

# Near-Diploidy: A New Prognostic Factor for Clinically Localized Prostate Cancer Treated with External Beam Radiation Therapy

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**Background.** DNA ploidy is a significant prognostic factor in patients with prostate cancer. Using DNA/nuclear protein flow cytometry, a subpopulation of tumors with near-diploid DNA is identifiable. The prognostic significance of near-diploidy was examined.

**Methods.** Paraffin-embedded formalin fixed prostate tumor tissue from patients treated at M. D. Anderson Cancer Center with external beam radiation therapy was processed for DNA/nuclear protein flow cytometry. All patients had pretreatment and follow-up serum prostate specific antigen (PSA) levels. Seventy-six specimens were suitable for flow cytometric analysis. Tumors were classified as either diploid (n = 30), near-diploid (n = 24), or nondiploid (n = 22, tetraploid and aneuploid). Median follow-up time was 36 months.

**Results.** Diploid tumors were associated with a significantly better actuarial outcome at 4 years, compared with near-diploid tumors, using either biochemical relapse (rising PSA) or a composite end point of a rising PSA or clinical relapse (16% versus 52% relapse,  $P < 0.05$ , log-rank). Moreover, patients who had nondiploid tumors had the worst prognosis (77% relapse, composite end point). No significant difference was observed between diploid and near-diploid neoplasms regarding actuarial local control, freedom from metastasis, freedom from clinical relapse, or overall survival time. A Cox proportional hazards model, using the composite end

point of a rising PSA or relapse, was performed with ploidy categorized as diploid, near-diploid, and nondiploid; pretreatment PSA, DNA ploidy, and tumor grade were found to be independent prognostic factors. When ploidy was categorized as diploid or near-diploid (nondiploid tumors excluded), pretreatment serum PSA and DNA ploidy were independent predictors of outcome. Ploidy remained an independent prognostic factor even when nondiploid tumors were excluded.

**Conclusions.** These data show that patients who have near-diploid tumors have an intermediate prognosis between the more favorable diploid tumors and the less favorable nondiploid tumors. *Cancer* 1994; 73: 1895-903.

**Key words:** prostate cancer, radiation therapy, flow cytometry, ploidy, DNA, nuclear protein.

Numerous studies have established that DNA ploidy, using flow or static cytometry, provides significant prognostic information for patients with prostate cancer.<sup>1-11</sup> Typically, single-parameter measurements of DNA have been used to classify tumors broadly as diploid or nondiploid, and nondiploid tumors have been further subdivided by some into tetraploid or aneuploid.<sup>5,7-9</sup> In some studies, the outcome associated with tetraploidy or aneuploidy was similar,<sup>4,5</sup> whereas in others, tetraploid tumors were associated with a better outcome compared with aneuploid tumors.<sup>7-9</sup>

Flow cytometry is a high resolution technique for measuring DNA content in large numbers of nuclei in a relatively short period. One limitation of this method is that diploid, or near-diploid, tumor nuclei are not distinguished from normal diploid nuclei. Multiparameter flow cytometry, whereby DNA and another parameter, such as RNA,<sup>12</sup> protein,<sup>13,14</sup> or an antigen,<sup>15</sup> are mea-

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sured simultaneously, offers a means for better separating overlapping populations.

In the late 1970s, Darzynkiewicz et al.<sup>12</sup> introduced new methods for cell cycle analysis by simultaneously measuring single- and double-stranded nucleic acid using flow cytometry after acridine orange staining. In subsequent reports by Roti Roti et al.<sup>16</sup> and Pollack et al.,<sup>13</sup> analogous results were obtained by simultaneously quantifying DNA and nuclear protein in nuclei isolated from unfixed tissue. More recently, Ciancio et al.<sup>17</sup> modified this technique for the DNA/nuclear protein staining of nuclei isolated from formalin fixed paraffin embedded tissue.

The advantage of DNA/nuclear protein analysis compared with DNA alone lies in the ability to (1) separate normal diploid cells from tumor diploid cells in some cases,<sup>14,17</sup> (2) recognize near-diploid populations that by single-parameter DNA histogram analysis are either not seen or are suggested by a shoulder on the diploid G<sub>1</sub> peak,<sup>14,17</sup> and (3) identify tissue degradation that can result in false peaks suggestive of aneuploidy.<sup>18</sup> Thus, DNA/nuclear protein analysis offers several potential advantages over DNA alone in retrospective studies of archival formalin fixed paraffin embedded tissue.

We recently confirmed, using a cohort of 76 patients treated at M. D. Anderson Cancer Center with external beam radiation therapy for clinically localized prostate cancer, that DNA ploidy is prognostic for disease outcome.<sup>19</sup> In that analysis, ploidy was defined using DNA content quantified by flow cytometry, and tumors were divided into diploid and nondiploid. Diploidy was associated with a significantly better outcome using such end points as local control, freedom from metastasis, freedom from clinical relapse, freedom from a rising prostate specific antigen (PSA), and freedom from a rising PSA or relapse. A Cox proportional hazards model, using the composite end point of a rising PSA or relapse, revealed pretreatment PSA, DNA ploidy, and tumor grade to be independent prognostic factors.

In the current study, we showed that near-diploidy, as identified by DNA/nuclear protein flow cytometry, provides prognostically significant information in addition to the diploid and nondiploid division described previously.

## Materials and Methods

### *Patient Characteristics*

A cohort of 314 men with available pretreatment PSA values were treated for adenocarcinoma of the prostate with external beam radiation therapy at M. D. Ander-

son Cancer Center between February 1987 and February 1991.<sup>20,21</sup> None of the patients received prior hormone ablation therapy or had prior radical prostate surgery. All pathology specimens were reviewed initially at M. D. Anderson Cancer Center. One hundred seventy of the formalin fixed paraffin embedded blocks were recovered and, of these, only 86 were deemed suitable for flow cytometric analysis using histologic criteria. Most of the tissue samples were from needle biopsies, and in many the amount of tumor material was deemed insufficient on histologic review. Of the 86 specimens analyzed, 8 yielded too few nuclei and 2 yielded uninterpretable histograms, leaving 76 with adequate flow cytometry results. These 76 patients are the subject of the current report.

The initial workup included a full history, complete physical examination, radionuclide bone scan, serum PSA and acid phosphatase determinations, routine blood tests (complete blood count and sequential multiple analyzer-12), chest radiographs, and biopsy of the prostate. Tumor grade was not obtained on one specimen because of insufficient material. Stage T1 (n = 27) was defined as multifocal or diffuse carcinoma in a gland having no palpable evidence of tumor, Stage T2 (n = 23) was defined as palpable cancer confined to the gland, and Stage T3 (n = 26) was defined as tumor that by palpation extended beyond the prostate either into the seminal vesicles or lateral sulcus. None of the patients had clinical-radiographic evidence of metastases. Patient age ranged from 54 to 80 years, with a mean of 67.4 years and a median of 68 years. Staging pelvic lymph node dissection was performed on some patients with intermediate and high-grade tumors without clinical-radiographic evidence of metastases. Fifteen patients in this cohort had a pelvic lymph node dissection: four with diploid, six with near-diploid, and five with nondiploid tumors. Patients with positive pelvic nodes were treated with androgen ablation and were not included in the current series.

Serum PSA levels were determined using the immunoenzymatic assay (Tandem-E PSA immunoenzymatic assay, Hybritech Inc., San Diego, CA). The lower limit of PSA detection is 0.3 ng/ml. Pretreatment and follow-up serum for PSA estimation was drawn before rectal examination, and all patients were ambulatory.

### *Radiation Therapy Treatment and Follow-up*

External beam radiation therapy was delivered using a four-field box technique with 18-MV photons, as described previously.<sup>20,21</sup> The initial 46 Gy was typically delivered through 11 by 11 cm anteroposterior and 11 by 9 cm lateral portals. The final 14–20 Gy were delivered through cone-down portals of approximately 9 by

9 cm. No attempt was made to cover the pelvic nodes. The mean dose was 64.4 plus or minus 1.6 Gy ( $\pm$  standard deviation; range, 60–66 Gy) and varied with stage: Stage T1, 63.0 Gy; Stage T2, 64.1 Gy; and Stage T3, 66.0 Gy. The treatments were administered at 2.0 Gy per day specified at isocenter.

Patients were followed up at 3-month intervals after the completion of radiation therapy with history, physical examinations, PSA and enzymatic prostatic acid phosphatase determinations, and routine blood tests. Radionuclide bone scans were done when indicated. Follow-up time ranged from 18 to 70 months, with a median of 36 months and a mean of 40 months. End-point times were calculated from the date of completion of radiation therapy.

Patients were considered disease-free if there was no clinical-radiographic evidence of metastases and if there was no clinical evidence of local recurrence. Because prostate cancer can take many months to regress completely at the primary site after radiation therapy, local control was defined as either a palpably normal prostate or one in which abnormalities were resolving. Stable residual prostate abnormalities on digital rectal exam, in the presence of a stable or declining PSA, were considered controlled until such time as clinical or biochemical evidence of progression was manifested. All local recurrences were confirmed by biopsy.

The definition of rising postradiation PSA values was that two or more consecutive values increased and that the last value was greater than 1 ng/ml. A rising PSA was not used as a criterion for clinical disease relapse. The timing and intensity of investigating patients with rising PSA values was at the urologist's discretion. In calculating actuarial curves for the incidence of rising PSA values with time, the time of onset of PSA rise was defined as the average time between the nadir PSA value and first elevated value. The following outcome end points were analyzed: local control, freedom from metastases, freedom from clinical relapse, freedom from a rising PSA, and a composite end point of freedom from a rising PSA or relapse.

### Sample Preparation

Tumor tissue specimens were obtained from archival paraffin embedded pathologic material, either from needle biopsy or transurethral resection of the prostate. Hematoxylin and eosin stained biopsy slides were reviewed by the study pathologist (A.K.E.) to ensure that tumor was present in sufficient quantity for flow cytometry. In all of the tissue samples that were analyzed, at least 30% of the specimen was replaced by tumor.

Because this was a retrospective study and, in many cases, limited tissue from transrectal biopsies was avail-

able, there is a risk that in some cases the biopsy was not representative of the most aggressive portion of the tumor. However, the techniques used are the same as those for assigning tumor grade, which appears consistently as an independent prognostic factor.<sup>20,21</sup> Thus, while sampling error may exist, it is probable that the overall effect on the results is minor. Multiple samples were prepared when sufficient tumor tissue was available (i.e., multiple blocks), and tumors were classified by the most aggressive DNA profile: diploid, near-diploid, tetraploid, aneuploid, with diploid tumors considered the least aggressive and aneuploid tumors the most aggressive.

The paraffin embedded tissue was prepared from blocks for flow cytometry using a method described by Ciancio *et al.*<sup>17</sup> Two 50- $\mu$ m thick sections were deparaffinized with two changes of xylene (5 ml each for 10 minutes) in glass centrifuge tubes. The sections were rehydrated by using decreasing concentrations of ethanol by incubating for 10 minutes in each of the following solutions: 100% ethanol times 2, 90% ethanol times 2, 70% ethanol times 1, and 50% ethanol times 1. The tissue was washed twice in isotonic calcium and magnesium-free phosphate-buffered saline (CMF-PBS). If there was minimal tissue available in the block, such as from a very small needle biopsy, then the second 100% ethanol and the second calcium and magnesium-free phosphate-buffered saline washes were not done in an attempt to decrease cell loss.

The tissue was then teased apart in 1 ml of 0.5% pepsin (pepsin A from porcine stomach mucosa [catalog number P7012, Sigma Chemical Co., St. Louis, MO] in 0.9% saline (adjusted to pH 1.5 with 2 normal hydrochloric acid). The teased tissue was incubated in 5 ml of 0.5% pepsin for 90 minutes at 37°C with intermittent vortex mixing every 10 minutes. The nuclei were then filtered through a 35- $\mu$ m nylon mesh before being washed twice in 2 ml CMF-PBS. The nuclei were counted using a hemocytometer and diluted to 1 times 10<sup>6</sup>/ml in nuclear isolation buffer (0.5% Nonidet P40; 0.05 molar Trizma base: Trizma-hydrochloric acid, pH 7.4; 0.05 molar sodium chloride; 1 mM ethylenediaminetetraacetic acid). Each sample was vortex-mixed for 2 seconds before staining.

The staining procedure has been described in detail previously.<sup>17</sup> To each 1 ml of nuclear suspension in nuclear isolation buffer was added 0.9 ml bicarbonate buffer, 0.1 ml fluorescein isothiocyanate solution (5  $\mu$ g/ml fluorescein isothiocyanate in bicarbonate buffer), and 0.7 ml propidium iodide solution (100  $\mu$ g/ml propidium iodide in bicarbonate buffer). Ribonuclease 1A (Sigma Chemical Co., St. Louis, MO) was added to each sample in 0.3 ml bicarbonate buffer at 1000  $\mu$ g/ml. The volumes of these solutions were halved

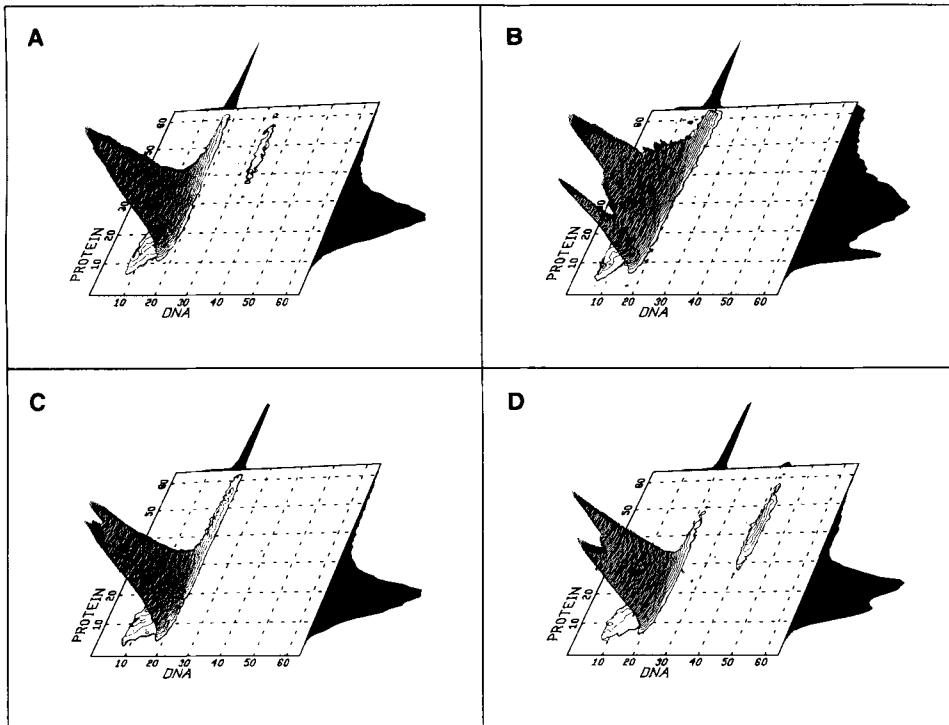


Figure 1. Representative bivariate flow histograms of diploidy, near-diploidy, and tetraploidy. (A) Diploid tumor, (B) near-diploid tumor, (C) near-diploid tumor, and (D) tetraploid tumor (this tumor also contains a near-diploid population).

when the number of nuclei was less than  $5 \times 10^5$ /ml. The suspension was vortex-mixed for 5 seconds and then left at  $4^\circ\text{C}$  for 18–24 hours. Before flow cytometric analysis, the samples were vortex-mixed and refiltered through  $37 \mu\text{m}$  nylon mesh. The pepsin and bicarbonate solutions were made up fresh every 2 weeks.

#### Flow Cytometric Analysis

Samples were analyzed on an EPICS 752 equipped with narrow-beam excitation optics ( $5 \mu\text{m}$ ) and a quartz flow cell. The flow cytometer (Coulter Electronics, Hialeah, FL) was set up with a 515-nm long-pass filter, a 560-nm dichroic filter, a 630-nm long-pass filter (red photomultiplier tube for DNA), a 530-nm short-pass filter (green photomultiplier tube for protein), and an argon ion laser emitting 200 mW at 488 nm. An attempt was made to analyze 30,000 nuclei from each specimen, but in some cases where there was little tissue available, fewer nuclei were analyzed. Samples were rejected if fewer than 5000 nuclei were accumulated in more than 2000 seconds at a flow rate set for optimal coefficient of variation or if the coefficient of variation was greater than 6.9. Exclusion of aggregates was accomplished by bit-mapped gating of the peak-red and integral-red signals. Of the 86 samples deemed to have sufficient tumor tissue histologically, 8 were rejected for having too few nuclei and 2 were rejected for having

broad coefficients of variation. The data were collected in list mode.

Tumors were classified as diploid, near-diploid, tetraploid, or aneuploid based on the DNA histogram profile. The  $G_1$  peak with the lowest DNA content was considered to be diploid. Tumors were classified as near-diploid when (1) a separate peak was seen in the bivariate histogram in the region of the diploid  $G_1$  peak, (2) the DNA content of nuclei in the second peak was higher than those of the diploid  $G_1$  peak, and (3) the DNA index (DI) was less than 1.12. In almost every case, the small difference in DNA content between diploid and near-diploid was not seen in the single-parameter DNA histogram. Near-diploid nuclei were separated from diploid nuclei in the bivariate histograms based in part on differences in nuclear protein; near-diploid  $G_1$  nuclei usually had higher nuclear protein levels than diploid nuclei. When another peak was seen with a DI of between 1.12 and 1.89 or greater than 2.1, then the tumor was considered to be aneuploid. The division between near-diploid and aneuploid at a DI of 1.12 is based on our experience that at this level, a separate second  $G_1$  peak is seen consistently in the single-parameter DNA histogram. The DI is the ratio of the peak red fluorescence channel of the abnormal  $G_1$  peak to the peak red fluorescence channel of the diploid  $G_1$  peak. The DI was analyzed using 256 channel single-parameter DNA histograms. When the percentage of nuclei was greater than 5% in the  $G_2\text{M}$  region (DI, 1.9–

**Table 1. Relationship of Near Diploidy to Other Potential Prognostic Factors**

Parameter	Percent diploid (n)	Percent near-diploid (n)	P *
Stage			
T1	30 (9)	50 (12)	
T2	33 (10)	33 (8)	
T3	37 (11)	17 (4)	0.2
Grade			
1	57 (17)	57 (13)	
2	40 (12)	30 (7)	
3 & 4	3 (1)	13 (3)	0.4
PSA (ng/ml)			
≤ 4	43 (13)	50 (12)	
> 4 ≤ 10	20 (6)	25 (6)	
> 10 ≤ 30	33 (10)	17 (4)	
> 30	3 (1)	8 (2)	0.5
PAP (mU/ml)			
≤ 0.4	79 (22)	70 (16)	
> 0.4 ≤ 0.8	21 (6)	30 (7)	0.5
TURP			
No	73 (22)	42 (10)	
Yes	27 (8)	58 (14)	< 0.02
Testosterone (ng/dl)			
≤ 400	55 (11)	44 (7)	
> 400	45 (9)	56 (9)	0.5
Age (yr)			
≤ 60	10 (3)	8 (2)	
> 60	90 (27)	92 (22)	0.8

PSA: prostate-specific antigen; PAP: prostatic acid phosphatase; TURP: transurethral resection of the prostate.

\* Chi-square statistic.

2.1), then the tumor was classified as tetraploid. We consistently visualized a sharp peak in  $G_2M$ , suggesting tetraploidy using this criterion. Benign and hyperplastic prostate tissue was found to have 3% or less in  $G_2M$ . Thirty patients had diploid tumors, 24 had near-diploid tumors, 11 had tetraploid tumors, and 11 had aneuploid tumors.

### Statistical Methods

The significance of differences between proportions was tested using the chi-square test.<sup>22</sup> Actuarial curves were calculated using the Berkson-Gage method, and tests of statistical significance were based on the log-rank statistic.<sup>23</sup> Multiple covariate actuarial analysis was done using the proportional hazards model with the log-linear relative hazard function of Cox.<sup>23</sup>

### Results

Figure 1 shows four examples of DNA/nuclear protein histograms. In some diploid tumors, a single  $G_1$  peak

was observed in the bivariate histogram (Fig. 1A), whereas in others, two peaks were seen with identical DNA contents that were separated based on differences in nuclear protein levels. The appearance of the latter type of histogram is similar to that of a near-diploid tumor (Fig. 1, B and C), except that in the case of near-diploidy, the second  $G_1$  peak by definition has a slightly higher DNA content. It should be noted that in these cases the near-diploid population is recognized as such only in the two-parameter histogram. The single parameter DNA profiles show a single population. Sometimes, two populations are seen in the single-parameter nuclear protein profile (Fig. 1B), however, one must refer to the bivariate histogram to determine if these have different DNA contents. In some cases, near-diploidy was seen in the presence of an elevated percentage of nuclei in  $G_2M$  (Fig. 1D); these tumors were classified as tetraploid. In 5 of the 11 tetraploid tumors, a near-diploid population was recognized in addition to the elevated percentage of nuclei in  $G_2M$ . For these five tumors, the percentage of nuclei in  $G_2M$  ranged from 5.3–26.9%, and two of the five were associated with failure using the composite end point of a rising PSA or relapse. The three tumors that were not associated with failure had 5.9%, 14.7%, and 15.1% in  $G_2M$ . Overall, diploidy was associated with failure using the composite end point in 4 of 30 patients, near-diploidy with failure in 10 of 24, and nondiploidy with failure in 16 of 22 ( $P < 0.05$ , chi-square test).

Table 1 shows the relationship of ploidy in terms of diploid and near-diploid to various prognostic factors. The groupings of PSA and prostatic acid phosphatase are based on prior studies.<sup>20,21,24</sup> The median value was used as the dividing point for serum testosterone. The dividing point for age was based on prior studies showing that patients over 60 years of age have a better prognosis.<sup>25</sup> Only transurethral resection of the prostate was significantly related to ploidy, in that more patients who had near-diploid tumors had transurethral resections of the prostate.

Figure 2 shows actuarial outcome based on DNA ploidy using the end points of a rising PSA, and a rising PSA or relapse. The results using these two end points are similar, because almost every patient who relapsed clinically had a rising PSA level (Table 2). The univariate analysis revealed three prognostically significant groups based on ploidy. Diploid tumors were associated with the best prognosis, near-diploid with an intermediate prognosis, and tetraploid or aneuploid (non-diploid) with the worst prognosis. The 4-year actuarial incidences of a rising PSA or relapse were 16% for diploidy, 52% for near-diploidy, and greater than 77% for nondiploidy ( $P < 0.0001$ , log-rank statistic). The differences between diploid versus near-diploid and near-

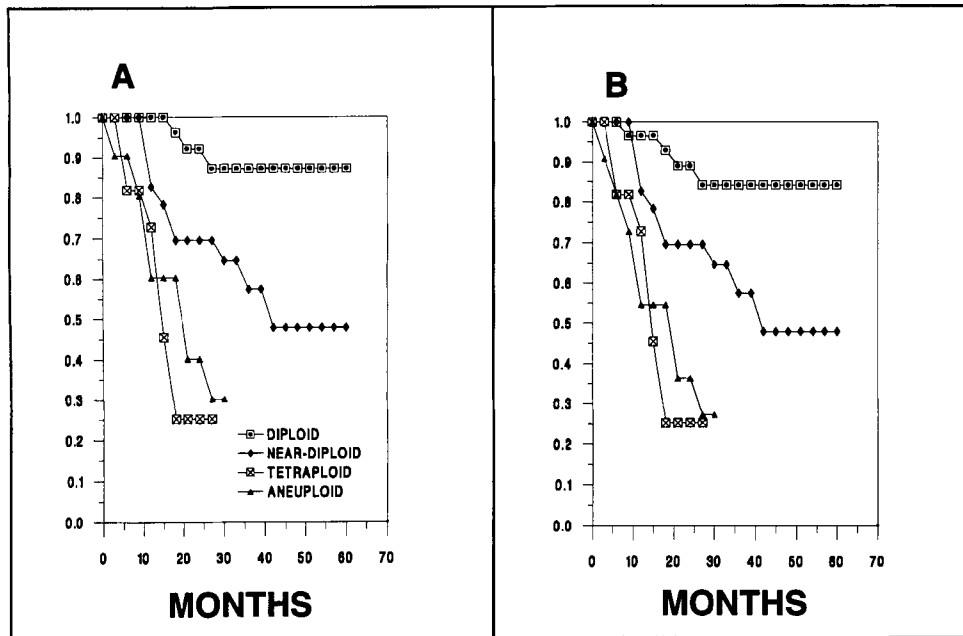


Figure 2. Actuarial outcome of patients with diploid, near-diploid, tetraploid, and aneuploid tumors in terms of (A) freedom from a rising PSA and (B) freedom from a rising PSA or relapse.

diploid versus nondiploid were significant ( $P < 0.05$ , log-rank).

In Figure 3, the association of diploidy and near-diploidy with local control, freedom from metastases, freedom from clinical relapse, and overall survival time are shown. No statistically significant differences between diploid and near-diploid were seen using any of these end points (Table 2).

Multivariate analysis using a Cox proportional hazards model was performed to determine if near-diploidy was an independent predictor of outcome. Initially, the whole group was included, and ploidy was categorized as diploid, near-diploid, and nondiploid (Table 3). In this analysis, pretreatment PSA, ploidy, and grade were found to be independent predictors of outcome. A second Cox proportional hazards model

was then tested with nondiploid tumors excluded, that is, ploidy categorized as diploid and near-diploid. In this analysis, ploidy and PSA were found to be independent predictors of outcome (Table 4). Thus, the significance of ploidy as a prognostic factor was retained even when nondiploid tumors were excluded. Grade was the least significant of the three factors and might have shown up as a significant factor in Table 4 if there were larger numbers of patients.

**Discussion**

DNA ploidy analysis using flow cytometry is useful in predicting outcome in patients with prostate cancer.<sup>1-11</sup> However, there are limitations in the technique, particularly when the tumor DNA content is similar to that of normal tissue components (i.e., stromal and lymphoid cells). Thus, any prognostic information that might result from the identification of near-diploid tumor cells is usually lost in single-parameter DNA histogram analysis.

Abnormalities in tumor DNA ploidy represent a spectrum, ranging from hypodiploid (less than 2C) to hypertetraploid (greater than 4C). However, not all malignant cells have measurable abnormalities in DNA content. For example, balanced translocations or minor deletions or duplications that would be easily identified by chromosome-banding techniques would not be detected using DNA content measurements. The ability to identify subtle abnormalities in DNA content is more problematic when paraffin embedded formalin fixed tissue is used; this is largely because of the greater coeffi-

**Table 2. Relationship of Ploidy to Disease Outcome**

Endpoint	4-Yr actuarial (%)		P*
	Diploid (n = 30)	Near-diploid (n = 24)	
Local relapse	10	26	0.193
Metastasis relapse	3	0	0.381
Clinical relapse	13	26	0.406
Survival	91	96	0.672
Rising PSA	13	52	0.014
Composite†	16	52	0.033

PSA: prostate-specific antigen.

\* Log-rank statistic.

† Rising PSA or relapse.

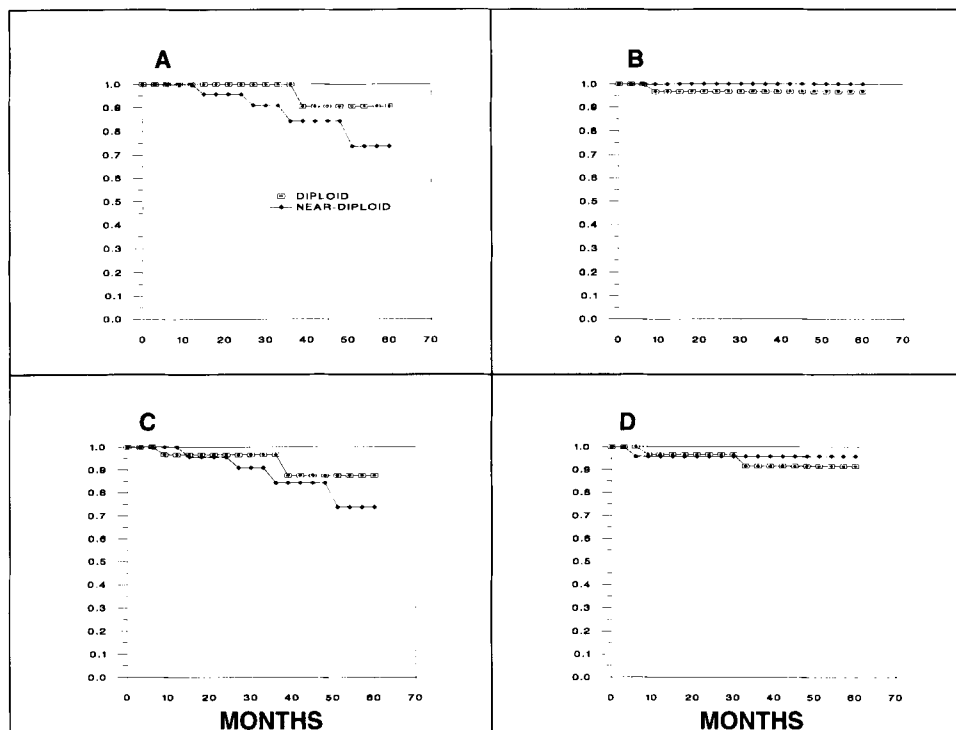


Figure 3. Actuarial outcome of patients with diploid and near-diploid tumors in terms of (A) local control, (B) freedom from metastasis, (C) freedom from clinical relapse, and (D) overall survival.

cient of variation in fixed compared with unfixed tissue.<sup>17,26</sup> In the analysis described in the current study, the threshold for detecting these abnormalities was lessened by (1) optimizing staining conditions,<sup>17</sup> (2) the ability to recognize tissue degradation,<sup>18</sup> (3) the use of high-resolution optics in the flow cytometer, and (4) the addition of the nuclear protein parameter. The best results were obtained when the yield of nuclei was optimal. Under these conditions, we were able to recognize near-diploid tumors with DI less than 1.12.

The finding of elevated numbers of nonaneuploid cells in G<sub>2</sub>M, and hence the group defined as tetraploid, may occur by three mechanisms. First, true tetraploidy means that cells have twice the normal complement of DNA; that is, 4C in G<sub>1</sub>. Tetraploid tumor cells in S or

G<sub>2</sub>M with greater than 4C DNA should also be seen; in prostate cancers, however, the percentage of tetraploid cells in S+ G<sub>2</sub>M is low, and these populations are less often identified. Second, near-diploidy may contribute to a higher percentage of cells in G<sub>2</sub>M. In this setting, two stem lines of cells with very similar DNA contents give the appearance of tetraploidy in the single-parameter DNA histogram analysis. Additionally, it is possible that some of these tumors contain more than one abnormal stem line, that is, near-diploid and tetraploid. Five tumors were identified as having near-diploidy and an elevated percentage of nuclei in G<sub>2</sub>M (range, 5.3–26.9) in this cohort. These tumors were classified as tetraploid, because they would have been classified as such by the single-parameter DNA histogram analysis. Larger numbers of patients are needed to determine whether it would be more appropriate to classify these tumors differently. Third, doublets may falsely elevate

Table 3. Cox Proportional Hazards Model Using the Composite Endpoint of a Rising PSA or Relapse: Ploidy Categorized as Diploid, Near-Diploid, and Non-Diploid

Variable	Likelihood ratio chi-square	Degrees of Freedom	P
PSA*	22.9	2	< 0.0001
Ploidy†	11.3	2	0.0035
MDA grade‡	7.5	2	0.0239

PSA: prostate-specific antigen; MDA: M.D. Anderson.

\* Grouped ≤ 4; > 4 but ≤ 30; > 30 ng/ml.

† Grouped diploid, near-diploid, nondiploid.

‡ Grouped Grade 1, Grade 2, Grades 3 & 4.

Table 4. Cox Proportional Hazards Model Using the Composite Endpoint of a Rising PSA or Relapse: Ploidy Categorized as Diploid and Near-Diploid

Variable	Likelihood ratio chi-square	Degrees of freedom	P
PSA*	12.9	2	0.016
Ploidy†	5.6	1	0.018

PSA: prostate-specific antigen.

\* Grouped ≤ 4, > 4 but ≤ 30, > 30 ng/ml.

† Grouped diploid, near-diploid.

the percentage of nuclei in G<sub>2</sub>M. To circumvent this problem, nuclei aggregates were excluded from the histograms by gating on the peak versus integral red signal. In addition, each sample was checked microscopically after staining with propidium iodide for aggregates, and this was not a problem. This explains in part why our cutoff for tetraploidy at greater than 5% in G<sub>2</sub>M is somewhat lower than that reported by others.<sup>7</sup>

Near-diploid tumors are associated with an intermediate prognosis using the actuarial end points of biochemical relapse or the composite end point of biochemical or clinical relapse. Other actuarial end points, such as local control, freedom from metastasis, freedom from clinical relapse, and overall survival time, did not reveal a significant difference between diploid and near-diploid tumors. This is anticipated, because there is a lead time from biochemical to clinical failure.<sup>27,28</sup> Such lead time is relatively short when biochemical failure promotes earlier, more aggressive investigations, including biopsy of the prostate.<sup>27,28</sup> A more realistic estimate of when clinically evident relapse (i.e., palpable changes on digital rectal exam, urinary obstructive symptoms, or bone pain) occurs in relation to biochemical relapse is obtained by comparing our newer series, where pretreatment and posttreatment PSA levels were available,<sup>21</sup> to our old series,<sup>25</sup> where this test was not used. In this setting, the lead time ranges from 2–5 years, depending on prognostic factors, for example, stage (unpublished observations). Thus, with longer follow-up, we expect the differences in biochemical relapse between patients who have diploid and near-diploid tumors to be manifested as differences in clinical relapse.

There is evidence that in untreated prostate tumors there is progression from diploid to tetraploid and then to aneuploid as time advances.<sup>8,29</sup> In support of this, some investigators have found tetraploidy to be associated with a better outcome compared with aneuploidy.<sup>7–9</sup> We have not observed any difference in outcome between tetraploidy and aneuploidy, although the numbers are small. The relationship of near-diploidy to this progression is unclear. Because some tumors that have a tetraploid pattern using single-parameter DNA histogram analysis are truly near-diploid, the mechanism of progression for some tumors might be from diploid to near-diploid and then to aneuploid.<sup>30</sup> Such a mechanism might explain the finding by Deitch et al.<sup>31</sup> of "abnormal diploid" DNA histograms that contained proportionally more cells in 4C. Also, this mechanism is more in line with our finding that near-diploidy is associated with an intermediate prognosis.

In summary, multiparameter flow cytometry was used to quantify DNA and nuclear protein in paraffin embedded prostate tumor tissue. Using this technique,

a subpopulation of tumors with near-diploid stem lines was identified. The near-diploidy profile was independently prognostic for biochemical failure. Thus, there are now three DNA histogram types that correlate with prognosis—diploid, near-diploid, and nondiploid.

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