Therapeutics, Targets, and Chemical Biology

Cancer Research

Dysregulation of Cholesterol Homeostasis in Human Prostate Cancer through Loss of *ABCA1*

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Abstract

Recent epidemiologic data show that low serum cholesterol level as well as statin use is associated with a decreased risk of developing aggressive or advanced prostate cancer, suggesting a role for cholesterol in aggressive prostate cancer development. Intracellular cholesterol promotes prostate cancer progression as a substrate for *de novo* androgen synthesis and through regulation of AKT signaling. By conducting next-generation sequencing–based DNA methylome analysis, we have discovered marked hypermethylation at the promoter of the major cellular cholesterol efflux transporter, *ABCA1*, in LNCaP prostate cancer cells. *ABCA1* promoter hypermethylation renders the promoter unresponsive to transactivation and leads to elevated cholesterol levels in LNCaP. *ABCA1* promoter hypermethylation is enriched in intermediate- to high-grade prostate cancers and not detectable in benign prostate. Remarkably, ABCA1 downregulation is evident in all prostate cancers examined, and expression levels are inversely correlated with Gleason grade. Our results suggest that cancer-specific *ABCA1* hypermethylation and loss of protein expression direct high intracellular cholesterol levels and hence contribute to an environment conducive to tumor progression. *Cancer Res; 73(3); 1211–8. ©2012 AACR.*

Introduction

Despite detection of prostate cancer at earlier stages and advances in the treatment of local as well as metastatic disease, there will still be an estimated 28,170 deaths due to prostate cancer in 2012 (1). Men who die of prostate cancer have cancers with aggressive pathologic features that increase the risk of tumor progression and metastasis, even if they undergo radical therapy with curative intent. As such, the investigation of novel strategies to prevent the development of aggressive or advanced prostate cancer will be critical to lowering the morbidity and mortality attributed to this disease.

Epidemiologic studies have described a positive correlation between high serum cholesterol level and prostate cancer aggressiveness (2, 3) as well as a protective effect of statin use in lowering the risk of advanced prostate cancer (2, 4–7). These reports, along with the discovery of *de novo* androgen synthesis in castration-resistant prostate cancer (CRPC), have fueled a renewed interest in intratumoral cholesterol homeostasis due to the central role of cholesterol in steroidogenesis (8–10). Furthermore, excess intracellular cholesterol is incorporated into membrane lipid rafts, thereby stabilizing the raft structure and enhancing AKT signaling in prostate cancer cells (11–15). Therefore, examining how prostate cancer cells manipulate intracellular cholesterol content is important for understanding prostate cancer biology.

To understand the role that altered DNA methylation patterns play in prostate cancer development, we used MBD-isolated genome sequencing (MiGS; ref. 16) to construct genome-wide DNA methylation profiles in the common prostate cell line models, PrEC, LNCaP, and DU 145. Using unbiased global analysis, we discovered dense hypermethylation in the 5′ regulatory regions of the cholesterol efflux transporter, ATP-binding cassette, sub-family A, member 1 (*ABCA1*), in LNCaP cells but not in PrEC or DU 145 cells. Given data suggest that loss of cellular cholesterol homeostasis is important in prostate cancer, we proceeded to delineate the biologic relevance of this epigenetic modification. We report that DNA hypermethylation at *ABCA1* promoter in LNCaP cells effectively suppresses basal expression and prevents full induction by a transactivator. Loss of ABCA1 expression results in retention of intracellular cholesterol. Furthermore, *ABCA1* hypermethylation is exclusively detected in intermediate- and high-grade prostate cancers, suggesting that epigenetic inactivation of *ABCA1* is involved in prostate cancer progression. Finally, significant decrease and complete loss of ABCA1 protein expression are evident in all prostate cancers examined in our study. Together, these findings indicate that ABCA1 is an important regulator of intracellular cholesterol levels in prostate epithelial cells and that its pervasive inactivation in prostate cancers likely provides a milieu favorable for tumor progression by permitting the accumulation of intracellular cholesterol.

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Materials and Methods

DNA methylation analysis

DNA methylome profiles for PrEC, LNCaP, and DU 145 were generated using MiGS as previously described (16). The sequencing reads generated and used in the manuscript are deposited in the NCBI Sequence Read Archive (SRA) under the accession number SRA049689.1. The raw sequencing reads for each sample were mapped to the reference human genome (UCSC Hg18) using Bowtie (17). Bisulfite sequencing and methylation-specific PCR (MSP) of the *ABCA1* promoter were conducted as previously described (18), and primers used are listed in Supplementary Table S1.

Cell culture, transfection, and luciferase assay

PrEC (Lonza) was cultured in PrEGM according to the manufacturer's instructions. LNCaP and DU 145 (American Type Culture Collection) were cultured in RPMI-1640 supplemented with 10% FBS. All 3 cell lines were obtained directly from the cell banks, and the identities of the cell lines were verified per the cell banks' protocols. Cells were treated with 10 μmol/L T0901317 (Sigma) for 24 hours or 5 μmol/L 5-aza-2′-deoxycytidine (Sigma) for 7 days, or a combination of the 2 compounds. *ABCA1* promoter (−1,132 to +112 relative to the transcription start site) was amplified by PCR and subcloned into the pGL4.20 (Promega). Methylated *ABCA1* promoter was *in vitro* DNA methylated using *Sss*I (NEB) and ligated into pGL4.20 before transfection. The reporter construct was co-transfected with pGL4.74 vector into DU 145 cells using Nucleofection (Lonza). Reporter luciferase activity was measured and normalized to control *Renilla* luciferase activity for each sample. The mean ± SEM from triplicate experiments for each experimental group was plotted for comparisons. The different groups were compared using one-way ANOVA with Bonferroni multiple comparison test.

Gene expression and Western blot

Expression of *ABCA1* and *GAPDH* mRNA was measured by real-time reverse transcription (RT)-PCR as previously described (18), and primers used are listed in Supplementary Table S1. The relative fold change in expression was calculated using the $2^{-\Delta\Delta C_T}$ method by normalizing to *GAPDH* mRNA expression in each sample and compared with LNCaP mock–treated cells. The mean ± SEM from triplicate experiments for each experimental group was plotted, and comparisons between each sample group against LNCaP mock treated cells were conducted using one-way ANOVA with Bonferroni multiple comparison test. For Western blot analysis of ABCA1 and β-actin (ACTB), 15 μg cell lysate per sample were resolved in 4–12% Bis-Tris gel (Life Technologies), transferred onto nylon membranes, and probed with rabbit anti-ABCA1 (Novus Biologicals) and mouse anti-ACTB (Sigma). For gene expression microarray analysis, total RNA was extracted with TRIzol (Life Technologies), followed by DNase I treatment. The RNA samples were labeled and hybridized according to the manufacturer protocol to the Illumina HumanRef-8 v3.0 expression beadchips (Illumina) in triplicates. The expression results generated and used in the manuscript are deposited with the Gene Expression Omnibus under the accession number GSE35401. Differential gene expression analysis was conducted using the Illumina GenomeStudio v2009.1 (Illumina).

Cellular cholesterol analysis

For filipin staining, LNCaP and DU 145 cells were grown on glass coverslips, fixed in 3% paraformaldehyde, and stained with 50 μg/mL filipin (Sigma). Images were acquired using QCapturePro software (QImaging) at the designated magnifications and fixed aperture and exposure time for both cell lines. Biochemical quantification of intracellular cholesterol was conducted as previously described (19). The different groups were compared using one-way ANOVA with Bonferroni correction. For analysis of cholesterol efflux, cells were labeled with 0.5 μCi/mL [^{3}H]-cholesterol in RPMI containing 1% FBS for 16 hours at 37°C. After labeling, cells were chased for 4 hours at 37°C in RPMI with or without acceptors (10 μg/mL APOA1 or 100 μg/mL HDL). At the end of this chase period, the radioactivity in the medium and cells was determined by liquid scintillation counting, and the percent efflux was calculated as 100 × (medium dpm)/(medium dpm + cell dpm). Percentage of efflux to acceptors was calculated as (percent efflux to acceptor) − (percent efflux to no acceptor). LNCaP treatment groups were compared with the mock-treated sample using one-way ANOVA with Dunnett correction. Unpaired *t* test with Welch correction was used to compare the T0901317-treated with the mock-treated DU 145 cells.

Human tissue specimens

Prostate cancer tissues were obtained from patients treated with radical prostatectomy at Cleveland Clinic (Cleveland, OH). Benign prostate tissues were obtained from patients treated with radical cystoprostatectomy for either malignant or benign bladder disease at Cleveland Clinic. All study specimens were collected under an approved Cleveland Clinic IRB protocol. All sections were retrieved and reviewed by dedicated genitourinary pathologists (C. Magi-Galluzzi and S.M. Falzarano) to confirm the original diagnosis. For MSP, formalin-fixed, paraffin-embedded sections were deparaffinized using xylene and rehydrated before gDNA extraction. Two μg gDNA from each sample was bisulfite-treated using the EpiTect bisulfite conversion kit (Qiagen) in 3 independent experiments. Only samples that show consistent methylation in all 3 experiments were deemed to harbor *ABCA1* promoter methylation. Immunohistochemistry was conducted on 4-μm sections. Antigen retrieval was conducted before incubation with a custom anti-ABCA1 rabbit polyclonal antibody raised against AA 104-125 in NP_005493.2 (Thermo), OmniMap secondary antibody (Ventana), and ChromoMap DAB (Ventana). ABCA1 staining patterns were evaluated by C. Magi-Galluzzi and S.M. Falzarano. The specificity of this custom antibody was tested using both Western blotting and immunohistochemistry staining of DU 145 and LNCaP cells (Supplementary Fig. S1). Scoring of ABCA1 staining was conducted using the *H*-score system as previously described (20) with the scale set from 0 to 3. *H*-score comparisons were conducted using the Mann–Whitney *U* test and Kruskal–Wallis test, with $P < 0.05$ considered to be statistically significant. ABCA1 staining was independently

analyzed by comparing the percentage of cancer cells stained positively for ABCA1 using the Kruskal–Wallis test, with $P < 0.05$ considered to be statistically significant.

Results

We assembled DNA methylome profiles for normal prostate epithelial cells, PrEC, and prostate cancer cell lines, LNCaP and DU 145, using MiGS. Unambiguously mapped sequencing reads were used for generating the individual DNA methylome profiles. At a false discovery rate of 5%, we identified the major cellular cholesterol efflux transporter, *ABCA1*, to be densely methylated in its 5′ regulatory region in LNCaP cells but not in PrEC or DU 145 cells (Fig. 1A).

While the *ABCA1* promoter region is one of several thousand novel differentially methylated loci among the three prostate cell lines, we focused on this gene because of its central role in intracellular cholesterol homeostasis. We verified this differential DNA methylation at the *ABCA1* promoter by targeted bisulfite (BSF) sequencing in PrEC and LNCaP cells (Fig. 1B; Supplementary Fig. S2). We also assayed this region using MSP (Fig. 1C). Both BSF sequencing and MSP results corroborated the robust DNA methylation at the *ABCA1* promoter in LNCaP cells as detected by MiGS.

To examine the functional consequence of *ABCA1* promoter methylation, we used RT-PCR to quantify *ABCA1* mRNA expression in PrEC, LNCaP, and DU 145 cells (Fig. 2A). Compared with LNCaP cells, *ABCA1* mRNA levels are at least 100-fold higher in PrEC and DU 145 cells where the *ABCA1* promoter is not DNA methylated. This finding is consistent with transcriptional repression caused by *ABCA1* promoter hypermethylation in LNCaP cells. We treated LNCaP cells with T0901317, a synthetic liver-x-receptor (LXR) α agonist known to induce *ABCA1* transcription, and found that *ABCA1* transcription was only modestly induced above baseline. Treatment of LNCaP cells with 5-aza-2′-deoxycytidine (5-aza), a demethylating agent, also did not strongly induce *ABCA1* transcription. However, when the *ABCA1* promoter in LNCaP is first demethylated with 5-aza, treatment with T0901317 resulted in robust activation of *ABCA1* transcription to levels comparable with PrEC cells. We confirmed demethylation of *ABCA1* promoter by 5-aza using MSP (Fig. 1C). These data show that *ABCA1* promoter hypermethylation renders it

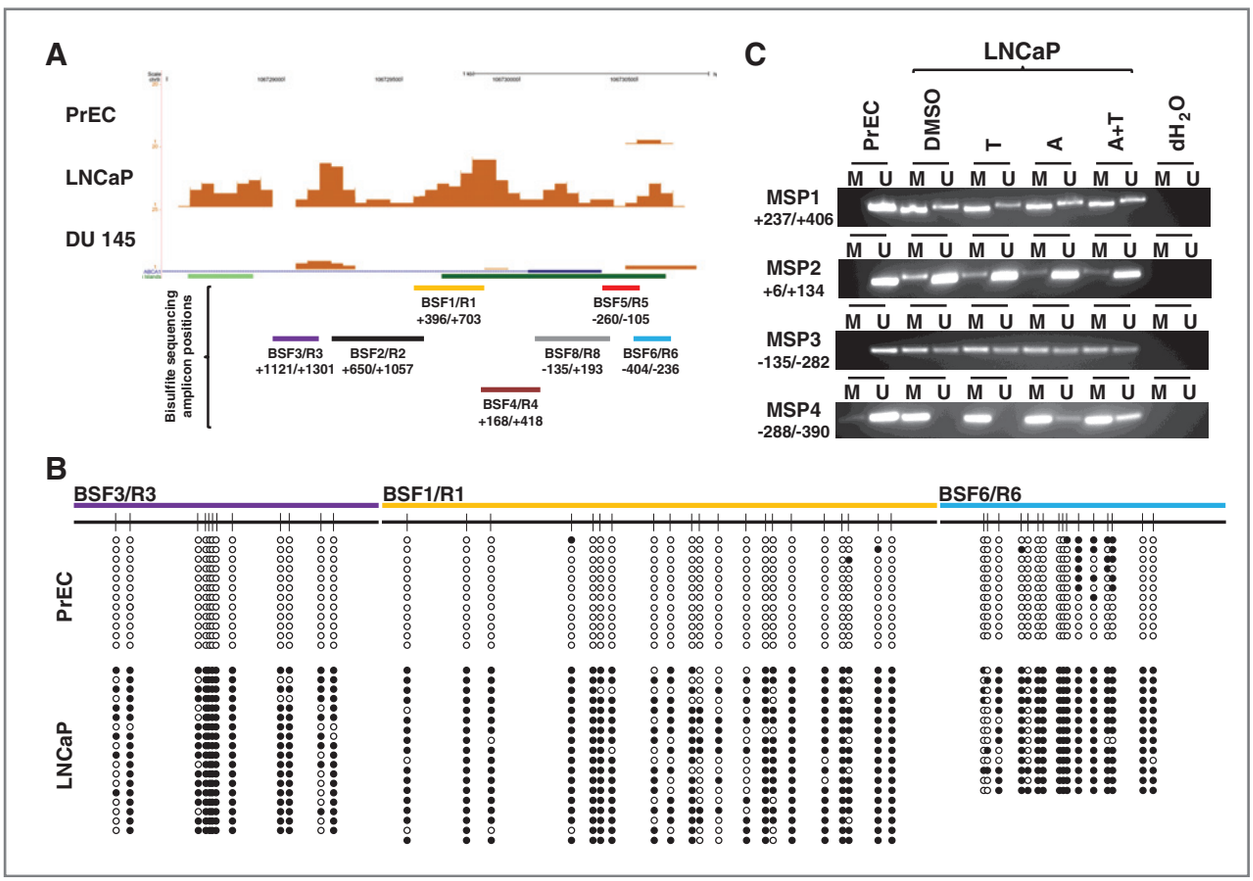

Figure 1. DNA methylation analysis of *ABCA1* 5′ regulatory sequences in prostate cell lines. A, UCSC genome browser snapshot displaying the DNA methylation sequencing signals in PrEC, LNCaP, and DU 145 cells at the *ABCA1* promoter region (UCSC Hg18, chr9:106,728,482-106,730,800). B, bisulfite sequencing validation in PrEC and LNCaP cells. Black circles represent methylated CpG sites, and white circles represent unmethylated CpG sites. C, MSP results in PrEC and LNCaP cells either mock-treated [dimethyl sulfoxide (DMSO)] or treated with 10 μmol/L T0901317 for 24 hours (T), 5 μmol/L 5-aza-2′-deoxycytidine for 7 days (A), or a combination of 5-aza-2′-deoxycytidine and T0901317 (A + T).

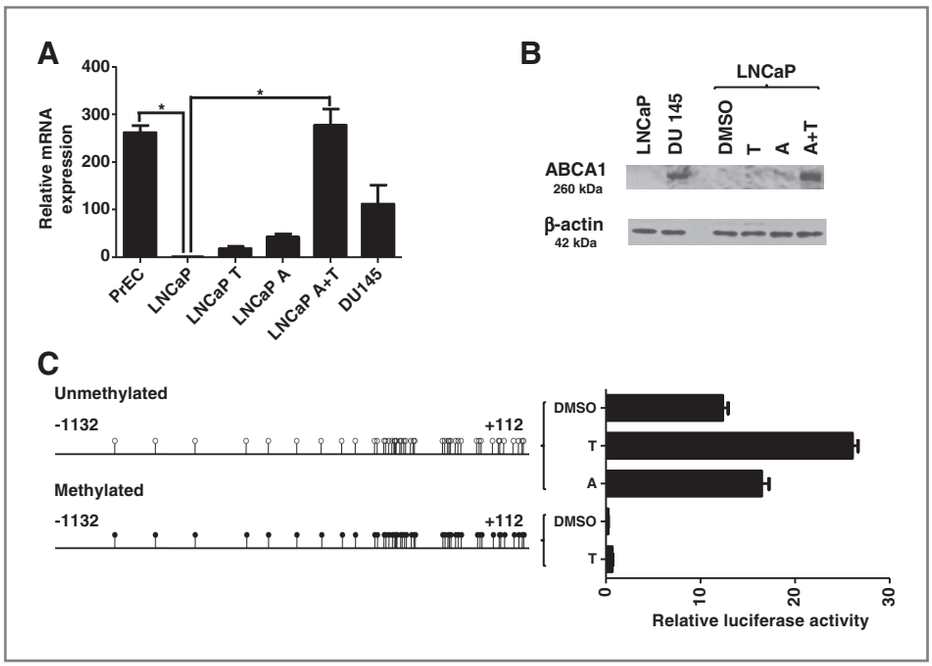

Figure 2. Effects of promoter DNA methylation on basal expression and inducibility of *ABCA1*. A, relative mRNA expression of *ABCA1* in prostate cells. LNCaP cells were treated identically as in Fig. 1. Data are represented as mean ± SEM from triplicate experiments. *, $P < 0.05$. B, Western blot analysis of ABCA1 and β-actin (ACTB) in LNCaP and DU 145 cells. LNCaP cells were treated identically as in Fig. 1. C, luciferase reporter assay of the unmethylated and the methylated *ABCA1* promoter in DU 145 cells. The cells containing the indicated reporter construct were either mock-treated [dimethyl sulfoxide (DMSO)] or treated with 10 μmol/L T0901317 (T) or 5 μmol/L 5-aza-2′-deoxycytidine (A) for 24 hours. Data are represented as mean ± SEM from triplicate experiments. All pairwise comparisons were statistically significant ($P < 0.05$) except for between the 2 methylated treatment groups.

unresponsive to transactivation. Not surprisingly, the lack of messenger RNA corresponds to a lack of ABCA1 protein expression in LNCaP cells whereas DU 145 cells clearly express ABCA1 (Fig. 2B). Again, demethylation of the *ABCA1* promoter with 5-aza followed by treatment with T0901317 resulted in robust ABCA1 protein expression in LNCaP. It is worth noting that minimal *ABCA1* expression and severely limited induction by transactivators in LNCaP cells have been independently reported by other groups without a mechanistic explanation (14, 21, 22).

Next, we ascertained the effect of *ABCA1* promoter hypermethylation on transcriptional activity (Fig. 2C). An *ABCA1* promoter/luciferase reporter construct was generated with sequences surrounding the *ABCA1* transcription start site. A fully methylated version was produced by treating the *ABCA1* promoter fragment with *Sss*I methylase and ligating it into the luciferase reporter construct before transfection. These vectors were transfected into DU 145 cells, which have the ability to express endogenous *ABCA1*. The unmethylated promoter expressed the luciferase reporter, and as expected, treatment with T0901317 resulted in a significant induction of reporter expression. Conversely, reporter activity from the methylated *ABCA1* promoter was almost undetectable, and it was not induced by T0901317. These data show that promoter hypermethylation of *ABCA1* is directly responsible for transcriptional repression and loss of responsiveness to activation by LXR agonist.

We examined the functional consequence of *ABCA1* promoter hypermethylation and transcriptional silencing. LNCaP and DU 145 cells were subjected to filipin staining, which allows visualization of free cholesterol, the major unesterified sterol in mammalian cells (Fig. 3A). Fluorescent microscopy showed that LNCaP cells have significantly elevated basal intracellular cholesterol levels when compared with DU 145. Total cellular cholesterol content was quantified biochemically (Fig. 3B), which confirmed that LNCaP has a higher basal level of intracellular cholesterol than DU 145 (51.2 ± 4.4 vs. 36.7 ± 3.0 μg/mg protein). When LNCaP was treated with either T0901317 or 5-aza alone, intracellular cholesterol did not decrease significantly. However, when LNCaP was treated with 5-aza before T0901317, intracellular cholesterol was significantly lower than untreated LNCaP cells (43.6 ± 1.4 vs. 51.2 ± 4.4 μg/mg protein). As expected, treatment of DU 145 with only T0901317 resulted in decreased intracellular cholesterol. We assessed whether *ABCA1* reactivation in LNCaP was responsible for the decrease in intracellular cholesterol content (Fig. 3C and D). We measured cholesterol efflux to apolipoprotein A-I (APOA1), which can accept cellular cholesterol only via ABCA1, and to HDL, which can accept cholesterol from both ABCA1 and other transporters such as SR-B1 and ABCG1. Using APOA1 as an acceptor, treatment of LNCaP with either T0901317 or 5-aza did not result in robust increases in cholesterol efflux. However, treatment with 5-aza followed by T0901317 led to a 2.5-fold increase in cholesterol efflux to APOA1. When HDL was used as an acceptor, the same overall trend was observed in cholesterol efflux in LNCaP after treatment with T0901317, 5-aza, or the 2 drugs combined. However, the magnitude of increase in cholesterol efflux after treatment with 5-aza followed by T0901317 was significantly lower when APOA1 as an acceptor. These data suggest that the decrease in cholesterol after treatment of LNCaP with 5-aza followed by T0901317 is mainly due to re-activation of *ABCA1*. Conversely, treatment of DU 145 with T0901317 alone led to a significant increase in cholesterol efflux to APOA1, suggesting that *ABCA1* was readily inducible in the absence of promoter methylation (Fig. 3E and F). Treatment of DU 145 with T0901317 also resulted in a small but statistically significant increase in cholesterol efflux to HDL. As ABCG1 is the other major

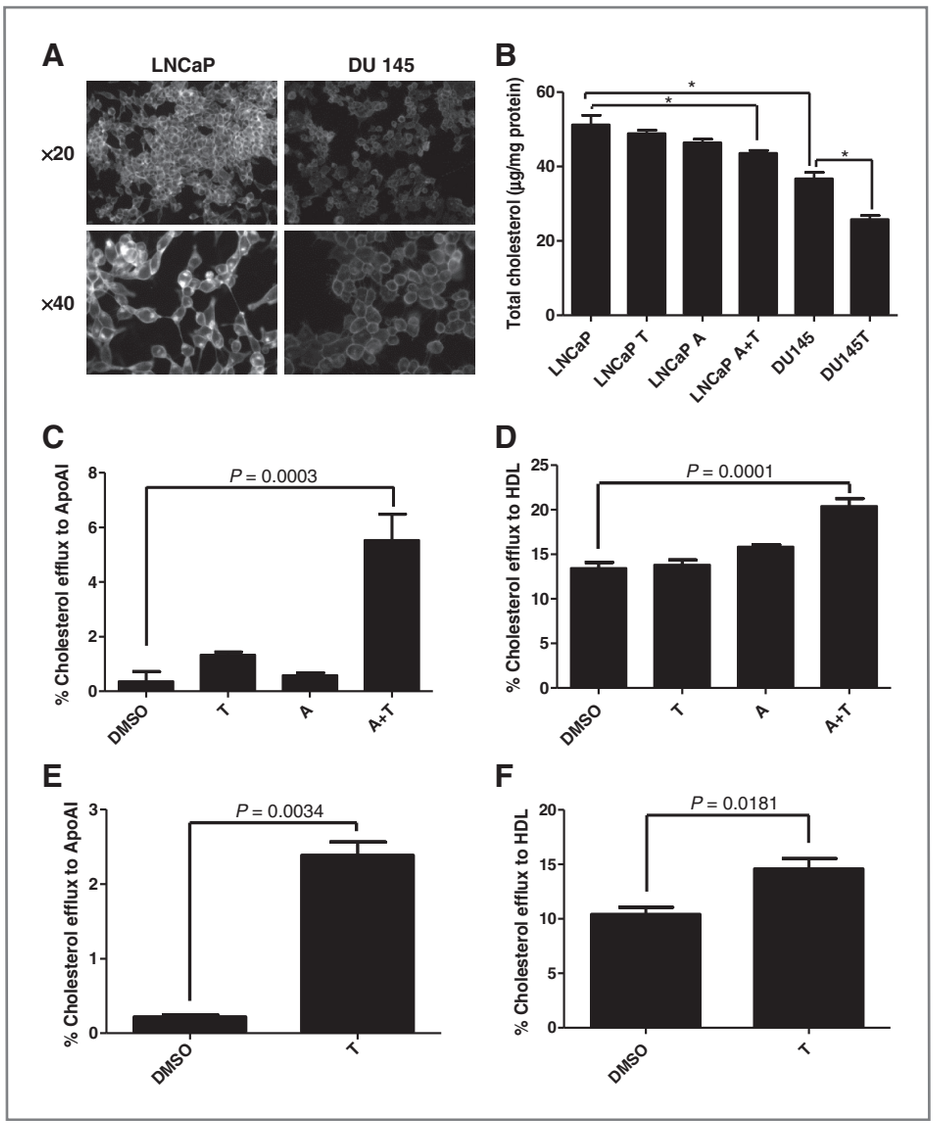

Figure 3. Functional analysis of *ABCA1* expression in prostate cancer cells. A, representative fields of filipin staining for LNCaP and DU 145. B, total cellular cholesterol content for LNCaP and DU 145 cells. LNCaP cells were treated identically as in Fig. 1, whereas DU 145 cells were either mock-treated [dimethyl sulfoxide (DMSO)] or treated with 10 μmol/L T0901317. C–F, cellular cholesterol efflux to APOA1 and HDL in LNCaP (C and D, respectively) and DU 145 (E and F, respectively) cells. Data are represented as mean ± SEM from triplicate experiments. *, $P < 0.05$.

contributor of cellular cholesterol efflux, we also examined *ABCG1* promoter methylation and expression in these cells. By MiGS analysis, the *ABCG1* promoter is free of DNA methylation in PrEC, LNCaP, and DU 145 cells (Supplementary Fig. S3). All 3 cell lines expressed *ABCG1* mRNA robustly with no statistically significant differences among them, whereas *ABCA1* expression showed previously validated differences by microarray analysis (Supplementary Table S2). Altogether, these data support the notion that hypermethylation and consequent loss of expression of ABCA1 in LNCaP cells contribute to the aberrant accumulation of intracellular cholesterol in these cancer cells.

To assess the prevalence of *ABCA1* hypermethylation in human prostate cancer, we conducted MSP on DNA extracted from 9 benign prostatic tissue samples from cystoprostatectomy specimens and 33 prostate cancers. Of the 33 prostate cancer samples, 30 yielded high-quality bisulfite-converted DNA for this analysis. We did not find *ABCA1* hypermethylation in any of the benign prostatic tissue samples; however, 4 of 30 prostate cancers (samples 21, 29, 34, and 36) showed *ABCA1* hypermethylation (Fig. 4A). Notably, *ABCA1* hypermethylation was only seen in men with intermediate- and high-risk prostate cancer [1 of 9 of Gleason score (GS) 7 cancers and 3 of 14 of GS 8–10 cancers]. Biochemical recurrence after radical therapy was documented in all of these men. Furthermore, we investigated the expression of ABCA1 in prostate tissue by developing a custom antibody to the protein and conducting immunohistochemistry on individual radical prostatectomy specimens and tissue microarrays containing prostate cancers (Fig. 4B). We used the *H*-score method to evaluate the ABCA1 expression in a semiquantitative fashion. There was significant heterogeneity of ABCA1 staining in each cancer specimen due to differences in tumor pattern. Thus, we determined the *H*-score of each of the following patterns observed: benign prostatic tissues ($n = 8$), high-grade prostatic intraepithelial neoplasia (HGPIN; $n = 27$), and Gleason patterns 3 ($n = 13$), 4 ($n = 24$), and 5 ($n = 14$;

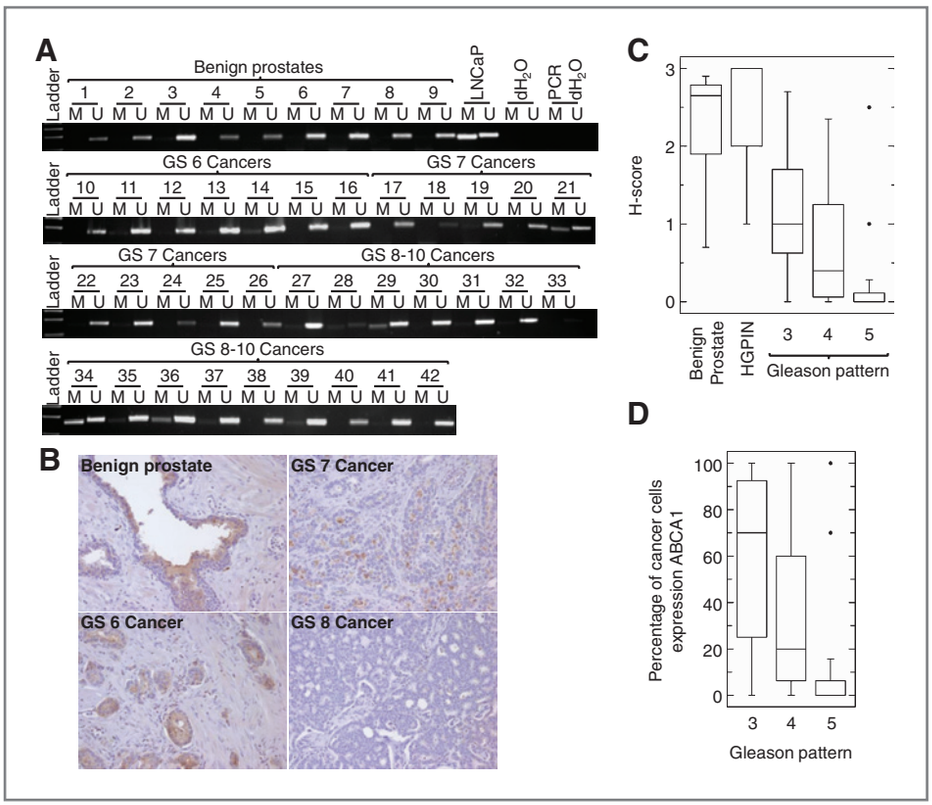

Figure 4. Analysis of *ABCA1* promoter methylation and expression in radical prostatectomy specimens. A, MSP was conducted using MSP1 primer set (Supplementary Table S1) on bisulfite-converted genomic DNA extracted from benign prostates (1–9), GS 6 (10–16), GS 7 (17–26), and GS 8–10 (27–42) prostate cancer specimens. LNCaP DNA was included as a positive control. Samples 18, 28, and 33 did not yield sufficient bisulfite-converted DNA to produce successful PCR results and therefore were excluded from further analysis. B, ABCA1 immunohistochemistry on benign prostate, GS 6, GS 7, and GS 8 prostate cancers. C, box plots of *H*-scores for ABCA1 staining in benign prostatic tissues ($n = 8$), HGPIN ($n = 27$), Gleason pattern 3 ($n = 13$), 4 ($n = 24$), and 5 ($n = 14$) tumors. The box shows the first quartile, median, and third quartile values. The whiskers show the minimum and maximum values. For Gleason pattern 5, outlier values, defined as 3 times the interquartile range, are present, and the whiskers denote 1.5 times the interquartile range with outliers plotted as individual black circles. The difference among the *H*-scores of Gleason patterns 3, 4, and 5 is statistically significant ($P = 0.0017$; Kruskal–Wallis test). D, box plots of percentages of cancer cells expressing ABCA1. Graphical representation is identical to C. The difference among the percentages of cells staining positive for ABCA1 in Gleason patterns 3, 4, and 5 is statistically significant ($P = 0.0013$; Kruskal–Wallis test).

Fig. 4C). No significant difference was observed in ABCA1 expression between benign prostatic tissues and HGPIN (median *H*-score = 2.65 vs. 3.00). ABCA1 expression was significantly lower for prostate cancers than for benign prostatic tissues (median *H*-score = 0.35 vs. 3.00; $P < 0.001$; Mann–Whitney test). Moreover, there was an inverse correlation between ABCA1 expression and Gleason pattern. Both Gleason pattern 4 and 5 cancers had a lower median *H*-score than Gleason pattern 3 cancer (0.40 and 0.00 vs. 1.00; $P = 0.0017$; Kruskal–Wallis test), and 71% of Gleason pattern 5 cancers completely lost ABCA1 expression. When we examined the percentage of cancer cells staining positively for ABCA1, we observed a statistically significant difference ($P = 0.0013$; Kruskal–Wallis test) among the Gleason patterns (Fig. 4D). Specifically, ranking by the percentage of cells expressing ABCA1, Gleason pattern 3 was the highest, Gleason pattern 4 was second, and Gleason pattern 5 was the lowest (median percentages = 70%, 20%, and 0%, respectively). These results show that *ABCA1* hypermethylation is specific to prostate cancer, and decrease in ABCA1 expression is associated with tumor aggressiveness.

Discussion

Perturbation in cholesterol homeostasis is a well-known characteristic of cancer that was described more than 50 years ago (23, 24). Subsequently, anecdotal reports described a beneficial effect of cholesterol lowering agents in the management of prostate cancer (25). The introduction and widespread use of statins as cholesterol lowering agents in the prevention of heart disease allowed the collection of epidemiologic data correlating prostate cancer risk and statin use. Although meta-analyses showed that statins had no effect on the overall risk of prostate cancer (26–29), other studies have shown that statin use is associated with a decreased risk of aggressive or advanced prostate cancer (6, 29–31). Importantly, these cancers are potentially life threatening even after radical treatment. Thus, focusing on preventing the development or progression of aggressive prostate cancer is of utmost importance, and cholesterol may provide an opportune target. Indeed, recent reports suggest that statin use protects against prostate cancer with adverse pathologic characteristics (32) and improves progression-free survival in men undergoing radiation therapy (33, 34).

On the basis of these observations, after compiling the methylomes for the 3 prostate cell lines, we initially focused on candidates involved in cholesterol homeostasis. As discussed previously, cholesterol has 2 proposed roles in the development of advanced prostate cancer: serving as a substrate in *de novo* androgen synthesis in CRPC and enhancing AKT signaling by stabilizing lipid raft structure. However, the exact mechanism by which cholesterol accumulates inside the cancer cells is not clearly defined. In this study, we have identified *ABCA1* promoter hypermethylation and subsequent transcriptional silencing as one mechanism that prostate cancer cells can use to maintain elevated intracellular cholesterol levels. As intracellular cholesterol level is the net sum of uptake, synthesis, and efflux, disruption of a major transporter involved in efflux will result in intracellular cholesterol accumulation. We have shown that this is the case through fluorescence microscopy as well as cholesterol quantification. When mechanisms responsible for cholesterol homeostasis are intact, excess cholesterol is converted to oxysterols, which bind to LXR so that *ABCA1* transcription is activated (35). We showed that in LNCaP cells, *ABCA1* promoter hypermethylation prevents *ABCA1* activation by the synthetic LXR agonist, T0901317, and demethylation of the promoter by 5-aza restores responsiveness to T0901317. As a result, cholesterol levels are significantly decreased when compared with untreated LNCaP cells or those treated with either agent alone.

When we examined human prostate tissue, we found that *ABCA1* promoter hypermethylation was seen in prostate cancer but not benign prostatic tissue. Interestingly, this epigenetic alteration has a higher prevalence in intermediate- and high-grade cancers than low-grade cancers. Importantly, immunohistochemistry revealed that loss of *ABCA1* expression is more prevalent in high-grade tumors than can be explained by promoter hypermethylation alone. These data suggest that *ABCA1* inactivation may be important in the development of or progression to aggressive and/or advanced prostate cancer. Identifying the exact mechanism underlying *ABCA1* inactivation is important, as promoter hypermethylation will render the gene unresponsive to LXR agonists but other mechanisms may not. Although it is presumed that statins protect against aggressive and advanced prostate cancers by inhibiting cholesterol synthesis, it would be interesting to see whether their use would be successful in prostate cancers with *ABCA1* inactivation.

In summary, *ABCA1* promoter hypermethylation and gene inactivation lead to the accumulation of cholesterol in prostate cancer cells. Thus, this cellular cholesterol efflux pathway may be an important determinant of prostate cancer aggressiveness and a potential therapeutic target.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: B.H. Lee, A.H. Ting
Development of methodology: B.H. Lee, M.G. Taylor, A.H. Ting
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B.H. Lee, M.G. Taylor, P. Robinet, J. Schweitzer, E. Sehayek, S.M. Falzarano, C. Magi-Galluzzi, E.A. Klein, A.H. Ting
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B.H. Lee, P. Robinet, J.D. Smith, E. Sehayek, S.M. Falzarano, A.H. Ting
Writing, review, and/or revision of the manuscript: B.H. Lee, M.G. Taylor, P. Robinet, J.D. Smith, S.M. Falzarano, C. Magi-Galluzzi, E.A. Klein, A.H. Ting
Study supervision: J. Schweitzer, A.H. Ting

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References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer J Clin 2012;62:10–29.
2. Platz EA, Till C, Goodman PJ, Parnes HL, Figg WD, Albanes D, et al. Men with low serum cholesterol have a lower risk of high-grade prostate cancer in the placebo arm of the prostate cancer prevention trial. Cancer Epidemiol Biomarkers Prev 2009;18:2807–13.
3. Batty GD, Kivimaki M, Clarke R, Davey Smith G, Shipley MJ. Modifiable risk factors for prostate cancer mortality in London: forty years of follow-up in the Whitehall study. Cancer Causes Control 2011;22:311–8.
4. Solomon KR, Freeman MR. The complex interplay between cholesterol and prostate malignancy. Urologic Clin North Am 2011;38:243–59.
5. Mondul AM, Selvin E, De Marzo AM, Freedland SJ, Platz EA. Statin drugs, serum cholesterol, and prostate-specific antigen in the National Health and Nutrition Examination Survey 2001–2004. Cancer Causes Control 2010;21:671–8.
6. Platz EA, Leitzmann MF, Visvanathan K, Rimm EB, Stampfer MJ, Willett WC, et al. Statin drugs and risk of advanced prostate cancer. J Natl Cancer Inst 2006;98:1819–25.
7. Farwell WR, D'Avolio LW, Scranton RE, Lawler EV, Gaziano JM. Statins and prostate cancer diagnosis and grade in a veterans population. J Natl Cancer Inst 2011;103:885–92.
8. Dillard PR, Lin MF, Khan SA. Androgen-independent prostate cancer cells acquire the complete steroidogenic potential of synthesizing testosterone from cholesterol. Mol Cell Endocrinol 2008;295:115–20.
9. Locke JA, Guns ES, Lubik AA, Adomat HH, Hendy SC, Wood CA, et al. Androgen levels increase by intratumoral de novo steroidogenesis during progression of castration-resistant prostate cancer. Cancer Res 2008;68:6407–15.
10. Montgomery RB, Mostaghel EA, Vessella R, Hess DL, Kalhorn TF, Higano CS, et al. Maintenance of intratumoral androgens in metastatic prostate cancer: a mechanism for castration-resistant tumor growth. Cancer Res 2008;68:4447–54.
11. Adam RM, Mukhopadhyay NK, Kim J, Di Vizio D, Cinar B, Boucher K, et al. Cholesterol sensitivity of endogenous and myristoylated Akt. Cancer Res 2007;67:6238–46.
12. Cinar B, Mukhopadhyay NK, Meng G, Freeman MR. Phosphoinositide 3-kinase-independent non-genomic signals transit from the androgen

receptor to Akt1 in membrane raft microdomains. J Biol Chem 2007;282:29584–93.
13. Oh HY, Lee EJ, Yoon S, Chung BH, Cho KS, Hong SJ. Cholesterol level of lipid raft microdomains regulates apoptotic cell death in prostate cancer cells through EGFR-mediated Akt and ERK signal transduction. Prostate 2007;67:1061–9.
14. Pommier AJ, Alves G, Viennois E, Bernard S, Communal Y, Sion B, et al. Liver X Receptor activation downregulates AKT survival signaling in lipid rafts and induces apoptosis of prostate cancer cells. Oncogene 2010;29:2712–23.
15. Zhuang L, Lin J, Lu ML, Solomon KR, Freeman MR. Cholesterol-rich lipid rafts mediate akt-regulated survival in prostate cancer cells. Cancer Res 2002;62:2227–31.
16. Serre D, Lee BH, Ting AH. MBD-isolated Genome Sequencing provides a high-throughput and comprehensive survey of DNA methylation in the human genome. Nucleic Acids Res 2010;38:391–9.
17. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol 2009;10:R25.
18. Yan H, Choi AJ, Lee BH, Ting AH. Identification and functional analysis of epigenetically silenced microRNAs in colorectal cancer cells. PLoS One 2011;6:e20628.
19. Robinet P, Wang Z, Hazen SL, Smith JD. A simple and sensitive enzymatic method for cholesterol quantification in macrophages and foam cells. J Lipid Res 2010;51:3364–9.
20. McCarty KS Jr, Szabo E, Flowers JL, Cox EB, Leight GS, Miller L, et al. Use of a monoclonal anti-estrogen receptor antibody in the immunohistochemical evaluation of human tumors. Cancer Res 1986;46 Suppl:4244s–8s.
21. Leon CG, Locke JA, Adomat HH, Etinger SL, Twiddy AL, Neumann RD, et al. Alterations in cholesterol regulation contribute to the production of intratumoral androgens during progression to castration-resistant prostate cancer in a mouse xenograft model. Prostate 2010;70:390–400.
22. Trasino SE, Kim YS, Wang TT. Ligand, receptor, and cell type-dependent regulation of ABCA1 and ABCG1 mRNA in prostate cancer epithelial cells. Mol Cancer Ther 2009;8:1934–45.
23. Potter VR. The biochemical approach to the cancer problem. Fed Proc 1958;17:691–7.
24. Siperstein MD, Fagan VM. Deletion of the Cholesterol-Negative Feedback System in Liver Tumors. Cancer Res 1964;24:1108–15.
25. Addleman W. Cancer, cholesterol and cholestyramine. N Engl J Med 1972;287:1047.
26. Browning DR, Martin RM. Statins and risk of cancer: a systematic review and metaanalysis. Int J Cancer 2007;120:833–43.
27. Dale KM, Coleman CI, Henyan NN, Kluger J, White CM. Statins and cancer risk: a meta-analysis. JAMA 2006;295:74–80.
28. Kuoppala J, Lamminpaa A, Pukkala E. Statins and cancer: a systematic review and meta-analysis. Eur J Cancer 2008;44:2122–32.
29. Murtola TJ, Tammela TL, Lahtela J, Auvinen A. Cholesterol-lowering drugs and prostate cancer risk: a population-based case-control study. Cancer Epidemiol Biomarkers Prev 2007;16:2226–32.
30. Jacobs EJ, Rodriguez C, Bain EB, Wang Y, Thun MJ, Calle EE. Cholesterol-lowering drugs and advanced prostate cancer incidence in a large U.S. cohort. Cancer Epidemiol Biomarkers Prev 2007;16:2213–7.
31. Shannon J, Tewoderos S, Garzotto M, Beer TM, Derenick R, Palma A, et al. Statins and prostate cancer risk: a case-control study. Am J Epidemiol 2005;162:318–25.
32. Mondul AM, Han M, Humphreys EB, Meinhold CL, Walsh PC, Platz EA. Association of statin use with pathological tumor characteristics and prostate cancer recurrence after surgery. J Urol 2011;185:1268–73.
33. Gutt R, Tonlaar N, Kunnavakkam R, Karrison T, Weichselbaum RR, Liauw SL. Statin use and risk of prostate cancer recurrence in men treated with radiation therapy. J Clin Oncol 2010;28:2653–9.
34. Kollmeier MA, Katz MS, Mak K, Yamada Y, Feder DJ, Zhang Z, et al. Improved biochemical outcomes with statin use in patients with high-risk localized prostate cancer treated with radiotherapy. Int J Radiat Oncol Biol Phys 2011;79:713–8.
35. Schmitz G, Langmann T. Transcriptional regulatory networks in lipid metabolism control ABCA1 expression. Biochim Biophys Acta 2005;1735:1–19.