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Relationship of Ki-67 labeling index to DNA-ploidy, S-phase fraction, and outcome in prostate cancer treated with radiotherapy.

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BACKGROUND: Our purpose was to evaluate the relationship of Ki-67 labeling index (Ki67-LI) to deoxyribonucleic acid (DNA) ploidy, S phase fraction (SPF), other clinical prognostic factors, and clinical outcome for patients with prostate cancer treated by external beam radiotherapy. METHODS: Tissue was retrieved from 42 patients who underwent transurethral resection of the prostate before treatment with external beam radiotherapy between 1987-1993. DNA histogram profiles were classified as diploid (diploid + near-diploid) and nondiploid (tetraploid + aneuploid). Immunohistochemical staining of Ki-67 by the MIB-1 monoclonal antibody was used to calculate Ki67-LI. Median patient follow-up was 62 months. Treatment failure was defined as two consecutive rises in serum prostate-specific antigen (PSA) or clinical evidence of disease recurrence. RESULTS: The mean and median Ki67-LIs were 3.1 and 2.4, respectively (range, 0-12.4). Mean Ki67-LI values were significantly associated with higher stage, Gleason score, and pretreatment PSA. Nondiploid tumors had significantly higher Ki67-LIs, as did patients who failed radiotherapy over the follow-up period. SPF was not significantly correlated with Ki67-LI. As a categorical variable, the most significant relationships were seen when Ki67-LI was subdivided into thirds around the median (Ki67-LI </=1.5%, Ki67-LI >1.5-3.5%, and Ki67-LI >3.5%). This trichotomous variable correlated significantly with pretreatment PSA (P = 0.0008), tumor stage (P = 0.016), Gleason score (P = 0.024), and treatment failure (P = 0.0015), but not with DNA-ploidy (P = 0.15). In actuarial univariate analyses, Ki67-LI appeared to be a more significant predictor of patient outcome (P = 0.003) than DNA-ploidy (P = 0.035). CONCLUSIONS: The Ki67-LI correlated with known prognostic factors such as pretreatment PSA, tumor stage, and Gleason score, and was also weakly related to DNA-ploidy. In comparison to DNA-ploidy, Ki67 LI seems to be a better correlate of treatment outcome.

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Relationship of Ki-67 Labeling Index to DNA-Ploidy, S-Phase Fraction, and Outcome in Prostate Cancer Treated With Radiotherapy

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RESULTS. The mean and median Ki67-LIs were 3.1 and 2.4, respectively (range, 0–12.4). Mean Ki67-LI values were significantly associated with higher stage, Gleason score, and pretreatment PSA. Nondiploid tumors had significantly higher Ki67-LIs, as did patients who failed radiotherapy over the follow-up period. SPF was not significantly correlated with Ki67-LI. As a categorical variable, the most significant relationships were seen when Ki67-LI was subdivided into thirds around the median (Ki67-LI 1.5%, Ki67-LI >1.5–3.5%, and Ki67-

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LI >3.5%). This trichotomous variable correlated significantly with pretreatment PSA (P = 0.0008), tumor stage (P = 0.016), Gleason score (P = 0.024), and treatment failure (P = 0.0015), but not with DNA-ploidy (P = 0.15). In actuarial univariate analyses, Ki67-LI appeared to be a more significant predictor of patient outcome (P = 0.003) than DNA-ploidy (P = 0.035).
CONCLUSIONS. The Ki67-LI correlated with known prognostic factors such as pretreatment PSA, tumor stage, and Gleason score, and was also weakly related to DNA-ploidy. In comparison to DNA-ploidy, Ki67 LI seems to be a better correlate of treatment outcome. *Prostate 41:166–172, 1999.*

KEY WORDS: **DNA-ploidy; Ki-67; MIB-1; prostate cancer; prostate-specific antigen; radiotherapy**

INTRODUCTION

The deoxyribonucleic acid (DNA) content of prostate tumors has repeatedly been shown to be predictive of disease outcome [1–15]. In our experience [16–18], DNA-ploidy is an independent correlate of biochemical and/or clinical failure after radiotherapy for clinically localized prostate cancer. The potential of DNA-ploidy for enhancing the prognostic classification of prostate cancer is evident from these studies; however, the application of such measurements clinically has been limited. Flow cytometry and image analysis, the two most common methods for quantifying DNA content, are technically demanding methods. The classification of histograms into diploid, tetraploid, and aneuploid is highly variable between investigators and not entirely objective. Some improvement in the resolution of overlapping cell populations is obtained by analyzing DNA in combination with other parameters, such as nuclear protein [17], but this adds complexity to an assay already difficult to standardize.

Immunohistochemical staining of the proliferation marker, Ki-67, has been shown to reasonably approximate the growth fraction in prostate cancers and other malignancies [19–22]. In contrast, DNA content histograms are strictly a freeze-frame of the proportion of cells distributed about the cell cycle phases. Although such histograms provide an approximation of the fraction of cells in S phase (SPF), the Ki-67 labeling index (Ki67-LI) is a more functional estimate of proliferation. The relationships between Ki67-LI and the DNA content parameters of DNA-ploidy and SPF are poorly documented for prostate cancer [23–25]. In addition, a number of reports indicate that Ki67-LI is significantly related to prostate cancer patient outcome after radical prostatectomy or androgen ablation therapy [26–32]. Preliminary results in radiotherapy-treated patients are also encouraging [33]. The purpose of this report was to explore the correlation of DNA content parameters and Ki67-LI, and to determine the relative predictive value of these factors for the outcome of patients treated with radiotherapy.

MATERIALS AND METHODS

Patient Characteristics

Sections from transurethral resection of the prostate (TURP) specimens were used for this study because the tissue requirements for MIB-1 immunohistochemical staining and DNA-ploidy by flow cytometry were beyond those of most needle biopsy specimens. There were 151 patients with prostate cancer diagnosed from TURP who were referred to the M.D. Anderson Cancer Center (MDACC) between 1987–1993. Paraffin-embedded prostatic sections were obtained from 42 of these patients. All patients were treated with definitive radiotherapy only; no patient received neoadjuvant or adjuvant androgen ablation, or underwent radical prostate surgery or surgical lymph node dissection. The workup of patients treated with radiotherapy at MDACC was described previously [34].

Pretreatment serum prostate-specific antigen (PSA) levels were determined in all patients. The median and mean pretreatment PSAs were 4.1 and 8.8 ng/ml, respectively (range, 0.3–92 ng/ml). The median and mean age was 68 years (range, 56–79 years). The median follow-up was 62 months (range, 19–121 months). The clinical stages for the study population were: stage T1 in 27 patients (64%); stage T2 in 8 (19%); stage T3 in 6 (14%); and stage T4 in 1 (2%). The Gleason scores were: 5 in 6 patients (14%); 6 in 15 (36%); 7 in 13 (31%); 8 in 3 (7%); 9 in 4 (10%); and 10 in 1 (2%).

The median external beam radiotherapy dose was 64 Gy, with a mean of 65 Gy and a range of 60–78 Gy. Radiotherapy was delivered via a four-field box with 18 MV photons, using a shrinking field technique in all but one patient, who received a conformal six-field boost after 46 Gy to a total dose of 78 Gy [34]. The dose was specified to the isocenter at 2 Gy per day. After the completion of radiotherapy, patients were followed at 3–6-month intervals with history, clinical examination, and repeat serum PSA for 2 years and then every 6–12 months thereafter.

Biochemical failure (a rising PSA profile) was defined as two or more consecutive rising PSA values following the postradiotherapy PSA nadir. The actuarial curves for the incidence of PSA rise were calculated from the average time between the nadir PSA value and the first elevated PSA value.

Immunohistochemistry Technique

The monoclonal antibody, MIB-1, was used as a proliferation marker (Immunotech, marketed by Coulter Corp., Miami, FL). The slide-mounted, paraffin-embedded prostatic tissue sections were deparaffinized in xylene before being rehydrated sequentially in ethanol (100%, 90%, and 70%) and placed in a 1% phostate-buffered solution (PBS, pH 7.4). The sections were heated in a conventional 600-W microwave oven at maximum power for 3 ×5 min. The sections were then allowed to cool to room temperature for 40 min before using 2% normal horse serum to block nonspecific protein binding. The sections were incubated with MIB-1 antibody (1:50) overnight at 4°C in a humidified chamber. Detection of the bound MIB-1 antibody involved applying the Vectastain Elite ABC reagents (Vector Laboratories, Inc., Burlingame, CA) using Avidin DH:biotin-ylated horseradish peroxidase H complex with 3,3′-diaminobenzidine (Polysciences, Inc., Warrington, PA) and Mayer's hematoxylin (Fisher Scientific, Fair Lawn, NJ). Appropriate positive controls (HeLa cells) were included in each immunohistochemical run to verify the specificity of MIB-1, and negative controls were produced by substituting the primary antibody with PBS in duplicate sections.

Tissue Specimens and MIB-1 Grading

All original TURP diagnostic material and additional sections were reviewed by the study pathologist (P.T.) and graded according to the Gleason system. Sections representative of the tumor with the highest grade were selected for immunohistochemistry. Within the selected tumor regions, random fields measuring at least 500 tumor cells (1,000 cells were counted when possible) were assessed using an eyepiece graticule at 400× magnification. Positive MIB-1 cells were counted by scoring any appropriate tumor nuclei staining, regardless of intensity. The labeling index of MIB-1 was expressed as a percentage of immunoreactive cells to the counted tumor cells. Scoring of these sections was performed by two of the investigators (V.S.K. and D.C.) without any prior knowledge of the patient data or treatment related outcomes. The mean (± SEM) Ki67-LIs for the two counts were 3.03 ± 0.43 and 3.15 ± 0.42, and were not statistically different (Student's *t*-test and Wilcoxon signed ranks test, for paired samples). The averages of these independent counts were used for the analyses.

Flow Cytometric Sample Preparation and Analysis

Paraffin-embedded tissue was prepared for flow cytometry using a method previously described in detail [16,17]. Briefly, 1–2 sections of 50 μm were deparaffinized with xylene, rehydrated with graded alcohols, and digested in pepsin, and the nuclei were isolated using nuclear isolation buffer (0.5% Nonidet P40; 0.05 M Trizma base: Trizma-HCl, pH 7.4; 0.05 M NaCl; 1 mM EDTA). The protein in the extracted nuclei was stained with flourescein isothiocyanate (FITC) and the DNA with propidium iodide (PI). Analyses of the samples were performed using an EPICS 752 flow cytometer (Coulter Electronics, Hialeah, FL), with an argon-ion laser set at 488 nm. Appropriate filters were used to resolve the green FITC signals from the red PI signals. The resultant DNA/nuclear protein histogram profiles were classified as diploid (n = 18), near-diploid (n = 9), tetraploid (n = 5), or aneuploid (n = 6), based on previously defined criteria [17]. Because the number of patients was small and we have shown [17] that the greatest differences for similar patients are between diploid/near-diploid (termed "diploid" here) vs. tetraploid/aneuploid (termed "nondiploid" here), these groups were used for the analyses described below.

As described previously, S-phase fraction (SPF) was derived from single-parameter DNA histograms using MODFIT-LT software [18]. The model corrected for single-cut debris, approximated S-phase using rectangles, and estimated G1 and G2M using Gaussians. A single composite SPF for the normal and tumor populations was determined when the DI was <1.3. In aneuploid or tetraploid cases, the SPFs of both populations were estimated, but only the aneuploid/tetraploid SPF was used in subsequent comparisons.

Statistics

The chi-square test was used to assess the significance of differences between proportions [35]. Nonparametric comparisons between independent groups were performed using the Mann-Whitney test. Actuarial curves were calculated using the Berkson-Gage method, with tests of statistical significance based on the log-rank statistic [36].

RESULTS

Table I shows the relationship of the mean Ki67-LI values to the potential prognostic factors of the study cohort. The groupings of PSA, Gleason grade, tumor stage, and DNA-ploidy were based on previous studies [16–18,34]. The mean Ki67-LI was 3.1%, with a median of 2.4% and a range of 0–12%. Significantly

TABLE I. Percent Ki-67 Staining by Various Potential Prognostic Factors

Grouping	N	% Ki-67 Mean ± SE	P*
All patients	42	3.1 ± 0.4	
Stage			
T1/T2	35	2.4 ± 0.3	
T3/T4	7	6.7 ± 1.4	0.003
Gleason score			
2–6	21	2.0 ± 0.3	
7–10	21	4.2 ± 0.7	0.017
Pretreatment PSA			
≤10	33	2.2 ± 0.3	
>10	9	6.4 ± 1.0	0.0003
DNA-ploidy			
Diploid	27	2.5 ± 0.4	
Nondiploid	11	4.7 ± 1.1	0.04
Percent S-phase			
≤2.5	14	2.9 ± 0.8	
>2.5	15	3.2 ± 0.7	0.53
Treatment failure			
No	29	2.3 ± 0.4	
Yes	13	4.9 ± 0.7	0.0009

*Mann-Whitney test; SE, standard error.

higher mean Ki67-LI values were seen with stage T3/T4 disease, Gleason score ≥7, pretreatment PSA >10, or nondiploidy. No association was seen between SPF (stratified by the median value) and Ki67-LI. Treatment failure correlated with higher Ki67-LIs.

As a categorical dichotomous variable, stratified around the median value, Ki67-LI was a correlate of palpable stage, Gleason score, and pretreatment PSA (Table II). A weaker, borderline-significant association was found with DNA-ploidy. With the exception of DNA-ploidy, these correlations were more significant when the patients were divided into thirds, based on Ki67-LI as a trichotomous variable (Table III). High Ki67-LIs above 3.5% were seen in significantly more patients with T3/T4 disease, Gleason scores ≥7, and pretreatment PSAs >10. Of the patients who failed biochemically, 62% had a Ki67-LI >3.5%.

The relationship of Ki67-LI with actuarial biochemical failure is shown in Figure 1. Ki67-LI predicted failure when used as either a dichotomous or trichotomous variable. The most significant correlation was seen with the latter (Table IV), in which no failures were evident at 4 years if the Ki67-LI was ≤1.5%, and 67% failed if the Ki67-LI was >3.5%. Only pretreatment PSA was a more significant determinant of failure. When Cox proportional hazards regression was performed, the only independent correlate of failure was pretreatment PSA.

TABLE II. Distribution of Patients by Ki-67 Staining as a Dichotomous Variable

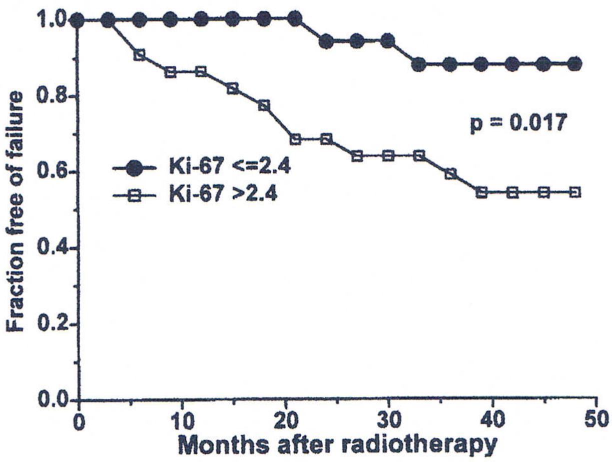
Grouping	Percent (n) patients by Ki-67[a] ≤2.4	>2.4	P*
Stage			
T1/T2	54 (19)	46 (16)	
T3/T4	14 (1)	86 (6)	0.05
Gleason score			
2–6	67 (14)	33 (7)	
7–10	29 (6)	71 (15)	0.01
Pretreatment PSA			
≤10	61 (20)	39 (13)	
>10	0 (0)	100 (9)	0.001
DNA-ploidy			
Diploid	59 (16)	41 (11)	
Nondiploid	27 (3)	73 (8)	0.07
Percent S-phase			
≤2.5	57 (8)	43 (6)	
>2.5	47 (7)	53 (8)	0.57
Treatment failure			
No	62 (18)	38 (11)	
Yes	15 (2)	85 (11)	0.005

[a]Patients were stratified by %Ki-67 staining of ≤2.4% and >2.4%.
[b]Chi-square test.

TABLE III. Distribution of Patients Stratified by Ki-67 Staining as a Trichotomous Variable

Grouping	Percent (n) patients by Ki-67[a] ≤1.5	>1.5–3.5	>3.5	P*
All				
Stage				
T1/T2	37 (13)	43 (15)	20 (7)	
T3/T4	0 (0)	29 (2)	71 (5)	0.016
Gleason score				
2–6	38 (8)	52 (11)	10 (2)	
7–10	24 (5)	29 (6)	48 (10)	0.024
Pretreatment PSA				
≤10	40 (13)	46 (15)	15 (5)	
>10	0 (0)	22 (2)	78 (7)	0.0008
DNA-ploidy				
Diploid	41 (11)	37 (10)	22 (6)	
Nondiploid	9 (1)	46 (5)	46 (5)	0.15
Percent S-phase				
≤2.5	36 (5)	36 (5)	29 (4)	
>2.5	27 (4)	40 (6)	33 (5)	0.87
Treatment failure				
No	45 (13)	41 (12)	14 (4)	
Yes	0 (0)	39 (5)	62 (8)	0.0015

[a]Patients were stratified by %Ki-67 staining of ≤1.5%, >1.5–3.5%, and >3.5%.
*Trended chi-square test.



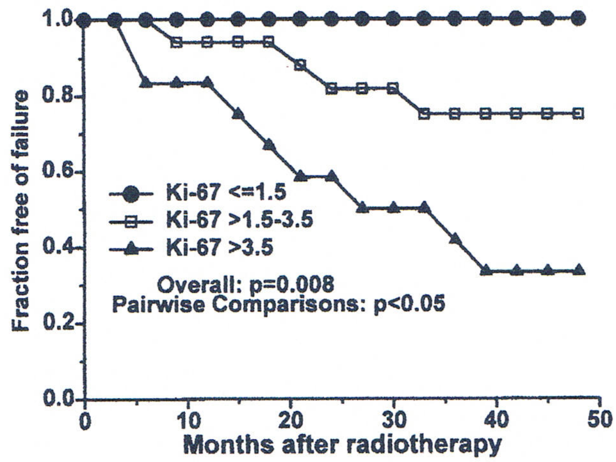


Fig. 1. Relationship of Ki67-LI as a dichotomous (**left**) and a trichotomous (**right**) variable to actuarial freedom from failure. The number of patients at risk at 24 and 48 months, respectively, were 17 and 7 for Ki67-LI ≤2.4%, 15 and 11 for Ki67-LI >2.4%, 11 and 5 for Ki67-LI ≤1.5%, 14 and 9 for Ki67-LI >1.5–3.5%, and 7 and 4 for Ki67-LI >3.5%.

TABLE IV. Correlates of Freedom From Biochemical Failure

Factor	4-year % free of failure	*P**
Stage		
T1/T2	78	
T3/T4	<29[a]	0.005
Gleason score		
2–6	90	
7–10	47	0.002
Pretreatment PSA		
≤10	86	
>10	<11[a]	<0.0001
DNA-ploidy		
Diploid	75	
Nondiploid	45	0.035
Percent S-phase		
≤2.5	78	
>2.5	62	0.46
Ki67-LI		
≤2.4	88	
>2.4	54	0.017
≤1.5	100	
>1.5–3.5	75	
>3.5	33	0.003

[a]The less-than sign indicates that the number of patients at risk at 4 years was small and the percentage shown less accurate.
*Log-rank test.

DISCUSSION

The development of MIB-1 for the staining of Ki-67 antigen in formalin-fixed tissues [37] has promoted the exploration of this marker of cell proliferation as a potential prognostic factor for patients with prostate cancer. Studies examining the relationship of Ki67-LI to other prognostic factors and patient outcome have, in general, indicated that this parameter may be useful in addition to Gleason score and stage. The majority of investigators have found that Ki67-LI correlates with tumor grade and/or stage [21,26,27,32,38–41]. In some reports these relationships were of borderline significance [28]. Rarely, no correlations with other prognostic factors were found [24]. For the cohort of TURP-diagnosed prostate cancer patients we investigated, highly significant associations between Ki67-LI and Gleason score, palpable stage, and pretreatment PSA were identified. Patients with high-risk features of Gleason score ≥7, stage T3/T4 disease, or pretreatment PSA >10 ng/ml had higher mean Ki-67-LIs and were comprised of a greater percentage with Ki67-LIs >3.5%.

The Ki67-LI is a static immunohistochemical estimate of tumor growth fraction [19–22]. Since a significant proportion of proliferating cells are in S phase, one would expect that the SPF obtained by flow cytometry would correlate with Ki-67-LI [41,42]. The data presented did not reveal any relationship between Ki67-LI and SPF. The discrepancy between Ki-67-LI and SPF is probably due in part to the difficulty in separating normal epithelial and stromal cells from tumor cells in the resultant flow cytometric DNA histograms. Although some investigators have found SPF to have potential as a correlate of patient outcome [15], we have never found SPF to be useful [16–18].

In terms of DNA-ploidy, a borderline-significant relationship with Ki67-LI was seen (Tables I–III). The 11 nondiploid tumors had a higher mean Ki67-LI. Coetzee et al. [24], Cher et al. [23], and Uzoaru et al. [25]

also reported associations between Ki67-LIs and DNA-ploidy. These results indicate that the parameters of Ki67-LI and DNA-ploidy are significantly, albeit weakly, related.

Several studies have examined the prognostic importance of Ki67-LI in patients with prostate cancer. In nearly every report, Ki-67-LI has been predictive of patient outcome in actuarial univariate analyses. While the majority have confirmed the independence of Ki67-LI as a correlate of patient outcome in multivariate analysis [26,27,28,29,32,33], others have not [24,43]. The number of patients in our study (n = 42) was inadequate to accurately assess the independence of Ki67-LI as a predictor of freedom from failure. Pretreatment PSA was the only correlate by Cox proportional regression in this series. Prior studies with larger numbers of patients have established that Gleason score, clinical stage, and DNA-ploidy are also independent correlates [16,17,34].

CONCLUSIONS

The Ki67-LI is significantly related to other prognostic factors, such as pretreatment PSA, Gleason score, and stage, and is a predictor of patient outcome. The data suggest that Ki67-LI is a stronger correlate of prostate cancer patient outcome following radiotherapy than DNA-ploidy or SPF. A Ki67-LI >3.5% was associated with a particularly poor prognosis. Prospective evaluation of pretreatment prostate tumor biopsy Ki67-LI will help to clarify the role of this potentially useful cell kinetic marker.

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