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Clinical Investigation

DNA Ploidy Measured on Archived Pretreatment Biopsy Material May Correlate With Prostate-Specific Antigen Recurrence After Prostate Brachytherapy

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Summary

Using core biopsy material form archived paraffin blocks, DNA ploidy can correctly classify the majority of failures and nonfailures. Aneuploid DNA patients had a significantly higher rate of failure (HR 5.13). "Excellent" dosimetry significantly increased the time to failure and decreased the overall failure rate among patients with aneuploid tumors but had no

Purpose: To explore whether DNA ploidy of prostate cancer cells determined from archived transrectal ultrasound-guided biopsy specimens correlates with disease-free survival.

Methods and Materials: Forty-seven failures and 47 controls were selected from 1006 consecutive low- and intermediate-risk patients treated with prostate ^{125}I brachytherapy (July 1998-October 2003). Median follow-up was 7.5 years. Ten-year actuarial disease-free survival was 94.1%. Controls were matched using age, initial prostate-specific antigen level, clinical stage, Gleason score, use of hormone therapy, and follow-up (all P nonsignificant). Seventy-eight specimens were successfully processed; 27 control and 20 failure specimens contained more than 100 tumor cells were used for the final analysis. The Feulgen-Thionin stained cytology samples from archived paraffin blocks were used to determine the DNA ploidy of each tumor by measuring integrated optical densities.

Results: The samples were divided into diploid and aneuploid tumors. Aneuploid tumors were found in 16 of 20 of the failures (80%) and 8 of 27 controls (30%). Diploid DNA patients had a significantly lower rate of disease recurrence ($P = .0086$) (hazard ratio [HR] 0.256). On multivariable analysis, patients with aneuploid tumors had a higher prostate-specific antigen failure rate (HR 5.13). Additionally, those with "excellent" dosimetry (V100 >90%; D90 >144 Gy) had a significantly lower recurrence rate (HR 0.25). All patients with aneuploid tumors and

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impact on diploid tumors. DNA ploidy may be a useful clinical marker for determining the aggressiveness of localized prostate cancer.

dosimetry classified as "nonexcellent" (V100 <90%; D90 <144 Gy) (5 of 5) had disease recurrence, compared with 40% of patients with aneuploid tumors and "excellent" dosimetry (8 of 15). In contrast, dosimetry did not affect the outcome for diploid patients.

Conclusions: Using core biopsy material from archived paraffin blocks, DNA ploidy correctly classified the majority of failures and nonfailures in this study. The results suggest that DNA ploidy can be used as a useful marker for aggressiveness of localized prostate cancer. A larger study will be necessary to further confirm our hypothesis. © 2013 Elsevier Inc.

Introduction

Prostate brachytherapy is a standard treatment for organ-confined prostate cancer (PCa). Although high rates of disease control and cure have been well documented (1, 2), these excellent outcomes come with well-documented treatment toxicity and impact on quality of life (3, 4). With widespread prostate-specific antigen (PSA) screening and increase in early disease detection, it is becoming evident that not all patients need to be treated up front. Therefore, active surveillance has become a favored approach for low-risk, low-volume disease (5, 6). Prostate cancer is a genetically heterogeneous disease (7). A reliable genetic signature of aggressive PCa as a predictor of biological behavior is still lacking, yet there is great clinical need to individualize treatment for PCa according to potential biological behavior. Individualization of treatment recommendations is still based on initial PSA level (iPSA), clinical stage, and Gleason score. It is well recognized that 5% to 10% of patients with low-risk disease treated up front with prostate brachytherapy or radical prostatectomy will have poor outcomes (1). Furthermore, more than 30% of the active surveillance patients will progress and require treatment, and 50% of those will ultimately fail the treatment (6).

Chromosome instability and genetic mutations are 2 hallmarks of cancer. It is still debated whether chromosome instability (gains or losses of chromosomes or their segments) is a cause or a consequence of genetic mutation. Large chromosomal changes often result in an increase of the DNA content of the nuclei. Increase in DNA content is an indirect measure of the sum of chromosomal abnormalities in the nuclei, likely a result of accumulated genomic alterations. Deoxyribonucleic acid ploidy is a quantitative measure of DNA in the nuclei. Diploid cells contain normal amounts of DNA, whereas all others are aneuploid (nondiploid) (Fig. 1). Tetraploid cells are presumed to be in cell division because they contain double the amount of DNA.

Deoxyribonucleic acid ploidy status, as prepared and measured according to accepted standards (8), by high-resolution DNA image cytometry, is an automated, quantitative, and objective measure of DNA quantity within the tumor specimen and can replace DNA ploidy measured using flow cytometry. There is evidence that DNA image cytometry is more sensitive in detecting small subpopulations of aneuploid cells and therefore more accurate overall in predicting disease outcomes (9). Others have used static microspectrophotometry to determine ploidy status (10, 11). Aneuploidy is a typical sign of malignancy and is often related to a poorer prognosis. Deoxyribonucleic acid ploidy has been shown to predict the therapeutic outcome in prostate (11-16) and other cancers (17, 18). Opinions differ as to whether DNA ploidy analysis is predictive of outcomes (19) and whether it could be implemented in routine clinical routine.

Over the last 10 years more than 4500 patients with low- and intermediate-risk PCa have undergone prostate brachytherapy in the province of BC. Our prostate brachytherapy program maintains a large clinical outcomes, toxicity, and dosimetry database. Using Fine and Gray's competing risks analysis, the 5-year and 10-year actuarial disease-free survival (DFS) was 96.7% and 94.1%, respectively (2). When applied to the whole cohort, none of the usual prognostic variables, including dosimetry, correlated

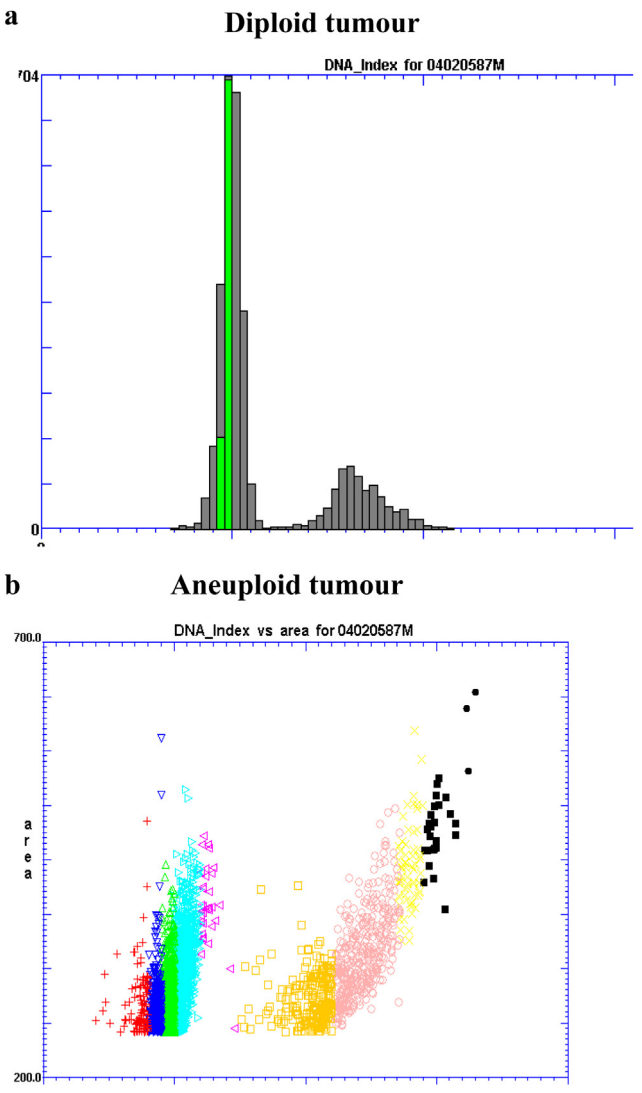

Fig. 1. Deoxyribonucleic acid histogram: scatter plot of nuclei by DNA index and area. Each ploidy group (DNA content) is marked in a different color. (a) Diploid tumor. (b) Aneuploid tumor.

with DFS. Increasing dose was the only covariate that correlated with improved DFS for the subset of men not receiving androgen deprivation therapy (ADT) ($P = .043$).

The aim of this study was to determine whether we can use DNA ploidy to predict DFS in favorable-risk PCa patients treated with low-dose-rate brachytherapy.

Methods and Materials

Patients for this study were selected from a consecutive cohort of 1006 uniformly selected low- and intermediate-risk patients treated with prostate brachytherapy between July 20, 1998 and October 23, 2003 (2). Median follow-up was 7.5 years. From this cohort we selected 47 of 49 patients (96%) with known failure and 47 controls (2 patients failed more recently and were not included in this analysis). Cohorts were matched using age, iPSA, clinical stage, Gleason score, use of hormone therapy, and length of follow-up. Control patients were not matched for dosimetry or percent positive cores. In fact a deliberate bias in favor of controls with relatively large percent positive cores was used, to ensure an adequate amount of tissue for ploidy determination. The site of first recurrence was established in 19 of 49 patients, including 8 who had clinical and/or histologic evidence of local relapse (only 4 biopsy proven) and 11 who had lymph node or distant metastatic relapse (2). Of the 94 specimens requested, 78 (83%) were received from pathology departments across the province and were successfully processed.

A pathologist outlined the tumor region on each of the hemotoxylin and eosin slides, which was then matched with the paraffin block. Four-micron-thick sections were cut from each patient's block, and one slide was stained with hemotoxylin and eosin. Tumor was removed using a scalpel and transferred into a nylon pouch. The tissue underwent deparaffinization and rehydration and enzymatic incubation (pepsin at 37°C). Each sample was shaken until the cells from the dissolving tissue diffused into the solution. Using a cytospin centrifuge, the cells were deposited as a monolayer onto a slide. The nuclei were quantitatively stained using the Feulgen-Thionin method and were scanned. Independent files were created for each scanned slide, and an in-house classifying tree was used to separate the nuclei into a DNA content groups. All cells from all files were manually verified by an experience cytotechnician. The DNA content of each individual cell was determined by measuring their integrated optical densities. Of the 78 specimens successfully processed, 27 controls and 20 failures contained more than 100 tumor cells, and these were used for the final analysis.

Controls were defined as those who had no biochemical, clinical, or radiologic evidence of recurrent or persistent PCa and had received no secondary treatment for PCa. Biochemical failure was defines using the Phoenix definition of PSA failure (nadir PSA +2). Because of the small number of patients, those with V100 >90% (V100 = volume of the prostate covered by 100% of the dose) and D90 of 100% to 125% (D90 = dose covering 90% of the prostate) were considered to have "excellent" dosimetry. All others were considered to have a "nonexcellent" dosimetry.

The sample was divided into diploid tumors and aneuploid tumors. The distribution of patient characteristics with respect to DNA ploidy and DNA determination was compared by Pearson χ^2 for Gleason score, tumor stage, and preimplantation androgen suppression. The t test was used for age, baseline PSA level, and positive cores, and Wilcoxon rank sums were used for dosimetry quality assurance (QA) categories. Time to PSA failure was estimated using the Kaplan-Meier method. Univariate comparisons of time to PSA failure were tested with the log−rank test. Multivariate Cox proportional hazard modeling was done to examine whether DNA content was of prognostic value after adjusting for characteristics associated with PSA failure. The SAS statistical package (SAS Institute, Cary, NC) was used for data analysis. The BC Cancer Agency Research Ethics Board reviewed and approved the project.

Results

Median follow-up is 7.5 years. There was no statistically significant difference in the distribution of pretreatment characteristics between patients with and without ploidy determinations (data not shown) or between diploid and aneuploid patients (data not shown). Age, iPSA, clinical stage, Gleason score, use of hormone therapy, and length of follow-up between failures and controls were well matched (t test, all P values nonsignificant). The control group had better dosimetry than PSA failures; however, there were no statistically significant differences when QA code was assigned (excellent vs nonexcellent dosimetry) (P value nonsignificant). For all 97 patients, mean V100 was 93.5% for controls and 90% for failures ($P = .0057$, t test). Mean D90 (%) was 110.5% for controls and 102.7% for failures ($P = .0014$, t test). Table 1 shows pretreatment characteristic for 20 failures and 27 controls, with DNA ploidy determined. Again, controls had better dosimetry than failures. Mean V100 was 93.8% for controls and 89.6% for failures, ($P = .0141$, t test). Mean D90 (%) was 111.9% for controls and 102% for PSA failures ($P = .0100$, t test), but there were no differences when QA code was assigned. The control group had a larger proportion of cores involved with cancer (t test, $P = .0021$). This was a consequence of deliberate bias by the investigators, in an effort to provide more tissue for analysis. Aneuploid tumors were found in 16 of 20 failures (80%) and 8 of 27 controls (30%) (Table 1).

The Kaplan-Meier curve (Fig. 2) shows the time to PSA failure by DNA ploidy for the 47 patients for whom ploidy was determined. Patients with diploid DNA had a significantly lower rate of PSA failure (log−rank test, $P = .0086$) than those with aneuploid tumors. Univariate Cox proportional hazard regression analysis hazard ratio (HR) was 0.256 (95% confidence interval [CI] 0.086-0.767) for diploid compared with aneuploid DNA patients ($P = .0149$).

Univariate analysis shows that DNA ploidy status ($P = .0086$) and dosimetry (V100, $P = .0008$; D90 [%], $P = .0001$) are the only significant factors for predicting the failure. Cox proportional hazards regression analysis was used in multivariable analysis (MVA) of failures for the 47 patients with DNA ploidy determinations. Covariates with a statistically significant association with failure—ploidy, dosimetry, and positive cores—were included in MVA modeling. Table 2 shows adjusted Cox proportional hazard regression results, whereby dosimetry was analyzed by QA codes ("excellent" vs "nonexcellent" dosimetry) and separately by D90 (as a continuous variable). Patients with aneuploid DNA tumors had a higher PSA failure rate (HR 5.13, 95% CI 1.61-16.30), and patients with higher D90% or excellent QA code experienced lower rates of PSA failure (HR 0.93, CI 0.89-0.97) and (HR 0.25, CI 0.09-0.72) respectively.

Table 1 Pretreatment characteristics for failures vs controls with known DNA ploidy (n=47)

Factor	PSA failure	Contorl	P*
Total	20	27	
Age (y)			.1523
Median, IQR	68.5, 65-70.5	65, 62-68	
Mean, SD	67.4, 5.5	65.1, 4.8	
Range	58-78	58-77	
Gleason score, n (%)			.5279
4-6	10 (50)	16 (59.3)	
7	10 (50)	11 (40.7)	
Clinical stage, n (%)			.6147
T1	6 (30)	10 (37)	
T2	14 (70)	17 (63)	
PSA (baseline)			.0592
Median, IQR	6.8, 5.3-8.6	7.1, 6.3-10	
Mean, SD	6.7, 2.1	7.9, 2.2	
Range	1.8-9.8	4-12	
Preimplant AS			.9051
No	7 (35)	9 (33.3)	
Yes	13 (66)	18 (16.7)	
Cores, % positive	n=16	n=27	.4714
Median, IQR	50, 33-67	50, 38-67	
Mean, SD	50, 18	55, 20	
Range	25-88	17-100	
V100	n=19	n=26	.0141
Median, IQR	91.4, 87.0-94.4	94.3, 92.2-96.8	
Mean, SD	89.6 (7.1)	93.8 (4.0)	
Range	69.1-97.9	82.0-99.1	
D90, %	n=19	n=26	.0100
Median, IQR	102.8, 95.3-108.8	112.0, 105.6-119.8	
Mean, SD	102.3 (11.5)	111.9 (11.9)	
Range	77.4-127.7	80.9-141.4	
Dosimetry QA codes,† n (%)	n=19	n=26	.1546
Excellent	13 (68.4)	22 (84.6)	
Good	2 (10.5)	3 (11.5)	
Suboptimal	3 (15.8)	1 (3.9)	
Poor	1 (5.3)	0	
Ploidy, n (%)	n=20	n=27	
Aneupolid	16 (80)	8 (30)	
Diploid	4 (20)	19 (70)	

Abbreviations: AS = androgen suppression; D90 = dose covering 90% of the prostate; IQR = interquartile range; PSA = prostate-specific antigen; QA = quality assurance; V100 = volume of the prostate covered by 100% of the dose.

* *t* test for age, PSA, cores, and V100 and D90; Wilcoxon rank sum tests for dosimetry QA; χ^2 test for all other factors.

† QA codes: Excellent: V100 ≥90%, D90 ≥100%-125% (145-180 Gy); good: V100 >85%-90% and D90 >90%-100% (130-145 Gy); suboptimal: V100 75%-85% and D90 80%-90%; poor: V100 <75% and D90 <80%.

Dosimetry

Both V100 and D90 were available for 91 patients. The *t* test was used to test for differences in mean dosimetry levels between controls and failures and between diploid and aneuploid patients. Controls showed a statistically significant higher mean D90 and V100 (mean D90 = 112% and V100 = 94%) than failures (mean D90 = 101.5% and V100 = 91%) (D90 P=.0014, V100 P=.0057). Neither measure showed a statistically significant difference between diploid and nondiploid patients. The distribution of dosimetry by QA codes in patients with aneuploid DNA (n=23) was 18 excellent, 2 good, 2 suboptimal, 1 poor; for diploid DNA patients (n=22) it was 17 excellent, 3 good, 2 suboptimal, 0 poor.

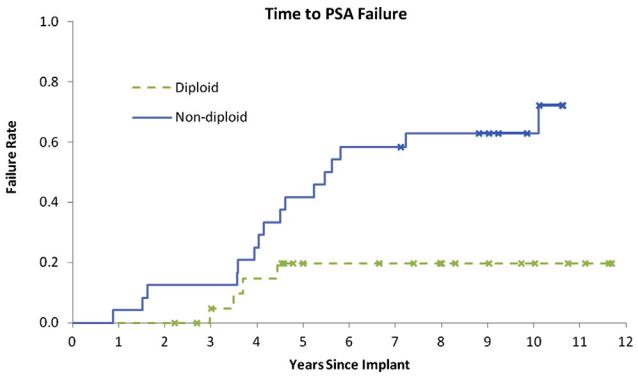

Fig. 2. Time to prostate-specific antigen (PSA) failure in 47 patients (20 failures and 27 controls) by DNA ploidy. Log−rank test, P=.0086.

Kaplan-Meier Curves of time to PSA failure in DNA aneuploid tumors (Fig. 3) shows a faster failure for patients with "non-excellent" dosimetry (log−rank test, P=.0007). Seven years after implant, 100% of aneuploid patients (5 of 5) with "nonexcellent" dosimetry (V100 <90% and D90 <100%) experienced failure. In patients with excellent dosimetry, only 44.4% (8 of 15) failed. In contrast, dosimetry seemed to make less difference in outcomes for diploid patients; 20% of patients (4 of 17) with diploid DNA tumors and "excellent" dosimetry relapsed to date, whereas 25% of patients (1 of 5) with "nonexcellent" dosimetry have relapsed (log−rank test, P=.9120) (Fig. 4).

None of the controls and none of the diploid DNA patients died. The 10-year overall survival and cause-specific survival for diploid patients was 100% and 100% and for aneuploid DNA patients was 96% and 96%, respectively.

Discussion

This study shows that measuring DNA ploidy has the potential to predict PSA failure after prostate brachytherapy. The number of patients in this study is small, and a larger study is needed to further investigate this hypothesis. However, to our knowledge, this has never been reported before. Of 47 failures and 47 controls, we were able to complete the DNA ploidy analysis on 20 failures and 27 controls, because only samples with >100 nuclei were included in the analysis. Most of the failures had aneuploid DNA (80%), and most of the controls had diploid DNA (70%). With 8 years median follow-up, 70% of the aneuploid DNA patients and only 20% of the diploid DNA patients had PSA relapse. Deoxyribonucleic acid ploidy and dosimetry were the only factors associated with higher risk of failure after prostate brachytherapy. Even though the number of patients was small, all patients (5 of 5) (100%) with aneuploid DNA tumors and "nonexcellent" dosimetry failed, compared with only 44% (8 of 15) if their dosimetry was "excellent." In contrast, for diploid patients, dosimetry did not influence the failure rate.

Table 2 Adjusted Cox proportional hazard regression for failures

Model	Correlates	Hazard Ratio	95% CI	*P**
Model 1 (n=45)	DNA ploidy (nondiploid vs diploid)	4.33	(1.40-13.41)	.0111
	Dosimetry QA* (not excellent vs excellent)	0.25	(0.09-0.72)	.0104
Model 2 (n=45)	DNA Ploidy (diploid vs aneuploid)	5.13	(1.61-16.30)	.0056
	D90† (as a continuous variable)	0.93	(0.89-0.97)	.0004

Abbreviation: CI = confidence interval. Other abbreviations as in Table 1.

* QA codes: Excellent: V100 ≥90%, D90 ≥100-125% (145-180 Gy); nonexcellent: V100 <90% and D90 <100%.

† Covariates V100 and D90 are highly correlated, so they were not included in multivariable analysis models simultaneously. Modeling was done with each successively. Both covariates were highly significant (data not shown).

The failure group had more suboptimal implants than controls. During the initial study design, analysis of PSA outcome for the entire cohort of 1006 patients did not find any dose–response relationship; for that reason, failures and nonfailures were not matched according to dosimetry. Our conclusions regarding the relationship between dosimetry and disease failure are applicable only to this small cohort but are not confirmed in an original 1006-patient cohort (2), because controls in this study are not representative of the population of all nonrelapsing patients.

It is of interest to mention that patients with aneuploid tumors relapsed relatively quickly after treatment (Fig. 3), implicating 2 possible mechanisms: either occult metastatic disease is more often present at presentation with aneuploidy, or early metastatic disease results from poor local control in aneuploid tumors owing to relative radioresistance (2).

Investigators from Oslo have reported postprostatectomy outcomes in 186 men with median follow-up of 73 months: 52% were diploid, 33% tetraploid, and 16% aneuploid. During the observation time, 23% of the diploid, 36% of the tetraploid, and 62% of the aneuploid cases had PSA relapse. In that study DNA ploidy and Gleason score were the only factors predictive of failure on multivariate analysis (HR 2.8 and 0.48, respectively). Among 68 Gleason score 7 cases, DNA ploidy was the only significant predictor of disease recurrence (12). Others also reported significantly higher risk of failure after radical prostatectomy for high-risk PCa, in patients with nondiploid tumors (HR 1.85 on MVA) (20). Pollack et al (13) reported a retrospective study assessing the predictive value of DNA ploidy determined in patients treated with RT alone versus RT plus 4 months of neoadjuvant and concurrent ADT, as part of Radiation Therapy Oncology Group protocol 8610. On MVA, patients with aneuploid tumors had shorter overall survival, which seemed to have been related to a reduced response to salvage hormone therapy for those previously exposed to short-term ADT (13). Isharwal et al (14) reported on 270 prostatectomy patients from John Hopkins Hospital, investigating DNA ploidy prognostic utilization as a predictor for differentiating between organ- and non-organ-confined tumors. When objectively measured, DNA ploidy was able to completely replace biopsy Gleason scores (subject to significant interobserver variability) for organ- versus non-organ-confined PCa predictions (14). Ahlgreen et al (21) found that DNA ploidy analysis with a proliferation index derived from DNA cytometry of imprints from core needle biopsies correlates with Gleason score ($P<.0001$), T stage ($P=.006$), M stage ($P=.009$), and disease progression ($P<.0001$). Our study showed that only approximately 30% of patients with low and low-tier intermediate risk have aneuploid tumors. A large population-based study of more than 60,000 patients showed that more than 50% of high-risk patents may in fact have aneuploid DNA (22). Taken together, these studies confirmed application of DNA ploidy as a predictor of PCa outcomes.

The most obvious limitation of our study is the small number of patients. This is due to a very low rate of PSA failure in our cohort (overall 5%). Therefore, conformation of the results will be necessary using a larger patient cohort. Only samples with >100 nuclei were included in the analysis. Deoxyribonucleic acid ploidy requires an adequate volume of tissue. Our study could only utilize the "left over" tissue available in archived tissue blocks. Original slides used for diagnosis were not used for this analysis. It is probable that a much larger number of biopsies will be suitable for DNA ploidy determination if included as part of the original pathology testing. Haggarth et al (23) caution that biopsies underestimated ploidy in 9 of 20 tumors (45%) with heterogeneous ploidy status, occurring when only 1 or 2 cores were analyzed. Analysis of multiple biopsies is important for accurate preoperative ploidy estimation.

Prostate cancer is genetically heterogeneous (7). The status of DNA ploidy represents a "bird's-eye view" of most accumulated genetic changes in the DNA. Our analysis suggests that cancers with aneuploid DNA have greater genetic alterations and a more aggressive biological behavior. The question remains whether knowledge of the ploidy status of a PCa before treatment with brachytherapy would select for a more aggressive treatment strategy. Furthermore, whether DNA ploidy as measured by automated high-resolution DNA image cytometry can separate newly diagnosed patients into groups that need immediate treatment and groups that are appropriate for active surveillance is

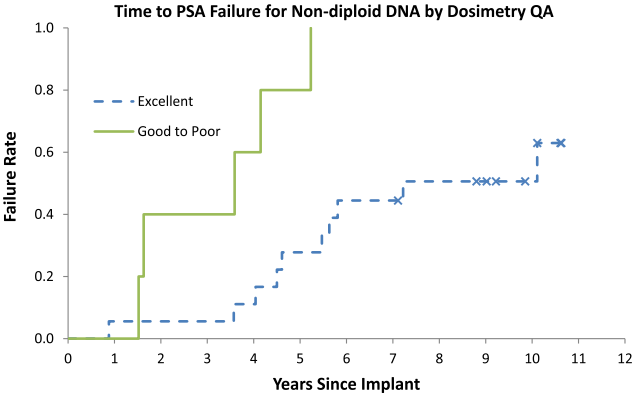

Fig. 3. Time to prostate-specific antigen (PSA) failure for aneuploid DNA patients (n=20) by dosimetry quality assurance (QA) codes. Patients with "nonexcellent" (good to poor) dosimetry (n=5) and patients with "excellent" dosimetry (n=15). Log–rank test, $P=.0007$.

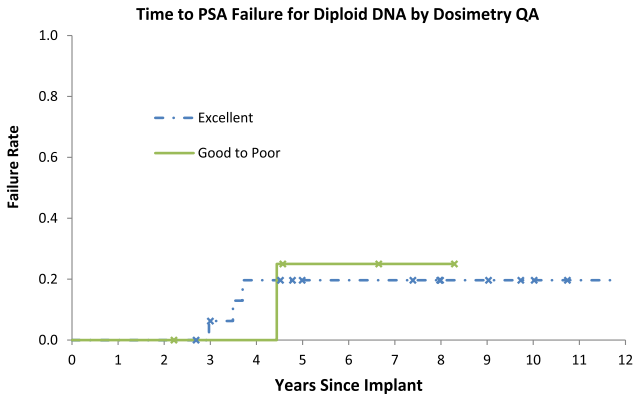

Fig. 4. Time to prostate-specific antigen (PSA) failure by dosimetry quality assurance (QA) for diploid DNA patients (n=22); patients with "nonexcellent" (good to poor) dosimetry (n=5) and patients with "excellent" dosimetry (n=17). Log−rank test, $P=.9120$.

another question that needs further research. Some authors suggest that this may be possible (24).

Last, tissue microarrays have been developed to address the problem of limited patient tissue samples and the cumbersome nature of tissue preparation. With tissue microarrays up to 1000 separate tissue cores imbedded in the paraffin blocks allow for histologic and gene analysis. Our work in progress focuses on investigating utility of image analysis to define specific nuclear morphometry features associated with poor outcome to assist management options in the early stages of PCa.

To summarize, using core biopsy material from archived paraffin blocks, DNA ploidy can correctly classify the majority of failures and nonfailures. Aneuploid DNA patients had a significantly higher rate of failure (HR 5.13). "Excellent" dosimetry significantly increased the time to failure and decreased the overall failure rate among patients with aneuploid tumors but had no impact on diploid tumors. Deoxyribonucleic acid ploidy may be a useful clinical marker for determining the aggressiveness of localized PCa.

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