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DAB2IP Status in High-Risk Prostate Cancer Correlates with Outcome for Patients Treated with Radiation Therapy

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Abstract

Purpose—This pilot study investigates the role of DAB2IP and EZH2 as prognostic biomarkers in high-risk prostate cancer patients receiving definitive radiation therapy.

Methods and Materials—Immunohistochemistry was performed and scored by an expert genitourinary pathologist. Clinical endpoints evaluated were freedom from biochemical failure (FFBF), castration resistance-free survival (CRFS), and distant metastasis-free survival (DMFS). Log-rank test and Cox regression were used to determine significance of biomarker levels with clinical outcome.

Results—Fifty-four patients with high-risk prostate cancer (stage T3a, or Gleason score 8, or PSA ≥ 20) treated with radiation therapy from 2005-2012 at our institution were evaluated. Nearly all patients expressed EZH2 (98%), whereas 28% of patients revealed DAB2IP-reduction and 72% retained DAB2IP. Median follow up was 34.0 months for DAB2IP-reduced patients, 29.9 months for DAB2IP-retained patients, and 32.6 months in the EZH2 study. DAB2IP reduction portended worse outcome compared to DAB2IP-retained patients, including FFBF (4-year: 37% vs. 89%, p

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= 0.04), CRFS (4-year: 50% vs. 90%, $p = 0.02$), and DMFS (4-year: 36% vs. 97%, $p = 0.05$). Stratified EZH2 expression trended toward significance for worse FFBF and CRFS ($p = 0.07$). Patients with reduced DAB2IP or highest intensity EZH2 expression exhibited worse FFBF (4-year: 32% vs. 95%, $p = 0.02$), CRFS (4-year: 28% vs. 100%, $p < 0.01$), and DMFS (4-year: 39% vs. 100%, $p = 0.04$) compared to the control group.

Conclusion—DAB2IP loss is a potent biomarker that portends worse outcome despite definitive radiotherapy for patients with high-risk prostate cancer. EZH2 is expressed in most high-risk tumors and is a less potent discriminator of outcome in this study. DAB2IP status in combination with degree of EZH2 expression may be useful for determining patients with worse outcome within the high-risk prostate cancer population.

Keywords

Biochemical failure; Castration resistance; DAB2IP; EZH2; Prostate cancer

Introduction

Prostate cancer is the most common male malignancy in the United States with 238,000 cases and 29,720 deaths expected in 2013,¹ of which 20-30% have high-risk features.² For high-risk prostate cancer defined by the National Comprehensive Cancer Network (NCCN) as Stage cT3a, Gleason score (GS) 8, or prostate-specific antigen (PSA) 20, standard treatment guidelines include definitive radiation therapy with neo-adjuvant, concurrent, and long-term androgen deprivation therapy in the United States.³ In spite of standard therapy, long-term outcomes are suboptimal with 5-year biochemical progression-free survival of 60-70% and 5-year overall survival of 75-85%.⁴⁻⁷

Identification of patients who are most likely to have poor outcomes despite dose escalated radiation therapy with long term androgen deprivation can be challenging. Being able to predict the patients that portend worse outcome at the time of diagnosis may provide means to offer patients an alternative, more effective therapy, possibly through auspices of clinical trial. Multiple biomarkers have been studied in the setting of high-risk prostate cancer,^{8,9} and while some have shown much promise, routine use of such markers in clinical practice has been limited.¹⁰ Additional biomarkers that can readily identify patients most likely to fail conventional therapy are warranted, and investigations of the molecular pathway involved in this poor outcome may further lead to future therapeutic interventions.

Disabled homolog 2-interacting protein (DAB2IP), a member of the RAS-GAP protein family, is a novel tumor suppressor. Decreased expression of DAB2IP is often detected in prostate cancer cells, and this loss of DAB2IP is primarily due to altered epigenetic regulation of its promoter.¹¹ A genome-wide screening of single nucleotide polymorphisms (SNPs) indicated that a genetic variant in DAB2IP is linked with aggressive prostate cancer.¹² Furthermore, a population based case-control study confirmed that DAB2IP was linked with an increased risk of aggressive prostate cancer.¹² Immunohistochemistry analysis of more than 200 patients with prostate cancer by Min et al. clearly demonstrated the causal role of DAB2IP loss in prostate cancer progression.¹³ The aggressive nature of DAB2IP-deficient tumors is explained by recent cell culture model studies which have

found that loss of DAB2IP can lead to radiation resistance, increased DNA repair ability, and decreased apoptosis after radiation.¹⁴⁻¹⁶

Enhancer of Zeste homolog 2 (EZH2), the catalytic unit of the polycomb repressor complex, is often upregulated in several cancers including breast, lung, and prostate.¹⁷ Our data indicate that DAB2IP is an EZH2 target gene in prostate cancer.¹⁸ Overexpression of EZH2 has been shown to drive prostate cancer progression, and studies have shown that clinically localized tumors that express high levels of EZH2 have poorer outcomes.^{19, 20} Additionally, it has been shown that in low-risk tumors EZH2 expression has been correlated with poor surgical outcomes.¹⁹

While several cell culture and animal based models have shown that DAB2IP and EZH2 are involved with increased radiation resistance,^{14, 15, 21} there is no study that evaluates the clinical relevance of DAB2IP-mediated radiation resistance. Therefore, we sought to determine the prognostic value of DAB2IP and its upstream regulator EZH2 in high-risk prostate cancer patients.

Methods and Materials

Patient selection

All patients with prostate cancer treated with external beam radiation at our institution between December 2005 and July 2012 were identified. Of the 658 patients identified, 138 patients met the NCCN guidelines for high-risk classification (Stage T3a, or Gleason score 8, or PSA ≥ 20) and did not receive any surgical management. Patients who had metastases prior to initial radiation therapy were excluded from the study. All patients were treated definitively with external beam radiotherapy using a variety of techniques, including dynamic arc therapy and all patients were treated with a dose exceeding 72 Gy, and 80% were treated to 79.2 Gy. Of the 138 patients identified, only those patients who provided signed consent, who had preserved pretreatment prostate biopsy tissue available, and whose biopsies had a sufficient tumor sample available for analysis were included in the study.

Specimen characteristics, staining, and quantification

Diagnostic needle biopsies were mounted in paraffin, and 3-4 µm sections were prepared for standard hematoxylin and eosin staining. Standard immunohistochemistry analysis was performed for DAB2IP and EZH2. Immunostain was performed using the Benchmark XT automated stainer (Ventana, Tuscan, AZ, USA). Briefly, formalin-fixed, paraffin-embedded tissue microarray sections were cut at 3-4 µm and air-dried overnight. The sections were deparaffinized, rehydrated, subjected to heat-induced epitope retrieval, and then incubated with anti-EZH2 (clone:SP129, Ventana, prediluted) or anti-DAB2IP²² (homegrown, 1:150) primary antibodies. UltraView universal detection system (Ventana) was used for signal detection. The slides were developed using 3-3'-diaminobenzidine chromogen and counterstained with hematoxylin. Specific positive and negative (slides incubated without primary antibody) controls were utilized for each run of immunostains and checked for validation of the assay. Controls for anti-EZH2 antibody included tissue from prostate and breast adenocarcinoma and normal lymph node that are known to have high EZH2

expression. Anti-EZH2 protocol was standardized according to the directions in the package insert. This antibody is intended for *in vitro* diagnostic use. Protocol for anti-DAB2IP was performed as published previously.²²

Tumor and benign prostate tissue stained with each marker were evaluated by an experienced genitourinary pathologist who was blinded to patient clinical information. Each case was evaluated for the extent (percentage of positive cells) and intensity of staining. The average intensity of positive tumor cells was given a grade (G) score: G0, none; G1, weak; G2, intermediate; and G3, strong. DAB2IP positivity was evaluated as cytoplasmic expression. EZH2 was evaluated as nuclear pattern of expression. Compared to DAB2IP expression in the benign 'control' prostate tissue surrounding the neoplastic cells, tumor DAB2IP status was categorized as retained (same or stronger expression) or reduced (weaker expression). EZH2 expression was scored as the product of percentage of cells staining and the intensity of staining.

Study design

The three primary clinical endpoints in this study were freedom from biochemical failure (FFBF), castration resistance-free survival (CRFS), and distant metastasis-free survival (DMFS). FFBF was determined using the Phoenix definition, which denotes a PSA increase of 2.0 ng/ml above the nadir PSA level.²³ CRFS was defined as 2 episodes of rising PSA while on standard hormone therapy in the setting of testosterone levels < 50 ng/ml, or if patients were started on second line therapy as first line hormone therapy was deemed to have failed at the clinical oncologist's discretion. DMFS was determined from clinical chart review documenting date of known metastasis by imaging. Time to each endpoint was calculated from the first day of radiation therapy.

Statistical analysis

In this study there were two main biomarkers: DAB2IP and EZH2. A two-sided log-rank test with an overall sample size of 46 for DAB2IP and 48 for EZH2 achieves 80% power at a 0.025 significance level to detect a significant difference in 4-year FFBF rates between the two groups. The sample size estimation was conducted using the sample size software PASS 11. Kaplan-Meier estimates and log-rank tests were computed to estimate the FFBF, CRFS, and DMFS. Univariate Cox regression analysis of T stage, N stage, highest Gleason score, pretreatment PSA, percent of positive biopsy cores, total radiation dose, hormone therapy, and duration of hormone therapy was performed. Finally, Fisher's exact test was used to evaluate statistical differences among these factors based on the status of the tumor biomarkers.

Results

Fifty-four patients with high-risk prostate cancer treated with definitive radiation therapy from 2005-2012 met the inclusion criteria and were evaluated. The median age of patients used for analysis was 66 years old. Twenty eight percent (13/46) of patients revealed DAB2IP-reduction while 72% (33/46) retained DAB2IP. Nearly all patients expressed EZH2 (98%) and the intensity level was G0 in 1 (2%), G1 in 9 (19%), G2 in 29 (60%), and

G3 in 9 (19%) patients. The median pretreatment PSA was 14.8 ng/ml (range 1.2-208.9). Gleason scores were 7 in 22% of patients and 8-10 in 78% of patients. Clinical T stage category was T1c in 19% of patients, T2a-c in 56% of patients, and T3a-b in 22% of patients. Table 1 displays a distribution of patient characteristics.

The median follow-up was 34.0 months (range 6.7-76.1) for DAB2IP-reduced patients, 29.9 months (range 6.1-84.6) for DAB2IP-retained patients, and 32.6 months (range 2.8-84.6) for patients in the EZH2 study. In the DAB2IP study, there was no statistically significant difference between use of hormone therapy ($p = 0.55$) or duration of hormone therapy ($p = 0.63$). Of the patients in the EZH2 study who received hormone therapy, there was no significant difference in duration ($p = 0.81$).

Interestingly, reduction of DAB2IP in patient's prostate cancer samples correlated strongly with several poor outcome measures. Reduction of DAB2IP portended a significantly lower FFBF (Fig. 1A, $p = 0.04$) and increased likelihood of progression to castration resistance ($p = 0.017$). Patients with reduced DAB2IP compared to patients retaining DAB2IP had a 4-year FFBF of 37% and 89%, respectively. The 4-year CRFS for DAB2IP-reduced patients was 50% while the 4-year CRFS was 90% for DAB2IP-retained patients. Loss of DAB2IP was not a statistically significant predictor of increased distant metastasis ($p = 0.05$), though it trended towards significance. Increased follow-up or a larger data set will most likely result in a significant outcome. The 4-year DMFS was 36% for DAB2IP-reduced patients and 97% for DAB2IP-retained patients. Median time to biochemical failure was 29.7 months and 23.7 months for the DAB2IP retained and reduced groups, respectively. The median time to castration resistance was 29.9 months and 27.6 months, while the median time to distant failure was 29.9 months and 26.7 months for the DAB2IP-retained and -reduced groups, respectively.

Analysis of EZH2 samples shows that nearly all high-risk patients exhibit expression of EZH2 (98%). Stratification of EZH2 expression was not significantly predictive of decreased FFBF (Fig. 1B, $p = 0.07$), increased castration resistance ($p = 0.07$), or decreased distant metastasis rate ($p = 0.2$), although it trended toward significance.

It is interesting to note, however, that combining patients who had either EZH2 G3 staining or decreased DAB2IP levels created a group found to have significantly worse outcomes. The 4-year FFBF for this combined group compared to the control group (retained DAB2IP and EZH2 G0-G2) was 32% vs. 95% (Fig. 1C, $p = 0.0153$), the 4-year CRFS was 28% vs. 100% ($p = 0.0019$), and the 4-year DMFS was 39% vs. 100% ($p = 0.04$), respectively. Also, the combined group of DAB2IP-reduced or EZH2 G3 patients accounted for six of the seven (86%) patients who were identified to have clinical castration resistance. Table 2 summarizes all patient outcomes by tumor biomarker status.

Of the 7 patients with castration resistance, their initial androgen deprivation therapy was leuprolide except for one patient who chose orchiectomy. Five of the patients had testosterone levels measured within one year prior to developing castration resistance and the remaining two patients did not have any serum testosterone levels recorded in their medical records. The five patients with serum testosterone data had the following levels

immediately prior to or on the day they were determined to have castration resistance: 3.7, 9, <12, 19, and 58 ng/ml. The patient with the testosterone level of 58 ng/ml as well as the two patients without measured testosterone levels were determined to have clinical evidence of castration resistance because of a continual rise in PSA despite adding a second agent to their androgen deprivation therapy.

Univariate analysis using Cox regression of typical prognostic variables versus FFBF revealed that only the highest Gleason score ($p = 0.0290$) was statistically significant (see Table 3). EZH2 grade ($p = 0.07$) trended toward significance. Although tumor DAB2IP status was nearly significant by Cox regression ($p = 0.055$), as stated earlier the log-rank test for DAB2IP versus FFBF was significant ($p = 0.04$). Further analysis showed that reduction of DAB2IP is strongly correlated with increasing Gleason score (Fig. 2, $p = 0.0087$), suggesting that DAB2IP reduction leads to more aggressive phenotypes of prostate cancer.

Discussion

DAB2IP, a tumor suppressor and member of the RAS-GAP family, has been linked with an increased risk of aggressive cancer.¹² While the exact function of DAB2IP has yet to be determined, studies have shown that loss of DAB2IP leads to hyper-activation of the PI3k-Akt axis.¹⁶ DAB2IP deficient cell lines are more resistant to ionizing radiation due an increased ability to repair double strand DNA breaks and promote robust G2/M cell cycle arrest.^{14, 21} Furthermore, cells which lack DAB2IP are more likely to undergo autophagy, rather than apoptosis, in response to ionizing radiation.¹⁵ Based on strong preclinical data indicating the role of DAB2IP in radiation response in prostate cancer cells, we sought to determine its clinical impact¹⁵. However, the tumor environment is a complex milieu with multiple interacting activators and inhibitors. Therefore, in addition to studying DAB2IP, we also sought to determine the potential prognostic role of its upstream regulator, EZH2, in high-risk prostate cancer patients treated with radiation therapy.

In this pilot study we show that DAB2IP status and not EZH2 appears to correlate with earlier biochemical failure rates after treatment with definitive radiation therapy in high-risk prostate cancer patients. Interestingly, we found that nearly all high-risk prostate cancer samples in this study express high levels of EZH2, confirming the notion that increased EZH2 precedes loss of DAB2IP. Our results are concordant with previous studies that show increased levels of EZH2 portends a poorer outcome, or more specifically, increased EZH2 levels can predict high-risk prostate cancer. It is worth emphasizing that the trend toward worse outcomes occurred despite the patients with higher EZH2 levels having been significantly more likely to receive hormone therapy ($p = 0.0417$). Varambally et al.²⁰ noted that EZH2 G3 staining or greater was associated with increased clinical failure (31%) while significantly fewer patients (9%) with low intensity staining had clinical failure. While the patients evaluated in Varambally's study received surgical management and not radiation, the results support the conclusions presented here.

Rather than predict cancers with poor therapeutic response, EZH2 levels may help screen for higher risk cancers. In addition, it is possible that if the cohort were expanded to include all prostate cancer patients (including low- and intermediate-risk patients), then EZH2 will

likely gain significance, and it may serve as an earlier marker of tumors that may have propensity towards high-risk differentiation. However, within the high-risk cohort of patients, DAB2IP appears to be a more suitable prognostic marker as it appears to be able to discriminate out the very worst players among this locally advanced aggressive prostate cancer group. We postulate that the higher the intensity of EZH2 staining, the more likely a tumor is to have decreased DAB2IP expression, and thus the more likely a patient is to have poor outcome. This theory is the logic behind using the “or” in our combined grouping system, that perhaps tumors with EZH2 G3 and retained DAB2IP will eventually manifest future DAB2IP loss and consequently become more aggressive.

There have been numerous studies into the use of biomarkers in prostate cancer, and while most are used for the evaluation of surgical patients, several studies have studied markers of radiation outcomes. The most successful studies have examined markers such as Ki-67, Bax, BCL-2, and BCL-xL which are markers of apoptosis.^{8,9} However, results from these studies are heterogeneous and conflicting. DAB2IP may be better able to differentiate the worst cases amongst the already high-risk group of prostate cancer patients. In this pilot study, DAB2IP appears to be a significant prognostic indicator of failure after radiation therapy; however, DAB2IP requires further studies to validate its role in this cohort of patients, and to further determine whether it may also be a predictive marker of response to radiation therapy. Limitations of this study include the small sample size, short follow up for patients with EZH2 G0 and G3, and the fact that not all 54 patients had adequate biopsy samples to be analyzed for both DAB2IP and EZH2. With increased sample size and longer follow up, it is possible that stratified EZH2 expression may also be a useful and statistically significant prognostic biomarker. As this is a pilot study, a prospective validation study with a larger sample size is warranted.

We report that loss of DAB2IP may predict worse FFBF and increased likelihood of progression to castration resistance. Decreased FFBF in DAB2IP-deficient cells may be explained by dysregulation of survival pathways in response to ionizing radiation as described in several pre-clinical models. In addition, high Gleason scores correlate with a loss of DAB2IP. These findings may indicate that loss of DAB2IP may be a driver of prostate cancer progression, or alternatively that DAB2IP loss may be a hallmark for aggressive prostate cancer behavior. A recently published study determined that androgen binding decreases EZH2 level with a concomitant increase in DAB2IP level,²⁴ which is consistent with our previous observation that DAB2IP is a target gene of EZH2 in prostate cancer.¹⁸ Another study found that DAB2IP expression was inversely related to androgen receptor activation in an androgen-independent manner.²⁵ The potential relation of DAB2IP to castration resistance may suggest an interaction of DAB2IP or its downstream proteins with the androgen receptor or interactions with androgen receptor elements.

As a prognostic biomarker, DAB2IP may identify patients at highest risk for treatment failure prior to initiation of standard therapy. Potential clinical application is that in the future we may be able to effectively use DAB2IP presence or absence from a patient's biopsy specimen as a means of determining curability of their cancer with standard radiation-based therapeutic regimens. Additionally, the DAB2IP pathway may be a potential target for improving treatment outcome and radiation response. Future research may be

directed toward creating molecularly-based therapeutic strategies to upregulate DAB2IP and restore the tumor suppressor's presence within the neoplastic cells, or by targeting downstream effectors such as clusterin.²⁶

This pilot study gives us a rationale to consider further validation study of DAB2IP and EZH2. DAB2IP status in combination with highest EZH2 expression (G3) may be useful for determining patients with worse outcome within the high-risk prostate cancer population.

Abbreviations

CRFS	Castration resistance-free survival
DAB2IP	Disabled homolog 2-interacting protein
DMFS	Distant metastasis-free survival
EZH2	Enhancer of Zeste homolog 2
FFBF	Freedom from biochemical failure
G	Grade
NCCN	National Comprehensive Cancer Network
PSA	Prostate-specific antigen

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Summary

Our pilot study demonstrates that tumor DAB2IP status may predict outcome after definitive radiation therapy in high-risk prostate cancer patients, and the prognostic value may be improved by also measuring EZH2 intensity levels.

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Figure 1.

Kaplan Meier and log-rank analysis comparing freedom from biochemical failure in patients based on (A) DAB2IP tumor status, (B) stratified EZH2 tumor status, and (C) reduced DAB2IP or grade 3 EZH2 expression compared to the control group of patients with both retained DAB2IP and grade 0-2 EZH2 expression.

Figure 2.
Decreased DAB2IP expression within prostatic adenocarcinoma correlates with a higher Gleason score.

Table 1

Patient Characteristics

Category		Percent (No.)
Median age (range)		66 (48-84)
T-Stage	T1c	18.5 (10)
	T2a-b	35.2 (19)
	T2c	20.4 (11)
	T3a	14.8 (8)
	T3b	7.4 (4)
	Unknown	3.7 (2)
N-Stage	N0	90.7 (49)
	N1	9.3 (5)
Pretreatment PSA	<10	37.0 (20)
	10-20	18.5 (10)
	>20	44.4 (24)
Gleason Score	7	22.2 (12)
	8	35.2 (19)
	9	33.3 (18)
	10	9.3 (5)
Total Radiation Dose	72-75.6 Gy	20.4 (11)
	79.2 Gy	79.6 (43)
Hormone Therapy	Yes	92.6 (50)
	No	7.4 (4)
Duration of Hormone Therapy	0 months	7.4 (4)
	4-18 months	35.2 (19)
	24-36 months	57.4 (31)
Tumor DAB2IP Status (n=46)	Reduced	28.3 (13)
	Retained	71.7 (33)
Tumor EZH2 Status (n=48)	Grade 0	2.1 (1)
	Grade 1	18.8 (9)
	Grade 2	60.4 (29)
	Grade 3	18.8 (9)

Table 2

Patient Outcomes by Tumor Biomarker Status

Biomarker status	FFBF		CRFS		DMFS	
	4 year	P-value	4 year	P-value	4 year	P-value
DAB2IP-retained	88.70%	0.0404	89.90%	0.0172	96.60%	0.0526
DAB2IP-reduced	36.50%		50.00%		36.00%	
EZH2 G0	100.00%	0.0732	100.00%	0.0722	100.00%	0.2035
EZH2 G1	88.90%		83.30%		88.90%	
EZH2 G2	65.50%		81.50%		100.00%	
EZH2 G3	57.10%		0.00%		83.30%	
DAB2IP-reduced or EZH2 G3	31.90%	0.0153	27.90%	0.0019	39.40%	0.0401
Control group	95.00%		100.00%		100.00%	

Table 3

Univariate Analysis

Variable		HR (95% CI)	P-value
DAB2IP Status	Reduced vs. Retained	3.7 (1.0-13.7)	0.055
EZH2 Grade	Continuous	2.9 (0.9-9.3)	0.073
T-Stage	Continuous	1.1 (0.7-1.6)	0.746
N-Stage	N0 vs. N1	2.9 (0.6-14.1)	0.182
Gleason Score	Continuous	2.1 (1.1-4.2)	0.029
PSA Pretreatment	Continuous	1.0 (1.0-1.0)	0.970
% Core Positivity	Continuous	3.7 (0.3-42.6)	0.299
Total Radiation Dose	Continuous	0.9 (0.7-1.3)	0.703
Hormone Therapy	No vs. Yes	3.5 (0.4-31.5)	0.261
Duration of Hormone Therapy	Continuous	1.0 (0.9-1.1)	0.512