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); 1–8. ©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 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.

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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 μM L0901317 (Sigma) for 24 hours or 5 μM 5-aza-2-deoxycytidine (Siga) for 7 days, or a combination of the 2 compounds. ABCA1 promoter (-1,132 to +122 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 SsI (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). Luciferase reporter 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^ΔΔCt 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 DNease 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 [3H]-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 tissue specimens 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...
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 SssI 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
contributor of cellular cholesterol efflux, we also examined ABGG1 promoter methylation and expression in these cells. By MiGS analysis, the ABGG1 promoter is free of DNA methylation in PrEC, LNCaP, and DU 145 cells (Supplementary Fig. S3). All 3 cell lines expressed ABGG1 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;
cancers had a lower median expression and Gleason pattern. Moreover, there was an inverse correlation between ABCA1 expression and Gleason pattern 4 and 5. 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). Furthermore, 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).
Loss of ABCA1 Promotes Prostate Cancer

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.

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