

ORIGINAL ARTICLE

Differential regulation of *PTEN* expression by androgen receptor in prostate and breast cancersY Wang¹, T Romigh¹, X He¹, M-H Tan¹, MS Orloff^{1,2}, RH Silverman^{2,3,4}, WD Heston^{2,3,4} and C Eng^{1,2,4,5,6}¹Genomic Medicine Institute, Lerner Research Institute, Cleveland Clinic, Cleveland, OH, USA; ²Taussig Cancer Institute, Cleveland Clinic, Cleveland, OH, USA; ³Department of Cancer Biology, Lerner Research Institute, Cleveland Clinic, Cleveland, OH, USA; ⁴CASE Comprehensive Cancer Center, Case Western Reserve University School of Medicine, Cleveland, OH, USA; ⁵Stanley Shalom Zielony Institute for Nursing Excellence, Cleveland Clinic, Cleveland, OH, USA and ⁶Department of Genetics, Case Western Reserve University School of Medicine, Cleveland, OH, USA

Prostate cancer and breast cancer are the most common malignancies in the western world. Androgen receptor (AR) and *PTEN* both have been well documented to have important roles in prostate carcinogenesis. In contrast, AR and *PTEN* in breast carcinogenesis have not been well studied. Furthermore, the crosstalk and connection between those two pathways remain unclear. Increased AR expression in prostate cancers, combined with decreased *PTEN* expression, portends a poor clinical outcome. Paradoxically, both high AR and high *PTEN* levels, detected by immunohistochemistry, in primary breast carcinomas have been associated with better disease-free survival. Here, we performed *in silico* analysis of publicly available microarray data sets from prostate or breast carcinomas. We found an inverse correlation between AR and *PTEN* transcript expression in prostate cancer tissues in contrast to the positive correlation in breast cancer. These data led us to hypothesize that AR may directly affect *PTEN* transcriptional regulation in prostate and breast cancer cells. Here, we show for the first time that AR inhibits *PTEN* transcription in prostate cancer cells, whereas AR upregulates *PTEN* transcription in breast cancer cells, which mechanistically explains both the immunohistochemical *PTEN*–AR expressional data noted in clinical trials and in our *in silico* analysis of the transcriptomes of breast and prostate cancers. In addition, we have fine-mapped the AR-binding motif within the *PTEN* promoter. Here we show that, in patients with Cowden syndrome, an inherited cancer syndrome caused by germline mutations scattered throughout *PTEN*, point variants affecting the 3' end of the AR-binding motif result in abrogation of androgen-mediated transcriptional regulation of *PTEN* expression. We may speculate that the differential AR effect on *PTEN* may begin to explain organ-specific and perhaps sex-specific neoplasia predisposition in Cowden syndrome, as well as why only a fraction of women with germline *PTEN* mutations develop

breast cancer, depending on the androgen steroid milieu and levels.

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Introduction

Androgen receptor (AR) belongs to the nuclear receptor superfamily and can function as a transcription factor. Presence of ligand, such as dihydrotestosterone (DHT), induces AR phosphorylation and a conformational change, which results in its nuclear translocation and target gene regulation. Androgen and the functional status of AR are important mediators of prostate cancer progression. Amplification of AR is often observed in advanced prostate cancer (Visakorpi *et al.*, 1995). Teleologically, this provides prostate cancer cells with a potential survival advantage under low androgen levels after androgen ablation treatment and, therefore, leads to progression to hormone-refractory disease. In females, AR regulates the development of the female reproductive tract, bone, kidneys and muscle. The ovaries stop making androgens after menopause, at which time a large percentage of female breast cancers are diagnosed. At least one report suggested that AR mediates an inhibitory effect on breast cancer cell proliferation through induction of apoptosis (Birrell *et al.*, 1995). In contrast to worse outcome in prostate cancer patients, expression of AR, detected by immunohistochemistry, has been found to be significantly associated with better survival among breast cancer patients (Agoff *et al.*, 2003; Ogawa *et al.*, 2008). Accordingly, androgens such as fluoxymesterone have been used as second-line hormonal therapy for advanced breast cancers, whereas most prostate cancer patients are treated with androgen antagonists to inhibit AR.

The tumor suppressor gene *PTEN*, located on chromosome 10q23, is one of the most frequently altered genes in a broad variety of human cancers

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(Eng, 2003). Through its phospholipid 3-phosphatase activity, *PTEN* negatively regulates the (PI3K)/Akt pathway, which is involved in cell proliferation, migration and apoptosis. Both monoallelic and biallelic deletions of *PTEN* occur in prostate cancer cell lines and non-cultured human prostate tumor specimens (Wang *et al.*, 1998; Bedolla *et al.*, 2007; Sircar *et al.*, 2009). Germline and somatic alterations of *PTEN* also have important roles in breast cancers (Zbuk and Eng, 2007). Germline *PTEN* mutations were first identified in Cowden syndrome (CS), which confers a high risk of breast, thyroid and other cancers (Liaw *et al.*, 1997). In fact, female CS patients with germline *PTEN* mutations have a significantly higher risk of breast cancers than those without identifiable mutations (Marsh *et al.*, 1998). Approximately, 85% of classic CS patients and 10% of Cowden-like (CSL) patients have germline *PTEN* mutations, with the most characterized ones being intragenic. Although proven pathogenic mutations in the promoter have been described (Zhou *et al.*, 2003b; Teresi *et al.*, 2007), there are many more 5'-UTR variations that are not well understood.

In both heritable and sporadic cancers, when *PTEN* is absent or dysfunctional, Akt phosphorylation and activity are significantly increased *in vitro* and *in vivo* (Stambolic *et al.*, 1998; Zhou *et al.*, 2003a; Agrawal *et al.*, 2005). *PTEN* inhibits phosphorylation of Akt, which, in turn, stimulates AR phosphorylation and activity in prostate cancers (Wen *et al.*, 2000). In addition, *PTEN* also directly interacts with the AR DNA-binding domain/Hinge domain and inhibits AR nuclear translocation and AR-mediated transcriptional activity in prostate cancer cells (Lin *et al.*, 2004). However, it is still unclear whether the converse, AR regulates *PTEN*, occurs, and if so, whether the AR-*PTEN* crosstalk is similar or different in prostate versus breast cancer models. Therefore, we sought to determine if and how AR regulates *PTEN*, addressing the hypothesis that AR directly regulates *PTEN* at the transcriptional level through an AR response element (ARE) in both breast and prostate cancer cells and in the Cowden-inherited cancer syndrome.

Results

AR-mediated PTEN transcriptional repression in prostate cancer cells

Amplification of AR and loss of *PTEN* protein expression during development and progression of prostate cancer suggest potential regulation between AR and *PTEN* at several possible levels, including transcriptional, translational or post-translational level. To begin to explore which of these mechanisms are involved, we investigated the correlation between increased AR and decreased *PTEN* using publicly available whole genome transcriptome data sets utilizing prostate carcinomas derived from patients. A search through the Gene Expression Omnibus was performed for prostate cancer samples and a total of seven data sets (345 patients) were identified for meta-analysis. The forest plot showed that the transcripts of *AR* and *PTEN*

in prostate carcinomas are clearly inversely correlated (correlation coefficient -0.291 , with the upper limit being -0.171 and the lower limit being -0.401 ; Figure 1a). Therefore, on the basis of these observations, we hypothesized that the decreased *PTEN* expression observed clinically might be a direct result of AR transcriptional regulation.

Before investigating whether AR regulates *PTEN*, we exposed the androgen-dependent prostate cancer cell line LNCaP and its androgen-independent subclone C4-2 cells to DHT, an AR ligand, or Casodex (also called bicalutamide), an AR antagonist used clinically to treat androgen-dependent prostate cancer. We transfected the relevant cells with a reporter plasmid containing a luciferase reporter, driven by a promoter comprising six copies of AREs. For both cell lines, the luciferase assay demonstrated that DHT stimulated AR transcriptional activity, whereas Casodex significantly decreased its transcriptional activity (Figure 1b).

To elucidate whether AR directly regulates *PTEN* expression in prostate cancer cells, AR-positive LNCaP and C4-2 cells, and AR-negative PC3 cells were transfected with *PTEN*-promoter luciferase reporter constructs and treated with DHT or Casodex. Reporter constructs included a full-length (-1344 to -1) and truncated (-1344 to -1001) *PTEN* promoter (Teresi *et al.*, 2008). Both *PTEN* promoters, the full-length as well as the -1344 to -1001 segments, could be inhibited by DHT and activated by Casodex in AR-positive LNCaP and C4-2 cells (Figure 1c). In contrast, neither *PTEN* promoter constructs responded to DHT or Casodex in PC3 cells (Figure 1c, lower panel), indicating that the androgen-associated *PTEN* promoter activity is AR-dependent. We also observed this androgen effect in LAPC4 cells (Supplementary Figure 1). To further confirm the AR-mediated androgen effect on *PTEN* promoter, we knocked down AR by transfecting cells with anti-AR siRNA. AR knockdown increased *PTEN* promoter activity by 2.5-fold, but DHT or Casodex had no further effect on *PTEN* promoter activity (Figure 1d, Supplementary Figure 2). Therefore, our data here suggest that AR, which represses *PTEN* transcription, mediates androgen-induced transcriptional inhibition of *PTEN* in prostate cancer cells.

Next, we used the chromatin immunoprecipitation (ChIP) assay to investigate whether AR directly binds to the *PTEN* promoter in prostate cancer cells in order to mediate the negative regulation. AR pulldown from ChIP assays was quantified by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and normalized to the 3% input. Our data showed that AR interacts with the *PTEN* promoter in AR-positive CWR22rv1 and LAPC4, but not in AR-negative DU145 cells, and that DHT significantly promotes this interaction in the AR-positive cells (Figure 1e, upper panel). We also extracted mRNA from these three cell lines to further confirm whether DHT represses endogenous *PTEN* transcription in prostate cancer cells. qRT-PCR data showed that this DHT-induced AR binding repressed endogenous *PTEN* transcription in both CWR22rv1 and LAPC4 cells, but not in DU145 cells

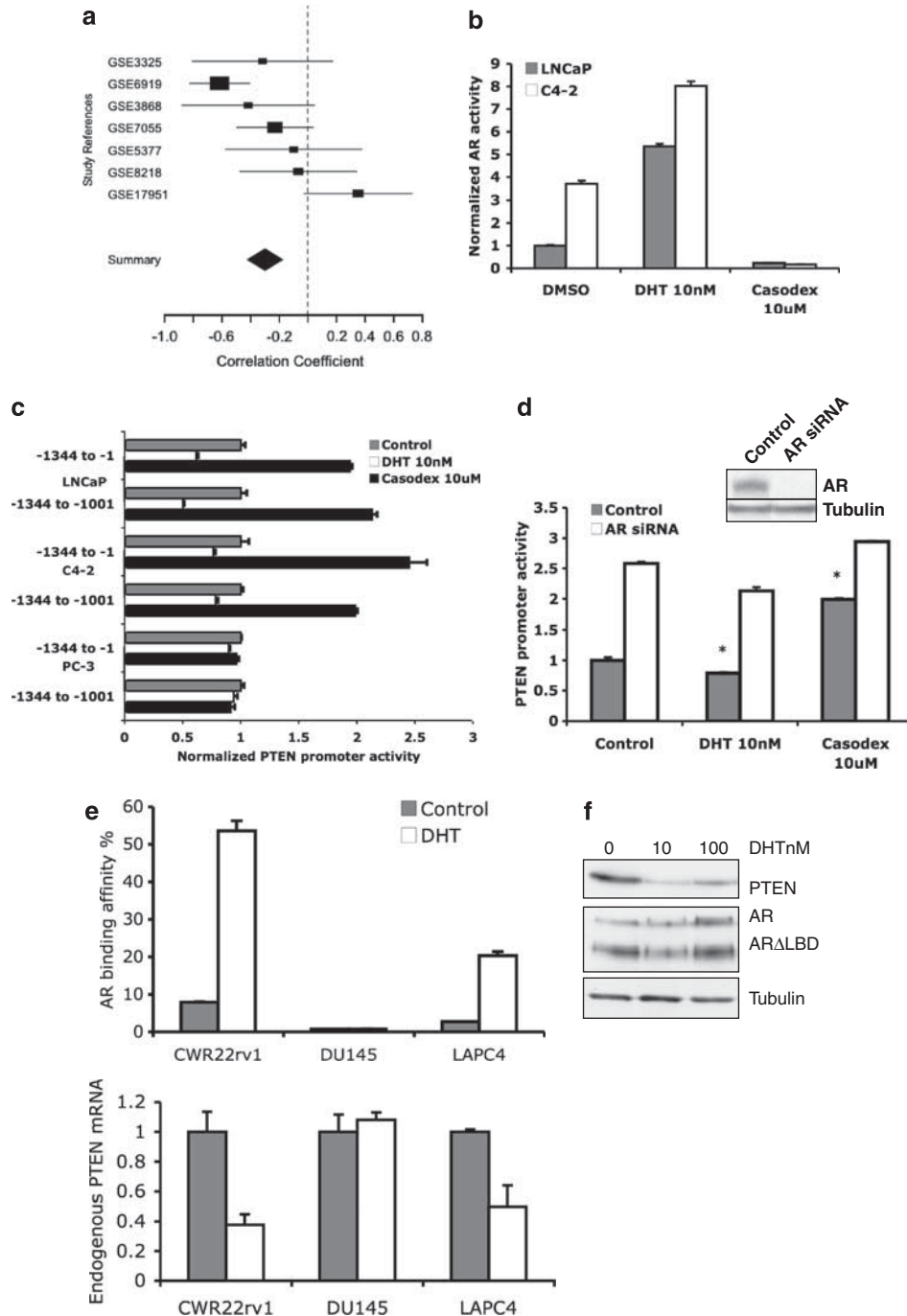


Figure 1 Androgen represses *PTEN* expression in prostate cancer tissues. (a) Forest plot showing an inverse correlation between the expression of *PTEN* and *AR* in prostate cancer tissues. The 95% confidence intervals are provided with boxes indicating the precision of the estimate. A total of eight sets of data were analyzed. The diamond represents the summary confidence interval. The summary correlation coefficient is -0.289 , with the upper limit being -0.173 and the lower limit being -0.396 . (b) LNCaP and C4-2 cells were co-transfected with plasmids expressing hARE-Luc and Renilla-Luc and treated with DMSO (control), DHT or Casodex for 48 h before lysis of cells for dual luciferase assay. All the data indicate the mean value of three independent experiments with the standard deviations shown as error bars, unless noted otherwise. (c) LNCaP, C4-2 and PC3 cells were co-transfected with *PTEN* promoter reporter plasmids and Renilla-Luc control. Luciferase assay showed *PTEN* promoter activity after 48-h treatment. (d) C4-2 cells were co-transfected with control siRNA or anti-AR siRNA and *PTEN* promoter reporter plasmids. After 48 h of treatment, *PTEN* promoter activity was measured by dual luciferase assay ($*P < 0.005$). (e) Upper panel: CWR22rv1, DU145 and LAPC4 cells were treated with control or with 10-nM DHT before being subjected to ChIP assay using anti-AR antibodies. AR pull-down from ChIP assay was quantified and normalized to 3% input in qRT-PCR by specific primers covering *PTEN* promoter. Lower panel: Total RNA was extracted from CWR22rv1, DU145 and LAPC4 cells after the same treatment for 12 h. After reverse transcription, the cDNAs were used as templates for qRT-PCR. Results show the average of three experiments using primers reflecting exon 3–4, 4–5 or 5–6 of *PTEN* unless noted otherwise. (f) CWR22rv1 cells were treated with increasing concentrations of DHT for 48 h. Western blots demonstrate *PTEN* and *AR* expression, whereas tubulin was used as control.

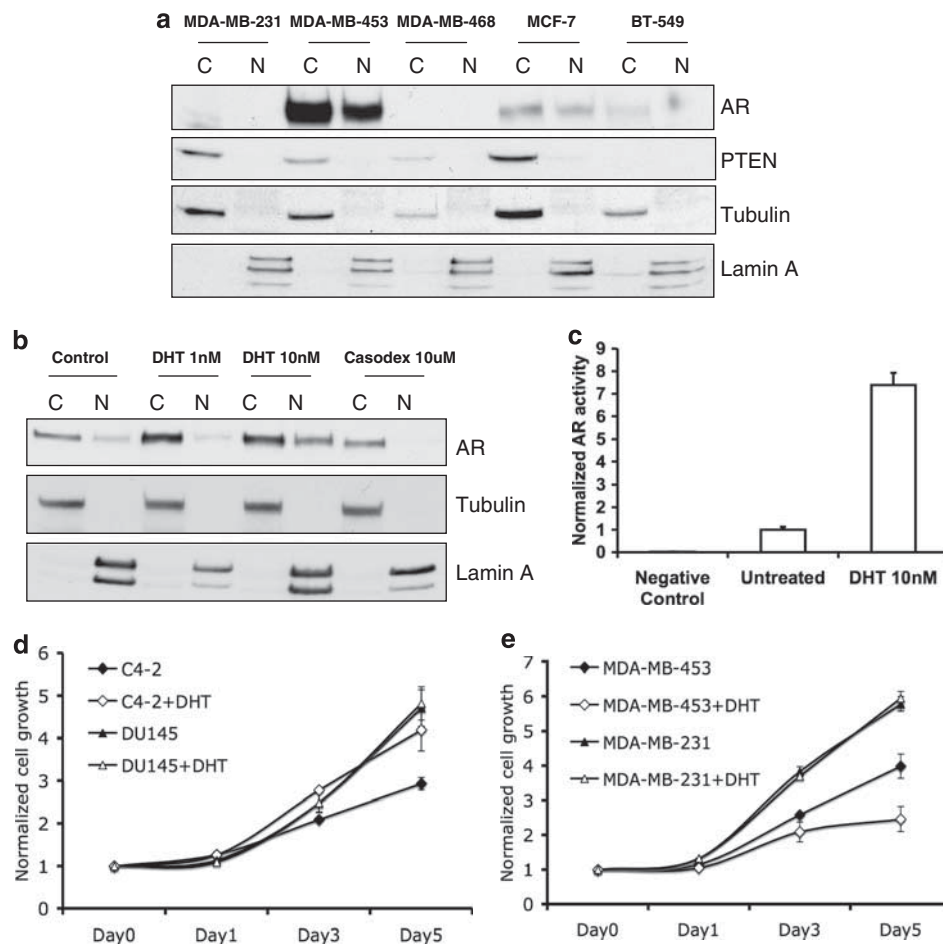


Figure 2 Androgen treatment decreases cell proliferation in breast cancer cell lines. (a) MDA-MB-231, MDA-MB-453, MDA-MB-468, MCF-7 and BT-549 breast cancer cells were subjected to subcellular fractionation. Western blots show AR expression and nuclear localization. Tubulin was used as a cytoplasmic (C) marker, and lamin A was used as a nuclear (N) marker. Note that MDA-MB-453 and MCF-7 cells do express AR. (b) MDA-MB-453 cells were treated with DHT and Casodex for 48 h before being subjected to subcellular fractionation, with western blotting revealing AR expression and nuclear localization. (c) MDA-MB-453 cell lines were treated with DMSO (control), 10-nM DHT for 48 h before lysis of cells, followed by luciferase assay. (d) Prostate cancer cell lines: C4-2 and DU145, and (e) breast cancer cell lines: MDA-MB-453 and MDA-MB-231, were treated with DMSO (control) or 10-nM DHT. Growth rates of the cells were assessed by the MTT assay, as previously described (Wang *et al.*, 2008). Note that DHT stimulated C4-2 cell proliferation, whereas the growth of MDA-MB-453 cells was inhibited by DHT. Neither DU145 nor MDA-MB-231 cells responded to DHT treatment.

(Figure 1e, lower panel). Knockdown of AR by siRNA resulted in increased endogenous *PTEN* transcript levels (Supplementary Figure 3). We then treated CWR22rv1 cells with different concentrations of DHT, which resulted in decreased protein expression (Figure 1f, Supplementary Figure 4). Our observations from this series of experiments demonstrate that AR, functioning as a transcriptional repressor, directly binds and down-regulates *PTEN* promoter activity, resulting in decreased *PTEN* mRNA, in prostate cancer cells. Thus, inhibition of AR by Casodex releases (and hence, increases) *PTEN* promoter activity, whereas the AR ligand DHT represses *PTEN* promoter activity.

Androgen stimulates *PTEN* expression through AR in breast cancer cells

Our data above showed that AR inhibits *PTEN* expression in prostate cancer cells, consistent with the

clinical observation of the inverse relationship between AR and *PTEN* expression in patients with prostate cancers. To investigate the AR pathway in breast cancer cells, we first surveyed for AR protein expression in several breast cancer cell lines. Among the cell lines expressing AR protein, MDA-MB-453 and MCF-7 cells have nuclear AR localization, indicating functional AR in these two lines (Figure 2a). Next, we treated the AR-positive MDA-MB-453 cells with DHT and Casodex. We found that DHT stabilized AR protein and induced its nuclear localization, whereas Casodex prevented AR nuclear localization (Figure 2b, Supplementary Figure 5). We then transfected the cells with ARE-luciferase plasmid combined with 10-nM DHT treatment, showing that DHT stimulated AR transcriptional activity eight-fold in MDA-MB-453 cells (Figure 2c).

To dissect the role of androgen exposure on proliferation in prostate cancer compared with breast cancer cells, AR-positive C4-2, MDA-MB-453 and

AR-negative DU145, MDA-MB-231 cells were treated with 10-nM DHT for 5 days. DHT exposure stimulated cell proliferation in AR-positive C4-2 prostate cancer cells, but not in AR-negative DU145 cells (Figure 2d, Supplementary Figure 6). Conversely, DHT inhibits cell growth in AR-positive MDA-MB-453 breast cancer cells, whereas knockdown of AR by siRNA promoted cell growth (Figure 2e, Supplementary Figure 7). The proliferation of AR-negative MDA-MB-231 cells was not affected by DHT (Figure 2e).

Clinical correlative data have shown that lower expression of AR or lower PTEN protein levels are correlated with breast cancer development and progression (Perren *et al.*, 1999; Ogawa *et al.*, 2008). To further elucidate a mechanism for this correlative observation, we again turned to whole transcriptome data sets from breast carcinomas, which were available publicly. A search through the Gene Expression Omnibus was performed for those breast cancer samples, the expression arrays of which were analyzed on Affymetrix HGU133Plus2.0 platform (GPL570). A total of 16 data sets (1180 patients) met our selection criteria and each individual data set was reviewed for meta-analysis. Forest plot showed a positive correlation between AR and PTEN transcript expression in breast cancer tissues (correlation coefficient +0.122, with the upper limit being 0.179 and the lower limit being 0.064; Figure 3a). From these data, we surmised that AR might directly effect PTEN expression at the transcriptional level.

To determine whether AR regulates PTEN in breast cancer cells, we transfected MDA-MB-453 cells with PTEN-luciferase reporter. In contrast to what we observed in prostate cancer cells, DHT increased the transcriptional activities of the PTEN promoters in the breast cancer cells (Figure 3b). ChIP assay quantified by qRT-PCR showed the interaction between AR and the PTEN promoter, and DHT significantly conferred this interaction in MDA-MB-453 cells (Figure 3c, left panel). This DHT exposure promoted AR binding that was associated with increased endogenous PTEN transcript levels in breast cancer cells, which is the converse of that observed in prostate cancer cells (Figure 3c, right panel). We also showed that DHT alone stabilized the AR, accompanied by subsequent increase in endogenous PTEN transcript and protein levels (Figure 3d, Supplementary Figure 8). The AR knockdown, which promoted breast cancer cell growth, not only decreased PTEN transcript and protein levels, but also abolished DHT-stimulated PTEN expression (Figure 3d, Supplementary Figure 8).

Previous reports have shown that p53 regulates PTEN transcription through the broad -1190 to -1157 promoter region (Stambolic *et al.*, 2001; Tang and Eng, 2006). To determine whether p53 is directly involved in AR-regulated PTEN expression, we used MDA-MB-453 cells expressing mutant p53 without transcriptional activity, compared with p53-wild-type (WT) MCF-7 cells (Figure 3e). DHT exposure resulted in similar PTEN transcriptional responses in both cell lines, suggesting that AR-regulated PTEN expression is independent of p53 (Figure 3e, right panel). Our results

here suggest that AR mediates the effect of the androgen by stimulating both PTEN transcription and protein expression in breast cancer cells.

Identification of the AR-binding element in the PTEN promoter

So far, our data show that AR can regulate both the -1344 to -1 and -1344 to -1001 regions of the PTEN promoter in prostate and breast cancer cells, suggesting that the ARE is located within the -1344 to -1001 region (Figures 1c and 3b). To identify the potential AR-binding site on the PTEN promoter, we used the ChIP assay to interrogate sequential overlapping of 100-bp segments of the PTEN promoter between -1344 and -1001. The ChIP assay data were quantified by qRT-PCR, which showed that AR had a high affinity to the -1244 to -1044 region of the promoter in CWR22rv1, LAPC4 and MDA-MB-453 cells (Figure 4a, Supplementary Figure 9). Our data suggest that this AR binding might also affect histone methylation of the PTEN promoter (Supplementary Figure 10). Deletion of the -1225 to -1172 region of the PTEN promoter resulted in loss of response to DHT in both the CWR22rv1 and MDA-MB-453 lines (Figure 4b), indicating that the ARE is located within the -1225 to -1172 region. Taking this observation (Figure 4b) together with the ChIP assay on AR in LAPC4 cells (Supplementary Figure 9), we may even be able to postulate that the ARE rests at the -1194 to -1172 region.

To precisely identify the ARE in this (more conservative) -1225 to -1172 region, we made a series of luciferase reporters, driven by PTEN promoters containing partially overlapping substitutions (Figure 4c, Sub A to G). C4-2 and MDA-MB-453 cells were transfected with each of the mutant PTEN promoter plasmids or the WT as control (Figure 4d). The mutant promoters Sub E, F and G (substitution from -1177 to -1152) showed significantly decreased baseline transcriptional activity in both cell lines, indicating the importance of this region for PTEN transcription (Figure 4d). In C4-2 prostate cancer cells, DHT decreased PTEN promoter activity for the control and each of the substituted promoters, except for Sub D (-1189 to -1178) and Sub E (-1177 to -1166) promoter regions, which demonstrate the least response to DHT (Figure 4d, left panel). The mean relative response ratios comparing DHT exposure with control were 0.9 for Sub D and 1.1 for Sub E, compared with 0.6–0.75 for WT and the Subs A–C constructs (Figure 4d, left 2 panels). From this experiment, the maximal ARE region would span from -1189 to -1166. In MDA-MB-453 cells, the control and most mutant PTEN promoters responded to DHT by increasing transcriptional activity, except for the promoter constructs Sub D (-1189 to -1178) and possibly Sub E (Figure 4d, right panels). It is important to note that Sub C overlaps with Sub D from -1189 to -1186, yet the response of Sub C to DHT is no different from the WT construct (Figure 4d, right panels). Although the base-

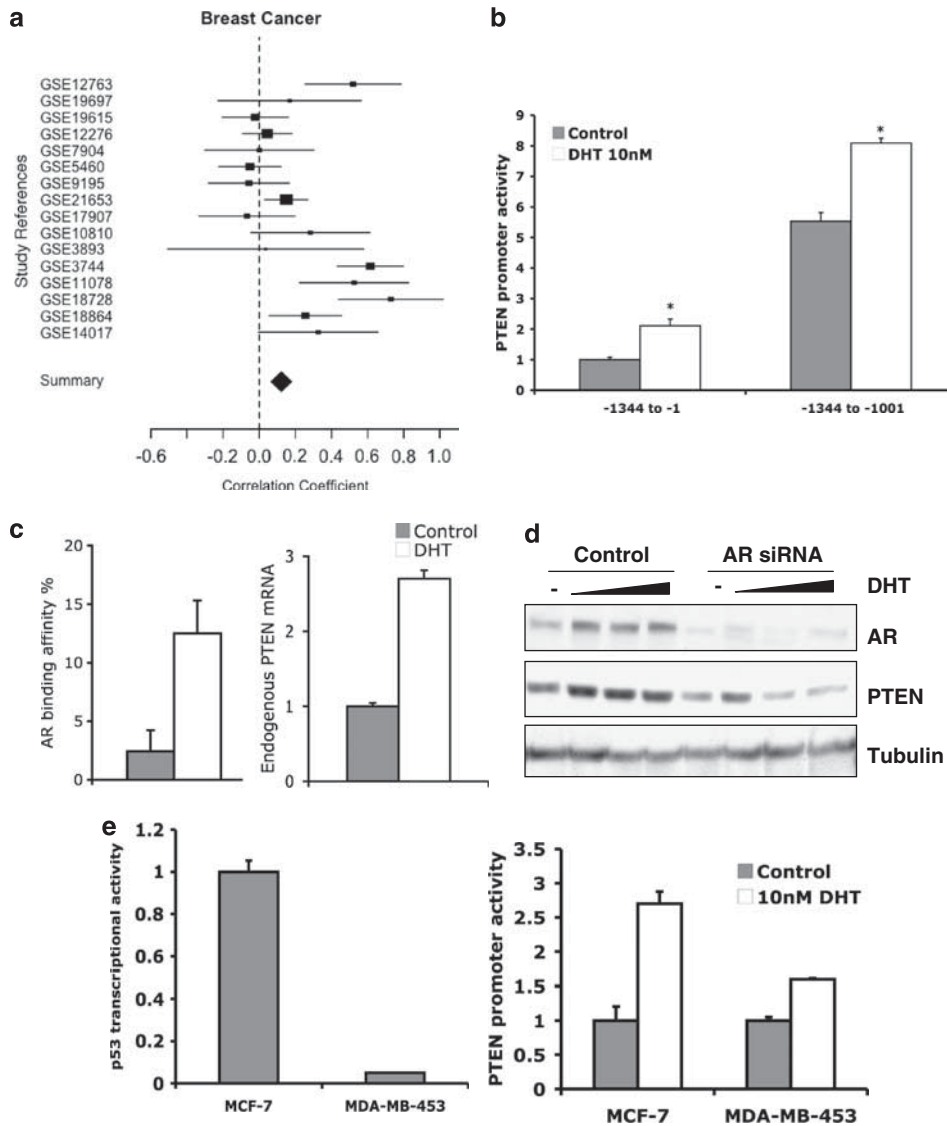


Figure 3 AR mediates androgen-stimulated *PTEN* expression in breast cancer cell lines. **(a)** Forest plots showing a positive correlation between the expression of *PTEN* and AR in breast cancer tissues. The 95% confidence intervals are provided with boxes indicating the precision of the estimate. A total of 16 data sets were analyzed. The diamond represents the summary confidence interval. The summary correlation between AR and *PTEN* expression for breast cancer is +0.122, with the upper limit being 0.179 and the lower limit being 0.064. **(b)** MDA-MB-453 cells were co-transfected with *PTEN* promoter reporter plasmids and Renilla-Luc control. Cells were treated as indicated for 48 h before *PTEN* promoter activity was assayed using a dual luciferase assay. Note that the activities of both full-length promoter and the -1344 to -1001 promoter region were stimulated by DHT ($*P < 0.001$). **(c)** Left panel: MDA-MB-453 cells were treated with control or 10-nM DHT before being subjected to ChIP assay using anti-AR antibodies. AR pull-down from ChIP assay was quantified and normalized to 3% input in qRT-PCR by specific primers covering *PTEN* promoter. Right panel: Total RNA was extracted from MDA-MB-453 cells after the same treatment for 12 h. After reverse transcription, the cDNAs were used as template for qRT-PCR. **(d)** MDA-MB-453 cells were transfected with control siRNA or anti-AR siRNA and treated with 0, 1 nM, 10 nM and 100 nM of DHT. Western blots show the expression of AR and *PTEN* proteins. Tubulin was used as a loading control. **(e)** MCF-7 and MDA-MB-453 cells were co-transfected with p53 binding element reporter plasmids and Renilla-Luc control. p53 transcriptional activity was assayed using a dual luciferase assay. MCF-7 and MDA-MB-453 cells were co-transfected with *PTEN* promoter reporter plasmids and Renilla-Luc control. Cells were treated, as indicated, for 48 h before *PTEN* promoter activity was assayed using a dual luciferase assay.

line transcriptional activity of Sub F and Sub G were depressed, both showed a response to DHT in both cell lines (Figures 4c and d). Sub E and Sub F share -1168 to -1166 region. Thus, combining the above data, a minimal ARE region within the *PTEN* promoter is -1184 to -1170 (AGTGCAGCTGCA GGC) (Figure 4c).

Germline alterations within and around the ARE in patients with CS and CSL

Although point mutations and variants can be found in the *PTEN* promoter in CS and CSL patients, small deletions appear to be less common. Nevertheless, large germline deletions and rearrangements involving the promoter including this ARE region are found in 10%

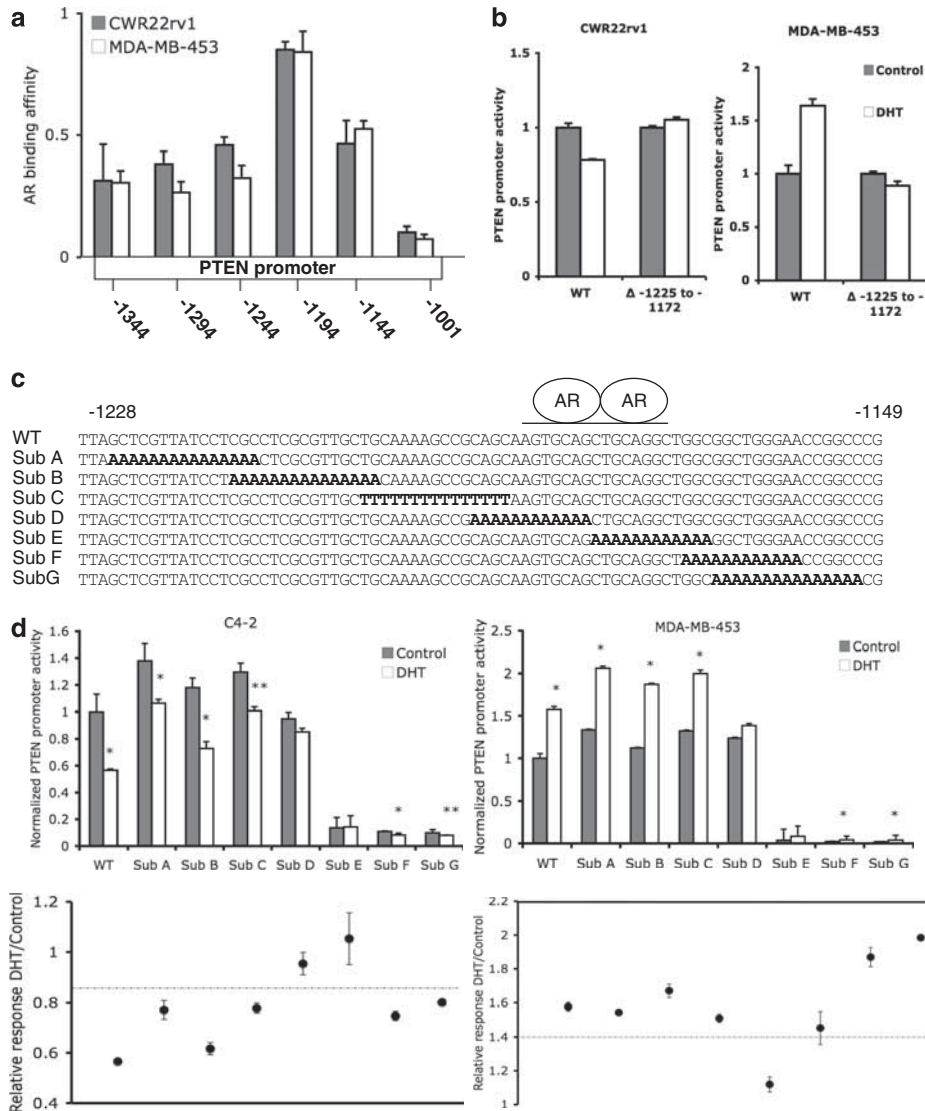


Figure 4 Identification of the AR response region in the *PTEN* promoter. (a) CWR22rv1 and MDA-MB-453 lysates were subjected to ChIP assay using anti-AR antibody. AR pull-down from ChIP assay was quantified and normalized to 3% input in qRT-PCR by specific primers covering different regions of *PTEN* promoter. (b) Cells were transfected with the *PTEN* WT or Δ-1225 to -1172 promoter reporter. Luciferase assay shows *PTEN* promoter activity after 48 h of treatment. (c) Constructs comprising each of WT and substitution *PTEN* mutants (Sub A to G). The location of the ARE is shown over the WT sequence. (d) C4-2 (left) and MDA-MB-453 (right) cells were transfected with WT or each mutant *PTEN* promoter reporter (A–G). After 48 h of treatment with 10-nM DHT, relative *PTEN* promoter activities were measured by comparing DHT treated cells with control (* $P < 0.001$, ** $P < 0.05$). The mean relative response ratio for each construct when exposed to DHT compared with control is shown in the lower panels. The dotted lines in these bottom panels represent the $P < 0.05$ significance level of mean response compared with that of the WT.

of CS/CSL patients (Zhou *et al.*, 2003b). However, we wondered whether a more limited germline deletion or point variant involving the ARE region exists. We scanned 2399 CS/CSL patient samples and found 299 (7.7%) patients who have germline variants in the *PTEN* promoter within the -1344 to -745 region (Figure 5a). Among those 299 patients, we found 11 patients with mutations/small deletions between -1225 and -1140 of the *PTEN* promoter, within or just at the ARE region that we identified above.

There were two patients with a germline heterozygous 12-bp deletion (-1199 del 12) in the *PTEN* promoter (Figure 5b). The 3' end of this deletion lies

2 bp upstream of the 5' end of the ARE. We also found three patients with germline heterozygous -1170 C>T point mutation located closest to the 3' end of the ARE region identified above (Figure 5b). We modeled these two mutants into our *PTEN*-promoter reporter constructs and used the luciferase assay to determine its transcriptional activity in response to androgen. The -1199 del 12 mutant *PTEN* promoter showed decreased baseline transcriptional activity compared with that of the control (Figure 5d). These results are consistent with the decreased *PTEN* protein levels in the patients' samples by western blot (data not shown). Interestingly, in C4-2 cells, this del-12 mutant did show

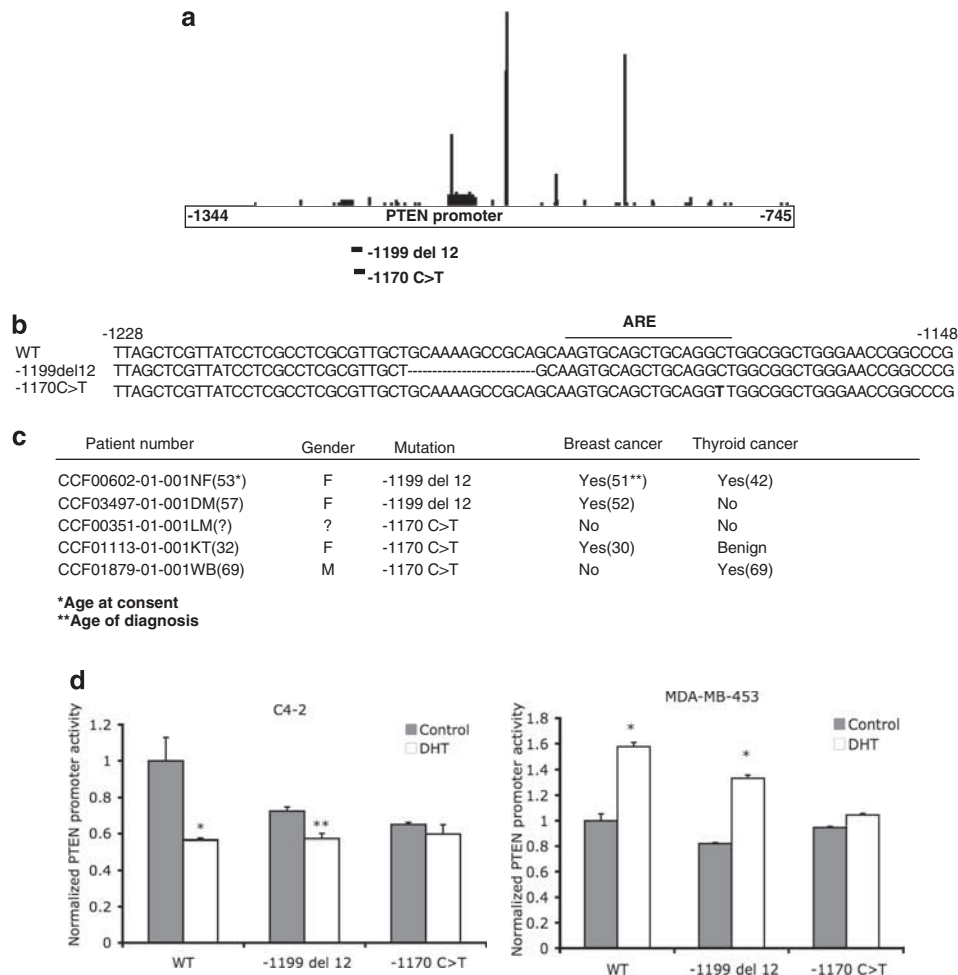


Figure 5 Germline ARE variants in patients with CS and CSL and *PTEN* response to DHT. **(a)** Among the 2399 patient samples that were screened for *PTEN* alterations, 299 have germline variants within the -1344 to -745 region. The relative frequency and location of the variants are shown on the *PTEN* promoter. The location of a 12-bp deletion mutant (identified in two patients: CCF00602-01-001NF and CCF03497-01-001DM) and a substitution variant at -1170 (identified in three patients: CCF00351-01-001LM, CCF01113-01-001KT and CCF01879-01-001WB) are shown by bars below the *PTEN* promoter. **(b)** The sequence and **(c)** phenotype of patients with -1199 del 12 and -1170 C>T mutations in *PTEN*. **(d)** C4-2 (left) and MDA-MB-453 (right) cells were transfected with mutant *PTEN* promoter reporters (-1199 del 12 or -1170 C>T) or WT *PTEN*-promoter reporter plasmids and the luciferase assay showed *PTEN* promoter activity after 48 h of treatment (* $P < 0.001$, ** $P < 0.05$).

a weakened response to DHT (normalized activity ratio for $-DHT/+DHT = 1.67$ for the WT construct, compared with 1.25 for the del 12 construct; Figure 4d, left panel). In contrast, DHT response in the del 12 mutant did not differ from the WT construct in the MDA-MB-453 cells. Of note, the -1170 C>T *PTEN* promoter was unable to respond to DHT in both C4-2 ($P = 0.141$) and MDA-MB-453 cells ($P = 0.298$; Figure 5d). In addition, the -1170 C>T mutant showed decreased baseline transcriptional activity in C4-2 cell (Figure 5d, left panel), whereas this baseline transcriptional activity did not change in MDA-MB-453 cells (Figure 5d, right panel).

Discussion

There exist ample data showing that the *PTEN*/Akt pathway can regulate AR phosphorylation, activity and

degradation (Manin *et al.*, 2002; Nan *et al.*, 2003; Lin *et al.*, 2004; Ghosh *et al.*, 2005; Wang *et al.*, 2007; Mikhailova *et al.*, 2008). However, it was not known whether AR could regulate *PTEN*. Thus, we set out to address the hypothesis that the feedback loop can be closed, such that AR can regulate *PTEN*. Here, we have shown that androgen regulates *PTEN* transcriptional expression, through AR, in both prostate and breast cancer cells, but in opposite directions (Figure 6). In prostate cancer cells, AR binds the *PTEN* promoter as a repressor, inhibiting its transcription. In contrast, AR stimulates *PTEN* gene expression as an activator in breast cancer cells. Therefore, it mechanistically explains both the differential correlations between the expression of AR and *PTEN* that we found in prostate and breast cancer tissues by *in silico* analysis of microarray expressional data and the observations between *PTEN* and AR protein levels in the clinical trial setting. In addition, we identified the ARE in the *PTEN* promoter,

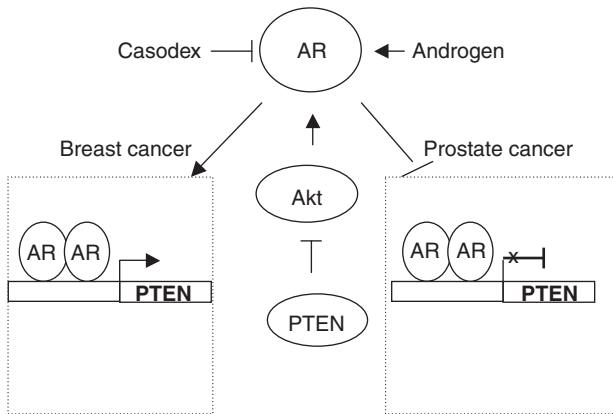


Figure 6 Schematic depiction of a proposed feedback loop between AR and PTEN. In breast cancer cells, AR activates the *PTEN* promoter and stimulates its expression. In prostate cancer, by contrast, AR represses *PTEN* transcription, which in turn results in higher PTEN expression.

through which AR regulates *PTEN* expression in both prostate and breast cancer cells. Our data indicate a new mechanism that steroid nuclear receptors can directly regulate the PTEN cell-signaling pathway in cancer cells. It also provides a novel mechanism, whereby androgen/AR mediates opposing effects in prostate and breast cancers.

It has been well documented that increased AR expression correlates with prostate cancer progression (Visakorpi *et al.*, 1995). Other reports also show that immunohistochemical loss of PTEN protein expression is associated with poorer clinical outcome for prostate cancer patients (Whang *et al.*, 1998; Bedolla *et al.*, 2007). In contrast, clinical reports associate decreased AR expression, by immunohistochemistry, with breast cancer progression (Agoff *et al.*, 2003; Ogawa *et al.*, 2008). Independently, PTEN-null status by immunohistochemistry in breast cancers is associated with poorer outcome and with a higher frequency of metastatic disease (Perren *et al.*, 1999). Our *in silico* analysis using 1180 patients' breast cancer samples from 16 data sets confirm this, and our functional work provides, for the first time, plausible mechanisms for these clinical correlative observations. Breast cancer is often diagnosed after menopause when the ovaries stop producing androgens. Our data suggest that changes in the androgenic hormonal milieu may modulate PTEN expression and lead to hormone-related neoplasias, such as breast and prostate cancers of AR regulation. More importantly, we found that androgen/AR regulate PTEN expression in opposite directions in prostate compared with breast cancer cells. As a nuclear receptor, AR binds AREs in the promoters of its target genes functioning as a transcription activator or repressor, depending on its cofactors. The formation of a preinitiation complex with AR coactivators, such as steroid receptor coactivator-1 (SRC1), transcriptional intermediary factor 2 (TIF2) and amplified in breast cancer 1 (AIB1), results in the activation of target gene transcription (Anzick *et al.*, 1997; Chen

et al., 1997; Li *et al.*, 1997). At the same time, AR can also downregulate target genes by recruiting corepressors, such as the well-known nuclear receptor corepressor (NCoR1) and silencing mediator of retinoid and thyroid hormone receptor (SMRT/NCoR2; Dotzlaw *et al.*, 2002; Hodgson *et al.*, 2005). The distinct effect of AR on *PTEN* expression may be due to the different, perhaps organ-specific, AR cofactors in prostate and breast cells. An investigation beyond the scope of this study is required to demonstrate the detailed mechanism of how AR differentially regulates *PTEN* expression in the two types of cancers. AR regulatory regions are not only located within proximal promoters, but also in distant promoter regions. Further investigation to identify additional AR regulatory regions in the *PTEN* promoter/enhancer region(s) is critical to fully understand the mechanism of AR-regulated *PTEN* expression in tumorigenesis.

Examining the germline mutations, variants and deletions in CS/CSL individuals reveal 299 (7.7%) patients who have germline variants in the *PTEN* promoter within the -1344 to -745 region (Figure 5a). Combined with our mutagenesis assay, we identified an ARE at -1185 to -1170 of the *PTEN* promoter (Figure 5b). The -1170 germline variant found in three CS/CSL patients mutates the nucleotide closest to the 3' end of the ARE, yet, *PTEN* transcriptional response to DHT is completely abrogated in both prostate and breast cancer cells, suggesting that the last base pair of the ARE in the *PTEN* promoter is critical for AR regulation in both prostate and breast cancer cell lines. The del 12-germline mutation adjacent to the ARE not surprisingly results in decreased promoter activity in both breast and prostate cancer cells. Moreover, this deletion partially weakened the promoter response to DHT only in prostate cancer cell line, but not in breast cancer cells. Combined with the decrease in the PTEN protein levels of the patients, the data suggest that the -1199 to -1188 region in the *PTEN* promoter is more important in baseline transcription instead of AR-induced regulation. This observation also confirms that the 5' extent of the ARE, as identified, is accurate. We noted that the overall frequency for variants or small deletions residing squarely in the midst of the ARE -1185 to -1170 region is very low (essentially 3 of 299) among CS/CSL patients, although it should be noted that *PTEN* is a tumor suppressor gene and germline mutations are scattered throughout the gene. There are two alternative interpretations. The first, and more plausible, is that the ARE has a vital role during human development; therefore, specific mutations within the ARE might result in *in utero* lethality. The second possibility is that the ARE region is functionally unimportant.

To date, little is known about the regulation of *PTEN* expression, especially that mediated by steroid hormone receptors. Here, we demonstrate a novel mechanism in which androgen transcriptionally regulates *PTEN*, in opposing directions, in the most common cancers in men and women, respectively. These data help elucidate the mechanism behind the clinical observation of the

inverse correlation of AR and *PTEN* expression in prostate cancer progression, compared with the relationships of AR and *PTEN* expression in breast cancer prognosis. We have found that this crosstalk between male sex steroid hormones and the *PTEN* pathway is tissue-dependent with different androgen-mediated *PTEN* responses between breast and prostate neoplasias. It is possible that the differential AR effect on *PTEN* may explain organ-specific and perhaps sex-specific neoplasia predisposition in CS. This phenomenon may begin to provide a testable hypothesis to explain why only a fraction of women with germline *PTEN* mutations develop breast cancer, depending on the androgen steroid milieu and levels.

Materials and methods

Meta-analysis of the correlation coefficient between AR and *PTEN*

All analyses were performed in R version 2.10.0 (Ihaka and Gentleman, 1996). A search through the Gene Expression Omnibus was performed for the words 'prostate cancer, patient samples' and 'breast cancer, patient samples'. Only those studies that contained more than 10 patients were included. We specifically excluded cultured cancer cells and the patients who received previous/on-going treatment. For prostate cancer studies, we also excluded the samples that contain less than 50% tumor content. For breast cancer studies, results were limited to the Affymetrix HGU133Plus2.0 platform (GPL570) (Affymetrix, Santa Clara, CA, USA). A total of seven prostate cancer data sets and 16 breast cancer data sets were identified and reviewed for meta-analysis. Meta-analysis of the correlation coefficients was performed using the library *rmeta*, and the R script is available at http://www.lerner.ccf.org/gmi/igac/published_data. Forest plots showing a summary estimate of the correlation between the expression of *PTEN* and *AR* in prostate cancer and breast cancer tumor tissue.

Cell culture and pharmacological treatments

AR-positive prostate cancer cell lines LNCaP, CWR22rv1 and AR negative PC-3, DU145 cells were purchased from the ATCC (American Type Culture Collection, Manassas, VA, USA). LAPC4 cells were available in the laboratory of Dr Heston. AR-positive C4-2 cells (UroCor, Oklahoma City, OK, USA) belong to an androgen-independent subline, developed from LNCaP xenografts in castrated nude mice. Breast cancer cell lines MDA-MB-231, MDA-MB-453, MDA-MB-468, MCF-7 and BT-549 were purchased from ATCC. 4, 5 α -Dihydrotestosterone (DHT) is from Sigma-Aldrich (St Louis, MO, USA; per Dr Silverman). Bicalutamide (Casodex) was purchased from Astra-Zeneca (Cheshire, UK RHS; per Dr Heston).

Patient mutation analysis

We utilized anonymized genomic DNA samples from CS/CSL patients ($n=2399$) archived in the Genomic Medicine Biorepository, Cleveland Clinic Genomic Medicine Institute, (Cleveland, OH, USA). Classic CS was diagnosed using the operational diagnostic criteria of the International Cowden Consortium (Liaw *et al.*, 1997). CSL defines a broad class of patients with combinations of features, but who do not meet the operational diagnostic criteria. We utilized genomic DNA from population controls ($n=48$). Informed consent was obtained for all subjects in accordance with procedures and

protocols approved by the respective Human Subjects Protection Committee of each participating institution. Genomic DNA from patient whole blood was amplified by PCR and subjected to direct sequencing (ABI3730 \times 1) of the *PTEN* promoter from -1344 to -745 region (Mutter *et al.*, 2000).

RNA inhibition

Control siRNA was a pool of four-scrambled non-specific siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Anti-AR siRNA (sc-29204, Santa Cruz Biotechnology) contains a pool of four target-specific siRNAs with the following sequences: (A) 5'-CAGUCCCACUUGUGUCAAAA-3', (B) 5'-CCUGAUCUGUGGAGAUGAA-3', (C) 5'-GUCGUCUUCGAAAUGUUA-3', (D) 5'-GACAGUGUCACACAUGAA-3'.

Western blotting

Western blotting was performed as described elsewhere (Ghosh *et al.*, 2005). Mouse monoclonal anti-AR (441) and anti-Lamin-A antibodies were obtained from Santa Cruz Biotechnology. Mouse monoclonal anti-tubulin antibody was obtained from Sigma-Aldrich. Mouse monoclonal anti-*PTEN* antibody was obtained from Cascade Biosciences, (Portland, OR, USA).

Promoter activity assay

AR transcriptional activity was measured by using the Cignal report assay kit (SA Bioscience, Frederick, MD, USA), according to the manufacturer's instructions. We generated *PTEN* promoters tagged with the firefly luciferase gene, as described in our previous study (Teresi *et al.*, 2008). Reporter gene activity was determined by dual luciferase assay using a luciferase enzyme assay system (Promega, Madison, WI, USA). P53-response-element reporter plasmid was obtained from Addgene (Cambridge, MA, USA).

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

qRT-PCR to quantitate *PTEN* mRNA expression was measured using SYBR Green (Applied Biosystem, Foster City, CA, USA), exactly according to the manufacturer's specifications. Expression of *GAPDH* was used as the internal control. The *PTEN* primer sequences are as follows: exon 3-4 Forward: 5'-ATATTCTCTGAAAAGCTCTGG-3', Reverse: 5'-GACAGTAAGATACAGTCTATC-3'; exon 4-5 Forward: 5'-CATTATAAAGATTCAGGCAATG-3', Reverse: 5'-TCCAGGAAGAGGAAAGGAAA-3'; exon 5-6 Forward: 5'-ACC TGTTAAGTTTGTATGCAA-3'; Reverse: 5'-GATATGGTTAAGAAAAGCTGTTTC-3'.

Chromatin Immunoprecipitation (ChIP) assay

ChIP assays were performed with the EZ Chromatin Immunoprecipitation assay kit (EZ ChIP, Upstate Biotechnology, Charlottesville, VA), according to the manufacturer's protocol. An amount of 5 μ g of antibody was used in every precipitation. The ChIP-eluted DNA was quantified by qRT-PCR, using primers specific for the full length and various regions of the *PTEN* promoter.

Conflict of interest

The authors declare no conflict of interest.

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