Prognostic Value of Preoperative Serum Cell-Free Circulating DNA in Men with Prostate Cancer Undergoing Radical Prostatectomy

Patrick J. Bastian,[1,6] Ganesh S. Palapattu,[1] Srinivasan Yegnasubramanian,[4] Xiaohui Lin,[2] Craig G. Rogers,[1] Leslie A. Mangold,[1] Bruce Trock,[1] Mario Eisenberger,[1,2,5] Alan W. Partin,[1] and William G. Nelson[1,2,3,4,5]

Abstract

Purpose: We evaluated the association of preoperative serum cell-free circulating DNA concentration in men with clinically localized prostate cancer who underwent radical prostatectomy with prostate-specific antigen (PSA) recurrence.

Experimental Design: One hundred and ninety-two men with clinically localized prostate cancer, who underwent radical prostatectomy at the Johns Hopkins Hospital and had preoperative serum available for analyses constituted our study population. All serum samples were collected before prostate biopsy or at least 4 months after prostate biopsy. The total amount of serum cell-free circulating DNA from each sample was calculated using a standard curve generated via quantitative real-time PCR. PSA recurrence was defined as a single postoperative PSA level of $\geq$0.2. The natural logarithm (ln) of the DNA concentration was used for statistical analyses.

Results: Of the 192 men in our study, 56 (29%) experienced PSA recurrence within the study period (median time to PSA recurrence 2 years). The median follow-up time for men free of disease at last follow-up was 3 years. The median serum cell-free DNA concentration of all men in the study was 5.3 ng/mL (mean 18.05 ng/mL; range 0.2-320 ng/mL). The mean serum DNA concentration for men who recurred and for those who did not was 3.8 $\pm$ 34.1 and 13.7 $\pm$ 33.6 ng/mL, respectively (P = 0.001). In a univariate analysis, ln DNA concentration was significantly associated with PSA recurrence (hazard ratio, 1.49; 95% confidence interval, 1.3-1.8; $P < 0.001$). In the multivariate model, ln DNA concentration was significantly associated with PSA recurrence (hazard ratio, 2.56; 95% confidence interval, 1.1-1.6; P = 0.003). Using bootstrap analyses, serum cell-free DNA concentrations $\geq$5.75 ng/mL were associated with an increased risk of PSA recurrence within 2 years of radical prostatectomy.

Conclusion: Our study suggests that preoperative serum cell-free DNA concentration may be a useful prognostic biomarker for men with clinically localized prostate cancer treated with radical prostatectomy.

Authors' Affiliations: The James Buchanan Brady Urological Institute, Departments of [1]Urology, [2]Oncology, [3]Pathology, and [4]Pharmacology, [5]The Sidney Kimmel Comprehensive Cancer Center, The Johns Hopkins University School of Medicine, Baltimore, Maryland; and [6]Urologische Klinik und Poliklinik, Universitätsklinikum Großhadern, Ludwig-Maximilians-Universität München, Munich, Germany

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Requests for reprints: Patrick J. Bastian, Urologische Klinik und Poliklinik, Universtätsklinikum Großhadern, Ludwig-Maximilians-Universität München, Marchioninistr. 15, 81377 Munich, Germany. Phone: 49-89-7095-2971; Fax: 49-89-7095-8890; E-mail: patrick.bastian@gmx.de.

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Prostate cancer is the most commonly diagnosed solid organ malignancy among men in the United States, with an estimated 244,000 new cases that will be diagnosed in 2006 alone (1). Primary therapy in the form of radical prostatectomy is one of the principal treatment modalities available for men with clinically localized disease. Approximately two thirds of patients treated with curative intent by surgery will remain disease free 15 years after radical prostatectomy (2). Men who experience early prostate-specific antigen (PSA) recurrence, particularly within the first 2 years of surgery, are likely to develop metastatic lesions and are prone to die because of prostate cancer (3, 4). Besides the commonly accepted risk factors for high-risk disease (i.e., biopsy Gleason sum 8-10, positive lymph nodes, and preoperative PSA >20 ng/mL; ref. 5), no established pretreatment laboratory test exists to identify men at risk for early disease progression after radical prostatectomy. A preoperative test that potentially identifies patients at risk for early PSA relapse postsurgery would be an important tool in the process of treatment planning.

The development of traditional tumor markers has been accelerated by the recent introduction of novel technologies

Table 1. Patient characteristics of 192 men with clinically localized prostate cancer treated with radical prostatectomy

	Study cohort, n = 192
Age (y), mean (range)	58.9 (40-71)
Preoperative PSA (ng/mL), mean (range)	7.68 (0.9-38)
Pathologic Gleason score*,† (%)	
5-6	89 (46)
7	77 (40)
8-10	26 (14)
Pathologic stage (%)	
Organ confined*	104 (54)
Nonorgan confined	87 (45)
Extraprostatic extension	86 (45)
Seminal vesicle invasion	18 (9)
Positive surgical margins	37 (19)
Pathologic lymph node positive	7 (4)

NOTE: The median follow-up time for men free of disease at last follow-up was 3 years (mean 4 y, range 1-14 y) and the median time to PSA recurrence was 2 years (mean 3 y, range 1-7 y).
*Two men with unknown data in the cohort.
†One man with unknown data in the cohort.

that can quantify small amounts of circulating proteins that are relatively specific for a certain type of cancer. The shortcomings of these methods, such as limited sensitivity and specificity, are well established (6). PCR-based methods have the ability to detect extremely minute amounts of nucleic acids and offer new molecular targets for the development of cancer biomarkers.

The presence of cell-free DNA and RNA in plasma/serum of cancer patients was described as early as the late 1970s (7, 8), but it was not until 1989 that the characteristics of plasma/serum DNA in cancer patients was recognized (9). In addition to quantitative changes, qualitative changes such as microsatellite alterations, decreased strand stability, and mutations have been shown in plasma or serum DNA in the cancer state (9–11). Although the clinical relevance of circulating cell-free DNA has not been completely elucidated, tumor-derived changes in circulatory nucleic acids may offer promising alternatives for cancer screening, detection, and treatment monitoring (12).

Despite the high prevalence of prostate cancer, there is little information on cell-free circulating DNA in the serum or plasma of prostate cancer patients. A recent analysis revealed limited use for cell-free circulating DNA derived from plasma in patients with metastatic prostate cancer as a biomarker of metastatic disease compared with serum PSA level (13). In addition, a recent report established reference ranges of cell-free circulating DNA in various types of cancer, but no correlation of cell-free circulating DNA in nonmalignant prostatic diseases as well as with clinical outcome was done (14).

Using a standard quantitative PCR, we sought to further characterize total cell-free circulating DNA, not specifically cancer nor specific gene DNA, in the preoperative serum of men with prostate cancer and to determine its association with PSA progression in men with clinically localized disease after radical prostatectomy.

Materials and Methods

Sample collection and DNA isolation. All serum samples were collected before or at least 4 months after prostate biopsy and stored at -80°C. All serum samples were stored on ice between the blood draw and further processing, which was done immediately afterward. Clotting of serum samples was allowed for 30 min on ice before centrifugation (1,800 × *g*, for 10 min). The study cohort consisted of 192 men who underwent radical prostatectomy at The Johns Hopkins Hospital for localized prostate cancer between 1990 and 2004 (Table 1). DNA was extracted from 600 μL of archived serum from each patient using the Qiagen Blood Mini Kit and diluted in a total volume of 25 μL. Thirty-five men without evidence of cancer on prostate biopsy served as a control population. Sera from 18 men with metastatic prostate cancer were also examined.

The primary end point of the study was PSA recurrence, which was defined as a single postprostatectomy PSA level of ≥0.2 ng/mL. Routine PSA follow-up consisted of assessment at 3 months postoperatively and annually thereafter. All of the men studied had an undetectable serum PSA level 3 months after radical prostatectomy. All studies were conducted with the approval of the Institutional Review Board and Health Insurance Portability and Accountability Act compliance.

Quantitative real-time PCR. Quantitative real-time PCR was done with primers flanking the promotor regions of three separate genes: *GSTP1*, *MDR1*, and *EBNRB*. All PCR reactions were carried out in an iCycler real-time thermal cycler (Bio-Rad) using specific primer sets [*GSTP1*: 5′ primer: ACTCACTGGTGGCGAAGACT; 3′ primer: GACCTGGGAAAGAGGGAAAG (15); *MDR1*: 5′ primer: AAACCG-TAGTGGCACTGGACCAT; 3′ primer: AGCATCTCCACGAAGGCA-GAGTT; *EDNRB*: 5′ primer: ATTCTCGCAGCGTTTCGT; 3′ primer: CGCGATTGAACTCGAAAGAC]. PCR conditions were set as follows: 95°C for 15 min followed by 45 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s. The within-run and between-run imprecisions for a DNA template were 2.9% and 6.8%, respectively. Calibration curve analysis was done after each PCR. PCR was accomplished using a 25 μL reaction mixture, consisting of 4 μL template DNA, 100 nmol/L of forward and reverse primer, and 12.5 μL of a 2× Sybr Green PCR Master Mix (Qiagen). In each reaction set, WBC DNA, the standard DNA (human male genomic DNA, Novagen), commercially available and not self-prepared (Novagen), served as a positive control; water served as negative controls. Each reaction was done in triplicate. A 10-fold dilution series of known DNA concentrations was used to generate a standard curve (100-0.001 ng). The total amount of DNA from each sample was calculated using this standard curve. All three assayed genes (i.e., *GSTP1*, *MDR1*, and *EBNRB*) are present in the genome of all cells.

All samples were tested for *GSTP1* CpG island hypermethylation and the total amount of cell-free circulating serum DNA using the three mentioned primer sets. The *GSTP1* CpG island hypermethylation was studied previously and the results of this analysis were included into the statistical analyses (categorical; ref. 16). We used the previously described restriction enzyme–quantitative PCR method to detect *GSTP1* CpG island hypermethylation (17). This method allows the quantification of the amount of total DNA and methylated DNA in each sample and is able to detect as few as one methylated *GSTP1* allele. Briefly, DNAs extracted from serum samples were subjected to restriction enzyme digestion with *Hpa*II (NEB). *Hpa*II cuts the sequence CCGG, but does not cut the methylated form of this sequence, $C^{5m}CGG$. If the CpG island does not contain $C^{5m}CGG$, the DNA is cut and no product can be detected after PCR amplification. Typically, 4 μL of DNA were incubated at 37°C for 5 h with 60 units of restriction enzyme, 2 μL of 10× restriction enzyme buffer (NEB), and the appropriate amount of DNase-free water to give a final reaction volume of 20 μL. To ensure complete digestion, an additional 30 units of restriction enzyme, 1 μL of restriction enzyme buffer, and 6 μL of DNase-free water were added to each reaction and incubated at 37°C overnight. As individual sample controls, PCR amplification was done

on undigested DNA and DNA digested with the restriction enzyme *Msp*I (NEB) using the above protocol. *Msp*I cuts both the unmethylated and methylated sequence; thus, no PCR product should be detected. All PCR reactions were carried out in an iCycler real-time thermal cycler (Bio-Rad) using a specific primer set (5′ primer: ACTCACTGGTGGCGAAGACT; 3′ primer: GACCTGGGAAAGAGGGAAAG) that brackets six *Hpa*II/*Msp*I recognition sites (15). PCR conditions were set as follows: 95°C for 15 min followed by 45 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s. Calibration curve analysis was done after each PCR. PCR was accomplished using a 25 μL reaction mixture, consisting of 4 μL template DNA, 100 nmol/L of forward and reverse primer, and 12.5 μL of a 2× Sybr Green PCR Master Mix (Qiagen). In each reaction set, restriction enzyme–treated universally methylated DNA (Chemicon) served as a positive control and restriction enzyme–treated WBC DNA and a water blank served as negative controls. Each reaction was done in duplicate.

Table 2. Association cell-free circulating cancer DNA and established pathologic factors (*GSTP1* primer set)

Prognostic factor	DNA GSTP1 mean (ng/mL)
Biopsy Gleason score	
<7	4.4
≥7	9.1
P	0.001
Pathologic Gleason score	
<7	3.8
≥7	7.6
P	0.001
PSA (ng/mL)	
<2.6	4.2
2.6-4.0	5.5
4.1-6.0	3.5
6.1-10.0	6.6
>10.0	8.0
P	0.071
PSA (binary)	
≤10.0	5.1
>10.0	8.0
P	0.108
Clinical stage	
T_{1c}	4.8
T_2	7.3
P	0.067
Lymph node	
Negative	5.5
Positive	10.3
P	0.270
Surgical margin	
Negative	5.0
Positive	9.0
P	0.008
Seminal vesicle involvement	
Negative	5.4
Positive	7.7
P	0.129
Organ confined	
Organ confined	3.8
Non–organ confined	8.8
P	<0.0001
Extraprostatic extension	
Negative	4.0
Positive	8.5
P	0.0004

NOTE: The prognostic factors considered were biopsy Gleason score, pathological Gleason score, PSA, clinical stage, lymph node metastases, surgical margin status, seminal vesicle involvement, organ-confined status, and extraprostatic extension. Results are expressed as geometric means of DNA levels. Analyses used *t* tests for binary prognostic factors, and ANOVA for factors with three or more categories (PSA was grouped into two or five categories).

Statistical analysis. The natural logarithm (ln) of the DNA concentration was used for statistical analyses. Univariate comparisons between recurrent and nonrecurrent patients consisted of χ^2 or Fisher's exact tests for categorical variables, and *t* tests for continuous variables. Hazard ratios and 95% confidence intervals were adjusted for multiple prognostic factors using the Cox proportional hazards model. Models were developed in a manual hierarchical approach, based on the likelihood ratio test and changes in the magnitude of hazard ratios. The effect of the ln of total DNA concentration on recurrence-free survival was evaluated using the proportional hazards model. The serum *GSTP1* hypermethylation status was coded as a categorical variable in this analysis. The optimal cut point for total DNA in the multivariable model was chosen using the minimum *P* value method with bootstrap resampling to correct for overfitting (18). For the multivariate analysis, we used the significant variables from the univariate model: total DNA level, surgical margin status, and lymph node status. A variable "DATA" was included into the multivariate model to correct for the fact that the cohort consisted of 55 matched pairs (110 patients) that have a much higher "recurrence rate" compared with the remaining 85 patients (consecutive patients) because recurrent patients were matched to nonrecurrent patients (based on pathologic Gleason score). Three patients were part of both cohorts, so the total cohort consisted of 192 patients. The concordance index was estimated using the modification for censored data, and was calibrated using bootstrap resampling (19). ANOVA was used to compare mean ln(DNA) levels among the four disease progression groups. Results are expressed as geometric means of DNA levels.

Results

Detection of cell-free circulating DNA and association with established prognostic features. The median serum cell-free DNA concentration of all men in the study was 5.3 ng/mL (mean 18.05 ng/mL; range 0.2-320 ng/mL). Table 2 shows the concentrations of cell-free circulating DNA detected with the *GSTP1* primer set. Mean levels of cell-free circulating DNA were similar for the three different primer sets (i.e., *GSTP1*, *MDR1*, and *EDNRB*; Figs. 1 and 2). Therefore, the calculated total DNA concentration based on the primer set for *GSTP1* PCR was used for statistical analysis. Total DNA levels were higher in association with each adverse prognostic feature (Table 2). Significant associations were observed for biopsy Gleason score, prostatectomy Gleason score, surgical margin status, organ-confined status, and extraprostatic extension in relation to total serum cell-free DNA concentration.

Of the 192 men in our study, 56 (29%) experienced PSA recurrence within the study period (median time to PSA recurrence 2 years, mean 4 years, range 1-14 years). The median follow-up time for men free of disease at last follow-up was 3 years (mean 3 years, range 1-7 years).

Association of cell-free circulating DNA levels with disease progression. The mean concentration of cell-free circulating DNA in patients without PSA recurrence (n = 136) and in patients with PSA recurrence (n = 56) was 3.8 ± 33.6 and 13.7 ± 34.1 ng/mL (P = 0.001), respectively. Univariate analysis with respect to PSA progression after radical prostatectomy revealed a hazard ratio of 1.49 (95% confidence interval, 1.3-1.8; P < 0.001; Table 3). The optimal calculated cut point for

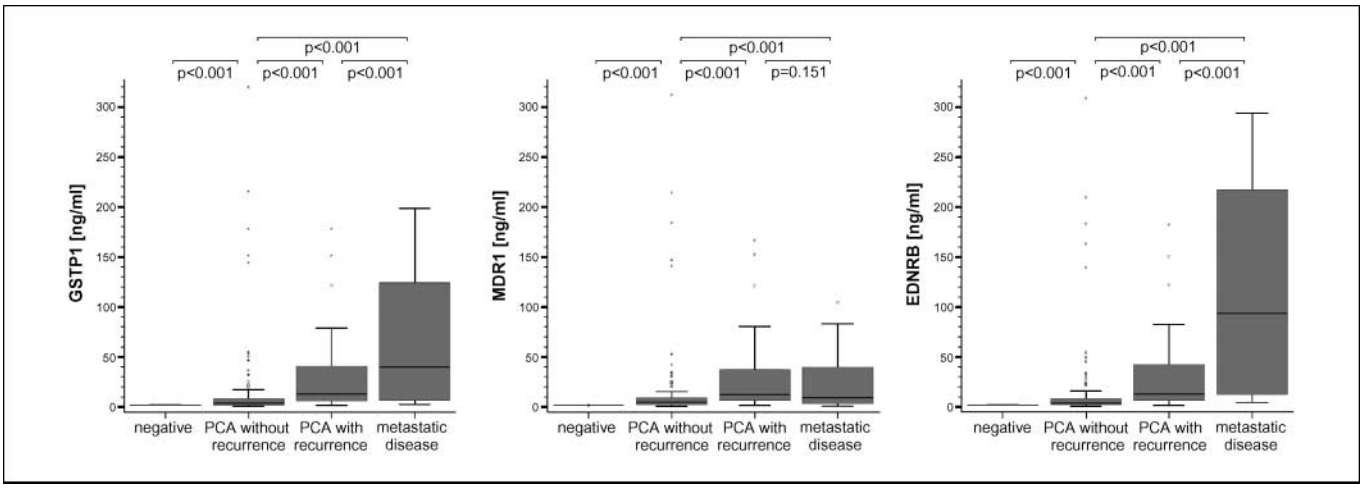

Fig. 1. Cell-free circulating serum DNA levels were determined as described in Materials and Methods by quantitative real-time PCR using primer sets for *GSTP1* (*A*), *MDR1* (*B*), and *EDNRB* (*C*). Mean levels of cell-free circulating DNA were similar for the three different primer sets. PCA, prostate cancer.

total preoperative cell-free circulating cancer DNA content with regard to PSA recurrence after radical prostatectomy was 5.75 ng/mL (ln 1.75 ng/mL) with a concordance index of 0.762 (also see Fig. 3).

Multivariable analysis incorporating the most powerful predictors of PSA recurrence. The multivariate model of the study cohort (n = 192) accounted for lymph node status, surgical margin status, biopsy and pathologic Gleason score, and pathologic stage. Because the univariate analyses revealed similar results for all three primer sets, only the *GSTP1* primer set was used in the analyses. In the multivariate Cox proportional hazard model, the total amount of cell-free circulating DNA was almost as predictive for PSA recurrence as positive lymph node status at the time of the operation (Table 4). The comparison of *GSTP1* hypermethylation status and level of cell-free circulating DNA did not reveal a statistically significant relationship (P = 0.21).

Comparison of total DNA concentration among disease progression groups. Table 5 shows the concentrations of total cell-free circulating DNA detected for the *GSTP1* primer set among disease progression groups. Disease progression groups consisted of patients undergoing prostate biopsies without histologic evidence of disease, patients with localized prostate cancer with or without biochemical recurrence, and patients with known metastatic prostate cancer. The amount of cell-free circulating DNA increased between disease progression groups to a significant extent (P < 0.0001).

Discussion

In the present study, we have shown that the content of cell-free circulating DNA, not cancer DNA, is higher in patients with prostate cancer than those with a negative prostate biopsy and that adverse pathologic findings in the surgical specimen are associated with elevated levels of circulating cell-free DNA. Significant associations were observed for biopsy Gleason score, prostatectomy Gleason score, surgical margin status, organ-confined status, and extraprostatic extension. In a

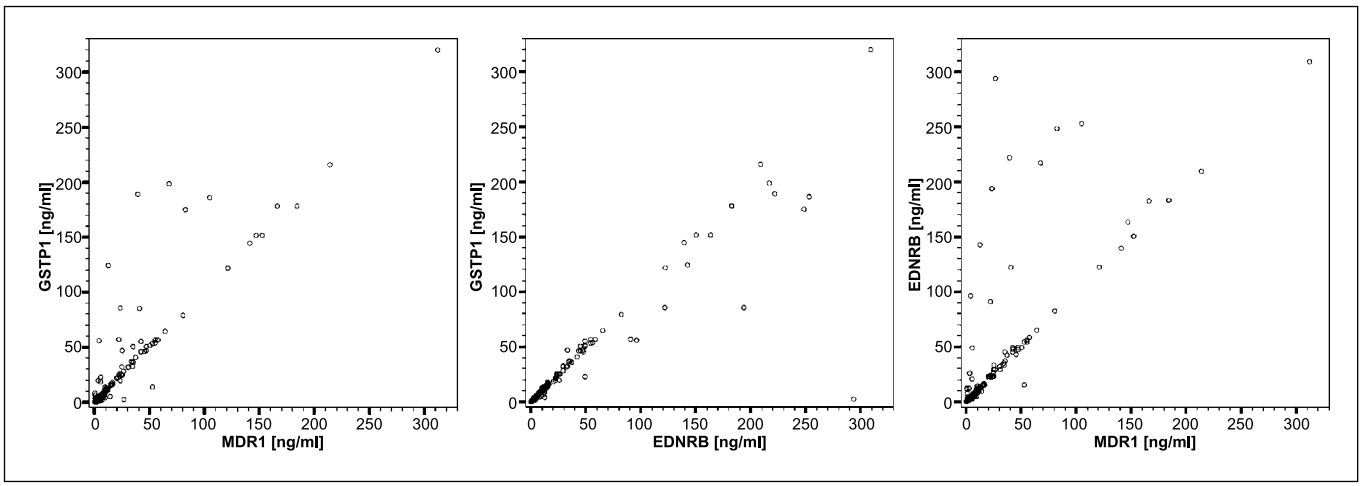

Fig. 2. Correlation plot revealing the degree of correlation in DNA levels in serum measured with *GSTP1, MDR1*, and *EDNRB*.

Table 3. Univariate model with respect to PSA progression

Variable	Hazard ratio (95% CI)	*P*
Cell-free serum DNA level	1.49 (1.3-1.8)	0.001
Preoperative PSA (continuous)	1.2 (0.9-1.7)	0.15
Clinical stage (T_{1c} vs T_{2c})	1.1 (0.5-2.0)	0.71
Biopsy Gleason score (<7 vs 8-10)	1.8 (1.2-3.1)	0.08
Pathologic Gleason score	1.6 (0.7-2.1)	0.07
Organ-confined disease	0.8 (0.4-1.2)	0.27
Positive lymph node(s)	3.2 (1.5-6.7)	0.005
Surgical margin status	1.8 (1.1-2.9)	0.03

Abbreviation: CI, confidence interval.

multivariate model, the content of cell-free circulating DNA was an independent predictor of early PSA recurrence in patients undergoing radical prostatectomy alone or in combination with other prognostic markers.

These results suggest that the content of cell-free circulating DNA before diagnosis may be a promising independent DNA-based prognostic serum biomarker for prostate cancer.

Presently, the true origin of cell-free circulating DNA is not fully understood. In addition to cell lysis, apoptosis, and necrosis, active DNA release has been advanced as possible sources of cell-free circulating DNA (20–22). Sozzi et al. (23) investigated the source of cell-free circulating DNA in lung cancer patients and concluded that the release of cell-free circulating DNA is not related to tumor necrotic rate or malignant cell proliferation. Active DNA release from fragile cells during the clotting process after blood sampling may be an additional source of serum cell-free circulating DNA (24) although a study by Jahr et al. (21) found this contribution to be negligible. Others, however, have shown that the amount of cell-free circulating DNA can be affected by blood sampling and processing (25, 26). For instance, Lee et al. (25) found that the amount of cell-free circulating DNA in serum significantly increased during longer storage at 4°C. A meticulous study by Jung et al. (27) also showed that preanalytic factors of blood sampling and processing may affect DNA concentrations in serum samples. They compared the influence of time delay in blood processing for plasma and serum samples at room temperature and at 4°C (27). From their analyses, it is obvious that there is a significant difference in the amount of serum DNA due to time delay and storage temperature. Also, Lui et al. (28) showed that the use of different centrifugation speeds ($400 \times g$–$16{,}000 \times g$) did not significantly alter the median total plasma DNA level in plasma samples. They feared that the use of high-speed centrifugation may destroy blood cells and thus lead to *ex vivo* release of DNA (28). How that may translate into serum samples remains unclear at this point. However, it is important to notice that not all serum samples can be compared with each other without acknowledging these major issues during the preanalytic sample collecting and preparation. Different DNA extraction protocols may also contribute to the *in vitro* release of cell-free circulating DNA (29, 30). Interestingly, a recent study by Umetani et al. (31) showed that the higher amount of cell-free circulating DNA in serum compared with plasma is not caused by contaminated extraneous DNA released from leukocytes or other sources during extraction and seems negligible and concluded that serum is the better specimen source for circulating DNA as a biomarker. Their possible explanation for this phenomenon was unequal distribution of DNA during separation from whole blood samples. They measured 6.1-fold more DNA in serum samples compared with plasma samples, which may result from electrostatic forces between DNA that is anionic and platelets that may also be anionic existing only in plasma (31). It is obvious that future studies have to be done to increase our understanding of these complex interactions.

The clearance of blood-borne DNA is also poorly understood. Plasma nucleases as well as hepatic and renal clearance mechanisms may play an important role in clearing the blood of free nucleic acids (32, 33). The half-life of circulating cell-free DNA is reported to be in the order of hours (34, 35).

Circulating DNA-based biomarkers have certain advantages over those using RNA or protein. DNA is more stable experimentally than RNA and is easier than protein to manipulate in the laboratory to identify changes. Multiple studies have shown that the amount of cell-free circulating DNA in the serum differs among normal and cancer patients. In healthy patients, the total amount of cell-free circulating DNA has been shown to be up to 50 ng/mL (14, 36, 37), compared with ~1,200 ng/mL in samples of patients with different types of cancer (21). Elevated levels of free nucleic acids in cancer patients are thought to originate, in part, from tumor cells. Evidence for this is based on cell-free circulating DNA having similar genetic and epigenetic properties as tumor DNA (16, 38–41). In a recent report by Jung et al. (13), patients with localized prostate cancer were observed to have similar amounts of cell-free circulating DNA in plasma compared with a healthy control group (~20 ng/mL). Higher levels of

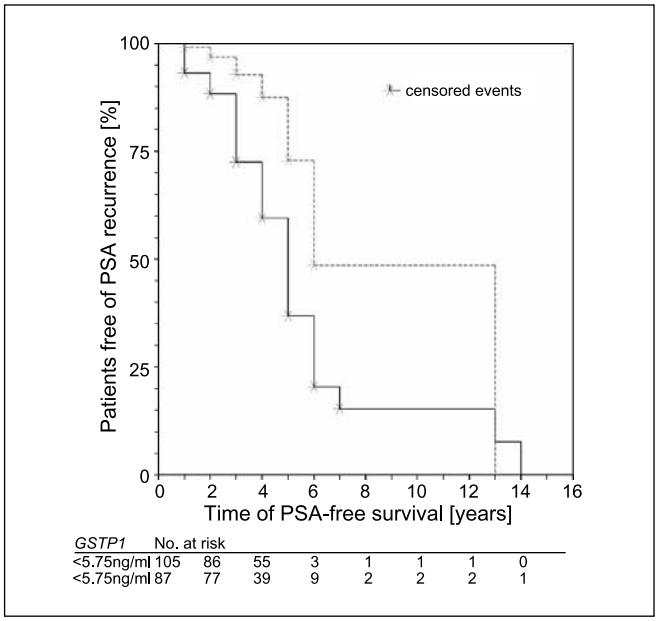

Fig. 3. Kaplan-Meier analysis cell-free circulating DNA in association with early PSA recurrence after radical retropubic prostatectomy for prostate cancer. The optimal calculated cutpoint for total preoperative cell-free circulating cancer DNA content with regard to PSA recurrence after radical prostatectomy was 5.75 ng/mL (ln 1.75 ng/mL) with a concordance index of 0.762.

Table 4. Multivariable model incorporating optimal predictors of PSA recurrence

Variable	Hazard ratio (95% CI)	*P*
Lymph nodes (metastases vs no metastases)	2.85 (1.27-6.40)	0.011
Total DNA (>5.75 vs ≤5.75; ng/mL)	2.56 (1.38-4.74)	0.003
Surgical margin status	2.03 (1.02-3.37)	0.010

NOTE: Likelihood ratio χ^2 (3 *df*) = 54.51, $P < 0.0001$. Pathologic stage, biopsy Gleason, and pathologic Gleason were nonsignificant in the model.

cell-free circulating DNA were found in patients with benign prostatic hyperplasia (28.1 ng/mL) as well as in patients with positive lymph nodes (25.8 ng/mL) and distant metastases (41.6 ng/mL; ref. 13). Chun et al. (42) reported on a total of 161 plasma samples from benign prostatic hyperplasia and prostate cancer patients (benign prostatic hyperplasia, $n = 19$; prostate cancer, $n = 142$) and found that DNA plasma level is an informative and highly accurate predictor for presence of prostate cancer. However, they do not provide specific information on the sample collection and processing as discussed above. Interestingly, those data are in contrast to the data by Boddy et al. (43), revealing a significantly higher level of plasma DNA in benign prostatic hyperplasia than in prostate cancer patients. However, Boddy et al. used different preanalytic techniques (i.e., centrifugation) that may contribute to their results.

In our study, we found that the total cell-free circulating DNA concentration of patients with localized prostate cancer was 3.8 and 13.7 ng/mL for men without and with PSA recurrence, respectively, after radical prostatectomy. We also noted that the ideal value of total DNA concentration that predicted which men who were destined to biochemically recur post surgery was 5.75 ng/mL.

In men with metastatic prostate cancer, serum PSA level and the amount of plasma DNA have been similarly associated with survival (13). Unfortunately, we were not able to correlate the amount of cell-free circulating DNA in serum with clinical or pathologic information for the subgroup of patients with metastatic disease. However, the amount of cell-free circulating DNA in patients with metastatic prostate cancer was significantly higher compared with men with localized prostate cancer with or without biochemical recurrence. This finding supports the finding by Wu et al. (14) of increased serum tumor markers (PSA in prostate cancer and CA 15-3 in colorectal cancer) in combination with the amount of cell-free circulating DNA in prostate cancer and colorectal cancer patients. Only a low serum tumor marker concentration correlated with the amount of cell-free circulating DNA (14). In our study, we were not able to show correlations of the amount of cell-free circulating DNA with the preoperative serum PSA value (binary or categorical; Table 2).

The group of Allen et al. (44) showed that the total amount of cell-free circulating plasma DNA in patients before and after prostate biopsy was significantly higher in prostate cancer and in benign prostatic hyperplasia 60 min postbiopsy. Interestingly, this increase in cell-free circulating DNA was not detected in PIN cases. They attributed this increase of cell-free circulating DNA to direct cell disruption during biopsy as has been described in a greater scale in trauma patients (45). As mentioned, the serum samples of our cohort were drawn before digital rectal examination or prostatic biopsy and are therefore not subject to these potential confounders.

Interestingly, Sozzi et al. (23) reported that the amount of cell-free circulating DNA in plasma rapidly decreased in patients undergoing lung resection for lung cancer. Furthermore, no increasing level of cell-free circulating plasma DNA in patients with recurrent disease was detected. The authors suggested that the simple quantification of cell-free circulating DNA may serve to monitor patients undergoing surgery or to assess treatment efficacy in lung cancer patients (23). No study on this topic exists for prostate cancer, but we are awaiting results in the very future.

One limitation of our study warrants further discussion. Because sufficient follow-up time has not yet elapsed for the majority of men in the entire cohort, we chose time to PSA recurrence as the end point and not time to metastasis or death. Regardless, PSA recurrence within the first 2 or 3 years of surgery has been associated with a higher probability of developing distant metastasis, a short PSA doubling time, and prostate cancer–specific mortality (4, 46, 47). Also, at this point, it remains unclear whether the amount of cell-free circulating DNA varies between samples drawn from the same patient at different times. Future studies are warranted to further evaluate this issue.

Furthermore, the role of *GSTP1* hypermethylation in serum samples of prostate cancer patients warrants discussion. As shown in our previous work, *GSTP1* hypermethylation was the strongest predictor of early PSA recurrence after surgical monotherapy. In this study, we compared the results of the *GSTP1* hypermethylation with the level of cell-free circulating DNA as a predictor and found that the level of cell-free circulating DNA is almost as predictive as the pathologic lymph node status for PSA recurrence (hazard ratios, 2.56 and 2.85, respectively). Because the true nature of cell-free circulating DNA and cell-free circulating cancer DNA (detected by *GSTP1* hypermethylation) remains unknown, future studies are necessary to address these uncertainties. Additionally, we do not know whether any of these markers may predict prostate cancer–specific mortality and need to wait longer follow-up periods. Other studies looking into the detection of

Table 5. Cell-free circulating DNA (*GSTP1*) levels among disease progression groups

Disease progression	DNA *GSTP1* mean (ng/mL)
Negative biopsy	1.6
Prostate cancer w/o recurrence	3.8
Prostate cancer with recurrence	13.7
Metastases	162.4
P	<0.0001

tumor-specific DNA in blood from prostate cancer patients have been published and this will be an emerging field with an interesting future (48).

Conclusion

In our study, preoperative serum cell-free DNA concentration was a powerful predictor of PSA progression after radical prostatectomy for localized prostate cancer. Men who experienced biochemical recurrence after prostatectomy had a significantly greater preoperative serum cell-free DNA concentration than those men who were free of disease at last follow-up. If corroborated by other prospective studies, DNA-based biomarkers, such as total DNA amount, may be helpful in identifying men who are most likely to fail after surgery and thus most likely to benefit from adjuvant therapy.

References

1. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2006. CA Cancer J Clin 2006;56:106–30.
2. Han M, Partin AW, Pound CR, Epstein JI, Walsh PC. Long-term biochemical disease-free and cancer-specific survival following anatomic radical retropubic prostatectomy. The 15-year Johns Hopkins experience. Urol Clin North Am 2001;28:555–65.
3. Freedland SJ, Humphreys EB, Mangold LA, et al. Risk of prostate cancer-specific mortality following biochemical recurrence after radical prostatectomy. JAMA 2005;294:433–9.
4. Pound CR, Partin AW, Eisenberger MA, Chan DW, Pearson JD, Walsh PC. Natural history of progression after PSA elevation following radical prostatectomy. JAMA 1999;281:1591–7.
5. Grossfeld GD, Latini DM, Lubeck DP, Mehta SS, Carroll PR. Predicting recurrence after radical prostatectomy for patients with high risk prostate cancer. J Urol 2003;169:157–63.
6. Nelson WG, De Marzo AM, Isaacs WB. Prostate cancer. N Engl J Med 2003;349:366–81.
7. 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.
8. 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.
9. Stroun M, Anker P, Maurice P, Lyautey J, Lederrey C, Beljanski M. Neoplastic characteristics of the DNA found in the plasma of cancer patients. Oncology 1989;46:318–22.
10. Lecomte T, Berger A, Zinzindohoue F, et al. Detection of free-circulating tumor-associated DNA in plasma of colorectal cancer patients and its association with prognosis. Int J Cancer 2002;100:542–8.
11. Kopreski MS, Benko FA, Gocke CD. Circulating RNA as a tumor marker: detection of 5T4 mRNA in breast and lung cancer patient serum. Ann N Y Acad Sci 2001;945:172–8.
12. Johnson PJ, Lo YM. Plasma nucleic acids in the diagnosis and management of malignant disease. Clin Chem 2002;48:1186–93.
13. 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.
14. Wu TL, Zhang D, Chia JH, Tsao KH, Sun CF, Wu JT. Cell-free DNA: measurement in various carcinomas and establishment of normal reference range. Clin Chim Acta 2002;321:77–87.
15. Bakker J, Lin X, Nelson WG. Methyl-CpG binding domain protein 2 represses transcription from hypermethylated pi-class glutathione *S*-transferase gene promoters in hepatocellular carcinoma cells. J Biol Chem 2002;277:22573–80.
16. Bastian PJ, Palapattu GS, Lin X, et al. Preoperative serum DNA GSTP1 CpG island hypermethylation and the risk of early prostate-specific antigen recurrence following radical prostatectomy. Clin Cancer Res 2005;11:4037–43.
17. Lee WH, Isaacs WB, Bova GS, Nelson WG. CG island methylation changes near the GSTP1 gene in prostatic carcinoma cells detected using the polymerase chain reaction: a new prostate cancer biomarker. Cancer Epidemiol Biomarkers Prev 1997;6:443–50.
18. Hollander N, Sauerbrei W, Schumacher M. Confidence intervals for the effect of a prognostic factor after selection of an "optimal" cutpoint. Stat Med 2004;23:1701–13.
19. Harrell FE, Jr., Califf RM, Pryor DB, Lee KL, Rosati RA. Evaluating the yield of medical tests. JAMA 1982;247:2543–6.
20. Stroun M, Lyautey J, Lederrey C, Olson-Sand A, Anker P. About the possible origin and mechanism of circulating DNA apoptosis and active DNA release. Clin Chim Acta 2001;313:139–42.
21. 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.
22. Vasioukhin V, Anker P, Maurice P, Lyautey J, Lederrey C, Stroun M. Point mutations of the N-ras gene in the blood plasma DNA of patients with myelodysplastic syndrome or acute myelogenous leukaemia. Br J Haematol 1994;86:774–9.
23. 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.
24. Lui YY, Chik KW, Chiu RW, Ho CY, Lam CW, Lo YM. Predominant hematopoietic origin of cell-free DNA in plasma and serum after sex-mismatched bone marrow transplantation. Clin Chem 2002;48:421–7.
25. Lee TH, Montalvo L, Chrebtow V, Busch MP. Quantitation of genomic DNA in plasma and serum samples: higher concentrations of genomic DNA found in serum than in plasma. Transfusion 2001;41:276–82.
26. Chiu RW, Lo YM. Recent developments in fetal DNA in maternal plasma. Ann N Y Acad Sci 2004;1022:100–4.
27. Jung M, Klotzek S, Lewandowski M, Fleischhacker M, Jung K. Changes in concentration of DNA in serum and plasma during storage of blood samples. Clin Chem 2003;49:1028–9.
28. Lui YY, Chik KW, Lo YM. Does centrifugation cause the *ex vivo* release of DNA from blood cells? Clin Chem 2002;48:2074–6.
29. Dixon SC, Horti J, Guo Y, Reed E, Figg WD. Methods for extracting and amplifying genomic DNA isolated from frozen serum. Nat Biotechnol 1998;16:91–4.
30. de Kok JB, Hendriks JC, van Solinge WW, Willems HL, Mensink EJ, Swinkels DW. Use of real-time quantitative PCR to compare DNA isolation methods. Clin Chem 1998;44:2201–4.
31. Umetani N, Hiramatsu S, Hoon DS. Higher amount of free circulating DNA in serum than in plasma is not mainly caused by contaminated extraneous DNA during separation. Ann N Y Acad Sci 2006;1075:299–307.
32. Botezatu I, Serdyuk O, Potapova G, et al. Genetic analysis of DNA excreted in urine: a new approach for detecting specific genomic DNA sequences from cells dying in an organism. Clin Chem 2000;46:1078–84.
33. Minchin RF, Carpenter D, Orr RJ. Polyinosinic acid and polycationic liposomes attenuate the hepatic clearance of circulating plasmid DNA. J Pharmacol Exp Ther 2001;296:1006–12.
34. Lo YM, Zhang J, Leung TN, Lau TK, Chang AM, Hjelm NM. Rapid clearance of fetal DNA from maternal plasma. Am J Hum Genet 1999;64:218–24.
35. To EW, Chan KC, Leung SF, et al. Rapid clearance of plasma Epstein-Barr virus DNA after surgical treatment of nasopharyngeal carcinoma. Clin Cancer Res 2003;9:3254–9.
36. Leon SA, Ehrlich GE, Shapiro B, Labbate VA. Free DNA in the serum of rheumatoid arthritis patients. J Rheumatol 1977;4:139–43.
37. Chen XQ, Stroun M, Magnenat JL, et al. Microsatellite alterations in plasma DNA of small cell lung cancer patients. Nat Med 1996;2:1033–5.
38. Chen X, Bonnefoi H, Diebold-Berger S, et al. Detecting tumor-related alterations in plasma or serum DNA of patients diagnosed with breast cancer. Clin Cancer Res 1999;5:2297–303.
39. Anker P, Mulcahy H, Chen XQ, Stroun M. Detection of circulating tumour DNA in the blood (plasma/serum) of cancer patients. Cancer Metastasis Rev 1999;18:65–73.
40. Wong IH, Lo YM, Zhang J, et al. Detection of aberrant p16 methylation in the plasma and serum of liver cancer patients. Cancer Res 1999;59:71–3.
41. Goessl C, Krause H, Muller M, et al. Fluorescent methylation-specific polymerase chain reaction for DNA-based detection of prostate cancer in bodily fluids. Cancer Res 2000;60:5941–5.
42. 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.
43. 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.
44. Allen D, Butt A, Cahill D, Wheeler M, Popert R, Swaminathan R. Role of cell-free plasma DNA as a diagnostic marker for prostate cancer. Ann N Y Acad Sci 2004;1022:76–80.
45. Lam NY, Rainer TH, Chan LY, Joynt GM, Lo YM. Time course of early and late changes in plasma DNA in trauma patients. Clin Chem 2003;49:1286–91.
46. Freedland SJ, Isaacs WB. Explaining racial differences in prostate cancer in the United States: sociology or biology? Prostate 2005;62:243–52.
47. D'Amico AV, Whittington R, Malkowicz SB, et al. Utilizing predictions of early prostate-specific antigen failure to optimize patient selection for adjuvant systemic therapy trials. J Clin Oncol 2000;18:3240–6.
48. Schwarzenbach H, Chun FK, Lange I, et al. Detection of tumor-specific DNA in blood and bone marrow plasma from patients with prostate cancer. Int J Cancer 2007;120:1457–63.

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