

Cell-Free DNA Modification Dynamics in Abiraterone Acetate-Treated Prostate Cancer Patients



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Abstract

Purpose: Primary resistance to abiraterone acetate (AA), a key medication for the treatment of metastatic castration-resistant prostate cancer, occurs in 20% to 40% of patients. We aim to identify predictive biomarkers for AA-treatment response and understand the mechanisms related to treatment resistance.

Experimental Design: We used the Infinium Human Methylation 450K BeadChip to monitor modification profiles of cell-free circulating DNA (cfDNA) in 108 plasma samples collected from 33 AA-treated patients.

Results: Thirty cytosines showed significant modification differences (FDR $Q < 0.05$) between AA-sensitive and AA-

resistant patients during the treatment, of which 21 cytosines were differentially modified prior to treatment. In addition, AA-sensitive patients, but not AA-resistant patients, lost inter-individual variation of cfDNA modification shortly after starting AA treatment, but such variation returned to initial levels in the later phases of treatment.

Conclusions: Our findings provide a list of potential biomarkers for predicting AA-treatment response, highlight the prognostic value of using cytosine modification variance as biomarkers, and shed new insights into the mechanisms of prostate cancer relapse in AA-sensitive patients. *Clin Cancer Res*; 24(14); 3317–24. ©2018 AACR.

Introduction

Prostate cancer is the second most common cancer and the fifth leading cause of cancer-related death in men (1). Despite castrate levels of testosterone following androgen-deprivation therapy, castration-resistant prostate cancer exhibits elevated levels of prostate specific antigen (PSA) and can progress rapidly. Currently, one of the most effective medications for treatment of castration-resistant prostate cancer is abiraterone acetate (AA). AA blocks CYP17A1 and inhibits both 17 α -hydroxylase-dependent

cortisol synthesis and C17,20-lyase activity, which are key pathways in androgen synthesis (2). AA treatment improves survival and a number of surrogate endpoints, including decrease in PSA levels (3, 4). However, 20% to 40% of patients show primary resistance to AA (5). The reasons for AA resistance are unclear, but prior history of treatment with ketoconazole, enzalutamide, or chemotherapy is a contributing factor (6). Understanding why a substantial fraction of patients do not respond to AA and identification of prognostic biomarkers are critical to improving its clinical utility.

In order to identify epigenetic biomarkers of AA-treatment response and understand its mechanisms, we conducted a plasma cell-free DNA (cfDNA) modification (largely the sum of 5-methylcytosines and 5-hydroxymethylcytosines) study using the Infinium Human Methylation 450K BeadChip. Samples were collected from a cohort of 33 men who were treated with AA as part of their routine clinical care (Fig. 1). cfDNA consists of short fragments of DNA released from apoptotic cells, including tumor cells, into the blood or other bodily fluids. Therefore, it can be utilized as a noninvasive source of tumor DNA for diagnostic and prognostic biomarker discovery (7–11). We identified cfDNA modification differences in AA-sensitive and -resistant patients that could act as prognostic biomarker of AA-treatment response. Furthermore, AA-sensitive patients exhibited temporary reduction of interindividual variation of cfDNA modification profiles in early treatment phases, which later reverted back to high variation. Our findings suggest a new perspective on the dynamics of malignant growth in response to AA treatment.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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Translational Relevance

Abiraterone acetate (AA) is a commonly used medication for the treatment of metastatic castration-resistant prostate cancer. A large proportion of AA-treated patients, however, do not show clinical improvement. We performed cell-free epigenetic DNA analysis in the plasma of AA-treated patients and identified a series of cytosine modification differences in AA-sensitive compared to AA-resistant patients. Our findings highlight the prognostic value of changes in cytosine modification variance, rather than the mean differences, and shed new insights into the molecular mechanisms of secondary AA resistance.

Materials and Methods

Patients and treatment

Blood samples were collected at two Canadian centers (Princess Margaret Cancer Centre and BC Cancer—Vancouver Centre) from patients with prostate cancer who had progressed through androgen deprivation therapy and were starting AA-treatment (1000 mg/day) under ethics approval from the University Health Network (NCT01857908) and University of British Columbia—British Columbia Cancer Agency Research Ethics Board (H12-02547). Written informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

Briefly, blood samples were collected by venipuncture at baseline (i.e., before starting AA), and on subsequent checkups, in BD Vacutainer CPT tubes (BD Biosciences). After collection, blood samples were immediately centrifuged for 20 minutes at 1,800 rpm, and the plasma layer was transferred into a 15-mL

Falcon tube (Thermo Fisher Scientific). Processed samples were stored in -80°C . cfDNA was extracted using a QIAmp Circulating Nucleic Acid Kit (Qiagen) following the manufacturer's protocol. PSA levels were measured using standard laboratory procedures.

Classification of samples

Patients were classified into AA-resistant and AA-sensitive based on PSA progression by a physician using prostate cancer clinical trial recommendations during patient treatment, independent of laboratory studies and results (12). PSA levels were preselected as an objective and quantitative estimate of the AA-treatment response. As suggested by an earlier study (13), individuals with PSA level reduction greater than 50% from the pretreatment level for at least two consecutive visits (median duration between visits = 29 days) were deemed as AA sensitive. In the cohort of 33 investigated individuals, we identified 20 AA-resistant and 13 AA-sensitive patients (Supplementary Methods; Supplementary Fig. S1A).

Microarray data processing

All microarray experiments were performed blinded to the clinical classification of the subjects. In all, 128 samples were collected and, together with an additional 64 technical replicates ($N = 192$), their cytosine modification levels were interrogated using Infinium Human Methylation 450K BeadChip (Illumina). The microarray experiment was performed according to the manufacturer's protocol. The positions of the samples on the microarray cells were randomized to account for potential batch effects. Eleven samples failed to pass the internal microarray quality control and were not considered for further processing.

All microarray data were processed using the minfi (14) package for R. Raw modification values were extracted using getM and preprocessRaw functions. At each of the 485,577 cytosines interrogated by the microarray, cytosine modification level was summarized by calculating the M value defined as $\log_2 \left(\frac{M + 100}{U + 100} \right)$, where M (modified) and U (unmodified) are signal intensities (15). Typically, the technical median absolute deviation is 0.75% for cytosine modification levels ranging from 1.5% to 98.8% (16). Cytosines were annotated using the Illumina HumanMethylation450 manifest file (GEO GPL13534) and those from sex chromosomes were removed. In addition, polymorphic cytosines and cross-hybridizing probes (17) were also removed (total remaining cytosines = 382,378).

Microarray outlier removal and normalization

Outlier samples were identified from the modification data using the following algorithm: first, a k-nearest-neighbor imputation algorithm (impute.knn; ref. 18) was used to fill missing cytosine values. Next, using the raw data matrix, each sample was projected into two-dimensional space using multidimensional scaling that preserves sample distances while reducing dimensionality. For each sample, its mean distance to the 15 nearest samples was computed. The samples that deviated from the mean of the resulting distribution by more than three standard deviations were identified as outliers and removed from subsequent analyses ($N = 18$; Supplementary Fig. S2A).

Identification of robust biological signals from the microarrays

Our final dataset consisted of 108 cytosine modification profiles, with 55 technical replicates (total $N = 163$), from 33

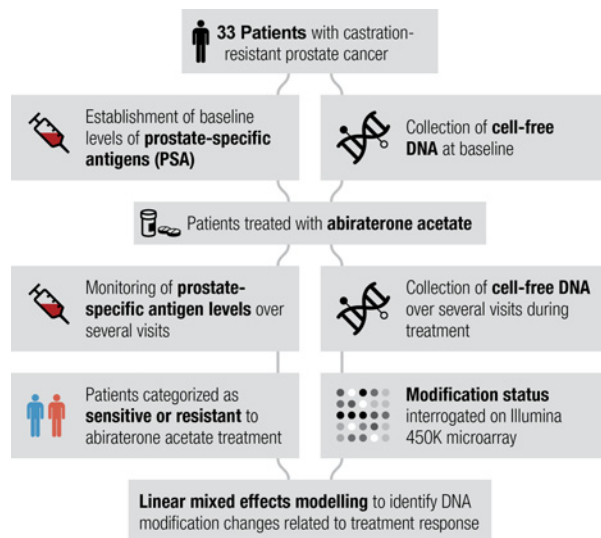


Figure 1.

Schematic illustration of experimental workflow. For each patient, levels of prostate-specific antigen (PSA) and cell-free DNA (cfDNA) modification profiles were obtained before AA administration, at multiple time points during treatment follow-up visits and after cessation of treatment. Plasma cfDNA was extracted and cytosine modification densities were interrogated on the Illumina Infinium Human Methylation 450K BeadChip.

individuals. After outlier removal, the modification profiles were normalized using stratified quantile normalization (preprocess-Quantile function; ref. 19) and M-values extracted from the normalized data were used further. Technical replicates were used to test whether global technical variability does not exceed biological variability (Supplementary Fig. S2B). In addition, we identified individual cytosines with biological variation exceeding technical variation and only cytosines with ANOVA $P < 0.05$ ($N = 237,300$) were retained for further analysis. The technical replicates were averaged for subsequent analyses.

Identification of confounding variables in the microarray data

Mean-centered modification data were used to perform principal component analysis (PCA). We excluded end-of-treatment samples due to their deviation from the general PSA trend. For each principal component, we fitted a mixed effects linear interaction model explaining the component score as the linear combination of the variables that might have confounded the study. Individual's age, white blood cell count estimates (20) from dnamage.ucla.edu, sample collection site, and sample collection batch were modeled as fixed effects, while individual's ID was modeled as a random effect. For each fixed effect variable, a null model without that variable was computed and models were compared using ANOVA. We found that the first 10 principal component scores were significantly associated with at least one confounding variable ($P < 0.05$; Supplementary Fig. S3), but none were associated with treatment-related variables. Therefore, in subsequent analyses, we used the scores of the first 10 principal components as surrogates to account for unwanted variability.

Mixed effects modeling of treatment effects on cytosine modification

Mixed effects linear interaction models were fitted for each cytosine across the visits (excluding end-of-treatment samples due to their deviation from the general PSA trend). Modification level was modeled as a response variable; visit time point, AA-sensitivity status, interaction of the two, individual's age, and the scores of the first 10 principal components were modeled as fixed effects; individual's ID was modeled as a random effect. Significant AA-differentially modified positions (DMPs) were identified by comparing a full model to a NoTreatmentEffects (NTE) model, which excluded AA sensitivity and interaction of sensitivity and visit time point variables using ANOVA. False discovery rate (FDR) was used to adjust P values for multiple testing (21). A *post hoc* analysis of significant AA-DMPs was performed as follows: (i) a model without the AA-sensitivity status variable and $Q < 0.05$ cutoff was used to identify AA-DMPs differentially modified at baseline and (ii) a null model without the interaction variable and $Q < 0.05$ cutoff was used to identify AA-DMPs that change modification level during treatment.

Permutation analysis was used to test whether more AA-DMPs with nominal $P < 0.05$ were observed than could be expected by chance in 100 iterations. At each iteration, the AA-sensitivity status was shuffled randomly across individuals and models were fitted for every cytosine as above. Permutation P value was defined as the fraction of iterations that yielded more cytosines with $P < 0.05$ than originally observed.

Enrichment analysis

Cytosines were associated with Refseq gene names using the microarray annotation file (GEO GPL13534). Whenever the

probe overlapped with two genes, both were used. The list of gene names was generated from AA-DMPs significant at FDR $Q < 0.2$ and uploaded to DAVID for functional analysis (22). Genes that were associated with any interrogated cytosine positions were used as the background. For GO annotation, we selected "GOTERM_BP_ALL" and "GOTERM_BP_DIRECT" in the "Gene_Ontology" section. For "Protein_Domains," only the "INTERPRO" category was used, where functionally significant protein domains were identified based on a predictive model to classify proteins into families and domains (23).

Analysis of differential variability

iEVORA (24) was used to compute differential variability given groups of samples. The test computes t test and Bartlett test for each cytosine. Cytosines with t test $P < 0.05$ and FDR corrected Bartlett test $Q < 0.05$ were deemed as differentially variable positions (DVPs). Next, two-dimensional representations of data were obtained for each visit using the DVPs identified between patient groups at that particular visit. Multidimensional scaling was used to obtain the representations as it preserves sample distances while reducing dimensionality.

Computational resources and data availability

Computations were performed on the CAMH Specialized Computing Cluster. All data have been deposited at Gene Expression Omnibus (GEO) under the accession code GSE108462.

Results

Thirty cytosines were differentially modified in the AA-sensitive patients compared with the AA-resistant patients

The summary of sample distribution across visits is provided in Supplementary Table S1 and sample clinical information is provided in Supplementary Table S2. We found that 26,874 (11.3%) cytosines were differentially modified (nominal $P < 0.05$) when comparing AA-sensitive with the AA-resistant patients, which we refer to as AA-differentially modified positions (AA-DMP). Permutations ($N = 100$) by randomly shuffling the AA-sensitivity status showed that the number of AA-DMPs was significantly higher than expected by chance ($P = 0.03$). After correction for multiple testing, we observed 30 AA-DMPs at FDR $Q < 0.05$ associated with 22 genes (Fig. 2) and 2,668 AA-DMPs associated with 1,820 genes at a more relaxed FDR $Q < 0.2$ (Supplementary Table S3).

We classified the 30 AA-DMPs into three categories based on the predictions of the fitted model: differentially modified at baseline (before treatment), during treatment, or both. Each category was further split by the direction of change in the AA-sensitive patients relative to the AA-resistant patients (Fig. 2). There were 21 AA-DMPs that were identified by the model to exhibit significant differences between the AA-sensitive and the AA-resistant patients at baseline. Eleven of them had higher density of modified cytosines among the AA-sensitive compared with the AA-resistant individuals (mean gain of modification \pm SD = 4.7% \pm 2.7%). These AA-DMPs were associated with cancer-related genes, such as PYD and CARD Domain Containing (PYCARD), Signal Transducer And Activator Of Transcription 5A (STAT5A), and Cathepsin S (CTSS). The remaining 10 showed lower degrees of modification in AA-sensitive individuals (mean loss of

IlmnID	Gene	CpG region	Baseline difference	Treatment change (per day)	Associated clinical outcome	Associated cancer type
cg16051361	<i>RAB21</i>	Shore	-6.115	-8.3×10^{-3}	Increased cancer cell migration, adhesion to collagen and bone	Multiple, including PCa
cg25617519	<i>KLHL29</i>		-2.488	-1.9×10^{-2}		
cg08112737	<i>KIAA1211</i>	Shelf	-2.488	-4.2×10^{-4}		
cg08889336		Island	-1.272	-5.8×10^{-3}		
cg08730348	<i>PYCARD</i>	Shore	2.737	-6.3×10^{-3}	Reduced tumor apoptosis, increased aggressiveness	Multiple, including PCa
cg11474701			3.761	2.5×10^{-2}		
cg20782117	<i>SLC25A13</i>		6.03	-7.4×10^{-4}		
cg00041599	<i>UNC5A</i>	Shore	-7.318	6.8×10^{-2}	Reduced tumor suppression	Multiple
cg25461186	<i>MLXIP</i>	Shore	-4.339	5.7×10^{-2}	Reduced tumorigenesis	Multiple
cg09185884	<i>KCTD2</i>		-4.126	8.9×10^{-2}		
cg12725706		Shore	-4.099	2.4×10^{-2}		
cg04028224	<i>RAB37</i>	Island	-3.843	4.2×10^{-2}	Disrupted metastasis and tumor angiogenesis suppression	Lung cancer, ESCC
cg01815536	<i>TSHB</i>		-2.045	2.7×10^{-2}	Increased PCa risk	PCa
cg14899522	<i>PRKCQ</i>		1.865	-3.4×10^{-2}	Induced tumorigenesis	Breast cancer
cg23331010	<i>ST3GAL6</i>		2.445	-1.3×10^{-2}		
cg08972357	<i>C5orf37</i>	Shelf	2.555	-3.1×10^{-2}		
cg11547104	<i>SORCS2</i>	Island	3.134	-2.0×10^{-2}		
cg03313364	<i>STAT5A</i>	Island	3.685	-3.6×10^{-2}	Increased sensitivity to AR	PCa
cg02121529		Island	6.775	-8.2×10^{-2}		
cg15900387	<i>CTSS</i>		8.287	-4.1×10^{-2}	Increased tumor angiogenesis and invasiveness	Multiple, including PCa
cg25610515	<i>LUZP1</i>		10.22	-8.9×10^{-2}		
cg20744163	<i>ZMIZ1</i>	Shelf	-1.438	-1.1×10^{-2}	Increased AR sensitivity and activity	PCa
cg11610350		Island	-0.64	-8.3×10^{-2}		
cg16771771		Shore	0.612	-7.3×10^{-2}		
cg09577144	<i>DNAH17</i>		-0.354	-5.0×10^{-2}		
cg10984178		Shelf	0.095	-2.9×10^{-2}		
ch.10.7244193R			-0.695	2.9×10^{-2}		
cg09927651	<i>ABR</i>		-1.109	3.9×10^{-2}		
cg00531453	<i>TMEM229A</i>	Shore	-1.953	7.9×10^{-2}		
cg07056285	<i>PRDM6</i>	Shore	-1.404	9.4×10^{-2}		

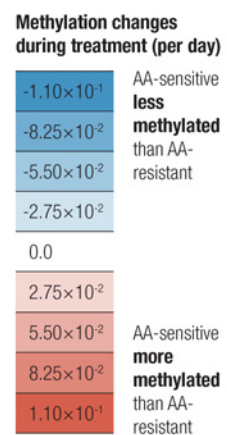
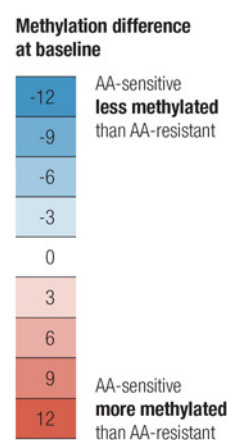


Figure 2. AA-DMPs identified at FDR $Q < 0.05$ and associated genes. Baseline difference is the difference in percentage of cytosine modification between groups before treatment. Treatment change represents methylation percentage change per day of treatment in AA-sensitive patients. Colored cells reflect significant changes (*post hoc* analysis $P < 0.05$). Associated clinical outcome and associated cancer type are based on literature search. P values and references are available in Supplementary Table S3. PCa, prostate cancer; ESCC, esophageal cancer; AR, androgen receptor.

modification \pm SD = $-3.8\% \pm 1.9\%$); these AA-DMPs were associated with genes *RAB21*, member RAS oncogene family (*RAB21*), Thyroid Stimulating Hormone Beta (*TSHB*), and MLX Interacting Protein (*MLXIP*), among others.

There were 23 AA-DMPs that emerged during treatment, 14 of which overlapped with AA-DMPs predicted to be differentially modified at baseline. Cytosines associated with *MLXIP*, *TSHB*, *RAB37*, and several other genes, showed increasing modification during treatment among the AA-sensitive patients, while cytosines associated with Zinc Finger MIZ-Type Containing 1 (*ZMIZ1*), *STAT5A*, *CTSS*, and several other genes lost modification.

Ten of the genes associated with AA-DMPs were previously identified to show differential steady state mRNA levels in tumor tissue, with six directly linked to prostate cancer (Fig. 2). We also performed functional enrichment analysis using DAVID (22) with the 1,820 associated genes identified at FDR $Q < 0.2$. The analysis showed an enrichment of genes in the Cadherin superfamily and genes containing Homeodomains, with gene ontology

(GO) enrichment for biological processes associated with cell adhesion and development (Supplementary Table S4).

AA-sensitive individuals acquire permanent changes at the end of treatment

Since the end-of-treatment samples were not included in the AA-DMP analysis, we performed hierarchical clustering of the pretreatment and the end-of-treatment samples using the 30 FDR significant AA-DMPs ($Q < 0.05$), to see if any systematic molecular changes had taken place following the treatment. Our analysis showed that among the AA-sensitive patients, pretreatment and end-of-treatment samples formed two distinct clusters, with one exception in each cluster (Fig. 3A). In contrast, among the AA-resistant patients, no distinguishable clustering pattern was detected (Fig. 3B). This further validates the significance of AA-DMPs and indicates that new distinct cytosine modification profiles have emerged in the cfDNA of AA-sensitive patients following the treatment.

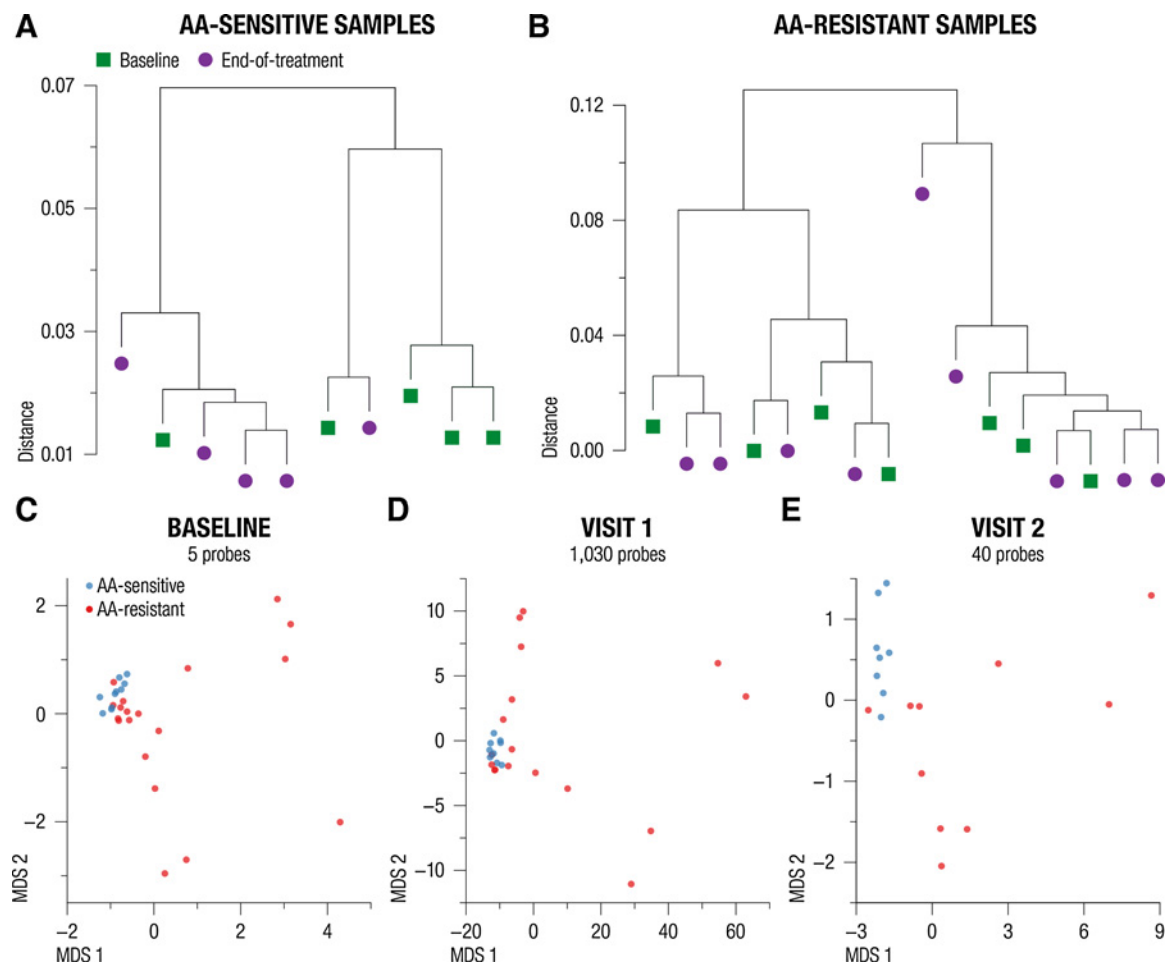


Figure 3.

Cytosine modification clustering of AA-DMPs and AA-DVPs. Hierarchical clustering dendrograms of the pretreatment and the end-of-treatment samples using the 30 AA-DMPs in (A) the AA-sensitive and (B) the AA-resistant patient groups. Green squares represent individual cytosine modification patterns of AA-DMPs at baseline, while purple circles show individual profiles at the end of treatment. Distance was measured as $1 - \text{Pearson correlation coefficient}$. C-E, Multidimensional scaling representation of the samples performed using AA-DVPs identified at each visit. AA-sensitive patients are marked in blue circles and AA-resistant patients in red circles. Compared with the AA-resistant group, AA-sensitive patients form a tighter cluster at (C) baseline and (D) visit 1, indicating lower interindividual variability. The cluster is diffused by (E) visit 2. AA, abiraterone acetate; MDS, multidimensional scaling.

Table 1. Number of DVPs measured between each visit and pretreatment for each group separately

Visit	AA-sensitive individuals			AA-resistant individuals		
	No. of DVPs	More variable at		No. of DVPs	More variable at	
		Baseline	Treatment		Baseline	Treatment visit
I	520	504	16	1	1	0
II	23	21	2	2	1	1
III and IV	4	4	0	2	2	0
End of treatment	0	0	0	8	8	0

Cytosine modification variability drops and surges back among AA-sensitive individuals

Higher cfDNA modification variability detected in prostate cancer and other tumors may reflect the epigenetic diversity of malignant cells (25). Increased variation of cytosine modification has been consistently detected in the tumors (26), as well as in the surrounding nonmalignant tissues (24) and premalignant cells (27). Motivated by this, we analyzed cytosine modification variability in cfDNA of AA-treated patients.

First, we checked whether cfDNA modification variability changed during AA treatment with respect to the baseline in each patient group (Table 1). Among the AA-sensitive patients, we detected 520 differentially variable positions (AA-DVPs; FDR $Q < 0.05$) between baseline and the first follow-up visit during treatment, of which 97% showed higher pretreatment variability. In contrast, the same comparison revealed only one AA-DVP among the AA-resistant patients. When comparing the second visit to baseline, we detected 23 AA-DVPs among the AA-sensitive patients, 21 of which were more variable at baseline. There were only two such AA-DVPs among the AA-resistant patients. Treatment visits three and four taken together yielded negligible numbers of AA-DVPs in both patient groups.

Next, we tested whether the variability differed between the AA-sensitive and AA-resistant patients across each visit. While only five AA-DVPs were identified pretreatment, this number rose to 1,030 during the first visit, and then declined to 40 during the second visit and eight beyond this time point (Table 2). Of the 1,030 AA-DVPs observed in the first visit, 1,025 were more variable among the AA-resistant patients. The same trend was observed during the second visit; 38 of 40 AA-DVPs were more variable among the AA-resistant patients.

There was a significant overlap between AA-sensitive DVPs (mainly identified during the first visit in the AA-sensitive group) with the AA-response DVPs (identified between AA-sensitive and AA-resistant groups; Fisher exact test OR = 67; $P = 1.28 \times 10^{-13}$; Supplementary Table S5), suggesting that these changes were driven by the same process. Like AA-DMPs, functional enrichment analysis of the 1,030 AA-DVPs mapping to 674 genes identified in the first visit showed enrichment of genes related to Cadherin and Homeodomain and processes associated with cell adhesion and development (Supplemen-

tary Table S4), suggesting that both AA-DMPs and AA-DVPs are driven by similar processes.

Finally, we asked if interindividual distances computed using AA-response DVPs can discriminate AA-sensitive and AA-resistant individuals during the early stages of treatment. We used multidimensional scaling for each visit separately to project and visualize the individuals in a two-dimensional space, while retaining their original pairwise distances (Fig. 3C–E). We found that AA-sensitive patients formed a dense cluster even prior to AA treatment but some AA-resistant patients were also a part of this cluster. During the first follow-up visit, AA-sensitive patients formed a distinctly dense cluster, whereas most AA-resistant patients were scattered in space. By the second follow-up visit, neither group of patients formed a dense cluster, but each occupied a distinct region of space. Our results suggest that cfDNA similarity computed from AA-DVPs has the potential to predict which patients will respond well to AA treatment; however, a formal sensitivity/specificity analysis using cross-validation was not feasible in this dataset due to small sample size.

Discussion

We investigated changes in cytosine modification associated with AA treatment in a cohort of patients with castration-resistant prostate cancer using plasma cfDNA. We identified 30 AA-DMPs, which occurred during treatment. Our unsupervised clustering analysis showed that cfDNA modification profiles diverged following AA treatment in the AA-sensitive but not in the AA-resistant patients. During the early stages of treatment, the AA-sensitive patients exhibited loss of variability of cytosine modification compared with the AA-resistant patients. Finally, we showed the potential for predicting AA-treatment response based on cfDNA modification variability.

Many of the genes containing cytosine modification changes were related to survival of malignant cells in low androgen environments. For instance, *STAT5A*, which is known to be up-regulated in prostate cancer (28), showed reduced modification during treatment in two cytosines covering 5'UTR region. *STAT5A/B* directly interact with androgen receptors and increase their activity, as well as protect antiandrogen-bound androgen receptors from degradation in proteasomes (29, 30). Consistently, *STAT5A/B* enable prostate cancer cells that do not express androgen receptors to survive by switching to an androgen-independent growth (28, 31). Similarly, *ZMIZ1*, an androgen receptor transcriptional coactivator (32) that is linked to development of androgen independence during treatment with antiandrogen agents, also showed reduction of cytosine modification during the AA treatment in two cytosines. Both of these genes showed a loss of cytosine modification only in the AA-sensitive patients during AA treatment, which may explain the secondary resistance to AA.

Table 2. Number of DVPs measured across patient groups for each visit

Visit	No. of DVPs	Less variable in	
		AA-sensitive	AA-resistant
Baseline	5	5	0
I	1,030	1,025	5
II	40	38	2
III and IV	3	0	3
End of treatment	0	0	0

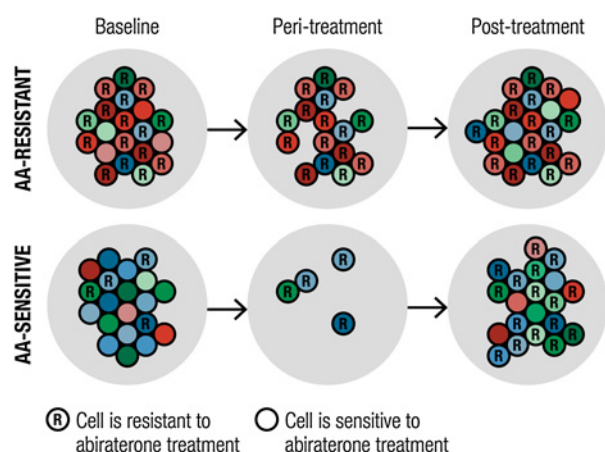


Figure 4.

Hypothetical dynamics of clonal complexity following AA treatment. At baseline, both AA-sensitive and AA-resistant patients have high clonal complexity, and no difference in cytosine modification variance is observed. However, patients differ dramatically in terms of the proportion of AA-sensitive and AA-resistant malignant cells. During treatment, most of the nonresistant cells are killed off in both groups, which results in drastic reduction of cytosine modification variability in the AA-sensitive patients. By the end of treatment, the resistant cells repopulate the tumor and high clonal diversity is restored.

Terms enriched in the gene ontology analysis also suggest that AA-treatment-associated cytosine modification changes contribute to cancer progression. Cadherin proteins, the genes of which included multiple AA-DMPs and AA-DVPs, are key factors in cancer progression, including metastasis, cell adhesion, drug resistance, and angiogenesis (33). AA-DMPs were also enriched for Homeobox (HOX) genes, which are upregulated in most solid tumors (34). Aberrant regulation of HOX genes in prostate cancer is associated with androgen-independence, loss of differentiation potential, and reduced apoptosis rate (35–37). Our results suggest that AA treatment is associated with cytosine modification changes at the genes accounting for carcinogenesis in AA-sensitive patients.

Because decreased cfDNA modification variability is followed by the emergence of new distinct profiles in AA-sensitive patients and a sharp increase in PSA levels (Supplementary Fig. S1B), we assume that this indicates a resurgence of malignant cell growth and clinical relapse. More aggressive malignant cells, which are resistant to androgen-deprivation therapy, may emerge and lead to secondary AA resistance (refs. 5, 13; Fig. 4). The initial decrease in variability of cytosine modification among the AA-sensitive patients supports selective elimination of malignant cell populations. Changes in the cellular complexity, however, may not be limited to the tumor but also involve tumor surrounding premalignant cells which are known to exhibit increased DNA modification variance, called "field

defects" (24). Even nonmalignant cells, such as ones expressing CYP17A1, may react to AA treatment via metabolic or other changes and contribute to the overall variation of cfDNA modification profiles. Identifying the true proportion of tumor DNA in cfDNA is of significant challenge, as each tumor can be heterogeneous with a complex spectrum of somatic mutations. Relatedly, studies that have investigated tumor DNA proportion in cfDNA show tremendous variability in the amount of tumor DNA in plasma, ranging from nearly 0% all the way to 23% (38). Dedicated experiments may be necessary to isolate the true source of cytosine modification and variability in cfDNA.

On a practical level, a complete understanding of the origins of AA-DMPs and AA-DVPs is not necessary for their clinical usage as biomarkers of AA-treatment response. Our study suggests that cfDNA modification analysis could be a productive line of research for the identification of clinically useful biomarkers for predicting drug response during the early stages of cancer treatment. Future efforts in this field of interest may benefit from the examination of associations between cfDNA modification and other treatment outcomes, such as radiographic response.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A. Kwan, R. Kustra, A.M. Joshua, A. Petronis

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