures with low levels of PTEN protein and mRNA resulted once again PTEN-negative in NHT group. The percentage of PTEN-positive cultures after azacitidine treatment was not significant comparing UNT and NHT groups (Fig. 4A) although in NHT group the percentage of PTEN-negative cultures was almost halved (63 vs. 37%). However, when we analyzed the densitometric amount of PTEN protein we found that PTEN expression was statistically higher both in UNT (P=0.035) and NHT (P<0.001) group.

**Androgen modulation by PTEN expression.** To obtain further demonstration of the role of anti-hormone therapy in the modulation of PTEN expression we cultured the PTEN- and AR-positive prostate cancer cell line, 22rv1, in presence or not of DHT or in presence of bicalutamide (casodex). In Fig. 6 we demonstrated that during the prolonged treatment with 1 and 10 μM bicalutamide the antiproliferative (Fig. 6A) and pro-apoptotic (Fig. 6B) effects of bicalutamide were significantly reduced and after 60 days of cultures 22rv1 cells were insensitive to bicalutamide. Parallely, bicalutamide significantly down-modulated PTEN expression in a time-dependent manner (Fig. 6C). As control, 22rv1 cells were cultured in presence of physiological concentrations of DHT. In these condition the expression of PTEN was increased. This is also associated to increased phosphorylation (and thus activation) of Akt.

**Discussion**

The behavior of many neoplasms appears to be correlated with the expression of positive (worse prognosis) or negative (better prognosis) regulators of the PI3K/Akt signaling pathway. PTEN has been found deleted in a substantial fraction of cancers. Amplification and overexpression of Akt is frequently observed in solid cancer including prostate cancer (30-33). Moreover, recent studies suggest that the dosage of PTEN in prostatic lesions is directly correlated to PCa progression, incidence, and overall biology (5). Strengthening this argument are other studies stating that prolonged androgen-ablation therapy can lead to heightened levels of PI3K-signaling activity in PCa cells (24,34). Consequently, efforts to regulate signals transduced through the PI3K pathway may provide therapeutic insight regarding progression and control of the disease. We have previously demonstrated that in vitro and in LuCaP35 xenograft in vivo experiments PTEN levels were regulated by androgen/antiandrogens (24). In vitro experiments performed on PTEN-positive AR-positive PCa cell lines indicated that PTEN expression was maintained at elevated levels in long-term androgen treatment whereas these levels were very low after long-term treatment with bicalutamide. The concomitant increase in the EGFR and Her2 expression synergizes with the reduction of PTEN in the upmodulation of Akt activity. Further we have previously demonstrated that the EGFR inhibitor, gefitinib, is able to synergize with BCLT in AR-positive cell lines in vitro (35,36) and that effectiveness of this treatment regimens was modulated by PTEN/Akt activity. In addition we have also demonstrated that inhibition of PI3K-dependent signaling alone induces proliferative arrest (26). The modulation of DNA methyltrasferase as well as histone acetylation/ deacetylation activities represents a central mechanism for the control of gene expression and we observed in this study that the demethylating agent 5-azacitidine increases the expression of PTEN in PTEN-positive prostate cancer cells and induces the expression of PTEN in ~50% of PTEN-negative prostate cancer cells. The induction of PTEN
expression, considering the mean difference in a 95% confidence limit by Tukey's all-pairs comparison, is statistically higher in prostate cancer primary cultures derived from patients who undergone neoadjuvant bicalutamide-based anti-hormone therapy respect to those observed in cultured derived from untreated patients suggesting that increased DNMT activity can be observed during bicalutamide treatment. We observed that DNMT1, DNMT3a and DNMT3b (unpublished data) expression was increased in tumors after NHT and in AR- and PTEN-positive 22rv1 cells in vitro during bicalutamide treatment.

In addition we observed also that the pan HDAC inhibitor, valproic acid (VPA) increased PTEN protein expression only in true PTEN-positive cultures. Also in this case the induction of PTEN expression is statistically higher in prostate cancer primary cultures derived from patients who undergone neoadjuvant bicalutamide-based anti-hormone therapy respect to those observed in cultured derived from untreated patients suggesting that increased HDAC activity can be observed during bicalutamide treatment. The induction of PTEN by VPA could potentize chemotherapy efficacy in prostate cancer after HRP development and this is in agreement with a recent study in which histone deacetylase inhibitor, trichostatin potentiates doxorubicin-induced apoptosis by upregulating PTEN expression (37). We demonstrated also that the inhibition of the proteasome with 17 AAG was able to maintain elevated the basal levels of PTEN protein showing additive effects with FK228 but not with VPA. It has been recently demonstrated that PTEN ubiquitization/deubiquitylation and intracellular trafficking modulate its function (38). Our results indicated that PTEN inactivation in prostate cancer is both a genetic (mutations or PTEN gene deletion) and an epigenetic (gene methylation and protein ubiquitization and degradation) event. The first seems is independent to androgenic therapy whereas the second is increased after the antiandrogenic therapy. We observed that DNMT1, DNMT3a and protein ubiquitinization and degradation event. The first is increased after the antiandrogenic therapy with bicalutamide whereas the second is increased after the antiandrogenic therapy with flutamide. The induction of PTEN by FK228 seems is independent to antiandrogenic therapy whereas the second is increased after the antiandrogenic therapy with flutamide. The induction of PTEN by FK228 seems is independent to antiandrogenic therapy whereas the second is increased after the antiandrogenic therapy.

References


