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DEOXYRIBONUCLEIC ACID PLOIDY ANALYSIS AS A PREDICTOR OF RECURRENCE FOLLOWING RADICAL PROSTATECTOMY FOR STAGE T2 DISEASE

MARNÉ J. CARMICHAEL, ROBERT W. VELTRI, ALAN W. PARTIN, M. CRAIG MILLER, PATRICK C. WALSH AND JONATHAN I. EPSTEIN*

From the Departments of Pathology and Urology, The Johns Hopkins University School of Medicine and the James Buchanan Brady Urological Institute, The Johns Hopkins Hospital, Baltimore, Maryland and Cytodiagnostics, Inc., Oklahoma City, Oklahoma

ABSTRACT

Deoxyribonucleic acid (DNA) ploidy image analysis was used postoperatively to predict recurrence of 112 clinically localized adenocarcinomas of the prostate. All men underwent radical retropubic prostatectomy between 1978 and 1991. Patients with positive lymph nodes or positive seminal vesicles were excluded because progression is nearly inevitable in these men. The minimum followup for men without progression was 5 years (range 5 to 15). Patients were considered to have clinically evident disease progression based on local recurrence (8%), distant metastases (4%) and/or an isolated elevation of serum prostate specific antigen (87%). Of the tumors 43% were diploid and 57% were nondiploid. In a multivariate analysis comparing grade, ploidy, capsular penetration and surgical margins, Gleason sum was the best predictor of progression ($p < 0.0001$). Nevertheless, a subset of patients remained with well to moderately differentiated Gleason grade tumors (Gleason sum 6 or less) who failed. DNA ploidy was able to predict recurrence in this particular group ($p = 0.034$). In addition, we compared different methods of tissue preparation to determine which best predicted progression. We found that ploidy analysis on tissue sections was more predictive than ploidy performed on disaggregated tissue. In summary, our study revealed that DNA ploidy analysis can offer additional prognostic information following radical prostatectomy for men with low grade prostatic adenocarcinoma.

KEY WORDS: DNA, ploidies, neoplasm staging, prostatic neoplasms

Approximately 25 to 41% of men have progression 10 years following radical prostatectomy for clinical stage T2 (stage B) adenocarcinoma of the prostate.[1–3] Although the use of postoperative adjuvant therapy is controversial, enhanced prediction of disease progression is desirable for prognostic purposes and for the design of trials investigating new treatment modalities. Currently, the standard pathological parameters of grade, margin status, capsular penetration, seminal vesicle invasion and lymph node metastases are used to estimate progression after radical prostatectomy.

In recent years, the advancement of computer technology has led to the development of several new analytical systems found to offer additional prognostic information contributing to the management and treatment of prostate cancer. Multiple studies have been performed using deoxyribonucleic acid (DNA) ploidy analysis of tumor on radical prostatectomy specimens, and the majority of these studies have assessed the relationship between DNA ploidy and pathological stage.[4–13] Although many of these studies have examined the relationship of ploidy with disease progression, many antedated the use of serum prostate specific antigen (PSA) and/or had a limited number of cases with short-term followup.[4,6–8,11,14]

We sought to determine whether the measurement of ploidy enhanced our prediction of progression following radical prostatectomy for early stage disease, beyond that provided by knowledge of the status of the grade, margins and capsular penetration. We also evaluated which method of tissue preparation for DNA ploidy image analysis (that is disaggregation versus tissue sections) correlated best with prognosis.

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* Requests for reprints: Department of Pathology, The Johns Hopkins Hospital, 600 North Wolfe St., Baltimore, Maryland 21287.

MATERIALS AND METHODS

We studied histological sections from 112 patients who had undergone radical prostatectomy for clinically localized adenocarcinoma of the prostate and who satisfied several criteria: 1) patients with positive lymph nodes and positive seminal vesicles were excluded, 2) no patient was treated postoperatively with radiation therapy, hormonal therapy or chemotherapy between the original operation and progression, and 3) all patients without progression had a minimum followup of 5 years (range 5 to 15, mean 8.5). Evidence of disease recurrence was noted if a patient had an elevated postoperative PSA level (greater than 0.2 ng./ml.) and/or needle biopsy proved local or distant failure, and/or positive metastatic surveys by bone scan. Following radical prostatectomy, the specimens were fixed in formalin, serially sectioned at 2 to 3 mm. intervals, submitted in their entirety and embedded in paraffin. All slides were reviewed by 1 pathologist (J. I. E.).

The Gleason grading system was used to grade the dominant tumor nodule. Because there were few tumors with a Gleason sum of 4 or less, those with Gleason sum 2 to 4 were considered together with Gleason sum 5 and 6 as well to moderately differentiated low grade lesions. For the purpose of this analysis, we combined the cases of Gleason sum 8 to 9 together with Gleason sum 7 tumors. There were only a few Gleason sum 8 to 9 tumors and the Gleason 7 tumors fared more similarly to the higher grade than they did to the lower grade lesions. In a prior larger study, the greatest difference in risk of progression was also between patients with a Gleason sum of 6 or less versus those with a Gleason sum of 7 or more.[1]

DNA analysis. One section from the largest diameter of the

dominant tumor nodule was chosen for ploidy analysis. Tissue sections from 62 of the 112 specimens judged to have an adequate amount of tumor on a single paraffin block were disaggregated and cyto-spins were prepared with 50 μm. sections using the Hedley technique.[15] Tissue sections (5 μ.) from these same 62 cases were deparaffinized, and the cyto-spins and tissue sections were Feulgen stained using the CAS* stain and rinse reagents. Tissue sections (5 μ.) from the remaining 50 of 112 cases were deparaffinized and Feulgen stained. The nuclear DNA content was analyzed using the CAS 200 image analyzer (tissue sections were analyzed using version 3.0 and disaggregated tissue was analyzed using version 2.0). The first 200 well preserved nuclei encountered in a systematic screening of each slide were analyzed. As an internal control, the first 10 well preserved fibroblasts were analyzed from the cyto-spins, and the first 25 well preserved prostatic epithelial nuclei from the normal area of the tissue sections were analyzed and designated the true normal diploid peak. Dejter et al used lymphocytes as an internal control for the diploid (2N) peak.[4] However, most other studies analyzing DNA ploidy of prostate cancer use only a separate calibration slide containing rat liver cells to standardize the true diploid peak.[8,9,14] While measuring the nuclear DNA ploidy, we used fibroblasts found on the same cyto-spin to mark the true diploid peak. Fibroblasts are much closer to the true diploid peak than lymphocytes and, because they were on the same slide as the cancer nuclei, we know they were processed and stained in the same manner. In preliminary studies, samples that were initially believed to be hypodiploid or hyperdiploid based upon the calibration slide were in actuality diploid when compared with the ploidy peak of the internal controls. For the tissue sections, histology was evident and, therefore, nuclei from normal prostatic epithelial cells were used to calculate the true diploid peak and adjust for the thickness of the tissue section.

Cells with a DNA index of 2N $\pm$ 10% were considered diploid and those with an index of 4N $\pm$ 10% were considered tetraploid. Cases with more than 13% of tetraploid cells (4N $\pm$ 10%) were classified as tetraploid. Peaks between 2N and 4N were considered aneuploid, although individual cells in this region were considered to be in S-phase. Histograms with any cells greater than 4N $\pm$ 10% were also considered aneuploid.

Statistics. The statistics graphic data measurement software program† was used to calculate various statistical tests. End points measured as interval to progression were measured by the Kaplan-Meier method for estimation. Differences between Kaplan-Meier curves were tested for statistical significance using the Wilcoxon-Gehan test. To account for more than 1 prognostic factor simultaneously, various pathological parameters were evaluated using a Cox proportional hazards model to determine which variables were independently correlated with progression.

RESULTS

Of the 112 cases 49 demonstrated positive surgical margins: 24 had progression and 25 did not. Of 63 specimens with negative surgical margins 21 had progression and 42 did not. Although there was a trend for increased progression with positive margins, this was not a statistically significant predictor of progression (p not significant) by the Wilcoxon-Gehan analysis of the Kaplan-Meier progression probability plot.

Ploidy was initially grouped into 3 categories: aneuploid (54 cases), tetraploid (10) and diploid (48). Tetraploid cases were first considered together with diploid cases for 1 analysis and then combined with aneuploid cases for another analysis. When tetraploid tumors were considered diploid, the difference in progression between diploid and nondiploid tumors was relatively weak (p = 0.02). In contrast, when tetraploid tumors were considered nondiploid, there was a more significant difference between diploid and nondiploid tumors (p = 0.009, fig. 1). Consequently, for the remaining analyses tetraploid tumors were combined with aneuploid tumors and considered nondiploid.

The mean Gleason sum for men with progression was 6.9 compared to 5.7 for those with no evidence of disease. The majority of patients in our study had either a Gleason sum of 5 (30), 6 (30) or 7 (41), with only 9 of 112 having a Gleason sum of 8 or 9 and 1 case each with Gleason sums 2 and 4. For statistical purposes, cases were divided into those with a Gleason sum of 6 or less and 7 or more. Gleason grade was highly significant in predicting progression (p <0.0001, fig. 2).

The relationship between ploidy and Gleason grade is illustrated in table 1. Using a multivariate Cox proportional hazards model comparing Gleason grade, DNA ploidy, surgical margins and capsular penetration for the entire group, ploidy did not offer any additional prognostic information beyond grade. However, when examining only the low grade tumors (62, Gleason sum 6 or less), ploidy provided additional prognostic information to the Gleason grade (p = 0.03, fig. 3).

Different methods of tissue preparation (disaggregated versus tissue sections) were compared in 62 of the 112 cases (table 2). There were 25 cases with discordance. Tissue sections identified 15 of the 25 discordant cases correctly (that is diploid with no progression and nondiploid with progression) while disaggregation identified only 10 of the 25 discordant cases correctly. In these 62 patients ploidy was statistically significant in predicting progression using tissue sections compared to the disaggregation method, which was not significant (fig. 4).

DISCUSSION

Before discussing the relationship of DNA ploidy analysis to disease progression, it is necessary to discuss the controversy regarding the classification of tetraploid tumors which make up a large proportion of prostate cancer. This controversy is highlighted by 2 major studies from the same institution. Of 283 pathological stage B prostatic adenocarcinomas reported by Montgomery et al 68% were diploid, 28% tetraploid and 4% aneuploid.[12] Although only a small fraction of the cases were aneuploid, all 10 men had progression.

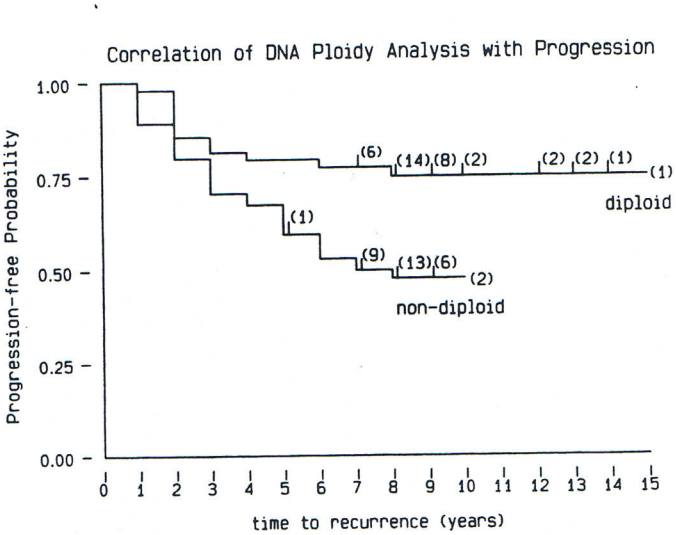

FIG. 1. Kaplan-Meier actuarial curves demonstrate likelihood of having undetectable postoperative serum PSA stratified by ploidy. Numbers in parentheses represent censored data.

* Cell Analysis System, Elmhurst, Illinois.
† STATA computing resource, Los Angeles, California.

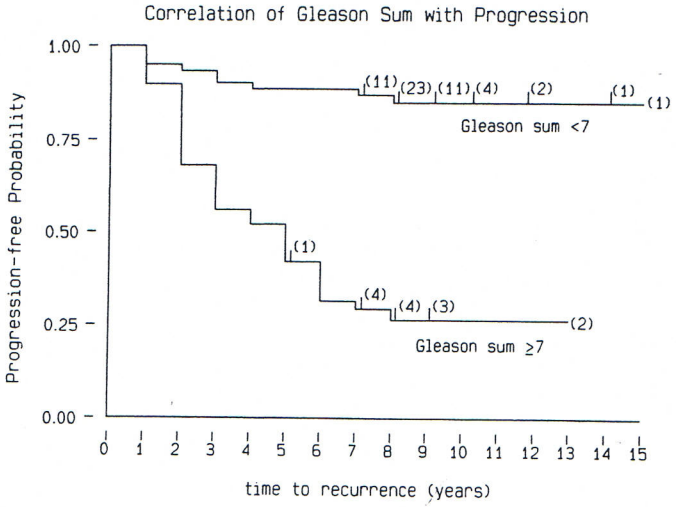

FIG. 2. Kaplan-Meier actuarial curves show likelihood of having undetectable postoperative serum PSA stratified by Gleason sum. Numbers in parentheses represent censored data.

TABLE 1. *Relationship between DNA ploidy and Gleason sum*

Gleason Sum	DNA Ploidy	
	Diploid	Nondiploid
6 or less	32	30
More than 6	16	34

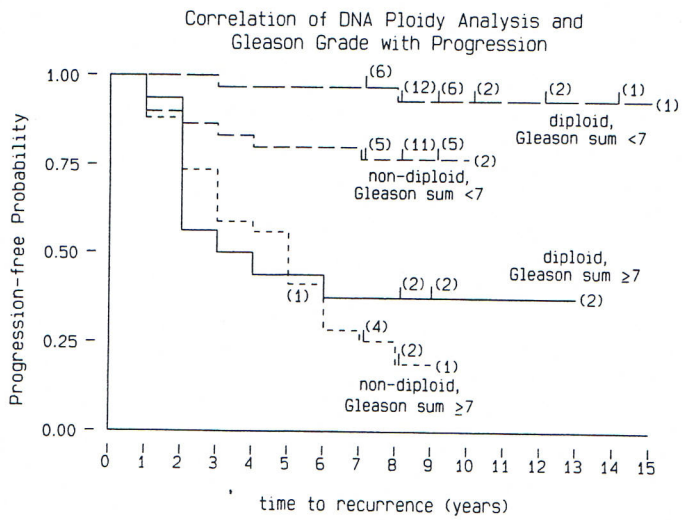

FIG. 3. Kaplan-Meier actuarial curves demonstrate likelihood of having detectable postoperative serum PSA stratified by DNA ploidy and Gleason sum. Numbers in parentheses represent censored data.

TABLE 2. *Comparison of different methods of tissue preparation*

Method of Tissue Preparation	DNA Ploidy	
	Diploid	Nondiploid
Tissue section	26	36
Disaggregated	43	19

In their series patients with tetraploid tumors had a favorable outcome, comparable to those with diploid tumors. The invariable progression seen in patients with aneuploid tumors was not confirmed in our study. Another report analyzing DNA ploidy following radical prostatectomy for clinically early stage disease was from Nativ et al.[8] However, in their study tetraploid tumors behaved closer to aneuploid tumors. Again, only 7% of tumors were classified as aneuploid, which was associated with a 64% progression rate.

The 2 previous studies bring up the controversy as to the classification of tetraploid tumors. In 1 study these tumors were considered comparable to diploid tumors and in the other comparable to aneuploid tumors. The European Organization for the Research and Treatment of Cancer (Genitourinary Group) recently studied 98 patients with lymph node metastases who either underwent radical prostatectomy immediately or had delayed endocrine therapy.[16] They concluded that tetraploid tumors should be combined with diploid lesions based on the similar rates of progression. In our study men with aneuploid tumors did not have progression at a significantly higher rate than those with tetraploid cancer. Also, when tetraploid tumors were considered nondiploid, better prognostication was achieved as opposed to combining tetraploid tumors with diploid tumors.

Most studies that have investigated DNA ploidy in radical prostatectomy specimens have demonstrated a correlation of ploidy with pathological stage.[4–6,8–11,13] However, some studies have not found ploidy to be proportional to stage.[17,18] Furthermore, there are conflicting results assessing whether ploidy adds information in predicting stage beyond the Gleason grade. In some studies ploidy was an independent predictor of stage,[8] while in others ploidy was not independently predictive of progression once grade was considered.[11]

Multiple studies have assessed ploidy on radical prostatectomy specimens to predict progression.[5,6,8,13,14,17–19] Early studies defined progression as biopsy proved local or distant metastatic disease, a positive bone scan or death from prostate cancer recurrence.[4,12,14] A few reports have found ploidy proportional to progression. However, these studies either did not examine the effect of grade[5] or they found that ploidy did not provide additional predictive information beyond grade.[10,17] Only a few studies have correlated ploidy with progression using serum PSA as the first sign of progression.[6,11,13] Ring et al found no correlation of ploidy with progression in 54 men yet followup was limited with a mean of 16 months (range 6 to 40).[18] Wirth et al correlated ploidy with progression, yet other important factors, such as grade and margin status, that might impact on progression were not evaluated.[6]

Several studies from the Mayo Clinic have found DNA ploidy to be predictive of progression in men independent of grade. Winkler et al demonstrated that ploidy was predictive of progression in men with stage D1 disease and was independent of grade.[14] Nativ et al found that in pathological stage C disease, ploidy was helpful to predict progression in men with low grade disease (Mayo Clinic grade 1 + 2) yet was not an independent predictor of progression in men with high grade disease (Mayo Clinic grade 3 + 4).[8] Progression in this case was defined as local or distant metastases. In men with pathological stage B carcinoma Montgomery et al found ploidy to be the best dependent predictor of progression.[12] Gleason grade, although less predictive, enhanced prognostication when combined with ploidy. In their study, grade may have not been as powerful a predictor as we found in our cases because of the method of grouping grades. Montgomery et al divided their patients according to the Gleason sums, that is sums of 5 or less versus 6 or more. However, we found that cases with a Gleason sum of 5 or 6 behaved similarly, and were significantly and statistically different from those with Gleason sums of 7 to 9. If one were to split Gleason sum into only 2 grades, those with a Gleason sum of 7 or more do significantly worse than those with a Gleason sum of 6 or less. This grouping is supported by the recognition that a Gleason component of 4 often characterizes a more aggressive tumor.[20,21] In the latter studies by Nativ[8] and Montgomery[12] et al an accurate assessment of pathological stage could have been hampered by assessing capsular penetration largely on frozen section. In these

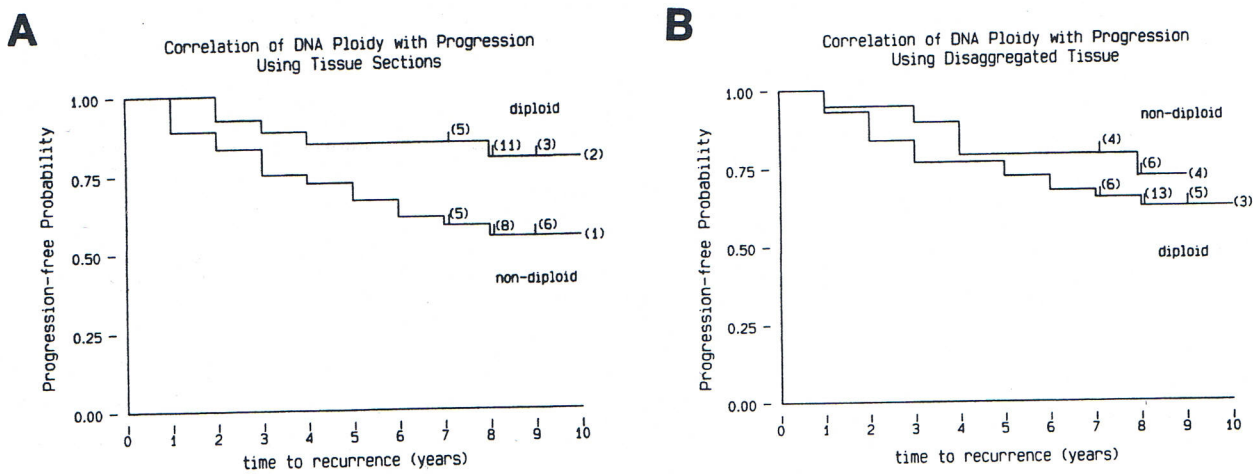

FIG. 4. Kaplan-Meier actuarial curves demonstrating likelihood of having detectable (*A*, ploidy analyzed using tissue sections) and undetectable (*B*, ploidy performed using disaggregated tissue sections) postoperative serum PSA stratified by ploidy in subset of 62 patients. Numbers in parentheses represent censored data.

studies margins were also not assessed as to the significance.

In our study DNA ploidy was helpful in predicting progression for patients with low grade tumors when tetraploid lesions were grouped with aneuploid tumors. These low grade neoplasms occupied a significant proportion of our total number of cases (50%). In patients with low grade diploid tumors there was virtually no progression. In contrast, low grade nondiploid disease progressed at a higher rate. Men with high grade tumors frequently had progression regardless of ploidy status. The behavior of these tumors was dictated by the poor differentiation, which overshadowed any effect ploidy may have had. Had we not analyzed the low grade tumors separately, similar to Nativ et al,[8] we would not have found ploidy to be predictive of progression for the group as a whole. Other studies that have not found ploidy to contribute additional information beyond the grade may also have had different results had they analyzed low and high grade tumors separately.

Although this was not the primary focus of our article, we analyzed in patients with low grade disease whether tumor volume was also predictive. The median tumor volume for those without progression was 2.1 cc, compared to 5.6 cc for those with progression. Tumor volume was predictive of progression using a Cox model with a p value of 0.002. In a multivariate analysis of low grade tumors, once tumor volume was known ploidy did not add any additional predictive information. This finding relates to the fact that ploidy and tumor volume were correlated with an R value of 0.24. In patients with low grade tumor one can enhance predictability using either tumor volume or ploidy, with tumor volume giving somewhat better discrimination. The 10-year progression-free probabilities for tumors with volumes of 4 cc or less and greater than 4 cc were 92% and 64%, respectively. Although tumor volume was predictive of progression in this smaller subset of men with low grade disease, in a previous study of 185 patients with clinical stage T2 adenocarcinoma of the prostate with 5 years of followup we found that, once the Gleason score and surgical margins were known, tumor volume did not provide additional prognostic information.[22]

To date there have been no studies on prostate cancer to compare directly different methods of tissue preparation (disaggregation versus tissue sections) for DNA ploidy image analysis. Our findings indicate that tissue sections were able to identify aneuploid tumors that were classified as diploid using the disaggregated method. The fact that the tissue section method was significantly predictive of progression ($p = 0.03$) over the disaggregated method (p = not significant) suggests that using tissue sections to analyze DNA ploidy may be more accurate. One reason for this finding could be due to the harsh treatment of cells resulting from disaggregation. Perhaps nondiploid cells are more fragile and not as able to withstand the disaggregation procedure. Another potential explanation could be that, despite attempting to trim benign prostate tissue away from the paraffin blocks, some benign prostate cells were inevitably analyzed using the disaggregation technique. In contrast, using histological sections one can accurately distinguish tumor nuclei from benign cells.

In conclusion, we found that Gleason grade was the most powerful predictor of progression following radical prostatectomy. In contrast to a prior study of 507 stages T1 and T2 cancer patients who underwent radical retropubic prostatectomy,[13] surgical margin status was not statistically significant as a predictor of progression in our study. This fact may be due to the substantially smaller number of patients in our study compared to the previous report. DNA ploidy helps to stratify the risk of progression after radical prostatectomy in men with low grade cancer (Gleason sum less than 7). In men with high grade tumor, ploidy does not contribute any additional prognostic information. Tissue sections are more sensitive in detecting nondiploid cells than disaggregated tissue. Furthermore, ploidy performed on tissue sections correlated better with progression following radical prostatectomy than ploidy performed on disaggregated tissue.

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