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THE PROGNOSTIC VALUE OF DEOXYRIBONUCLEIC ACID FLOW CYTOMETRIC ANALYSIS IN STAGE D2 PROSTATIC CARCINOMA

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ABSTRACT

This study was designed to compare the prognostic potential of tumor grade and ploidy status in patients with stage D2 prostate cancer. Two outcome groups were selected on the basis of survival after orchiectomy: a bad outcome group consisting of 66 patients who died of the disease within 12 months and a good outcome group comprising 37 patients who survived beyond 5 years. Tumors were classified histologically as well (17%), moderately (17%) or poorly (66%) differentiated. Tumor grade was a significant predictor of outcome, with 76% of poorly differentiated tumors in the bad outcome group and 65% of well differentiated tumors in the good outcome group ($p < 0.005$).

Deoxyribonucleic acid (DNA) ploidy analysis was performed on formalin fixed, paraffin embedded samples of the primary tumor to yield 97 final tracings that were classified using set criteria for DNA ploidy status. Over-all, 54% of the tumors were nondiploid (33% aneuploid and 21% tetraploid) and the remaining 46% were diploid. DNA ploidy status was a significant indicator of outcome ($p < 0.001$), with 64% of diploid tumors in the good outcome group and 88% of the nondiploid tumors in the poor outcome group. Tetraploid tumors behaved no differently from other nondiploid tumors. We conclude that DNA ploidy status and tumor grading are significant independent predictors of outcome after orchiectomy and when combined yield important additional prognostic information.

KEY WORDS: prostate, carcinoma, DNA, flow cytometry

Prostatic adenocarcinoma is the most common malignant tumor in men resulting in more than 30,000 male deaths per year in the United States.[1] Since first described in 1941, androgen ablative therapy has been the treatment of choice for disseminated or locally invasive carcinoma of the prostate.[2] Numerous investigators have commented on the unpredictability of extent and duration of response to this form of therapy.[3–5] Stage D2 disease is common, with more than 40% of the patients assigned to this advanced stage at diagnosis. Although 80% of the patients with stage D2 disease have an initial response to androgen withdrawal, it is estimated that 10% die within 6 months, 50% within 3 years and 80% within 5 years. Only 10% of the patients survive 10 years after treatment.[6] It has been shown that once a carcinoma becomes unresponsive to androgen ablative therapy no subsequent treatment has any meaningful impact on disease progression and over-all survival.[7,8] Consequently, the ability to identify at diagnosis those patients destined to respond poorly to conventional therapy would enable alternative or adjuvant therapy to be instituted. Prognostic features adequately fulfilling this role have not yet been determined.

We examined whether flow cytometry of nuclear deoxyribonucleic acid (DNA) content may fulfill this role. We used suspensions of cell nuclei prepared by limited pepsin digestion of archival tumor tissues from patients with known clinical outcome. Although the over-all DNA histogram quality from paraffin embedded tissue is not as good as that obtained from fresh tissue, the tracings are usually sufficient for evaluation of DNA ploidy status.[9–11] There have been a number of reports in which flow cytometry has been used to demonstrate correlations between DNA ploidy and prognosis or clinical stage but data on survival probabilities for patients with advanced prostatic cancer are sparse.[10,12–14] Klein et al in 1988 showed flow cytometry to be an accurate, objective means to quantify nuclear DNA and cellular growth fraction, and to identify benign from malignant prostatic tissues.[15] The studies of McIntire,[16] Tribukait,[17] Fordham[18] and Frankfurt[19] et al indicate that aneuploidy is an important parameter to assess disease progression in patients with prostate cancer. Seppelt and Sprenger,[20] and Lundberg et al[10] have also correlated DNA ploidy status with tumor progression in biopsies taken after treatment. However, Ritchie et al in 1988 cast doubt on these earlier findings.[21] After correcting for histological grade they did not find any significant prognostic value in tumor DNA ploidy status. Our study was done to clarify the prognostic role of DNA ploidy status in patients with stage D2 carcinoma of the prostate. To this end we have designed a retrospective study relating DNA ploidy status to histological grading and patient survival after orchiectomy.

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MATERIALS AND METHODS

Patient selection. Patient tissues entered into our study fulfilled a number of selection criteria. All patients presented with clinical stage D2 disease and underwent prostatectomy followed by bilateral orchiectomy. Critical to the study was the presence of adequate neoplastic tissue on the histological blocks confirmed by pathologist examination and the selection into 2 outcome groups based on survival after orchiectomy. Patients who survived longer than 5 years were considered to be in a good outcome group, while those who died of prostate cancer within 12 months comprised the bad outcome group. Between 1978 and 1988, 103 patients from 3 institutions fulfilled these selection criteria: 36 were in the good and 67 the bad outcome groups. Patient age ranged from 56 to 94 years (median age 74 years).

Pathological tissue. All tumors were obtained by transurethral resection with the exception of 2 open prostatectomy specimens. Sections 5 μm. thick were cut from each available paraffin block, and stained with hematoxylin and eosin. The carcinomas were classified by 2 pathologists (D. M. R. and A. S.-Y. L.) into well, moderately or poorly differentiated tumor grades according to the system of Böcking et al.[22] For each patient representative blocks with the greatest percentage of tumor-bearing tissue were selected for analysis.

Preparation of nuclei for flow cytometry. Nuclei were isolated from paraffin embedded tissues using the technique of Hedley[11] with some minor modifications. Four to 6 sections 30 μm. thick were cut from each paraffin block and placed into a glass test tube. The sections were then dewaxed by 3, 10-minute immer-

sions in xylene and progressively rehydrated to distilled water using graduated ethanol concentrations of 100, 95, 70 and 50% for 10 minutes each. The sections were then incubated in 2 ml. pepsin saline solution (0.5 mg./ml.) at pH 1.5. Tissue digestion was performed in a water bath at 37C for 60 minutes with intermittent vortexing and agitation. At the completion of this procedure, 1 drop of solution was removed and stained with 1 drop of trypan blue (1.5 mg./ml.), and a microscopic nuclear count was done with a hemocytometer grid. If the nuclear count was less than 1×10^5/ml. digestion was continued until this level was obtained. Each digest was then sieved through a 50 μm. sieve and centrifuged at 1,700 revolutions per minute for 10 minutes. The pellet was resuspended in 400 μl. calcium magnesium free Hank's solution to prevent clumping of the nuclei.

DNA flow cytophotometry. Nuclear suspensions were stained with 4'6-diamidino 2-phenylindole dihydrochloride (DAPI) at a concentration of 5 μg./ml. in phosphate buffer and incubated for 20 minutes at room temperature before assaying in a FACS analyzer.* Formalin fixed chicken red blood cells were used as an internal standard for each assay (5 μl. at a concentration of 1×10^6/ml.) and added before DAPI staining to each sample. The DNA flow data incorporating 25,000 events were transferred to microcomputer and analyzed as a 256 channel intensity histogram, where staining intensity was proportional to the DNA content.

Optical conditions used ultraviolet excitation from a 100 watt ercury arc lamp, teamed with the standard ultraviolet filters pplied by the manufacturer for the analyzer. Excitation filters re a 360 nm. band pass filter and a 375 nm. short pass blocking filter. The short pass filter was used as a dichroic mirror to direct the fluorescence energy to the emission filters, which were a single 490 nm. band pass and 2, 400 nm. long pass blocking filters.

Preparation of formalin fixed chicken red blood cell standard. Chicken red blood cells were obtained by washing 50 ml. whole blood in 0.9% saline centrifuged at 1,700 revolutions per minute for 10 minutes, followed by repeat centrifugation through a Ficoll cushion to separate the leukocytes. The chicken red blood cell pellets were then resuspended in 2 ml. saline and washed twice before resuspension in 10 ml. 10% buffered formaldehyde solution for 30 minutes. The chicken red blood cells were subsequently washed twice in saline and resuspended to a concentration of 1×10^7 cells per ml.

Assignment of ploidy status. Ploidy status was determined from our tracings using set parameters. A diploid tumor was defined as possessing a single G0/G1 (resting cell) peak and a G0/G1-to-chicken red blood cell peak ratio of 3.25 ± 1.25 (95% confidence limits). DNA index was also calculated for each tumor by assignment of the diploid value (2N) to each G0/G1 peak. Thus, the G2 (dividing cell) peak of a diploid tumor was delineated by a DNA index of 4N ± 0.15N.

Aneuploid tumors were assigned to DNA histogram tracings th more than 1 G0/G1 or G2 peaks; bifid G0/G1 or G2 peaks or histograms with skewed peaks indicating the presence of more than a single population of cells. When the DNA index calculated for the G2 peak was outside normal range (4N ± 0.15N) and comprised 20% or more of the total number of nuclei counted, aneuploid status was also assigned. Tetraploid status was specifically assigned when a peak was located at the normal diploid cell G2 position but contained 20% or more of the total cells in the G0/G1 and G2 peaks. Tumors assigned this status occasionally demonstrated a further (G2) peak close to 8N. Aneuploid and tetraploid stem cell lines were collectively defined as the nondiploid tumor cell lines. Representative tracings of our common tumor histograms are demonstrated in figure 1. The statistical analysis of our findings was performed using chi-square tables.

RESULTS

Of the 103 patients fulfilling our selection criteria 97 yielded DNA histogram tracings suitable for ploidy analysis (35 good and 62 bad outcomes). The remaining 6 tissues did not yield suitable tracings despite repeated attempts at digestion with pepsin. When re-examined these blocks were devoid of extensive carcinoma deposits and, consequently, these patients were omitted from this study.

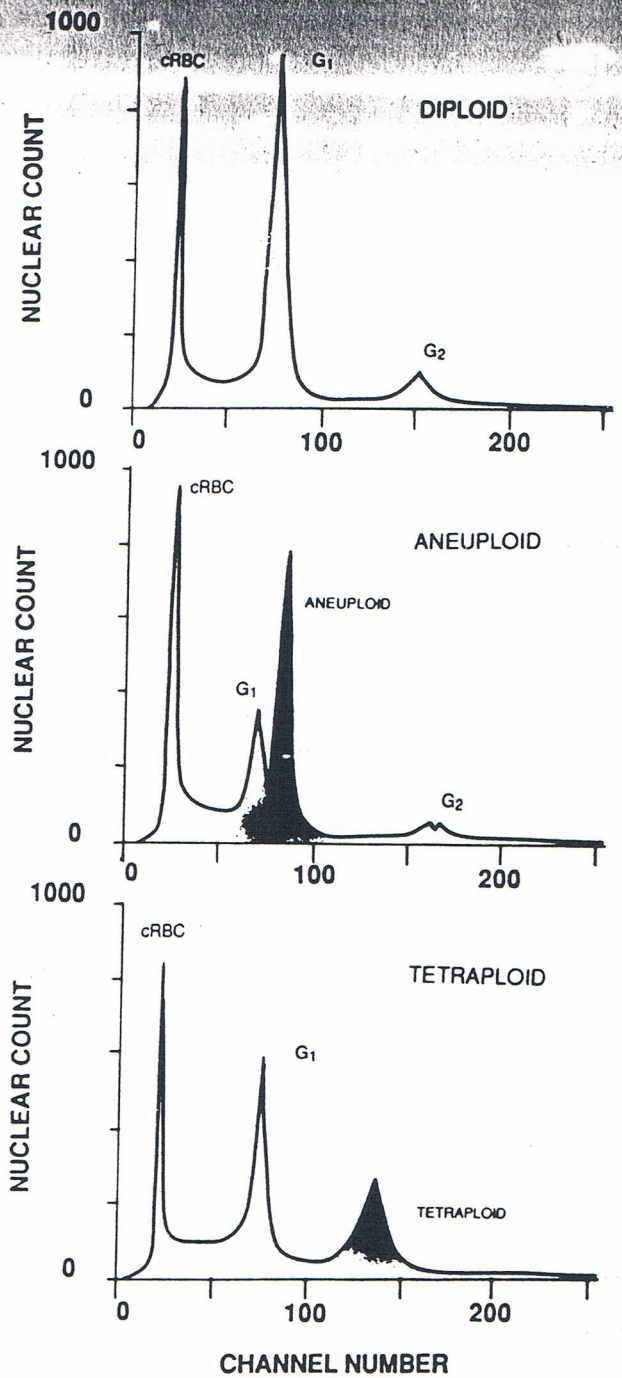

FIG. 1. Representation of DNA ploidy histograms. *cRBC*, chicken red blood cell peak. G_1, resting cell peak. G_2, dividing cell peak.

Tumor grade and prognosis. Over-all, 17 tumors were graded into each of the well and moderately differentiated groups, with 63 tumors in the poorly differentiated group. Within the good outcome group 11, 9 and 15 tumors were graded as well, moderate and poorly differentiated, respectively. Within the bad outcome group the tumors were graded as 6 well, 8 moderately and 48 poorly differentiated. Therefore, tumor grade was a significant indicator of prognosis ($p < 0.005$), with 76% of poorly differentiated tumors in the poor outcome group and, conversely, 65% of well differentiated tumors in the good outcome group of patients. Moderately differentiated tumors were di-

* Becton Dickinson, Mt. View, California.

vided evenly between the good and bad outcome groups (53 and 47%, respectively) and were of no predictive value (fig. 2).

Ploidy status and prognosis. Over-all, 52 of 97 tumors (54%) were nondiploid (33% aneuploid and 21% tetraploid). Within the good outcome group only 6 of 35 tumors (17%) were nondiploid (5 aneuploid and 1 tetraploid). Conversely, in the bad outcome group 46 of 62 tumors (74%) were nondiploid (27 aneuploid and 19 tetraploid). The balance in all cases were diploid tumors. Figure 3 demonstrates that DNA ploidy status was a highly significant indicator of outcome ($p < 0.001$). Of the 45 diploid tumors 29 (64%) were in the good outcome group, whereas 19 of 20 tetraploid (95%) and 27 of 32 aneuploid (83%) tracings, together representing 46 of 52 (88%) nondiploid tumors, were in the poor outcome group.

Prognosis by combination of tumor grading and ploidy status. The predictive ability of patient outcome was significantly improved by combination of tumor grading and ploidy status ($p < 0.001$). No significant association was detected linking these 2 variables. Figure 4 displays, in histogram form, subsets formed by the combination of these tumor characteristics and the chance of being in the good outcome group after orchiectomy. Patients with a well differentiated diploid tumor had a 91% (10 of 11, $p < 0.005$) chance of being in the good outcome group while a patient with a poorly differentiated nondiploid tumor had a 5% (2 of 37, $p < 0.001$) chance of being in this group. Those with a well differentiated but nondiploid tumor had only a 17% (1 of 6, $p < 0.005$) chance of being in the good outcome group, compared to 50% (13 of 26) of those with a poorly differentiated but diploid tumor. Patients with a moderately differentiated and diploid tumor had a 75% (6 of 8) chance of being in the good outcome group, compared to 33% (3 of 9) of those with a moderately differentiated but nondiploid tumor.

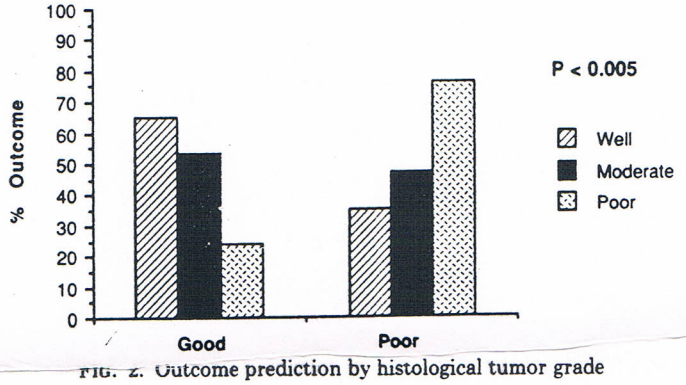

FIG. 2. Outcome prediction by histological tumor grade

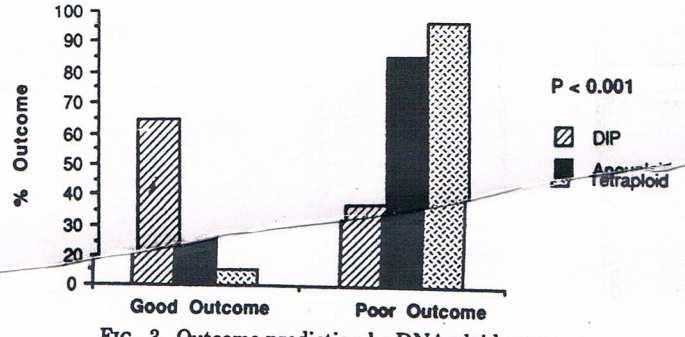

FIG. 3. Outcome prediction by DNA ploidy status

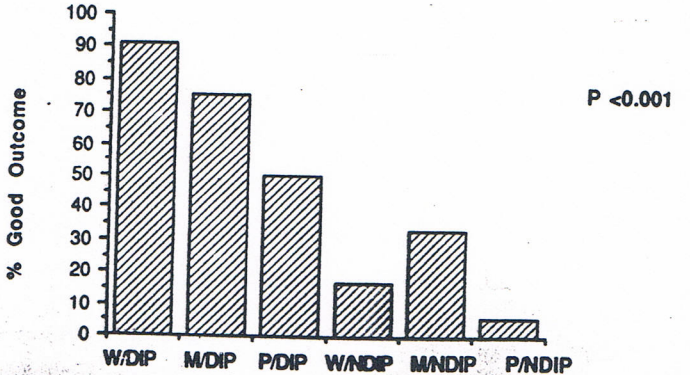

FIG. 4. Outcome prediction by DNA ploidy and histological grading. *W*, well differentiated. *M*, moderately differentiated. *P*, poorly differentiated. *DIP*, DNA diploid tumor. *NDIP*, DNA nondiploid tumor.

DISCUSSION

Numerous questions remain unanswered concerning prostate cancer. Why are some prostate cancers indolent while others demonstrate overtly aggressive behavior despite attempts at therapeutic control? Can features be identified at initial diagnosis that accurately predict the subsequent outcome of a specific treatment in a given patient? More importantly, can we identify a group of patients who are destined to respond poorly to conventional hormonal manipulation and, therefore, allow examination of alternative treatment regimens?

A major value of prognostic indicators is their potential to distinguish responders from nonresponders, which is particularly relevant for prostate cancer, since all forms of antiandrogen therapy have significant side effects. Isolation of this patient group would also allow for early introduction of adjuvant therapy not presently used until conventional modalities have failed. Such an approach may allow for greater success of these alternative therapies.

In localized prostate cancer nuclear morphometric features (shape, roundness and so forth) and nuclear ploidy status have proved to be significant predictors of long-term disease-free survival after radical prostatectomy.[12,16,18,19,21,23-27] However, the role of these features in prediction of disease outcome for patients with metastatic prostate cancer is unclear. Some researchers found that ploidy status correlates well with disease progression independent of histological grade,[10,24] while others state that ploidy estimation has no role in predicting outcome once disease stage[17,18] and grade[21,28] are considered. Our study was designed to maximize any prognostic information gained from ploidy analysis in 97 patients with known clinical outcome by selecting patients at the 2 extremes of outcome, representing the upper 15% of survivors treated by orchiectomy for stage D2 disease.

DNA histograms were produced according to the currently accepted methods described by Hedley.[11] As with other researchers, we found it to be a time-consuming, inefficient technique for DNA ploidy analysis, requiring up to 4 specimen preparations to obtain suitable tracings. Problems of interpretation also existed due to inclusion of benign tissue in the specimen and, as expected, evidence of a diploid cell line was found in histograms taken from specimens with a low tumor percentage due to benign cells present in the tissue block. This problem has been addressed by others[10-12,19] as has the question of appropriate nuclear staining. In our series DAPI[10,19] provided the best results at an optimal concentration of 5 μl./ml. (we examined 1 to 20 μl./ml. concentrations of stain) and yielded better clarity of tracing than either ethidium bromide[17,26] or propidium iodide stains.[9,12,13-15,18]

Some doubts have been cast on the malignant potential of polyploid cell lines (tetraploid and so forth). However, we could not demonstrate any significant survival advantage to patients with a tetraploid tumor over those with an aneuploid tumor. Thus, the union of these patients with the aneuploid tumor group to form a nondiploid group is justified, giving an over-all distribution of 54% nondiploid and 46% diploid tumors. Other studies vary in their content of nondiploid tumors from 40 to 72% and the reasons for this variation could be 2-fold: 1) variation in patient populations in terms of grade, stage and treatment, and 2) due to the lack of uniformity in the assessment of DNA histograms.[9-15,18,19] The subjectivity of ploidy

assessment has been discussed recently in an article by Joensuu and Kallioniemi, who called for establishment of an optimal classification method especially when dealing with DNA histograms derived from paraffin embedded tissues.[29] This is important if prognostic information gained from DNA histograms is to be substantiated in further studies.

The majority of our tracings (70 of 97) could be designated clearly as diploid or nondiploid on initial blind viewing of the histograms. However, problems occurred in designating some tetraploid cell lines along with asymmetrical G0/G1 peaks and small abnormal peaks. Often, these tumors needed to be reexamined after further tissue preparation to clarify their ploidy status (up to 4 times). We chose a G2 peak volume of more than 20% of the total cells analyzed to indicate the presence of a separate tetraploid stem cell line. Other researchers have used levels as low as 5% but, since no clear guidelines exist in the literature, we believe a level of 20% was justified in view of the debris present after enzymatic digestion of archival tissue. The presence of an appropriate internal standard may help with the interpretation of asymmetrical G0/G1 and small abnormal peaks. However, since the majority of work on prognostic ability of DNA ploidy in human cancers has been on paraffin embedded material such an internal standard is lacking in many cases. For this reason we chose formalin treated chicken red blood cells as our standard marker to run with each specimen. Although far from perfect, the chicken red blood cells, containing far less DNA than human cells, did allow for clearer identification of aneuploid and polyploid cell lines, while providing a basis for standardization of results reported from histograms derived using paraffin embedded tissue.

Our study has demonstrated, similar to others, that histological grading has significant prognostic information to offer the patient at diagnosis ($p < 0.005$). This is particularly true for well differentiated and poorly differentiated tumors. However, prediction of survival with moderately differentiated tumors remains poor. Our study confirmed the earlier experiences of Lundberg et al, who found no significant correlation between DNA ploidy and histological grade, indicating that DNA ploidy status and histological grade are independent prognostic variables.[10] Nuclear ploidy status has recently been shown to have prognostic importance in patients with lymph node involvement (stage D1). Our study currently extends this, demonstrating a highly significant link between DNA ploidy status and disease outcome in patients with bony secondaries (stage D2, $p < 0.001$), nondiploid tumors indicating a poor prognosis (88% in the bad outcome group) and diploid tumors offering a much improved outlook (64% in the good outcome group) irrespective of histological grade.

In our study, the combination of histological grading with ploidy status enhanced disease outcome prediction for all patients, including the moderately differentiated group. Furthermore, although we are unable to conclude that well differentiated, diploid tumors respond to hormonal manipulation, it is possible to state that patients with poorly differentiated, nondiploid tumors almost universally fail to respond to antiandrogen therapy. In this regard ploidy status appears to select a patient group that may benefit from early adoption of adjuvant therapy. Although based on a retrospective study of selected patient outcome groups, we would advocate that DNA ploidy estimation be instituted for all patients with prostate cancer, to examine whether an over-all improvement in patient outcome prediction is possible through standardization of multicenter treatment trials based on this prognostic indicator.

Mr. J. Webster provided technical assistance.

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EDITORIAL COMMENTS

This is an important contribution to understanding the prognostic association of nuclear DNA ploidy for patients with prostate carcinoma. The results of this investigation demonstrate that even for patients with clinically detectable osseous metastases those who happen to have a DNA diploid primary tumor have a remarkably good long-term prognosis when treated by castration. This is particularly clear from the data presented in figure 4. This figure shows that the probability of the patient being alive at 5 years ranges from 90% for patients with well differentiated diploid tumors to 50% with poorly differentiated diploid tumors. The contrasting results for patients with nondiploid tumors illustrated at the right side of figure 4 are easily evident.

Such a result for patients with stage D2 disease treated by castration turns out to be highly congruent for the end results found for patients with stage D1 disease treated at my own institution. Patients with DNA diploid stage D1 prostate carcinoma treated by radical prostatectomy, pelvic lymphadenectomy and early medical or surgical castration also have turned out to have an unexpectedly excellent prognosis with even long-term followup (reference 13 in article).[1,2] Conversely, patients with stage D1 disease with DNA tetraploid or aneuploid tumors have had a much poorer prognosis despite early endocrine therapy. Moreover, the authors found little difference in prognosis between patients with tetraploid or aneuploid tumors, which is similar to previous results at the Mayo Clinic for patients with stage D1 disease (reference 13 in article).

It is important to note that the authors characterized the DNA content of the tumors by using transurethral resection chips from the primary tumors. The fact that the metastatic disease progression and lethality are associated so strongly with the ploidy of the primary tumor suggests that in most instances the metastases bear a close behavioral similarity to the ploidy characteristics of the primary tumor and that heterogeneity in this regard is not common.

Finally, the authors demonstrate that for these patients with stage D2 prostate cancer treated by castration DNA ploidy pattern and histological grade are each independent and important factors associated with prognosis. Our Mayo Clinic research group has found a similar synergistic prognostic association effect of ploidy and histological grade for patients with pathological stage C prostate carcinoma.[3] We could not demonstrate it for patients with stage D1 disease in which ploidy pattern and the associated response to early endocrine therapy dominate end results. Thus, the current report supports a working hypothesis that for prostate carcinoma (in general) the prognosis is highly associated with the 3 variables of stage, histological grade and DNA ploidy. Description of results for the treatment of patients with prostate cancer by an operation, radiotherapy or hormonal ablation currently must take into account the DNA ploidy characteristics of the tumors studied. As clearly indicated by this study, DNA ploidy information (which currently is widely available from clinical pathology laboratories) can make an important and unique contribution to estimating the probability of successful therapy in patients with prostate cancer.

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The authors state in their introduction that a purpose of the study was to clarify the prognostic role of DNA ploidy status in patients with stage D2 prostatic cancer. To achieve this goal they selected a group of patients who survived for greater than 5 years from diagnosis and the initiation of therapy and a group who died within 12 months after initiation of therapy. Of the 103 patients 36 lived more than 5 years after the diagnosis was established and therapy was initiated, and 67 died less than 12 months after initiation of therapy. The authors then, in a retrospective manner, set out to determine the ploidy status of the long-term and short-term survivors.

To determine truly the effect of ploidy on outcome a more reasonable study design would have been to select 50 patients with diploid tumors and 50 with aneuploid tumors, each group having had metastatic disease at diagnosis and each patient in each group having received androgen deprivation by similar means at diagnosis. Then, a survival analysis based on ploidy would truly test the hypothesis that was posed, that is whether DNA ploidy had a prognostic role in patients with stage D2 carcinoma of the prostate. Thus, from my perspective the study design was flawed from initiation.

In their analysis the authors demonstrated that only 17% of the 36 long-term survivors had nondiploid tumors, compared to 74% of the short-term survivors. Thus, the authors demonstrated that patients who tend to be long-term survivors have a higher probability of diploid disease, whereas short-term survivors have a higher probability of nondiploid tumors. They fail to show that diploid disease patients who present with metastatic disease at diagnosis have a survival experience significantly different from patients who have aneuploid tumors at presentation. In concordance with my interpretation of these data, the authors fail to select a patient group that may benefit from early adoption of adjuvant therapy, a position that they establish in their discussion. It is unfortunate that this flaw in study design failed to provide the authors with the opportunity to test the hypothesis that they proposed.

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REPLY BY AUTHORS

This study was purposefully designed to maximize any prognostic value that could be attributed to the ploidy status of stage D2 prostate cancer patients, since before the study its value for stage D2 was unknown. Subsequent determination of the ploidy status of 2 patient groups extracted from the wider D2 population using defined survival criteria demonstrated a strong association between survival and ploidy, which was reinforced by an independent association between survival and tumor grade. We believe that the test for this association now is not to use just 50 diploid and 50 nondiploid patients as suggested by Doctor Paulson but to perform a survival analysis on our total D2 patient population, including the 60 to 70% of the patients who died between 1 and 5 years, which in effect should total in excess of 300 patients. This study is now in progress. However, we are aware that the associations we observed previously may be much weaker because of the inclusion of those patients who died 1 to 5 years after orchiectomy. Furthermore, it is unlikely that ploidy, alone or in combination with grade, will suffice to predict prognosis in the individual stage D2 patient with absolute certainty because of the dependence on other, as yet unknown, factors.