Research Paper

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Pathologic Parameters and Flow Cytometric Ploidy Analysis in Predicting Recurrence in Carcinoma of the Prostate

G.E. Voges[a]
E.B. Eigner[b]
W. Ross[c]
H. Sussman[c]
M. Stöckle[a]
F.S. Freiha[b]
T.A. Stamey[b]

[a] Departments of Urology, Mainz Medical School, Mainz, FRG;
[b] Department of Urology and
[c] Clinical Laboratories, Stanford University School of Medicine, Stanford, Calif., USA

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Abstract
Recurrence of prostate cancer following radical prostatectomy is determined by the extent of local disease. Tumor volume and grade have improved our ability to predict extraprostatic extension, but tumors of intermediate volume and grade vary in their biologic behavior. To assess the prognostic significance of DNA ploidy, we performed flow cytometry in 85 patients with prostate cancer volumes >4 cm^3. Post-radical prostatectomy serum prostate-specific antigen was used to prove recurrence of cancer. Mean follow-up was 35 months (median 31 months). 26 patients (30%) had diploid histograms, 55 (65%) non-diploid histograms. In 4 cases (5%) the histograms were uninterpretable. Tumor volume and percent of Gleason grades 4 or 5 separated the recurrent from nonrecurrent groups in a highly significant manner ($p < 0.001$). When tested alone, ploidy had no ability to predict recurrence ($p = 0.26$). However, in a subset of patients with 4–8 cm^3 of cancer with $<30\%$ Gleason grade 4 or 5 tumor, ploidy conferred significant additional prognostic information ($p < 0.005$).

Introduction

The natural history of prostate cancer, including the recurrence of disease following radical prostatectomy, has until recently been highly unpredictable. Previous studies have been hindered by the long (10–15 years) follow-up necessary to document clinical recurrence and less than optimal examination of the prostate specimen. The addition of prostate specific antigen (PSA) as a marker has telescoped the time necessary to detect the presence of malignant cells following radical prostatectomy [1]. Analysis of deoxyribonucleic acid (DNA) content of malignant cells by flow cytometry has been proposed as a useful additional prognostic measure which has been the subject of several recent investigations [2–9]. We have shown that tumor volume and Gleason grade are reliable predictors of capsular penetration, seminal vesicle invasion, lymph node metastasis and recurrence [10, 11]. The present study was undertaken to assess the value of tumor ploidy to predict prognosis both alone and in conjunction with the tumor volume and Gleason grade.

Gunther E. Voges, MD
Department of Urology
Mainz Medical School
Langenbeckstrasse 1
D-W-6500 Mainz (FRG)

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Materials and Methods

Two hundred and seventy radical prostatectomies were performed for clinically localized disease at Stanford University Medical Center between 1/84 and 2/89. All patients who received preoperative radiation or hormonal therapy were excluded from review, as were any patients who received postoperative radiation or hormonal therapy prior to the documentation of clinical or biochemical recurrence. All patients with cancers <4 cm^3 in volume were also excluded as well as any patients who had a positive margin caused by inadvertent incision into the prostate since these latter patients do not reflect the natural history of prostate cancer [12, 13]. Included were only patients with a minimum follow-up of 15 months. With these exclusions, 85 patients were available for study. Patients were routinely examined at 3-month intervals for the first year and every 4–6 months thereafter. All follow-up visits consisted of physical examination including a careful rectal examination. A serum PSA (Yang) was obtained at each visit beginning in August, 1986. Bone scans were obtained 6 months following radical prostatectomy, and yearly thereafter.

Pathologic Analysis. Immediately after removal, prostates were inked over the entire surface and fixed overnight in undiluted (37%) formalin. All specimens were processed according to the Stanford technique [9, 13, 14] with serial sectioning of the specimen at 3-mm intervals in transverse planes perpendicular to the rectal surface. A single coronal cut was prepared from each seminal vesicle. A 5-µm slide was cut from each tissue block and stained with hematoxylin and eosin. The outlines of carcinoma, prostate capsule, and anatomic landmarks were marked in ink on each slide and transferred by tracing to serial maps. Areas of complete capsule penetration by the cancer were precisely marked by an ink line drawn along the capsular surface in the area of capsule penetration. In this way, each prostate was completely reconstructed, allowing the calculation of cancer volume by computer planimetry, the estimation of proportion of poorly differentiated elements in the cancer (tabulated as percent of Gleason grade 4 and/or 5) and the estimation of seminal vesicle invasion. Capsular penetration into the periprostatic fat was quantified as the sum of measured line lengths on the slides.

Flow Cytometric Analysis. For flow cytometry, 50-µm sections were cut from tissue blocks where the presence of cancer was verified by inspection of the adjacent transverse sections. One to three blocks were analyzed per case (mean 1.9). Formalin-fixed, paraffin-embedded benign prostatic tissue taken from cystoprostatectomy specimens were utilized as an external control. These sections were extensivley reviewed to exclude the presence of occult cancer. Additionally, the vast majority of sections submitted for flow cytometry included microscopically normal tissue to serve as an internal diploid control. Sample preparation was performed using a modification of the technique described by Hedley et al. [15]. Samples were dewaxed in two changes of Histoclear and then rehydrated in sequential rinses of 100 (times 2), 95, 70 and 50% ethanol. Distilled water was then added until the sample was covered and remained in water overnight. The water was aspirated and 1 ml of pepsin solution added. The resulting nuclear suspension was filtered through a 40-µm mesh and centrifuged. The supernatant was discarded and 1 ml of staining solution (containing propidium iodide) and 100 µl of RNAse added to the pellet. After incubation the samples were vortexed and centrifuged and the pellet was resuspended in 750 µl of staining solution and filtered through a 35-µm mesh. Samples were placed on ice and run within 1 h.

Table 1. Pathologic parameters of 85 radical prostatectomy specimens with tumor volumes greater than 4 cm^3

Factor	Mean	Median	SD
Volume, cm^3	10.3	7.8	6.9
% Gr 4–5	39	30.0	31.5
Caps. penetr., cm	2.9	1.5	2.4
Preop. PSA, ng/ml	36.5	23.8	44.1
SV Inv.	34/85 (40%)	–	
Pos. LNs	19/85 (22%)	–	
F/U, months	34.5	31.0	13.8

% Gr 4–5 = Proportion of Gleason grade 4 and/or 5 in the cancer; caps. penetr. = amount of disease outside the prostatic capsule; SV Inv. = seminal vesicle invasion; pos. LNs = positive lymph nodes; preop. PSA = preoperative serum PSA (Yang); F/U = follow-up after radical prostatectomy.

Samples were run on a FACSCAN (Becton Dickinson) flow cytometer equipped with a doublet discrimination module (pulse processor). Excitation wavelength was 488 nm. Instrument performance (linearity and coefficient of variation) was confirmed using a DNA Quality Control Kit (Becton Dickinson) which included chicken erythrocyte nuclei, calf thymocyte nuclei, and 2-µm beads. Data analysis was performed using the CELLFIT program (Becton Dickinson). DNA aneuploidy was defined as a sample with a DNA index not equal to 1.0. As a mean 1.9 samples were run per patient, in cases where the ploidy of the samples was discordant, the more abnormal ploidy was assigned for further analysis. This occurred in 15 cases (17%).

Results

The morphometric data from our analysis of these 85 radical prostatectomy specimens are presented in table 1. Tumors varied in volume from 4.15 to 45.5 cm^3 (mean 10.3 cm^3). The mean percentage of cancer with Gleason grade 4 or 5 was 39% and ranged from 0 to 100%. Seventeen patients (20%) had tumors confined within the capsule, 49 (58%) had cancer involving the seminal vesicle and/or the periprostatic fat, and 19 patients (22%) were classified as stage D1 when final pathology revealed occult micrometastases to the pelvic lymph nodes. Minimum follow-up was 15 months and mean follow-up was 34.5 months (median 31 months).

Recurrent versus Nonrecurrent Cases. Of the 85 patients, 43 (51%) have developed biochemical and sometimes clinical evidence of residual or recurrent disease during follow-up. The difference in pathologic parameters between the recurrent and nonrecurrent groups are presented in table 2. Differences in tumor volume, percent of

Table 2. Comparison of pathologic data in recurrent versus nonrecurrent cases of carcinoma of the prostate (mean ± SD)

Factor	Recurrent	Nonrecurrent	p value
n	43	42	–
Volume, cm^3	13.7 ± 8.2	6.9 ± 2.2	<0.0001
% Gr 4–5	50.5 ± 29.2	26.9 ± 29.7	0.0004
Caps. penetr., cm	4.7 ± 4.3	1.1 ± 1.5	<0.0001
Preop. PSA, ng/ml	48.2 ± 57.6	24.4 ± 18.7	0.018
SV Inv.	25/43	9/42	<0.005
Pos. LNs	17/43	2/42	<0.005
Ploidy[1]	11 D/31 ND	15 D/24 ND	>0.05
F/U, months	37.0 ± 14.0	31.1 ± 13.0	0.05

Recurrent tumors had larger volumes and more high grade cancer than nonrecurrent tumors, but no difference in tumor ploidy was seen. D = Diploid histograms; ND = non-diploid histograms. For other abbreviations see table 1.

[1] 81 DNA histograms evaluable.

Table 3. Comparison of pathologic data in diploid versus nondiploid cases (mean ± SD)

Factor	Diploid	Non-diploid	p value
n[1]	26 (30%)	55 (65%)	–
Volume, cm^3	11.0 ± 6.2	10.3 ± 7.4	0.65
% Gr. 4–5	40.8 ± 33.3	38.7 ± 31.4	0.78
Caps. penetr, cm	3.7 ± 4.8	2.6 ± 3.1	0.31
Preop. PSA, ng/ml	32.9 ± 52.5	37.2 ± 41.1	0.73
SV Inv.	11/26	21/55	>0.05
Pos. LNs	5/26	14/54	>0.05
Recurrence	11/26 (42%)	31/55 (56%)	>0.05

There were no significant differences between the groups. See table 1 for abbreviations.

[1] 5% were not interpretable, despite multiple runs.

Table 4. Recurrence rates when combining tumor volume plus ploidy (81 cases with evaluable histograms)

Factor	Tumor ploidy				Total	
	diploid		non-diploid			
	n	%	n	%	n	%
Any	11/26	42*	31/55	56*	42/81	52
4–8 cm^3	0/10	0**	13/31	42**	13/41	32
≥8 cm^3	11/16	69*	18/24	75*	29/40	73

A statistically significant difference in recurrence rate is noted in tumors <8 cm^3, but not in tumors ≥8 cm^3.

* $p > 0.05$ (not significant); ** $p < 0.025$ (significant).

Gleason grade 4 or 5, degree of capsular penetration, seminal vesicle invasion and lymph node metastases were all highly significant. In the 43 recurrent cases, total tumor volume and percent of tumor involved with Gleason grades 4 or 5 were nearly twice the mean values obtained for the 42 nonrecurrent cases; the amount of capsular penetration in the recurrent group was over 4 times that of the nonrecurrent group.

Comparison of Diploid versus Non-diploid Groups. Flow cytometric ploidy analysis was performed in all 85 cases. Despite multiple runs from several different blocks, 4 patients (5%) had uninterpretable histograms, leaving 81 patients for ploidy analysis. Samples in 26 patients (30%) were found to be diploid, while 55 patients (65%) were found to have non-diploid tumors. There was no statistical difference in total volume, percent Gleason grade 4 or 5, capsular penetration, or rates of seminal vesicle or pelvic lymph node involvement between diploid and non-diploid groups (table 3). While there was a slight trend towards greater incidence of tumor recurrence in the non-diploid group, this was not statistically significant (χ^2 = 1.4 with 1 degree of freedom). Kaplan-Meier analysis of patients divided by ploidy alone is presented in figure 1. Overall, no significant differences between the diploid and non-diploid groups were recognized either clinically or pathologically.

Comparative Ability to Predict Recurrence. An effort was made to determine the single parameter most likely to separate recurrent from nonrecurrent patients. As noted above, ploidy alone showed no ability to accurately predict biochemical or clinical recurrence. However, when patients were divided into two groups by total cancer volume, a significant trend was noted. Of 45 patients with tumor volumes of 4–8 cm^3, 14 (31%) have experienced recurrence of disease. Of the remaining 40 patients with greater than 8 cm^3 of tumor volume, 29 (72.5%) have evidence of disease recurrence ($p < 0.005$). Time to disease recurrence in relation to cancer volume in a Kaplan-Meier analysis is shown in figure 2. Of 44 patients with 30% or less of Gleason grade 4 or 5 cancer, 14 (32%) have recurred. Of the remaining 41 patients with more than 30% Gleason grade 4 or 5 cancer, 29 (71%) have recurred ($p < 0.005$).

Combination of Ploidy and Pathological Parameters. Though we were unable to differentiate between the recurrent and nonrecurrent groups using ploidy status alone, total cancer volume and percent Gleason grade 4 or 5 differed significantly between the recurrent and nonrecurrent patients. However, there were several exceptions where recurrence developed in tumors with volumes of

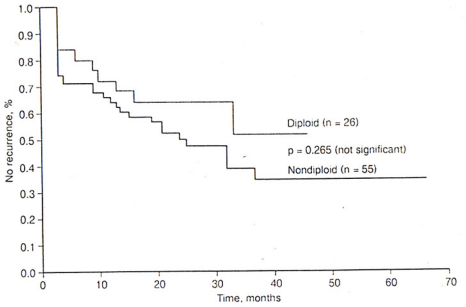

Fig. 1. Kaplan-Meier analysis of time to first recurrence comparing diploid to non-diploid cases. While a trend towards earlier recurrence in non-diploid is noted, this is not statistically significant (p = 0.265, log-rank statistics).

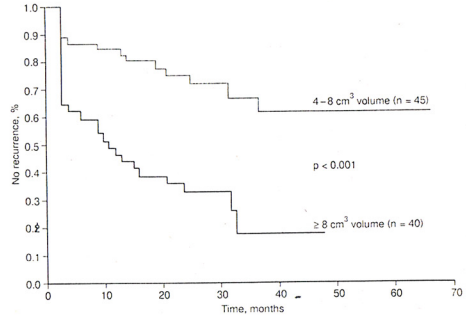

Fig. 2. Kaplan-Meier analysis of time to first recurrence comparing smaller volume tumors (4–8 cm^3) to larger tumors (≥8 cm^3). The difference is highly significant ($p < 0.001$, log-rank statistics).

4–8 cm^3 which had less than 30% Gleason grade 4 or 5 cancer. In an attempt to resolve these exceptions we combined ploidy with volume in a separate analysis of disease recurrence (table 4). Of 45 patients with total tumor volume of 4–8 cm^3, 14 (31%) have recurred. An interpretable histogram was available in 41 of the 45 total patients and in 13 of 14 with disease recurrence. All 13 patients with recurrence had non-diploid tumors ($p < 0.025$). In the 31 patients with no recurrence out of the group of 45 who had cancer volumes of 4–8 cm^3, samples were non-diploid in 18 (58%), diploid in 10 (32%), and indeterminate in 3 (10%). This is not significantly different from the study population as a whole. Twenty-nine of 40 patients (72.5%) with tumors larger than 8 cm^3 suffered recurrences. Of these, 11 were diploid and 18 were non-diploid ($p > 0.05$). Of the 11 nonrecurrent cases in this group, 6 were non-diploid and 5 diploid. When ploidy was combined with Gleason grade, the results were nearly identical to those seen with the combination of ploidy and tumor volume. Of 44 patients with 30% or less Gleason

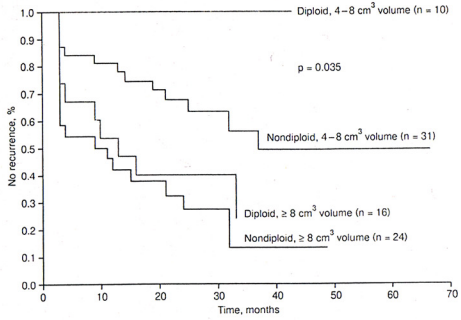

Fig. 3. Kaplan-Meier analysis of time to recurrence dividing patient groups by tumor volume and ploidy. Of 10 patients with tumor volumes $<8\ cm^3$ and diploid histograms, none have recurred. Of 31 patients with tumor volumes $<8\ cm^3$ and non-diploid histograms, 13 have recurred ($p = 0.035$, log-rank statistics).

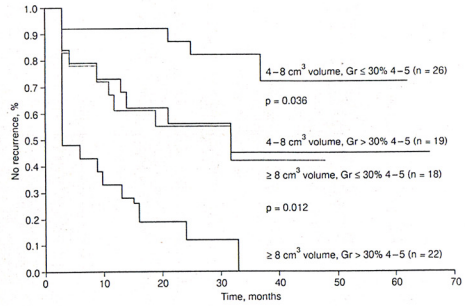

Fig. 4. Kaplan-Meier analysis comparing time to recurrence when combining tumor volume and Gleason grade.

grade 4 or 5 in their cancer, 14 (32%) experienced recurrence. Excluding 1 patient in whom ploidy could not be assigned, the tumors of 12 patients contained non-diploid elements while only 1 patient had a diploid tumor ($p < 0.025$). In the remaining 41 patients with greater than 30% grade 4 or 5, 29 (71%) recurred. Of these, 10 were diploid and 19 non-diploid ($p < 0.05$). Figure 3 illustrates time to recurrence in patients separated by tumor volume plus ploidy status.

Logistic Regression Analysis. Because of the interdependency of pathologic parameters, which we have previously demonstrated [11], a logistic regression was performed to identify the most accurate predictors of lymph node metastasis and recurrence of disease. The parameters tested included tumor volume, percent of tumor involved with Gleason grade 4 or 5, and tumor ploidy. Tumor grade was the single most accurate predictor of lymph node metastasis. The next most accurate predictor was tumor volume, followed by ploidy. When both volume and grade were combined, a highly significant improvement in predictive value was achieved ($p < 0.025$). The addition of ploidy as a third variable did not add additional information or improve the ability to predict lymph node invasion. The ability to predict recurrence was studied in a similar logistic regression. Tumor volume was the variable most predictive of recurrence by a large margin. The log volume was an even better single predictor of recurrence. The next best single predictor of recurrence was grade, followed by ploidy. The addition of grade to volume again conferred a highly significant improvement in ability to predict recurrence ($p < 0.005$; fig. 4). The further addition of ploidy also added significantly to the ability to predict prognosis ($p < 0.005$).

Discussion

The complex natural history of prostate cancer has long confounded efforts to predict prognosis. The importance of tumor volume and Gleason grade has greatly improved our ability to predict risk of recurrence following radical prostatectomy [10, 11]. Even so, tumors of intermediate volume and grade still vary widely in their biologic behavior. The analysis of DNA content (ploidy) by flow cytometry has been a valuable addition to the management of leukemias and transitional cell carcinoma of the urothelium. The shorter natural history and ease of specimen collection (already in single cell suspension) has lent itself well to this technology. The long natural history of prostate cancer has made its study more difficult. However, the use of PSA as a marker indicating persistent or recurrent disease following radical prostatectomy has shortened the time necessary to detect recurrence [1]. The usefulness of ploidy to study solid tumors was greatly enhanced by Hedley's method of deparaffinizing previously fixed specimens [15], which allowed the use of archival material, in which the outcome was already known. Several investigations have studied the role of aneuploidy in prostate cancer with varying results. Frankfurt et al. [3] noted that only 7% of diploid tumors with intermediate Gleason scores formed metastases, while 80% of aneuploid tumors with high Gleason scores were noted to metastasize. Fordham et al. [4] combined ploidy and Gleason score in a survival analysis and noted an increased incidence of death in the aneuploid groups. However, no statistical significance was demonstrated in these reports. Lee et al. [5] in the Duke experience, found that aneuploidy, Gleason grade and seminal vesicle involvement correlated with disease recurrence, but were not independent of each other. The combination of ploidy plus seminal vesicle status further separated the recurrence from nonrecurrent groups. Finally a series of studies from the Mayo Clinic [6–8] found that markedly higher rates of progression occurred in non-diploid tumors as compared to diploid tumors. However, in a few other series, ploidy was no or only a weak predictor of tumor progression [16–18].

In the present study we have limited our review in several ways. Only patients at significant risk for recurrence, those with 4 cm^3 or more of cancer volume, were selected. Patients who received preoperative therapy (which might impact on recurrence or time to recurrence) have been excluded. Similarly, patients treated postoperatively with adjuvant therapy before documentation of biochemical or clinical recurrence have also been excluded. Of more importance, we have excluded all cases of positive surgical margins where the margin was caused by inadvertent incision (by the surgeon) into cancer within the prostate capsule [12, 13]. This has allowed us to study the natural biologic potential of each tumor to recur in relation to pathologic parameters and ploidy analysis of the excised prostate.

Our technique of measuring flow cytometric ploidy also deserves comment. Consistent, reproducible results require an experienced operator and rigorous standardization of technique [19]. Despite this, there is evidence that even among experienced centers there is measurement variability both among laboratories and within the same laboratory on different days; this had occurred even using standardized replicate samples [20]. Additionally, the interpretation of the DNA histograms obtained from flow cytometry is far from standardized. As in this study, it must be performed without knowledge of patient outcome. The identification of an abnormal peak clearly separate from the G0/G1 and G2/M peaks was easily categorized as aneuploid. More difficult were the numerous cases where abnormal populations were very close to the 2C or 4C peaks. In some cases, only a wide coefficient of variation of the G0/G1 peak was noted. Despite evidence that in many cases this represents 'near diploid aneuploidy' [21], we have categorized these cases as uninterpretable. Similarly, only those cases with a sharp 4C peak containing 20% or more of cells and with an identifiable 8C peak were categorized as tetraploid. These cases were so infrequent that they have been combined with the aneuploid cases and commonly referred to as 'non-diploid' throughout this review.

In the present study, 85 patients were subjected to analysis: 26 (30%) had diploid and 55 (65%) had non-

diploid tumors. Four patients (5%) had uninterpretable histograms. Previous studies have identified aneuploidy in 44–66% of patients [2–8]. Forty-three patients (51%) in our study have biochemical and sometimes clinical evidence of recurrent cancer; in this recurrent population, 74% were non-diploid, which is not statistically different from the 65% non-diploid samples in the overall study population of 85 patients. Of 42 patients (49%) who have not recurred 61% were non-diploid, again not statistically different from the study population as a whole. It is clear that when taken alone, ploidy had no value in making a prognosis as defined by biochemical or clinical recurrence. Time to first documented recurrence, as judged by Kaplan-Meier analysis, was also unaffected by DNA content when tested alone (fig. 1).

While ploidy, taken alone, had no predictive value in identifying those destined to recur, the pathologic parameters of total cancer volume and percent of tumor involved with Gleason grades 4 or 5 separated the recurrent from nonrecurrent groups. However, some exceptions were noted, decreasing the clinical utility of these measures. When we combined *ploidy with tumor volume* we obtained a better separation, with nonrecurrence in 10 prostates with 4–8 cm^3 of cancer which had diploid DNA; of 31 non-diploid cases of the same volume, 13 have recurred (42%). This is highly statistically significant. Interestingly, however, this relationship did not hold in larger volume tumors of greater than 8 cm^3. One possible explanation is that above a certain size, the sheer volume of the tumor makes local spread so likely that it overwhelms the contribution of ploidy to malignant potential.

Regression analysis also indicated that tumor volume and grade are the most important parameters in predicting recurrence. Using both factors together was far more accurate in predicting recurrence than use of a single factor alone. The addition of ploidy to these parameters added significant information, although to a smaller degree than volume and grade.

In conclusion we have tested the contribution of ploidy to the development of recurrent disease. When taken alone, ploidy analysis added nothing to the prognosis. However in intermediate volumes (4–8 cm^3) and intermediate grades (less than or equal to 30% Gleason grade 4 or 5) the addition of ploidy analysis allowed further separation of recurrent from nonrecurrent patients. When combined with careful assessment of total cancer volume and percent of tumor of Gleason grades 4 or 5, ploidy analysis may identify a subgroup of patients with volumes between 4 and 8 cm^3 which are at a high risk for disease progression and allow institution of appropriate adjuvant protocols. Since cancers under 4 cm^3 are usually cured by radical prostatectomy, and since ploidy in this study could not discriminate progression from nonprogression in cancers over 8 cm^3, the use of flow cytometry as a prognostic index appears limited. Indeed, even its usefulness in the subset of patients with cancer volumes between 4 and 8 cm^3 depends upon the development of technique to accurately estimate preoperatively cancer volume and Gleason's grade.

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