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Androgen receptor antigen density and S-phase fraction in prostate cancer: *a pilot study*

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Purpose: To determine whether quantitative flow cytometric androgen receptor density expression (MFC ratio) in prostate cancer was associated with S-phase fraction.

Methods: Flow cytometry was performed to determine DNA aneuploidy, S-phase fraction, percentage of androgen receptor (AR)-positive cells, and MFC ratio in prostate cancer patients.

Results: MFC ratio showed distinct clustering. Eight patients had a low MFC ratio of 1.78–2.74, while 10 patients had high MFC ratios between 4.99 and 6.48. The S-phase fraction had average values of 11.05 *vs* 4.92 in tumors with high *vs* low MFC ratio ($P < 0.01$).

Conclusion: S-phase fraction was significantly higher in tumors with high AR density.

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Keywords: antigen density; androgen receptors; S-phase fraction

Abbreviations: PSA, prostate-specific antigen; T stage, primary tumor stage per American Joint committee TNM recommendations; H&E, hematoxylin and eosin; FBS, fetal bovine serum; IgG, immunoglobulin G; MFC, mean channel fluorescence; DFS, disease free survival; AR, androgen receptors; GS, Gleason score; TTR, time to relapse; MAb, monoclonal antibody

Introduction

Stimulation of growth in response to androgens, as well as inhibition in the absence of androgens has long been recognized as a unique feature of prostate cancer. The underlying mechanisms of action that lead to hormone refractory disease have been less understood.

What is known is that the androgen receptor (AR) is a regulator of DNA transactivation and cellular growth[1] and its upregulation has been implicated in the pathogenesis of prostate cancer.[2] The length of the CAG trinucleotide repeat on the transcription activation domain is associated with activity, with an increase in length being associated with a decrease in activity.[3] Upregulation of AR protein may also be involved in prostate cancer progression or recurrence. Men with prostate cancer, who were at a low risk for recurrence by conventional prognosticators, were found to have a relative risk of recurrence of 8.07 if they had 18 or less CAG repeats.[6] Several authors have reported on the role of AR expression as a prognostic marker for response to hormone therapy, time to tumor progression, and as an indicator of tumor aggressiveness.[7–24] A review of the literature on clinical studies involving AR expression shows a wide variation in tissue sampling, methods used for estimation of AR expression and correlation of the data.[7–24] Biochemical methods for quantitation of receptor expression in cytosolic tissue homogenates (ligand binding assays) cannot distinguish between AR expression of the tumor cells and that of the nonmalignant epithelial and stromal cells. Immunohistochemical methods are, in general, not quantitative.

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Flow cytometric methods for multiparametric analysis of hormone receptor expression and DNA content in formalin-fixed/paraffin-embedded human breast and prostate tumors have been developed.[25,26]. These methods allow for rapid estimation of the percentage of cells with receptor expression, as well as determination of antigen density. These techniques measure nuclear AR, which is important since total AR binding and nuclear AR binding significantly predict time to progression while cytosolic AR binding does not predict time to relapse.[22] The additional advantage to this technique is the ability to measure S-phase fraction and ploidy, which have been found to have prognostic value in several publications.[32–36] ARs may stimulate proliferation through their mechanism of action. However, it is not known whether high AR density correlates with a higher S-phase fraction, with its adverse prognostic implications. The purpose of this pilot study was to determine if S-phase fraction correlates with AR density.

Patients and methods

Clinical methods

The pathologist (CM) systematically determined the availability of paraffin blocks with sufficient tissue for flow cytometry in patients with localized, nonmetastatic prostate cancer diagnosed between December 1994 and September 1995. AR data obtained from samples from 19 patients included in the study will be described in this report. Specific inclusion criteria used included the presence of pathologically confirmed adenocarcinoma of the prostate, completion of definitive treatment in the form of radical or modified radical prostatectomy, and the presence of paraffin-embedded blocks with sufficient tissue for study. Exclusion criteria included presence of clinically detectable metastatic disease at initial diagnosis, insufficient clinical data, or inadequate amount of tissue in the archived paraffin blocks for the study.

Clinicopathologic data were retrieved including date of diagnosis and surgery, Gleason score, T stage, pathologic T stage, number and percentage of positive cores, margin status, initial and nadir psa, date of nadir psa and of psa increase, date of clinical evidence of local recurrence or metastases, and site of metastases.

AR expression was then measured in the archived paraffin-embedded tissue in the manner described below.

Laboratory methods

Isolation and immunostaining of nuclei from paraffin blocks. A H&E-stained slide was used to confirm and select areas with predominant tumor tissues. Sections (50 μm thick) were dewaxed and processed for enzyme digestion and antigen retrieval as described earlier in prior publications.[25,26] First, the sections were cut, deparaffinized and rehydrated in a descending ethanol/water series followed by two washes in distilled water. For nuclear isolation, deparaffinized sections were digested in pepsin (0.05% in normal saline, pH 1.65) for 60 min at 37°C. After 60 min of incubation, samples were vortexed every 5 min for an additional 30 min. The proteolytic reaction was terminated by the addition of chilled 10% FBS in PBS. The resulting digest was filtered through a 40 μm nylon mesh (Small Parts Inc., Miami, FL, USA), washed in PBS and centrifuged at 200 *g* for 10 min. Antigen unmasking was achieved by heating of the nuclear suspension in citrate buffer for 15 min at 90°C in a water bath. For AR staining, nuclei were incubated with 150 : 1 of anti-AR monoclonal antibody (MAb, F39.4.1 BioGenex, San Ramon, CA, USA) at 1 : 25 dilution (in PBS) for 2 h at 37°C. Processing and flow analysis were performed as described in our recent publication.[26] Negative isotype control used for anti-AR antibody was normal mouse IgG (Sigma, St Louis, MO, USA), adjusted to the same concentration as anti-AR MAb. Following incubation with the primary MAb, cells were washed 2 × with 2 ml 0.1% Triton X-100 and stained with 150 l of fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody (Sigma) at 1 : 80 dilution (in PBS) for 35 min at 37°C. After incubation, the cells were washed with 3 ml 0.1% Triton X-100 and centrifuged at 200 *g* for 10 min. The pellets were resuspended in 1 ml PBS for flow cytometric analysis.

For simultaneous monitoring of AR expression and nuclear DNA content, propidium iodide (PI, final concentration: 25 μg/ml+RNase 0.5 mg/ml) was added to the AR-FITC-stained samples for 15 min at 37°C.

Flow cytometry. For routine two-color flow cytometry, we used a Coulter XL/*mcl* cytometer equipped with an argon laser to record the AR-FITC and DNA-PI fluorescence. Data were collected as a list mode. The percentage of AR-positive cells in a tumor sample was determined by the following method. WinMidi software program obtained from the internet (Joseph Trotter, Scripps Institute, La Jolla, CA, USA) was used for gated analysis. It was used to exclude 95% of fluorescent cells from the isotype controls and then copy these gates on the antibody-treated samples to determine the percent of AR-positive cells. Antigen density was calculated after measuring mean log fluorescence channel (MFC). MFC was determined for the isotype and the antibody-treated samples and the ratio of the two used as a measure of antigen density. ModFit program (Verity Software House) was used for determination of aneuploidy and S-phase fraction (SPF).

Statistical analysis

Scatter plots and summary statistics were used to examine the distribution of the percent of AR-positive cells in a sample (by WinMidi gated analysis) as well as the distribution of MFC ratio, which measured the antigen density of the samples. We examined the strength of association between these variables by computing linear correlation coefficients and, where data showed distinct clustering, testing for differences between clusters. Relationships between the AR variables and patient characteristics were examined with linear correlation coefficients, *t*-tests, or Fisher's exact test according to whether the variables of interest were continuous or dichotomous.

Disease-free survival (DFS) was calculated by the product limit method of Kaplan and Meier.[27] DFS was defined as time from surgery to recurrence or last follow-up. Deaths from noncancer causes were treated as censored observations. We examined prognostic factors using the Mantel–Haenszel test (dichotomous variables) and the likelihood ratio test in a univariate Cox proportional hazards model (continuous variables). The factors considered included the two measures of AR expression as well as the following variables: age, primary pathologic T stage, Gleason score, presurgery psa, margin status, number of positive cores, and S-phase fraction.

Results

AR expression

Distribution. The distributions of the two measures of AR expression are summarized in Table 1. Overlay histograms (of the isotype and the anti-AR mAB stained nuclei) demonstrate the differences in AR measurements in specimens from three different patients using the WinMidi method (Figure 1). The distribution of MFC ratio showed distinct clustering with a gap between the clusters that was wider than the range of either one. Specifically, eight patients had an MFC ratio in the range from 1.78 to 2.74, while another 10 patients had MFC ratios between 4.99 and 6.48. Thus, the remainder of our analysis was carried out with AR density characterized as high (MFC ratio ≥ 4.99) *vs* low (MFC ratio ≤ 2.74).

Associations between AR measurement parameters. Average percent of AR-positive cells as measured by the WinMidi method was 85.6 in the 10 tumors with high MFC ratio compared with an average of 67.3 in the eight tumors with low MFC ratio; the difference is statistically significant ($P<0.01$). Thus, a higher percent of AR-positive cells was associated with higher AR density. In addition, the group of 10 high-density tumors exhibited less variability with respect to percent AR-positive cells than did the eight low-density tumors (standard deviation 5.5 *vs* 12.6, $P=0.03$). These findings are shown in Table 1 and Figure 2.

SPF and AR expression. SPF as determined by the ModFit software was found to range from 2.2 to 17.4 (median 6.6, mean 8.0, s.d. 4.6). S-phase measurements were not significantly correlated with percent of AR-positive cells (WinMidi: $R=0.39$; $P=0.10$). S-phase was associated with density as measured by MFC ratio with significantly higher S-phase values observed in tumors with high MFC ratio as compared to those with low MFC ratio (average S-phase fraction—11.05 *vs* 4.92; $P<0.01$; Table 3). All but one patient had diploid tumors. Thus, ploidy could not be investigated further in this study.

With regard to AR density (MFC ratio), which was available in 18 of the studied patients, we found that patients in the high-MFC cluster were on the average older than those in the low-MFC cluster and that they also had a higher average Gleason score, pSA, and S-phase value. Only the difference in average S–phase value was statistically significant (Table 3).

As regards survival, two patients were known to have died by the time of the last follow-up. One of these patients died of metastatic transitional cell carcinoma of the renal pelvis without recurrence of prostate cancer. The cause of death was unknown in the second patient. Their deaths occurred after follow-up periods of 4 years, 10 months and 1 year, 8 months. The analysis of overall survival was not possible due to the small number of deaths.

Recurrence status was known for 17 patients, including one who died of noncancer causes. The Kaplan–Meier estimates for the probability of remaining disease-free for 1, 2, and 5 years were 0.94 (95% CI: 0.82–1.0), 0.88 (95% CI: 0.71–1.0), and 0.75 (95% CI: 0.54–0.96), respectively (Figure 3).

Status of the margins was significantly associated with disease recurrence ($P=0.05$). The data also suggest that stage is a poor prognostic factor because all six instances

Table 1 AR levels

AR level	Patients	Range	Median	Mean	s.d.
% AR-positive cells (WinMidi)	19	54.66–91.03	80.75	77.31	12.64
MFC ratio—all patients	18	1.78–6.48	5.04	4.16	1.91
Low MFC cluster	8	1.78–2.74	2.14	2.14	0.36
High MFC cluster	10	4.99–6.48	5.85	5.77	0.57

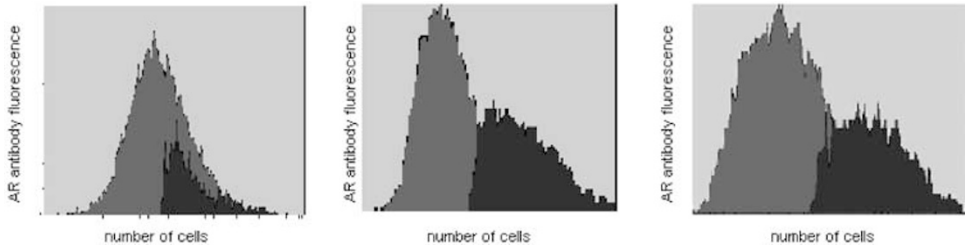

Figure 1 *Overlay histograms of the isotype (light) and the anti-AR mAB-stained nuclei (dark) with the lowest (26%), intermediate (45%), and the highest (60%) percent of AR-positive cells (WinMidi method).*

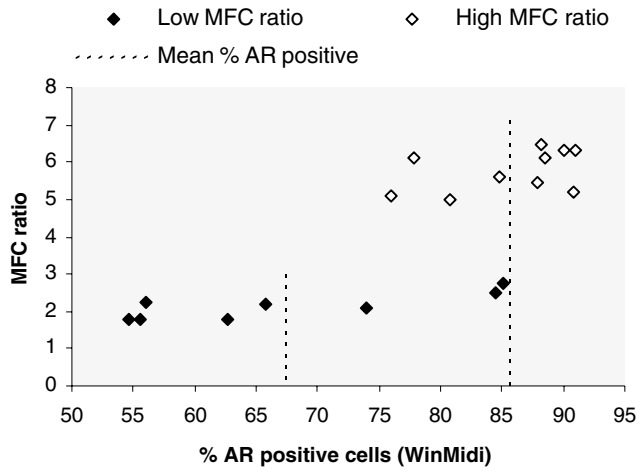

Figure 2 *AR level measurements.*

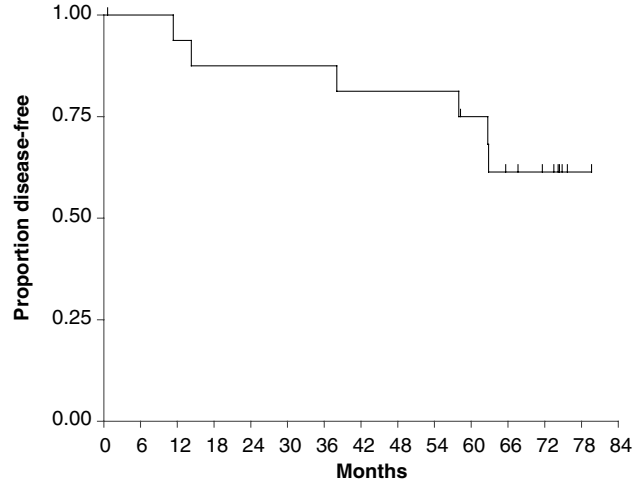

Figure 3 *Disease-free survival in prostate cancer patients.*

of disease recurrence were observed in patients with stage T3 disease ($P<0.01$). No association was found between disease recurrence and any of the remaining known prognosticators (described in Table 2), or with either of the two AR measurements.

Discussion

In this report, we describe the patterns seen on flow cytometric measurement of AR expression in 19 patients with localized prostate cancer. The investigation of quantitative levels and patterns of androgen receptors in prostate cancer, using this technique, has not been reported previously. Two distinct measures of AR positivity are the percentage of positive cells and the amount of AR within each cell. The measurement of percentage of cells that express ARs is a general gauge of the presence of ARs in the tissue and is used to quantitate AR in studies using IHC techniques.[29] However, the measure of the amount of AR positivity in each cell, or density, by MFC ratio may point to a distinct subgroup of cells that may have upregulated ARs.

Table 2 Characteristics of 19 men treated for prostate cancer with prostatectomy

	Number (%)
Age at surgery (years) (median 64)	
54–59	4 (21)
60–64	6 (32)
65–69	8 (42)
70–74	1 (5)
pT-stage	
1, 2	9 (50)
3, 4	9 (50)
Unknown	1
Gleason score (median 7)	
5–6	5 (28)
7	7 (39)
8–9	6 (33)
Unknown	1
PSA (mean 9.9, median 10.4)	
Less than 5	4 (24)
5 to less than 10	4 (24)
10 to less than 15	6 (35)
15 or higher	3 (18)
Unknown	2
Nodal status	
Negative	15 (79)
Unknown	4 (21)
Margins	
Positive	7 (39)
Negative	9 (50)
Close	2 (11)
Unknown	1
Number of positive cores	
1	8 (53)
2 or 3	7 (47)
Unknown	4
% involved	
0–14%	4 (25)
15–29%	3 (19)
30–44%	5 (31)
45–100%	4 (25)
Unknown	3
Risk group	
1	3 (17)
2	6 (33)
3	5 (28)
4	4 (22)
Unknown	1

Table 3 Association of AR MFC with continuous covariates

	Average by AR density		
	Low	High	*P*-value
Age	62	64	0.38
Gleason score	6.75	7.33	0.28
S-phase	4.92	11.05	<0.01
Presurgery psa	7.41	11.26	0.20

This upregulation may be due to a mutation, such as in progressive hormone insensitive prostate cancer cells, or due to stimulation by androgens or other possible substrates. Thus, the two AR measures may actually assess separate parameters. The heterogeneity of ARs has been reported to be of greater value than AR positivity alone.[30] This improved predictive value may be related to the ability of heterogeneity measures to identify such

Table 4 Association of AR level with dichotomous covariates*

	% AR-positive cells (WinMidi)		
	Pts.	Average	*P*-value
Age 65 to 74 *vs* age 54 to 64	9, 10	74.19 *vs* 80.12	0.32
Stage T3 *vs* T2	9, 9	75.93 *vs* 77.16	0.84
Gleason score $\geqslant 8$ *vs* $\leqslant 7$	6, 12	83.90 *vs* 72.87	0.03
Presurgery PSA >5 *vs* $\leqslant 5$	13, 4	77.79 *vs* 70.53	0.34
Positive *vs* negative margins	7, 9	79.18 *vs* 76.47	0.67
2–3 positive cores *vs* 1	8, 7	80.03 *vs* 72.08	0.28
Risk group 3–4 *vs* 1–2[a]	9, 9	80.01 *vs* 73.09	0.25

[a]Risk group 1 patients had a Gleason score (GS) of 2–6 and a stage of T1–2Nx. Group 2 had a GS of 2–6 and T3Nx; or GS of 2–6 and positive nodes (N+); or GS=7 and a stage of T1–2Nx. Group 3 had T3Nx and GS=7; or N+ and GS=7; or T1-2Nx and GS=8–10. Group 4 patients had T3Nx and GS=8–10; or N+ and GS=8–10.
*P-values refer to a two-sided t-test for equal means; variances were first tested for equality.

abnormal groups of cells, but not to actually quantify them. Quantification of AR density is possible with this technique. Upregulated ARs may or may not be functional, depending on the factors precipitating upregulation, such as exposure to androgens or genetic mutations.

The high levels of AR positivity that ranged from 55 to 91% (WinMidi method) are consistent with that in the literature, where 85% of prostate cancer had high androgen expression as defined as over 50%.[31] Of greater interest is the presence of two distinct groups or *clusters* of patients, one with high MFC ratio and another with low MFC ratio—with a definite gap between the two groups. Thus, two clearly distinguishable subsets were present.

The overexpression of AR has been described in more aggressive, hormone-insensitive prostate cancer. This may be due to point mutations or amplification of the AR gene.[29] The current technique may allow us to detect more aggressive tumors that are undergoing an early transformation to hormone insensitivity, or that may be more aggressive or may need different adjuvant treatments like chemotherapy.

Of note is that these aggressive tumors, in general, were found to also exhibit elevated S-phase values in various published studies.

Both S-phase fraction (5% or greater) and aneuploidy were associated with high tumor grade,[32,33] advanced stage,[34] large prostate size, and presence of distant metastases.[35] Higher SPF is associated with shorter overall survival,[36] psa failure,[37] time to progression.[36,38] Others found that S-phase (<4.2 *vs* ≥ 4.2) was a significant predictor of local control in patients with well or moderately differentiated tumors (100 *vs* 51%, $P=0.03$).[39] Visakorpi *et al* demonstrated that high SPF predicted short 10-year progression-free survival ($P=0.0002$), overall survival ($P<0.0001$), as well as prostate cancer survival ($P<0.0001$). In their study, patients with SPF above 12% had 0/7 endocrine responses as compared to 26/49 responses in patients with an SPF of $<12\%$.[35] Whether the higher AR density was related to upregulation of AR in certain cells that have undergone AR mutations to become hormone resistant, remains to be seen. Of interest is that recurrent tumors with AR gene amplification have a significantly higher ($P=0.02$) S-phase fraction (14 ± 6.5) than those with no amplification (9 ± 2.9).[40]

In this study, S-phase was significantly associated with density as measured by MFC ratio. Higher S-phase values were observed in tumors with high MFC ratio as compared to those with low MFC ratio (average S-phase fraction—11.05 *vs* 4.92; $P<0.01$; Table 3).

This significant association between S-phase measurements and AR density, as measured by MFC ratio, was interesting. The increase in AR in low-grade tumors as measured by immunohistochemistry is not quantitative and is a better measure of percentage[29] rather than intensity of staining. The fact that AR density is related to S-phase fraction, which has been shown in prior studies[32–36] to be associated with poor prognostic features and outcome, may indicate that the significance of AR density may be different from that of the percentage of AR-positive cells. For example, the amount of ARs per cell may be increased due to the presence of nonfunctioning AR with upregulation, a mutation leading to sensitivity of AR to other substrates or simply increased AR due to androgenic stimulation leading to more division.

The results of our literature search did not reveal any reports describing any correlation between AR expression and S-phase. Thus, the current study is the first to study of the relationship between AR and S-phase. The fact that the majority of AR research was carried out using immunohistochemical techniques makes correlation with S-phase difficult, since this would require a separate effort through the flow cytometry lab and such practical constraints.

Correlation with other factors or outcome, although explored in this study, was limited by our small sample size and will be examined in a future study with more tumor samples.

As for the correlation of AR measurements with higher Gleason score, we found significantly different average values of WinMidi measure of percentage of AR-positive cells when we analyzed Gleason score dichotomously, with Gleason 8 as the cutoff. Such averages were not used in the same manner in other studies, which makes comparisons difficult, as regards Gleason score. For example, Takeda *et al*[19] studied 62 patients with stage D2 disease on hormonal therapy and reported that patients with $>48\%$ AR-positive cells had a significantly better outcome (progression-free survival and cause-specific survival). However, they studied a different patient population that received hormonal treatment for stage

D, and AR levels may have been affected differently by the treatment as compared to our population. Upregulation of AR by androgen deprivation has been described.[41]

Henshall *et al*[42] reported that patients with higher pretreatment psa had overexpression of AR in tumor cells. A higher AR expression in patients who presented with higher pretreatment psa was observed in the current study, although the small number of patients in this study may have contributed to the lack of significance.

Although the main purpose of this study was to determine the *patterns* of AR expression in these patients, we also explored a possible prognostic role for AR expression with respect to disease recurrence. There is evidence in the literature regarding the prognostic value of AR measurements in patients with prostate cancer.[7–24] The small number of patients in this pilot study results in limited power to detect significance. Even some well-known prognostic factors, such as Gleason score, were not significantly associated with recurrence in our data. In the literature, AR positivity (above 10 %) has been shown to influence endocrine response ($P = 0.03$), progression ($P = 0.0016$), and survival ($P = 0.002$).[12] Since the minimum value of WinMidi-gated AR measurement in our patient population was 54.66%, we could not carry out a similar analysis based on a cutoff of 10%. Furthermore, the differences in technique may not allow for a valid comparison between the studies.

Variance of AR staining intensity within a certain specimen was found to discriminate between poor responders with a short time to relapse (TTR) and good responders (long TTR).[15] Quadrimet image analysis can be used to analyze nuclear immunostaining into four receptogram patterns of AR expression that correlate with response to treatment.[18] The different technique is a limiting factor in our attempt to compare their findings with those from our study.

The finding, by these authors, that *variance of AR intensity* and *patterns of AR expression* were significant predictors of response to therapy is important in the interpretation of our results. In our study, there were distinct groups of high and low AR density, as measured by MFC. These groups may, in fact, represent areas of heterogeneity on immunohistochemical analysis, where actual percent of cells expressing AR may be constant, while the amount per cell varies. These differences would be better reflected by flow cytometry density measurements and would be lost if measurement of the percent of positive cells were the only parameter for the evaluation. The fact that flow cytometry allows quantification of the amount per cell, or antigen density (MFC) is an important advantage to this technique. The presence of two distinct groups and the significant correlation with S-phase fraction is interesting and should be tested for clinical significance in future studies.

Transcriptional integrity of the AR is another confounding factor. A possible approach in future studies would be measurement of the number of CAG repeats in tandem with absolute AR levels. Studies exploring the effect of number of CAG repeats on outcome have shown correlation with outcome.[6,23] Some studies suggest that shorter CAG repeats are associated with a worse overall prognosis[10,11] and likelihood of developing a biochemical response.[23]

In order to address the questions raised by the current study, a new study is being undertaken using specimens from the RTOG 86-10 study. This study is a randomized phase III study composed of two arms. The first consisted of local radiation alone and the second included hormonal ablation in conjunction with the radiation.[43]

Conclusion and future direction

The prostate tumors studied had two distinct levels of AR density, and significantly higher S-phase values were found in tumor samples characterized by high AR density. Further study of a larger number of evaluable patients will be carried out to determine whether tumors that differ in AR density measured by flow cytometry have correspondingly different responses to hormonal manipulation or outcome.

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