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Novel RNA hybridization method for the *in situ* detection of *ETV1*, *ETV4* and *ETV5* gene fusions in prostate cancer

Lakshmi Priya Kunju, MD^{1,2,3}, Shannon Carskadon, MS^{1,2}, Javed Siddiqui, MS^{1,2}, Scott A Tomlins, MD., PhD^{1,2,3,4}, Arul M Chinnaiyan, MD., PhD^{1,2,4,5,#}, and Nallasivam Palanisamy, Ph.D^{1,2,4,5,#}

¹Michigan Center for Translational Pathology, Ann Arbor, MI, USA

²Department of Pathology, University of Michigan, Ann Arbor, MI, USA

³Department of Urology, University of Michigan, Ann Arbor, MI, USA

⁴Comprehensive Cancer Center, University of Michigan, Ann Arbor, MI, USA

⁵Howard Hughes Medical Institute, University of Michigan, Ann Arbor, MI, USA

Abstract

The genetic basis of 50–60% of prostate cancer is attributable to rearrangements in ETS (*ERG*, *ETV1*, *ETV4*, and *ETV5*), *BRAF* and *RAF1* genes and overexpression of *SPINK1*. The development and validation of reliable detection methods are warranted to classify various molecular subtypes of prostate cancer for diagnostic and prognostic purposes. ETS gene rearrangements are typically detected by fluorescence *in situ* hybridization (FISH) and reverse transcription PCR methods. Recently, monoclonal antibodies against ERG have been developed that detect the truncated ERG protein in immunohistochemical assays where staining levels are strongly correlated with *ERG* rearrangement status by FISH. However, specific antibodies for *ETV1*, *ETV4* and *ETV5* are unavailable, challenging their clinical use. We developed a novel RNA *in situ* hybridization based assay for the *in situ* detection of *ETV1*, *ETV4*, and *ETV5* in formalin fixed paraffin embedded tissues from prostate needle biopsies, prostatectomy, and metastatic prostate cancer specimens using RNA probes. Further, with combined RNA *in situ* hybridization and immunohistochemistry we identified a rare subset of prostate cancer with dual ETS gene rearrangements in collisions of independent tumor foci. The high specificity and sensitivity of RNA *in situ* hybridization provides an alternate method enabling bright field *in situ* detection of ETS gene aberrations in routine clinically available prostate cancer specimens.

Keywords

RNA in situ hybridization; ETS gene fusion; Immunohistochemistry; fluorescence in situ hybridization

[§]**Corresponding Author** Nallasivam Palanisamy, Ph.D., Michigan Center for Translational Pathology, Department of Pathology, Comprehensive Cancer Center, University of Michigan Medical School, 2900, Huron Parkway, Traverwood IV, Suite 100, Room 1219, Ann Arbor, MI 48105, nallasiv@med.umich.edu, Phone: 734-615-1417, Fax: 734 232 0805.

[#]Sharing Senior Authorship

DISCLOSURE/CONFLICT OF INTEREST

The remaining authors declare no conflicts of interest.

Introduction

Prostate cancer (PCa) remains the most commonly diagnosed cancer in American men with an estimated incidence of 238,590 new cases and accounting for 29,720 deaths in 2013. (www.cancer.org). Currently the clinical diagnosis of PCa relies on serum prostate specific antigen (PSA) testing that has well- documented limitations including its unreliability for distinguishing aggressive disease from benign conditions. The identification of PCa specific molecular biomarkers, driving molecular alterations and robust molecular subtypes suggest more accurate diagnosis and prognosis. Using a novel bioinformatics approach, our group reported the first recurrent chromosomal rearrangement in prostate cancer involving fusion of the androgen-regulated *TMPRSS2* gene with *ERG* or *ETV1*, members of the ETS transcription factor family. Identification of recurrent gene fusions and other molecular aberrations in prostate cancer helped to classify prostate cancer into distinct molecular subtypes [12]. Subsequent studies confirmed the presence of *E26 transformation specific* (ETS) gene fusions in approximately 50% of prostate specific antigen screened PCa. Among the ETS genes, *ERG*, *ETV1*, *ETV4* and *ETV5* overexpression result from the fusion of these genes with various androgen regulated 5' partner genes[3–5]. Overexpression of *SPINK1* has been identified in 5–10% of cases that do not harbor ETS gene rearrangements [6]. Our group also recently reported the identification of “druggable” RAF kinase gene rearrangements (*SLC45A3-BRAF* and *ESRP1-RAF1*) and FGFR gene fusions [7] in 1–2% of PCa patients who are negative for the known ETS family gene rearrangements [8]. The genetic basis of the remaining 30–40% of prostate cancer is yet to be identified.

Considering the emerging therapeutic agents targeting prostate specific markers[9],[10], reliable, efficient methods are warranted for the detection of various markers in both clinical setting for diagnostic or sub classification purposes, as well as in the research setting. Further, the multifocal nature of prostate cancer is well- recognized and several studies have documented the interfocal heterogeneity of *ERG* rearrangements in PCa harboring multiple tumor foci[11],[12] [13]. Hence genetically distinct tumor foci within the same patient are not unusual.

ETS gene rearrangements were initially detected by fluorescence *in situ* hybridization (FISH) and reverse transcription polymerase chain reaction (PCR) methods. Recently, monoclonal antibodies against ERG have been developed which are strongly correlated with *ERG* rearrangement as detected by FISH [14],[15],[6]. We recently developed a novel dual color immunohistochemistry (IHC) assay for the simultaneous detection of *ERG-PTEN* and *ERG-SPINK1* in prostate cancer[16]. *ETV1*, *ETV4* and *ETV5* rearrangements, when assessed, are generally detected by FISH and/or reverse transcription PCR due to the lack of specific antibodies for these genes. Since multiple 5' partner genes are involved in the fusion with *ETV1*, *ETV4* and *ETV5* genes, development of PCR based methods require synthesis of multiple primers and reactions. FISH analysis is time consuming, laborious, and can be challenging to identify small foci of interest in biopsies or prostatectomy specimens. In order to overcome the technical limitations associated with PCR and FISH, we have developed a novel RNA based *in situ* hybridization method (RNA-ISH) for the *in situ* detection of *ETV1*, *ETV4* and *ETV5* in formalin fixed paraffin embedded PCa samples. In

this study we validate this novel RNA-ISH method that is comparable to IHC and a viable alternate method for *in situ* detection of ETS gene rearrangements.

MATERIALS AND METHODS

Study Design

To assess the feasibility of RNA-ISH method for the reliable detection of ETS rearrangement positive cases with high specificity and sensitivity, we initially evaluated RNA-ISH approach by comparing with ERG-IHC on previously confirmed ERG-positive and ERG-negative cases from tissue microarray (TMA) samples. We then tested the *ETV1*, *ETV4* and *ETV5* RNA-ISH probes on a cohort of previously confirmed positive cases (needle biopsies and TMAs). Lastly we screened a large cohort of localized and metastatic prostate cancer cases from TMAs where the molecular status of these genes was unknown. Each case in the TMA was represented in triplicate cores, 0.6mm in size.

Tissue Selection

Multiple TMAs were used in this study including cases from prostatectomy cases and distant metastases (details of TMAs are provided in Table 1). All tissue samples were collected at the University of Michigan with informed consent and Institutional Review Board approvals. The metastatic prostate carcinoma samples were obtained from patients with hormone-refractory prostate cancer who were part of our posthumous tissue donor program. To date, 60 such autopsies have been performed. Normal and malignant tissues from multiple sites including bone were collected and incorporated in tissue microarrays used in this study.

FISH and IHC validated *ERG*, and fluorescence *in situ* hybridization validated *ETV1*, *ETV4* and *ETV5* rearrangement positive PCa samples (radical prostatectomy) were used to establish the RNA *in situ* hybridization method for the reliable detection of ETS rearrangement positive cases in prostate[16–19] cancer.

Immunohistochemistry

ERG IHC was performed using the anti-ERG (EPR3864) rabbit monoclonal primary antibody (1:100) (Cat#790-4576, Ventana Medical Systems, Inc., Tucson, AZ, USA). IHC was performed using an automated protocol developed for the DISCOVERY XT automated slide staining system (Ventana Medical Systems, Inc.,) using Ultramap anti-rabbit HRP (Cat#760-4315, Ventana Medical Systems, Inc.,) as secondary antibody and detected using ChromoMap DAB (Cat#760-159, Ventana Medical Systems Inc.,) for ERG. Hematoxylin (Cat#790-2208 Ventana Medical Systems, Inc.,) was used as the counterstain. ERG immunohistochemistry staining was evaluated by board certified pathologist LPK. Staining of vessels was used as a positive control and slides without staining of vessels were excluded from further analysis. ERG staining in tumor foci was either absent or diffusely strong (2–3+), unless otherwise indicated, and was reported as present/absent. Cases with documented *ERG* rearrangement by fluorescence *in situ* hybridization were used as positive control.

ETV1, ETV4, ETV5 RNA ISH probes

RNA scope target probes were designed by Advanced Cell Diagnostics (Hayward, CA) using custom software. The software automatically select from the target sequence a series of oligonucleotides which have an average length of 25 bases and uniform melting temperature and do not cross-hybridize with any off-target sequence in the human transcriptome under the RNA scope assay conditions. The oligonucleotides are synthesized and quantified by measurement of UV absorbance at 260nm. Equi-molar amounts of oligonucleotides were pooled into a single probe set for each target. The human *ERG* (cat#604021), *ETV1* (cat # 311411), *ETV4* (cat # 491521), and *ETV5* (cat # 590371) probes were used in this study.

RNA in situ Hybridization

RNA-ISH in situ hybridizations were performed essentially as described previously[20]. Briefly, formalin fixed and paraffin embedded sections and tissue microarray slides were baked at 60° C for one hour. RNA-ISH was performed using RNA scope FFPE Reagent Kit 2.0 (Advanced Cell Diagnostics, Hayward, CA) according to manufacturer's instructions. Briefly, tissues were deparaffinized by immersing in xylene twice for 15 minutes each with periodic agitation. The slides were then immersed in 100% ethanol twice for 3 minutes each with periodic agitation, then air-dried for 5 minutes. Tissues were circled using a pap pen (Vector, #H-4000), allowed to dry, and treated with Pretreatment 1 buffer for 10 minutes. Slides were rinsed in deionized water, and then boiled in 1X Pretreatment 2 for 15 minutes. Slides were rinsed again in deionized water, and then treated with Pretreatment 3 buffer for 30 minutes at 40° C in a humidity chamber. Slides were rinsed twice in deionized water, and then incubated with target probes for either *ERG* (NM_001136154, region 2933–3913), *ETV1* (NM_004956.4, region 998–2031), *ETV4* (NM_001079675.2, region 431–1891), *ETV5* (NM_004454.2, region 2638–3839), (Figure 1) *DapB*, bacterial gene (as negative control) or *POLR2A* (as positive control) for 2 hours at 40°C in a humidity chamber. Slides were then washed in 1X Wash Buffer twice for 2 minutes each. Slides were then treated with Amp 1 solution for 30 minutes, Amp 2 solution for 15 minutes, Amp 3 solution for 30 minutes, and Amp 4 solution for 15 minutes, all at 40 C in a humidity chamber with 2 washes in 1X Wash Buffer for 2 minutes each after each step. Slides were then treated with Amp 5 solution for 30 minutes and Amp 6 solution for 15 minutes at room temperature in a humidity chamber with 2 washes in 1X Wash Buffer for 2 minutes each after each step. Color was developed by adding a 1:60 solution of Fast Red B: Fast Red A to each slide and incubating for 10 minutes. Slides were washed twice in deionized water and then immersed in a 50% Hematoxylin (Fisher, #SH26-4D) solution for 2 minutes. Slides were rinsed several times in deionized water, and then immersed in a 0.01% ammonium hydroxide solution. Slides were rinsed in deionized water then dehydrated by dipping 5 times in 70% ethanol twice, dipping 5 times in 95% ethanol twice, immersing twice in 100% ethanol for 5 seconds each, and immersing in xylene for 5 seconds. The slides were mounted in Eco Mount (Fisher, 50–828-32) for viewing under bright-field microscopy.

RNA-ISH evaluation criteria

RNA-ISH scoring guidelines were established to classify tumor foci as described previously[20]. In positive cases, RNA-ISH staining appeared as distinct punctate dots within the tumor cells ranging from 0- >10 in each cell. All tumor foci were evaluated and scanned at 20X magnification. Scoring for a tumor focus was based on the highest intensity observed. Based on the number of dots/cell we established five grading levels ranging from 0 to 4: tumor foci with no staining or less than 1 dot/cell under 20X magnification are scored as 0 or negative; tumor foci with 1–3 dots/cell in > 5% of the tumor were scored as 1; tumor foci with 4–10 dots/cell with no or very few dot clusters (fused overlapping dots) in >5% of tumor were scored as 2; tumor foci with more than 10 dots/cell with <10% of positive cells having dot clusters were scored as 3; tumor foci with > 10 dots/cell with >10% of positive cells having dot clusters were scored as 4. For the purposes of this study, tumor foci showing scores of 2–4 were classified as positive whereas tumor foci with scores 0–1 were considered negative. All slides were reviewed by a study pathologist (LPK).

Fluorescence in situ hybridization

BAC clones were used to generate the dual color break-apart FISH probes for *ERG* (RP11-476D17- 3' probe, RP11-95I21-5' probe), *ETV1* (RP11-79G16-3' probe, RP11-661L15-5' probe), *ETV4* (CTD-3215I16-3' probe; RP11-147C10-5' probe) and *ETV5* (RP11-480B15-3' probe; RP11-822O23-5' probe). All clones were tested on normal human metaphase chromosomes to validate map position and these clones have been used extensively in various studies from our laboratory and others [17],[19].

BAC DNA Preparation—200 ml overnight cultures for each BAC clone were grown in LB medium containing 12.5g/ml of chloramphenicol at 37°C for 14–16 hours with constant shaking. DNA was prepared using Qiagen- midiprep kit using Qiatip-100 according to the protocol provided by the manufacturer (Qiagen, USA).

Probe labeling—All FISH probes were prepared by nick translation labeling using modified nucleotides conjugated with biotin or digoxigenin utilizing biotin nick translation mix (11745824910, Roche, USA) for 3' *ERG* and *PTEN* locus probes; digoxigenin nick translation mix (11745816910, Roche, USA) for 5' *ERG* and chromosome 10 control probes. Probe DNA was precipitated and dissolved in hybridization mixture containing 50% formamide, 2XSSC, 10% dextran sulphate, and 1% Denhardt's solution. Approximately 200ng of each labeled probe was used for hybridization. Fluorescent signals were detected with Streptavidin Alexa fluor 594 (S-32356, Invitrogen, USA) and anti-digoxigenin fluorescein Fab fragments (11207741910, Roche, USA) for red and green colors, respectively.

Image capture and FISH signal analysis

FISH analysis was performed by experienced cytogeneticist (NP) and pathologist (LPK). *ERG* rearrangement by translocation and/or deletion was recorded when the corresponding abnormal signal pattern was observed in more than 10–15% of cells. Fluorescent images were captured using a high resolution CCD camera controlled by ISIS image processing software (Metasystems, Germany).

Sequential Hybridization: RNA-ISH and ERG IHC

We developed standardized protocols to sequentially perform RNA-ISH and ERG IHC on radical prostatectomy formalin fixed paraffin embedded whole sections and/or TMA cores with multifocal tumor foci. The criteria for scoring the RNA-ISH and ERG IHC remained identical to that described above.

RESULTS

Comparison of ERG expression at RNA and Protein level by RNA-ISH and IHC

RNA-ISH *in situ* hybridization was positive in all (100%) of the 12 cases with ERG rearrangement confirmed by IHC and FISH. Additionally, RNA-ISH was negative in all the 70 ERG-negative cases as determined by IHC, indicating a 100% concordance between IHC and RNA-ISH (Figure 2) with a 0% false positive rate. For the 12 ERG-positive cases, RNA-ISH results were scored as follows: 2 (n=2), 3 (n=3) and 4 (n=7).

Validation of ETV1, ETV4 and ETV5 expression by RNA-ISH in situ

hybridization—Based on results from the RNA-ISH and FISH/IHC comparison for *ERG*, we subsequently tested *ETV1*, *ETV4*, and *ETV5* RNA-ISH probes on known positive cases and screened a large cohort of cases where the rearrangement status was unknown. We selected 11 positive cases, (5 *ETV1*, 4 *ETV4* and 2 *ETV5*) identified by FISH and performed RNA-ISH separately with *ETV1*, *ETV4* and *ETV5* probes. Positive RNA *in situ* staining was observed in cases with corresponding probes only in the fluorescence *in situ* hybridization confirmed positive cases. No cross reactivity or nonspecific hybridization of any of the probes other than the designated positive cases (Figure 3) was observed in either localized or metastatic PCa samples. The RNA-ISH intensity scores for positive cases were as follows: *ETV1* 3 (n=2), 4 (n=3); *ETV4* 3 (n=1) and 4 (n=3) and *ETV5* 2 (n=1) and 4 (n=1).

We further tested *ETV1*, *ETV4* and *ETV5* probes on 319 cases represented on different TMAs comprising 265 localized PCa and 54 metastatic PCa samples with unknown ETS rearrangement status (details of TMA listed in Table 1). We identified 10 *ETV1* (5 localized and 5 metastatic PCa) (3.4%) and 3 *ETV4* (localized PCa) (1%) and no *ETV5* positive cases. The RNA-ISH intensity scores for *ETV1* positive cases were, 2 (n=2), 3 (n=5) and 4 (n=3); RNA-ISH intensity scores for *ETV4* were as follows: 3 (n=1) and 4 (n=2). No non-specific background signals were observed for any of these three probes in any of the negative cases indicating the high specificity and sensitivity of the RNA probes. All RNA-ISH in positive cases were independently validated by respective break-apart FISH.

Identification of dual ETS gene rearrangements by Immunohistochemistry and RNA in situ hybridization

In the majority of the positive cases, the RNA-ISH was homogeneous within the tumor focus; a small subset of cases (three *ETV1*-positive cases and in one *ETV4*-positive case) of localized PCa from a large cohort (n=319) showed heterogeneous staining pattern within the triplicate cores of the TMA tissue microarray i.e 1–2 cores of a case were positive while the remaining cores were negative.

Sequential RNA-ISH and ERG-IHC were performed on these 4 cases on representative whole block sections from radical prostatectomy specimens (3 cases) and TMA (1 case). RNA-ISH for *ETV1* was repeated and small foci of tumor were negative on the whole sections. ERG-IHC sequentially performed on the same blocks found that small *ETV1* negative tumor foci were positive with ERG staining (See Figure 4 and 5).

Sequential ERG-IHC performed on the TMA sample with one core negative with *ETV4* RNA-ISH was confirmed to be ERG-positive (Figure 6) and thus dual ETS rearrangement (ERG rearrangement and *ETV1/ETV4* rearrangement in separate tumor foci/ areas were confirmed in 4 cases. The three ERG/*ETV1* and one ERG/*ETV4* rearrangement cases were observed in localized, multifocal, and organ confined PCa with Gleason Score 3+4=7 (n=2), 4+3=7 (n=1) and 3+3=6 (n=1) respectively.

DISCUSSION

In the present study, we demonstrate the utility of a novel RNA-ISH in-situ hybridization technique to detect *ETV1*, *ETV4* and *ETV5* rearrangements in formalin-fixed paraffin embedded PCa samples with high sensitivity and specificity. We systematically validated this technique initially by comparing the sensitivity and specificity of ERG RNA-ISH in *situ* hybridization with previously validated techniques such as FISH and IHC. We subsequently screened a large cohort of localized and metastatic PCa samples to identify the rare *ETV1*, *ETV4* and *ETV5* rearrangements using this novel technique.

Since the initial discovery of recurrent gene fusions in PCa, other prostate-specific molecular aberrations have been identified that stratify prostate cancer into distinct molecular subtypes, many of which are associated with altered clinical behavior[2]. Recent studies demonstrated that in addition to protein coding genes, non-coding RNA[21 22] and lineage- and cancer-specific pseudogenes[23 24] may serve as potential new biomarkers for disease classification and patient stratification in PCa.

RNA-based in situ detection methods are a viable approach to evaluate tissue level expression of non-coding RNAs and genes for which specific antibodies are not available. *ERG*, *ETV1*, *ETV4* and *ETV5* are highly expressed only in the presence of ETS gene fusions in PCa while benign prostate and ETS fusion-negative PCa show low or complete absence of ETS gene expression. This unique expression pattern is optimal for the identification of ETS rearrangement by RNA-ISH without any ambiguity based on expression. The utility of RNA-ISH is particularly evident for the detection of *ETV1*, *ETV4* and *ETV5* gene fusion at the tissue level as no specific antibodies exist unlike for *ERG* fusions.

Importantly, we identified the existence of rare molecular subsets of prostate cancer with dual ETS gene rearrangements in different tumor focus of the same tumor. Given the multifocal nature of prostate cancer, it has been shown that prostate tumors showed inter tumoral molecular heterogeneity with ERG rearrangement by deletion in one focus and translocation in other focus and intra tumoral heterogeneity with more than one 5' ERG fusion partner gene (*NDRG1*, *SLC45A3*, *TMPRSS2*) rearrangement based on FISH evaluation [25–27]. Here, we observed heterogeneous expression pattern of *ETV1* and *ETV4*

in a small subset of positive cases (3/11 ETV1 and 1/3 ETV4 cases) from the large cohort (n=319 cases) screened for these rearrangements. In samples with heterogeneous expression of *ETV1* and *ETV4*, of the triplicate cores in tissue microarray, 1–2 were positive while the remaining cores were negative. We were intrigued by this observation and hypothesized that such small foci may have rearrangement with other ETS family gene or unknown gene (s). Since ERG accounts for the vast majority of ETS rearrangements, we performed ERG immunohistochemistry for these cases on whole sections and found ERG positivity in the small tumor foci that were negative for ETV1 or ETV4. Using RNA-ISH and ERG-IHC sequentially, we demonstrated small ERG-positive foci admixed with large ETV1 rearranged prostate cancer tumor focus (Figure 4 and 5). We also found one case where of the triplicate TMA cores; one core was ERG-positive by IHC and 1 core strongly positive for ETV4 by RNA-ISH (Figure 6). No tumor foci had concomitant staining with ERG and ETV1 or ETV4. While inter-focal heterogeneity of ERG rearrangements within multifocal prostate cancer is well described[28], to the best of our knowledge this is the first time dual ETS rearrangement in prostate cancer has been demonstrated in formalin fixed and paraffin embedded samples by sequential RNA in situ hybridization and ERG immunohistochemistry methods (Supplemental Figure 1). On morphologic evaluation these tumor foci appeared to be a single focus. Hence our results demonstrate that in such rare instances, what appears to be a single tumor focus by routine histologic evaluation are actually collisions of two or more distinct tumor foci.

Gene expression based microarray studies have shown high level expression of *ERG*, *ETV1*, *ETV4* and *ETV5* almost exclusively in ETS positive prostate cancers while benign prostate and ETS negative prostate cancers show very low or complete absence of ETS gene expression. This unique expression pattern is optimal for the identification of ETS rearrangement without any ambiguity based on RNA-ISH. Its utility is specifically critical in the recognition of *ETV1*, *ETV4* and *ETV5* genes which lack specific antibodies at the tissue level by a methodology analogous to immunoperoxidase detection.

Although ETS gene fusions are common in PCa, and are known to be an early event in prostate carcinogenesis, the role of these genes in the prostate tumor development is not known. ERG, ETV1 and ETV4 belong to the ETS gene family and in the unusual cases with dual ETS rearrangement in a given prostate cancer specimen, the overall clinical course of the disease may be driven by the ETS rearrangement that is dominant by tumor grade and/or volume. For example in the case depicted in Figures 4 and 5 the larger tumor harboring ETV1 rearrangement may drive disease progression in contrast to the smaller ERG-positive tumor foci with similar or lower grade. Recent data suggests that ETV1 rearrangement may be associated with a more adverse prognosis. Baena et al.,[29] showed that ETV1 under PTEN deletion background in mice developed a more invasive carcinoma compared to ERG with PTEN deletion background implicating differential oncogenic potential of ERG and ETV1 in prostate cancer development.

The cases we observed herewith dual ETV1 and ERG rearrangement, a majority of the tumor focus was ETV1 positive whereas smaller foci were ERG-positive suggesting independent clonal origin of the tumor harboring distinct molecular aberration. It is however not clear whether such cases have altered clinical behavior. Given that the majority of the

tumor foci are ETV1 positive, such cases can be easily misdiagnosed as only ETV1-positive which underscores the importance of *in situ* detection methods for the simultaneous identification of multiple molecular subtypes of prostate cancer in a single specimen. Our preliminary studies here revealed the hitherto unidentified molecular subset of prostate cancer that warrants further in depth investigations to understand the molecular heterogeneity and associated clinical behavior.

In this study, we evaluated tissue microarrays containing predominantly specimens selected from major tumor foci and identified 4 cases with dual ETS rearrangement. Although, our small cohort of dual ETS rearranged tumors failed to reveal any specific histomorphologic features, further studies of larger cohorts of these unusual carcinomas are required to fully elucidate their clinical behavior and implications for prognosis. In addition to IHC and FISH, RNA-ISH *in situ* would be a valuable addition to the repertoire of tools used for the molecular sub classification of PCa. We are currently successfully using this RNA-ISH technique for determining the ETV1 status in an ongoing multi-institutional clinical trial that is testing the role of ETS fusions status from metastatic tumor biopsy as predictive biomarkers for response to abiraterone (ABI) alone or with ABT-888 (a PARP1 inhibitor) in metastatic castrate resistant prostate cancer patients (NCI 9012, a randomized ETS fusion –stratified Phase II trial).

In summary, this study shows that RNA-ISH is a viable alternative for the *in situ* detection of genes for which no specific antibodies are available or for non-coding RNAs. Further, RNA-ISH assays can be easily performed on formalin fixed and paraffin embedded tissues from needle biopsies, radical prostatectomies and tissue microarrays that can be standardized and scaled for high-throughput screening of large number of samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The University of Michigan has been issued a patent on ETS gene fusions in prostate cancer, on which S.A.T. and A.M.C. are co-inventors. The diagnostic field of use has been licensed to Gen-Probe Inc. /Hologic, who has sublicensed some rights to Ventana Medical Systems Inc. /Roche. A.M.C. serves on the advisory board of Ventana/Roche. S.A.T. has received honoraria from and consults for Ventana/Roche. Ventana/Roche did not play a role in the design and conduct of this study, in the collection, analysis, or interpretation of the data, or in the preparation, review, or approval of the article. N.P. receives research funding from Ventana/Roche but this funding did not play a part in development of the assays.

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ERG

Chr 21 - 39751950 40033704 NM_001136154

ETV1

Chr 7 - 13930856 14029642 NM_004956.4

ETV4

Chr 17 - 41605211 41623800 NM_001079675.2

ETV5

Chr 3 - 185764106 185826901 NM_004454.2

Figure 1.

Schematic diagram showing the genomic organization of A) *ERG*, B) *ETV1*, C) *ETV4* and D) *ETV5* genes. Horizontal black bar indicate the location of the probe in the gene and the numbers in the bar indicate the probe coverage region.

Figure 2.

Comparison of ERG RNA ISH and immunohistochemistry. Left panel showing the images of ERG RNA ISH and right panel showing images of ERG immunohistochemistry in benign prostate tissue (A&B); strong positive ERG by RNA ISH with score 4 (C) and positive ERG by immunohistochemistry (D) in a localized prostate carcinoma; weak staining of ERG by RNA ISH with score 1 and weak ERG by immunohistochemistry in a metastatic prostate carcinoma (E and F); ERG negative both by RNA ISH (G) and immunohistochemistry (H) in a metastatic prostate carcinoma.

Figure 3.

Unique expression of ETV1, ETV4 and ETV5 genes detected by RNA ISH in corresponding positive cases (ETV1 RNA-ISH score 2, ETV4 RNA-ISH score 4 and ETV5 RNA ISH score 2 with no cross reactivity or nonspecific hybridization of the probes in the negative cases).

Figure 4.

Whole section view of a prostate carcinoma tissue showing tumor molecular heterogeneity with a small foci showing ERG expression by immunohistochemistry (A) and a large tumor foci showing ETV1 expression by RNA ISH (Score 4). Confirmation of ERG and ETV1 rearrangements by FISH (Insets) in the corresponding tumor foci. Green and red color signals indicate 5' and 3' probes, respectively.

Figure 5.

Whole section view of a second prostate carcinoma tissue showing tumor molecular heterogeneity with a large tumor foci showing ETV1 expression by RNA ISH (Score 4) and two independent small foci showing ERG expression by immunohistochemistry (B). Confirmation of ERG and ETV1 rearrangements by FISH (Insets) in the corresponding tumor foci. Green and red color signals indicate 5' and 3' probes, respectively.

Figure 6.

A prostate adenocarcinoma sample showing ERG and ETV4 rearrangement in two independent tumor foci. A) ERG IHC; B) ETV1 RNA ISH; C) ETV4 RNA ISH with score 4; D) ETV5 RNA ISH.

Table 1

Types of tissue microarrays and distribution of cases for detection of *ETV1*, *ETV4* and *ETV5* rearrangement

Typeas of TMA	Number of patients
Localized PCa	160
Salvage prostatectomy	105
Metastatic PCa	54