Association of Tissue Promoter Methylation Levels of APC, TGFβ2, HOXD3, and RASSF1A with Prostate Cancer Progression

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Abstract
Aberrant promoter methylation is known to silence tumor-suppressor genes in prostate cancer (PCa). We correlated quantitative promoter methylation levels of \textit{APC}, \textit{TGFβ2} and \textit{RASSF1A} in 219 radical prostatectomies diagnosed between 1998-2001 with clinicopathological follow-up data available including, Gleason Pattern (GP), Gleason Score (GS) and pathological stage, and explored their potential in predicting biochemical recurrence using univariate and multivariate analyses.
We observed that the average methylation levels of \textit{APC} increased significantly from GS\textless{}6 to GS7, and pT2 to pT3a, and that of \textit{TGFβ2} increased from GS\textless{}6 to GS7, but not for \textit{RASSF1A}. PCa samples were also stratified into high methylation (HM) and low methylation (LM) groups based on the PMR scores of all cases analyzed for each marker. The HM frequency of \textit{APC} was greater in pT3a than pT2, and in GS\textgreater{}8 than GS\textless{}6. The HM frequency also increased significantly from GP3 to GP4 for \textit{APC}, \textit{TGFβ2} and \textit{RASSF1A}. \textit{APC} methylation level was a significant predictor of biochemical recurrence in univariate analysis (p-value=0.028). Finally, we combined methylation data of these three genes with the previously reported novel methylation biomarker \textit{HOXD3}. Quantitative methylation assessment of a multiplex panel of markers, consisting of \textit{APC}, \textit{HOXD3} and \textit{TGFβ2}, outperforms any single marker for the prediction of biochemical recurrence (p-value=0.017).
This is the first study demonstrating that quantitative increase in promoter methylation levels of \textit{APC}, \textit{HOXD3} and \textit{TGFβ2} are associated with PCa progression.
Introduction:

Prostate cancer (PCa) is the most commonly diagnosed non-skin cancer in North America, affecting 1 in 6 men with an estimated 192,280 new cases and 27,360 deaths reported in the United States in 2009 [1]. The routine use of serum PSA testing has greatly increased PCa detection since the 1990s [2]. However, there are significant limitations to the PSA test as the test lacks specificity and sensitivity and fails to discriminate between low-grade and high-grade prostate cancers [3].

PCa progression is assessed by the Gleason Score (GS), which is the sum of two assigned histopathologic glandular patterns (Gleason pattern) [4]. The Gleason pattern (GP) ranges from 1 to 5 and the GS therefore ranges from 2 to 10. Prostate tumors with GS≤6, usually composed of GP3, are defined as low-grade cancers and tend to be slow-growing and localized. GS 7 tumors, constituted by a mixture of GP3 and GP4, are considered intermediate while tumors with GS≥8 represent high-grade cancers with fast-growing and aggressive phenotype, which are likely to metastasize [5]. PCa is a heterogeneous cancer and tends to be multifocal. One prevailing theory to explain the presence of different GPs in the same tumor is that prostatic tumors de-differentiate during their development and progress from low grade to high grade [6]. Unfortunately, as a consequence of sampling bias, GS obtained on a biopsy often underestimates the GS in the corresponding prostatectomy specimen, which may result in suboptimal treatment choices [7, 8]. In this context, identification of biomarkers related to prostate cancer progression would be beneficial to improve pretreatment diagnostics of prostate cancer.

DNA methylation of gene promoters is a common and usually tumor-specific event in PCa. To date, aberrant methylation of CpG island-containing promoters of over 50 hypermethylated loci has been identified in prostate cancer [9-12]. There are important advantages of using DNA methylation as cancer biomarkers. In particular, methylated DNA can be detected with a high degree of specificity and sensitivity with a ratio of tumor to normal DNA of less than 1:1000 [13], enabling its application on minimal samples available from PCa patients. Previous studies have also demonstrated that hypermethylation of a combination of genes rather than a single gene is most prognostic for PCa behavior [14, 15].

APC (adenomatous polyposis coli) and RASSF1A (Ras association domain family protein 1 isoform A) have been shown to be consistently hypermethylated in PCa development, with well-established diagnostic and prognostic significance [15-20]. APC is a well-characterized tumor suppressor gene which regulates the Wnt-signaling pathway via ubiquitin mediated beta-catenin degradation. RASSF1A is a cell cycle regulator that controls transition from G1 to S phase. Methylation statuses of both these genes have been reported to discriminate benign tissues from primary prostate adenocarcinomas [21, 22]. In addition, hypermethylation of APC has been observed to correlate significantly with clinicopathological variables including tumor stage, grade [23], time to progression in GS7 patients [14], and prostate cancer–specific mortality [15, 24]. Although not consistently, RASSF1A promoter methylation has been correlated with advanced tumor grade and stage [25-27]. However, the majority of published studies have examined gene methylation as a
dichotomous variable: any methylation versus no methylation. Depending on the extent of gene promoter methylation, the resultant gene expression levels and the consequent downstream signaling effects are likely to differ. Thus, quantitative assessment of $APC$ and $RASSF1A$ gene methylation levels may provide better insights into their contribution to prostate carcinogenesis. This point is underscored in a previous study of the best characterized diagnostic methylation marker in PCa, $GSTP1$ methylation, in which quantitative GSTP1 methylation levels were found to significantly correlate with Gleason grade and tumor volume in prostate needle biopsies[28].

By genome-wide differential methylation array profiling of GS6 versus GS8 prostate tumors, we had previously identified aberrant methylation of $TGF\beta2$ and $HOXD3$ in PCa. [29]. Our group subsequently showed a significant association of quantitative $HOXD3$ methylation levels with prostate cancer progression and biochemical recurrence [30]. TGF\beta2 methylation profiles differed significantly between GS6 cases and GS8 cases on our microarrays, and therefore could potentially be implicated in cancer progression.

Our study employs 219 primary PCa tumors spanning the entire spectrum of primary prostate cancer progression from low grade, prognostically favorable primary prostate tumors to aggressive and highly metastatic ones. Consequently, alterations in candidate gene methylation profiles could be monitored closely at every stage of PCa tumorigenesis. Biochemical recurrence, as determined by increased PSA levels, is the first sign of cancer recurrence and could lead to clinical relapse if left untreated. It is used in the current study to indicate patient outcome.

In this study, we analyzed quantitative methylation levels of $APC$ and $RASSF1A$ as well as the newly discovered methylation biomarkers $TGF\beta2$ and $HOXD3$ and examined their association with PCa progression either as individual markers or in combination.
Materials and Methods:

Patient Cohort

A total of 219 formalin-fixed, paraffin-embedded radical prostatectomy patient specimens, diagnosed between 1998 and 2001, were collected at the University Health Network (UHN) in Toronto. All patients who received neo-adjuvant therapy prior to radical prostatectomy were excluded from the study. All patients had consented to tissue collection, banking and utilization in research studies according to the protocols approved by the Research Ethics Board at The University Health Network and Mount Sinai Hospital, Toronto. Patient tissue and clinicopathological data collection, storage and processing were described previously [30]. The total number of patients was reduced from 232 in our previous study to 219 due to lack of complete methylation data. The clinicopathological characteristics of these 219 patients are listed in Supplementary Table 1. A significant proportion of prostate tumors analyzed in the current study were composed of multiple GPs, and each GP was assayed individually. Matched normal tissues are derived from the same patient cohorts, mostly peripheral zone tissue surrounding the cancerous areas, containing at least 50% glandular content.

DNA Extraction and the MethyLight Assay

Genomic DNA was isolated, bisulfite treated and subsequently used for MethyLight assay as described before [30]. Primer and probe sequences used for APC and RASSF1A were from published sequences[31], and the ones for TGFβ2 and HOXD3 were self-designed: 1) For APC: (Forward) 5’-GAA CCA AAA CGC TCC CCA T -3’; (Reverse) 5’-TTA TAT GTC GGT TAC GTG CGT TTA TAT -3’; (Probe) 5’FAM-CCC GTC GAA AAC CCG CCG ATT A -BHQ1-3’. 2) For TGFβ2: (Forward) 5’-TTT TAG GAG AAG GCG AGT CG-3’; (Reverse) 5’- CTC CTT AAC GTA ATA CTC TTC GTC G-3’; (Probe) 5’- FAM-TCT CGC GCT CGC AAA CGA CC-BHQ1-3’. 3) For RASSF1A: (Forward) 5’-ATT GAG TTG CGG GAG TTG GT-3’; (Reverse) 5’- ACA CGC TCC AAC CGA ATA CG -3’; (Probe) 5’FAM-CCC TTC CCA ACG CGC CCA -BHQ1-3’. 4) For HOXD3: (Forward) 5’-TTA AAG GTT TAT GGT TGC GC-3’; (Reverse) 5’-TTA CGA ACA CTA AAC TAC ACC CG-3’; (Probe) 5’FAM-ACA AAA CGT TCC CGA CGC TTC TAA AA-BHQ1-3’. A percentage of methylated reference (PMR) score was calculated for each specific gene locus by dividing the GENE:Alu-C4 ratio of a sample by the GENE:Alu-C4 ratio of commercially available fully methylated DNA and multiplying by 100.

Statistical Analysis

The final PMR score of each sample was obtained by averaging results from duplicate runs. If a patient had multiple tumor samples, a final methylation level was assigned by averaging all tumor PMRs of that individual as described previously [30]. We analyzed the prognostic significance of known clinical and pathological characteristics (including GS, stage and surgical margin) in the patient series by constructing Kaplan-Meier curves and by Logrank test. For statistical purposes, PCa samples were divided into four pattern categories: normal, GP2, GP3 and GP4/5, or into three grade categories: GS≤6, GS7, and GS≥8, or into three stage categories: pT2, pT3a, and pT3b/pT4. GP 4 and 5 were grouped together for statistical analysis due to
a small number of observations for GP 5 carcinomas (n=8), and pT3b and pT4 were combined for the same reason (n=5 for pT4). PCa samples were also classified into two methylation groups: high methylation (HM) group, which was equal or greater than the third quartile PMR values of the gene of interest, and low methylation (LM) group, which accounted for the rest of the samples [14, 15]. Association between methylation and GS, GP, and pathological stage was examined using the Mann-Whitney U test (for mean) and Pearson Chi-square (for frequency). If any spreadsheet cell had an expected outcome less than 5, then Fisher’s Exact Test was used instead of Pearson Chi-square. Paired t-test was used to assess methylation differences between matched normal and cancerous tissues and between different tumors from the same patient. Candidate gene methylation markers were grouped in different combinations and tested for their ability to predict biochemical recurrence using both Kaplan-Meier curves and Cox multivariate regression model. Using a multivariate Cox-regression model comprising clinicopathological and methylation variables, the relative contribution of each variable to biochemical recurrence was assessed. The sensitivity and specificity of quantitative methylation in discriminating cancer versus normal tissue was determined by receiver operator curve (ROC) analysis. For all statistical analyses, a p-value of $\leq 0.05$ was considered significant. All analyses were done using SPSS (Chicago, IL) and R statistical software.
**Results:**

**Correlation of Methylation Status with Gleason Pattern**

We established PMR threshold values using ROC curves that allowed optimal distinction between benign and malignant tissue with maximum sensitivity and specificity (Supplementary Figure 1). The cutoff PMR values were 9.13 for APC, 0.05 for TGFβ2, and 42.82 for RASSF1A. Both APC and RASSF1A could correctly classify patients with and without PCa, as demonstrated by excellent sensitivity, specificity and areas under the curve (Supplementary Table 2). TGFβ2 methylation showed poor sensitivity due to low frequency of TGFβ2 methylation in the patient cohort (Supplementary Table 2).

The average methylation level and HM frequency of APC, RASSF1A, and TGFβ2 in prostate adenocarcinomas (stratified by GP), and their matching normals are described in Table 1. The average methylation and the proportion of HM cases of all three genes were significantly higher in tumor than that observed in normal (Table 2). Substantial increases in HM frequency and average methylation of APC were observed for GP2 versus GP4/5 and GP3 versus GP4/5 comparisons (Table 2, Mann-Whitney U p=0.028 and p=0.004, Chi-square p=0.008 and p=0.031). TGFβ2 exhibited elevated HM frequency when comparing GP3 versus GP4 (Table 2, p=0.008). For RASSF1A, both the average methylation and the HM frequency differed between GP2 versus GP3 and GP2 versus GP4 (Table 2, Mann-Whitney U p=0.001 and p<0.001, Chi-square p=0.006 and p<0.001), while HM frequency was only significantly different for GP3 versus GP4 (Table 2, p=0.017).

Among paired samples, where multiple different patterns came from the same individual, the methylation levels of APC, TGFβ2 and RASSF1A were significantly higher in tumor than that in normal (Supplementary Table 3, p<0.001), and in GP4/5 compared with GP3 (Supplementary Table 3, p<0.001 for APC, p=0.005 for TGFβ2, and p=0.004 for RASSF1A).

**Correlation of Methylation Status with Gleason Score**

The quantitative methylation levels of APC, RASSF1A and TGFβ2 were analyzed in relation to GS. The average methylation level and the proportion of HM cases for each analyzed gene associated with different subgroups are described in Table 1. For APC, the average methylation was significantly increased in GS7 PCa compared with GS≤6 PCa (Table 2, p=0.018), and both the average methylation and proportion of HM cases were significantly greater in GS≥8 PCa compared with GS≤6 PCa (Table 2, Mann-Whitney U p=0.036 and Fisher’s Exact p=0.013). In addition, TGFβ2 average methylation was considerably higher in GS7 compared to GS≤6 (Table 2, p=0.029). On the contrary, for RASSF1A methylation, no difference was observed when comparing the three GS groups (Table 2).

**Correlation of Methylation Status with Pathological Stage**

We also investigated the relationship between quantitative methylation level and pathological stage. Average PMR values and frequency of HM are shown in Table 1. For APC methylation, we observed considerable differences in the average PMR between pT2 versus pT3a (Table 2, p<0.001). The frequency of APC HM methylation
was shown to differ significantly when comparing pT2 versus pT3a and pT2 versus pT3b/pT4 PCa cases (Table 2, \(p=0.006\) and \(p=0.035\) respectively). However, no considerable difference was observed for TGF\(\beta\)2 and RASSF1A methylation when comparing the three pathological stages (Table 2).

**Promoter Methylation and Biochemical Recurrence**

We next analyzed the prognostic significance associated with \(APC\), \(RASSF1A\) and TGF\(\beta\)2 promoter methylation. Univariate log-rank analysis demonstrated that hypermethylation of \(APC\) was significantly correlated with higher biochemical recurrence rate (Supplementary Figure 2A, \(p=0.028\)). However, such an association was not observed for TGF\(\beta\)2 and RASSF1A promoter methylation levels (Supplementary Figure 2B-C, \(p=0.156\) and \(p=0.556\) respectively). Next, we performed Kaplan-Meier curves after stratification for GS and pathological stage. \(APC\) was a significant predictor of biochemical recurrence in pT2 stage patients (\(p=0.028\), Supplementary Figure 3), and TGF\(\beta\)2 for both pT3a stage and GS \(\leq 6\) patients (\(p=0.019\) and \(p=0.024\) respectively, Supplementary Figure 3).

Multivariate analyses comprising of five variables (methylation category of gene of interest, GS, stage, surgical margins and age) were performed to test the contribution of each predictor to biochemical recurrence in the presence of other variables. GS, pathological stage and surgical margin status were all significant predictors, but none of the three methylation markers was an independent predictor of biochemical recurrence (See Figure 1 and Supplementary Tables 4-6). After stratifying for stage, \(APC\) could independently predict biochemical recurrence of pT2 stage patients in the presence of other clinicopathological variables, while TGF\(\beta\)2 could predict that of pT3a patients (See Figure 2 and Supplementary Tables 7-9, \(p=0.038\) and \(p=0.020\), respectively).

**Multiparametric Analysis of Different Combinations of Epigenetic Markers**

We have previously demonstrated that promoter methylation of a homeobox gene, HOXD3 is significantly associated with PCa progression and biochemical recurrence [30]. Next, we incorporated HOXD3 methylation profile together with that of \(APC\) and TGF\(\beta\)2 in multiparametric analysis to assess its ability to predict biochemical recurrence. Since RASSF1A methylation did not show additional prognostic association, it was not included in the multiparametric analysis. To evaluate if a multigene panel could improve predictive accuracy over any single biomarker, methylation profiles of \(APC\), HOXD3 and TGF\(\beta\)2 were analyzed in two different combinations as follows: presence of at least 2 of the 3 HM markers versus no HM and <2 HM markers, and presence of all 3 HM markers versus no HM and <3 HM markers. Univariate Kaplan-Meier Curves demonstrated that both HM marker combinations predicted significantly earlier biochemical recurrence (Supplementary Figure 4). The presence of any two or more HM markers exhibited the most significant correlation with patient biochemical recurrence (\(p<0.001\), Supplementary Figure 4, panel A and B). Using a multiparametric analysis we observed that the presence of any two or more HM markers could predict biochemical recurrence independent of all existing clinicopathological variables (\(p=0.017\), detailed in Table 3, panel A). Furthermore, we separated GS7 patients into two categories: patients with
predominantly GP3 and patients with predominantly GP4 because they had been associated with distinct prognostic outcomes. A multivariate model consisting of four separate GS groups, GS6, GS7=3+4, GS7=4+3, and GS8 was performed. The presence of any two or more HM markers remained to be a significant predictor of biochemical recurrence independent of all existing clinicopathological variables (p=0.014, detailed in Table 3, panel B)
Discussion:

One of the major current challenges in PCa care is to find a reliable marker to identify rapidly progressing tumors. In this study, we systematically investigated methylation levels, independently and in combination, of a panel of four genes, \textit{APC}, \textit{TGFβ2}, \textit{HOXD3} and \textit{RASSF1A}, and examined their relationships with clinicopathological features of primary prostate adenocarcinoma.

Methylation of \textit{APC}, \textit{RASSF1A} and \textit{TGFβ2} demonstrated diagnostic potential. Both \textit{APC} and \textit{RASSF1A} methylation levels increased significantly from normal to tumor, which is consistent with observations reported earlier [23, 32, 33]. \textit{TGFβ2} was also able to accurately differentiate normal from cancerous prostate tissues.

We observed a significant increase in \textit{APC} promoter methylation level going from GS≤6 (low grade) to GS7 (intermediate grade) groups, and from pathological stage pT2 (confined) to pT3a (extends beyond prostate capsule) in our series of archival prostate specimens. Both pathological transitions are indicative of progression from relatively indolent, slow-growing tumors to intermediate stage tumors with the potential to invade. Taken together, these observations suggest that \textit{APC} methylation level is likely to be elevated in biologically more aggressive tumors.

Promoter hypermethylation of \textit{TGFβ2} as well as \textit{HOXD3} was first discovered by our group as a potential epigenetic marker in genome-wide methylation profiling studies [29]. The increased level of \textit{TGFβ2} methylation with higher Gleason score cancers initially observed in our CpG island array data [29] was independently validated in the current series of prostatectomy specimens. Promoter hypermethylation is expected to lead to gene silencing. Interestingly, \textit{TGFβ2} protein levels have been shown to be higher in PCa patients with low pathological stages and low-grade disease[34]. This result is consistent with the \textit{TGFβ2} methylation profile observed in our study. Our findings demonstrate the diagnostic and prognostic significance associated with \textit{TGFβ2} promoter methylation in PCa.

Steady increase in \textit{RASSF1A} methylation levels was observed moving from the well-differentiated GP2 to the poorly differentiated GP5, despite similar profiles of \textit{RASSF1A} methylation across pathological stage and GS groups. Although \textit{RASSF1A} has been reported to discriminate normal from cancerous tissues with high sensitivity and specificity, its role remains controversial in prostate cancer progression. Inconsistent results have been reported in exploring association of \textit{RASSF1A} methylation level with either GS or stage [15, 23, 26, 27, 33]. Our study has also demonstrated the highest sensitivity and specificity of RASSF1A among all genes examined for discriminating cancerous from normal tissue. Taken together, these observations suggest that \textit{RASSF1A} methylation may serve as a potential diagnostic marker in PCa.

For PCa cases with HM of \textit{APC}, there was a significant trend to a higher probability of biochemical recurrence in univariate analysis. This provides some interesting potential insights into underlying biological mechanisms. One possibility is that patients with apparently organ-confined cancer at the time of diagnosis may possess neoplastic or pre-neoplastic cells that exist in the histological normal fields surrounding the tumors predicted by high \textit{APC} methylation. Even though \textit{APC} and
TGFβ2 failed to independently predict biochemical recurrence in multivariate analysis, when stratified for pathological stage, they became significant predictors. It could possibly be a result of overlapping predictive power between DNA methylation markers and pathological stage.

Cancer progression course and aggressiveness are expected to be better recapitulated when taken into account the behavior of a combination of markers, instead of any single marker. Accordingly, we established a small panel of epigenetic markers, including APC, TGFβ2, and a previously identified prognostic marker, HOXD3, whose quantitative methylation levels closely correlated with PCa progression [30]. In line with the previous view, the presence of HM in any two or more of the above markers significantly predicted patient biochemical recurrence, independent of any existing clinicopathological variables. The presence of HM in any two or more markers remained to be significant when we examined GS7=3+4 and GS7=4+3 patients separately, demonstrating the strong prognostic significance of our finding. Therefore, incorporating combined methylation profiles of APC, TGFβ2 and HOXD3 into pre-existing nomograms could potentially improve current estimations of PCa progression and biochemical recurrence.

A unique aspect of our study is that we collected matched normal and multiple tumor foci from the same patient whenever available and analyzed the methylation status of each focus. For matched GP comparisons, methylation levels of all three genes APC, RASSF1A and TGFβ2, increased significantly from normal to tumor, and also from GP3 to GP4. This indicated that the general correlation between methylation level and GP also held true on a patient by patient basis. The final methylation PMR associated with every tumor was obtained by averaging all tumor PMRs of that individual. Tumor average methylation level is more clinically useful because DNA methylation levels detected in tissue biopsy, serum or urine samples are more likely to reflect an average of the many tumor foci present.

In conclusion, we have examined the extent of promoter methylation in APC, TGFβ2, HOXD3 and RASSF1A genes in a large series of archival prostatectomy specimens, and correlated these findings with clinicopathological variables. Aberrant DNA methylations of APC and RASSF1A have been demonstrated to be readily detected in available clinical specimens obtained through non-invasive procedures, such as bodily fluids. Successful detection of these epigenetic markers might serve as a non-invasive diagnostic for early-stage, curable PCa tumors screening and risk evaluation.

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