

Serum Free Circulating DNA Is a Useful Biomarker to Distinguish Benign versus Malignant Prostate Disease

Edna Gordian, Kavitha Ramachandran, Isildinha M. Reis, Murugesan Manoharan, Mark S. Soloway, and Rakesh Singal

Abstract

Background: Free circulating DNA (fcDNA) has been shown to be elevated in serum of prostate cancer patients compared with benign controls. However, studies evaluating the role of fcDNA as a biomarker in a “representative” patient group who have undergone prostate cancer screening are lacking. Our study examined the use of serum fcDNA levels as a biomarker of prostate cancer in such a setting.

Methods: The study included 252 men, with prostate-specific antigen (PSA) levels >4 ng/mL and/or abnormal digital rectal exam. fcDNA levels in serum before prostate biopsy were quantitated by real-time PCR amplification of the *glutathione S-transferase, pi*, gene.

Results: Patients with PSA ≤ 10 ng/mL with fcDNA > 180 ng/mL were at increased risk for prostate cancer compared with those with fcDNA ≤ 180 ng/mL (odds ratio, 4.27; 95% confidence interval, 2.05–8.88; $P < 0.001$; area under the curve, 0.742). The multivariate model including age, race, PSA, fcDNA, and interaction between fcDNA and PSA yielded a high negative predictive value of 93.1% and increased specificity of 33.1% compared with negative predictive value of 73.3% and specificity of 6.7% in the model excluding fcDNA.

Conclusions: Our results indicate that fcDNA may improve the specificity of prostate cancer screening.

Impact: Our study shows that adding fcDNA to prostate cancer screening can reduce the number of unnecessary prostate biopsies. *Cancer Epidemiol Biomarkers Prev*; 19(8); 1984–91. ©2010 AACR.

Introduction

Prostate cancer is the second leading cause of death in men in the United States, with an estimated 192,280 new cases and 27,360 prostate cancer related deaths for 2009 (1). Although prostate-specific antigen (PSA) test along with digital rectal examination is the currently used prostate cancer screening method in men >50 years of age, it is still unclear whether the benefits of PSA screening outweigh its limitations. The predictive value of elevated serum PSA levels (>4 ng/mL) is greater than that of digital rectal exam. However, PSA screening alone results in a large number of false positives and false negatives because elevated PSA levels do not represent the presence of prostate cancer alone but may also reflect certain non-malignant conditions such as benign prostatic hyperplasia (BPH), as well as prostatitis (2, 3). In men undergoing prostate biopsy for elevated PSA levels between 4.1 and 9.9 ng/mL, 25% to 35% were diagnosed with prostate

cancer, indicating that the remaining 65% to 75% of the men were subjected to unnecessary prostate biopsy (4). This high rate of false positives results in additional medical procedures with potential health risks, significant healthcare expenses, and anxiety to the patients and their family. On the contrary, prostate cancer was detected in 22% of men who underwent prostate biopsy with PSA level between 2.6 and 4 ng/mL (5). Moreover, 30% of men with PSA levels between 4.1 and 10 ng/mL who are diagnosed with prostate cancer are likely to have an extraprostatic extension of prostate cancer. Based on these findings, it has been suggested that the PSA cut point for prostate cancer screening be lowered to 2.5 ng/mL (6, 7). Although this reduction in PSA cutoff value has improved the detection rate of prostate cancer, it has also led to increased number of false positives who are then subjected to unnecessary prostate biopsy (8). A noninvasive test with higher specificity is required to eliminate unnecessary prostate biopsies.

The ratio of free PSA to total PSA has been shown to improve the diagnostic accuracy in the group with slightly elevated serum PSA values (9–11). However, the clinical significance of free to total PSA ratio remains controversial. Hence, there is a desperate need for novel biomarkers that, when used independently or in combination with PSA, are capable of increasing the specificity and sensitivity of prostate cancer detection.

The presence of circulating cell-free nucleic acids in cancer patients has been well documented, and the correlation between elevated levels of free circulating

Authors' Affiliation: Sylvester Comprehensive Cancer Center, University of Miami, Miami, Florida

Note: E. Gordian and K. Ramachandran contributed equally to this work.

Corresponding Author: Rakesh Singal, Sylvester Comprehensive Cancer Center, University of Miami School of Medicine, 1475 NW, 12th Avenue, Miami, FL 33136. Phone: 305-243-9544; Fax: 305-243-5977. E-mail: rsingal@med.miami.edu

doi: 10.1158/1055-9965.EPI-10-0287

©2010 American Association for Cancer Research.

DNA (fcDNA) and cancer has been established. In 1977, Leon et al. (12) reported increased levels of serum DNA in cancer patients compared with healthy controls. This was followed by other studies showing elevated levels of circulating serum or plasma DNA in patients with malignant tumors compared with benign controls (13-19). Sozzi et al. (17) examined plasma fcDNA levels as a marker for lung cancer and found that the median fcDNA levels in plasma were 8 times greater in cancer than that in control samples (area under the curve, 0.94). The use of fcDNA as a biomarker for prostate cancer has been evaluated in several studies (20-25). Jung et al. (23) described no difference in fcDNA levels between patients with localized prostate cancer and BPH, although fcDNA levels were found to be significantly elevated in metastatic prostate cancer patients. Another study examined levels of a noncancer gene *prostaglandin-endoperoxidase synthase 2* in 168 prostate cancer, 42 BPH, and 11 healthy individuals and found significantly elevated fcDNA levels in prostate cancer (median, 70.2 ng/mL) compared with that of BPH and healthy controls (10.5 and 7.1 ng/mL, respectively; ref. 26). Altimari et al. (27) showed that plasma fcDNA quantitation distinguished between patients with prostate cancer and healthy controls and correlates with pathologic tumor stage. Chun et al. (22) prospectively assessed plasma fcDNA in 142 men with localized prostate cancer and 19 BPH controls and found that fcDNA is an accurate and informative predictor of prostate cancer independent of the established clinical variables such as age and PSA. Bastian et al. (28) showed a significant association of increased preoperative serum fcDNA levels in men with localized prostate cancer with PSA recurrence after radical prostatectomy, indicating that serum fcDNA levels may be a useful prognostic biomarker in patients undergoing radical prostatectomy. Although the above reports indicate the potential clinical application of fcDNA as a diagnostic and prognostic marker of prostate cancer, contrary results have been published, showing that fcDNA levels are higher in BPH controls compared with prostate cancer patients (21, 23).

However, there have been no studies evaluating the role of fcDNA as a biomarker in a "representative" patient group who have undergone prostate cancer screening. In the present study, we prospectively collected blood samples and assessed fcDNA levels before prostate biopsy in patients referred for the procedure based on PSA and digital rectal exam screening. We analyzed fcDNA levels in relation to the demographic and clinicopathologic features and report results of this analysis.

Materials and Methods

Study patients

Patient group consisted of men with elevated PSA levels >4 ng/mL and/or abnormal digital rectal exam, undergoing 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. This comprised of a racially diverse population that included non-Hispanic Blacks, Hispanic Whites, non-Hispanic Whites, and Hispanic Blacks. Two hundred fifty-two men who fit the study inclusion criteria were enrolled in the study before their prostate biopsy. Baseline demographics and medical history were collected. The pathologic and clinical staging information was obtained from the hospital charts.

Blood collection and DNA isolation

Blood was collected in 10 mL serum plus blood collection tubes (BD Vacutainer) before patients undergoing prostate biopsy. Serum was separated by centrifugation at 3,500 rpm for 15 minutes at room temperature, after which DNA was extracted immediately. DNA was extracted from 1 mL serum using QIAamp UltraSens virus kit (Qiagen) following the manufacturer's protocol and stored at -20°C until further analysis.

Quantitation of fcDNA

Serum DNA was quantitated by real-time PCR for *glutathione S-transferase, pi (GSTP1)*, based on the previous study by Bastian et al (28). We used the amplification primers (forward, 5' AGG CCT TCG CTG GAG TTT C 3'; reverse, 5' CCA TGC TGG GAG CTC TGA G 3') and an amplicon-specific fluorogenic hybridization probe (6FAMCGC CGC AGT CTT CGC CAC CTAMRA). The PCR was carried out in an iCycler (Biorad). The PCR mixture consisted of 12.5 µL of Taqman Universal master mix (Applied Biosystems), 5 pmoles of probe, and 5 pmoles each of the forward and reverse primer in a 25 µL reaction volume. Each sample was analyzed in triplicate. All PCR runs included a negative control using water blanks. A standard curve was generated for each PCR run using serial dilutions of human placental DNA (Sigma) at concentrations ranging between 160 ng/µL to 160 pg/µL. For calculation of DNA concentrations, the standard curve was interpolated with the threshold cycle (C_T) of unknown target samples.

Statistical analysis

Data were tabulated by biopsy result: 89 prostate cancer, 59 prostatitis, and 104 BPH patients. Groups were compared using Fisher's exact test or χ^2 test for categorical variables and ANOVA and pairwise comparison by contrasts with Bonferroni's adjustment for continuous variables. Given the large variability of PSA and fcDNA data, corresponding data were log 2 transformed for better normal approximation and stabilization of variance. Univariate and multivariate logistic regression was used to examine the effect of fcDNA (log 2-transformed data and binary indicators of

various cut points) on risk for disease comparing prostate cancer versus a combined benign group of prostatitis and BPH patients. Multivariate models were adjusted for age (years), race (Black or White), and PSA (log₂ PSA and binary indicator of PSA > 10 ng/mL). Bivariate interactions were tested. Results for selected logistic models are presented as age- and race-adjusted odds ratio estimates with corresponding 95% confidence intervals (95% CI) and *P* values for the association between fcDNA or PSA and risk for disease [prostate cancer versus benign (prostatitis + BPH)]. In addition, for selected models, we report area under the curve of receiver operating characteristic, which is a popular measure of model classification accuracy, and predictive probability cut point associated with sensitivity about 95%, as well as the corresponding estimates of specificity, positive predictive value, and negative predictive value. All analyses were done in SAS 9.2.

Results

Patient characteristics

Characteristics of patients grouped according to prostate biopsy are reported in Table 1. Prostate cancer was diagnosed in 35.3% of the patients. The mean age was 66 years in prostate cancer, 63 in prostatitis, and 63 in BPH patients. The prostate cancer group had a significantly higher percentage of Blacks (48%) than prostatitis (17%) and BPH (34%) groups. Most Blacks (75%) were non-Hispanic, and most Whites (96%) were Hispanic. Comparison of log₂-transformed PSA in prostate cancer versus BPH and prostate cancer versus benign (BPH + prostatitis) showed statistically significant mean differences, whereas there was no significant difference in mean of log₂-transformed PSA between BPH and prostatitis. A similar pattern was observed with respect to the proportion of patients with PSA >10 ng/mL, which was significantly higher in prostate cancer (57%) than in

Table 1. Characteristics of study patients

		Prostate cancer	Prostatitis	BPH	<i>P</i> *		
		(89 patients)	(59 patients)	(104 patients)	PCA vs BPH	PTIS vs BPH	PCA vs PTIS + BPH
		<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)			
Age	Mean (SD)	65.5 (7.5)	63.5 (4.9)	62.9 (6.9)	0.007	0.580	0.010
	Md (range)	64 (52-85)	63 (52-75)	63 (42-86)			
Race [†]	Black	43 (48.3)	10 (16.9)	35 (33.7)	0.039	0.022	0.001
	White	46 (51.7)	49 (83.1)	69 (66.3)			
Ethnicity [†]	Hispanic	54 (60.7)	50 (84.7)	75 (72.1)	0.092	0.067	0.007
	Non-Hispanic	35 (39.3)	9 (15.3)	29 (27.9)			
PSA, ng/mL	Mean (SD)	171.2 (768.6)	7.7 (5.7)	7.0 (4.1)	<0.0001	0.993	<0.0001
	Md (range)	9.2 (0.02-5000)	5.7 (0.4-24.9)	6.3 (0.4-26.7)			
Log ₂ PSA	Mean (SD)	3.7 (2.6)	2.5 (1.2)	2.5 (1.0)	<0.0001	0.993	<0.0001
	Md (range)	3.2 (-5.6-12.3)	2.5 (-1.4-4.6)	2.6 (-1.4-4.7)			
PSA	≤2.5	6 (6.7)	9 (15.2)	9 (8.6)	<0.001	0.119	<0.001
	>2.5-4	6 (6.7)	3 (5.1)	9 (8.6)			
	>4-8	26 (29.2)	25 (42.4)	54 (51.9)			
	>8	51 (57.3)	22 (37.3)	32 (30.8)			
	≤10	47 (52.8)	42 (71.2)	85 (81.7)			
	>10	42 (47.2)	17 (28.8)	19 (18.3)			
fcDNA	Mean (SD)	263.4 (383.5)	202.5 (315.5)	188.2 (298.0)	0.036	0.546	0.058
	Md (range)	149.4 (0.1-2798.6)	96.9 (0.1-1781.4)	81.8 (0.1-1886.1)			
Log ₂ fcDNA	Mean (SD)	6.6 (2.7)	6.1 (2.6)	5.8 (2.8)	0.042	0.337	0.007
	Md (range)	7.2 (-3.3-11.5)	6.6 (-4.1-10.8)	6.4 (-3.3-10.9)			
fcDNA	≤180 ng/mL	49 (55.1)	45 (76.3)	72 (69.2)	0.042	0.337	0.007
	>180 ng/mL	40 (44.9)	14 (23.7)	32 (30.8)			
Gleason score	6	52 (58.4)					
	7	15 (16.8)					
	8-10	22 (24.8)					

Abbreviations: Md, median; PCA, prostate cancer; PTIS, prostatitis.

*Fisher's exact test or χ^2 test for categorical variable. ANOVA and pairwise comparison by contrasts for continuous variables.

[†]Most Blacks (75%) were non-Hispanic, and most Whites (96%) were Hispanic.

prostatitis (37%) and in BPH patients (31%). Most prostate cancer patients (58.4%) had a Gleason score of 6 (3 + 3).

fcDNA levels and association with prostate cancer

The median fcDNA levels were 149.4 ng/mL in prostate cancer, 96.9 ng/mL in BPH, and 81.8 ng/mL in prostatitis. There was no statistically significant difference between prostatitis and BPH about fcDNA original measurements or log₂-transformed PSA. Figure 1 shows the distribution of fcDNA levels in different patient groups. There was significant difference in fcDNA distributions for prostate cancer versus benign (prostatitis + BPH), considering all patients ($P = 0.0129$) and in the subgroup of patients with PSA ≤ 10 ng/mL ($P = 0.023$); however, there was no statistically significant difference in fcDNA distributions for prostate cancer versus benign (prostatitis + BPH) in patients with PSA > 10 ng/mL ($P = 0.485$). There was no significant association between fcDNA levels and Gleason score among prostate cancer patients (data not shown).

fcDNA levels as a potential marker of prostate cancer

Univariate logistic models were computed as a first step in examining the effect of fcDNA (log₂ fcDNA or

fcDNA high versus low), PSA (log₂ PSA or PSA > 10 versus ≤ 10 ng/mL), age (in years), and race (Black versus White) on the risk for prostate cancer in comparison with benign (prostatitis + BPH) patients. Based on the area under the curve criteria, the best discrimination between prostate cancer and benign (prostatitis + BPH) using fcDNA was found to be a cut point of 180 ng/mL. This characterization of fcDNA was then included in a multivariate model together with indicator of PSA > 10 ng/mL, age, and race. All two-way interactions were tested, and only fcDNA and PSA interaction was found to be significant ($P = 0.003$). Odds ratios for fcDNA or PSA comparing prostate cancer cases versus controls (prostatitis + BPH) based on various fitted logistic models, with adjustment for age and race, are reported in Table 2. Model 4 including age, race, fcDNA (> 180 versus ≤ 180 ng/mL), PSA (> 10 versus ≤ 10 ng/mL), and the interaction between fcDNA and PSA provides the best fit to data by the area under the curve criteria (area under the curve, 0.742). Based on this model, patients with PSA ≤ 10 ng/mL with fcDNA > 180 ng/mL were at increased risk for prostate cancer compared with those with fcDNA ≤ 180 ng/mL (odds ratio, 4.27; 95% CI, 2.05-8.88; $P < 0.001$), whereas there was no significant effect of fcDNA among patients with PSA > 10 ng/mL (odds ratio, 0.66; 95% CI, 0.25-1.78;

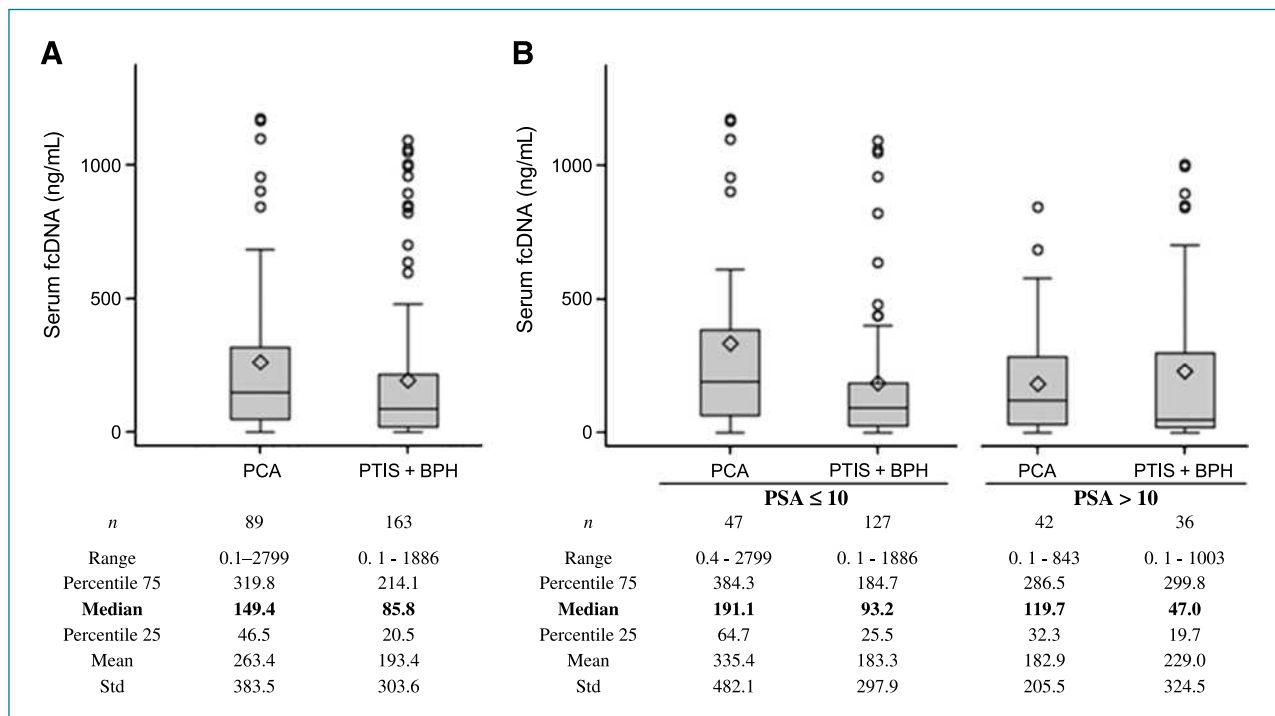


Figure 1. Distribution of serum fcDNA by patient group overall (A) and by PSA category (B). Three fcDNA outlier values are not displayed in box plots; these are the largest fcDNA value in group prostate cancer with PSA ≤ 10 (2,799) and the two largest values in benign group (prostatitis + BPH) with PSA ≤ 10 (1,781 and 1,886). Based on Wilcoxon–Mann–Whitney two-sample test, there was significant difference in fcDNA distributions for prostate cancer versus benign, considering all patients ($P = 0.0129$) and in the subgroup of patients with PSA ≤ 10 ($P = 0.023$); however, there was not statistically significant difference in fcDNA distributions for prostate cancer versus benign in the subgroup of patients with PSA > 10 ($P = 0.485$). PCA, prostate cancer; PTIS, prostatitis; Std, standard deviation.

Table 2. Effect of fcDNA and/or PSA on risk for prostate cancer versus prostatitis + BPH

Logistic regression models, with adjustment for age and race		OR* (95% CI)	P	AUC
1. fcDNA	>180 vs ≤ 180	2.15 (1.22-3.79)	0.008	0.676
2. PSA	>10 vs ≤10	2.67 (1.50-4.77)	<0.001	0.687
3. fcDNA + PSA	fcDNA > 180 vs ≤180	2.25 (1.26-4.03)	0.005	0.726
	PSA > 10 vs ≤10	2.78 (1.54-5.02)	<0.001	
4. fcDNA + PSA + fcDNA * PSA [†]	fcDNA > 180 vs ≤180, in PSA ≤ 10	4.27 (2.05-8.88)	<0.001	0.742
	fcDNA > 180 vs ≤180, in PSA > 10	0.66 (0.25-1.78)	0.417	
	PSA > 10 vs ≤10, in fcDNA ≤ 180	5.42 (2.55-11.52)	<0.001	
	PSA > 10 vs ≤10, in fcDNA > 180	0.84 (0.32-2.24)	0.733	

NOTE: Since the interaction fcDNA * PSA was significant, effects of fcDNA and PSA are reported within categories of the other variable.

Abbreviations: AUC, area under receiver operating curve; OR, odds ratio.

*Odds ratio, estimate relative risk of prostate cancer associated with fcDNA or PSA; relative risk, ratio between odds of prostate cancer and odds of prostatitis + BPH comparing two categories (or values) of explanatory variable. Using log 2-transformed data, the odds ratio estimate for 1-unit increase in log 2 fcDNA represent the change in risk corresponding to a 2-fold increase in the original variable.

[†]Fitted logistic regression equation:

$$\text{Log}[p/(1-p)] = -5.397 + 0.057 \text{ Age} + 0.813 \text{ Race} + 1.690 \text{ PSA} + 1.452 \text{ fcDNA} - 1.860 \text{ fcDNA} * \text{PSA},$$

in which *P*, probability of prostate cancer, PSA (>10 versus ≤10), fcDNA (>180 versus ≤180), race (Black versus White), and age in years. *P* < 0.001 for intercept, 0.015 for age, 0.006 for race, <0.001 for PSA, <0.001 for fcDNA, and 0.003 for fcDNA * PSA interaction.

P = -0.417). Subset analyses of low and high PSA patients confirmed estimates obtained from model 4 (data not shown). Table 3 shows finding from analyses carried out in each of the three subsets defined by PSA ≤ 4, 4 to 10, and >10 ng/mL. The corresponding odds ratios were 3.12 (95% CI, 0.56-17.22; *P* = 0.192), 4.32 (95% CI, 1.83-10.21; *P* < 0.001), and 0.65 (95% CI, 0.24-1.79; *P* = 0.406). Esti-

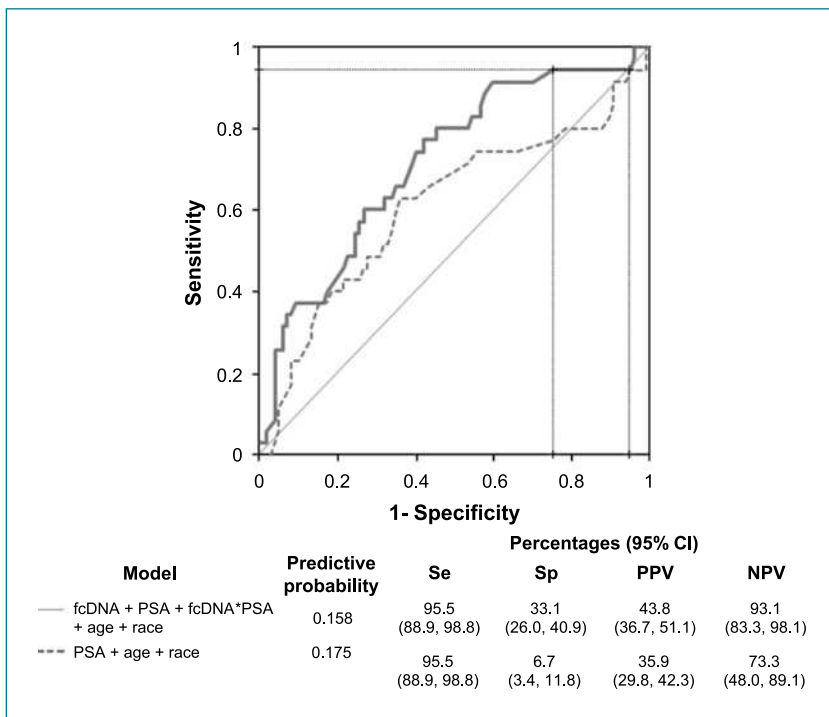
mates in the low and intermediate PSA groups were similar, although statistical significance was not achieved in the low PSA group, which included only 12 cases and 30 controls. Furthermore, inclusion of fcDNA and the interaction of fcDNA and PSA to a model with PSA, age, and race significantly improved predictivity (area under the curve, 0.742 versus 0.687; *P* = 0.029; Fig. 2).

Table 3. Distribution of fcDNA by biopsy result in three PSA categories and estimate effect of fcDNA on risk for prostate cancer

PSA, ng/mL	fcDNA, ng/mL	Biopsy result		Total	OR* (95% CI)	P	AUC
		PCA	PTIS + BPH				
≤4	>180	7 (58.3%)	8 (26.7%)	15	3.12 (0.56-17.22)	0.192	0.775
	≤180	5 (41.7%)	22 (73.3%)	27			
	Total	12	30	42			
4 to ≤10	>180	20 (57.1%)	25 (25.8%)	45	4.32 (1.83-10.21)	<0.001	0.715
	≤180	15 (42.9%)	72 (74.2%)	87			
	Total	35	97	132			
>10	>180	13 (30.9%)	13 (36.1%)	26	0.65 (0.24-1.79)	0.406	0.660
	≤180	29 (69.1%)	23 (63.9%)	52			
	Total	42	36	78			

*Odds ratio, age and race adjusted estimates of relative risk of prostate cancer associated to fcDNA (ratio between odds of prostate cancer and odds of prostatitis + BPH for men with fcDNA > 180 as compared with those with fcDNA ≤ 180) from models by PSA category.

Figure 2. Comparison of receiver operating curves for two fitted models. Dotted line corresponds to model 2 (PSA + age + race) and solid line corresponds to model 4 (fcDNA + PSA + fcDNA * PSA + age + race) in Table 2. Adding fcDNA and the interaction of fcDNA and PSA to a model with PSA, age, and race significantly improved predictivity (area under the curve, 0.742 versus 0.687; $P = 0.029$). At a predicted probability of ≥ 0.158 , inclusion of fcDNA yields a sensitivity of 95% and specificity of 33.1% as compared with 95% sensitivity and 6.7% specificity at predicted probability of ≥ 0.175 when fcDNA was not included. Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value.



Although, in terms of area under the curve, the overall gain is modest, there is considerable gain in specificity at cut points defining high sensitivity. As shown in Fig. 2, based on the model including fcDNA, PSA, interaction of fcDNA and PSA, race, and age, a predicted probability

≥ 0.158 yields sensitivity and specificity of 95.5% and 33.1%. The model without fcDNA at a predictive probability ≥ 0.175 yields similar sensitivity of 95.5% but a much smaller specificity of 6.7%. Furthermore, we observed a considerable increase in negative predictive

Table 4. Summary of previous studies comparing fcDNA levels between prostate cancer and controls

Study	DNA source	No. of patients		Median fcDNA, ng/mL		Key findings	Comments
		PCA	Control	PCA	BPH		
Allen et al. (20)	Plasma	15	22	11.75	6.12	<ul style="list-style-type: none"> fcDNA levels in PCA were significantly higher than in BPH 	<ul style="list-style-type: none"> Limited sample size
Boddy et al. (21)	Plasma	78	74	5*	6.25*	<ul style="list-style-type: none"> fcDNA levels in PCA were significantly higher than that of healthy controls fcDNA levels were found to be higher in BPH than that of PCA 	<ul style="list-style-type: none"> Provided limited clinicopathologic data Results not consistent with other studies
Chun et al. (22)	Plasma	142	19	709	267	<ul style="list-style-type: none"> Levels of fcDNA were significantly higher in PCA vs BPH 	<ul style="list-style-type: none"> High rate of PCA diagnosis in study population, which is not representative of a typical patient distribution Plasma fcDNA levels reported were much higher than the level reported in other studies

*Approximate values derived from the figure.

value in the model, including fcDNA (93.1%) compared with the model without fcDNA (73.3%).

Discussion

We designed this prospective study to evaluate the role of fcDNA as a biomarker to distinguish prostate cancer patients from those with benign prostatic conditions. The patients included in this study were suspected to have prostate cancer based on their PSA values (≥ 4 ng/mL) and/or abnormal digital rectal exam. Serum fcDNA levels were evaluated in 252 patients (89 prostate cancer, 59 prostatitis, and 104 BPH). Our results indicate that at a cut point of 180 ng/mL, fcDNA levels can differentiate prostate cancer from benign prostatic conditions such as prostatitis and BPH in patients with PSA ≤ 10 ng/mL. After adjustment for age and race, patients with PSA ≤ 10 ng/mL with fcDNA > 180 ng/mL were at increased risk for prostate cancer compared with those with fcDNA ≤ 180 ng/mL (odds ratio, 4.27; 95% CI, 2.05-8.88; $P < 0.001$). The multivariate model with fcDNA, at a predicted probability ≥ 0.158 , yields sensitivity of 95.5% and specificity of 33.1%, whereas the multivariate model without fcDNA, at a predicted probability ≥ 0.175 , yields sensitivity of 95.5% and specificity of 6.7%. We also show that inclusion of fcDNA yields a large increase in negative predictive value as compared with when fcDNA was not included (93.3% versus 73.3%). The role of fcDNA as a biomarker of prostate cancer has been evaluated in about 10 studies (20-29). Of these, only three studies have examined the usefulness of fcDNA quantitation as a diagnostic marker in prospective settings (20-22). The results are summarized in Table 4. The common drawback of these studies is the lack of a "representative patient distribution" in a prostate cancer screening scenario. Our study represents a typical distribution of patients referred for prostate biopsy based on elevated PSA and/or abnormal digital rectal exam (DRE) because 35% were diagnosed with prostate cancer, similar to results obtained in other studies (4). In addition, we maintained stringent patient inclusion criteria. Our control cohort comprised of BPH and prostatitis patients. Because elevated fcDNA levels are not prostate cancer specific and may be related to a variety of cancers, we excluded patients who were diagnosed with other malignancies.

Median fcDNA levels have been found to vary across different studies. These variations can be attributed to the source of fcDNA, as well as variation in methods of sample processing and fcDNA quantitation. A critical factor for determining a standard fcDNA cutoff value is to establish uniform methodologies for sample collection, processing, DNA extraction, and fcDNA quantitation and analysis (30). To rule out differences in DNA concentrations arising from variation in sample processing and DNA extraction, we uniformly processed all samples. DNA was extracted immediately after serum separation to avoid variations in fcDNA levels because

of the sample storage (31). Among different methods of fcDNA quantitation, PCR based assays for quantitation of target genes have been found to be highly sensitive and thus offer a simple and fast method for fcDNA analysis (32).

Cell lysis and apoptosis have been postulated to be the predominant mechanisms of release of DNA into circulation (19, 33). Ellinger et al. (26) examined levels of a non-cancerous gene, *prostaglandin-endoperoxidase synthase 2*, as a fcDNA marker to distinguish between prostate cancer and BPH. In this study, *GSTP1* hypermethylation was used as a marker of tumor derived DNA, and it was found that tumor DNA accounted only for about 1.9% of the total DNA. These data supported the theory that a large proportion of fcDNA was derived from noncancerous cells because of the induction of apoptosis by proapoptotic cytokines released from prostate cancer cells (34). Therefore, levels of non-cancer-specific gene for quantitation of fcDNA levels may be more useful for cancer detection. Genetic heterogeneity between tumor cells hinders the use of a cancer-specific gene mutation as a fcDNA marker. As stated previously, Bastian et al. (28) previously assayed three genes, *GSTP1*, *EDNRB*, and *MDR1* that were present in the genome of all cells to quantitate fcDNA and found that means levels of fcDNA were similar for the three genes. Because *GSTP1* has not been shown to be deleted or mutated in prostate cancer, it is a suitable non-cancer-specific marker for quantitation of fcDNA.

Thus far, fcDNA analysis has not been done in patients stratified according to PSA in a prospective study. Total PSA above 10 ng/mL is associated with a high likelihood of prostate cancer, and a prostate biopsy is commonly recommended in this situation. In contrast, PSA levels between 4.1 and 9.9 ng/mL is associated with prostate cancer in only 25 to 35% of men (4). We compared fcDNA analysis in patients with PSA levels ≤ 10 ng/mL and those with PSA > 10 ng/mL. In patients with PSA ≤ 10 ng/mL, fcDNA levels ≤ 180 ng/mL was associated with reduced risk for prostate cancer. Although there is a relatively high incidence of prostate cancer diagnosis in the high PSA group, the proportions with high fcDNA were comparable in prostate cancer versus benign (prostatitis + BPH; 31% versus 36%). Our results indicate that fcDNA levels could act as a discriminating marker in patients with PSA ≤ 10 ng/mL.

In our study cohort, 27% of men with PSA ≤ 10 ng/mL were diagnosed with prostate cancer by prostate biopsy. Hence, a test with high negative predictive value is required to identify men who can forgo biopsy in this group of patients. Our results indicate that, with a high negative predictive value of 93.1%, adding fcDNA can spare 33% of patients with PSA ≤ 10 ng/mL of unwanted prostate biopsies. This finding is of major clinical significance because of the fact that ~65% to 75% of patients with intermediate PSA levels between 4 and 10 ng/mL are subjected to unnecessary prostate biopsies (4). If validated in independent studies, fcDNA assay could potentially

improve the specificity of prostate cancer screening in this group of patients. Future prospective studies using a standardized technique of isolation and quantitation of fcDNA are needed to evaluate the role of fcDNA as a prostate cancer biomarker.

References

- Jemal A, Siegel R, Ward E, et al. Cancer statistics. *CA Cancer J Clin* 2009;59:225–49.
- Nadler RB, Humphrey PA, Smith DS, Catalona WJ, Ratliff TL. Effect of inflammation and benign prostatic hyperplasia on elevated serum prostate specific antigen levels. *J Urol* 1995;154:407–13.
- Partin AW, Carter HB, Chan DW, et al. Prostate specific antigen in the staging of localized prostate cancer: influence of tumor differentiation, tumor volume and benign hyperplasia. *J Urol* 1990;143:747–52.
- Smith DS, Humphrey PA, Catalona WJ. The early detection of prostate carcinoma with prostate specific antigen: the Washington University experience. *Cancer* 1997;80:1852–6.
- Catalona WJ, Smith DS, Ornstein DK. Prostate cancer detection in men with serum PSA concentrations of 2.6 to 4.0 ng/mL and benign prostate examination. Enhancement of specificity with free PSA measurements. *JAMA* 1997;277:1452–5.
- Hernandez J, Thompson IM. Prostate-specific antigen: a review of the validation of the most commonly used cancer biomarker. *Cancer* 2004;101:894–904.
- Thompson IM, Pauler DK, Goodman PJ, et al. Prevalence of prostate cancer among men with a prostate-specific antigen level \leq 4.0 ng per milliliter. *N Engl J Med* 2004;350:2239–46.
- Gilbert SM, Cavallo CB, Kahane H, Lowe FC. Evidence suggesting PSA cutpoint of 2.5 ng/mL for prompting prostate biopsy: review of 36,316 biopsies. *Urology* 2005;65:549–53.
- Oesterling JE, Jacobsen SJ, Klee GG, et al. Free, complexed and total serum prostate specific antigen: the establishment of appropriate reference ranges for their concentrations and ratios. *J Urol* 1995;154:1090–5.
- Catalona WJ, Smith DS, Wolfert RL, et al. Evaluation of percentage of free serum prostate-specific antigen to improve specificity of prostate cancer screening. *JAMA* 1995;274:1214–20.
- Higashihara E, Nutahara K, Kojima M, et al. Significance of serum free prostate specific antigen in the screening of prostate cancer. *J Urol* 1996;156:1964–8.
- Leon SA, Shapiro B, Sklaroff DM, Yaros MJ. Free DNA in the serum of cancer patients and the effect of therapy. *Cancer Res* 1977;37:646–50.
- Flamini E, Mercatali L, Nanni O, et al. Free DNA and carcinoembryonic antigen serum levels: an important combination for diagnosis of colorectal cancer. *Clin Cancer Res* 2006;12:6985–8.
- Ren N, Qin LX, Tu H, et al. The prognostic value of circulating plasma DNA level and its allelic imbalance on chromosome 8p in patients with hepatocellular carcinoma. *J Cancer Res Clin Oncol* 2006;132:399–407.
- Shapiro B, Chakrabarty M, Cohn EM, Leon SA. Determination of circulating DNA levels in patients with benign or malignant gastrointestinal disease. *Cancer* 1983;51:2116–20.
- Silva JM, Dominguez G, Garcia JM, et al. Presence of tumor DNA in plasma of breast cancer patients: clinicopathological correlations. *Cancer Res* 1999;59:3251–6.
- Sozzi G, Conte D, Leon M, et al. Quantification of free circulating DNA as a diagnostic marker in lung cancer. *J Clin Oncol* 2003;21:3902–8.
- Taback B, O'Day SJ, Hoon DS. Quantification of circulating DNA in the plasma and serum of cancer patients. *Ann N Y Acad Sci* 2004;1022:17–24.
- Wu TL, Zhang D, Chia JH, et al. Cell-free DNA: measurement in various carcinomas and establishment of normal reference range. *Clin Chim Acta* 2002;321:77–87.
- Allen D, Butt A, Cahill D, et al. Role of cell-free plasma DNA as a diagnostic marker for prostate cancer. *Ann N Y Acad Sci* 2004;1022:76–80.
- Boddy JL, Gal S, Malone PR, Harris AL, Wainscoat JS. Prospective study of quantitation of plasma DNA levels in the diagnosis of malignant versus benign prostate disease. *Clin Cancer Res* 2005;11:1394–9.
- Chun FK, Muller I, Lange I, et al. Circulating tumour-associated plasma DNA represents an independent and informative predictor of prostate cancer. *BJU Int* 2006;98:544–8.
- Jung K, Stephan C, Lewandowski M, et al. Increased cell-free DNA in plasma of patients with metastatic spread in prostate cancer. *Cancer Lett* 2004;205:173–80.
- Papadopoulou E, Davilas E, Sotiropoulos V, et al. Cell-free DNA and RNA in plasma as a new molecular marker for prostate cancer. *Oncol Res* 2004;14:439–45.
- Cherepanova AV, Tamkovich SN, Bryzgunova OE, Vlassov VV, Laktionov PP. Deoxyribonuclease activity and circulating DNA concentration in blood plasma of patients with prostate tumors. *Ann N Y Acad Sci* 2008;1137:218–21.
- Ellinger J, Bastian PJ, Haan KI, et al. Noncancerous PTGS2 DNA fragments of apoptotic origin in sera of prostate cancer patients qualify as diagnostic and prognostic indicators. *Int J Cancer* 2008;122:138–43.
- Altimari A, Grigioni AD, Benedettini E, et al. Diagnostic role of circulating free plasma DNA detection in patients with localized prostate cancer. *Am J Clin Pathol* 2008;129:756–62.
- Bastian PJ, Palapattu GS, Yegnasubramanian S, et al. Prognostic value of preoperative serum cell-free circulating DNA in men with prostate cancer undergoing radical prostatectomy. *Clin Cancer Res* 2007;13:5361–7.
- Schwarzenbach H, Alix-Panabieres C, Muller I, et al. Cell-free tumor DNA in blood plasma as a marker for circulating tumor cells in prostate cancer. *Clin Cancer Res* 2009;15:1032–8.
- Xue X, Teare MD, Holen I, Zhu YM, Woll PJ. Optimizing the yield and utility of circulating cell-free DNA from plasma and serum. *Clin Chim Acta* 2009;404:100–4.
- Sozzi G, Roz L, Conte D, et al. Effects of prolonged storage of whole plasma or isolated plasma DNA on the results of circulating DNA quantification assays. *J Natl Cancer Inst* 2005;97:1848–50.
- Jen J, Wu L, Sidransky D. An overview on the isolation and analysis of circulating tumor DNA in plasma and serum. *Ann N Y Acad Sci* 2000;906:8–12.
- Jahr S, Hentze H, Englisch S, et al. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. *Cancer Res* 2001;61:1659–65.
- Furuya Y, Fuse H, Masai M. Serum soluble Fas level for detection and staging of prostate cancer. *Anticancer Res* 2001;21:3595–8.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Received 03/18/2010; revised 04/26/2010; accepted 05/14/2010; published OnlineFirst 07/20/2010.