Serum *GADD45a* methylation is a useful biomarker to distinguish benign *vs* malignant prostate disease

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Background: Prostate-specific antigen (PSA) screening for prostate cancer results in a large number of unnecessary prostate biopsies. There is a need for specific molecular markers that can be used in combination with PSA to improve the specificity of PSA screening. We examined *GADD45a* methylation in blood DNA as a molecular marker for prostate cancer diagnosis.

Methods: The study included 82 men, with PSA levels $>4\,\mathrm{ng\,ml}^{-1}$ and/or abnormal digital rectal exam, who underwent prostate biopsy. We compared *GADD45a* methylation in DNA from serum and buffy coat in 44 patients (22 prostate cancer and 22 benign). *GADD45a* methylation in serum DNA was examined in 82 patients (34 cancer and 48 benign).

Results: There was no significant difference in buffy coat *GADD45a* methylation between cancer and benign patients. Serum *GADD45a* methylation was significantly higher in cancer than in benign patients. Classification and regression tree predictive model for prostate cancer including risk groups defined by PSA, free circulating DNA (fcDNA) level and *GADD45a* methylation yielded specificity of 87.5%, sensitivity of 94.1% and receiver operator characteristic curve area of 0.937.

Conclusions: Serum *GADD45a* methylation in combination with PSA and fcDNA level was useful in distinguishing benign from prostate cancer patients.

Prostate cancer is the second most diagnosed cancer and the second leading cause of cancer-related death in the United States with estimated 220 800 new cases and 27 450 deaths in 2015 (Siegel *et al*, 2015). Serum prostate-specific antigen (PSA) levels and digital rectal exam (DRE) are currently used in prostate cancer screening. Although PSA screening is highly sensitive, it lacks specificity and results in a large number of false positives. Only about 25–35% of patients with elevated PSA levels (4.1–9.9 ng ml^{-1}) undergoing prostate biopsy are diagnosed with prostate cancer (Smith *et al*, 1997) indicating that two-third or more of patients have elevated PSA caused by non-malignant conditions (Partin *et al*, 1990; Nadler *et al*, 1995). Widespread PSA

screening has led to a large proportion of patients being subjected to the discomfort of prostate biopsies resulting in increased health-care costs. Moreover, PSA screening results in overdetection of indolent prostate cancer on one hand (Etzioni *et al*, 2002) and missing high-grade prostate cancers in patients with normal PSA levels on the other (Thompson *et al*, 2004). Hence, there is an unmet need for specific molecular markers that can be used in combination with PSA to improve the specificity of prostate cancer-screening tests.

A blood-based test that can accurately predict the presence of prostate cancer may greatly help prostate cancer screening by eliminating unnecessary prostate biopsies. Free circulating DNA

(fcDNA) in serum/plasma has provided an attractive tool in cancer diagnosis due to the non-invasive nature of sample procurement. We have previously demonstrated that serum fcDNA levels increases the specificity of the PSA test in prostate cancer diagnosis (Gordian *et al*, 2010). Although fcDNA levels may be helpful in prostate cancer diagnosis, it is not presently used in the clinical setting. One of the limitations of using elevated levels of fcDNA as a marker is that it is not specific to prostate cancer and can occur in other cancers as well (Shapiro *et al*, 1983; Silva *et al*, 1999; Wu *et al*, 2002; Sozzi *et al*, 2003; Taback *et al*, 2004; Flamini *et al*, 2006; Ren *et al*, 2006). Moreover, studies have shown that increasing levels of fcDNA in plasma and serum can be detected in benign pathological conditions as well (Chang *et al*, 2003; Okkonen *et al*, 2011).

Because of their chemical and biological stability, DNA methylation-based biomarkers have greater clinical applications in cancer detection, diagnosis and targeted therapies compared with RNA or protein. DNA methylation occurs primarily in CpG dinucleotides and is a common event in cancer. Methylation patterns that are specific to prostate cancer have been previously identified in tumour tissue (Goering *et al*, 2012; Yang and Park, 2012). However, this is limited by the invasiveness of the sample procurement method and increased costs. DNA methylation patterns in blood of prostate cancer patients and have been shown to be similar to that from tissues (Bastian *et al*, 2008; Ellinger *et al*, 2008). *GSTP1* is the most studied DNA methylation marker in prostate cancer (Heyn and Esteller, 2012). *GSTP1* hypermethylation was identified as an early marker of prostate cancer as it was detected in a large proportion of proliferative inflammatory atrophy lesions (Nakayama *et al*, 2003). *GSTP1* hypermethylation was also detected in various bodily fluids such as urine, serum and ejaculate (Goessl *et al*, 2000; Reibenwein *et al*, 2007; Ellinger *et al*, 2008; Sunami *et al*, 2009). Other genes that exhibit a high incidence of hypermethylation in prostate cancer include *APC*, *RASSFIA*, *PTGS2*, *TIG1* and *MDRI* (Bastian *et al*, 2008; Ellinger *et al*, 2008; Ellinger and Bastian, 2010).

In our previous study on *GADD45a* methylation in prostate cancer (Ramachandran *et al*, 2009), we found that methylation of four CpGs at positions -737, -723, -706 and -690 with respect to the transcription start site correlated inversely with *GADD45a* expression levels in prostate cancer cell lines. The 5′ four-CpG region was also frequently methylated in prostate cancer tissues compared with benign tissues. Methylation of this region correlated inversely with gene expression in prostate cancer and benign prostate tissues. So far, methylation of *GADD45a* has not been studied as a blood-based biomarker of prostate cancer.

In this study, we examine the usefulness of *GADD45a* methylation in blood DNA as a marker of prostate cancer alone and in combination with PSA and fcDNA in 82 patients. We also compared *GADD45a* methylation in matched DNA from serum and buffy coat in 44 patients.

MATERIALS AND METHODS

Study patients. We have previously conducted a prospective study that examined the use of serum fcDNA levels as a biomarker of prostate cancer in a 'representative' group of patients that underwent prostate cancer screening (Gordian *et al*, 2010). This study included 252 men, with PSA levels $>4\,\mathrm{ng\,ml}^{-1}$ and/or abnormal DRE, who underwent prostate biopsy at the Urology Clinic of University of Miami/Jackson Memorial Hospitals between 2004 and 2008 under an institutional review board protocol. Patients with other malignancies and patients not willing to sign an informed consent form were excluded. A racially diverse population comprising of non-Hispanic blacks, Hispanic whites,

non-Hispanic whites and Hispanic blacks was included. Baseline demographics, medical history and other pertinent information were obtained from the hospital charts. Levels of fcDNA in serum before prostate biopsy were quantitated by real-time PCR amplification of the *GSTP1* gene (Gordian *et al*, 2010). The present study included a subset of 82 patients with sufficient serum for *GADD45a* methylation analysis. We first determined whether *GADD45a* methylation in serum or buffy coat or both correlate with prostate biopsy results and whether there is correlation between *GADD45a* methylation in serum and buffy coat. For this, we compared *GADD45a* methylation at CpG 1 site in matched DNA from serum and buffy coat in 22 prostate cancer and 22 benign patients. This assay was done using Ms-SNuPE analysis. Following this, we examined *GADD45a* methylation at four CpG sites in serum in the larger group of 82 patients (34 cancer and 48 benign) using a more robust pyrosequencing analysis.

Blood collection and DNA isolation. Blood was collected in 10-ml serum plus blood collection tubes (BD Vacutainer, Franklin Lakes, NJ, USA) before patients undergoing prostate biopsy. Serum was separated by centrifugation at 3500 r.p.m. for 15 min at room temperature. DNA was extracted from 2 ml serum using QIAamp UltraSens virus kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol and stored at $-20\,^{\circ}\mathrm{C}$ until further analysis.

Ms-SNuPE analysis of *GADD45a* methylation. Bisulphite conversion of the serum DNA was carried out as previously described (Das *et al*, 2006). After bisulphite treatment, the DNA template was amplified with primer sets: forward: 5′-TTGG GTTGTTAGGGATTTTTATATG-3′; reverse: 5′-AAAATCTTT TCCACAAAAAACAAAA-3′ for the 5′ four-CpG region (Ramachandran *et al*, 2009). Ms-SNuPE analysis was done with the primer spanning CpG 1: 5′-AAACTAATCTAC-3′. Ms-SNuPE reactions were carried out using 1 μl of the PCR product in a 25 μl reaction mixture containing 1 × PCR buffer (Sigma, St Louis, MO, USA), 1 μM of Ms-SNuPE primer, 1 μCi of ^{32}P-labelled dATP or ^{32}P-labelled dGTP and 1 U of Taq Polymerase (Sigma) (Gonzalgo and Jones, 1997; Ramachandran *et al*, 2007, 2009). Percent methylation in each CpG was calculated as $\mathrm{G}/(\mathrm{G}+\mathrm{A})\times 100$.

Pyrosequencing analysis of *GADD45a* methylation. Methylation levels were quantified using pyrosequencing (EpigenDx, Hopkiton, MA, USA), to examine the methylation status at four CpG sites in the *GADD45a* 5′-UTR located between –737 and -690 relative to the transcription start site. Bisulphite-treated DNA template was amplified by nested PCR. The outer PCR product of 116 bp was generated using primers, forward: 5′-TTGGGTTGTTAGGGAT TTTTATATG-3′ and reverse: 5′-AAAATCTTTTCCACAAAAAA CAAAA-3′. This was then purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) after agarose gel electrophoresis. The resulting DNA was amplified using forward primer: 5′-TGGGTTGTTAGGGATTTTTATAT G-3′ and the biotinylated reverse primer: 5′-AAAATCTTTTCCA CAAAAAACA-3′. The biotinylated primer was used to capture one single-stranded DNA template for pyrosequencing (Royo *et al*, 2007). The PCR products (10 μl) were sequenced using the forward sequencing primer: 5′-GATTTTTATATGTGGTTATT-3′ on the Pyrosequencing PSQ96 HS System (Biotage, Uppsala, Sweden). Loci were analysed as a T/C SNP using QCpG software (Biotage). Percent methylation was calculated as the relative peak height of T *vs* C at each CpG site. Each plate contained unmethylated, partially methylated and heavily methylated DNAs (SssI-treated DNA) for quality control.

Statistical analysis. Patient groups (prostate cancer *vs* benign) were compared with respect to categorical variables using the Fisher's exact test or the χ^2-test. For continuous variable approximately normal distributed, such as age, log2 PSA and

log2 fcDNA, patient groups were compared by the two-sample Student's *t*-test. Patient groups were compared with respect to PSA and fcDNA original data using the Wilcoxon–Mann–Whitney two-sample rank-sum test. This test is based on the ranks, as opposed to the actual measurements, and is recommended when data is not approximately normal distributed. Multivariate predictive model for prostate cancer was generated using Classification and Regression Tree (CART) (Breiman *et al*, 1984) analysis implemented in the R-package RPART (recursive partitioning for classification, regression and survival trees), version 4.1–8 (http://CRAN.R-project.org/package=rpart) (Therneau *et al*, 2014). In assessing the performance of the decision tree model, we report misclassification error rate considering all data as well as the misclassification error rate obtained by leave-one-out cross-validation. We employed logistic regression analysis to estimate odds ratios (ORs) and corresponding 95% confidence intervals (CIs) associated to individual binary predictors and risks groups identified by the RPART analysis. The Firth's penalised likelihood approach was employed owing to small sample size and quasi-complete separation of data resulting in sparse data for some risk groups. (Heinze and Schemper, 2002; Heinze, 2006). Statistical analyses were performed using SAS version 9.3 (SAS Institute, Inc., Cary, NC, USA) and R package 3.1.1.

RESULTS

Patient characteristics. Characteristics of patients grouped according to prostate biopsy are reported in Table 1. Of the total 82 patients analysed in this study, 34 were diagnosed with prostate cancer and 48 were benign based on prostate biopsy. The mean age was 64.9 years in prostate cancer and 64.5 in benign patients. The prostate cancer group had a higher percentage of blacks (44%) than the benign group (29%). The majority of blacks (79%) were non-Hispanic and the majority of whites (96%) were Hispanic. Following the exclusion of the two largest PSA values (1981 and 5000 ng ml^{-1}), the data follows normal distribution; mean PSA in the cancer group (mean = 34.7 ng ml^{-1}; s.d. = 62.6, max = 306.4 ng ml^{-1}) was significantly different from that of benign group (mean = 7.1 ng ml^{-1}; s.d. = 4.1) using *t*-test ($P = 0.018$). The number of patients with PSA > 10 ng ml^{-1} was greater in prostate cancer (47%) than in benign (27%). The majority of prostate cancer patients (47.1%) had a Gleason score of 6 (3 + 3).

Correlation of serum and buffy coat *GADD45a* methylation with biopsy result in 44 matched samples. First, we determined whether serum and/or buffy coat *GADD45a* methylation correlated with prostate biopsy results. For this, we analysed methylation of a CpG site located at −737 (CpG 1) with respect to transcription start site by Ms-SNuPE (Ramachandran *et al*, 2009) in a subset of 44 patients comprising of 22 prostate cancer and 22 benign patients. Patient groups (prostate cancer *vs* benign) were compared using the two-sample *t*-test. Difference in CpG 1 methylation levels in serum between cancer *vs* benign was statistically significant ($P = 0.004$), with mean levels higher in prostate cancer (33.9%) as compared with benign (14.6%) (Figure 1A). Similar results were obtained using log base 2-transformed data ($P = 0.027$; data not shown). There was no statistically significant difference in methylation between cancer (18.5%) and benign (20.1%) in buffy coat DNA ($P = 0.686$; Figure 1B). This was also true for log base 2-transformed data ($P = 0.405$; data not shown). No significant correlations were seen between serum and buffy coat in both prostate cancer ($r = 0.047$; $P = 0.836$) and benign groups ($r = 0.273$; $P = 0.219$; Figure 1C). As we found low correlation of buffy coat and serum *GADD45a* methylation levels and also lack of significant difference in buffy coat *GADD45a* methylation levels

	Biopsy				
	Cancer		**Benign**		
Variable	***N***	**%**	***N***	**%**	***P***
Total	34	100.0	48	100.0	
Age (years)					
Mean (s.d.)	64.9 (7.0)		64.5 (6.1)		0.741
Median (range)	63 (56, 84)		64 (50, 75)		
Ethnicity					
Hispanic	20	58.8	37	77.1	0.077
Non-Hispanic	14	41.2	11	22.9	
Race					
Black	15	44.1	14	29.2	0.163
White	19	55.9	34	70.8	
Ethnicity/race					
H					
Black	2	5.9	4	8.3	
White	18	52.9	33	68.8	
NH					
Black	13	38.2	10	20.8	
White	1	2.9	1	2.1	
PSA					
≤2.5	3	8.8	6	12.5	0.331
2.5–|4	1	2.9	2	4.2	
4–|10	14	41.2	27	56.3	
>10	16	47.1	13	27.1	
≤10	18	52.9	35	72.9	0.062
>10	16	47.1	13	27.1	
Mean (s.d.)	238.0 (907.2)		7.1 (4.1)		
Median (range)	9.1 (0.6, 5000)		5.9 (0.4, 15.9)		0.003[a]
Log2 PSA					
Mean (s.d.)	4.1 (2.8)		2.5 (1.1)		0.003
Median (range)	3.2 (−0.7, 12.3)		2.6 (−1.2, 4.0)		
fcDNA					
Mean (s.d.)	277.5 (490.8)		128.2 (294.1)		
Median (range)	174.8 (0.1, 2798.6)		43.2 (0.1, 1886.1)		0.009[a]
Log2 fcDNA					
Mean (s.d.)	6.3 (3.1)		5.2 (2.7)		0.088
Median (range)	7.4 (−3.1, 11.5)		5.4 (−4.1, 10.9)		
Gleason Score					
6	16	47.1			
7	9	26.5			
8–10	9	26.5			

Table 1. Patient characteristics

[a]*P*-value from Wilcoxon–Mann–Whitney two-sample rank-sum test.
P-value from Fisher's exact test or χ^2-test, or two-sample Student's *t*-test. Note: Excluding two largest PSA values (1981 and 5000 in cancer group) data approaches normal distribution; in this case, cancer group PSA mean of 34.7 (s.d. = 62.6, max = 306.4) is significant different from benign group mean of 7.1 (s.d. = 4.1) using *t*-test ($P = 0.018$).

between the benign and prostate cancer groups, we decided that further analysis would be limited to serum DNA. Despite high reproducibility and ease of being conducted in the lab, Ms-SNuPE has a few limitations. Only one CpG could be analysed per assay and it also required use of radioisotopes. Pyrosequencing is a more robust high-throughput assay that allows analysis of multiple CpGs at a time. We measured methylation levels in all four CpGs (−737 (CpG 1), −723 (CpG 2), −706 (CpG 3) and −690 (CpG 4) with respect to transcription start site) in serum in this subset of patients. Our results for CPG 1 were similar to that observed by Ms-SNuPE assay. Significantly higher levels of methylation were observed in prostate cancer group compared with that of benign (mean: 19.3% *vs* 6.4%, $P = 0.006$; Figure 1D). The correlation between serum *GADD45a* methylation levels measured by Ms-SNuPE and that measured by pyrosequencing were very high in both groups, prostate cancer ($r = 0.972$, $P < 0.0001$) and benign ($r = 0.776$, $P < 0.0001$; Figure 1E).
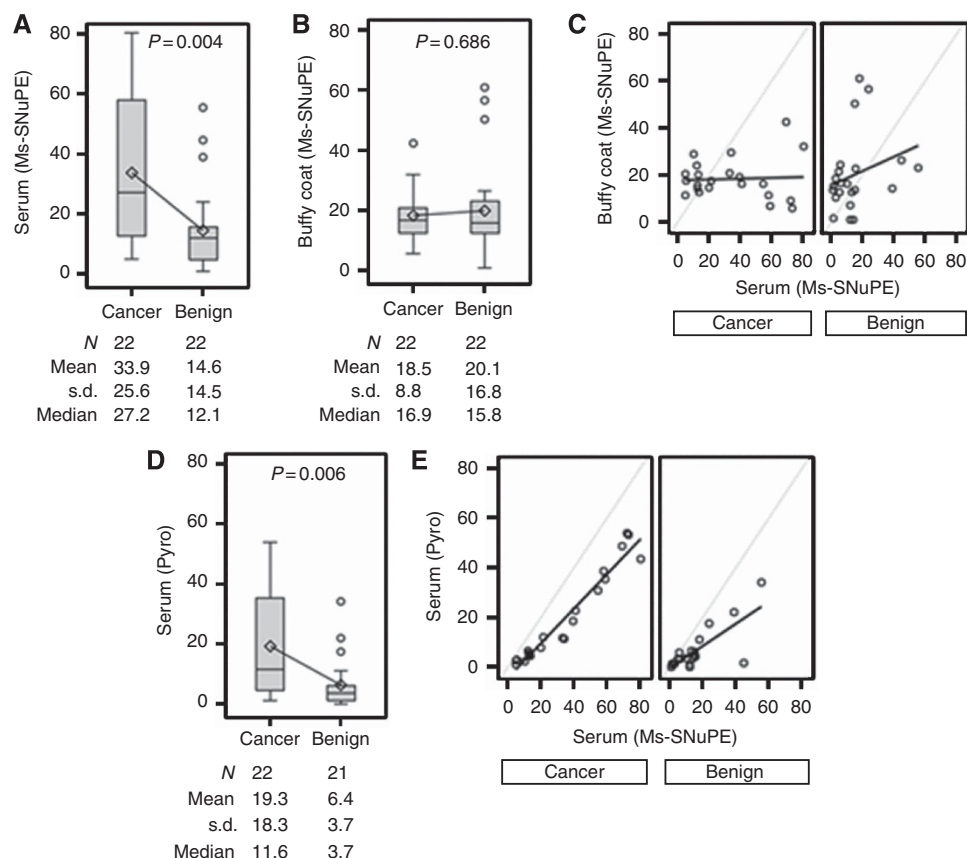
Figure 1. Methylation in *GADD45a* CpG 1 in matched serum and buffy coat DNA samples. Box plots of percent *GADD45a* methylation in serum (**A** and **D**) and buffy coat (**B**) by biopsy result. Low correlation was seen between *GADD45a* methylation levels in serum and buffy coat by Ms-SNuPE analysis (**C**). *GADD45a* serum methylation was higher in cancer as compared with benign patients measured by Ms-SNuPE (**A**) and pyrosequencing (Pyro) (**D**). High correlation was observed between serum *GADD45a* methylation analysed by Ms-SNuPE and pyrosequencing (**E**).

Serum *GADD45a* methylation in benign and prostate cancer patients. We examined the pattern of methylation of four CpGs in 34 cancer and 48 benign patients by pyrosequencing analysis. The distributions of serum *GADD45a* methylation in biopsy-positive and -negative patients were compared using the two-sample Student's *t*-test and also using the non-parametric Mann–Whitney–Wilcoxon rank-sum test. In all CpGs, means and medians of *GADD45a* methylation were significantly higher in cancer than in benign patients. Greater variability of *GADD45a* methylation was seen among the cancer patients compared with benign patients (Figure 2A). Serum *GADD45a* methylation measured at each of the four CpGs, and also sum of four CpGs were log base 2-transformed for easy visualisation and to improve the approximation to normal distribution. Log-transformed data by biopsy result were compared using the two-sample Student's *t*-test. Mean values at each CpG, and also the sum of four CpGs were significantly higher in cancer than in benign group (Figure 2B). The results are summarised in Table 2.

Correlation of serum *GADD45a* methylation with Gleason score in prostate cancer patients. In order to assess the prognostic value of *GADD45a* methylation in serum, we examined whether the levels of serum *GADD45a* methylation correlates with Gleason score among the 34 prostate cancer patients. Of these, there were 16 patients with Gleason score 6, 9 with Gleason score 7 and 9 patients with Gleason score 8–10. We did not observe correlation of *GADD45a* methylation levels at any of the four CpG sites with Gleason score. There was no statistically significant difference between *GADD45a* means of the three Gleason score groups

considering the individual CpG or the sum of four CpGs. ANOVA *P*-values were 0.320 for CpG 1, 0.380 for CpG 2, 0.500 for CpG 3, 0.785 for CpG 4 and 0.443 for sum of four CpGs (data not shown).

Prostate cancer predictive models based on PSA, fcDNA levels and *GADD45a* methylation. In order to understand the relationship between potential predictors of prostate cancer and to develop a multivariate predictive model, we employed Classification and Regression Tree (CART) analysis. As shown in Figure 3A, the RPART analysis resulted in classification trees defining risk groups based on cut-off points for PSA, fcDNA and *GADD45a* methylation (sum of four CpGs). Age, race and ethnicity were also included in the RPART model statement; however, none of these demographic characteristics was predictive to the extent of defining partition groups in the presence of the other variables. The strongest predictor was PSA level 16.9 ng ml^{-1}. All 13 patients with PSA $\geqslant$16.9 ng ml^{-1} had cancer. Using this PSA cutoff of 16.9 ng ml^{-1} resulted in 100% specificity, but only 38.2% sensitivity. The next strongest predictor was fcDNA level $\geqslant$188 ng ml^{-1}. Of note, the fcDNA cutoff point by CART analysis is consistent with previous study with 252 patients (Gordian *et al*, 2010), in which we found that the cutoff point of 180 ng ml^{-1} best discriminated between cancer and benign patients. Among the 69 patients with PSA <16.9 ng ml^{-1}, 51 had fcDNA level <188, among which 43 were benign and 8 had cancer. Of the 18 patients with fcDNA $\geqslant$188 ng ml^{-1}, 13 had cancer and 5 were benign. The combination of PSA with fcDNA had a specificity of 89.5% and resulted in improvement of sensitivity to 76.5%. The third
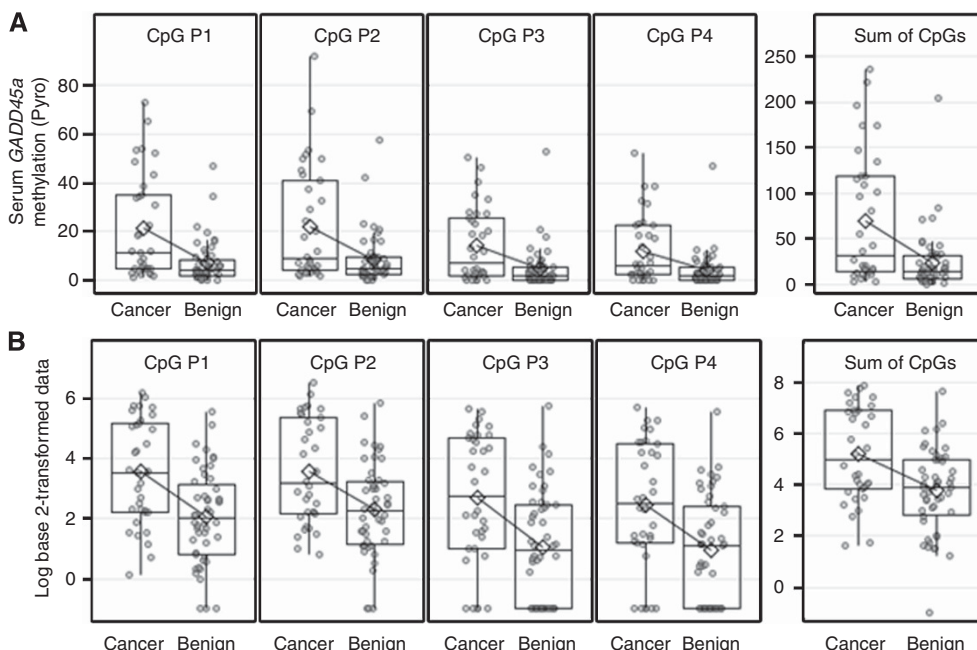
Figure 2. Percent *GADD45a* methylation in serum (pyrosequencing) by biopsy result. Jittered box plots of original data (**A**), and of log base 2-transformed data (**B**).

Table 2. Pyrosequencing serum percent *GADD45a* methylation in four CpGs by biopsy result										
	CpG P1		CpG P2		CpG P3		CpG P4		Sum of four CPGs	
	Cancer	Benign	Cancer	Benign	Cancer	Benign	Cancer	Benign	Cancer	Benign
N	34	48	34	48	34	48	34	48	34	48
Percent *GADD45a* methylation										
Min	1.10	0.00	1.74	0.00	0.00	0.00	0.00	0.00	3.11	0.00
Max	73.22	46.85	92.00	57.60	50.30	52.68	52.52	46.79	235.08	203.92
s.d.	21.16	8.91	23.27	10.73	14.83	8.51	13.86	7.31	70.72	32.69
Median	11.60	4.02	8.99	4.81	7.06	1.93	5.75	2.16	31.59	14.91
Median difference	7.58 $P=0.005$		4.18 $P=0.0031$		5.15 $P=0.001$		3.59 $P=0.0017$		16.68 $P=0.002$	
Mean	21.42	7.19	22.20	8.44	14.37	4.63	12.23	4.00	70.22	24.25
Mean difference	14.23 $P=0.006$		13.76 $P=0.0025$		9.75 $P=0.0012$		8.23 $P=0.0027$		45.97 $P=0.001$	
Log base 2-transformed data										
Min	0.14	−1.00	0.80	−1.00	−1.00	−1.00	−1.00	−1.00	1.64	−1.00
Max	6.19	5.55	6.52	5.85	5.65	5.72	5.71	5.55	7.88	7.67
s.d.	1.75	1.56	1.74	1.55	2.19	1.85	2.15	1.77	1.84	1.63
Median	3.54	2.00	3.17	2.26	2.74	0.93	2.52	1.11	4.96	3.90
Mean	3.54	2.05	3.56	2.28	2.68	1.04	2.43	0.93	5.19	3.77
Mean difference	1.50 $P=0.0001$		1.28 $P=0.0008$		1.64 $P=0.0005$		1.50 $P=0.0009$		1.42 $P=0.0004$	
P-value from two-sample Student's *t*-test.										

strongest predictor was *GADD45a* methylation (sum of four CpGs) at a cutoff point of 89.6. Seven patients had *GADD45a* methylation ⩾89.6 among those with PSA <16.9 and fcDNA <188; of these six had cancer and one was benign. Of the 44 patients with sum of four CpG methylation level <89.6, 42 were benign and 2 had cancer. The regression tree model misclassified 8 patients (9.8%), 6/48 benign (12.5%) and 2/34 cancer (5.9%), yielding a specificity and sensitivity of 87.5% and 94.1%, respectively. The leave-one-out cross-validation misclassification error was 9.9%.

Next, we employed univariate and multivariate logistic regression analysis to estimate ORs and corresponding 95% CIs associated to risks groups identified by the RPART analysis. (Table 3). Univariate logistic models were computed for examining the effect of individual variables: PSA >16.9 *vs* ⩽16.9 ng ml^{-1}, fcDNA >188 *vs* ⩽188 ng ml^{-1} and *GADD45a* methylation >89.6 *vs* ⩽89.6, on the risk of prostate cancer in comparison with benign patients. Odds ratios for PSA, fcDNA or *GADD45a* methylation comparing prostate cancer *vs* benign patients based on various fitted logistic models are reported in Table 3. Univariate
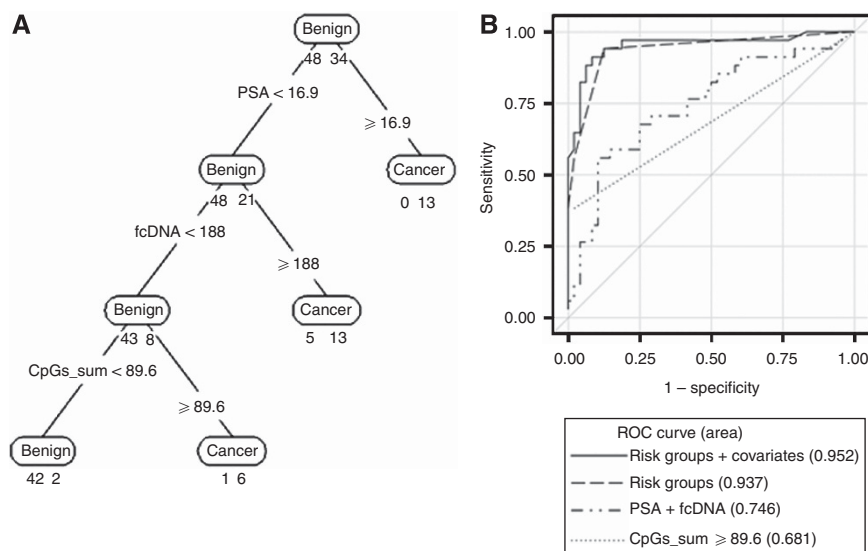
Figure 3. (**A**) Classification tree based on cut points for PSA, fcDNA and sum of *GADD45a* four-CpG methylation (CpGs_sum). Age, race and ethnicity were also included in each model statement under RPART but none defined partition. Below each prediction node label (corresponding to majority vote) is shown the number of observations from benign and cancer groups, respectively. The corresponding misclassification rates were 8/82 (9.8%) overall, 6/48 (12.5%) in benign and 2/34 (5.9%) in cancer group. These implied specificity 42/48 (87.5%) and sensitivity 32/34 (94.1%). In addition, the leave-one-out cross-validation misclassification error was 9.9%. (**B**) Receiver-operating curves for comparison of the classification tree models including PSA, fcDNA and *GADD45a* methylation (solid and dashed lines) to other fitted models.

Table 3. Estimated odds ratios for prediction of prostate cancer status from logistic regression models evaluating the effect of risk groups and cutoffs for PSA, fcDNA and *GADD45a* methylation obtained from RPART analysis

Risk groups defined by univariate analysis of cutoffs for PSA, fcDNA and *GADD45a* methylation (sum of four CpGs)

Risk group	PSA	fcDNA	*GADD45a* methylation	Benign (*N*)	Cancer (*N*)	Total (*N*)	Prediction	Models		
								OR (95% CI)	*P*	AUC
1	<16.9	—	—	48	21	69	Benign	Reference		0.691
2	⩾16.9	—	—	0	13	13	Case	60.9 (3.11, >999)	0.007	
1	—	<188	—	43	17	60	Benign	Reference		0.698
2	—	⩾188	—	5	17	22	Case	8.6 (2.7, 27.0)	0.0002	
1	—	—	<89.6	47	21	68	Benign	Reference		0.681
2	—	—	⩾89.6	1	13	14	Case	29.1 (3.57, 237.1)	0.002	

Risk groups defined by PSA, fcDNA and *GADD45a* methylation (sum of four CpGs)

Risk group	PSA	fcDNA	*GADD45a* methylation	Benign (*N*)	Cancer (*N*)	Total (*N*)	Prediction	Model 1 (AUC = 0.937)		Model 2 (AUC = 0.952)	
								OR (95% CI)	*P*	OR (95% CI)	*P*
1	<16.9	<188	<89.6	42	2	44	Benign	Reference		Reference	
2	<16.9	<188	⩾89.6	1	6	7	Case	73.7 (7.4, 731)	<0.001	77.8 (6.7, 902)	<0.001
3	<16.9	⩾188	—	5	13	18	Case	41.7 (8.1, 215.9)	<0.0001	36.4 (8.1, 188)	<0.0001
4	⩾16.9	—	—	0	13	13	Case	458.9 (18.7, >999)	<0.001	348.7 (18.7, >999)	<0.001

Abbreviations: OR = odds ratio; CI = confidence interval. Model 1: effect of the composite variable risk group. Model 2: effect of the composite variable risk group adjusted for race (Black *vs* White; OR = 0.57, *P* = 0.636), ethnicity (non-Hispanic *vs* Hispanic; OR = 4.25, *P* = 0.262) and age (1-year increase; OR = 0.97, *P* = 0.607).

analysis including only PSA >16.9 *vs* ⩽16.9 ng ml^{-1} yielded an OR of 60.9 (95% CI: 3.11, >999; *P* = 0.007; AUC = 0.691), model including only fcDNA >188 *vs* ⩽188 ng/ml yielded an OR of 8.6 (95% CI: 2.7, 27.0; *P* = 0.0002; AUC = 0.698), and model including only *GADD45a* methylation >89.6 *vs* ⩽89.6 yielded an OR of 29.1 (95% CI: 3.57, 237.1; *P* = 0.002; AUC = 0.681). Figure 3B shows the ROC curves for various fitted models. There were significant

improvements (*P* < 0.001) in prediction comparing models including risk groups *vs* model including PSA + fcDNA or model including *GADD45a* alone. Receiver-operating characteristic AUCs for the multivariate models defined by PSA, fcDNA levels and *GADD45a* methylation derived from regression trees analysis without and with adjustment for race, ethnicity and age were 0.937 and 0.952, respectively, and these were significantly higher than

those for the univariate models and the model including PSA + fcDNA (AUC = 0.746). Thus, the composite models including these three markers were effective in discriminating biopsy positive from biopsy negative patients.

DISCUSSION

The present study included 82 subjects who were part of our previous study that examined the usefulness of fcDNA as a biomarker of prostate cancer (Gordian *et al*, 2010). These subjects had sufficient stored serum for methylation analysis. We first determined whether *GADD45a* methylation in serum or buffy coat or both correlate with prostate biopsy results and whether there is correlation between *GADD45a* methylation in serum and buffy coat. For this, we compared *GADD45a* methylation in matched DNA from serum and buffy coat in 22 prostate cancer and 22 benign patients. This assay was done using Ms-SNuPE analysis (Ramachandran *et al*, 2009). We found statistically significant difference in serum methylation between cancer and benign groups. However, we did not observe statistically significant difference in buffy coat methylation between cancer and benign groups. There was no significant correlation between serum and buffy coat in both prostate cancer and benign groups. Hence, we proceeded to study serum *GADD45a* methylation in 82 patients comprising of 34 cancer and 48 benign patients using a more robust pyrosequencing analysis.

Although there has been a lot of interest in using DNA derived from peripheral blood as biomarkers for prostate cancer diagnosis and prognosis, the relative significance of serum *vs* buffy coat DNA is currently unknown. Several studies have examined DNA methylation in buffy coat as a biomarker of various cancers including prostate cancer (Goessl *et al*, 2000; Wong *et al*, 2000; Suga *et al*, 2008; Marsit *et al*, 2011; You *et al*, 2012). The advantages of using buffy coat include recovery of large amounts of DNA as compared with serum or plasma, and the ability to store for prolonged periods of time (Mychaleckyj *et al*, 2011). Buffy coat DNA is comprised of cell-bound DNA including blood cell DNA as well as DNA from circulating tumour cells. On the other hand, the source of serum/plasma fcDNA is not clear. Despite several possible sources such as apoptosis/necrosis of tumour cells, blood cells, lysed circulating tumour cells and active release of fcDNA by living cells (van der Vaart and Pretorius, 2008), tumour DNA constitutes a large proportion of fcDNA in cancer patients.

There are conflicting reports in literature regarding the correlation of buffy coat and serum DNA methylation with tumour. Goessl *et al* (2000) examined *GSTP1* methylation in prostate tissue and compared with bodily fluids such as serum, plasma, buffy coat and ejaculate. They found higher correlation of *GSTP1* methylation in serum and plasma with that of prostate tissue compared with that of buffy coat. Contrary to this, Wong *et al* (2000) examined *p15* methylation in tumours, plasma, serum and buffy coat samples from hepatocellular carcinoma patients and found that buffy coat *p15* methylation showed a higher correlation with that of tumour DNA compared with serum and plasma. We found that *GADD45a* methylation in serum fcDNA but not buffy coat DNA distinguishes between benign and prostate cancer patients.

The first study of non-invasive methylation biomarker of prostate cancer was the analysis of *GSTP1* promoter methylation in prostate cancer and benign patients. *GSTP1* promoter hyper-methylation was observed in 72% of prostate cancer patients, but none of the patients with benign prostatic hyperplasia (BPH) (Goessl *et al*, 2000, 2001). The usefulness of hypermethylation of other genes including *RASSF1A*, *RARβ2*, *MDR1*, *EDNRB*, *TIG1*

and *Reprimo* in serum as a diagnostic marker of prostate cancer has also been shown (Ellinger *et al*, 2008; Hoque, 2009). A pooled analysis of about 900 prostate cancer cases in several studies that examined the presence of *GSTP1* hypermethylation in fcDNA showed that the assay specificity was high (>93%), but the sensitivity was varying (ranging from 11 to 100%). Moreover, it was found that the sensitivity was increased when methylation of multiple genes was analysed (Ellinger and Bastian, 2010). To our knowledge, ours is the first study to examine the role of *GADD45a* methylation in early detection of prostate cancer. The cutoff level of 89.6 for sum of four CpG methylation could distinguish between biopsy-positive and biopsy-negative patients with a high specificity of 97.9% and moderate sensitivity of 38.2% (AUC = 0.681). There are only a limited number of reports on quantitative analysis of serum DNA methylation in prostate cancer. Our results are comparable to one such study that showed that *GSTP1* hypermethylation could discriminate prostate cancer and BPH patients at a high specificity of 92.9% and sensitivity of 42.3% (AUC = 0.684; Ellinger *et al*, 2008).

Prognostic value of DNA methylation markers in fcDNA has been previously reported. Reibenwein *et al* (2007) showed correlation between *GSTP1* methylation and Gleason score. Sunami *et al* (2009) reported that hypermethylation of *RASSF1A*, *RARβ2* and *GSTP1* correlated with Gleason score and serum PSA. However, in the study by Ellinger *et al* (2008), there was no correlation between methylation of *GSTP1, TIG1, PTGS2 and Reprimo* either in combination or individually with the patholo-gical stage or grade. Among the 34 biopsy-positive patients, we did not find correlation between *GADD45a* methylation levels and Gleason score. This lack of correlation may be related to the limited sample size.

Prostate cancer screening has remained controversial as it has been associated with a large risk of overdetection (Andriole *et al*, 2009; Schroder *et al*, 2009). A large majority of the prostate biopsies performed for increased PSA alone are negative. There have been attempts to reduce the number of unnecessary biopsies by using PSA derivatives as markers. However, many of these markers have not gained acceptance owing to the lack of sensitivity or specificity or both. Garzotto *et al* (2005) used CART analysis to develop a prostate biopsy algorithm in patients with elevated PSA/abnormal DRE or both. Their CART model included PSA, PSA density, hypoechoic lesions, age and prostate volume. This model had a specificity of 31.3%, sensitivity of 96.6% and AUC of 0.74. In our previous study we found that inclusion of fcDNA and the interaction of fcDNA and PSA to a model with PSA, age and race significantly improved predictivity compared with model without fcDNA yielding a sensitivity and specificity of 95.5% and 33.1%, respectively, AUC 0.742, positive predictive value of 43.8 and negative predictive value of 93.1 (Gordian *et al*, 2010). To our knowledge, there are no reports on CART models that have included molecular markers for the prediction of prostate cancer. In the present study, the CART model had sensitivity and specificity of 94.1% and 87.5%, respectively, and the AUC was 0.937, indicating high prediction accuracy (positive predictive value = 84.2% and negative pre-dictive value = 95.5%). We show that if we apply this model including PSA, fcDNA and *GADD45a* methylation in our patient group, we would eliminate the need for biopsy in 53.6% patients (44 out of 82), as a consequence of our estimated high specificity of 87.5%. Furthermore, only 2 out 34 true positives were missed (false negative rate of 5.9%). However, a major limitation of this study is the small sample size. Owing to the lack of sample availability, this study only included a subset of patients of the larger group that was representative of prostate cancer screening setting. Thus, the results of this study need to be validated in a larger prospective group of patients that undergo prostate biopsy after prostate cancer screening.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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