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Analytic Validation of RNA *In Situ* Hybridization (RISH) for AR and AR-V7 Expression in Human Prostate Cancer

Liana B. Guedes¹, Carlos L. Morais¹, Fawaz Almutairi¹, Michael C. Haffner¹, Qizhi Zheng¹, John T. Isaacs³, Emmanuel S. Antonarakis^{2,3}, Changxue Lu³, Harrison Tsai¹, Jun Luo³, Angelo M. De Marzo^{1,2,3,*}, and Tamara L. Lotan^{1,2,*}

¹Pathology, Johns Hopkins University School of Medicine, Baltimore, MD

²Oncology, Johns Hopkins University School of Medicine, Baltimore, MD

³Urology, Johns Hopkins University School of Medicine, Baltimore, MD

Abstract

Purpose—RNA expression of androgen receptor splice variants may be a biomarker of resistance to novel androgen deprivation therapies in castrate resistant prostate cancer (CRPC). We analytically validated an RNA *in situ* hybridization (RISH) assay for total AR and AR-V7 for use in formalin fixed paraffin embedded (FFPE) prostate tumors.

Experimental Design—We used prostate cell lines and xenografts to validate chromogenic RISH to detect RNA containing AR exon 1 (AR-E1, surrogate for total AR RNA species) and cryptic exon 3 (AR-CE3, surrogate for AR-V7 expression). RISH signals were quantified in FFPE primary tumors and CRPC specimens, comparing to known AR and AR-V7 status by immunohistochemistry and RT-PCR.

Results—The quantified RISH results correlated significantly with total AR and AR-V7 levels by RT-PCR in cell lines, xenografts and autopsy metastases. Both AR-E1 and AR-CE3 RISH signals were localized in nuclear punctae in addition to the expected cytoplasmic speckles. Compared to admixed benign glands, AR-E1 expression was significantly higher in primary tumor cells with a median fold increase of 3.0 and 1.4 in two independent cohorts ($p < 0.0001$ and $p = 0.04$, respectively). While AR-CE3 expression was detectable in primary prostatic tumors, levels were substantially higher in a subset of CRPC metastases and cell lines, and were correlated with AR-E1 expression.

Conclusions—RISH for AR-E1 and AR-CE3 is an analytically valid method to examine total AR and AR-V7 RNA levels in FFPE tissues. Future clinical validation studies are required to determine whether AR RISH is a prognostic or predictive biomarker in specific clinical contexts.

To whom correspondence should be addressed: Tamara Lotan, MD, 1550 Orleans St CRB2 Rm 343, Baltimore, MD 21231, (410) 614-9196 (ph), tlotan1@jhmi.edu.

*Co-senior authors

Conflicts of Interest: ESA, CL and JL are co-inventors of a related technology that has been licensed to Tokai Pharmaceuticals.

Keywords

Prostatic adenocarcinoma; androgen receptor; androgen receptor splice variants; RNA in situ hybridization

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Introduction

Primary hormone-sensitive and most castration resistant prostate cancers (CRPC) require signaling through the androgen receptor (AR) for survival (1). Despite the pivotal role of the AR in prostate tumor progression, it remains unclear whether total or full length AR expression at the protein or RNA level is a prognostic or predictive biomarker in either primary prostate cancer or CRPC (2–10). In part, this may be due to the variable immunostaining protocols and RNA quantification methods employed in prior studies with diverse clinical endpoints. In addition to the full length canonical AR, recent work has identified a number of structurally and functionally distinct splice variants of the AR mRNA in prostate cancer cells (11–17). Many of these variants code for truncated androgen receptor proteins, where the entire ligand-binding domain is replaced with a short variant-specific peptide coded by a transcribed intronic sequence (i.e., cryptic exon). Though these splice variants are generally only expressed at a fraction of the level of full length AR (AR-FL), they are capable of signaling independent of ligand binding, and may represent an important cellular mechanism mediating castration resistance (14, 18–24).

Of these variants, AR-V7, where the ligand binding domain is replaced by a peptide encoded by cryptic exon 3 (CE3), is one of the most commonly expressed and one of only a few splice variants proven to be expressed at the protein level (12, 13). AR-V7 is generally expressed at very low but detectable levels in primary tumors (by RT-PCR in bulk tumors), but increased by approximately 20 fold in CRPC (12). In primary tumors, the clinical significance of AR-V7 expression remains unclear, with one study showing it correlated with poor prognosis and another finding that it did not (12, 25). Importantly, RNA expression of AR-V7, as detected by RT-PCR in circulating tumors cells (CTCs), correlated with resistance to abiraterone and enzalutamide, novel forms of androgen deprivation therapy (ADT) currently used in the modern treatment of CRPC (26). This drug resistance appears to be specific for ADT, since levels of AR-V7 in CTC do not predict outcomes to taxane-based chemotherapies (27–30). AR-V7 RNA levels are generally correlated with AR-FL RNA expression, suggesting that one or both may be a useful predictive biomarker for response to novel ADT in CRPC (12, 26). However, the clinical success of enzalutamide and abiraterone has moved these treatments earlier in the course of CRPC (1), when only a fraction of patients may have adequate numbers of CTCs to study. Moreover, even in patients with advanced high-burden disease, CTCs are not always detected. Thus a validated tissue-based method that may be applied to routinely obtained biopsy samples to query AR-V7 status is an area of unmet clinical need.

Though much work is underway to develop immunohistochemistry-based assays to detect AR-V7, many of the currently available antibodies remain suboptimal (31). The recent emergence of highly sensitive RNA in situ hybridization (RISH) methodologies for use in

formalin fixed paraffin embedded (FFPE) tissues has revolutionized our ability to examine RNA expression in tissues (32). The novel RISH assays provide a higher level of target sensitivity and specificity when compared with many immunostaining protocols (33). The increased specificity is achieved by requiring two separate oligonucleotides (Z-pairs) to hybridize near each other in order to generate a signal, and the increased sensitivity is achieved by having a series of these Z-pairs bind to the target as well as having a branched DNA amplification step prior to enzymatic amplification (32). Previously, we reported preliminary data on the development of a RISH assay for AR-FL and AR-V7 expression in metastatic CRPC tissues that correlated with AR-V7 RT-PCR results in a small sample of CRPC cases from the previous study that was focused primarily on CTCs (26). Here, we extend this work to present a more comprehensive analytical validation of these assays and to quantify expression of AR-FL and AR-V7 across a wide spectrum of primary prostate tumors and CRPC specimens. We show that RISH is a sensitive and specific method for querying AR-FL and AR-V7 status and may be useful in future clinical trials seeking to validate these potentially predictive biomarkers in CRPC.

Materials and Methods

Tissue selection

Tissue collection protocols were approved by the Johns Hopkins School of Medicine Institutional Review Board. Transrectal ultrasound (TRUS) guided needle biopsies containing high grade (n=17, Gleason 9–10) were randomly selected from the surgical pathology archives of the Johns Hopkins Hospitals between 2011 and 2014. Recent radical prostatectomy specimens were assessed on standard slides (n=5) or on tissue microarrays (TMA) where primaries were divided into low grade (Gleason score 3+3=6 or 3+4=7; n = 9) and high grade (Gleason score 4+3=7 or higher; n = 9). Tissue from 2–3 castrate resistant prostate cancer (CRPC) metastases were obtained from each of 4 recent rapid autopsies performed at the Johns Hopkins Hospitals on TMA. One of these autopsies has been recently published as a case report (34) and two others were included in our previous AR-V7 study (26). Finally, tissue was obtained from an additional 12 biopsies of metastatic CRPC in living patients starting treatment with abiraterone, enzalutamide or taxane chemotherapy.

Patient derived xenografts

Formalin fixed paraffin embedded (FFPE) LuCaP patient-derived xenografts were kindly provided by the University of Washington researchers. The LuCaP xenograft lines have been previously described and consist of prostate tumor tissues obtained from radical prostatectomies or rapid autopsies and serially passaged after grafting subcutaneously or in the subrenal capsule of 6–8 week old male SCID mice (35).

RNA in situ hybridization (RISH)

RNA in situ hybridization (RISH) was performed to detect the total AR RNA level and AR-CE3 using the ACD RNAscope 2.0 Brown kit (Advanced cell Diagnostics, Hayward, CA) as previously described (26). ACD target probes, a series of paired oligonucleotides forming a binding site for a preamplifier, were custom designed to detect RNA corresponding to exon 1 of the human AR (ACD 401211), or the cryptic AR exon 3 sequence that is part of the

mature human AR-V7 mRNA transcript (ACD 401221). In some experiments, probes for peptidylprolyl isomerase B (cyclophilin B, PPIB) were used as a positive control (ACD 313901). Formalin-fixed paraffin-embedded (FFPE) tissue or cell pellet blocks were sectioned and the slides baked for one hour at 60°C. The slides were subsequently deparaffinized with xylene for 20 min at room temperature, and allowed to air dry following two rinses using 100% ethanol. Following a series of pretreatment steps, the cells were permeabilized using protease to allow probe access to the RNA target. Hybridization of the probes to the AR RNA targets was performed by incubation in the oven for 2 hours at 40°C. Following two washes, the slides were processed for standard signal amplification steps per manufacturer's instructions.

Immunohistochemistry (IHC) for AR protein

TMA sections were deparaffinized, rehydrated and briefly equilibrated in water. Antigen unmasking was performed by steaming in HTTR (Target Retrieval Solution; DAKO, Glostrup, Denmark) for 50 minutes. Endogenous peroxidase activity was quenched by incubation with peroxidase block for 5 minutes at room temperature. Non-specific binding was blocked by incubating in 1% bovine serum albumin in Tris-HCl pH 7.5 for 20 minutes at room temperature. Slides were incubated with an N-terminal-specific, rabbit polyclonal anti-human AR antibody (Clone N-20, Santa Cruz Biotechnology, Santa Cruz, CA; 1:1000 dilution) for 45 minutes at room temperature. A horseradish peroxidase-labeled polymer (PowerVision, Leica Microsystems, Bannockburn, IL) was applied for 30 minutes at room temperature. Signal detection was performed using 3,3'-diaminobenzidine tetrahydrochloride (DAB) as the chromogen. Slides were counterstained with hematoxylin, dehydrated, and mounted.

RISH and IHC Image Quantification

For image quantification, two different methods were used: Aperio software for standard tissue sections and TMAJ software for TMAs. For standard slides, including all biopsies and cell line pellets, as well as all TMAs, slides were scanned at 40x magnification on the Aperio Scanscope AT Turbo. Quantification on standard slides was performed with the Aperio Digital Pathology software. For each sample, a mean of 6 areas (range: 1–11) of benign, non-atrophic epithelium with minimal intervening stromal tissue were selected for analysis. Similarly, a mean of 8 areas of tumor glands (range: 4–16) with minimal intervening stromal tissue were selected for analysis. Image quantification was performed for each case by applying an automatic quantification algorithm that measures blue (negative) and brown (positive) pixels within a previously defined range (See Figure 5B for example of processed image). The algorithm consists of a positive pixel counter that quantifies a positive pixel according to a specified color (range of hue and saturation). Brown pixels with a hue value of 0.1, a hue width of 0.5 and color saturation of at least 0.03 were considered positive. Negative stained pixels or blue pixels were the ones that did not meet the hue/saturation limits and have intensity less than lower limit of intensity for weak-positive pixels. The positivity, or percent of positive pixels, is the number of positive pixel divided by the total number of pixels in each section (positive plus negative pixels), of the tumor areas and the benign areas was measured for each slide for AR-E1 or AR-CE3 RNA expression by RISH or AR protein expression by IHC. In addition, for AR IHC, an H-score

equivalent for each case was calculated by the sum of: number of weak positive/total number positive (NWP/NTotal) x 100 + the number of positive/total number positive (NP/NTotal) x 200 + number of strong positive/total number positive (NSP/NTotal) x 300.

For TMA sections, AR RISH analysis was performed with Frida TMAJ software (an open-source software using ImageJ available at <http://tmaj.pathology.jhmi.edu/>) on an independent sample set of metastases from autopsies, radical prostatectomy specimens or cell line pellets scanned at 20x on an Aperio Scanscope AT Turbo scanner. Representative tumor areas and non-atrophic benign glands from several cores were selected for each patient. All 8 malignant cores and all 4 benign cores available for each cases were utilized for analysis. Areas of benign or malignant glands with minimal intervening stroma on each core were circled for analysis (example image in Figure 4B). The positivity was determined for each tumor and non-atrophic benign glands areas using a similar positive pixel counter algorithms, considering the brown pixels as positive and the blue pixels as negative.

RT-PCR for AR-FL and AR-V7

Quantitative RT-PCR analysis for full length AR and AR-V7 in metastatic CRPC specimens was carried as described previously. For each tested sample, 5–10 cryosections of 6 µm thickness were prepared from frozen metastatic CRPC tissue blocks. Total RNA was extracted and purified by TRIZOL (Invitrogen, Carlsbad, CA) followed by RNeasy Mini Kit (Qiagen, Valencia, CA). Briefly, 1mL TRIZOL was added into the tube containing cryosections. Following repeated vortexing, 200 µl chloroform was added to the dissolved tissue sections. The mixture was vortexed and centrifuged at 13,000 rpm x 10 min at 4°C. The top aqueous layer was carefully transferred to a new tube and mixed with an equal volume of 70% ethanol. The mixed lysate was onto RNeasy Mini Spin Column to further purification by RNeasy Mini Kit according to manufacturer's instructions. The quality of total RNA was ascertained by RNA 6000 Nano kit on Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). The RNA concentration was determined by checking UV absorbance at 260 nm on spectrophotometer (Nanodorp, Wilmington, DE). Reverse transcription (RT) (500 ng input total RNA) was performed using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. The ensuing quantitative PCR (qPCR) was performed using 1x iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), 400 nM forward primer and 400 nM reverse primers on CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Primer sequences are RPL13A (control gene), full length AR (AR-FL), and AR-V7 are as follows:

RPL13A-forward: 5'-CCTGGAGGAGAAGAGGAAAGAGA-3'

RPL13A-reverse: 5'-TTGAGGACCTCTGTGTATTTGTCAA-3'

AR-FL-forward: 5'-CAGCCTATTGCGAGAGAGCTG-3'

AR-FL-reverse: 5'-GAAAGGATCTTGGGCACTTGC-3'

AR-V7-forward: 5'-CCATCTTGTCGTCTTCGGAAATGTTA-3'

AR-V7-reverse: 5'-TTTGAATGAGGCAAGTCAGCCTTTCT-3'

Detection of Nuclear and Cytosolic AR RNA in Cell Lines

Nuclear and cytosolic RNAs were extracted from LNCaP and LAPC4 cells using the PARIS fractionation kit (Life Technologies). Extracted RNAs were used as a template for cDNA synthesis using random primers (Life Technologies). AR transcript levels were analyzed by real-time PCR (Biorad SYBR Green Master Mix, Biorad) with primers spanning the intron-exon junction of exon 1 of the AR (unspliced, F: GGTGAGCAGAGTGCCCTATC, R: CAGGGGCAATCTGAGTGTTC) or flanking intron 1 (primers anchored in exon 1 and exon 2) (spliced, F: GGTGAGCAGAGTGCCCTATC, R: ACCCAGAAGCTTCATCTCCA). The transcript abundance in the nuclear and cytosolic fractions was normalized to the transcript abundance in unfractionated (total input) samples.

Bioinformatic Analysis of RNA-seq data from GSE50630

Reads spanning selected AR junctions were identified and counted in the RNA-seq data of CRPC samples from GSE50630 (36), which was depleted of ribosomal RNA but not enriched for polyA transcripts. Since this dataset sequenced 200–300bp ds-cDNA fragments using paired-end reads, 600bp regions surrounding each junction based on AR transcript models from GENCODE Release 24 were constructed. Raw fastq reads were aligned against these regions using Bowtie2, keeping only concordantly mapped read-pairs whose alignments spanned across at least 30bp surrounding a junction, with no more than 2 mismatches and at most a 1bp insertion per individual read. Reads were also identified containing 30bp junction sequences exactly, which produced similar read counts, and we confirmed that these 30bp sequences did not align to other areas of the genome.

Results

AR-E1 and AR-CE3 levels by RISH correlate with AR and AR-V7 levels by RT-PCR and IHC in cell lines and LuCaP xenografts

We previously showed that AR-E1 and AR-CE3 RISH results from cell lines were generally concordant with AR-FL and AR-V7 levels by RT-PCR (12). For analytic validation of the assays, we reproduced these results using a biological replicate subset of the same lines and image quantified these results using Frida (Figure 1) and Aperio image analysis software (data not shown). To examine the specificity of the assay, we included PC3 and DU145 cells, which were largely negative for AR-E1 and AR-CE3 expression with only faint intranuclear signals. This was expected since these cell lines express only very low or undetectable levels of AR at the RNA and protein level (12, 37, 38) (Figure 1A, 1B). Additional controls, including untransfected, AR-negative M12 and HeLa cells also showed undetectable AR-E1, however when transfected with AR constructs, AR-E1 was detectable by RISH (Supplementary Figure S1A, S1B). Across AR-positive cell lines, both AR-E1 and AR-CE3 RISH signals were detected as cytoplasmic speckles and intranuclear punctae. Of note, the intranuclear punctae for both AR-E1 and AR-CE3 were most commonly seen as one or two punctae per nucleus, and were almost invariably present adjacent to the nuclear envelope (Figure 1A). The intranuclear punctae increased in intensity across cell lines with known increasing expression of AR-E1 and AR-CE3 by RT-PCR (Figure 1A). Similar intracellular RISH signal distribution was seen across tissues (see Figures 2, 3, 4, 5, Supplementary

Figure S1A). The intranuclear punctae were not seen with other positive control gene probes, such as PPIB (cyclophilin B) (Supplementary Figure S2A).

To exclude the possibility that the intranuclear signals represented cross-reaction of the RISH probe with DNA, treatment of primary tumor samples with intranuclear AR-E1 (Supplementary Figure S2B) or AR-CE3 (data not shown) signals with RNase prior to RISH was conducted. RNase pre-treatment abrogated both intranuclear and cytoplasmic RISH signals, confirming that these intranuclear signals represent RNA. Because the RISH probes do not span a splice junction and could theoretically detect both spliced and unspliced transcripts containing AR-CE3, we cannot exclude the possibility that these intranuclear punctae represent nascent or partially spliced pre-mRNAs. Indeed, qRT-PCR analysis of LNCaP and LAPC4 cells performed on nuclear and cytosolic fractions of RNA revealed detectable unspliced AR RNA in the nuclear compartment of both cell lines (Supplementary Figure S2C).

Using Frida to quantify the RISH signals across cell lines (this quantification does not discriminate between intranuclear and cytoplasmic signals), there was a high correlation between AR-E1 expression and known AR-FL mRNA and AR protein levels in all lines. LAPC4, LNCaP, CWR22rv1 and VCaP cells were increasingly positive for AR-E1, correlating with the known increasing expression of AR-FL and AR protein across this series of cell lines (12) (Figure 1A, 1B). Similarly, there was a significant correlation between AR-E1 and AR-CE3 expression in these lines ($R^2=0.991$, $p=0.0004$; Figure 1B). VCaP showed substantially higher expression of AR-CE3 compared to LNCaP (Figure 1B), similar to what has been previously demonstrated by RT-PCR (12).

Next we examined expression in the LuCaP series of patient derived xenografts. LuCaP 49, a small cell carcinoma that lacks androgen receptor expression at the RNA or protein level (39) was negative for AR-E1 and AR-CE3, further supporting the specificity of the RISH assay (Figure 1C). In two series of parental hormone sensitive and castrate resistant (CR) xenografts (LuCaP 96 and 35), relative AR-E1 and AR-CE3 expression was roughly correlated with previously reported results for AR protein and AR-V7 protein and mRNA by RT-PCR (12). As expected, both CR xenografts showed higher expression of AR-E1 and AR-CE3 compared to the parental. Of note, levels of AR-E1 and AR-CE3 were quite low in the parental xenografts overall.

As additional evidence of the specificity of the RISH assays, we examined a prostate needle core biopsy containing concurrent AR-positive and AR-negative prostate cancer (40). In the same contiguous biopsy core, AR-E1 was easily visible as both cytoplasmic speckles as well as intranuclear punctae in the AR protein-expressing tumor component (Figure 2, arrows). Of note, AR-E1 expression was dramatically lower in the AR protein-negative tumor component, where it was not detectable in the cytoplasm and only detectable as barely visible intranuclear punctae. AR-CE3 was present in only small intranuclear punctae in both tumor components. Murine prostate tissue provided an additional negative control for AR-E1 RISH because the RISH probes were designed for the human sequence (Supplementary Figure S1C). Overall, we conclude that RISH for AR-E1 and AR-CE3 is generally

analytically validated versus RT-PCR for AR-FL and AR-V7, respectively, in cell lines and xenografts.

AR-E1 and AR-CE3 expression correlate with AR-FL and AR-V7 mRNA levels by RT-PCR in metastatic CRPC specimens

To provide further analytic validation of the RISH assay in tissue specimens, we next examined metastases from 4 recent autopsy cases of CRPC. For two of these cases (A002 and A003), a single metastasis from each case had been previously qualitatively analyzed by RISH (26). Here, we image quantified AR-E1 and AR-CE3 RISH on FFPE samples of 2–3 metastases from each of 4 autopsy cases and correlated with RT-PCR performed on fresh metastatic tissue from the same location. Relative RISH results (normalizing all tissues to the A001 liver metastasis sample) were generally similar between different metastases sampled from the same case, both for AR-E1 and AR-CE3, suggesting relatively little inter-metastatic variation in AR expression at the time of autopsy (Figure 3). The same was true for RT-PCR. One notable exception was the paraaortic lymph node from A003, which showed dramatically lower AR-E1 and AR-CE3 expression compared to the liver metastasis sampled from this case (Figure 3). However, inspection of the FFPE section of the lymph node used for RISH did not demonstrate any viable tumor cells, likely explaining the RT-PCR results which varied dramatically from the RT-PCR results for the liver metastasis in this case. However, additional larger studies in earlier stage disease are required to formally address whether tumor heterogeneity could limit the utility of the RISH assay.

Providing further analytic validation for the assay, quantified RISH for AR-CE3 and the RT-PCR for AR-V7 levels were significantly correlated ($R^2=0.539$, $p=0.02$). Similarly, quantified RISH for AR-E1 and RT-PCR for AR-FL levels were significantly correlated ($R^2=0.444$, $p=0.03$) (Supplementary Figure S3). A notable exception to the high correlation between RISH and RT-PCR was case A004, where RISH results for AR-E1 and AR-CE3 were relatively high, and RT-PCR results were quite low for both the liver metastasis and the residual prostate tumor in this case (Figure 3, Supplementary Figure S3). In fact, removal of case A004 from the regression plots increased the R^2 for the AR-E1 RISH vs AR-FL RT-PCR to 0.709 ($p=0.009$) and the R^2 for the AR-CE3 RISH vs AR-V7 RT-PCR to a remarkable 0.984 ($p<0.00001$). The cause for the discrepancy in this case remains unclear, however it is notable that both the liver and prostate tumor tissues from A004 showed a predominant intranuclear punctate labeling for the AR-CE3 RISH probes, with relative less cytoplasmic labeling. This raises the possibility that much of the AR-CE3 RISH signal detected in this case might be nascent or unspliced RNA (41, 42), which would not be detected by RT-PCR which uses primers to span splice sites for AR-V7. Indeed, RNAseq re-analysis of a recently reported set of 8 CRPC samples (36) confirmed frequent reads of AR-CE3 within unspliced transcripts of AR that were present at a similar level as spliced AR transcript. Most of these cases did not express mature AR-V7 RNA, consistent with the fact that all were pre-abiraterone/enzalutamide (Supplementary Table S1). Taken together, these data suggest that detection of unspliced, intranuclear RNA is an important potential pitfall of the RISH assay that does not span splice junctions and thus does not distinguish spliced and unspliced transcripts.

Though digital image analysis generally shows much less inter-observer variability than analyzing intensities by eye, operator-dependent issues such as circling the tumor area and setting the thresholds for analysis could introduce error. To examine inter-observer variability in RISH scoring, we had a second, independent operator circle tumor areas and perform the scoring for AR-E1 and AR-CE3 in Frida across a subset of the autopsy samples (Supplementary Figure S4). In one experiment, the second operator made their own thresholds for brown and blue intensity scoring and in another experiment, the second operator used the intensity thresholds set by the first operator. In both cases there was negligible variability between operators, with highly correlated values across all specimens.

AR-E1 and AR-CE3 expression are correlated and generally higher in primary tumors compared to intermingled benign glands

Having analytically validated the RISH assays across 6 cell lines, 5 xenografts and 10 CRPC metastases from autopsy, we next examined expression of AR-E1 and AR-CE3 by RISH in primary tumors and intermingled benign glands sampled on radical prostatectomy (n=18) or transrectal ultrasound guided needle biopsy (n=17) (Figures 4A and 5A, respectively). Both AR-E1 and AR-CE3 RISH showed the expected cytoplasmic speckles and nuclear punctae. The nuclear punctae were seen in tumors as well as in benign glands for both AR-E1 and AR-CE3 and were more noticeable when overall expression levels were low, as was the case for most of the AR-CE3 signals. Next, we quantified AR-E1 and AR-CE3 expression levels in benign and malignant glands in radical prostatectomy specimens using Frida (Figure 4B) and in needle biopsy specimens using Aperio software (Figure 5B). AR-E1 and AR-CE3 expression levels were correlated with one another in both benign and tumor tissues, as measured in radical prostatectomy specimens quantified by Frida ($R^2=0.778$; $p<0.0001$; Figure 4C) and needle biopsy specimens quantified with Aperio software ($R^2=0.368$; $p=0.01$; Figure 5C).

In a number of cases, we noticed that AR-E1 expression appeared substantially lower in non-atrophic benign glands compared to surrounding tumor glands (Figure 4A, 5A), particularly in high Gleason grade cases. To quantify this, we compared mean AR-E1 and AR-CE3 expression in tumor glands to that in admixed non-atrophic benign glands in radical prostatectomies (Figure 4D) and needle biopsies (Figure 5D). In radical prostatectomies of all grades, AR-E1 expression was slightly higher in tumor glands compared to non-atrophic benign (median fold difference = 1.4), though this result was only weakly significant ($p=0.04$ on matched pair T-test) and more apparent for high grade (Gleason 4+3=7 and higher; n=9) tumors compared to low grade (Gleason 3+3=6 and 3+4=7; n=9) tumors. For AR-CE3, there was a non-significant but similar trend ($p=0.07$). To test whether higher relative expression of AR message in tumor compared to non-atrophic benign glands was more prominent among high Gleason grade tumors, we examined 17 Gleason score 9–10 tumors on needle biopsy. Interestingly, among these high grade tumors, there was a highly significant increase in AR-E1 and AR-CE3 expression among tumor glands compared to matched intermingled non-atrophic benign glands (median fold difference = 3.0; $p<0.0001$ for AR-E1) (Figure 5D).

To examine whether AR-E1 expression level correlated with AR protein expression by IHC, we quantified AR IHC in tumor and benign glands from the high grade biopsies using Aperio image analysis software (Supplementary Figure S5). AR IHC was positive in almost all benign and tumor nuclei as expected, and by visual inspection, there were some cases with higher AR protein expression in tumor glands compared to intermingled non-atrophic benign glands (Figure 5A). On quantification with Aperio software to examine the percent positive pixels by IHC or RISH, there was a weak correlation between AR IHC and AR_E1 RISH expression across benign and tumor glands in these cases ($R^2=0.352$; $p=0.01$; Supplementary Figure S5A), though there was a small but statistically significant increase in IHC staining in tumor compared to benign ($p<0.0001$ on matched pair t-test, Supplementary Figure S5B). Similar results were obtained using a modified H-score to take into account intensity of staining on IHC (Supplementary Figure S5C).

Quantified AR-E1 and AR-CE3 expression is substantially higher in CRPC metastases and cell lines compared to benign prostate tissue and primary tumors

Though we studied autopsy-derived metastatic CRPC specimens for analytic validation of the RISH assays, these late-stage tissues may not be representative of the dynamic range of expression of AR message in earlier stage metastatic CRPC biopsies. To compare with expression in primary biopsy tissues and cell lines, we queried AR-E1 and AR-CE3 expression by RISH across 12 metastatic CRPC biopsy specimens (Figure 6). Importantly, these patients were treated with varying therapeutics (docetaxel, degarelix, goserelin, abiraterone, enzalutamide). AR-E1 and AR-CE3 expression were both seen in the cytoplasm as well as in intranuclear punctae, which were quite prominent in some cases (Figure 6A, TB_010). AR-E1 and AR-CE3 expression were clearly correlated in most cases, with a few notable exceptions (Figure 6A, TB_020).

Because a number of pre-analytic variables (such as time to tissue fixation and decalcification) may affect analysis of biopsies from bone in particular, and this is a relatively common site of metastatic biopsies in prostate cancer, we paid particular attention to the RISH results among bone biopsies. Of note, TB-015 was a bone biopsy, with interpretable RISH results based on reasonable intensity of AR-E1 RISH. To expand our analysis of bone biopsies in this setting, we examined two additional clinical bone metastatic specimens as well as two additional autopsy bone biopsies. We found that AR RISH results were interpretable in three out of four of these additional cases, including two autopsies where the AR RISH intensities in the bone biopsy were largely comparable to the intensities seen in other soft tissue sites from the same patients (Supplementary Figure S6). Of note, the one case where both AR-E1 and AR-V7 RISH were negative, making the results uninterpretable, was a large orthopedic metastatic resection specimen which likely had increased time to fixation and prolonged decalcification due to its large size.

Overall, there was a considerable range of expression for both AR-E1 and AR-CE3 in metastatic biopsies, with some cases having dramatically higher expression than primary tumors (compare to Figure 5) and even higher expression than seen in autopsy CRPC cases (compare to Figure 3). To compare relative AR-E1 and AR-CE3 levels across prostate cancer tissue types, we plotted the quantified Aperio scores for AR-E1 and AR-CE3 for cell

lines (Figure 1), benign prostate glands, high grade primary tumor specimens (Figure 5) and 3 metastatic biopsies from CRPC patients which showed prominent AR-CE3 signal and would be considered clearly “positive” by the RISH assay (TB_014, TB_016, TB_017) (Figure 6B). As previously reported for RT-PCR, quantified AR-E1 expression was generally higher than AR-CE3 expression in both benign and primary tumor tissues on each image analysis platform, with a less prominent difference seen in CRPC metastatic biopsies. It is important to note, however that a limitation to comparing RISH assays for different targets exists since the number and hybridization efficiency of probes may not be equivalent for different targets such as AR-E1 and AR-CE3. Comparing between tissue types, mean AR-CE3 was more than 20-fold increased among CRPC metastases compared to high grade primaries and benign tissues, with a less dramatic difference for AR-E1 between tissue types. Overall, the AR-CE3 levels among the AR-CE3-positive metastases were comparable to what is observed in AR-positive cell lines. Similar results were seen when examining specimens with image quantification by Frida (benign and primary tumor tissue from radical prostatectomies, the 4 autopsy CRPC cases from Figure 3 and AR-positive cell lines).

Discussion

Potent anti-androgen therapies and chemotherapy are being used at an earlier stage in prostate cancer therapy and biomarkers that predict resistance to these therapies are sorely needed to realize the promise of precision medicine. Recent work has suggested that AR-V7 mRNA expression, as measured by RT-PCR in CTCs, may be a specific biomarker of resistance to abiraterone and enzalutamide. However the use of these drugs earlier in the disease course has meant that substantial numbers of patients lack measurable CTCs on which to perform AR-V7 expression analysis, while other patients may have undetectable CTCs despite high tumor burden. A number of additional, potentially more sensitive techniques have emerged rapidly to improve the utility of the “liquid biopsy” for measuring AR and AR-V7 status, including enrichment-free CTC characterization platforms (43) and measurement of AR genomic status in cell-free plasma DNA (44). However, a tissue-based measurement of AR-V7 expression in metastatic tumor biopsies in CRPC may also be a useful alternative when patients lack CTCs. Using such an assay, patients who have failed standard ADT, or even first line therapy with abiraterone/enzalutamide and who have a potential metastatic site available for biopsy could have their metastatic tissue queried for AR-V7 expression. If positive, the oncologist might have a low threshold for switching to other lines of therapy if a rapid response is not seen on abiraterone/enzalutamide, or perhaps to avoid these therapies altogether, favoring chemotherapies or other treatment regimens instead.

However before clinical validation studies to support the utility of the assay for directing these decisions can be considered, it is paramount to develop a highly *analytically* validated tissue-based assay for AR-V7 mRNA (45). Here, we expanded on prior work (26) to perform a full analytic validation of RNA in situ hybridization (RISH) assays to detect total AR species (AR-E1) and a surrogate for AR-V7 (AR-CE3). Using cell lines, xenografts and autopsy derived metastatic CRPC tissues with known AR and AR-V7 levels by RT-PCR, we demonstrate that our RISH assays on formalin fixed paraffin embedded (FFPE) samples correlate remarkably well with RT-PCR results from the corresponding fresh-frozen tissues.

In addition, using AR-negative prostate cancer cell lines, xenografts and biopsies, we show that our RISH assays have a high specificity for AR mRNA expression, with one potentially important caveat. Across almost all of our samples, even in a tissue known to be AR-protein negative, we noted that both AR-E1 and AR-CE3 signal was present to varying degrees in the nucleus, often as a single punctate dot. This dot did not overlap with the nucleolus by hematoxylin staining and did not appear similar to the distribution of any known nuclear subcompartment. This signal almost certainly represents RNA (rather than DNA) because pre-treatment of tissues with RNase before RISH nearly completely eliminates this signal. Next generation RISH assays used for FFPE tissue require multiple oligonucleotide probe pairs for each RNA target to enhance specificity and sensitivity. Thus, it is not usually possible to use the assays in their current form to span exon-exon boundaries, enabling detection of only spliced mRNA species. Because nuclear export of RNA is tightly regulated to include only spliced species in most contexts, it is unlikely that cytoplasmic RISH signals correspond to unspliced RNA species (41, 42). However, it is possible that intranuclear AR-E1 and AR-CE3 RISH signals may detect nascent or pre-spliced RNA species in the nucleus. Indeed, inefficient splicing with intronic retention has been reported for multiple transcripts in CRPC and we have shown that many unspliced transcripts contain AR-CE3 in this same dataset (36). Thus, we suspect that these intranuclear RISH signals may represent nuclear retention of unspliced RNA species at the transcription site, a phenomenon that has been reported for other transcripts that are inefficiently processed (46).

The possibility that RISH assays may detect intranuclear, potentially unspliced RNAs is an important limitation that must be acknowledged and further studied. Our image quantification algorithms did not distinguish between intranuclear and cytoplasmic RISH signal since this is quite technically difficult in cases with high RISH signal and would likely not be feasible for clinical use. Remarkably, there was still a good correlation between the quantified RISH signals and RT-PCR results (which span splice junctions and measure only spliced AR-FL and AR-V7 transcripts). This may be due to the fact that the cytoplasmic and nuclear RISH signals were highly correlated in most cases. Indeed, one case that appeared to be a bit of an exception to this rule (A004, with high intranuclear signals and lower cytoplasmic signals) was a significant outlier and had quite poor correlation between RISH and RT-PCR results, consistent with the hypothesis that much of the RISH signal derived from intranuclear, unspliced RNAs. To mitigate this weakness of the RISH, it may be most practical to screen cases with these assays and to follow-up any positive results with additional testing. Cases with low AR-CE3 expression could be assumed to be true negatives, since the RISH assay should inclusively detect all RNA species containing CE3, including AR-V7. On the other hand, cases with high AR-CE3 expression, particularly if there is a high level of intranuclear signal relative to cytoplasmic signal (such as A004 or TB_010 or TB_014) would require further study by other methodologies, such as RT-PCR on fresh tissue. Regardless of these technical limitations in analytic validation, the RISH assays described herein will ultimately require large clinical validation studies to support their use, and these studies will clarify the appropriate screening and scoring protocols for these markers.

In addition to the analytic validation, our study was the first to use new and highly sensitive chromogenic RISH assays to measure AR mRNA expression in primary prostate tumors.

Consistent with previous studies (12, 25, 47, 48), we confirmed that AR-V7 is expressed in primary prostate tumors, and we demonstrated that expression of AR-CE3 and AR-E1 are highly correlated in this context (as has been reported previously for metastases). Importantly, we also confirmed that both AR-E1 and AR-CE3 expression in primary tumors are orders of magnitude lower than that seen in positive CRPC metastases, which suggests that AR-CE3 expression may not be biologically significant in primary tumors as has been shown previously (48). Indeed, there is currently no evidence that AR-E1 or AR-V7 expression could be used to inform clinical decisions prior to the development of CRPC as the low level of AR-CE3 expression, tumor heterogeneity and lack of informative AR genomic alterations are limitations that would likely be difficult to surmount.

One novel finding in the current study is that AR-E1 expression (and AR-CE3 expression) is up to three-fold higher in primary tumor epithelium compared to surrounding benign glandular cells. This finding was particularly prominent in higher grade primary tumors (Gleason 9 and 10). Previous studies have reported higher AR protein in primary tumor cells compared to intermingled benign epithelium (49), however higher levels of AR message in tumor compared to benign have not been reported previously to our knowledge. However the biological significance of this relatively upregulated AR message expression in primary tumors remains unclear. Though AR protein expression by IHC was a bit lower in the benign compared with the tumor epithelium, this difference was quite small and may not be biologically significant (with the important caveat that our AR IHC assay may mask significant differences in AR protein expression as it has been optimized to distinguish negative and positive cells, but not to detect gradation of AR-positivity). Further studies will be required to follow-up on this intriguing finding.

In conclusion, next generation chromogenic RISH assays, such as those utilized in this study, represent a major advance in tissue-based biomarker development. Herein, we have analytically validated one such putative biomarker in prostate cancer, and identified potentially important limitations of the current generation of RISH assays as they may detect intranuclear and potentially nascent RNAs. However, much work still lies ahead before these tests can inform clinical decision-making. Pre-analytic validation studies will be required since RNA species are not optimally preserved in FFPE tissues, and RNA degradation may occur over time. Issues of tissue fixation duration and methodology, as well as tissue age and other pre-analytic variables are critical to assess before moving forward with any clinical assay and may be a significant barrier to clinical application of any biomarker. If the RISH assays appear reasonably robust in these pre-analytic studies, large clinical validation studies, optimally in the context of prospective clinical trials, will be required to further examine the utility of these markers and resolve the clinical significance of intranuclear RNA labeling. Ultimately, large biomarker-driven clinical trials that examine the association of both AR-V7 measured by CTCs and by tissue-based measures with outcomes to novel AR-targeted agents will likely be required and many are currently in the early stages. Trials such as these will address a number of other important clinical questions, including the correlation between CTC and tissue-based measurements for AR-V7 and the effects of tissue heterogeneity. In the context of such large studies, it will be feasible to choose the assay that shows the highest correlation with clinical outcomes to take forward to routine clinical use.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

Primary prostate cancer is androgen dependent, and most castration resistant prostate cancers (CRPC) continue to require signaling through the androgen receptor (AR). Recent work has demonstrated that both primary tumors and CRPC may express varying levels of AR splice variant RNA, which when translated into a protein may function in a ligand independent manner. Accordingly, detection of the AR-V7 splice variant RNA in circulating tumor cells (CTCs) was recently shown to be a potential biomarker for resistance to androgen deprivation therapies (ADT) but not chemotherapies. However, not all patients have CTCs available for AR-V7 assessment. Since a tissue-based assay may address this limitation, we analytically validated an RNA *in situ* hybridization (RISH) assay to measure total AR and AR-V7 RNA in formalin fixed paraffin embedded (FFPE) tissues and report relative expression in primary and metastatic prostate tumors. If clinically validated, this assay may identify patients who are resistant to ADT.

Figure 1. AR-E1 and AR-CE3 mRNA by RISH and AR protein levels by IHC in prostate cancer cell lines and xenografts

(A) AR-E1 levels are essentially undetectable in PC3 and DU145 cell lines, known to be negative for AR by RT-PCR and at the protein level by IHC, supporting the specificity of the RISH assay. AR-E1 levels increase concordant with AR protein levels by IHC in LAPC4, LNCaP, CWR22RV1 and VCaP cells. Both intranuclear punctate labeling (arrows) and speckled cytoplasmic labeling are identified for the RISH assays. (B) AR-E1 and AR-CE3 levels are highly correlated across cell lines when quantified by image quantification, as expected. (C) LuCaP 49 is a patient derived xenograft known to be negative for AR expression and it is also negative for AR-E1 and AR-CE3 by RISH. LuCaP 96 and LuCaP

35 show increasing levels of AR-E1 and AR-CE1 from parental to castrate resistant (CR) sublines, consistent with previously reported results for AR-FL and AR-V7 by RT-PCR (12).

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Figure 2. Prostate needle core biopsy containing concurrent AR-positive and AR-negative prostate cancer

In the same contiguous biopsy core, AR-E1 is visible as both cytoplasmic speckles as well as intranuclear punctae (arrows) in the AR protein-expressing tumor component. Of note, AR-E1 expression is dramatically lower in the AR protein-negative tumor component, where it was not detectable in the cytoplasm and only detectable as barely visible intranuclear punctae. AR-CE3 was present in only small intranuclear punctae in both tumor components, perhaps representing AR-CE3 present in nascent or unspliced RNA species.

Figure 3. AR-E1 and AR-CE3 expression correlate with AR-FL and AR-V7 mRNA levels by RT-PCR in metastatic CRPC specimens

(A) RISH for AR-E1 and AR-CE3 on 4 separate autopsy cases with two metastatic sites examined for each. A004 shows high levels of AR-CE3 expression, however much of the labeling is intranuclear (arrows). **(B)** Relative AR-CE3 quantified RISH levels and relative AR-V7 by RT-PCR correlate significantly. AR-CE3 expression is calculated relative to the A001-liver metastasis. A004 metastases are notable outliers to the correlation with RT-PCR.

Figure 4. Measured in radical prostatectomy specimens, AR-E1 and AR-CE3 expression are correlated and generally higher in primary tumors compared to intermingled benign glands (A) Representative primary tumor (T) and benign gland (B) labeling with AR-E1 and AR-CE3 RISH and AR IHC for AR protein. (B) RISH image quantification algorithm using Frida. The marked area is enriched for tumor epithelium with minimal intervening stromal tissue and is quantified for RISH signals. Pre-set thresholds divide images into blue (unlabeled nuclei and some cytoplasm) and brown (RISH signal) pixels. Within the marked area, quantified RISH signal is expressed as the ratio of blue signals to (brown+blue signals). (C) Quantified AR-CE3 versus AR-E1 for high grade (Gleason 4+3=7 and higher; HG) and low grade (Gleason 3+3=6 and 3+4=7; LG) primary tumors and benign epithelium. There is a significant correlation between AR-CE3 and AR-E1 levels. (D) AR-E1 levels are significantly increased in tumor glands versus intervening benign glands in each case. This trend appears slightly stronger in high grade primary tumors compared to low grade primary tumors. A similar, though insignificant, trend is seen for AR-CE3.

Figure 5. Measured in prostate needle biopsy specimens, AR-E1 and AR-CE3 expression are correlated and higher in Gleason 9–10 primary tumors compared to intermingled benign glands (A) Representative primary tumor (T) and benign gland (B) labeling with AR-E1 and AR-CE3 RISH and AR IHC for AR protein. In case #1 and #2, both AR-E1 RISH and AR IHC are higher in tumor compared to adjacent benign glands. Both AR-E1 and AR-CE3 are localized in the cytoplasm as well as in nuclear punctae (arrows). (B) RISH image quantification algorithm using Aperio Digital Pathology Software. The marked area (green) is enriched for tumor epithelium with minimal intervening stromal tissue and is quantified for RISH signals. Pre-set thresholds divide images into blue (unlabeled nuclei and some cytoplasm) and brown (RISH signal) pixels. Within the marked area, quantified RISH signal is expressed as the ratio of blue signals to (brown+blue signals). (C) Quantified AR-CE3

versus AR-E1 for high grade (Gleason 9–10) primary tumors and benign epithelium. There is a significant correlation between AR-CE3 and AR-E1 levels for the tumors. **(D)** AR-E1 and AR-CE3 levels are significantly increased in tumor glands versus intervening benign glands in each case.

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Figure 6. Quantified AR-E1 and AR-CE3 expression is substantially higher in CRPC metastases and cell lines compared to benign prostate tissue and primary tumors

(A) Biopsies of metastatic CRPC labeled by AR-E1 and AR-CE3 RISH assay. Significant variation for both markers is seen between cases. In general, AR-E1 and AR-CE3 appear highly correlated with some notable exceptions (TB_020). Of note, TB_015 is a bone biopsy. Some cases show prominent intranuclear punctate accumulation of AR-CE3 labeling. (B) Left panel: Mean ($\pm$ SEM) quantified AR-E1 and AR-CE3 levels using Aperio Digital Pathology Software for benign epithelium and high grade primary tumors sampled on needle biopsy (same samples as in Figure 5) compared to high expressing AR-CE3 CRPC metastatic biopsies (TB_014, TB_016, TB_017) and AR-positive cell lines (Figure 1). Right panel: Mean ($\pm$ SEM) quantified AR-E1 and AR-CE3 levels using Frida for benign epithelium and low/high grade primary tumors sampled on radical prostatectomy (same samples as in Figure 4) compared to CRPC metastases from autopsies (samples in Figure 3) and AR-positive cell lines (Figure 1).