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DNA Ploidy and Proliferation Heterogeneity in Human Prostate Cancers

T. Vincent Shankey, Jia-Kuan Jin, Scott Dougherty, Robert C. Flanigan, Sharon Graham, and Joseph M. Pyle

Departments of Urology (T.V.S., S.D., R.C.H., S.G.) and Pathology (T.V.S., J-K.J., J.M.P.), Loyola University Medical Center, Maywood, IL 60153, and Research Services, Edward J. Hines, Jr. V.A. Medical Center, Hines, Illinois (T.V.S., S.G.)

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DNA ploidy determinations have been shown to have clinical application in predicting disease progression, survival, or response to anti-androgen therapies in prostate carcinomas. Since intra-tumor heterogeneity may have a profound effect on DNA measurements, we determined the frequency of DNA ploidy and proliferation (here S-phase fraction) heterogeneity in early prostatic carcinomas, and estimated the potential impact of heterogeneity on predicting disease course, survival, or response to therapy.

Using image and flow cytometric analysis of archival, paraffin-embedded prostate tumors, we measured DNA ploidy in individual foci of prostatic carcinoma in stage T1a, T1b and T1c disease. Image analysis studies included the use of Feulgen stained tissue sections, and a comparison of these results with flow cytometric DNA ploidy determinations on nuclei isolated from the same tumor foci. Flow cytometry was also used to measure DNA Index and tumor S-phase fraction, in some cases using multiparameter analysis of isolated nuclei to determine DNA content and the level of the proliferation-associated antigen, p105.

Our results indicate that DNA aneuploid foci of prostate carcinoma are infrequently seen in stage T1a disease (13% of the individuals studied), and that the presence of both DNA diploid and aneuploid foci in the same sample is seen in less than 10% of these individuals. Stage T1b and T1c tumors containing only DNA diploid nuclei are seen, though these are likely most common in low volume, low Gleason grade tumors. By using flow cytometry to compare these results with those using image analysis of the same tumor foci, we demonstrated that the majority (>75%) of these aneuploid tumors are DNA tetraploid. Our data on prostate tumor S-phase fractions indicate that DNA diploid tumors generally have a lower S-phase than DNA aneupoloid foci (including comparisons of DNA diploid and aneuploid foci in the same prostate tumor). These results support the model that early prostate tumors are DNA diploid and have a low S-phase, and that these tumors likely evolve to DNA tetraploid tumors with a similar low S-phase fraction. © 1995 Wiley-Liss, Inc.

Key terms: Tumor heterogeneity, prostate cancer, DNA ploidy, tumor proliferation, flow cytometry, image analysis, proliferation-related antigens

At the current time, the natural history of prostate cancer remains poorly understood. Factors which initiate prostatic carcinoma, cause it to grow or remain indolent, penetrate the prostatic capsule, spread to metastatic sites, and become refractory to anti-androgen therapy remain largely unknown. These cancers are known to have a variable clinical course, with some individuals progressing rapidly to metastatic disease, while in others the disease remains indolent. Autopsy studies have demonstrated a high incidence of foci of prostate cancer in males over 50 years of age (5,18) This is significantly higher than the rate of clinically apparent cancer in men in the same age group (17). Together, these lines of evidence indicate that prostate cancer progresses at different rates in different individuals. What is not known is whether all prostate cancers start with the same rate of progression, with the rate increasing in some cancers but not in others, or whether the initial cancer progression is uniform throughout its disease course within a single individual, with some individuals starting with cancers hav-

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Address reprint requests to T. Vincent Shankey, Department of Urology, Loyola University Medical Center, 2160 South First Ave., Maywood, IL 60153.

Abbreviations used: FBS, Fetal Bovine Sera; FITC, Fluorescein Isothiocyanate; HBSS, Hank's Balanced Salts Solution; PBS, Phosphate Buffered Saline (0.01M Phosphate/0.15M NaCl); PEG, Polyethylene Glycol; TUR, Transurethral Resection.

; a higher natural progression rate. Since the incidence histologically defined prostate cancer is significantly eater (four-fold) than the incidence of clinical disease age-matched groups, it is likely that in many individu- the disease progresses at a very slow rate, and in many these individuals remains clinically undetected. Since ease progression becomes significantly more rapid in ne individuals, the regulation of tumor cell growth ely plays an important role in prostate cancers which come clinically symptomatic. The underlying question whether prostate cancers maintain roughly the same e of growth throughout their existence, or whether ne tumors progress from lower to higher rates of tu- r cell growth. In addition, the genetic differences be- een slowly and rapidly progressing groups of prostate cinomas are unknown. It is possible that rapidly pro- essing prostate tumors represent a genetically distinct ease compared with indolent prostate carcinoma. In dition, it is not known whether the two groups are netically related, with changes in one or a few key nes involved in genetic evolutionary steps in the nat- l progression from slow growing to rapidly progress- , metastatic prostate carcinoma.

MATERIALS AND METHODS

Surgical Specimens

Patients with biopsy proven prostate carcinoma under- ing radical prostatectomy at Loyola University Medical nter were used as a source of tissue for DNA ploidy idies using whole mount sections. In some of the stud- s described here, transurethral resections (TUR) of the rostate were used from patients presenting with voiding mptoms and no prior history of prostate cancer. All ssues were obtained for routine histopathological eval- ation and were obtained prior to any therapy (hormonal radiation). Histopathological evaluation was per- med using hematoxylin and eosin stained thin sec- ns. Patients were staged according to current system posed by the American Joint Committee on Cancer, ere stages T1a and T1b represent incidental prostatic cinoma found in TUR specimens with a tumor volume ess than 5% of the tissue removed and a Gleason's re <5 (stage T1a), or tumor volume >5% of the tissue oved or Gleason's score of 5 or greater (stage T1b). ical prostatectomy specimens were from patients h stage T1c disease, defined as disease diagnosed only to an elevated serum PSA level which led to further ly and biopsy.

Image Analysis

ix μm thick tissue sections were prepared from par- n-embedded whole mount sections (5 mm) from rad- prostatectomy (stage T1c) patients, or from TUR ples embedded in paraffin. Tissues were de-paraf- ed, rehydrated, and stained with Feulgen reagent ll Analysis Systems, San Jose, CA) as previously de- bed (8). DNA content image analysis was performed Pathologist (J-K J) using a CAS 200 (Cell Analysis ems) image analysis system. A control slide contain- ing rat liver cells (CAS) was stained with each batch of prostate tissue sections, and was used for instrument calibration. For all prostate tissue sections, areas of BPH (benign prostatic hypertrophy) were used as an internal diploid DNA content standard for that tissue section. Analysis of whole mount sections was performed by first dividing the tissue into four equal quadrants by marking the coverslip with a felt-tipped marking pen, and independently measuring the DNA content of a minimum of 400 BPH nuclei in each quadrant (as close to the prostatic capsule as possible). If the DNA content of BPH control nuclei was different in any two quadrants by more than 15%, that tissue section was discarded, and another section was prepared for image analysis. All areas of prostatic carcinoma within the whole mount section were outlined by marking the coverslip with a marking pen. For large continuous areas of prostate carcinoma (occupying more than 25% of the area of the section), two or more areas of the tumor were marked for independent analysis. Separate areas of prostatic carcinoma were analyzed by acquiring images for a minimum of 400 nuclei of carcinoma (by morphologic appearance). Acquisition was performed using a minimal gating "mask" (to include minimal IOD, area, and volume nuclei), and images from all analyzed fields were stored on an optical disk to allow later re-analysis. Nuclei tangentially touching adjacent nuclei were manually separated during analysis, and all visibly overlapping nuclei were excluded from analysis. DNA ploidy analysis was performed using the CAS DNA ploidy analysis software. DNA diploid tumors were defined here as tumor nuclei having the same modal DNA content as BPH nuclei in the same tissue section ± 25%. All tumor nuclei with a modal DNA content (presumably the tumor G_0/G_1 population) in excess of 25% (2.5C) were considered to be DNA aneuploid. A minimum of 15% of the tumor nuclei had to contain 2.5C or higher DNA content for a tumor to be considered to contain a DNA aneuploid population. When both DNA diploid and DNA aneuploid tumor nuclei were measured within the same tumor containing region, the tumor was considered to be heterogeneous by DNA ploidy criteria. Similarly, a tumor was considered to be heterogeneous when two different regions contained either DNA diploid or DNA aneuploid tumor nuclei. All of the DNA aneuploid prostate tumors analyzed in this study from radical prostatectomy (whole mount) samples had a modal DNA content of 3.0C or greater. Prostate tumors from stage T1b TUR specimens had either a modal DNA content of 2.0C (DNA diploid), or a modal DNA content of 3.0C or greater. No distinction was made in this study for DNA aneuploid tumors with different measured DNA contents (DNA tetraploid versus other DNA aneuploidies) as slice artifacts have been demonstrated to significantly impact on the modal DNA content of DNA aneuploid tumors measured in tissue sections (22).

Flow Cytometry

Samples used for flow cytometric analysis included nuclei from blocks of TUR tissue (stage T1b) and nuclei

from selected portions of whole mount sections. After image analysis of whole mount sections, different areas of prostate cancer from selected whole mount sections were dissected out of the 5 mm whole mount sections, re-embedded, and processed for isolation of nuclei. Paraffin-embedded tissues were deparaffinized and dissociated by the method of Hedley and co-workers (7) with slight modification. Fifty μm thick sections (3 to 7) were placed in 15 ml glass centrifuge tubes and deparaffinized using three incubations (30 min each) with 10 ml Americlear (Scientific Products). Samples were rehydrated using a sequence of 10 ml of 100%, 95%, 70%, and 50% ethanol incubations for 30 min each at room temperature. The tissue was washed three times in distilled water, and placed in distilled water at 4°C for 1-3 days prior to enzymatic digestion. Tissue sections were minced into small pieces using dissecting scissors then suspended in 1 ml of 0.1% pepsin (Worthington, Freehold, NJ) solution in saline adjusted to pH 1.5 with 1N HCl. This mixture was incubated for 30 min at 37°C with brief vortexing at 5-10 min intervals. At the end of incubation, samples were filtered through 37 μm nylon mesh (Small Parts, Inc.), washed with cold HBSS/10 mM HEPES/5% PEG-6000, and stored at 4°C until used for staining.

Some samples of isolated nuclei were stained immunohistochemically using an antibody to a proliferation-associated protein marker (p105 antibody, provided by Dr. Wade Bolton Coulter Corp, Hialeah, FL) which was originally reported by Clevenger and co-workers (3). The epitope recognized by this antibody has been shown to be resistant to enzymatic digestion used for isolation of nuclei (11). Proliferation-associated protein p105 staining was performed in conjunction with DNA staining using methods adapted from Bauer and co-workers (2,11). Briefly, nuclei were resuspended in 0.5 ml PBS/3% FBS at a concentration of 0.5-1 X 10^6 nuclei/0.5 ml and were incubated with an optimal concentration of p105 antibody (antibody concentration 3-5 μg/ml). An identical aliquot of nuclei was incubated with isotype matched normal mouse Ig at an identical protein concentration for a background fluorescence (FITC) control. After incubation for 1 h at 4°C, nuclei were washed two times with PBS/0.1% Triton X-100, and resuspended in 0.5 ml of FITC labeled goat anti-mouse Ig (Southern Biotechnolgy, Birmingham, AL) using an optimal antibody concentration previously determined by titration. Following a 30 min incubation at 4°C, nuclei were washed with PBS/0.1% Triton X-100. DNA staining was accomplished by resuspending antibody stained nuclei in RNAse (180 Kunitz Units/ml, Worthington, Freehold, NJ) in 1 ml PBS containing 10 mM $MgCl_2$ for 30 min at 37°C. After the addition of propidium iodide to a final concentration of 50 μg/ml (Molecular Probes, Eugene, OR), nuclei were incubated overnight at 4°C prior to analysis by flow cytometry.

Fluorescence of FITC and/or propidium iodide stained nuclei were monitored using an Epics V flow cytometer (Coulter Corp.). Immediately prior to analysis, nuclear suspensions were filtered through 37 μm nylon mesh to remove aggregates. Laser excitation (400 mW) was at 488 nm. Fluorescence signals were collected from individual nuclei using a 550 nm long pass dichroic filter to separate DNA content (propidium iodide) signals from antibody (FITC) signals; a 630 long pass interference filter in front of the red (PI) photomultiplier, and a 525 nm band pass plus 530 nm short pass interference filter in front of the green (FITC) photomultipliers. List mode files were collected from 10,000 to 25,000 nuclei (gated on foreword scatter plus red fluorescence signals to remove debris below ~channel 15 on a 256 channel histogram). Histogram files used for the analysis of individual samples include DNA content (integrated red fluorescence), and DNA content versus p105 (log and linear green fluorescence) with or without gating for cells positive for proliferation antigen expression (for tumor S-phase analysis). Cell cycle analysis was performed using MultiCycle software (Phoenix Flow Systems, San Diego, CA), and S-phase determinations were performed in accordance with previously published guidelines (20).

RESULTS

Patients with stage T1c prostate cancers analyzed using image analysis of whole mount sections have demonstrated only DNA diploid tumor in some prostates, while in others both DNA diploid and aneuploid (heterogeneity by DNA ploidy) tumor, or only DNA aneuploid tumor was seen. As shown in Figure 1a, small volume tumors (including samples with multiple foci of prostate carcinoma in different quadrants of one section) sometimes had only DNA diploid tumor. Here, four different foci of tumor were found on one 5 mm section, and all four had the same DNA content (histogram results from two of the four areas are shown here) as the BPH standard from this same section (results not shown). In other individuals with low volume tumors (Fig. 1b) part of the single focus of tumor (adjacent to the prostatic capsule) contained only DNA diploid nuclei (area 3) while part of this same tumor focus (area 1) had both DNA diploid and aneuploid tumor nuclei. Prostates containing large volume tumors were divided into multiple areas for analysis, and as shown in Figure 1c, these tumors generally showed DNA aneuploid tumor, even in areas adjacent to the prostatic capsule (area 1, Fig. 1c).

The results of DNA ploidy analysis of whole mount sections from 10 radical prostatectomy patients (Table 1) demonstrated the presence of both DNA diploid and DNA aneuploid foci of prostatic carcinoma in four of the ten prostatectomy samples analyzed thus far. Five samples showed only DNA aneuploid tumor, while one had only DNA diploid tumor. Whole mount sections studied thus far with large volume tumors (10 cc or greater) contained DNA diploid and DNA aneuploid tumor in some cases, or only DNA aneuploid tumor in others. Tumors of smaller volume (<10 cc) contained either a mixture of DNA diploid plus aneuploid, or only DNA diploid tumor.

We define histograms in this study which contain both 2C and a significant perecnt of 4C nuclei (25% or more, Figure 1B, area 1) as evidence for both DNA diploid and

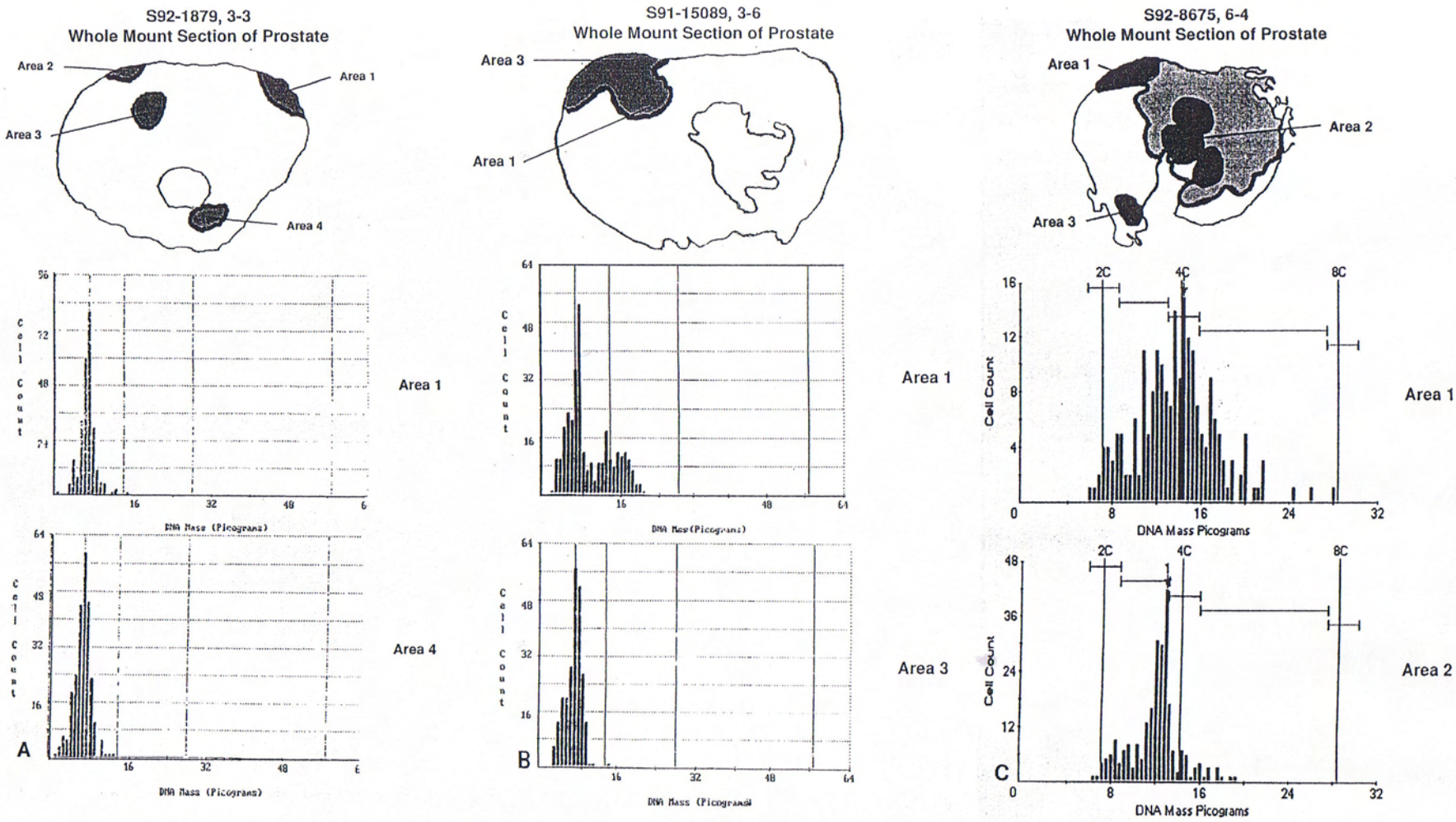

FIG. 1. DNA content image analysis of whole mount sections from three different radical prostatectomy specimens from stage T1c patients. Tumor maps illustrate the location of tumor foci, and independent areas of tumor analyzed within each section. **A:** Four different tumor foci were identified, and histograms from all areas (two shown here) had only DNA diploid events. **B:** Section from another radical prostatectomy sample with a single focus of tumor. Two different areas were analyzed; the area adjacent to the prostatic capsule (Area 3) had only DNA diploid nuclei, while the tumor in Area 1 contained both DNA diploid and aneuploid nuclei. **C:** A section with a large focus of tumor (total volume 6 cc) had only DNA aneuploid nuclei in all areas analyzed (results shown from image analysis of two areas).

Table 1
DNA Ploidy in Stage T1c Whole Mount Sections Measured by Image Analysis

Sample	Gleason score	Tumor volume[a]	Pathologic stage	Number of foci analyzed[b]	DNA ploidy[c]
4941	3 + 4	12cc	T3N0	4	D + A
14140	3 + 4	6cc	T2aN0	3	A
15089	2 + 3	2cc	T3N0	1	D + A
561	3 + 4	4cc	T2N0	3	D + A
539	3 + 4	4cc	T2N0	3	D + A
2456	3 + 4	15cc	T3N1	5	A
1879	2 + 3	6cc	T3N0	4	D
994	4 + 5	10cc	T3N0	4	A
4518	3 + 4	10cc	T2bN0	3	A
8675	4 + 4	15cc	T3N0	3	A

[a]Tumor volume was calculated for all tumor foci in all tumor containing sections.

[b]Number of individual, noncontiguous areas of prostatic carcinoma found on each tumor containing section. No attempt was made to determine if foci on adjacent sections were from the same foci.

[c]Diploid or aneuploid tumor foci or a single focus of tumor containing both DNA diploid and aneuploid prostate tumor nuclei. See Materials and Methods section for DNA ploidy definitions

aneuploid tumor within the same foci. As shown in Figure 1c, foci from large volume tumors show very few (<10%) events in the 2C region (from 1.6C-2.3C), which is likely caused by sliced DNA aneuploid nuclei in these histograms. In contrast, foci which we have interpreted as containing both DNA diploid and DNA aneuploid tumor contained at least 50% of the nuclei in the DNA diploid (1.6C-2.3C) region (Fig. 1b, area 1).

The presence of both DNA diploid and aneuploid nuclei in foci of prostate cancer led us to study the frequency of DNA aneuploid foci, and heterogeneity of DNA ploidy in low stage disease. For these studies, we selected cases from stage T1a disease, which represents low volume tumors with Gleason grade of 1 or 2 (hence, Gleason sum of 4 or less).The results of image analysis of 15 stage T1a patients, presented in Table 2, demonstrated that DNA aneuploid tumor was seen in two (case 1 and 3) of the 15 individuals studied thus far. For these studies, we used areas of BPH in the same tissue section as a DNA diploid standard. As shown in Table 2, BPH nuclei with 1.6C to 2.3C DNA content represent 94% or more of all nuclei measured. In the majority of foci of prostate carcinoma from these samples, the majority of nuclei (80% or more) fall within this same 1.6C-2.3C region. In two cases (1 and 3) the percent of tumor nuclei with 2.4C or greater DNA content was more than 50% of the nuclei measured. The DNA ploidy histogram results for case 1, shown in Figure 2, demonstrate a pattern with major peak at 2C, a peak at 4C, and significant numbers of nuclei with DNA content between these peaks. This pattern could be the result of two different populations of nuclei (one 2C and one 4C), with intermediate values caused by sliced

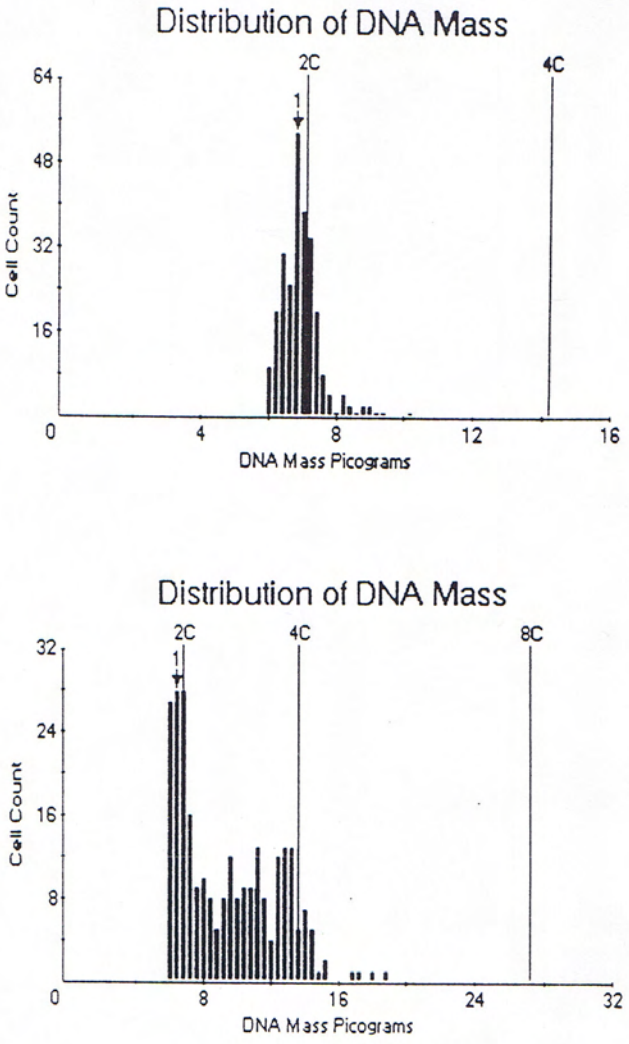

FIG. 2. DNA content image analysis of tissue section from a patient with stage T1a prostate carcinoma. Top panel: Histogram results from the analysis of BPH nuclei found in this tissue section and used for the internal DNA diploid standard. Bottom panel: Histogram results from the analysis of prostate carcinoma nuclei in tissue section, showing the presence of a peak at 2C DNA content, a smaller peak at roughly 4C, and nuclei with measured DNA content extending between these two peaks. Sample shown is Case 1 in Table 2.

and overlapping nuclei, or by the presence of a proliferating 2C tumor, with events between the 2C and 4C events represented by S-phase nuclei. Careful analysis of this sample has demonstrated the presence of two adjacent foci of prostate carcinoma (same Gleason grade), one with 2C, the other with 3C to 4C nuclei (Fig. 3). While these results do not rule out the possibility of S-phase events contributing to this histogram pattern, they demonstrate that adjacent tumor foci in stage T1a disease can be heterogeneous by DNA ploidy. The other DNA aneuploid T1a sample (case 3) demonstrated a peak at 3-3.5C (containing 54% of all nuclei) from the analysis of all nuclei found in a single focus of tumor in this sample. These results indicate that DNA aneuploid foci are

Table 2
VA Ploidy in Stage T1a Prostatic Carcinoma. Results of Image Analysis of TUR Tissue Sections

ber	BPH	Prostatic Carcinoma			
	pg. DNA	1.6C–2.3C	1.6C–2.3C[a]	2.4C–3.5C[a]	3.6C–4.4C[a]
	6.9	97.3	43.6	31.8	20.5
	6.9	94.4	93.3	4.9	1.8
	7.2	97.7	38.7	54.4	14.6
	6.6	99.5	96.0	4.0	
	6.9	98.1	96.7	3.3	
	6.8	95.9	88.2	10.1	
	6.7	99	79	21	
	6.9	99	90.3	7.5	2.2
	7.1	96.3	98.6	1.4	
	6.7	99.1	99	1	
	6.7	99	81.4	15.3	2.1
	7	96.4	98.9	1.1	
	6.9	98.1	82.9	8.3	7
	6.8	100	96.1	3.0	0.9
	7.1	96.4	77.7	13.4	8.4

Percent of prostate carcinoma nuclei in uncorrected DNA ogram falling between values shown, where BPH nuclei are 1 as an internal DNA diploid standard. Samples marked with are considered to contain DNA aneuploid nuclei. See Mate- and Methods section for DNA ploidy definitions.

n at low frequency (13% in this study) in stage T1a state carcinoma, and that the pattern of DNA ploidy erogeneity (DNA diploid and aneuploid tumor foci) is n with low frequency in small tumor lesions with low eason grade.

Further evidence for DNA ploidy heterogeneity within single tumor was seen by the use of multiparameter w cytometric analysis of nuclei isolated from either 1b samples, or from selected portions of stage T1c hole mount sections. As shown in Figure 4, flow cyto- tric analysis of a stage T1b tumor foci (left panels) that s DNA diploid by image analysis demonstrated a single jor peak by DNA content analysis, with low percent hase and G_2M events. Here, the DNA diploid nuclei ubated with p105 antibody (lower left panel) demon- ted a p105 positive and negative DNA diploid peak 05 negative events have the same FITC fluorescence he Ig control, top left panel). The DNA diploid G_2M nts are all p105 positive (bottom left panel), with C fluorescence significantly above the Ig control. In a ilar analysis of a portion of a stage T1c sample (dis- ted fromm the whole mount section), the p105 ned sample (bottom left panel) showed p105 positive A aneuploid (here, tetraploid), and DNA diploid nu- i. In this sample, some of the nuclei in the first (DNA loid) peak are p105 negative (same FITC fluorescence n Ig control, top right panel) and presumably repre- t G_0 cells (normal, stromal, and reactive cell nuclei, l some of the diploid tumor nuclei). In the 4C peak ntaining both diploid G_2M events and DNA tetraploid nuclei) all nuclei were p105 positive in this sample. e results of image analysis of the tissue section from same sample demonstrated the presence of both DNA diploid and aneuploid tumor nuclei (right panels). Interpretation of the single parameter DNA content histogram would correctly identify the presence of a DNA tetraploid tumor. However, in this case, the DNA diploid peak would be interpreted as normal or reactive cell nuclei. The use of multiparameter analysis using the p105 antibody allows the identification of proliferating DNA diploid cells. While these could include normal or reactive cells, in all cases of prostate carcinoma analyzed thus far, the presence of p105 positive DNA diploid nuclei was seen only in samples from tissues shown to contain DNA diploid tumor by image analysis. As summarized in Table 3, all tumors that contain DNA aneuploid nuclei by image analysis of tissue sections were correctly interpreted as containing aneuploid events (here, all DNA tetraploid) by flow cytometry. However, in eight tissues that contained both DNA diploid and aneuploid tumor by image analysis, the use of p105 antibodies and multi-parameter flow cytometric analysis correctly identified the presence of the heterogeneous DNA ploidy populations not appreciated by single parameter DNA content analysis alone.

The presence of DNA ploidy heterogeneity within a single prostate raises the question whether similar heterogeneity in tumor proliferation is present within a prostate tumor. To investigate this, we have analyzed multiple samples from stage T1b patients thus far using single parameter DNA content flow cytometry of nuclei from paraffin-embedded tissues. A total of 24 cases (of 54 analyzed to date) have contained both DNA diploid and DNA aneuploid tumor foci within the same prostate sample (some within the same block, others from the analysis of multiple blocks from the same TUR sample). Of these, ten samples have provided S-phase data from both the DNA diploid and aneuploid samples that is of suitable reliability to provide a comparison of the S-phase of these different ploidy populations in the same tumor. DNA tetraploid tumors which had low reliability of the S-phase values caused the rejection of many of these samples from the comparison. This was due to either low percent DNA tetraploid events (<20%), low percent of DNA tetraploid G_2M events, or unacceptable percent BAD (background aggregates and debris) in the DNA tetraploid portion of the histogram (20).

The comparison of S-phase fractions of DNA diploid and aneuploid (here, all tetraploid) foci from the same stage T1b tumors is shown in Table 4. In all but one case, the S-phase of the DNA aneuploid (tetraploid) foci was higher than that of the DNA diploid foci in the same prostate. In addition, the mean value for S-phase of DNA diploid tumors (4.4%) was significantly lower than the mean S-phase for the DNA aneuploid tumors (12.4%). This difference in means was significant ($p>0.05$) when tested using a student's T-test. These results also indicate that heterogeneity in S-phase (as a measure of tumor proliferation) is also seen within a single prostate.

DISCUSSION

The role of DNA ploidy in predicting disease course or survival in prostate carcinoma has been somewhat con-

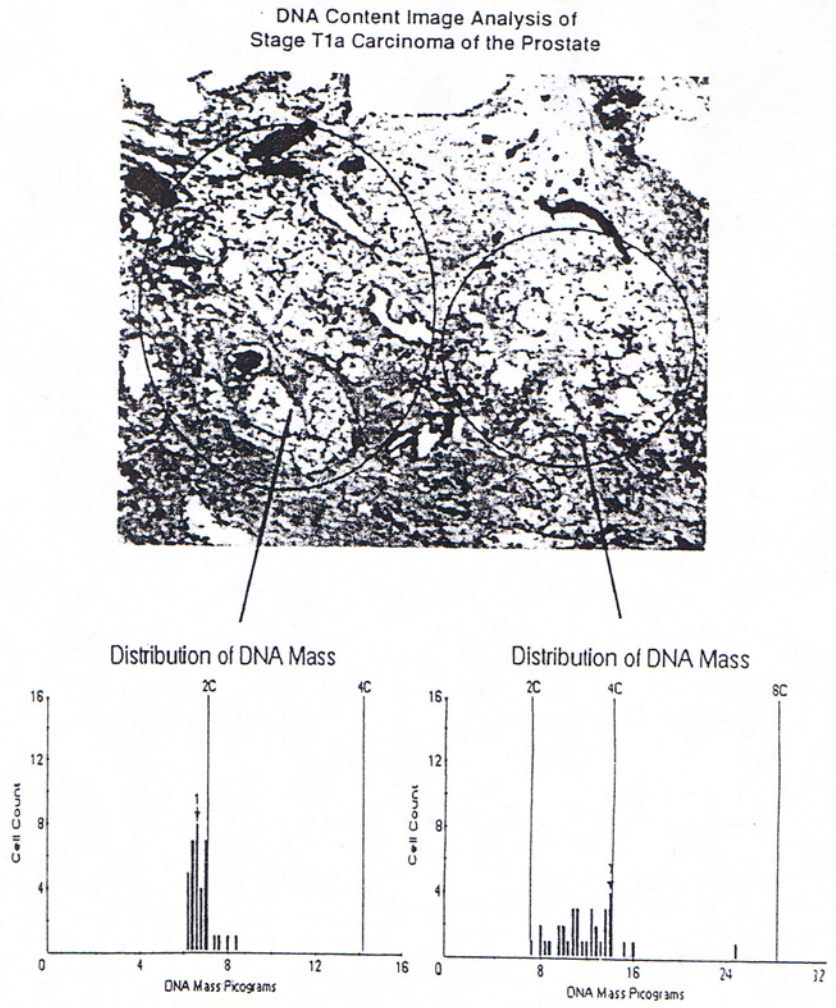

FIG. 3. DNA content image analysis of tissue section from same stage T1a prostate carcinoma as shown in Figure 2. Hematoxylin and eosin stained tissue section (**top**) demonstrating two adjacent areas of prostatic carcinoma. Histogram results from the independent analysis of these two areas demonstrated the presence of DNA diploid nuclei in the left area, and DNA aneuploid nuclei in the right area.

troversial, with some studies demonstrating a strong predictive value (10,14,15,25), while others have failed to demonstrate significance (13,16). A recent consensus conference (21), in reviewing the published literature on DNA cytometry of prostatic carcinomas has concluded that DNA ploidy provides a strong prediction of progression, survival and response to anti-androgen therapy. This concensus also commented that some evidence existed at that time that prostate carcinomas could contain both DNA diploid and aneuploid tumor foci (tumor heterogeneity). The concensus noted that the evidence at that time was consistant that such heterogeneity was likely seen in clinically early disease, and that the existence of such heterogeneity could have a significant impact on the predictive value of DNA ploidy determinations in these tumors. Since the published reports which fail to provide evidence that DNA ploidy is predictive of disease course or survival include a number of studies on clinical stage A and B disease (13,16), we have undertaken a study of clinically early disease in order to: 1) determine the frequency of DNA ploidy heterogeneity (and proliferation or S-phase heterogeneity) in early prostate carcinomas; and, 2) to determine the impact this heterogeneity has on predicting disease course, survival, or response to therapy.

The DNA concensus also indicated that S-phase might provide a clinically important predictive marker for prostate carcinoma (21). However, the consensus thought these conclusions were preliminary in nature as they were based on limited numbers of publications which have analyzed prostate carcinoma proliferation, and correlated S-phase with outcome (4, 24).

Our results of DNA ploidy analysis of prostatic carcinomas demonstrates that tumor heterogeneity, as seen by DNA ploidy, is seen in prostate cancers. Using image analysis of tissue sections from stage T1a patients, this study has shown that DNA aneuploid tumors are infrequently noted (13% of the T1a tumors studied thus far) in low volume and low Gleason grade tumors, and that

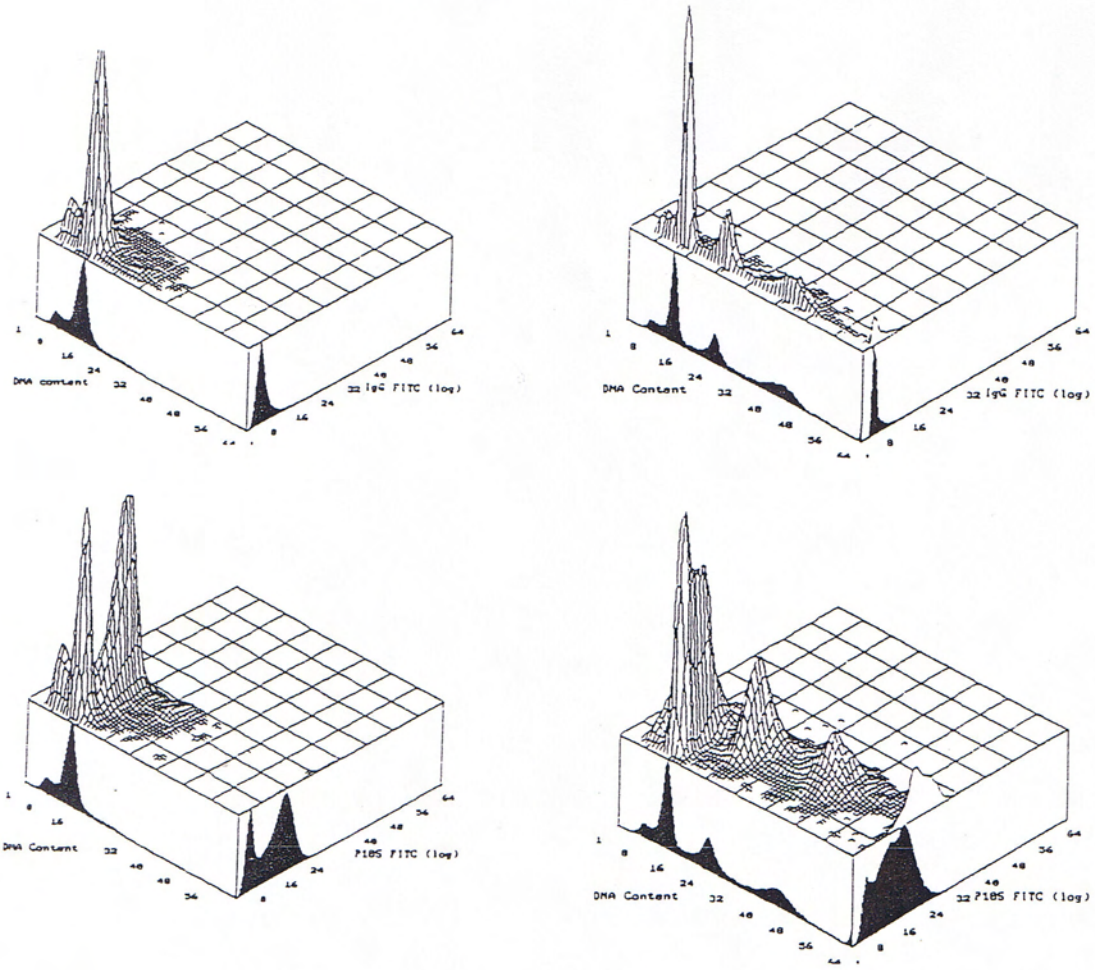

Fig. 4. Multiparameter flow cytometric analysis of nuclei isolated from a DNA diploid (**left panels**), or a DNA aneuploid (**right panels**) case of prostatic carcinoma. Top panels: histogram results for nuclei incubated with Ig (FITC control) demonstrating DNA content (horizontal axis) and background FITC fluorescence. Bottom panels: histogram results for nuclei incubated with p105 (proliferation associated protein) antibody, demonstrating p105 positive and negative DNA diploid nuclei in both diploid and aneuploid foci.

DNA diploid and DNA aneuploid tumor foci in the same tumor are seen in less than 10% of the stage T1a tumors analyzed. Here, the use of image analysis of tissue sections provides clear evidence that adjacent foci of prostatic carcinoma in the same section can be heterogeneous by DNA ploidy. While there is some concern that DNA ploidy analysis in tissue sections leads to artifacts due to sliced and overlapped nuclei, and other errors due to non-spherical nuclei, uneven distribution of DNA, etc., the results of our studies outlined in part below compare the results of DNA ploidy results using image analysis of tissue sections and flow cytometric analysis of nuclei isolated from identical tumor foci supports the conclusion that DNA ploidy heterogeneity (here the presence of both diploid and aneuploid tumor) can be found in the same prostate carcinoma.

Table 3
Multi-Parameter Flow Cytometry Using p105 Antibodies for the Identification of DNA Diploid and DNA Aneuploid Prostate Carcinomas From Paraffin-Embedded Tissues.

	Single parameter (DNA content only)	Dual parameter (DNA plus p105)
DNA diploid only[a]	3	3
DNA aneuploid only	12	4
DNA diploid + aneuploid	0	8

[a] Tissues from a total of 15 stage T1b or T1c samples were analyzed by image analysis to determine presence of only DNA diploid or DNA aneuploid tumor, or both DNA diploid and aneuploid nuclei in tumor foci. The identical tumor containing area was dissected out of tissue, and subsequently analyzed using single (DNA content only) or dual parameter (DNA plus p105) flow cytometry as shown in Fig. 4.

Table 4
DNA Content Flow Cytometry Analysis of Stage T1b Prostate Carcinoma Samples Containing DNA Diploid and Aneuploid Tumor Foci Within the Same Prostate

Case number	Gleason score	DNA diploid foci[a,b]	DNA aneuploid foci[a,c]
1	2 + 1	2.7%	10.2%
2	2 + 1	3.4%	20.9%
3	2 + 2	1.2%	5.2%
4	2 + 3	1.0%	16.8%
5	2 + 3	1.0%	6.0%
6	3 + 4	10.5%	6.9%
7	3 + 4	9.8%	19.6%
8	4 + 4	1.0%	6.9%
9	4 + 5	6.0%	19.4%
10	4 + 5	7.6%	12.4%

[a]Percent S-Phase.
[b]Mean = 4.42 +/− 3.7.
[c]Mean = 12.4 +/− 6.2.

The use of flow and image analysis for DNA content measurements in stage T1c disease has demonstrated that DNA ploidy heterogeneity is seen in 40% of the tumors analyzed thus far. While these results are somewhat preliminary, they indicate that lower volume tumors (<10 cc) are more frequently heterogeneous, and that larger volume tumors frequently contain only DNA aneuploid tumor. In this study, it was not possible to make a significant correlation of DNA ploidy and Gleason grade, as the majority of tumor foci were of intermediate grade (3 to 4). The only tumor analyzed thus far which contained only DNA diploid nuclei had a Gleason score of 2+3, and a volume of <10 cc. These results are consistent, however with previously reported results showing a positive correlation of Gleason grade and tumor volume (1).

The studies reported here extend our previous investigations on heterogeneity in stage T1b prostate cancers (8). In these studies, we have demonstrated that roughly 25% of the 54 samples studied thus far had both DNA diploid and aneuploid tumor foci in the same TUR sample. Using flow cytometry to study these same T1b samples, we have demonstrated that more than 85% of the DNA aneuploid tumors are tetraploid. These tumors represent a larger volume and higher Gleason score than stage T1a tumors, and together with the results of our studies of DNA ploidy in stage T1a reported here, suggest that as prostate tumors reach larger volume, the probability of the tumor containing DNA aneuploid foci increases significantly.

A previous study of DNA ploidy using image analysis of Feulgen stained whole mount tissue sections from prostatectomy samples (6) has demonstrated similar DNA ploidy heterogeneity in prostate carcinomas. In this study, 63 different foci were analyzed in a total of 30 radical prostatectomy samples from stage A2 (T1b) and B patients. The definition of tumor ploidy heterogeneity used in this and in our study was different, in that here we have defined histograms from single tumor foci which contain both 2C and a significant percent of 4C nuclei as evidence for both DNA diploid and aneuploid tumor within the same tumor foci. The major implication of this interpretation is that DNA ploidy heterogeneity is more commonly seen within single foci of prostatic carcinoma than reported by Greene and co-workers (6). This interpretation of DNA ploidy heterogeneity within tumor foci suggests that genetic (DNA ploidy) heterogeneity is commonly seen, particularly in lower stage (T2) organ confined prostatic carcinomas, and that ploidy evolution can progress throughout an entire tumor, most likely from DNA diploid to DNA aneuploid prostate cancers.

In these studies, we have been careful to identify tumors as either DNA diploid or aneuploid based on the results of image analysis studies. As indicated above, several important factors make the assignment of significant DNA Index values from the results of image analysis highly uncertain. However, in previous studies on stage T1b tumors, and here in the analysis of stage T1c samples, we have compared the DNA ploidy results of flow cytometric and image analysis of the same tumor foci. These results demonstrate that the majority (>75%) of the DNA aneuploid tumors analyzed thus far in stage T1b and c samples are DNA tetraploid. As discussed below, our results (and those of others) are consistent with the interpretation that the common evolutionary pathway in prostatic carcinomas is from a DNA diploid to tetraploid tumor.

McNeal and co-workers (12) have shown that many small stage T1a and T1b cancers in radical prostatectomy specimens occur in the transition zone of the prostate. In contrast, stage T2 cancers are generally located in the peripheral zone of the prostate. Greene and associates (6) have reported that most lesions arising in the transition zone of the prostate are DNA diploid while most peripheral zone lesions are DNA aneuploid. Our results indicate that 50% of stage T1b tumors are DNA aneuploid (tetraploid by flow cytometric analysis), and as indicated above, half of these tumors contain foci of both DNA diploid and aneuploid tumor. These results are supported by the results of our image analysis of whole mount sections from stage T1c patients, which included some transition zone tumors with DNA aneuploid nuclei. A recent publication by Kucuk and co-workers (9) have similarly demonstrated the presence of DNA aneuploid transition zone tumors using flow cytometric analysis.

The presence of tumor heterogeneity by proliferation measurements (here, S-phase fraction) could have similar impact on the predictive value for prostate carcinomas. Our results of stage T1b tumors containing both DNA diploid and aneuploid foci indicate that the S-phase fraction in these different ploidy populations are significantly different in the cases analyzed thus far. As in other tumor systems, DNA aneuploid (here, all tetraploid) T1b tumors generally have a higher S-phase fraction than the DNA diploid tumors. If a similar pattern of relationship of DNA ploidy and S-phase fraction holds for stage T1c tumors (currently being studied), then either DNA ploidy or

se fraction provide an useful predictive marker for ite carcinomas.

erall, our data on ploidy heterogeneity and prostate r S-phase are consistent with a model that predicts arly prostate carcinomas are most frequently DNA id, with low S-phase fraction. These progress first by lication of all chromosomes ("tetraploidization") to ice DNA tetraploid tumors with low S-phase, similar : DNA diploid precursor. These tumors likely then ess following a mutation or mutations to growth atory pathways to DNA tetraploid tumors with in- ed proliferation rates, in a fashion similar to that cted by Shackney and co-workers using computer lations of tumor evolutionary pathways (19). DNA id tumors may also be capable of evolving from low se early tumors to high S-phase tumors that may ess at a rate similar to high S-phase DNA tetraploid rs. Since DNA aneuploid (other than tetraploid) tu- are seen with increasing frequency in stage T2 to T4 cate tumors (14, 25), and these have high S-phase ions, these tumors likely represent genetic evolu- ry steps from DNA tetraploid (high S-phase) tumors. all, the progression in DNA ploidy evolution reflects results of work published by Tribukait (23). These els predict that prostate carcinomas evolve over time in a single individual, and that multiple tumor sub- lations are likely to be found in any one tumor, par- arly early in the disease course. What remains to be stigated is the underlying genetic mechanisms which late tumor evolution (and tumor growth) and the ns to utilize this information to best predict disease rse and outcome for individual prostate carcinoma ients.

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