

# Flow Cytometry of Prostate Cancer: Relationship of DNA Content to Survival<sup>1</sup>

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## ABSTRACT

Between March 1970 and December 1978 there were 366 patients with prostatic cancer treated by <sup>125</sup>I seed implants and pelvic lymph node dissection. All had a minimum of 5 years follow-up. One hundred thirty-three patients had metastatic prostatic cancer in lymph nodes (Stage D1) at the time of lymph node dissection and seed implantation. Ninety-one of the 133 patients were judged to have sufficient metastatic prostatic cancer in their nodal tissue (greater than 50% replacement with tumor) to justify flow cytometric cellular DNA measurements on the involved paraffin-embedded nodal tissue. Nine patients were excluded due to uninterpretable DNA histograms leaving 82 patients for analysis.

Forty-nine patients had aneuploid and 33 had diploid tumors. There was no statistical bias between the aneuploid and diploid groups due to age ( $P = 0.970$ ,  $\chi^2$  test), time between diagnosis and implantation ( $P = 0.217$ ,  $\chi^2$  test), number of positive nodes ( $P = 0.669$ , two-sample  $t$  test of means), or tumor grade ( $P = 0.332$ ,  $\chi^2$  test).

Median survival time of the aneuploid and diploid groups was 5.0 and 8.8 years, respectively ( $P = 0.0109$ , log rank test). Cox regression analysis confirmed the effect of aneuploidy *versus* diploidy on survival by controlling for other potentially confounding variables (age, time from diagnosis to implantation, number of positive nodes, and grade). Grade as a predictor of survival did not approach statistical significance in this series of relatively small size ( $P = 0.116$ ).

Thirty-eight of the 82 patients had moderately differentiated neoplasms. Nineteen of these were aneuploid and 19 diploid. The median survival was 5.8 and 9.1 years, respectively, for these grade-matched aneuploid and diploid groups ( $P = 0.039$ , log rank test).

We conclude that flow cytometric DNA measurements on archived paraffin-embedded tumor in nodal metastases appear to be a strong predictor of survival for Stage D1 prostatic cancer.

## INTRODUCTION

Accurate prediction of tumor progression and patient survival remains a major problem in the management of most human solid tumors. In the case of prostatic cancer several clinical parameters have proved useful, the most widely accepted being clinical stage and histological grade. Yet the course of disease may still be uncertain, due in part to the subjective nature of these parameters and to the variable natural history of this tumor. In a search for new descriptors, the DNA content of human solid tumors has been measured and found to vary over a wide range of values. Whether measured by single cell microdensitometry of Feulgen stained tissue sections (1) or by flow cytometry (2-6), there is mounting evidence of a more favorable prognosis for diploid compared with aneuploid tumors. Unfortunately, sufficient prostatic tissue is not easily obtained for prospective studies, and the prolonged natural history of prostatic cancer tends to discourage these investigations. Thus, reports correlating survival with DNA ploidy of prostatic cancer have been few, and based on small numbers of patients for

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whom other factors such as tumor grade and stage were not controlled (7-10).

A recent report by Hedley *et al.* has demonstrated the feasibility of measuring DNA content in nuclear suspensions prepared from archival paraffin-embedded tumor tissue (11). This technique has been applied to specimens from patients with metastatic ovarian cancer (12), and breast cancer (13), in whom the clinical outcome was already known, and DNA ploidy was reported to be a statistically significant prognostic variable.

The present study reports the prognostic value of DNA ploidy for a group of patients with Stage D1 prostatic cancer who were uniform with respect to major prognostic factors (*i.e.*, age, time from diagnosis to treatment, local stage, primary grade, and number of positive lymph nodes). All were treated by interstitial irradiation to the prostate, using <sup>125</sup>I seeds and simultaneous pelvic lymph node dissection. The measurements of DNA content were carried out by flow cytometry of tumor cell nuclei recovered from lymph node metastases in archival paraffin blocks.

## PATIENTS AND METHODS

**Patient and Tissue Selection.** Between March 1970 and December 1978, 366 patients with carcinoma of prostate were treated at Memorial Hospital by interstitial irradiation (<sup>125</sup>I) and pelvic lymph node dissection. Lymph node metastases were confirmed by pathological examination in 133 patients; no nodal metastases were found in the remaining 233. The most involved lymph node from each case was selected for study; those with less than complete replacement by tumor were trimmed to remove as much nonneoplastic tissue as possible. The histological sections were reexamined to select 82 cases with at least 50% involvement of the selected lymph node after trimming; 15 specimens had 50-75% involvement, 27 had 75-90%, and 40 had 90-100% of at least one lymph node replaced by tumor. In each case a noninvolved lymph node was selected to provide a control specimen of lymphocytes with normal diploid DNA. Forty-two patients with nodal metastases were excluded because of the small amount of tumor in nodes, and 9 patients because of poor quality DNA histograms that were judged uninterpretable presumably due to poor tissue preservation.

**Preparation of Paraffin-embedded Tissues.** Two or three 50- $\mu$ m sections were cut from paraffin blocks of the previously selected lymph nodes, with and without tumor from each case. Each set of sections was placed in a separate fine mesh bag, deparaffinized, and rehydrated according to conventional protocol. Briefly, the sections were immersed in three changes of xylene for 10 min each, followed by two 10-min changes each in 100, 95, 70, and 50% ethanol, and distilled water. The rehydrated tissues were then transferred into 1 ml of 0.5% pepsin (Sigma, St. Louis, MO) in 0.9% NaCl adjusted to pH 1.5 with HCl, and incubated for 1-2 h at 37°C with occasional vortexing. Whole nuclei were released from the tissues, washed twice with Hank's balanced salt solution, and filtered through a 53- $\mu$ m mesh filter. The resulting nuclear suspension was adjusted to 10<sup>5</sup> to 10<sup>7</sup> nuclei/ml and stored overnight at 4°C prior to measurement.

**DNA Staining and Flow Cytometry.** A 0.2-ml suspension of cells in Hank's balanced salt solution (approximately 10<sup>5</sup> cells) was resuspended in 2 ml of ice-cold 4,6-diamidino-2-phenylindole dihydrochloride (2  $\mu$ g/ml) in 0.1 M piperazine-N,N'-bis(2-ethanesulfonic acid) buffer (pH 6.0) with 2 mM MgCl<sub>2</sub> and 0.1% Triton X (Packard Instrument Co., Downers Grove, IL). The cells were kept in the staining solution on ice until measured on a standard ICP-22 flow cytometer

(Ortho Instruments, Westwood, MA) equipped with a 100-w mercury arc lamp with UGI UV excitation filter. At least 20,000 cells were examined in each specimen, and the DNA content per cell measured by 4,6-diamidino-2-phenylindole dihydrochloride fluorescence intensity (480–530 nm), recorded and displayed in a histogram.

**Analysis of DNA Histograms.** Each DNA histogram was designated as diploid, tetraploid, near diploid, or aneuploid. In other studies of benign prostatic tissue the normal DNA histograms demonstrated a mean value of 4.2% cells in G<sub>2</sub>M. Up to 7% tetraploid cells were considered within the normal range in this analysis (2 SD above the mean) (14). As noted, we used lymph node lymphocytes as control cells taken from uninvolved nodes of the cases studied. Because of an electronic offset in the flow cytometer, the mean DNA content for the G<sub>2</sub>M peak was 4.12c. Two standard deviations from the mean gave a range of 4.01c to 4.26c, which was defined as the range of tetraploidy. Tumor histograms displaying fewer than 7% tetraploid cells were designated diploid, while those with 7% or more were designated tetraploid (14). Since the suspension contained only extracted nuclei there were no doublets of cells, and it was sufficiently dilute to make coincidence of nuclei negligible. Near-diploid histograms were defined as those with a nondiploid peak of DNA content of 2.6c or less. Aneuploid histograms were those with a peak above 2.6c but outside the tetraploid range.

**Statistical Analysis.** Preliminary statistical analysis revealed no significant difference in survival or recurrence of tetraploid compared with aneuploid groups. Therefore they were combined and are subsequently referred to as aneuploid cases. Similarly, survival and recurrence data were no different for diploid and near-diploid cases, which also were combined and are subsequently referred to as diploid cases.

Patient group covariates (age, time from diagnosis to implant, primary grade, metastatic grade, local stage, number of positive nodes, and percentage of specimen involved with tumor) were tested to ensure comparability of the diploid and aneuploid groups using standard  $\chi^2$  and two-sample  $t$  tests of means, as appropriate. Survival curves were generated by the Kaplan-Meier product limit method, with nonparametric comparison of univariate survival groups achieved by the log rank test. Cox regression models were used to control for bias in multivariate survival analyses and comparisons.

**Grading of Primary and Metastatic Tissue.** Histological grading was based on nuclear grade and performed on both the primary prostatic biopsies and the nodal metastases: Grade I, well differentiated; Grade II, moderate differentiation; Grade III, poor differentiation; and Grade IV, anaplastic.

## RESULTS

**Group Comparison.** Between March 1970 and December 1978, 366 patients with prostatic cancer were treated at Memorial Sloan-Kettering Cancer Center with pelvic lymph node dissection and <sup>125</sup>I seed implants into the prostate; 133 patients had metastatic prostatic cancer in pelvic lymph nodes (Stage D1). When these 133 node positive patients were compared to the remaining 233 node negative patients, no statistical bias was noted due to patient age ( $P = 0.856$ ,  $\chi^2$  test) or time from diagnosis to treatment ( $P = 0.393$ ,  $\chi^2$  test). However, histological grade of the primary tumor ( $P = 0.00027$ ,  $\chi^2$  test) and local extension as determined by local stage ( $P = 0.00001$ ,  $\chi^2$  test) were significantly different for node positive versus node negative patients.

Of the 133 node positive patients, 51 were excluded for technical reasons as noted previously. A comparison of the remaining 82 with the 51 patients who were excluded revealed no statistical difference with respect to age ( $P = 0.224$ ,  $\chi^2$  test), time from diagnosis to implant ( $P = 0.659$ ,  $\chi^2$  test), local stage ( $P = 0.3289$ ,  $\chi^2$  test) or number of positive nodes ( $P = 0.343$ ,  $\chi^2$  test). However, there was a statistically significant difference in grade of the primary tumor, which was less differentiated for the nonexcluded cases ( $P = 0.0153$ ,  $\chi^2$  test).

Lymph node metastases in the 82 patients suitable for analysis were classified as follows: 27 were diploid, six were near diploid, 23 were tetraploid, and 26 were aneuploid. As mentioned above, the diploid and near-diploid cases were combined, as were the tetraploid and aneuploid cases, and referred to as diploid and aneuploid, respectively. There was no statistical bias in the percentage of lymph node replacement by metastatic cancer in the aneuploid versus diploid cases ( $P = 0.112$ ,  $\chi^2$  test) (Table I). Finally, a comparison of the 33 patients with diploid tumors versus the 49 patients with aneuploid tumors revealed statistical uniformity with respect to age ( $P = 0.970$ ,  $\chi^2$  test), time from diagnosis to treatment ( $P = 0.314$ ,  $\chi^2$  test), grade of the primary tumor ( $P = 0.667$ ,  $\chi^2$  test), grade of the metastatic tumor ( $P = 0.509$ ,  $\chi^2$  test), local stage ( $P = 0.856$ ,  $\chi^2$  test) and number of positive nodes ( $P = 0.669$ ,  $\chi^2$  test).

**Survival Analysis.** Five-year survival of the patients with diploid tumors was 87.9% compared to 49.5% for those with aneuploid tumors. Median survival was 8.8 and 5.0 years, respectively, for the diploid and aneuploid groups, and was significantly different ( $P = 0.0109$ , log rank test). Median follow-up time for living patients was 7.65 years for the diploid group and 7.73 years for the aneuploid group. Kaplan-Meier curves for survival are seen in Fig. 1.

It has been observed previously that the likelihood of survival varies considerably among patients with moderately differentiated prostatic cancer. Of the 38 patients with moderately differentiated primary tumors, the nodal metastases in 19 were diploid and 19 were aneuploid. The median time of survival for the diploid and aneuploid groups was 9.1 and 5.8 years, respectively. This difference achieves statistical significance ( $P = 0.0393$ , log rank test). Kaplan-Meier curves are seen in Fig. 2.

Survival relationships within metastatic grades were also examined. Of the 23 patients with moderately differentiated nodal metastatic tumors 11 were diploid and 12 were aneuploid. The median time of survival for the aneuploid group was 5.0 years. Median survival for the diploid group has not been reached. This difference in median survival did not achieve statistical significance ( $P = 0.1532$ , log rank test) however the sample size is quite small. Of the 56 patients with poorly differentiated nodal metastatic tumors 22 were diploid and 34 were aneuploid. The median time of survival was 8.1 and 4.7 years, respectively. This difference achieves statistical significance ( $P = 0.0360$ , log rank test).

Table 1 Distribution of specimens according to microscopically estimated percentage of tumor involvement versus ploidy<sup>a</sup>

Specimens	Tumor involvement			Total
	50–75%	75–90%	90–100%	
Diploid	9	12	12	33
Aneuploid	6	15	28	49
Total	15	27	40	82

<sup>a</sup>  $P = 0.112$  ( $\chi^2$  test).

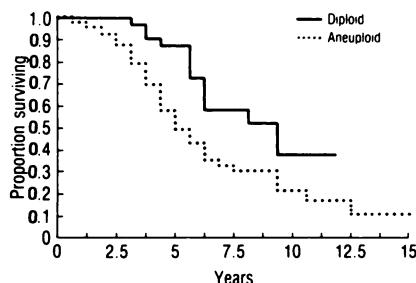


Fig. 1. Survival of all 82 patients with stage D1 prostatic cancer by flow cytometry. Kaplan-Meier plots of 49 aneuploid versus 33 diploid tumors.

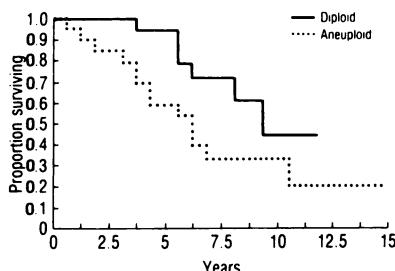


Fig. 2. Survival of 38 patients with moderately differentiated stage D1 prostate cancer. Kaplan-Meier plots of 19 aneuploid versus 19 diploid tumors.

**Time to Recurrence Analysis.** The 5-year disease-free survival was 33.4 and 13.9% in the diploid and aneuploid groups, respectively. Median disease-free survival was 4.0 and 2.1 years in the diploid and aneuploid groups, respectively, approaching statistical significance ( $P = 0.0608$ , log rank test).

**Time from Recurrence to Death Analysis.** Use of hormonal therapy in these patients was reserved until after the development of clinically detectable recurrence. In order to assess the importance of hormonal therapy on survival, we compared the length of survival from time of recurrence to time of death. Median duration of survival from time of recurrence was 4.0 and 2.5 years for the diploid and aneuploid groups, respectively ( $P = 0.055$ , log rank test). This borderline significant result suggests that response to hormonal therapy is, at most, partially responsible for the observed difference in patient survival between diploid and aneuploid groups.

## DISCUSSION

The use of paraffin-embedded tissue for flow cytometric DNA determinations offers one distinct advantage over fresh tissue in the study of solid tumors. Namely, survival and disease progression data which are already available can be compared with the DNA measurements. This is particularly important in the case of prostatic cancer because of the prolonged natural history of this neoplasm. Flow cytometric assays can be carried out on archived paraffin-embedded tissue, and patients who were operated on 10 or 15 years ago are candidates for clinical correlative studies.

Nevertheless, there are difficulties with this technique. The overall quality of DNA histograms obtained from paraffin-embedded tissue is generally inferior to that obtained with fresh tissues (15). In this study and others (12) 5–10% of histograms were judged to be uninterpretable. In at least some cases poor quality histograms may have been due to autolytic changes in the tissue, or to variables induced by delayed or prolonged formalin fixation or other differences in tissue handling, but as yet there are few studies of these factors (16).

Indeed, DNA stainability of tissues extracted from paraffin, *i.e.*, accessibility to dye molecules, also may be variable from one specimen to another. Thus a single control specimen of normal cells may not be sufficient for an entire series of specimens. The interpretation of histograms obtained from paraffin-embedded tumor tissue in this study was based on a separate control specimen for each tumor sample. The control specimen consisted of lymphocytes from an uninvolved node obtained from the same patient at the same surgical procedure and processed in the same way as the node with tumor. The modal channel number for DNA of the control lymphocytes was defined as 2.0c.

Once the channel number for diploidy (2.0c) has been determined, objective but at this time still arbitrary criteria were

applied to classify the DNA distribution in each case as diploid, tetraploid, or aneuploid, as previously described. There were no significant differences in patient survival with tetraploid compared with aneuploid tumors; nor were there differences in survival between diploid and near-diploid tumors. Because of the possibility of misclassifying tetraploid tumors as diploid when lymph nodes were only partially replaced by tumor, particularly with relatively little tumor (*e.g.*, 10% tumor cells), we arbitrarily excluded cases in which less than 50% of the lymph node was replaced by tumor.

As noted, there was a significant difference in clinical behavior of diploid versus aneuploid tumors. The absence of differences in other potential covariates (age, time from diagnosis to treatment, local stage, primary grade, metastatic grade, and number of positive nodes) was confirmed with Cox regression analysis. Thus, DNA ploidy has prognostic impact that is independent of these variables, and in this group of patients was the only significant prognostic indicator identified. Further, if the subgroup of moderately differentiated primary tumors only is analyzed, thus excluding histology as a variable, statistically significant differences between the diploid and aneuploid groups are still identified.

Survival analysis based on DNA measurements of primary rather than metastatic prostatic cancer remains to be done. Other workers have found that low stage (surgically resectable) carcinomas tend to be predominantly diploid (17). Diploidy also is associated with low grade carcinomas (17). However, in a recent study of renal cell carcinoma, DNA measurements of metastatic tumor had significant impact on survival while DNA measurements of the primary tumor from the same patients had no significance (18).

It is the policy of this institution to reserve hormone therapy for patients who develop symptomatic recurrence or show progression of disease. In this study the difference in hormone responsiveness of diploid versus aneuploid tumors was indirectly assessed by comparing time from first evidence of recurrence or progression to death. A marginal statistical difference was observed. In view of this we concluded that (a) ploidy is not clearly predictive of hormone responsiveness and (b) differences in survival in the diploid and aneuploid groups are not accounted for by differences in hormone response alone.

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 (1–6). Although little further light is shed upon the basic biological mechanism, these measurements may permit better clinical judgments of prognosis and affect recommendations of treatment.

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