Rani Crowe. *Mother Love.* Oil, 30" × 40".

*DNA ploidy determined by flow cytometry helps to predict prostate cancer aggressiveness in some groups of patients.*

# Stage B Prostate Cancer: Correlation of DNA Ploidy Analysis With Histological and Clinical Parameters

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***Background:*** *The ability to accurately predict tumor behavior and patient survival is a problem in managing patients with prostate cancer. Prognostic variables in predicting death from tumor include prostate-specific antigen (PSA) level, histological grade, and clinical stage. Observer subjectivity is inherent in determining grade and stage; thus, criteria that are more objective are needed to identify patients for appropriate treatment.*

***Methods:*** *The authors correlated flow cytometric nuclear DNA ploidy with Gleason score, PSA level, and recurrence risk in patients who underwent radical retropubic prostatectomy and bilateral pelvic lymphadenectomy between 1987 and 1993 for histopathologic stage B prostate cancer (T2, N0, M0).*

***Results:*** *Of the tumors analyzed, 64% were DNA diploid with a low proliferative fraction, 25% were DNA diploid with a high proliferative fraction, and 11% were DNA aneuploid. DNA aneuploidy was associated with high Gleason grade (7-10). All Gleason grade 10 tumors were DNA aneuploid. Both DNA aneuploidy and high proliferative fraction ($S+G_2M$) were statistically correlated with high Gleason grade and adverse prognosis but not with PSA level or patient age.*

***Conclusions:*** *A direct relationship is shown between both DNA aneuploidy and a high proliferation index with aggressive biological behavior in stage B prostatic cancer. Objective tumor criteria are needed to choose treatment more selectively for individual patients.*

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## Introduction

Prostate cancer is the most common malignant tumor in American men, resulting in more than 41,800 deaths per year in the United States and an estimated 185,500 new cases in 1999.[1] The ability to accurately predict tumor behavior and patient survival remains a major problem in management. In the case of prostate

cancer, prostate-specific antigen (PSA) level, histological grade, and clinical stage are powerful prognostic variables in predicting death from tumor.[2-5] Since grade and stage are influenced by observer subjectivity, criteria that are more objective are ultimately needed to stratify patients for more selective treatment.

Analysis of tumor DNA content (DNA index: DI) and proliferation index (PI) by flow cytometry has been demonstrated to be useful in predicting the clinical course of patients with various malignant urologic lesions.[6-13] A direct correlation among increasing Gleason grade, DNA aneuploidy, and elevated PI has been shown,[12-22] but little is known of the relative prognostic validity of these indicators.

In this study, we evaluated by multivariate analysis the relative prognostic contribution of histological Gleason grade, DNA ploidy status, and PI in prostatectomy specimens from patients with stage B prostate cancer. We demonstrate a direct relationship between both DNA aneuploidy and high proliferative fraction with aggressive biological behavior in this type of neoplasia.

## Materials and Methods

### *Patients*

A total of 130 patients underwent radical retropubic prostatectomy and bilateral pelvic lymphadenectomy between 1987 and 1993. For this study, we chose 65 consecutive patients who had circumscribed tumors that were greater than 5 mm in size and amenable for dissection from paraffin blocks. They ranged in age range from 40 to 80 years (average = 69 years), and all had histopathological stage B prostate cancer (T2, N0, M0). Patients were staged clinically according to rectal examination, serum PSA levels, and bone scan. Postoperatively, patients were followed regularly with physical examination, PSA determination every six months, and bone scan when clinically indicated due to symptoms or change in laboratory parameters. The chemical disease-free interval was defined as the postoperative period until the first PSA elevation (>0.1 ng/mL). Because clinical follow-up was possible in only 55 cases, 10 cases were excluded from further analysis. Mean follow-up was 80 months (range = 48-120 months).

### *Histopathology*

Paraffin-embedded archival specimens were retrieved from the files of the Division of Surgical Pathology at our institute. From these cases, the most representative stained slides and respective paraffin blocks were selected for this study (105 total sections). The original hematoxylin and eosin (H&E) stained slides were evaluated for adequacy and the presence or absence of carcinoma. One block containing only hyperplastic prostate and one block containing the maximum amount of tumor relative to normal or hyperplastic prostate were selected from each patient when applicable. Between one and three 50-µm sections were recut from each block, the nonpertinent areas of sections were scraped off the slides and discarded, and the remaining localized tumor or hyperplastic area was processed for flow cytometry. An additional 5-µm section was subsequently cut and stained with H&E to verify the presence of tumor and localize it for microdissection. In addition, histological grade was evaluated from this slide according to the Gleason system by an experienced uropathologist who had no knowledge of the patient's outcome or DNA status. The following groups of patients were established: Gleason 2-4 (n=13), Gleason 5-6 (n=16), Gleason 7 (n=13), and Gleason 8-10 (n=13). Benign prostatic hyperplasia was used as control (n=50).

### *Flow Cytometry*

For each selected block, one or more 50-µm sections were cut and placed into a labeled 16 × 125-mm glass culture tube. Nuclear suspensions of tumors were prepared by use of a modified version of the method of Hedley et al.[23] Briefly, 50-µm sections were deparaffinized, rehydrated, and digested with 0.5% pepsin solution in phosphate-buffered saline at pH 1.5 for 60 minutes at 37°C with intermittent vortex mixing. After centrifugation at 2,000 rpm for 10 minutes, the supernatant was removed and the pellet resuspended, washed, and filtered through a 50-µm-pore nylon mesh. The nuclear suspension was incubated with 1.5-mL RNAse (30 U/mL) for 10 minutes at 37°C. It was then centrifuged and the pellet stained for 10 minutes at 4°C with 1 mL propidium iodide (50 µg/mL). The stained nuclei were analyzed using a FACScan flow cytometer (Becton-Dickinson, San Jose, Calif). CellFit software (Becton-Dickinson) was used to acquire and analyze the gated data. The fluorescence signals from 10,000 nuclei were plotted on a 1,024 channel linear scale and displayed as a frequency histogram.

### *Interpretation of DNA Histograms*

DNA histograms were classified as either normal or abnormal based on the DNA content of the $G_0/G_1$ peak (DI) relative to endogenous normal cells and the percentage of proliferating cells ($S+G_2M$ phases). Normal or diploid histograms were those with a single peak exhibiting a DI of 1.00 ± 0.10 and 14% or fewer cells in $S+G_2M$ phases. Abnormal histograms included those exhibiting one of the following patterns: (1) DNA

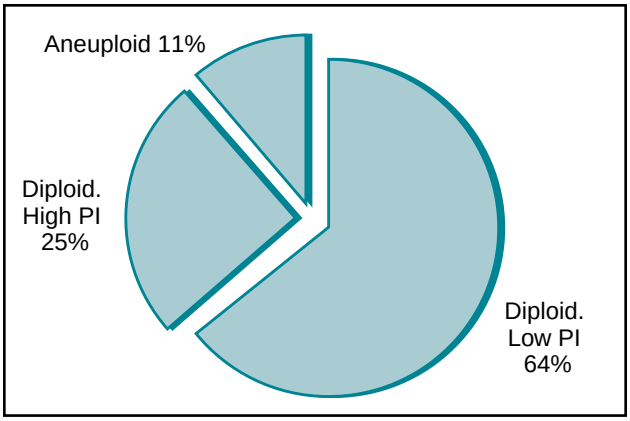


Fig 1. — The distribution of DNA ploidy patterns for the entire group.

diploidy and 15% or more of cells in S+$G_2$M phases (high proliferative fraction) or (2) DNA aneuploidy with one peak or multiple peaks outside the diploid range. Determination of cell proliferation (S+$G_2$M phases) was not attempted in aneuploid samples. No patient showed a DNA tetraploid histogram (>20% of cells in $G_2$+M).

### *Statistical Analyses*

The mean percent of proliferating cells (S+$G_2$M phases) was compared among patients with benign prostatic hyperplasia and the various Gleason score carcinoma groups. The comparisons were analyzed by a one-way analysis of variance test using the general linear models procedure (Proc GLM) of the Statistical Analysis System software program. A Bonferroni test controlling the experiment type I error rate at 0.05 was then performed for each pair of groups. The rate of PSA failure and aneuploidy among the various Gleason score carcinoma groups and the rate of PSA failure in the diploid and aneuploid tumor groups were compared using a standard chi-square contingency table. The mean percent of proliferating cells (S+$G_2$M phases) was compared between the PSA failure and nonfailure groups using the pooled *t* test. In addition, the effects of Gleason score, DNA ploidy, and PI (S+$G_2$M phases) on PSA failure were analyzed using survival analytic methods (Cox regression, log-rank tests, and Kaplan-Meier estimation) that take into consideration the time to failure as well as its occurrence. All statistical analyses were done using SAS statistical software.

## Results

At the time of surgery, patient ages ranged from 40 to 80 years (mean = 66 ± 0.8). Of the 55 evaluated patients, 13 had Gleason scores 2 to 4, 16 had scores of 5 or 6, and 26 had scores of 7 or more. Age and initial PSA level were not significantly correlated with Gleason score (*P*>0.08 and 0.06, respectively).

A total of 105 paraffin-embedded samples of pathologic stage B prostate cancer (n=55) or hyperplastic prostate (n=50) were available for evaluation by flow cytometry and provided high-quality DNA histograms. The coefficient of variation (CV) for the diploid peak varied from 2% to 9% (mean = 5.8 ± 0.5%). All samples had diploid CVs of less than 10%. The distribution of DNA ploidy patterns for the entire group was as follows: 64% were DNA diploid with a low proliferative fraction, 25% were DNA diploid with a high proliferative fraction, and 11% were DNA aneuploid (Fig 1).

The distribution of DNA patterns according to Gleason grade is shown in Fig 2. Well-differentiated carcinomas (Gleason 2-4) exhibiting a uniform growth pattern and little cellular atypia showed DNA histograms similar to those observed in benign hyperplasia. No

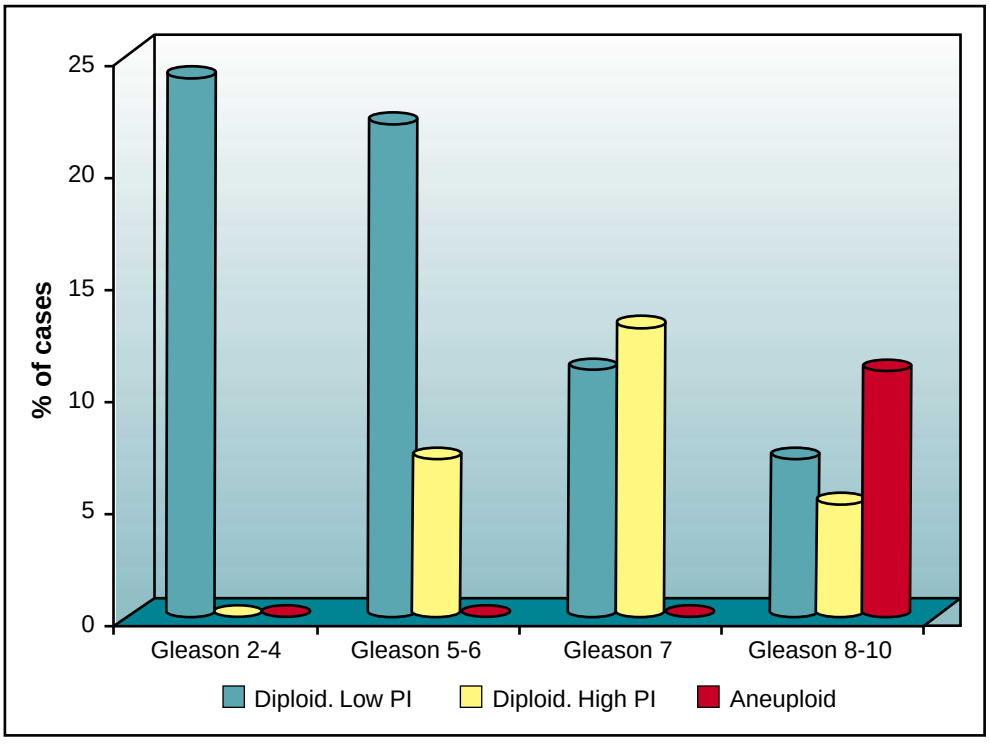


Fig 2. — The distribution of DNA patterns according to Gleason grade.

hypodiploid tumors were observed. The mean DI of these specimens was 0.95 ± 0.05. The proportion of tumors with a high proliferative fraction increased significantly with increasing Gleason score. All DNA aneuploid tumors (11%) were found within the high Gleason groups (8-10). During the follow-up period, three (5.25 %) patients died, all from prostate cancer. Three patients with Gleason score 7-10 developed early bone metastases. Five patients were treated subsequently with castration and or hormonal therapy.

Cox regression analyses of each variable (univariately) found Gleason score, DNA ploidy, and PI to be prognostic for PSA failure. The estimated hazard ratio associated with each unit increase in Gleason score was 1.3 (*P*=0.007, 95% confidence interval [CI] 1.3 to 2.4). The estimated aneuploid vs diploid hazard ratio was 4.7 (*P*=0.002, 95% CI 1.9 to 10.5). The estimated hazard ratio for each increase of 1% in $S+G_2M$ was 1.2 (*P*<0.0001, 95% CI 1.10 to 1.28). When a multivariate, stepwise approach to model selection was employed using $S+G_2M$ and Gleason score, $S+G_2M$ — but no other variables — entered the model. However, this analysis excluded aneuploid tumors, as $S+G_2M$ could not be calculated in these cases. Thus, two groups of poorer prognosis could be easily defined.

To further investigate the effects of this variable on PSA failure, $S+G_2M$ was dichotomized by cutpoints of 15.0% and 20.0%, respectively, and Gleason score by less than 7 and by 7 or greater. Kaplan-Meier curves for both $S+G_2M$ groups, both Gleason score groups, and for the diploid and aneuploid groups were produced and log-rank tests performed. The results are shown in Figs 3 and 4. The superior discrimination produced by PI measures compared with DNA ploidy and Gleason score is apparent.

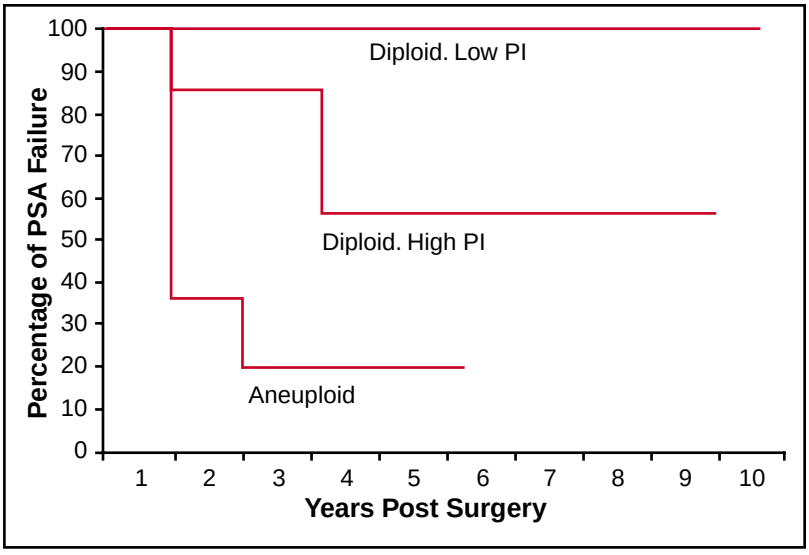


Fig 3. — Kaplan-Meier survival curves of time to PSA failure by DNA ploidy.

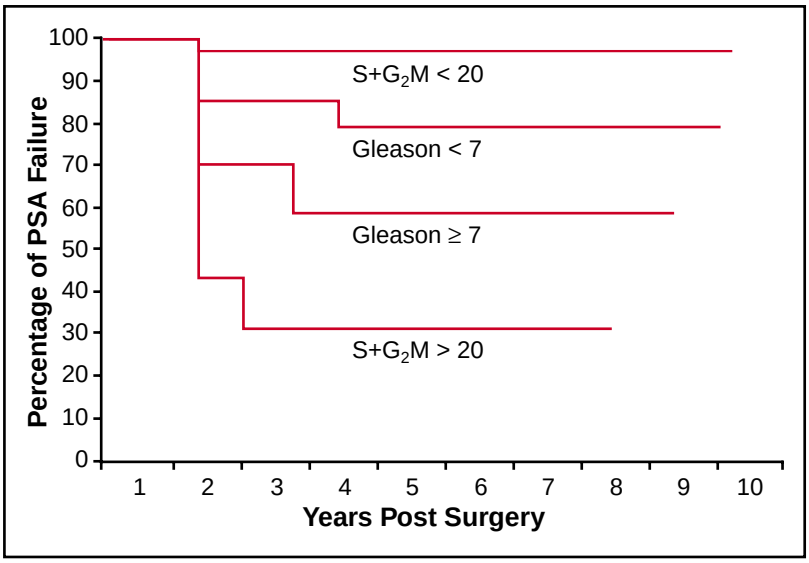


Fig 4. — Kaplan-Meier survival curves of time to PSA failure by percent of $S+G_2M$ phase and Gleason score.

## Discussion

The natural course of prostate cancer is extremely variable, and numerous questions remain unanswered concerning the determination of prognosis in prostate cancer. Tumor grades assessed by histopathology and tumor stage are the standard prognostic parameters used, although the significance of these has been challenged.[2-5] The analysis of tumor DNA content (DI) and PI by flow cytometry has been demonstrated to be useful in predicting the clinical course of patients with prostatic carcinoma and other malignant urologic lesions.[4-12,16] A direct correlation among increasing Gleason grade, DNA ploidy, and PI has been shown.[8-12,22] In some investigations, DNA ploidy and PI were of prognostic significance, while in others the added value of DNA ploidy or PI to tumor stage and grade has been questioned.[2-4,8,15,20-22,24,25] Many of these studies were retrospective flow cytometric analyses of disaggregated paraffin-embedded, formalin-fixed specimens. In most studies, there is a good correlation of DNA diploidy with histological grade. Low-grade tumors are generally DNA diploid, and high-grade tumors are more frequently DNA aneuploid.[4,5,14] In our study, all of the patients who developed metastases during the observation period had aneuploid prima-

ry tumors. The urologic literature suggests that there is heterogeneity in DNA ploidy with both diploid and aneuploid population in an individual patient.[2,13] Prostate cancer of the diploid type is less aggressive than aneuploid cancer. Whether progression of diploid tumors is associated with a change in the modal DNA value has not yet been evaluated.

Survival and disease progression data were available from the institutional tumor database and could be compared with the DNA measurements. As noted, there was a significant difference in the clinical behavior of diploid vs aneuploid tumors. Furthermore, if the subgroup of moderately differentiated primary tumors (Gleason 7-10) is analyzed, thus excluding histology as a variable, statistically significant differences between the diploid and aneuploid groups are still identified.[2] As indicated in this and other studies, DNA measurements in human solid tumors appear to be a useful parameter in the assessment of risk for individuals with cancer. In localized prostate cancer, nuclear morphometric features (shape, roundness, and nucleoli) and nuclear ploidy status prove to be significant predictors of long-term disease survival after radical prostatectomy.[14-27] We demonstrate a direct relationship between both DNA aneuploidy and high PI with aggressive biologic behavior in prostatic cancer. DNA ploidy measurements could play an important role in stratifying patients for consolidative radiation and chemotherapy regimens, most notably as an adjuvant treatment for DNA aneuploid tumors that also infrequently have long-term responses to antiandrogen therapy.

Analysis of the literature and our current study have identified areas where further research is justified with respect to the clinical application of DNA ploidy for prostatic carcinoma. The extent and clinical significance of DNA heterogeneity within a single tumor needs investigation.

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