Research Article

TMPRSS2-ERG gene fusion is rare compared to *PTEN* deletions in stage T1a prostate cancer[†]

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Running Head: Molecular Alterations in T1a Prostate Cancer

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Abstract

T1a prostate cancers (cancer found incidentally in transurethral resection, <5% of the tissue) are indolent tumors of the transition zone. The overexpression of ERG and the inactivation of PTEN have been shown to be important drivers of carcinogenesis in large series of prostate cancer, but the genetics of transition zone tumors have not been well characterized. We evaluated the status of ERG and PTEN in formalin-fixed paraffin-embedded tissue using immunohistochemical and FISH analysis in 54 T1a transition zone tumors. The protein expression of ERG was determined using a rabbit monoclonal antibody and nuclear staining was scored as positive or negative. The genomic status of *ERG* was determined using 3 colored FISH using an *ERG-TMPRSS2* tri-color probe set. The protein expression of PTEN was determined using a rabbit monoclonal antibody and cytoplasmic and nuclear staining was scored as positive or negative. The genomic status of *PTEN* was determined using dual color FISH with a *PTEN* probe and a CEP10 probe. We found *ERG* rearrangement in 2 of 54 tumors (4%), one with protein overexpression by immunohistochemistry. PTEN inactivation was seen in 13 of 54 tumors (24%). Nine of the 13 *PTEN* alleles were inactivated by hemizygous deletion. No homozygous *PTEN* deletion was observed. *PTEN* deletion and *ERG* rearrangement were mutually exclusive. *ERG* rearrangement was rare compared to peripheral zone tumors and to *PTEN* inactivation in T1a transition zone tumors.

Introduction

Adenocarcinomas of the prostate gland arise within different anatomic zones that have varying clinical and molecular characteristics.[1,2] The vast majority of clinically significant prostatic adenocarcinomas arise in the peripheral zone, which have been widely investigated. Transition zone tumors are estimated to make up 16-20% of all prostate tumors, but these tumors have been less extensively studied. Compared to peripheral zone tumors, transition zone tumors have a lower Gleason scores, lower Ki-67 labeling indices, less extraprostatic extension, seminal vesicle invasion, and lymphovascular invasion, suggesting they may have a limited malignant potential.[1-4] However, approximately 20% of transition zone tumors progress to disease that invades beyond the prostate, and approximately 5% have lymph node metastases.[5] Currently, there is no method to predict which transition zone tumors will follow an aggressive course.

Recent discoveries have shown that peripheral zone tumors show a high prevalence of *TMPRSS2–ERG* gene fusions and PTEN inactivation, and the use of these two genetic events may help predict the clinical prognosis. Translocations between the *TMPRSS2-ERG* genes creates a constitutively active transcription factor that is uniquely found in approximately 50% of prostate tumors and thought to be essential for the carcinogenesis in this subset of tumors.[6-9] Large series of prostate cancer estimate PTEN inactivation occurs in approximately 18-23% of all tumors [6,9,10], and its loss allows for uninhibited activation of the PI3K/Akt/mTOR pathway and additional downstream targets. Mouse models that constitutively over-expressed ERG in a PTEN null background lead to highly penetrant prostate cancer that that arises much quicker than PTEN loss alone.[11] *PTEN* deletions have been associated with higher histologic grades, lymph node metastases and lower overall survival in *TMPRSS2-ERG* gene fusion positive and negative cancers.[6,9,12,13] Prostate cancers that lack *TMPRSS2-ERG* gene fusions and *PTEN* deletion have been shown to have a better prognosis, and the use of both gene rearrangements is the basis of a predictive model of disease reoccurrence.[14]

Clinical course of T1a prostate cancers (tumors found incidentally in transurethral resection without clinically suspected tumor, making up <5% of the tissue) is variable with long term followup.[15-17] The status of *TMPRSS2-ERG* fusions and *PTEN* and have not been well characterized in transition zone T1a tumor. These tumors typically do not receive treatment, but have the risk of progression to clinically significant disease. We assessed the genetic status of *TMPRSS2-ERG* fusions and *PTEN* in order to further understand the biology of prostate cancer in an attempt to find genetic predictors of aggressive behavior. Here we describe the frequency of PTEN inactivation and *TMPRSS2-ERG* gene fusions and their relationship in T1a tumors using human prostatic tissue removed by transurethral resection of the prostate (TURP). We analyzed 54 cases of Gleason score 7 or lower adenocarcinoma for the status of *PTEN* and *TMPRSS2-ERG* using both immunohistochemistry and fluorescence in situ hybridization.

Methods

Patients

We identified 54 T1a prostate adenocarcinomas by reviewing TURP specimens from participating institutions (Indiana University, Indianapolis, USA; Polytechnic University of the Marche Region, United Hospitals, Ancona, Italy; Case Western Reserve University, Cleveland, USA; Henry Ford Health System, Detroit, MI; Cordoba University, Cordoba, Spain) between 2003 and 2014. All TURP samples with carcinoma diagnoses were reviewed by 2 anatomic pathologists (KWF and LC) to confirm tumor volume was less than 5% of the resected specimen, meeting the criteria of the American Joint Committee on Cancer for T1a tumor staging. No Gleason grade 8 or higher cancers were found to have less than 5% involvement of a TURP sample. This research was approved by the Institutional Review Board.

Immunohistochemistry

We evaluated the status of ERG and PTEN proteins in formalin-fixed, paraffin-embedded tissue using immunohistochemical staining. Briefly, 4-μm-thick sections were heated in a PT module (DAKO, Carpinteria, CA) in Tris/EDTA (pH 9.0) for 20 minutes and then cooled down to room temperature. Samples were incubated at 1:200 dilution with PTEN antibody (rabbit monoclonal antibody, clone D4.3 XP, Cell signaling technology, Danvers, MA) or ready-to-use ERG antibody (rabbit monoclonal antibody, clone EP111, Dako Carpinteria, CA.) for 30min and 20min. respectively. This was followed by incubations with DAKO Envision Flex+rabbit linker, Envision HRP, and DAB+ chromogen. All other step followed the manufacturers' provided protocols. A tumor was considered to have PTEN protein loss if the intensity of cytoplasmic staining was markedly decreased or entireley lost in more than 10% of tumor cells compared with surrounding benign glands.[18-21]. ERG nuclear staining was scored as positive or negative and any nuclear staining of ERG was considered as indicative of ERG expression.[22,23]

Fluorescence in situ hybridization

Four-micrometer-thick sections were obtained from formalin-fixed, paraffin-embedded specimen blocks and deparaffinized with two 15-minute washes in xylene, subsequently washed twice with 100% ethanol for 10 minutes each, and air-dried. The sections were heated at 95°C in 0.1 mM citric acid (pH 6) solution (Invitrogen, Carlsbad, CA) for 10 min, rinsed with distilled water for 3 min, and washed with 2x saline-sodium citrate (SSC) for 5 min. Tissue digestion was performed by applying 0.4 ml of pepsin (Sigma, St Louis, MO, USA) solution (4 mg/ml in 0.9% NaCl in 0.01N HCl) to each slide and incubating the slides in a humidified box for 40 min at 37°C. The slides were rinsed with distilled water for 5 min, washed with 2xSSC for 5 min, and then air dried.

For *PTEN* copy number assay, a probe cocktail containing BAC clone RP11-383D9-Orange (*PTEN*, Empire Genomics, Buffalo, NY, USA) and CEP10-Green (Abbott Molecular, Abbott Park, IL) diluted 1:25 in tDenHyb2 (Insitus, Albuquerque, NM, USA). The genomic status of *ERG* was

determined using 3 colored FISH using a *TMPRSS2* aqua probe, a 5' *ERG* green probe, and a 3' gold *ERG* probe. Five microliters of diluted probe were applied to each slide. Coverslips were placed over the slides and sealed with rubber cement. The slides were denatured at 80°C for 10 min and hybridized at 37°C overnight. The coverslips were removed and the slides were extensively washed with two 0.1xSSC/1.5M urea solutions at 45°C for 20 min, in 2xSSC at 45°C for 10min, and then in 2xSSC/0.1% NP40 at 45°C for 10 min. Finally, the slides were washed with 2xSSC at room temperature for 5 min, air dried, counterstained with 10 µl DAPI/Antifade (DAPI in Fluorguard, 0.5 g/ml, Insitus, Albuquerque, NM, USA) and sealed with nail polish.

The hybridized slides were observed and documented using a MetaSystem imaging system and ISIS software (Belmont, MA, USA) under x100 oil objective. The images were acquired with a CoolCube 1 camera (MetaSystem) and analyzed with Isis software (Belmont, MA). The following filters were used: SP-100 for DAPI, FITC MF-101 for spectrum green, Gold 31003 for spectrum orange. Signals from each color channel (probe) were counted under false color, with computerized translation of each color channel into blue, green, red, or aqua. Four sequential focus stacks with 0.3 µm intervals were acquired and integrated into a single image to reduce thickness-related artifacts. For each case, 100 non-overlapping cancer cells nuclei were evaluated. Preparations were considered valid if >90% of the cells showed bright signals. Hemizygous deletion of *PTEN* was defined as ≥50% of tumor nuclei containing one *PTEN* signal, and with the presence of CEP 10 signals. Homozygous deletion of *PTEN* was defined as in ≥30% of tumor nuclei simultaneous loss of both *PTEN* signal, and with the presence of CEP 10 signals.[13,24,25] Cases with *ERG* signal abnormalities in ≥20% of the tumor cell population were considered to be positive.[22,26]

Statistical methods

Fisher exact tests were used to determine the association between PTEN protein expression and allele deletion status. Statistical significance was defined as $p < 0.05$ and all p values were two-sided.

Results

The samples we analyzed were composed of 51 Gleason grade 3+3=6 and 3 Gleason grade 3+4=7 prostatic adenocarcinomas, all of which involved less than 5% volume per sample. The average age was 73 years (range: 52-92 years), and the average specimen mass was 58 grams (range: 3-260 g) (**Table 1**).

We found *ERG* rearrangement by FISH in 2 of 54 tumors (4%), one with corresponding protein overexpression by immunohistochemistry **(Figure 1 and Table 1**). We did not find any ERG overexpression or *ERG* rearrangements in adjacent benign prostatic glands. We found PTEN protein loss in 13 of 54 (24%) tumors using immunohistochemistry (**Figure 2 and Table 2**). The PTEN protein loss status was highly correlated with *PTEN* allele deletion detected by FISH method (p=0.0001). Nine of the 13 cases (69%) with PTEN protein loss showed hemizygous deletion of *PTEN* by FISH. No homozygous *PTEN* deletion was observed. We did not find any PTEN protein loss in adjacent benign tissue.

Discussion

TMPRSS2-ERG rearrangements can be found in approximately 50% of peripheral zone tumors [6-9] and large series of prostate cancer estimate PTEN inactivation to occur in approximately 18-23% of all tumors [6,9,10]. Using a combination of IHC and FISH, we found that ERG overexpression (4%) is dramatically underrepresented in small T1a transition zone tumors. However, PTEN inactivation (24%) in stage T1a prostate cancer was similar to the most recent estimates of PTEN inactivation in large series of prostate cancer [6,9,10].

It is possible that our study using only immunohistochemistry and FISH studies might underestimate the true incidence of genetic *PTEN* inactivation, but other studies has shown a 75-89% correlation between FISH and IHC.[6,18] A recent study has shown inactivating point mutations of

PTEN occur in approximately 5% of samples, which would not be detected by FISH and IHC.[6] Even a minor upward adjustment in our estimation of PTEN inactivation in T1a tumors would not change the conclusion that the prevalence is similar to PTEN inactivation seen in peripheral zone tumors. *PTEN* deletion has been associated with a worse prognosis in peripheral zone cancers and the similar frequency of PTEN inactivation in our sample does not seem to be an explanation for the indolent behavior of transition zone tumors.[6,9,18,24] Larger data sets and prospective studies will be needed to assess the prognostic value of PTEN inactivation in transition zone tumors. More recent work has been done to optimize PTEN analysis by immunohistochemistry and that optimized four color FISH probes have been identified and applied in other cohorts of prostate cancer. These optimized assays have now been applied to a large multicenter cohort with rigorous statistical analysis. [21] The development of a clinical-grade, automated, and cost effective PTEN assay will facilitate further validation of PTEN as an important prognostic and predictive biomarker for prostate cancer.

TMPRSS2-ERG fusion proteins are seen in approximately 50% of prostate cancers, but these were dramatically underrepresented in our sample population. *ERG* is a member of the ETS family, which has 28 unique genes. Of these, *FLI*, *ERG*, *ETV1* and *ETV4* are commonly deregulated in cancer.[27] *TMPRSS2* and *ERG* are located within 3 megabases of each other on chromosome 21, and large deletions, and less commonly translocations, help to explain the high prevalence of fusions involving *ERG* compared to other ETS family members.[28] ETS family members can also be fused to proteins other than *TMPRSS2*, but represent a tiny fraction of all fusion proteins.[29-32] ERG immunohistochemistry has approximately 85% sensitivity and specificity for *ERG* fusions confirmed by RT-PCR and can be used in conjunction with *ERG* FISH to increase confidence in identifying *ERG* fusion positive tumors up to 98.5%.[6,7,33,34] It is possible in our sample population that a different ETS family member or different partner other than TMPRSS2 is involved in rearrangements in some cases; however, this is unlikely to add a substantive fraction of ETS family-rearranged tumors, since

other partners have a much lower incidence than *ERG* and *TMPRSS2* was not aberrantly disrupted in any sample.

The best defined role for *TMPRSS2-ERG* fusions appears to be in the initiation of carcinogenesis, as it is found in early lesions and typically homogenously maintained within high grade tumors.[22,35-37] However, there are emerging molecular pathways to carcinogenesis that appear to be mutually exclusive to *ERG* fusions: 1) Speckle-type POZ protein (*SPOP*) is the most commonly mutated gene in prostate cancer and acts as an E3 ubiquitin ligase adaptor that directly binds target proteins and promotes their cullin 3-dependent ubiquitination and proteolysis.[38,39] Mutations in *SPOP* occur in the substrate binding domain and prevent the interaction with target proteins leading to increased levels of oncogenic steroid receptor co-activator-3 (SRC-3/AIB1) and the androgen receptor.[40-42]; 2) Chromodomain helicase DNA-binding protein 1 (*CHD1*) is a tumor suppressor located at 5q21 that is inactivated, mainly through deletion, in 13-26% of prostate cancers.[43-45] The loss of CHD1 inhibits AR-dependent signaling, which is required for *TMPRSS2-ETS* family gene rearrangements, so CHD1 inactivation and *TMPRSS2*-ETS family gene rearrangements are seldom identified in the same tumor.[46,47] Inactivation of CHD1 forces the developing cancer into a pathway of carcinogenesis that does not require *TMPRSS2*-ETS family rearrangements. Deletion of *CHD1* alone in cell line models of prostate cancer was insufficient to cause invasive carcinoma, and the additional genetic events required for malignancy remain undetermined [44]; 3) Serine peptidase inhibitor, Kalal type 1 (SPINK1) is overexpressed in approximately 6% of all prostate cancers and 10% of *TMPRSS2*-ETS family gene fusion negative cancers.[48,49] SPINK1 overexpression appears largely to be mutually exclusive with *ERG* fusion, and is highly associated with 6q15 and 5q21 deletions, suggesting it represents a unique pathway to carcinogenesis.[49] SPINK1 overexpressing tumors as sensitive to inhibition of EGFR and may be clinically amenable to targeted inhibition of EGFR.[50,51]

PTEN inactivation is relatively common compared to ERG rearrangement in T1a prostate cancers. The low prevalence of *TMPRSS2-ERG* gene fusion positive cancers in our study suggests that

the alternative molecular pathways to carcinogenesis may play a crucial role in T1a cancers. Further study is needed to define the role of these alternate pathways to tumorigenesis and assess their role in prognosis and targeted treatment regimens.

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Figure Legends

Figure 1 Histological and ERG status by immunohistochemistry and *ERG* FISH in T1a prostate cancer: Only a single example (1 of 54, 2%) of T1a prostate cancer (A) exhibited ERG protein expression by immunohistochemistry (B), and only 2 of 54 (4%) demonstrated *ERG* rearrangement by FISH (C). In each nucleus, one red-green-aqua signal triplet is closely juxtaposed, whereas the other copy exhibits a widely separated green signal (C). In most T1a prostate cancers (D), neither ERG protein expression (E) nor *ERG* rearrangement was present (F), the latter evidenced by two copies of closely juxtaposed red-green-aqua signals.

Figure 2 PTEN loss in a subset of T1a prostate cancers: Among the 54 T1a prostate cancers (A), 76% exhibited normal PTEN protein expression (B) and normal *PTEN* copy number (C) as indicated by 2 red (*PTEN*) and 2 green (CEP10) signals. A subset of 24% of T1a prostate cancers (D) showed loss of PTEN expression (E), which correlated with hemizygous PTEN deletion as indicated by the loss of 1 red signal (*PTEN*) and 2 normal green signals (CEP10).

Table 1. Immunohistochemical and FISH assessment of ERG status

ERG STATUS	IHC +	IHC -	Total
FISH Rearrangement +	**1**	**1**	**2**
FISH Rearrangement -	**0**	**52**	**52**
Total	**1**	**53**	**54**

IHC: immunohistochemistry.

Table 2. Immunohistochemical and FISH assessment of PTEN status

PTEN STATUS	IHC +	IHC -	Total
Deletion -	**41**	**4**	**45**
Deletion +	**0**	**9**	**9**
	41	**13**	**54**

IHC: immunohistochemistry.

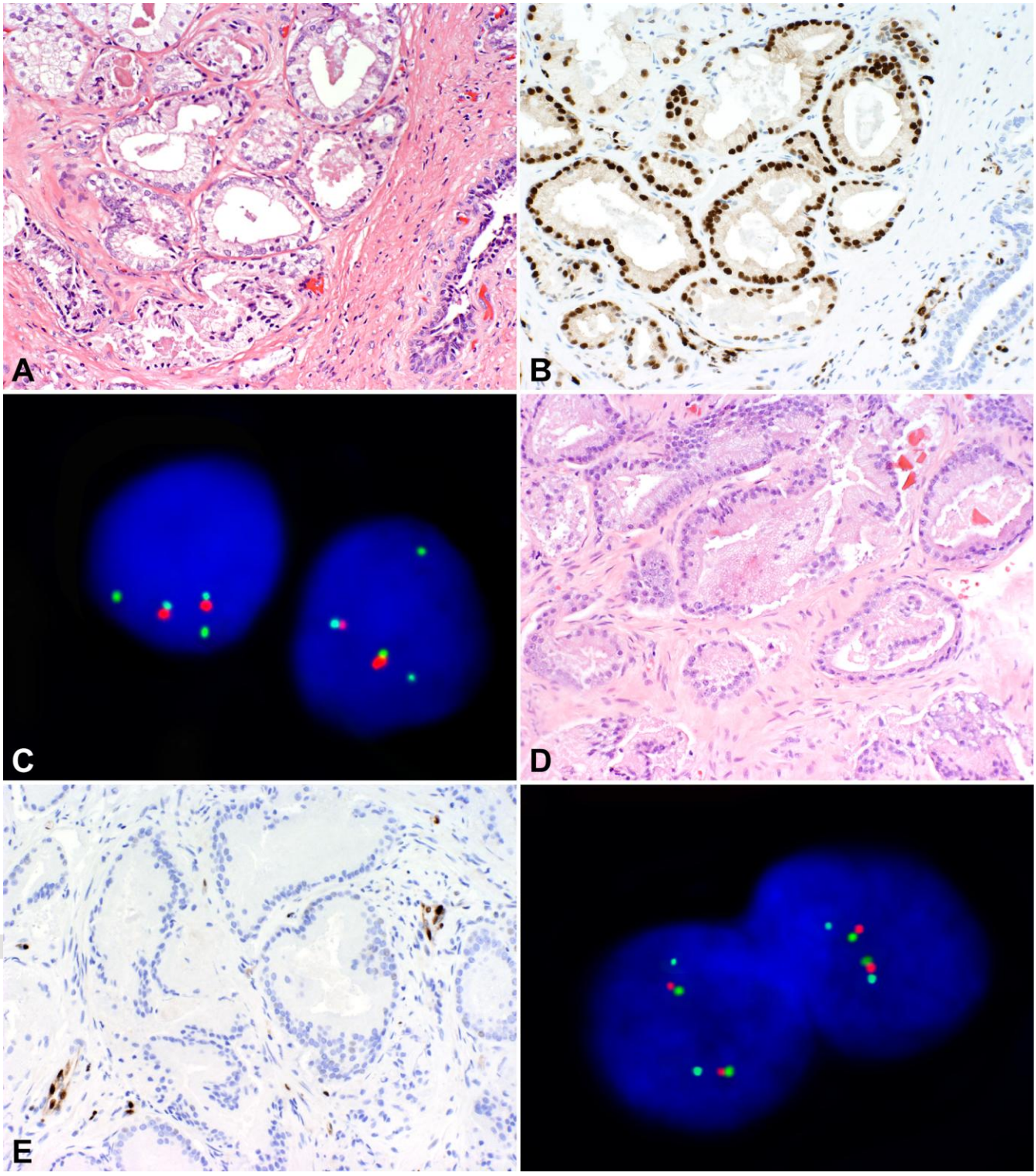

Figure 1

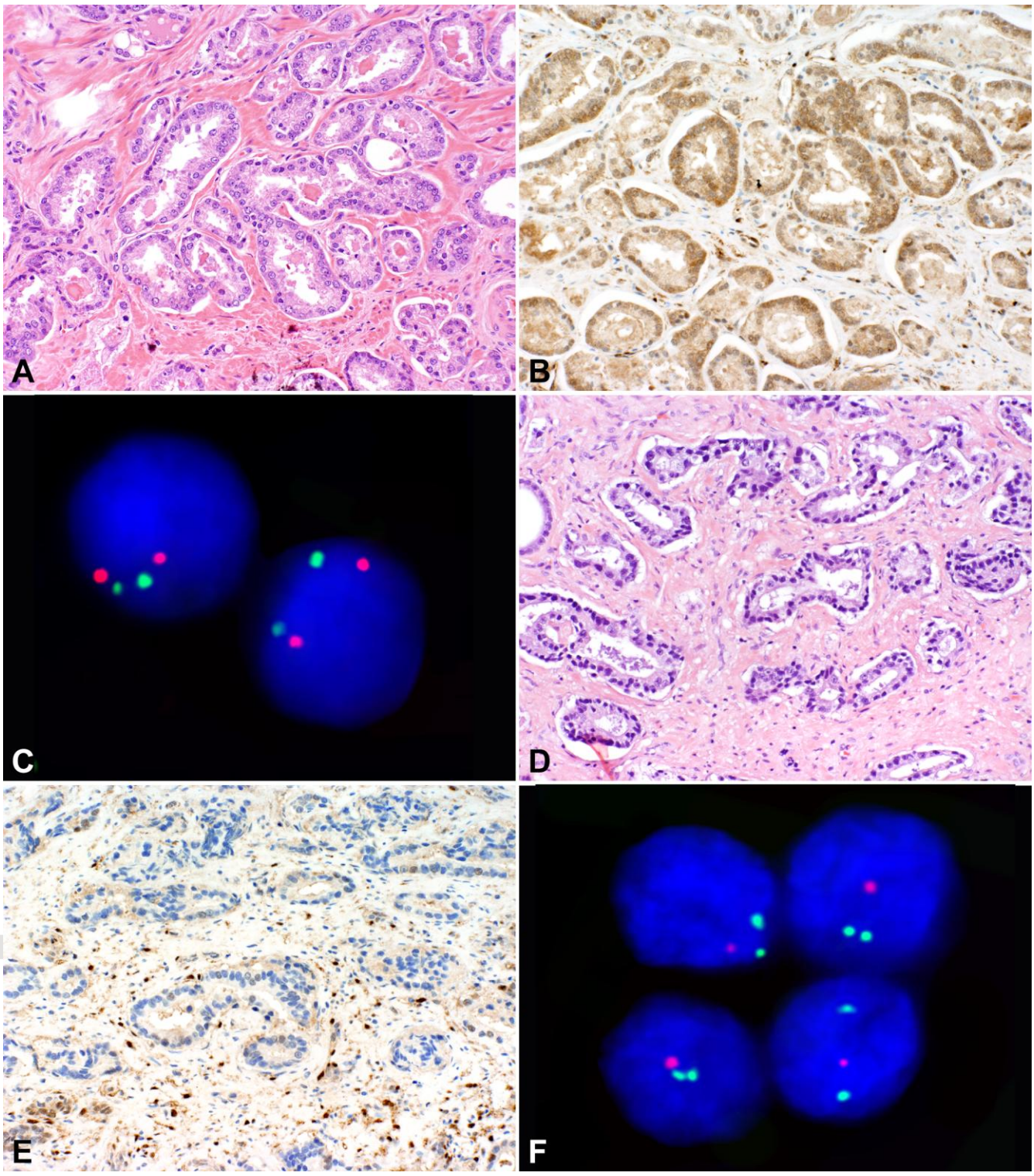

Figure 2