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Research Paper

Correlation between *ERG* Fusion Protein and Androgen Receptor Expression by Immunohistochemistry in Prostate, Possible Role in Diagnosis and Therapy

Amir Hassan Navaei[1], Beatriz A Walter[1], Vanessa Moreno[1], Svetlana D Pack[1], Peter Pinto[2], Maria J Merino[1]✉

1. Translational Surgical Pathology, Laboratory of Pathology;
2. Urologic Oncology Branch, CCR, NCI, NIH, Bethesda, MD, USA.

✉ Corresponding author: Maria J. Merino, MD. mjmerino@mail.nih.gov 10 Center Drive, Room 3S235C, Bethesda, Maryland 20892, Phone 301-480-8483.

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Abstract

Background: Recent discovery of gene rearrangements have brought a new look to the molecular pathogenesis of cancer. Gene fusions occur in nearly 60% of prostate adenocarcinoma, being the *TMPRSS2-ERG* one of the most common. Evidence supports the role of *ERG* fusion in tumorigenesis, progression and invasion via effecting pathways such as *WNT*, *MYC*, *uPA*, *PI3K/AKT/PTEN*, *RAS/RAF/MAPF*, *NKX3.1*, *GST-pi* and androgen receptor (AR) mediated signaling. Most of the *ERG* fusions involve 5'-partners androgen responsive. Therefore, we aimed to evaluate AR and *ERG* fusion protein expression on prostate tissue to find clinicopathological applications and possible role in therapy.

Methods: One hundred three samples, including prostate core biopsies and radical prostatectomy specimens, were evaluated for *ERG* and AR expression by immunohistochemistry (IHC). *ERG* rearrangement was done by fluorescence *in situ* hybridization (FISH) on 11 randomly selected cases and correlated with IHC results.

Results: From the total of 103 samples, eight (8/103) were benign, fourteen (14/103) had atypical glands, two (2/103) had prostatic intraepithelial neoplasia (PIN), and seventy nine (79/103) showed prostate adenocarcinoma. Forty four (44/79) tumor cases were Gleason score (GS) 6-7 (lower GS), and thirty five (35/79) were GS of 8-10 (higher GS). *ERG* immunoreaction was observed in 27.8% (22/79) of the tumor cases, showing higher expression in those with lower GS (68.2%, 15/22) compared to higher GS (31.8%, 7/22). Neither benign glands nor PIN stained with *ERG*. AR expression was observed in 75% of benign samples, 78.5% of atypical glands, 100% of PIN, and in 87.3% of tumor cases with no significant difference based on GS. Co-expression of *ERG* and AR was evaluated on all the tumor samples. *ERG*+/AR+ was seen in 77.3% (17/22) of the *ERG*+ tumor cases, with higher frequency in lower GS (64.7%, 11/17) compared to those with higher GS (35.3%, 6/17). All but five corresponding *ERG*+ tumor samples were negative for AR. Only 5 samples were *ERG*-/AR- corresponding to adenocarcinoma GS of 6. Presence or absence of *ERG* rearrangement was confirmed by FISH and correlated with IHC results.

Conclusions: Characterization of *ERG* status by IHC in prostate tissue has an excellent correlation with FISH. It may also assist in diagnosis since none of the benign glands stained with *ERG*. Co-expression of *ERG*+/AR+ in prostate tumor by IHC may suggest gene fusion between *ERG* and a 5'-partner driven by androgen signaling such as *TMPRSS2*, which it could represent an important ancillary test for clinical management and development of new therapeutic targets.

Key words: *ERG* Fusion Protein, Androgen Receptor Expression

Introduction

In the United States, prostate cancer is the most common primary neoplasm in males with an estimated of 180,890 new cases in 2016. Despite diagnostic and therapeutic advances in medicine, prostate cancer remains the second leading cause of cancer related deaths among males with 26,120 deaths expected to occur in 2016[1]. In the last decade, progress has been made to search for molecular pathways and oncoproteins associated to the progression of prostate cancer, most of them driven by gene rearrangements and gene fusion, helping to identify new markers and modalities of treatment.

ETS-related gene (*ERG*) is a transcription factor from the Erythroblastosis virus E26 (*ETS*) oncogene family located on chromosome 21[2]. The *ETS* family of proteins shows a wide variety of expression patterns in human tissues[3]. Members of the ETS family are known to be the key regulators of embryonic development, cell proliferation, differentiation, angiogenesis, inflammation, and apoptosis. The protein encoded by *ERG* is mainly expressed in the nucleus and contains and *ETS* DNA-binding domain and a PNT (pointed) domain which is implicated in the self-association of chimeric oncoproteins[4]. *ERG* is expressed in endothelial tissues, hematopoietic cells, kidney, and in the urogenital track[5]. This protein regulates hematopoiesis, and differentiation and maturation of megakaryocytic cells[5]. It is also required for platelet adhesion to the subendothelium, inducing vascular cell remodeling[6]. This gene is also involved in chromosomal translocations, resulting in different fusion gene products. One of the first findings was the identification of a nonrandom translocation of *ERG* from chromosome 21 to 8 in t(8; 21)(q22; q22) associated with acute myelogenous leukemia of the subgroup M2 (AML-M2)[2]. Later, other various contributions identified were the fusion of *ERG* with the *TLS/FUS* gene in acute myeloid leukemia and the *EWS* gene in Ewing's sarcomas[7].

Discovery of the role of *ERG* in prostate cancer began in 2005 when Petrovics et al identified that *ERG* was frequently overexpressed in prostate cancer[3]. Subsequently, Tomlins et al, in 2005, discovered the *ETS* family transcription factor gene fusions, changing dramatically the field of solid tumor biology[8]. Recurrent gene fusions of the transmembrane protease serine 2 (*TMPRSS2*) gene to five members of the *ETS* family of transcription factor genes (*ERG, ETV1, ETV4, ETV5, and ELK4*) has been reported and confirmed in human prostate cancer, resulting in overexpression of normal or truncated *ETS*-proteins[8-12].

The most common rearrangement in prostate cancer is the *TMPRSS2-ERG* fusion with a reported range from 15% to 80%, depending on the clinical cohorts investigated and methods of detection[8-19]. There is also a small percentage (<10%) of fusions that occur between *ERG* and other three additional androgen responsive 5′ partners such as solute carrier family 45, member 3 (*SLC45A3)*[19-21], homocysteine inducible endoplasmic reticulum protein with ubiquitin-like domain member 1 (*HERPUD1)*[22] and *N-myc downstream regulated gene 1 (NDRG1)*[15,19].

With the identification of *ERG* new studies emerged demonstrating its potential role as a prognostic indicator and as a marker for prostate cancer[23-25]. In 2006, Perner et al found that the presence of *ERG* rearrangements accompanied by 5′-*ERG* deletion has a significant correlation with higher tumor stage and the presence of metastatic disease involving pelvic lymph nodes[13]. Later in 2007, Demichelis et al, in a watchful waiting cohort of 111 patients, reported a significant association between the presence of a *TMPRSS2-ERG* fusion and prostate cancer-specific death, and a link between the presence of *ERG* alterations and higher Gleason score[14].

Multiple molecular pathways have been implicated in the pathogenesis, development, differentiation, progression, invasion, and metastasis of prostate cancer. Several studies have revealed details about the mechanisms of *ERG* fusion in the development and progression of prostate cancer, such as: activation of c-Myc abrogating prostate epithelial differentiation[26]; regulation of the 15-hydroxy-prostaglandin dehydrogenase (*HPGD*) gene, a tumor suppressor, and prostaglandin catabolizing enzyme leading to altered levels of the *HPGD* and prostaglandin E2 (PGE2) as well as urokinase-type plasminogen activator (uPA)[27]. Also, *ERG* fusion has been involved in promoting cell proliferation through the PTEN/AKT/PIK3/mTOR pathway, and it has been suggested that its presence along with PTEN deficiency may cause the development of invasive cancer[28]. The androgen receptor (AR) plays a central role in any of those key-signaling pathways[12].

Androgen is an important regulator of normal development and maintenance of the prostate gland[29,30]. Approximately 80-90% of prostate cancers are initially dependent on androgen requiring endocrine therapy directed toward the reduction of serum androgens and inhibition of AR[31]. AR is a DNA-binding nuclear transcription factor that regulates gene expression, which is maintained throughout prostate cancer progression, and persists in the majority of patients with hormone refractory disease[25,32-34]. As we know, in the majority of the prostate carcinomas there are fusion genes with oncogenic potential involving 5′-end elements composed of androgen regulated genes and the *ETS*

gene transcription factor family[25,35,36]. Data has shown that overexpression of the androgen receptor does not give rise to hyperplastic lesions, but when combined with high levels of *ERG*, it promotes the development of a more poorly differentiated, invasive adenocarcinoma[28]. Some other studies have also demonstrated that androgen overstimulation of prostate cells cause development of fusion between *TMPRSS2* and *ERG* genes[18].

During the last decade, immunohistochemistry (IHC) has been introduced as a promising tool to detect *ERG* expression on the protein level. Furusato et al reported a highly specific mouse monoclonal *ERG* antibody performing a comprehensive evaluation of *ERG* protein expression using whole mount prostate sections from 132 prostate carcinoma cases, where *ERG* protein expression showed a strong concordance with *ERG* fusion transcripts by branched DNA assay or *ERG* rearrangement by fluorescence *in situ* hybridization (FISH) in selected specimens[37]. Similarly, other authors have provided a comprehensive data showing that virtually all *ERG* rearranged tumors exhibit *ERG* overexpression on the transcriptional and translational level[19,38]. To the best of our knowledge the evaluation of *ERG* fusion gene product and the AR at the expression level has not been well established in prostate specimens. Therefore, here we studied the correlation of *ERG* fusion product with AR expression by immunohistochemistry (IHC) in a wide range of prostate biopsies and prostatectomies in order to determine the clinical and pathological impact and possible therapeutic targets.

Materials and Methods

Samples

Formalin fixed paraffin embedded sections of a total of 116 prostate tissue specimens, including 102 biopsies and 14 radical prostatectomies, were retrieved from the archives of the Laboratory of Pathology at the NIH/NCI. These samples corresponded to 46 patients with a median age of 62 year-old. All these patients had been evaluated at the Urologic Oncology Branch of the National Cancer Institute, under approved protocol by the Institutional Review Board of the National Cancer Institute. Appropriate informed consents were obtained.

ERG and AR evaluation was performed on 103 samples from the total cases retrieved due to lack of material from thirteen (13/116) specimens. Tumors with various Gleason score (GS) were included in this study. Each tumor locus was evaluated separately.

Immunohistochemistry

Protein expression of *ERG* was performed in all the 103 cases by immunohistochemistry using a rabbit monoclonal antibody against human *ERG* (Clone EPR3864; Epitomics, Burlingame, CA). Formalin-fixed paraffin-embedded sections (5 µm) from core biopsies and whole tissue sections were deparaffinized in three Xylene baths and rehydrated in graded ethanol concentrations. Antigen retrieval was performed with Tris-EDTA buffer, pH 9.0, for 20 minutes under microwave and then allowed to cool down at room temperature for other 20 minutes. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in phosphate-buffered saline (PBS) for 15 minutes. Sections were then quenched with protein blocking buffer at room temperature for 30 minutes followed by one-hour incubation with a 1:100 dilution in PBS of the *ERG* antibody. For negative controls, sections were incubated in parallel with PBS instead of primary antibody. After rinsing, the sections were incubated with a peroxidase-labeled polymer conjugated to goat anti-mouse and goat anti-rabbit immunoglobulins as secondary antibody for 45 minutes (EnVision®+ Dual Link System-HRP Kit, DakoCytomation, Carpinteria, CA). The staining was visualized with 3, 3'-diaminobenzidine (DAB) as chromogen and slides were counterstained with hematoxylin, dehydrated and finally mounted. Vascular endothelial cells that were uniformly and strongly positive for *ERG* expression were used as the internal positive control.

Protein expression for AR was done similarly as the staining protocol for *ERG*. However, antigen retrieval was performed with citrate buffer, pH 6.0, for 20 minutes under microwave and then allowed to cool down at room temperature for other 20 minutes. Slides were incubated with mouse monoclonal antibody against human AR (1:50 dilution, Clone AR441, Dako, CA) for 30 minutes.

Both antibodies, *ERG* and AR, showed nuclear immunoreaction and it was subsequently graded using a semiquantitative scoring system as 0 (negative), 1 (weak), 2 (moderate) and 3 (strong) according to the intensity and percentage of stained glands (Figure 1). Appropriate internal and external controls were used. The histopathologic evaluation was performed by one experienced pathologist (MJM).

Fluorescence *in situ* hybridization

FISH analysis was performed on eleven (11) samples using dual-color intherphase break-apart probes for the 5′ and 3′ regions of *ERG* gene. Briefly, sections of 5 µm-thick, paraffin-embedded tissues were heated at 60°C for 1-hour, then deparaffinized and rehydrated using xylene and graded ethanol, respectively. Antigen retrieval was performed with

epitope retrieval solution (Catalog IW-1100, IHC-Tek, MD, USA) under electric steamer for 25 minutes and then allowed to cool down at room temperature for other 20 minutes. For optimum digestion, slides were incubated in pepsin solution (0.1mg/ml pepsin in 0.01 *N* HCl) at 37ºC for 30 minutes. Assessment was done every 3 to 5 minutes for optimal extent of tissue digestion. After rinsing in saline sodium citrate (SSC) buffer, dehydration was performed in increasing orders of ethanol and set for drying on heating block for 10 minutes. Subsequently, slides were incubated with the *ERG* Break-apart FISH probes (Empire genomics, Buffalo, NY) and covered with cover slip and sealed with rubber cement. After being denatured at 78ºC for 10 minutes they were incubated overnight at 37ºC in dark moist chamber. Later, the rubber cement was removed and soaked twice in the SSC buffer and then washed in pre-warmed 0.4xSSC/0.03% Tween 20 at 73ºC and followed by counter staining with 0.25µg/mL of 4,6-diamidino -2phenylindole-2-hydrochloride (DAPI). After incubating the slides at -20°C they were analyzed using fluorescein microscope. The hybridization was performed in low-stringency conditions with 50% formamide/2xSSC/10% dextran sulfate co-denaturation of the slide/probe, 1-hour hybridization at 37°C, followed by a 2 min wash in 0.4×SSC/0.3% Tween 20 at room temperature. Slides were counterstained with DAPI.

FISH signals were scored with a Zeiss epifluorescence microscope (Zeiss Axio Imager-2, Carl Zeiss Microimaging LLC, Thornwood, NY) equipped with a DAPI/FITC/Rhodamine single band pass filters (Semrock, Rochester, NY) using 40–60x objectives. In each case, a minimum of 100 cells were scored for the presence/absence of *ERG* gene rearrangement through deletion or split and it was considered positive if present in more than 50% of the nuclei. On each nucleus, the scoring was based on the number of unrearranged *ERG* loci (twinned red and green signals forming yellow signals), separated 5′-*ERG* sequences (labeled green) and separated 3′-*ERG* sequences (labeled red). Tumors were defined as positive for *ERG* rearrangement if a break occurs between the two probes giving two separated color signals (signal split) and/or deletion of the 5′ probes (absence of one or more green signals), indirectly suggesting *ERG* fusion (*TMPRSSS2-ERG*).

Results

A total of 103 samples were evaluated for both antibodies, *ERG* and AR, by IHC. From these, eight (8/103) cases were benign, fourteen (14/103) had atypical glands, two (2/103) had prostatic intraepithelial neoplasia (PIN), and seventy nine (79/103) showed prostate adenocarcinoma. The cases with adenocarcinoma included twenty six (26/79) with GS of 6, eighteen (18/79) with GS of 7, twenty one (21/79) with GS of 8, and fourteen (14/79) with GS of 9 – 10. Table 1, 2 and 3 summarizes the results for *ERG* and AR expression by IHC.

Table 1. *ERG*/AR expression by IHC staining prostate tissue from 103 samples.

Samples (n = 103)	Expression			
	ERG+/AR+ (n = 19)	*ERG*+/AR- (n = 5)	*ERG*-/AR+ (n = 69)	*ERG*-/AR- (n = 10)
Benign (n = 8)	0	0	6	2
PIN (n = 2)	0	0	2	0
Atypical glands (n = 14)	2	0	9	3
Adenocarcinoma (n = 79)	17	5	52	5

Abbreviations: IHC, immunohistochemistry; n, total number of cases; *ERG*, transcription factor from the *ETS* family (Erythroblastosis virus E26 oncogene); AR, androgen receptor; PIN, prostatic intraepithelial neoplasia.

Table 2. Correlation of *ERG*-positive tumor samples (based on GS) with AR expression by IHC.

Adenocarcinoma samples, *ERG*+ (n = 22)	Expression	
	ERG+/AR+ (n = 17, 77.3%)	*ERG*+/AR- (n = 5, 22.7%)
GS of 6 (n = 9)	7 (41.2%)	2 (40%)
GS of 7 (n = 6)	4 (23.5%)	2 (40%)
GS of 8 (n = 4)	3 (17.6%)	1 (20%)
GS of 9 – 10 (n = 3)	3 (17.6%)	0

Abbreviations: IHC, immunohistochemistry; n, total number of cases; *ERG*, transcription factor from the *ETS* family (Erythroblastosis virus E26 oncogene); AR, androgen receptor; GS, Gleason score.

Table 3. Correlation of *ERG*-negative tumor samples with AR expression by IHC.

Adenocarcinoma samples, *ERG*- (n = 57)	Expression	
	ERG-/AR+ (n = 52, 91.2%)	*ERG*-/AR- (n = 5, 8.7%)
GS of 6 (n = 17)	12 (23.1%)	5 (100%)
GS of 7 (n = 12)	12 (23.1%)	0
GS of 8 (n = 17)	17 (32.7%)	0
GS of 9 – 10 (n = 11)	11 (21.1%)	0

Abbreviations: IHC, immunohistochemistry; n, total number of cases; *ERG*, transcription factor from the *ETS* family (Erythroblastosis virus E26 oncogene); AR, androgen receptor; GS, Gleason score.

ERG Immunohistochemistry

ERG immunoreaction was observed in 24 cases from the total of 103 samples, where two (2/103) had atypical glands and twenty two (22/103) were adenocarcinomas. A moderate to strong nuclear staining was present in most of the cases.

The expression of *ERG* in adenocarcinoma was 27.8% (22/79), and based on the GS it was more frequently seen on those tumors showing GS of 6 with 40.9% (9/22), followed by GS of 7 (27.3%, 6/22), GS of 8 (18.2%, 4/22), and GS of 9-10 (13.6%, 3/22). In

general, *ERG* expression was 68.2% (15/22) in tumors with lower GS (6 and 7), and 31.8% (7/22) in those with higher GS (8 through 10). The majority of the tumor samples were *ERG* negative (72.1%, 57/79).

No immunoreaction to *ERG* was observed in PIN, benign samples, and in benign glands adjacent to adenocarcinoma in those tumor samples (Figure 2).

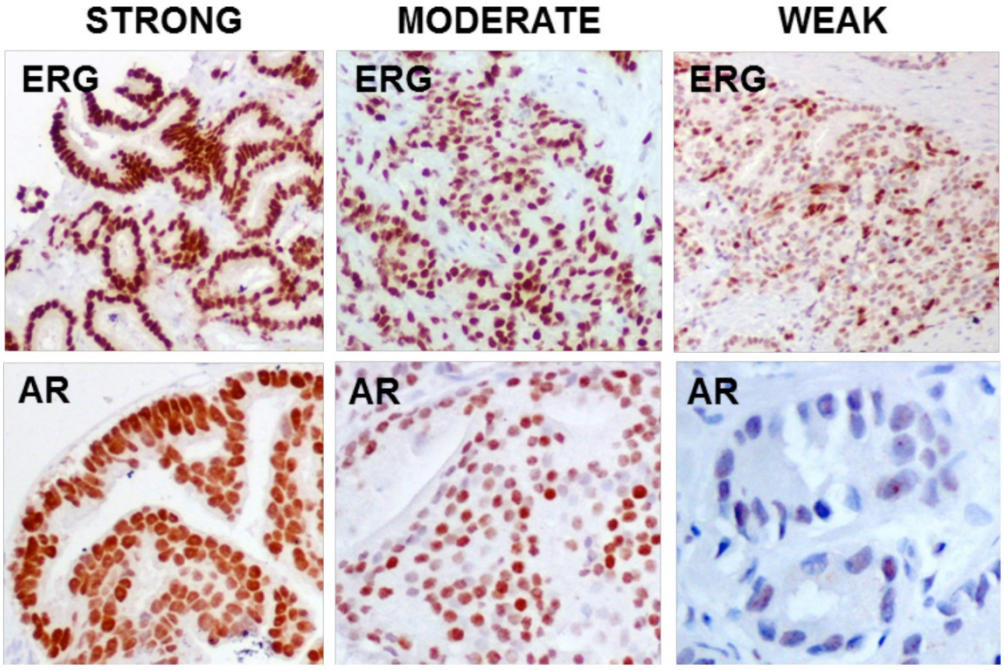

Figure 1. IHC staining strength for *ERG* (EPR3864) and AR

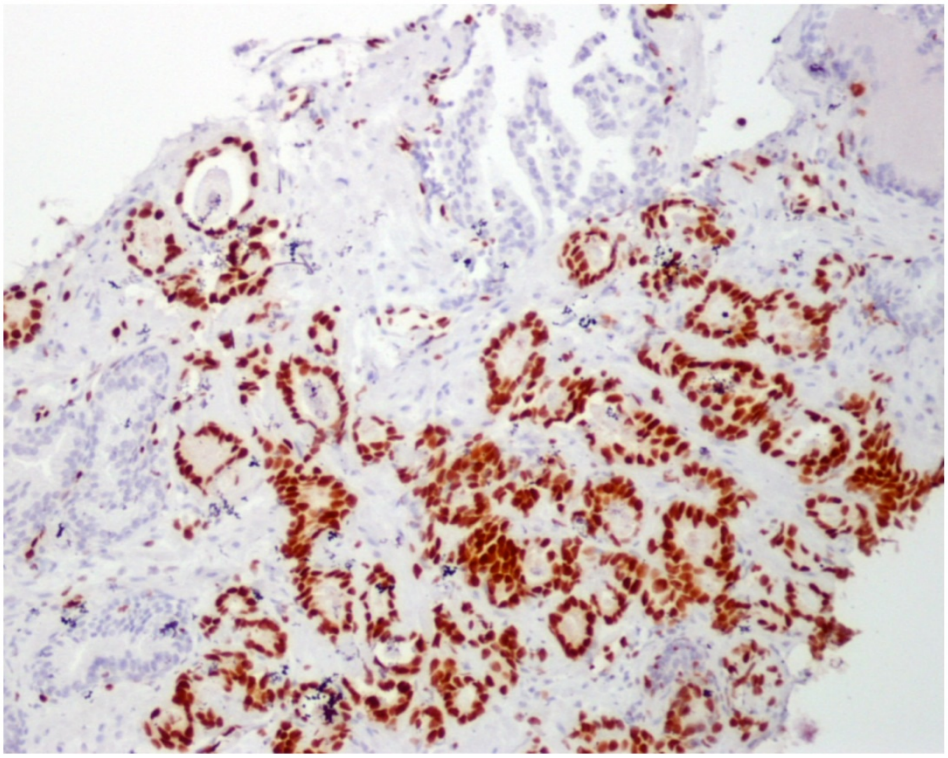

Figure 2. *ERG* immunoperoxidase staining showing the strong nuclear staining in the tumoral glands, while the benign glands have no nuclear immunoreaction to *ERG* (10X).

AR Immunohistochemistry

AR immunoreaction was observed in 88 cases from the total of 103 samples, where six (6/103) had benign glands, 2 (2/103) had PIN, eleven (11/103) had atypical glands and sixty nine (69/103) were adenocarcinomas. The strength of staining was mostly scored as 1 to 2 (weak to moderate). Few sections showed strong (score of 3) staining, and most of them were tumors with higher GS (8 through 10).

Among the total number of tumor cases, 87.3% (69/79) showed positive immunoreaction to AR antibody, and based on the GS it was as follows: 27.5% (19/69) on GS of 6, 23.2% (16/69) on GS of 7, 29% (20/69) on GS of 8, and 20.3% (14/69) on GS of 9-10. In general, AR expression was present in 50.7% (35/69) of the tumors with lower GS (6 and 7), and 49.3% (34/69) of those with higher GS (8 through 10), demonstrating no significant differences.

In addition, AR expression was seen in 75% (6/8) of benign samples, 78.6% (11/14) of atypical glands and in 100% (2/2) of PIN.

ERG/AR Immunohistochemistry Correlation

The majority of the cases showed *ERG*-/AR+ expression as follows: six benign samples (6/69), two samples with PIN (2/69), nine with atypical glands (9/69), and fifty-two cases (52/69) with prostate adenocarcinoma. Nineteen specimens were ERG+/AR+, being more frequently seen in samples with adenocarcinoma (89.5%, 17/19), and only in 2 cases with atypical glands (see Table 1).

Among the total number of *ERG*-positive (*ERG*+) tumor cases, 77.3% (17/22) showed expression for both, *ERG* and AR (*ERG*+/AR+); and based on the GS the co-expression was present as follows: 41.2% (7/17) on GS of 6, 23.5% (4/17) on GS of 7, 17.6% (3/17) on GS of 8, and 17.6% (3/17) on GS of 9-10. In general, *ERG*+/AR+ immunoreaction was more frequently seen in tumors with lower GS of 6-7 (64.7%, 11/17) than in those with higher GS of 8-10 (35.3%, 6/17) (see Table 2).

ERG+/AR- immunostaining was observed in a total of 5 tumor samples (5/22, 22.7%). Two of them were GS of 6, another 2 were GS of 7, and only 1 corresponded to GS of 8 (see Table 2).

From the total samples with *ERG*-negative (*ERG*-) tumors, 91.2% (52/57) showed *ERG*-/AR+ immunoreaction, and based on the GS it was as follows: 23.1% (12/52) on GS of 6, 23.1% (12/52) on GS of 7, 32.7% (17/52) on GS of 8, and 21.1% (11/52) on GS of 9-10. In general, *ERG*-/AR+ immunoreaction was seen in 46.2% (24/52) of the tumors with lower GS of 6-7, and 53.8% (28/52) in those tumors with higher GS of 8-10, suggesting no significant difference between GS (see Table 3).

ERG-/AR- was only seen in 9.7% (10/103) from the total samples evaluated. Among these, two (2/10) cases were benign, three (3/10) showed atypical glands, and 5 (5/10) were prostate adenocarcinoma with GS of 6.

Fluorescence *in situ* hybridization (FISH)

A break-apart assay was performed for the indirect detection of *TMPRSS2-ERG* fusion. One benign sample (*ERG*- by IHC) and 10 tumor sections (3 *ERG*-, 4 *ERG*+/AR+ and 3 *ERG*+/AR- by IHC) were included for the evaluation and correlation of *ERG* fusion by FISH. Benign glands in each sample were consistently negative for *ERG* fusion and were used as our internal control. Areas of tumor showing positive immunoreaction to *ERG* consistently demonstrated splitting or deletion of signal in more than 50% of the nuclei evaluated. Neither split of signal nor deletion was observed on the other 3 samples with *ERG*-negative tissue by IHC (1 benign and 2 tumor samples). *ERG* IHC overexpression (*ERG*+) was detected in all the cases with *ERG* rearrangement (7/7, 100%), while the other cases with no *ERG* rearrangement were also *ERG*-negative (*ERG*-) by IHC (4/4, 100%), indicating high sensitivity and specificity to detect *ERG* fusion protein expression by IHC. Detailed results are demonstrated in Table 4.

Table 4. Correlation of *ERG* gene rearrangement by FISH with *ERG* expression by IHC on 11 randomly selected samples.

ERG gene rearrangement by FISH (n = 11)	*ERG* expression by IHC		
	ERG+/AR+(n = 4)	*ERG*+/AR-(n = 3)	*ERG*- (n = 4)
Positive (n = 7)	4	3	0
Negative (n = 4)	0	0	4

Abbreviations: *ERG*, transcription factor from the *ETS* family (Erythroblastosis virus E26 oncogene); IHC, immunohistochemistry; FISH, fluorescence *in situ* hybridization; n, total number of cases; AR, androgen receptor.

Discussion

Discovery of the fusion genes in prostate cancer has brought a new perspective in our understanding of the pathophysiology of these tumors. The most common rearrangement in prostate cancer is the *TMPRSS2-ERG* fusion, which is androgen-driven and its prevalence has been confirmed to occur in 40% to 70% of prostate adenocarcinoma by several independent studies[8-19]. Although most studies have focused on the dominant rearrangement *TMPRSS2-ERG* fusion, a variety of other fusions and other androgen 5'-partners (*SLC45A3*, *HERPUD1* and *NDRG1*) have been described, but appear to be less common[8-22].

TMPRSS2-ERG fusion joins *TMPRSS2* exons 1 or 2 usually to *ERG* exons 2, 3 or 4, which results in the

activation of the *ERG* transcription factor. This fusion separates the 3′-*ERG* centromeric regions from the 5′-*ERG* telomeric ends; however, deletions of the 5′ region can also occur and has been reported to be more common in prostatic small cell carcinomas with a more aggressive behavior[39]. The result of the fusion of *ERG*, either by translocation or deletion, is the production of a duplicated protein[40]. *ERG* protein expression has been detected in lymphocytes and endothelial cells of small vessels from benign and cancerous prostate tissue, regardless of the *ERG* rearrangement status. However, *ERG* rearrangements have never been detected by FISH in endothelial cells or lymphocytes[19,41].

The recent literature, described *ERG* positivity in 26 – 90% of prostate carcinoma[25,42-45]. In our experience, *ERG* immunoreaction was observed in 27.8% (22/79) of the tumor cases, as described in the literature. Additionally, some authors described a link between the presence of *ERG* alterations and higher GS[14,45], while others did not find any significant differences[25,46]. Our data reflects the opposite; we found that *ERG* expression is twofold more frequent in those tumors with lower GS (68.2%, 15/22) compared to those with higher GS (31.8%, 7/22). Similar data was observed in another study by Kron et al[44]. Also, endothelial cell nuclei have positive immunoreaction to *ERG* in all samples, and they were used as our internal control, keeping in consistency with previous studies. The *ERG* antibody used in our study demonstrated a very strong nuclear staining in most of the cases.

Interestingly, gene fusions have not been observed in benign prostate biopsies or in those normal prostatic glands adjacent to the tumor areas, benign prostatic hyperplasia (BPH) or even in atrophic glands through different molecular analysis[13,46,47]. However, Clark et al detected *TMPRSS2-ERG* fusions in 2 out of 31 BPH samples by reversed transcriptase-PCR (RT-PCR). But, when these two specimens were subjected to a repeated histopathological examination, no prostatic malignancy was identified[17]. *ERG* fusion has been consistently reported to be highly specific to the tumor tissue[19,40]. On the contrary, in previous studies, assessment of *ERG* protein expression by IHC in high grade PIN and benign glands that were adjacent to the *ERG*-positive tumor glands have been reported to be positively immunoreactive to the antibody[19,48,49]. Our experience demonstrated no expression of *ERG* in PIN, benign samples, and in those benign glands that were adjacent to the tumor areas. However, only 2 cases from a total of 14 with atypical glands demonstrated *ERG*+/AR+ immunoreaction. In both cases, the atypical glands were adjacent to *ERG*-positive, low GS (6 and 7), prostate adenocarcinoma. This finding may suggest the role of *ERG* gene fusion along with androgen stimulation in early development of prostate cancer. Also, identification of *ERG* immunoreaction in atypical glands should trigger the suspicion of the pathologist to evaluate the specimen thoroughly.

AR expression was observed in 75% of benign samples, 78.5% of atypical glands, 100% of PIN, and in 87.3% of tumor cases with no significant difference based on GS. However, if we correlate the expression of *ERG* and AR instead of analyzing each one by itself, our findings are similar to most of the published data[3,12]. For instance, Hermans et al evaluated the presence of *ERG* fusion in androgen-sensitive and androgen-independent xenografts by qPCR and FISH[18]. It was reported that in all tested androgen-sensitive cell lines, with the exception of one, the overexpression of *ERG* correlated with the presence of *TMPRSS2-ERG* fusion transcripts; however, in all androgen-independent xenografts no correlation has been observed. As we know *TMPRSS2* is AR dependent, and androgen signaling induces proximity of the *TMPRSS2* and *ERG* genomic loci, rendering fusion events most likely[18]. Moreover, in a recent study performed in prostatic small cell carcinomas, Wang et al demonstrated that there is an increased in the AR gene copy number and AR protein expression frequently associated with *TMPRSS2-ERG* fusion[50]. In our study, 77.3% (17/22) of the total *ERG*-positive tumors showed co-expression with AR (*ERG*+/AR+), being more frequently observed in lower GS (64.7%, 11/17) compared to those with higher GS (35.3%, 6/17). At the same time, four tumor cases randomly selected and expressing both proteins (*ERG*+/AR+) by IHC showed positive break-apart split indicating presence of *ERG* rearrangement (Figure 3), indirectly suggesting a fusion between an androgen driven 5′-partner and the 3′-*ERG* (most likely TMPRSS2-*ERG*). Although our study did not show statistical significance, based on our results it is possible to conclude that *ERG*+/AR+ expression by IHC is highly correlated to the TMRPSS2-*ERG* fusion at the expression level.

In vitro and *in vivo* models have demonstrated that *ETS* genes have an effect on tumor progression, but alone do not appear to be sufficient for transformation into cancer. For instance, Zong et al, demonstrated that overexpression of both AR and *ERG* promoted the development of poorly differentiated invasive adenocarcinomas[28]. However, in our experience the co-expression of *ERG* and AR was higher in those tumors with low GS compared to those with high GS.

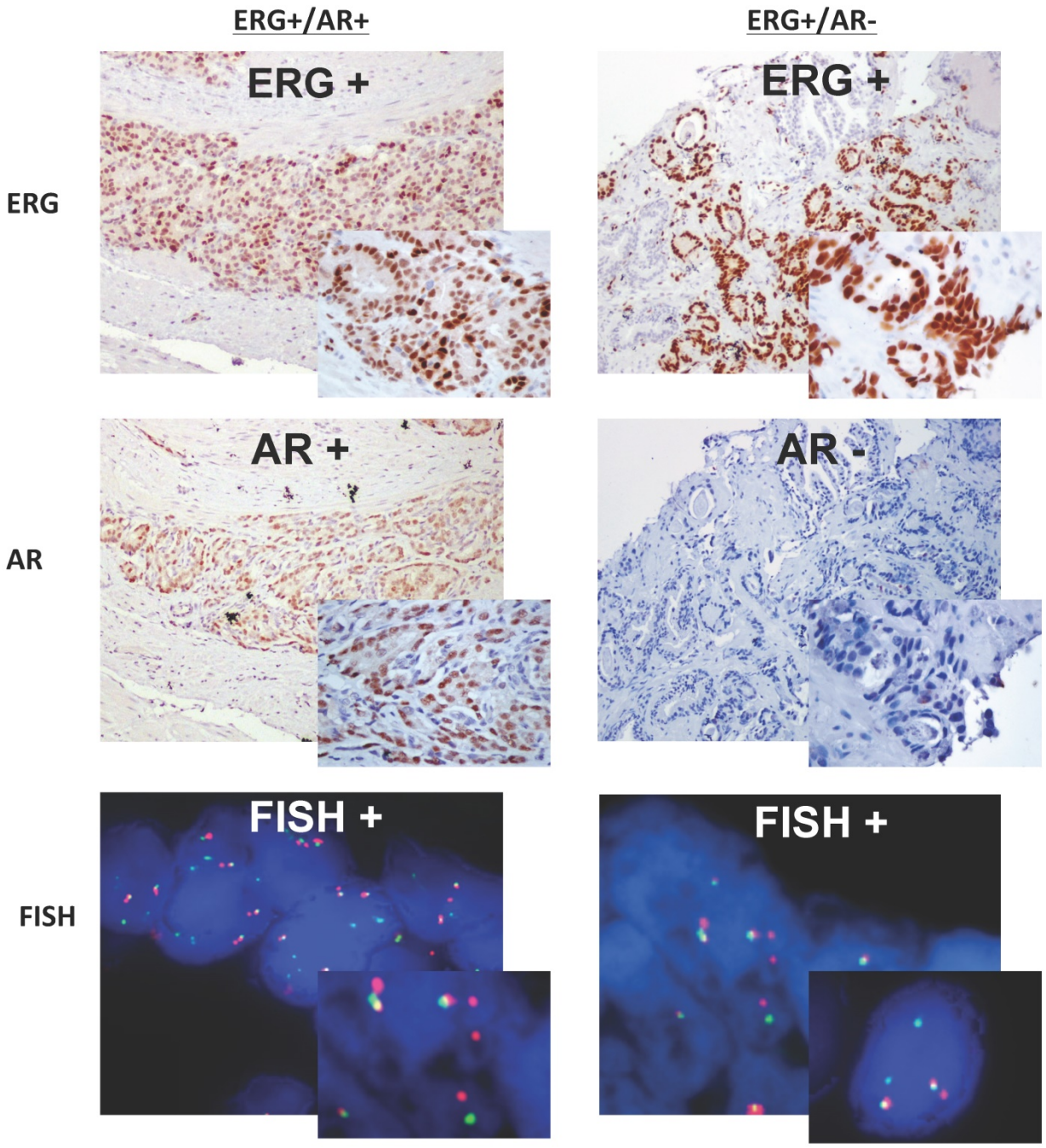

Figure 3. Two cases, right and left panel, with prostate adenocarcinoma demonstrating *ERG*/AR expression by IHC (low and high magnification) correlated with their FISH results. The left panel shows positive expression of both proteins *ERG* and AR by IHC, and the right panel depicts the positive expression of *ERG* and lack of expression for AR. Both cases, show positive FISH analysis for *ERG* rearrangement with red and green signals split apart and spatially separated in different regions of the nuclei, indirectly suggesting *TMRPSS2-ERG* fusion.

Furthermore, 22.7% (5/22) of the *ERG*-positive tumors were negative for AR (*ERG*+/AR-) by IHC. Two of them had a GS of 6, the other 2 had a GS of 7 and only 1 had a GS of 8. FISH analysis in three of these cases confirmed the presence of *ERG*-rearrangement (split signal) (Figure 3). One might speculate that the lack of expression of AR in those *ERG*-positive tumors could be explained by the fact that there are some 5′ partners that are androgen-independent genes (*DDX5* and *C15orf21*). These androgen-independent 5′ partners are known to be ubiquitously expressed and found to be fused in frame with other ETS family members, leading to the expression of fusion proteins such as DDX5-ETV4 and C15orf21-ETV1[21,51]. We might hypothesize that these 5′ partner androgen-independent genes could be present in those tumors showing *ERG*+/AR- by IHC or we could think that these tumors may no longer be reliant on androgen signaling leading to a lack of staining for the AR. Further analysis and studies need

to be done in order to clarify this issue.

Since the initial discovery of *ETS* fusions in prostate cancer there are multiple types of gene fusions described. On the basis of these discoveries, Rubin et al have developed a classification system composed by three categories: 1) fusions involving *ETS* gene family members (*ERG*, *ETV1*, *ETV4*, *ETV5*, and *ELK4*); 2) *RAF* kinase family fusions; and 3) *SPINK1*-positive prostate cancers[12]. In our study, the majority of the tumor samples were *ERG*-negative (72.1%, 57/79), and all of these but five cases were AR positive. Three (3) of these *ERG*-negative, randomly selected, tumor cases were confirmed to be negative for *ERG* rearrangement by FISH analysis. One could speculate that the *ERG*-/AR+ tumor cases could represent any of those categories described by Rubin et al, where the fusion gene involved corresponds to a *RAF* kinase family fusion, or a *SPINK1* fusion, or still, it could belong to another gene of the *ETS* family member that is not expressing the *ERG* protein. More studies need to be done in order to find more answers to these findings.

Recent advances in next generation transcriptome sequencing have facilitated the discovery of the second category-*RAF* kinase gene fusions *SCL45A3-BRAF*, *ESRP1-RAF1*, and *RAF1-ESRP1* in advanced prostate cancers. Although rare, detected in approximately 1 to 2% of prostate cancers, *RAF* kinase fusions represent the first "driver" fusions in prostate cancers that do not involve an *ETS* family member. The third category, *SPINK1*-positive prostate cancers, is included in the classification since the outlier expression of *SPINK1* occurs in *ETS*-rearrangement-negative prostate cancers, and therefore defines a specific subclass of prostate cancer[12].

In addition, we found that only 5 five cases of prostate adenocarcinoma with GS of 6 showed *ERG*-/AR- by IHC, which they could probably represent any of the less frequent previously described fusion genes (*DDX5-ETV4* or *C15orf21-ETV1*) or even they could belong to any of those categories described by Rubin et al. Further analysis need to be done as well for these rare cases for better characterization, since they may have different behavior and clinical implications.

A limitation of our study is that due to the lack of material in the majority of the cases from our cohort, FISH analysis could not be done to confirm a significant correlation between the presence or absence of *ERG* rearrangement and the expression of *ERG* protein by IHC. Ideally, it would be to confirm the type of rearrangement or gene fusion involved by PCR or other methods in order to have a more clear evidence of the molecular pathways and corresponding levels of expression by IHC. Despite these limitations, we could provide data showing the prevalence of *ERG* positivity in 26.7% of the prostate carcinoma samples evaluated as well as we could show that *ERG* expression is inversely related to the GS. It is also expressed in a subset of atypical glands that are immediately adjacent to cancer. Additionally, there is a strong correlation between FISH and IHC for the *ERG* antibody with 100% sensitivity and specificity, which is similar to the reported data by Park et al where the sensitivity and the specificity of IHC is approximately 97% and 96%, respectively, a comparison done by FISH, PCR and IHC[19].

In summary, based on our findings indicate that IHC may have an important function in determining truncated *ERG* protein expression as a result of *ERG* gene fusion and can be an efficient alternative to FISH. In addition, examination and correlation of *ERG* and AR have diagnostic significance and may be useful in assessing the biological character of the prostate cancer as well as selecting the best treatment with therapeutic molecular targets. Also, the co-expression of *ERG*/AR in prostate cancer could indirectly suggest that the patient harbors a gene fusion between *ERG* and a 5'-partner driven by androgen signaling such as *TMPRSS2, SLC45A3* or *NDRG1.* Finally, is important to be aware of the expression of *ERG* protein in benign glands, atypical glands and/or high grade PIN since may warrant further workup to identify a possible adjacent tumor either in the same core or other cores. Further studies to establish the relevance of all these findings with the clinical outcome would be highly suggested.

Competing Interests

The authors have declared that no competing interest exists.

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