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A*IM1* PROMOTER HYPERMETHYLATION AS A PREDICTOR OF DECREASED RISK OF RECURRENCE FOLLOWING RADICAL PROSTATECTOMY

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Abstract

Purpose—To evaluate the prognostic significance of six epigenetic biomarkers (*AIM1, CDH1, KIF1A, MT1G, PAK3* and *RBM6* promoter hypermethlation) in a homogeneous group of prostate cancer patients, following radical prostatectomy.

Patients and Methods—Biomarker analyses were performed retrospectively on tumors from 95 prostate cancer patients all with a Gleason score of 3+4=7 and a minimum follow up period of 8 years. Using Quantitative Methylation Specific PCR (QMSP), we analyzed the promoter region of six genes in primary prostate tumor tissues. Time to any progression was the primary endpoint and development of metastatic disease and/or death from prostate cancer was a secondary endpoint. The association of clinicopathological and biomolecular risk factors to recurrence was performed using the Log-rank test and Cox proportional hazards model for multivariate analysis. To identify independent prognostic factors, a stepwise selection method was used.

Results—At a median follow-up time of 10 years, 48 patients (50.5%) had evidence of recurrence: biochemical/PSA relapse, metastases, or death from prostate cancer. In the final multivariate analysis for time to progression, the significant factors were: older age, HR=0.95 (95% CI: 0.91, 1.0) (P=0.03), positive lymph nodes HR=2.11 (95%CI: 1.05, 4.26) (P=0.04) and decreased hypermethylation of *AIM1* HR=0.45 (95%CI: 0.2, 1.0) (P=0.05).

Conclusions—Methylation status of *AIM1* in the prostate cancer specimen may predict for time to recurrence in Gleason 3+4=7 patients undergoing prostatectomy. These results should be validated in a larger and unselected cohort.

INTRODUCTION

Prostate cancer accounts for a quarter of all new cancer cases diagnosed in men and remains the most common cancer diagnosed in men in the US (1). The widespread use of the PSA test leads to a significant increase in the proportion of patients diagnosed with early stages of disease. Approximately 90% of newly diagnosed patients present with no evidence of metastatic disease (1) and most of them are treated with potentially curative local modalities (radical prostatectomy, external radiation, brachytherapy, etc.) Uncontrolled studies suggest

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that within a 10-year follow-up period, 30-50% of patients treated with these local modalities will demonstrate a biochemical (PSA) relapse (2-5).

Outcome of localized prostate cancer patients is heterogeneous. Some patients undergoing potentially curative treatments who develop a recurrence may have benefited from immediate adjuvant treatment. Treatment decisions in early stage prostate cancer patients are based mainly on old, well known prognostic factors (stage, Gleason score, PSA variables etc). These factors are helpful but are far from being perfect due to significant clinical heterogeneity. Clearly, new biologic markers are needed to predict more accurately the risk of relapse. Such markers may aid clinicians and patients with the primary treatment selection and may also help with selection of patients at high risk for recurrence who may benefit most from trials of adjuvant therapy.

Epigenetic changes are changes in gene expression not caused by alterations in the primary sequence of the nucleotides that compose the gene. DNA hypermethylation is the most common epigenetic change and one of the most common molecular alteration in human cancer (6-7). A methyl group is added to cytosine only when it precedes a guanosine. CpG dinucleotides can be found in clusters called CpG islands often in promoter regions. CpG islands of many genes including tumor suppressor genes (TSG) are unmethylated in normal tissues but are methylated to a varying degree in multiple types of cancer causing silencing of gene transcription and inactivation of these TSGs (8-11). Promoter regions of several genes were found to be hypermethylated in prostate cancer using conventional methylation-specific PCR (MSP) (12-13). The ratio between the number of methylated genes and the total number of genes analyzed, known as the Methylation Index, was found to correlate with clinicopathological parameters of poor prognosis, though it was not shown to have independent prognostic value in a multivariate analysis. However, conventional MSP (14) is also of limited usefulness for specific cancer detection because benign lesions can be weakly positive and cannot be distinguished from cancer cases. This distinction has become possible by the development of quantitative assays (quantitative MSP, QMSP) (15-16).

In a previous study, we evaluated the possibility that the methylation status of different genes, that were found to be hypermethylated in prostate cancer (in comparison to benign hyperplasia) (17), may predict the clinical outcome of prostate cancer patients. That study has demonstrated that hypermethylation of *APC* and a profile of *APC* and *cyclinD2* may predict the time to recurrence in patients undergoing a radical prostatectomy (18).

In this study, we evaluated the methylation status of the promoter regions in a new set of genes. These genes are involved in different pathways: modulating apoptosis (*RBM6*), cell growth and proliferation where methylation was associated with a higher prostate cancer stage (*MT1G*) (19), a metastatic suppressor gene shown to be more frequently methylated in prostate cancer patients than in controls (*CDH1*) (12) and genes found to be hypermethylated in various tumors (*AIM1 (20), KIF1A (21), PAK3(21)*). Promoter hypermethlation was evaluated by QMSP. The purpose of this study was to evaluate the significance of the methylation status of six genes by QMSP in predicting the outcome of prostate cancer patients following a potentially curative radical prostatectomy (RP).

PATIENTS AND METHODS

Study population

We retrospectively identified 95 patients with an identical Gleason score of 3+4=7 for whom both tissue samples and median follow-up period of more than 8 years from radical prostatectomy (RP) were available. All patients underwent a RP for clinically localized prostate cancer at the Brady Urological Institute at Johns Hopkins University. All available

tissue blocks were reviewed by a pathologist (JE), and had adequate tumor present in the surgical specimen. Full clinical and pathological data were collected and known for all patients (including age, pre RP PSA, clinical and pathological stage, Gleason grade and surgical margin status). All patients were followed for at least 8 years and data were recorded in a coded database (NED, biochemical relapse, metastatic progression and/or death). Biochemical progression was defined as PSA values of 0.2mg/ml and rising. The date of progression was assigned to the date of the first PSA > 0.2mg/ml. None of the patients received any form of adjuvant treatment or hormonal treatment at time of biochemical relapse prior to the development of metastatic disease. By selecting all patients with the identical Gleason grade we could evaluate the ability of these epigenetic methylation markers to predict recurrence in a relatively homogenous group of patients.

This study was granted an exemption from the Johns Hopkins institutional review board since samples were evaluated without any identifiers (03-11-12-06e).

DNA extraction

After initial patient deidentification, all original histologic slides from the prostatectomy specimens were reviewed to reconfirm the diagnosis and the Gleason grade by a senior pathologist (J.E.). A representative block was retrieved for DNA extraction. Histologic slides from the formalin fixed, paraffin embedded tissue were taken. Slides were microdissected to obtain greater than 80% neoplastic cells. DNA was extracted using standard protocols as previously described (22).

Bisulfite treatment and QMSP

Sodium bisulfite conversion of unmethylated (but not methylated) cytosine residues to uracil of genomic DNA obtained from patient tissue samples was performed as described previously, with little modification (23). Two μg of DNA were used for the chemical treatment. DNA samples were then purified using the Wizard purification resin (Promega, Madison, WI), treated with sodium hydroxide, precipitated with ethanol, and resuspended in water and stored at −80°C. The modified DNA was used as a template for real-time fluorogenic MSP. The primers and probes used for *AIM1, CDH1, KIF1A, MT1G, PAK3* and *RBM6,* are described in Supplemental Table 1. In addition, primers and a probe were used to amplify areas without CpG nucleotides of*β-actin*, an internal reference gene. To determine the relative levels of methylated promoter DNA in each sample, the values of each gene of interest were compared with the values of the internal reference gene to obtain a ratio that was then multiplied by 1000 for easier tabulation (target gene/reference gene x1000). Fluorogenic quantitative MSP assays were carried out in a reaction volume of 20μL in 384-well plates in an Applied Biosystems 7900HT Sequence Detector (Applied Biosystems, Foster City, CA). PCR was performed in separate wells for each primer/probe set and each sample was run in triplicate. The final reaction mixture consisted of 600 nM of each primer (Invitrogen, Carlsbad, CA); 200 nM probe (Applied Biosystems, Foster City, CA); 0.6 unit of platinum Taq polymerase (Invitrogen); 200 μM each of dATP, dCTP, dGTP, and dTTP; 16.6 mM ammonium sulfate; 67 mM Trizma; 6.7 mM magnesium chloride; 10 mM mercaptoethanol; 0.1% DMSO, and 3 μL bisulfite-converted genomic DNA. PCR was performed using the following conditions: 95 °C for two minutes, followed by 50 cycles at 95 °C for 15 seconds and 60 °C for one minute. Each plate included multiple water blanks, a negative control, and serial dilutions of a positive control for constructing the calibration curve on each plate. Leukocyte DNA collected from healthy individuals was utilized as the negative control. The same leukocyte DNA was methylated *in vitro* with SssI bacterial methyltransferase (New England Biolabs Inc., Beverly, MA) and used as positive control for all studied genes.

Statistical Methods

The major statistical endpoint of this study was time to progression. Progression included PSA elevations >0.2ng/ml, metastasis and or death. Event time distributions for this endpoint were estimated with the method of Kaplan and Meier and compared using the log-rank statistic or the proportional hazards regression model. The simultaneous effect of two or more factors was studied using multivariate Cox proportional hazards models.

A second major statistical endpoint of this study was the probability of metastasis or death. Factors associated with this outcome were based on cross tabulations and logistic regression modeling. Cross tabulations were analyzed using chi square or Fisher's Exact tests where appropriate. Multivariate logistic regression models were used to determine the effects of multiple factors on the probability of metastasis or death.

All statistical computations were performed using SAS or EGRET (Statistics and Epidemiologic Research Corp.) PC packages. All confidence intervals (CI) are at the 95% level and all p values are two sided.

RESULTS

We evaluated the methylation status of several genes (*AIM1, CDH1, KIF1A, MT1G, PAK3* and *RBM6*) in radical prostatectomy specimens from 95 patients by QMSP. The median age at diagnosis was 60 years (range 46-72). Demographics, clinical and pathological classic risk factors for predicting recurrence in these patients are summarized in table 1.

Overall median follow up time of patients without progression was 10 years (range 8-14). At the time of this study, 48 patients (50.5%) have experienced a recurrence. Twenty two patients (23%) had biochemical relapse only, while 17 (18%) developed metastatic disease and 9 (9.5%) have died of prostate cancer. The median TTP for the whole group was 8 years.

The potential of methylation status in each tested gene alone or in combination for predicting time to recurrence was investigated. In a univariate cox proportional hazard model, all clinical and pathological risk factors (age, pre RP PSA, lymph node status, surgical margins, seminal vesicle involvement, extra prostatic extension, and organ confined disease) and methylation status were evaluated. The significant factors for predicting time to progression were younger age, positive LN, and the methylation status of *AIM1*. Extra capsular extension was of borderline significance (Table 2).

In the final multivariate COX proportional hazards model the significant factors predicting time to progression were: older age, HR=0.95 (95% CI: 0.91, 1.0) (P=0.03), positive lymph nodes HR=2.11 (95%CI: 1.05, 4.26) (P=0.04) and decreased hypermethylation of *AIM1* HR=0.45 (95%CI: 0.2, 1.0) (P=0.05). (Table 3)

An additional factor was calculated combining the best known clinical and pathological risk factors for recurrence. Based on the Kattan nomogram for postoperative probability of freedom from recurrence at 7 years (24), each of the 95 patients was scored. Higher nomogram probabilities were associated with an improved TTP outcome, HR=0.59 (95% CI: 0.23, 1.53), but it did not reach significance. (p=0.28).

The methylation status was also evaluated in its ability to predict the probability of developing metastases or death. None of the methylation factors alone or in combination were found to significantly predict for this endpoint.

DISCUSSION

We evaluated the methylation status of 6 genes promoters and their ability to add to known risk factors in predicting time to recurrence in prostate cancer patients following prostatectomy. These genes were chosen based on their ability to differentiate between benign hyperplasia of the prostate and prostate cancer (19), correlation to certain clinicopathological characteristics (12), or presence of hypermethylation in different types of cancer (20-21).

All patients in this study had the same Gleason grade of 3+4=7. This unique cohort allowed us to evaluate the significance of hypermethylation in predicting aggressiveness of prostate cancer in a relatively homogeneous group of patients without interference in the natural history of the disease (No treatment was given until the development of metastatic disease).

A Gleason score of 7 was chosen since it represents the most heterogeneous group of patients in terms of outcome. Most patients with a Gleason score of ≤6 will be cured with RP only. In patients with a Gleason score of ≥8, the probability of recurrence is very high. In the multivariate analysis including the most significant clinical and pathological prognostic factors, hypermethylation of *AIM1* was found to be a significant predictor for TTP. The HR of *AIM1* hypermethylation was as significant as the HR given by LN status. It was more significant than the risk calculated from the Kattan nomogram (24). The latter combines the best known clinical and pathological risk factors for recurrence. These findings are especially significant since we evaluated a relatively homogenous group of patients with the same Gleason score. The nomogram may have lost significance as it lost one of its most important variables which is the Gleason score.

Adding significant biomarkers to models predicting recurrence were shown to override commonly known risk factors by others as well. When Kattan et al. added IL-6 soluble receptor and TGFβ to their nomogram which included all known clinical and pathological risk factors, clinical stage lost its significance in predicting recurrence (25) in their multivariate analysis. Similarly, Yegnasubramanian et al. demonstrated in a sub group of their study population analysis, that hypermethylation of *PTGS2* predicted an increased risk of recurrence following RP, while pathological stage lost its significance as well (26).

The upper quartile of hypermethylation of *AIM1* was a significant predictor of longer TTP in our population. We expected that the upper quartile group in this group might have a worse prognosis; however our results showed the opposite. It may be that the patients in the lower quartiles develop their prostate cancer through alternative pathways that cause more aggressive disease. Another explanation may be that we know from different studies that the same gene may have different expression in various tumors. The same gene can be over expressed in one tumor and silenced in another tumor (27). *AIM1* is known to act as a tumor suppressor gene in melanoma but it may act as an oncogene in prostate cancer explaining the better prognosis for those patients who have hypermethylation of *AIM1* in their primary tumor. This may also suggest that these cancers may have originated by a different pathway or possibly from an earlier cancer progenitor stem cell.

Functional studies using *in vitro* and *in vivo* model will clarify the role of *AIM1* in prostate cancer. Clinical correlation from this study with *AIM1* may be by chance only and we have to validate this finding in an independent cohort. Prospective study with careful follow-up will corroborate *AIM1* clinical use.

Biochemical relapse and time to this relapse is a controversial endpoint. On the one hand, it is not as strong an endpoint as development of metastasis or survival. Not all patients who have a biochemical relapse develop metastasis or die from prostate cancer. On the other

hand, patients undergoing a radical prostatectomy are interested in their chance of cure and not only in reducing their chance of dying from their disease. Biochemical relapse alone causes great anxiety and a significant reduction in quality of life. Furthermore, the endpoint of TTP includes not only the probability of progression but also the time to its development, which is one of the predictors for further development of metastasis and death.

The failure of the methylation status to predict the probability of metastasis or death may be due to a relatively short follow-up. Pound et al. have shown that the median time from biochemical progression to the development of metastases is 8 years and the median time from clinically evident metastases to death is additional 5 years (28). Some of the patients with a biochemical relapse at the time of the study may harbor an aggressive disease and may develop metastasis with further follow-up. Some may even have more aggressive disease than some of the patients already found to have metastasis with longer follow-up. Therefore this endpoint should be optimally evaluated many years later, when full survival data of this cohort has been achieved. It may also be that since we evaluated molecular markers from the initial tissue this may reflect more on the first outcome of biochemical relapse and that time to metastases and death are dependent also on molecular changes that may develop with time, and are not evident at the time of the initial prostatectomy.

In conclusion, this study suggests that hypermethylation of *AIM1* may predict the time to recurrence in patients undergoing a radical prostatectomy. These results should be validated in a larger and unselected group of patients with all Gleason scores. Study of the predictive value of the methylation status of other candidate genes (e.g. *APC*, *PTGS2* etc.) and evaluating their possible addition to these results should also be pursued. Further studies in earlier stages may provide an important tool for identifying patients who have an indolent cancer not requiring any form of treatment or in later stages in identifying those patients with biochemical relapse who may have a more aggressive course requiring early intervention. Thus, such studies may eventually help clinicians and patients in assessing the probability of cure more accurately and therefore also help in selecting better candidates for possible adjuvant treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The abbreviations used are

QMSP	Quantitative methylation specific PCR
TSG	Tumor suppressor genes
PCR	Polymerase Chain Reaction

RP	Radical prostatectomy
TTP	Time To Progression

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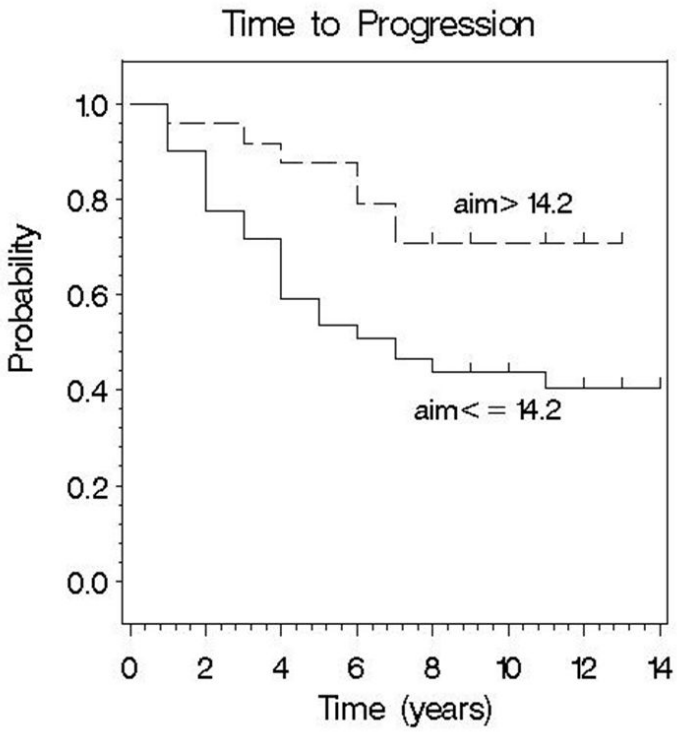

Figure 1.
Time to progression stratified by hypermethylation of the *AIM1* promoter. *AIM1* hypermethlation predicted longer time to progression (HR 0.45, P= 0.05).

Table 1

Patient characteristics_(Each patient may have more than one surgical risk factor)

Number of patients	**95**
Age	46-72 (Mean = 60)
Gleason 3 + 4 = 7	95 (100%)
Lymph nodes (+)	12 (16%)
Surgical Margins (+)	21 (28%)
Extra-prostatic extension	41 (55%)
Seminal vesicle (+)	15 (20%)
Organ confined	16 (22%)
Follow up	8+ years

Table 2

Univariate analysis for TTP (Time To Progression) including significant clinical/pathological variables and all upper quadrant hypermethylation of evaluated genes

Variable	Hazard Ratio (HR)	%HR Confidence Limits		Pr>ChiSq
		Lower	Upper	
Age	0.94	0.9	0.99	0.01
EPE	1.72	0.95	3.11	0.07
LN	2.43	1.21	4.9	0.01
AIM1	0. 4	0.18	0.89	0.02
MT1G	0.89	0.28	2.87	0.85
KIF1A	0.8	0.4	1.61	0.54
CDH1	1.41	0.44	4.56	0.56
PAK3	1.15	0.36	3.72	0.81
RBM6	0.88	0.45	1.73	0.71
Kattan nomogram	0.59	0.23	1.53	0.28

– ECE = extra-prostatic extension

– LN = Histologically positive lymph nodes

■ Kattan nomogram= prediction of probability of recurrence by combing most clinical and pathological risk factors (stage, grade, PSA variables etc.)

Tables 3

Multivariate analysis for TTP:

Variable	Hazard Ratio (HR)	%HR Confidence Limits		Pr>ChiSq
		Lower	Upper	
AIM1	0.45	0.2	1	0.05
LN	2.11	1.05	4.26	0.04
age	0.95	0.91	1	0.03