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의학박사 학위논문

전립선암에서 ERG 단백발현의 이질성과 TMPRSS2-ERG 유전자 융합 양상의 상관관계 및 그 임상적, 병리학적 의미

2016년 2월

서울대학교 대학원

의학과 병리학 전공

서 자 희

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지도교수 문 경 철

이 논문을 의학박사학위논문으로 제출함

2016년 2월

서울대학교 대학원
의학과 병리학 전공
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By JA HEE SUH

A thesis submitted in partial fulfillment of the Requirements for the Degree of Doctor of Philosophy in Medicine (Pathology) at the Seoul National University College of Medicine

February, 2016

Approved by thesis committee:

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국문초록

전립선암에서 ERG 단백발현의 이질성과 TMPRSS2-ERG 유전자 융합 양상의 상관관계 및 그 임상적, 병리학적 의미

서 자 희

서울대학교 의과대학원 의학과

병리학 전공

학번: 2011-30602

전립선 암에서 높은 빈도로 발생하는 것으로 알려진 유전자 융합 (gene fusion) 은 TMPRSS2-ERG 유전자 융합이며, 이 유전자 융합에 의하여 ERG 단백 과발현이 일어난다. 이는 서양에서는 그 빈도가 50%에 이르는 것으로 보고되고 있으나 한국 및 일본인 환자에서는 20% 초반의 빈도로 발생하여 서양보다 훨씬 낮은 빈도를 보인다. 전립선 암의 특성상 유전자 융합에 의한 ERG 단백 발현의 정도가 한 증례 안에서도 부위에 따라 서로 다른 이질성이 보고되었으나 이러한 ERG 단백발현의 이질성이 어떤 이유로 일어나며, 임상적 혹은 병리학적으로 어떠한 의미를 가지는지에 대한

연구는 발표된 바가 없다. 이에 저자는 서울대병원의 전립선 암 증례를 대상으로 ERG 단백 발현의 이질성과 TMPRSS2-ERG 유전자 융합 패턴의 이질성을 확인하고 이와 관련된 임상적 혹은 병리학적 의미를 확인하고자 하였다.

두 개의 전립선 암 환자 군을 대상으로 하여 ERG 단백 발현의 이질성을 확인하였다. 첫 번째 환자 군은 2012 년 서울대병원에서 전립선 암으로 수술 받은 172 증례를 대상으로, 두 번째 환자 군은 1999 년부터 2006 년까지 서울대병원에서 전립선 암으로 수술 받은 303 증례를 대상으로 하였다. 이 중 ERG 면역조직화학염색 상 양성인 104 증례에서 각각의 대표적인 절편으로부터 2-6 개의 코어를 얻어, Tissue microarray(TMA)를 제작하였다. 이렇게 제작된 TMA 에서 동일한 종양 내에서 ERG 단백발현 이질성 확인을 위한 면역조직화학 염색 시행하였으며 fluorescence in situ hybridization (FISH)를 시행하여 ERG 유전자 융합 pattern 의 이질성을 파악하였다. 또한 ERG 단백 발현 이질성 및 ERG 유전자 융합 pattern 의 이질성과 임상적, 병리학적 특성들과의 상관관계를 살펴보았다.

그 결과 ERG 면역 염색 상 양성인 104 증례는 모두 FISH 에서 ERG 유전자 융합이 관찰되었다. 이 중 34 증례 (32.7%) 에서는 ERG 유전자 융합상의 이질성이 관찰되었으며, 이러한 이질성은

22 개의 서로 다른 ERG 유전자 융합 양상 (FISH 상 break apart 와 interstitial deletion)이 혼재되어 있는 경우와 12 개의 ERG 유전자 융합 양성 및 음성이 혼재되어 있는 경우가 포함되었다. 또한 ERG 면역염색의 강도에 따라 ERG FISH 의 양상은 break apart 와 interstitial deletion 이 비교적 균일하게 분포하였다. ERG 단백 발현의 이질성 및 ERG 유전자 융합 pattern 의 이질성은 임상적, 병리학적 특성과 무관하였으며, 생화학적 재발율 역시 이질성에 따른 유의한 차이를 보이지 않았다.

주요어: 전립선암, 유전자 융합, 종양, 이질성

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Introduction

As prostate cancer is associated with a wide spectrum of histologic variants and biologic behaviors, it is regarded as a heterogeneous disease. Prostate cancer is the 2nd most common cancer in the western male population (up to 14%) and the 6th leading cause of cancer-related mortality (up to 6%) (1). In addition, the incidence rate and the mortality rate of prostate cancer in the Korean population are rapidly increasing (2, 3). The incidence of prostate cancer differs between the Asian and the Caucasian population, and the prognosis is significantly associated with ethnicity (4).

A fusion gene between ERG (21q22.2) (avian v-ets erythroblastosis virus E26 oncogene homolog) and TMPRSS2 (21q22.3) (transmembrane protease serine 2) is the most common recurrent genetic rearrangement in prostate cancer. The TMPRSS2-ERG fusion gene in prostate cancer was first reported by Tomlins et al in 2005 (5). The frequency of the fusion gene varies among study groups, from approximately 15.3% (6) to 72% (7) in the western population to approximately 20.9% (8) to 28% (9) in the Korean and Japanese populations. Various detection methods for the TMPRSS2-ERG fusion gene have been described

in previous studies.

TMPRSS2-ERG fusion results in androgen-induced over-expression of ERG, a gene that encodes erythroblast transformation-specific (ETS) transcription factors (5). The most common ERG fusion partner is TMPRSS2, although rare cases of other ERG fusion partners such as solute carrier family 45, member 3 (SLC45A3) and n-myc downstream regulated 1 (NDRG1) have been reported (10, 11).

Reverse transcription polymerase chain reaction (RT-PCR) and quantitative reverse transcription PCR have been used as the principle methods for the quantitative detection of genetic rearrangements (12), although fluorescence in situ hybridization (FISH) is considered the standard and confirmative molecular method for identifying gene fusions in single tumor cells and for quantitative assessment of gene fusions (13-16). Park et al. reported an efficient method for detecting the TMPRSS2-ERG fusion gene using the ERG-specific antibody EPR-3864 in formalin-fixed paraffin-embedded (FFPE) tissue (17). Detecting the TMPRSS2-ERG fusion gene using immunohistochemical staining (IHC) with antibodies against ERG is a relatively simple,

low-cost method that employs a routine laboratory procedure. The sensitivity and specificity of this approach was verified with FISH and other molecular laboratory methods in various studies; therefore, ERG IHC is regarded as a validated technique for detecting the TMPRSS2-ERG fusion gene (18-20).

Several studies that investigated the relationship between the TMPRSS2-ERG fusion gene and clinicopathological factors of prostate cancer, including clinical outcomes, in various groups (21-23) have reported inconsistent results. Although recent studies demonstrated that the ERG fusion gene is associated with a low Gleason score (15, 24, 25).

ERG and TMPRSS2 are located in close proximity to one another on chromosome 21. Loss of the chromosomal region between the TMPRSS2 and ERG genes (an interstitial deletion) is frequently associated with the TMPRSS2-ERG gene fusion and is associated with a more advanced tumor stage (21). Attard et al. later reported various ERG FISH patterns, including interstitial deletion and split signal (sometimes referred to as break apart) (26), and some previous studies reported the prognostic value of ERG FISH patterns (22, 26, 27).

Recently, multiple studies reported an association between the heterogeneity of TMPRSS2-ERG fusion gene rearrangement patterns and ERG expression (22, 27-29). Mehra et al. revealed that different tumor foci in multifocal prostate cancers frequently exhibited different patterns of TMPRSS2 rearrangement (22). Minner et al. reported that intrafocal heterogeneity in ERG expression, defined as heterogeneous ERG expression within one tumor focus, was observed in 69 out of 103 ERG-positive cases evaluated by IHC (29). However, some of these studies were associated with limitations. Mehra et al. observed only interfocal heterogeneity, i.e., ERG heterogeneity in different tumor foci, but did not observe ERG heterogeneity within a single tumor focus (22). In contrast, Minner et al. observed intrafocal heterogeneity in ERG expression but did not identify a correlation between specific gene rearrangement patterns and heterogeneity in ERG expression, nor did they identify the clinicopathological significance of heterogeneous ERG expression (29).

We hypothesized that heterogeneous ERG expression identified by IHC might be associated with different ERG gene fusion patterns identified by FISH. We also aimed to elucidate the

clinicopathological significance of the heterogeneity in the expression pattern of the ERG fusion gene.

Material and method

1. Patient information and tissue microarray construction

A total of 475 prostate cancer cases were screened in this study. Group 2 included samples from 303 patients who had undergone a radical prostatectomy at Seoul National University Hospital from 1999 to 2006 that had been used for ERG IHC in a previous study (9). Group 1 included 172 patients who had undergone a radical prostatectomy at Seoul National University Hospital in 2012. Information regarding age, Gleason score, primary Gleason grade, tumor volume (%), pT staging, preoperative PSA level and biochemical recurrence was obtained from patient medical records, pathologic records and the analysis of clinical slides. The Gleason score and primary Gleason grade were assigned according to the criteria recommended by the 2005 International Society of Urological Pathology (ISUP) Consensus Conference (30). Biochemical recurrence was defined as a postoperative prostate specific antigen (PSA) level greater than 0.2 ng/ml in 2 consecutive measurements.

A representative tumor block was immunostained using an ERG antibody, and each ERG IHC slide was reviewed to evaluate the ERG signal and staining intensity. ERG immunostaining intensity was scored from 0 to 3+. Among the 475 cases screened, 114 cases were ERG-positive according to IHC, and 10 cases from Group 2 were excluded due to insufficient tissue available for the tissue microarray (TMA) construction. A total of 104 ERG positive cases were ultimately included in this study. For the construction of the TMAs, 2 to 6 representative cores (1 mm in diameter) from a single tumor focus were used. In samples with heterogeneous ERG immunostaining intensity, areas of different intensity were separated for TMA construction (Figure 1). A total of 428 cores were constructed into 4 TMA blocks using a trephine apparatus (Superbiochips Laboratories, Seoul, Korea) (31).

2. ERG immunohistochemical stainings (IHC).

Sections mounted on superfrost slides were deparaffinized. IHC was conducted using the Ventana Benchmark XT automated staining system (Ventana Medical Systems, Tucson, AZ, USA) using

Ventana reagents. We used the anti-ERG rabbit monoclonal antibody (RTU, clone EPR3864, Ventana, Tucson, AZ, USA) (17, 20). Heat-induced epitope retrieval was performed with anti-ERG antibody. The slides were incubated with primary antibodies for 16 minutes at room temperature. For IHC using the anti-ERG rabbit monoclonal antibody (rabbit ERG-MAb), a secondary antibody (Ultraview anti-Rabbit HRP; Ventana Medical Systems, Tucson, AZ, USA) was applied for 16 minutes at room temperature. Secondary antibody detection was performed using the Ultraview DAB detection kit (Ventana Medical Systems). Slides were counterstained with Hematoxylin II for 4 minutes followed by Bluing Reagent (Ventana Medical Systems) for 4 minutes at 37 ℃. ERG staining in the nuclei of endothelial cells served as the endogenous positive control, as previously described (10). The intensity of ERG expression was scored as negative (0; no staining), weak (1+; only visible at high magnification), moderate (2+; visible at low magnification) and strong (3+; striking at low magnification).

3. ERG dual color break apart FISH.

FISH was performed as previously described (32, 33) using a dual color probe (Zytolight® SPEC ERG Dual Color FISH probes, ZytoVision GmbH, Bremerhaven, Germany). Briefly, 2 differentially labeled probes that provided coverage of the neighboring telomeric and centromeric regions of each locus were designed. The following telomeric/centromeric BAC clones were used to design the break-apart assays for assessing chromosomal rearrangement status (i.e., rearrangement vs. no rearrangement): target sequences mapping to 21q22.2, distal to the ERG breakpoint region (green signal); and target sequences mapping to 21q22.13-q22.2, proximal to the ERG breakpoint region (orange signal). Briefly, 2-μm sections were deparaffinized and dehydrated, and the slides were incubated in 0.2 N HCl at room temperature for 20 minutes and subsequently incubated in 8% sodium thiocyanate at 80 ℃ for 30 minutes. The slides were treated with 1% pepsin at 37 ℃ for 20 minutes. The probe set was applied to the slides, and a coverslip was added and sealed with rubber cement. The slides were incubated in ThermoBrite (Abbott Molecular, Des Plaines, IL) at 75 ℃ for 5 minutes to denature the probe and subsequently incubated at 37 ℃

for 16 hours to allow for hybridization. After the post-hybridization washing, the samples were counterstained with 4,6-diamidino-2-phenylindole (DAPI). The samples were analyzed using a 1000x oil immersion lens on an Olympus BX-51TRE microscope (Olympus, Tokyo, Japan) equipped with DAPI, green, orange and triple-pass (DAPI/Green/Orange) filters (Abbott-Vysis). A minimum of 100 tumor nuclei per case were assessed. A positive FISH result was defined as the presence of a break apart or interstitial deletion in at least 50% of the nuclei of epithelial cells. The break apart was defined as the combination of 1 green and orange signal, and 1 separated green and orange signal. The interstitial deletion was defined as one combined signal and one orange signal with the loss of a green signal (8). If both interstitial deletion and break-apart co-existed in a single core, we classified the sample according to the pattern observed in more than 50% of nuclei.

4. Determination of heterogeneity.

Heterogeneity of ERG IHC was defined as the presence of both ERG-positive and -negative cores or of 1+ and 3+ cores in the

same tumor focus. Heterogeneity of ERG FISH was defined as the presence of both positive and negative cores or of break apart and interstitial deletion cores in the same tumor focus (Figure 2).

5. Statistics

All statistical analyses were conducted using SPSS v. 23.0 (IBM Inc., Chicago, IL). Fisher extract test was used for identifying significant correlations, and Kaplan-Meier survival analysis was used to evaluate biochemical recurrence-free survival analysis

6. Institutional review board (IRB) permission

IRB of this study was deliberated and approved by the Center for Human Research Protection of Seoul National University Hospital / Biomedical Research Institute. (IRB approval number: 1212-054-450).

Results

1. Clinicopathologic characteristics and ERG IHC.

In Group 1, samples from 172 patients were evaluated by ERG IHC. The mean age of the patients was 66 years, with a standard deviation of 6.9 years. The mean value of the initial PSA level was 11.1 ng/ml, and 40 cases (23.2%) were confirmed to be ERG-positive by IHC. In Group 2, samples from 303 cases were evaluated by ERG IHC. The mean age was 65.7 years with a standard deviation 6.4 years. The mean value of the initial PSA level was 11.2 ng/ml, and 74 cases (24.4%) were confirmed to be ERG-positive by ERG IHC (9). ERG protein overexpression was related to a low Gleason grade only and to the Gleason score in Group 1 and 2 (Table 1 and 2). Other clinicopathological parameters were not associated with ERG expression levels.

ERG heterogeneity was observed in 47 of the 104 ERG-positive cases (45.2%). Among the 47 cases, 16 exhibited both positive and negative foci, and the remaining 31 cases exhibited both 1+ and 3+ foci.

2. Correlation between ERG IHC and ERG FISH in TMA cores

A total of 428 cores were constructed from Group 1 and 2. The ERG FISH results associated with 11 cores could not be interpreted due to a lack of sufficient tissue. Ultimately, 417 cores were analyzed and used to investigate the correlation between the results of ERG IHC and ERG FISH (Table 3). All of the ERG-positive cores exhibited ERG break apart (positive) in the FISH analysis. Discrepancies between the ERG IHC and FISH results were observed in 14 out of 39 ERG-negative cores (35.9%), i.e., these cores exhibited positive FISH results. ERG FISH patterns (break apart or interstitial deletion) were not associated with ERG IHC intensity and demonstrated a relatively even distribution (Table 3). The distribution of TMA cores according to IHC intensity, FISH pattern and Gleason score is described in Table 4, and representative images are presented in Figure 3.

3. ERG FISH heterogeneity.

All 104 IHC positive cases were confirmed to be ERG FISH positive. Seventy cases exhibited homogeneous ERG FISH patterns with either break aparts (n=37) or interstitial deletions (n=33). The remaining 34 cases exhibited heterogeneous FISH patterns, including 22 cases with both break aparts and interstitial deletions and 12 cases with both FISH positive and negative cores within a single tumor focus (Figure 4).

ERG FISH heterogeneity was not associated with clinicopathological parameters (Table 5). ERG FISH patterns (break apart and interstitial deletion) were also not associated with clinicopathological parameters (Table 6).

The cases exhibiting ERG heterogeneity in either the IHC or FISH experiments did not experience significantly different biochemical recurrence-free survival periods (Figure 5 and 6).

Table 1. Correlation with clinicopathological factors and ERG IHC results in Group 1

Parameter	ERG IHC (n=168*)		*p* value
	Negative	**Positive**	
Age (years)			
< 66 (n=67)	49 (73.1%)	18 (26.9%)	0.449
≥ 66 (n=101)	79 (78.2%)	22 (21.8%)	
Initial PSA level (ng/ml)			
< 10 (n=96)	64 (66.7%)	32 (33.3%)	0.076
≥ 10 (n=72)	64 (88.9%)	8 (11.1%)	
Tumor volume (%)			
< 10% (n=59)	39 (66.1%)	20 (33.9%)	0.083
≥ 10% (n=96)	78 (81.3%)	18 (18.7%)	
Primary Gleason grade (pattern)			
3 (n=121)	87 (71.9%)	34 (28.1%)	**0.036**
4 & 5 (n=47)	41 (87.2%)	6 (12.8%)	
Gleason score (sum of primary and second Gleason grade)			
≤ 7 (n=158)	118 (74.7%)	40 (25.3%)	0.068
> 7 (n=10)	10 (100%)	0 (0%)	
pT-stage (AJCC 7th edition)			
pT2 (n=100)	70 (70%)	30 (30%)	0.056
pT3 (n=68)	58 (85.3%)	10 (14.7%)	

IHC: immunohistochemical staining, PSA: prostate specific antigen, AJCC: American Joint Committee on Cancer.

* In 172 cases, 4 cases were excluded in this analysis due to preoperative hormonal therapy.

Table 2. Correlation with clinicopathological factors and ERG IHC results in Group 2 from previous study (9)

Parameter	ERG IHC (n=303)		*p* value
	Negative	**Positive***	
Age (years)			
< 66 (n=147)	109 (74.1%)	38 (25.9%)	0.595
≥ 66 (n=156)	120 (76.9%)	36 (23.1%)	
Initial PSA level (ng/ml)			
< 10 (n=191)	140 (73.3%)	51 (26.7%)	0.268
≥ 10 (n=112)	89 (79.5%)	23 (20.5%)	
Tumor volume (%)			
< 10% (n=100)	78 (78%)	22 (22%)	0.570
≥ 10% (n=203)	151 (74.4%)	52 (25.6%)	
Primary Gleason grade (pattern)			
3 (n=218)	155 (71.1%)	63 (29.9%)	**0.004**
4 & 5 (n=85)	74 (87.1%)	11 (12.9%)	
Gleason score (sum of primary and second Gleason grade)			
≤ 7 (n=253)	184 (72.7%)	69 (27.3%)	**0.011**
> 7 (n=50)	45 (90%)	5 (10%)	
pT-stage (AJCC 7th edition)			
pT2 (n=165)	125 (75.8%)	40 (24.2%)	0.521
pT3 (n=138)	104 (75.4%)	34 (24.6%)	

IHC: immunohistochemical staining, PSA: prostate specific antigen, AJCC: American Joint Committee on Cancer.

Table 3. Tissue microarray core analysis focused on IHC intensity variation and FISH pattern

IHC	Total core number	
Negative	**39**	
(0)	FISH Negative	25(64.1%)
	FISH Positive	**14 (35.9%)**
Weak positive (1+)	115	
(1+)	FISH Break apart	FISH Interstitial deletion
	66 (57.4%)	49 (42.6%)
Moderate positive (2+)	103	
(2+)	FISH Break apart	FISH Interstitial deletion
	56 (54.4%)	47 (45.6%)
Strong positive (3+)	160	
(3+)	FISH Break apart	FISH Interstitial deletion
	81 (50.6%)	79 (49.4%)
	417*	

FISH: fluorescence in situ hybridization.

*Total core number was 428, but 11 cores are not available for FISH because of limitation of tumor tissue.

Table 4. Tissue microarray core analysis: immunohistochemical staining (IHC) , FISH and Gleason score

	Total core number = 428			
FISH result	**Negative**	**Break apart**	**Interstitial deletion**	**NA**
	25	213	179	11
IHC result	**Negative**	**1+**	**2+**	**3+**
	39	115	103	160
Gleason score	**6**	**7**		**8**
	276	124		28

FISH: fluorescence in situ hybridization, IHC: immunohistochemical staining, NA: not available due to lack of tissue, 1+: weak positive, 2+: moderate positive, 3+: strong positive.

Table 5. Correlation between clinicopathological factors and ERG FISH heterogeneity

Parameter	ERG FISH Heterogeneity (n=104)		*p* value
	Homogeneous (N=70)	**Heterogeneous (N=34)**	
Age (years)			
< 66 (n=56)	41 (73.2%)	15 (26.8%)	0.165
≥ 66 (n=48)	29 (60.4%)	19 (39.6%)	
Initial PSA level (ng/ml)			
< 10 (n=75)	47 (62.7%)	28 (37.3%)	0.105
≥ 10 (n=29)	23 (79.3%)	6 (20.7%)	
Tumor volume (%)			
< 10% (n=38)	27 (71.1%)	11 (28.9%)	0.537
≥ 10% (n=66)	43 (65.2%)	23 (34.8%)	
Primary Gleason grade (pattern)			
3 (n=79)	53 (67.1%)	26 (32.9%)	0.933
4 & 5 (n=25)	17 (68%)	8 (32%)	
Gleason score (sum of primary and second Gleason grade)			
≤ 7 (n=96)	63 (65.6%)	33 (34.4%)	0.205
> 7 (n=8)	7 (87.5%)	1 (12.5%)	
pT-stage (AJCC 7th edition)			
pT2 (n=64)	41 (64.1%)	23 (35.9%)	0.372
pT3 (n=40)	29 (72.5%)	11 (27.5%)	

FISH: fluorescence in situ hybridization, PSA: prostate specific antigen, AJCC : American Joint Committee on Cancer.

Table 6. Correlation between clinicopathological factors and ERG FISH pattern

Parameter	ERG FISH Pattern in homogeneous ERG FISH result (n=70)		*p* value
	Break apart (n= 37)	**Interstitial deletion (n=33)**	
Age (years)			
< 66 (n=41)	23 (56.9%)	18 (43.1%)	0.518
≥ 66 (n=29)	14 (48.3%)	15 (51.7%)	
Initial PSA level (ng/ml)			
< 10 (n=47)	28 (59.6%)	19 (40.4%)	0.108
≥ 10 (n=23)	9 (39.1%)	14 (60.9 %)	
Tumor volume (%)			
< 10% (n=27)	15 (55.6%)	12 (44.4%)	0.720
≥ 10% (n=43)	22 (51.2%)	21 (48.8%)	
Primary Gleason grade (pattern)			
3 (n=53)	27 (50.9%)	26 (49.1%)	0.571
4 & 5 (n=17)	10 (58.8%)	7 (41.2%)	
Gleason score (sum of primary and second Gleason grade)			
≤ 7 (n=63)	31 (49.2%)	32 (50.8%)	0.066
> 7 (n=7)	6 (85.7%)	1 (14.3%)	
pT-stage (AJCC 7th edition)			
pT2 (n=41)	18 (43.9%)	23 (56.1%)	0.074
pT3 (n=29)	19 (65.5%)	10 (34.5%)	

FISH: fluorescence in situ hybridization, PSA: prostate specific antigen, AJCC : American Joint Committee on Cancer.

Figure 1. TMA construction by multiple cores from ERG IHC in full section

After representative full section ERG IHC, heterogeneity of ERG expression was reviewed. And multiple cores were extracted from heterogeneous or homogeneous area of tumor with ERG expression.

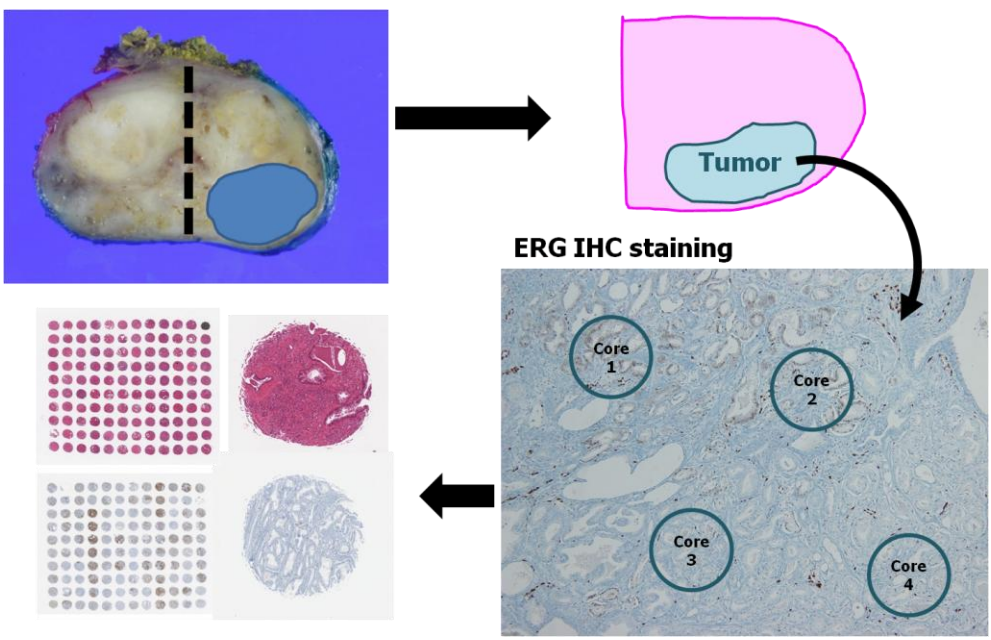
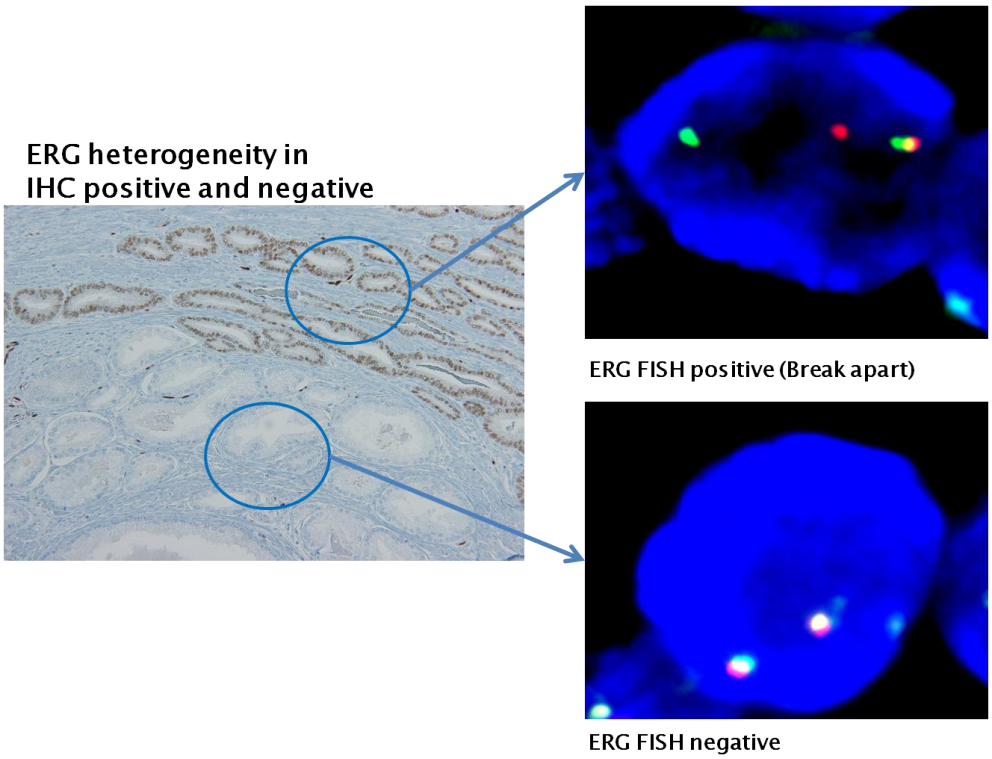
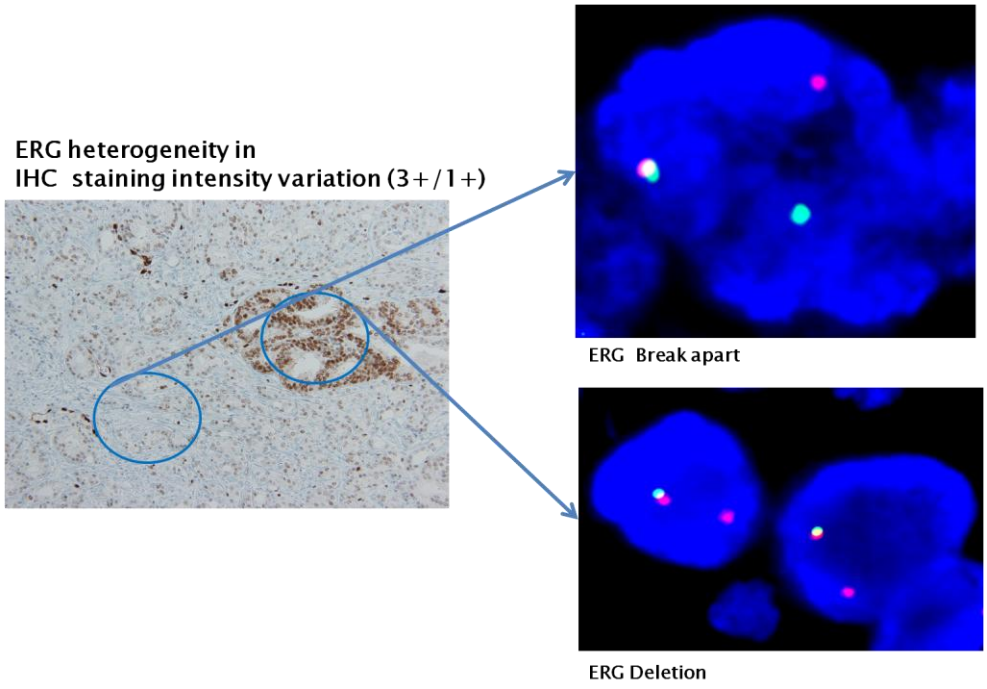


Figure 2. ERG heterogeneity determined as positive and negative (A) and ERG IHC intensity variation and mixed pattern in ERG FISH (B)



(A)


ERG heterogeneity in
IHC staining intensity variation (3+/1+)
ERG Break apart
ERG Deletion

(B)

Figure 3. ERG IHC intensity (A−D), Gleason score (E−G) and FISH result (H−J) of prostate cancer

A: ERG negative, B: ERG weak positive(1+), C: ERG moderate positive (2+), D: ERG strong positive (3+), E: Gleason score 6(3+3), F: Gleason score 7(3+4), G: Gleason score 8(4+3), H: TMPRSS2−ERG FISH negative (fusion signal), I: TMPRSS2−ERG FISH positive (break apart signal), J: TMPRSS2−ERG FISH positive (interstitial deletion signal)

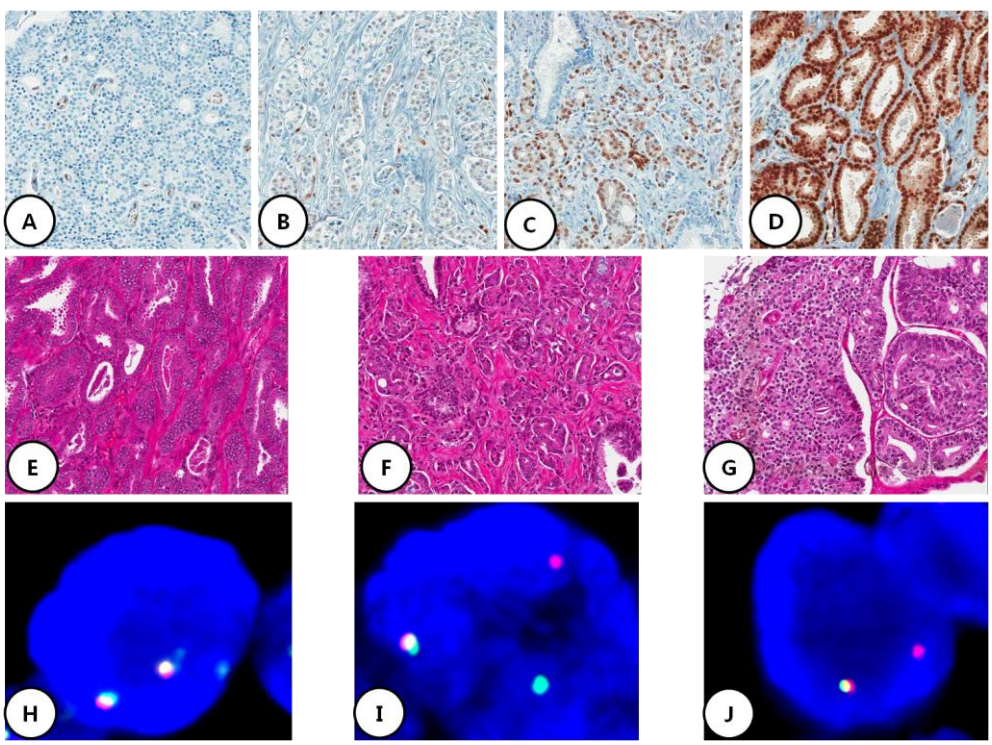


Figure 4. Flowchart of data evaluation in ERG FISH results of 104 ERG IHC positive cases

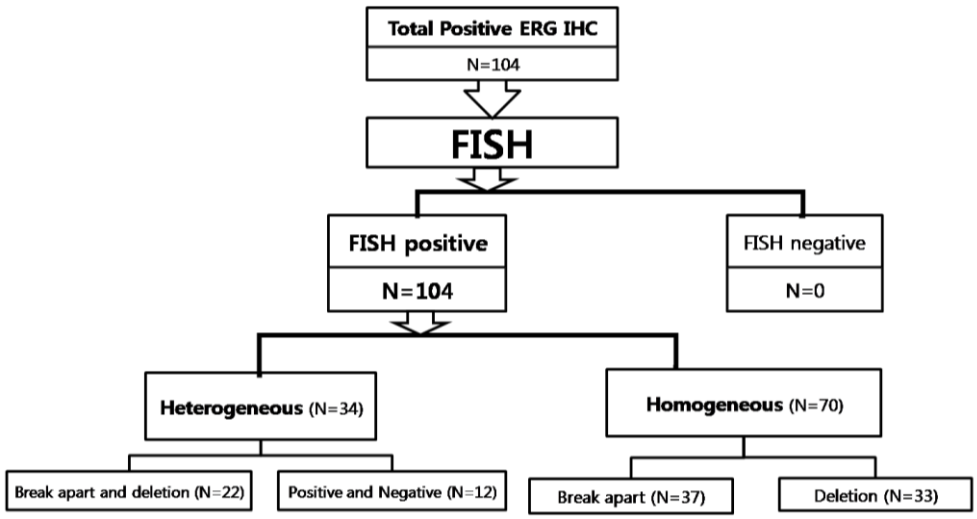
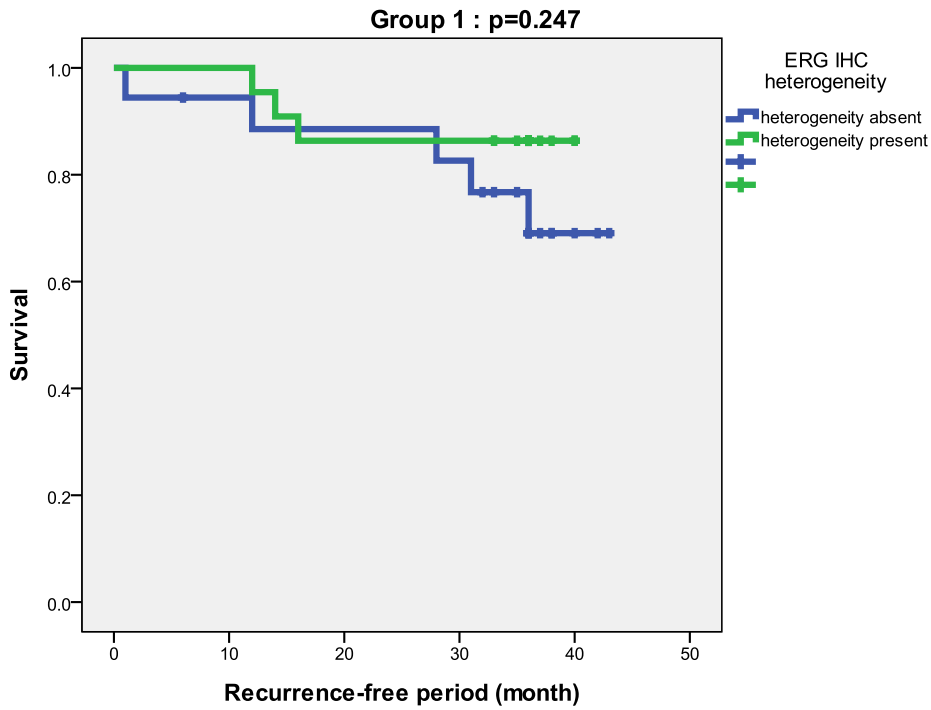
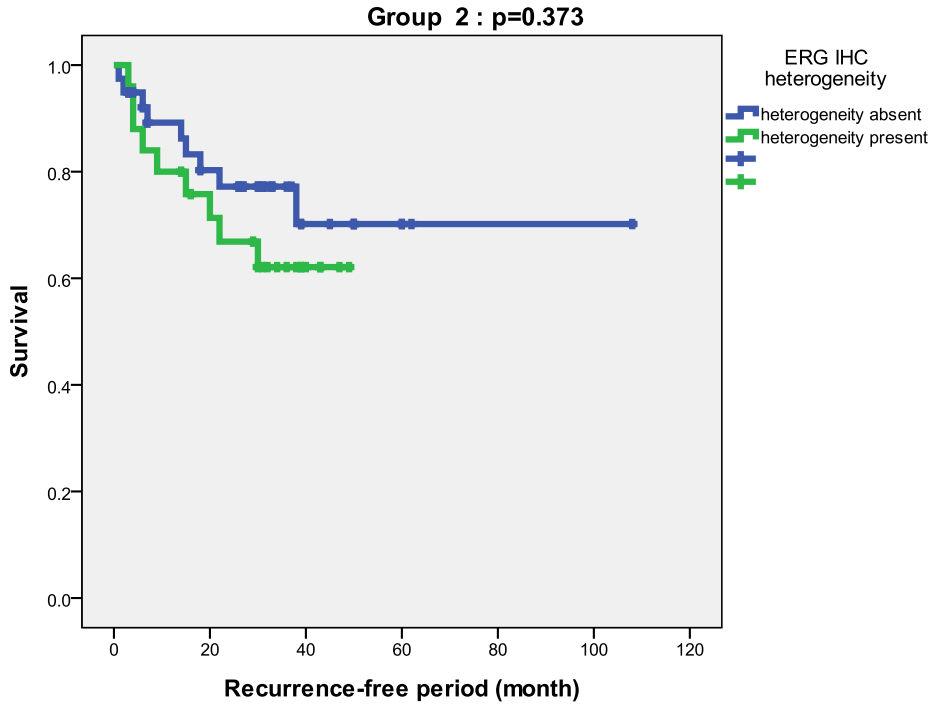


Figure 5. Kaplan-Meier curves of biochemical recurrence in Group 1 (A) and 2 (B) according to ERG IHC heterogeneity

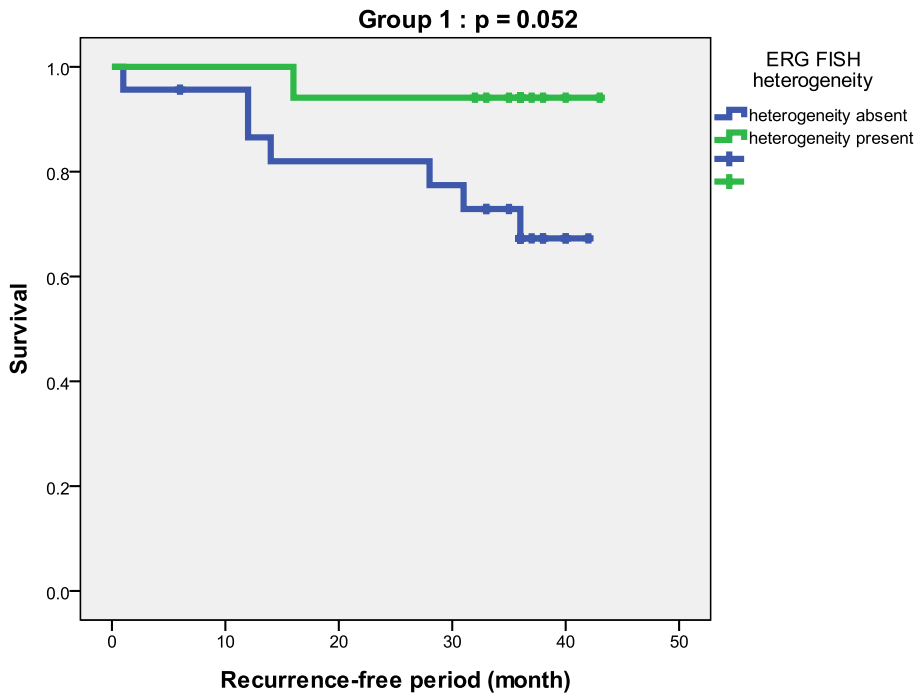


(A)

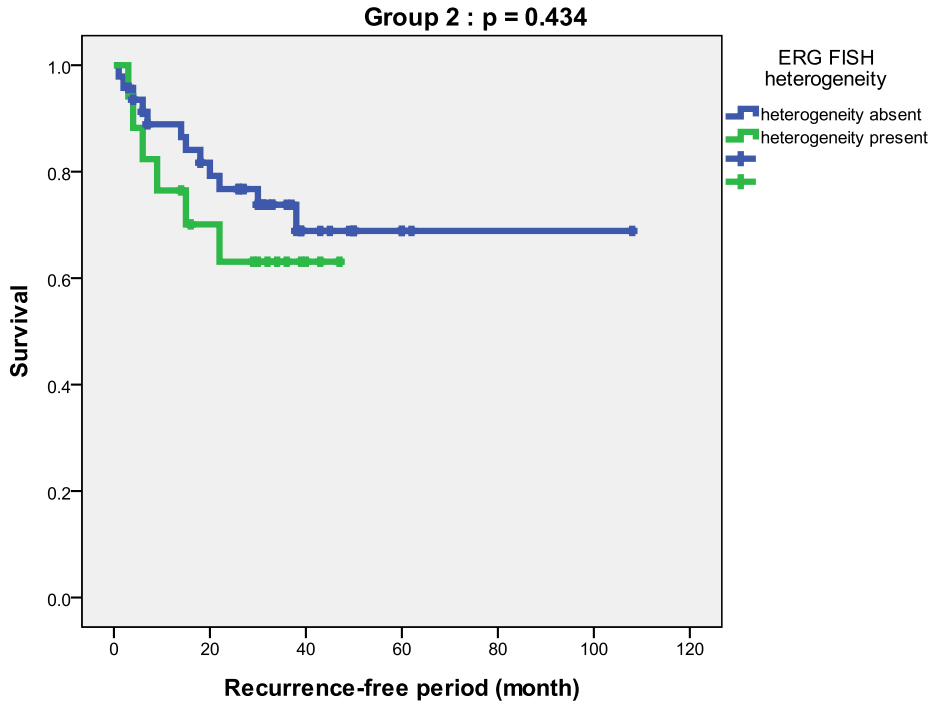

Group 2 : p=0.373
ERG IHC heterogeneity
heterogeneity absent
heterogeneity present
Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
120
Recurrence-free period (month)

(B)

Figure 6. Kaplan-Meier curves of biochemical recurrence in Group 1 (A) and 2 (B) according to ERG FISH heterogeneity

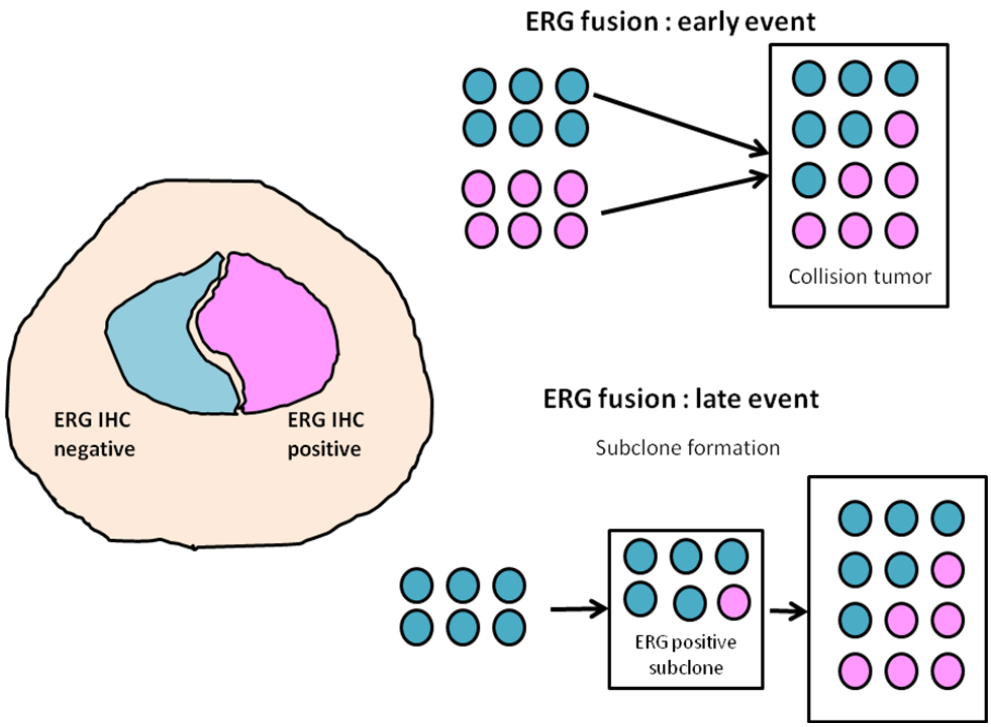


(A)

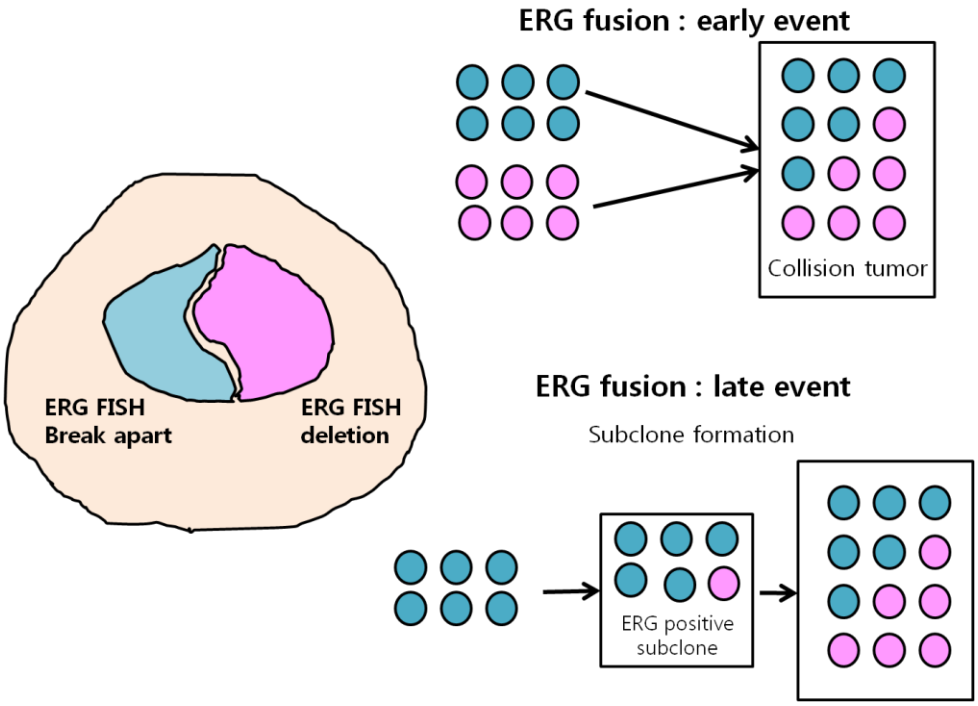

Group 2 : p = 0.434
ERG FISH heterogeneity
heterogeneity absent
heterogeneity present
Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
120
Recurrence-free period (month)

(B)

Figure 7. Heterogenity of ERG fusion status (A) and ERG FISH pattern (B) in prostate cancer. Both collision tumor and subclonal formation are considered as the interpretation of heterogeneity of ERG FISH patterns.



(A)


ERG fusion : early event
Collision tumor
ERG FISH
Break apart
ERG FISH
deletion
ERG fusion : late event
Subclone formation
ERG positive
subclone

(B)

Discussion

In this study, we studied the heterogeneity of EGR expression and gene fusion patterns in prostate cancer. 24% of cases were determined to be ERG-positive by IHC, a frequency lower than that reported in the overall western population (approximately 50%). A similar frequency was reported in the Japanese population (20.1%) (34). ERG status as measured by IHC was associated with a lower Gleason score and Gleason grade in this study. It has previously been established that the ERG fusion gene fusion is associated with low Gleason pattern (8).

In the TMA core analysis, a subset of cores exhibited negative ERG IHC and positive ERG FISH results (14 cores out of 39 ERG IHC negative cores, 35.9%). This result indicates that ERG IHC can potentially generate false negatives, and this should be taken into account when interpreting ERG IHC.

Regarding ERG expression heterogeneity, Minner et al. reported that 74 ERG-positive cases out of 103 cases (72%) evaluated by IHC exhibited ERG expression heterogeneity (29). In this study, we

observed ERG expression heterogeneity in 45.2% of ERG-positive cases evaluated by IHC, a frequency lower than that reported in a previous study (29).

We also evaluated the differences of FISH patterns in regions with heterogeneous ERG expression. Our results demonstrated that the intensity of ERG IHC was not associated with ERG FISH patterns (break apart and interstitial deletion). All ERG IHC positive cores exhibited the ERG fusion gene in FISH analysis. These results suggest that ERG IHC is correlated with ERG gene fusion status; however, the staining intensity observed in IHC and the specific ERG fusion gene patterns has no influence on ERG protein expression.

In this study, we analyzed ERG FISH patterns in multiple areas of the same tumor focus and found that 34 out of 104 FISH positive cases (32.7%) exhibited different FISH patterns in the same tumor focus. This if the first study to report this result, and our findings revealed that a single tumor focus can be associated with intrafocal heterogeneity of ERG gene fusion patterns (break apart and interstitial deletion). Mehra et al. also studied the heterogeneity of the TMPRSS2 fusion gene using FISH analysis (22). They reported

a high frequency (70%) of interfocal heterogeneity for the TMPRSS2 rearrangement, and they reported that individual tumor cells within a single tumor focus exhibited homogeneous TMPRSS2 rearrangement patterns (22). In contrast, we observed intrafocal heterogeneity in ERG fusion gene patterns. The explanation for this discrepancy is not entirely clear, although there were a few differences between the previous study by Mehra et al. and the present study that are worth noting. Our study used an ERG break apart FISH probe, whereas Mehra et al. used a TMPRSS2 FISH probe. In addition, we constructed all TMA cores from one tumor focus from each case, whereas Mehra et al. selected TMA cores from different tumor foci. Finally, we focused our attention on intrafocal heterogeneity rather than interfocal heterogeneity.

We found that the intrafocal heterogeneity of ERG FISH patterns included positive/negative heterogeneity and break apart/interstitial deletion heterogeneity. These results can potentially be explained by 1 of 2 mechanisms. One possibility is that 2 different tumors with different ERG fusion patterns collide to form a single tumor (the collision tumor) with a heterogeneous ERG gene fusion pattern. Another possibility is that the existence of subclones with different

ERG fusion gene patterns (Figure 7). Previous studies reported that the ERG fusion gene was observed in high grade prostate intraepithelial neoplasm (HGPIN). Mosquera et al. reported that the ERG fusion gene in HGPIN is analogous to the ERG fusion gene in prostate cancer (35). Tomlins et al. evaluated an in vivo and in vitro modeling system using transgenic mice expressing ERG to investigate the development of HGPIN and the mechanism of cellular invasion. They suggested that the ERG fusion gene mediates the invasion process in HGPIN and prostate cancer (36). These previous results support the hypothesis that the ERG fusion gene is an early genetic aberration in tumorigenesis and support the collision tumor mechanism rather than subclone mechanism.

A relationship between ERG heterogeneity and clinicopathological characteristics, such as biochemical recurrence, has not been reported in previous studies. In our study, a comparison of cases with heterogeneous FISH patterns (including mixed positive-negative results and mixed break apart and interstitial deletion FISH patterns) and cases with homogeneous FISH pattern did not reveal any significant differences in clinicopathological parameters between the 2 Groups. The frequency of biochemical recurrence

was also not significantly different between heterogeneous and homogeneous IHC and FISH groups. This result suggests that ERG heterogeneity has no clinical significance.

In the group with a homogeneous ERG FISH pattern (70 cases), 37 cases exhibited a break apart pattern and 33 exhibited an interstitial deletion pattern. The proportion of cases with break apart and interstitial deletion was fairly similar. In addition, clinicopathological factors are not correlated with different ERG FISH patterns. Attard et al. reported that interstitial deletions with polysomy were associated with a poor prognosis compared with other FISH patterns (37). In contrast, we did not identify any clinical significance of ERG FISH patterns.

Conclusion

Heterogeneity of ERG expression was observed in 32.7% of the cases evaluated by IHC and FISH. However, clinicopathological factors, including age, initial PSA level, pathological staging, tumor volume, Gleason score, primary Gleason grade and biochemical recurrence were not significantly correlated with ERG heterogeneity.

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Abstract

The relationship between ERG protein expression heterogeneity and TMPRSS2-ERG fusion gene patterns in prostate cancer

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2011-30602

Prostate cancer is considered to be a heterogeneous tumor with various morphologic features and biologic behaviors. The TMPRSS2-ERG fusion gene is the most frequently observed genetic aberration observed in prostate cancer. The aim of this study is to determine the correlation of ERG protein expression heterogeneity and TMPRSS2-ERG fusion gene patterns in prostate cancer.

We analyzed 2 groups of prostate cancer samples. The first group

was composed of 172 radical prostatectomy specimens that were performed at Seoul National University Hospital in 2012, and the second group was composed of 303 radical prostatectomy specimens that were performed at Seoul National University Hospital from 1999-2006. We constructed tissue microarray blocks composed of 2 to 6 representative samples from each ERG-positive case to use for immunohistochemistry. Using the tissue microarray block, ERG immunohistochemical staining (IHC) and ERG fluorescence in situ hybridization (FISH) were conducted to evaluate the heterogeneity of ERG protein expression and ERG fusion gene patterns, respectively, in a single tumor focus. In addition, we analyzed the clinicopathological significance of ERG heterogeneity in prostate cancer.

A total of 104 ERG-positive cases were identified among the 475 prostate cancer cases evaluated by IHC, and presence of the ERG fusion gene was confirmed by FISH in all 104 cases. Heterogeneous ERG FISH patterns were observed in 34 cases (32.7%), including 22 cases with a mixed FISH pattern (break apart and interstitial deletion) and 12 cases of a mixed positive-negative FISH pattern. Clinicopathological factors, including age, initial prostate-specific

antigen (PSA) level, Gleason score, primary Gleason grade, tumor volume and pathological staging were not significantly associated with ERG FISH heterogeneity. Biochemical recurrence was not associated with heterogeneity in either ERG IHC or ERG FISH results.

In summary, ERG heterogeneity was observed in 32.7% of cases with the ERG fusion gene. However, clinicopathological factors and biochemical recurrence were not significantly correlated with ERG heterogeneity.

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전립선암에서 ERG 단백발현의 이질성과 TMPRSS2-ERG 유전자 융합 양상의 상관관계 및 그 임상적, 병리학적 의미

2016년 2월

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의학과 병리학 전공

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By JA HEE SUH

A thesis submitted in partial fulfillment of the Requirements for the Degree of Doctor of Philosophy in Medicine (Pathology) at the Seoul National University College of Medicine

February, 2016

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국문초록

전립선암에서 ERG 단백발현의 이질성과 TMPRSS2-ERG 유전자 융합 양상의 상관관계 및 그 임상적, 병리학적 의미

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병리학 전공

학번: 2011-30602

전립선 암에서 높은 빈도로 발생하는 것으로 알려진 유전자 융합 (gene fusion) 은 TMPRSS2-ERG 유전자 융합이며, 이 유전자 융합에 의하여 ERG 단백 과발현이 일어난다. 이는 서양에서는 그 빈도가 50%에 이르는 것으로 보고되고 있으나 한국 및 일본인 환자에서는 20% 초반의 빈도로 발생하여 서양보다 훨씬 낮은 빈도를 보인다. 전립선 암의 특성상 유전자 융합에 의한 ERG 단백 발현의 정도가 한 증례 안에서도 부위에 따라 서로 다른 이질성이 보고되었으나 이러한 ERG 단백발현의 이질성이 어떤 이유로 일어나며, 임상적 혹은 병리학적으로 어떠한 의미를 가지는지에 대한

연구는 발표된 바가 없다. 이에 저자는 서울대병원의 전립선 암 증례를 대상으로 ERG 단백 발현의 이질성과 TMPRSS2-ERG 유전자 융합 패턴의 이질성을 확인하고 이와 관련된 임상적 혹은 병리학적 의미를 확인하고자 하였다.

두 개의 전립선 암 환자 군을 대상으로 하여 ERG 단백 발현의 이질성을 확인하였다. 첫 번째 환자 군은 2012 년 서울대병원에서 전립선 암으로 수술 받은 172 증례를 대상으로, 두 번째 환자 군은 1999 년부터 2006 년까지 서울대병원에서 전립선 암으로 수술 받은 303 증례를 대상으로 하였다. 이 중 ERG 면역조직화학염색 상 양성인 104 증례에서 각각의 대표적인 절편으로부터 2-6 개의 코어를 얻어, Tissue microarray(TMA)를 제작하였다. 이렇게 제작된 TMA 에서 동일한 종양 내에서 ERG 단백발현 이질성 확인을 위한 면역조직화학 염색 시행하였으며 fluorescence in situ hybridization (FISH)를 시행하여 ERG 유전자 융합 pattern 의 이질성을 파악하였다. 또한 ERG 단백 발현 이질성 및 ERG 유전자 융합 pattern 의 이질성과 임상적, 병리학적 특성들과의 상관관계를 살펴보았다.

그 결과 ERG 면역 염색 상 양성인 104 증례는 모두 FISH 에서 ERG 유전자 융합이 관찰되었다. 이 중 34 증례 (32.7%) 에서는 ERG 유전자 융합상의 이질성이 관찰되었으며, 이러한 이질성은

22 개의 서로 다른 ERG 유전자 융합 양상 (FISH 상 break apart 와 interstitial deletion)이 혼재되어 있는 경우와 12 개의 ERG 유전자 융합 양성 및 음성이 혼재되어 있는 경우가 포함되었다. 또한 ERG 면역염색의 강도에 따라 ERG FISH 의 양상은 break apart 와 interstitial deletion 이 비교적 균일하게 분포하였다. ERG 단백 발현의 이질성 및 ERG 유전자 융합 pattern 의 이질성은 임상적, 병리학적 특성과 무관하였으며, 생화학적 재발율 역시 이질성에 따른 유의한 차이를 보이지 않았다.

주요어: 전립선암, 유전자 융합, 종양, 이질성

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Introduction

As prostate cancer is associated with a wide spectrum of histologic variants and biologic behaviors, it is regarded as a heterogeneous disease. Prostate cancer is the 2nd most common cancer in the western male population (up to 14%) and the 6th leading cause of cancer-related mortality (up to 6%) (1). In addition, the incidence rate and the mortality rate of prostate cancer in the Korean population are rapidly increasing (2, 3). The incidence of prostate cancer differs between the Asian and the Caucasian population, and the prognosis is significantly associated with ethnicity (4).

A fusion gene between ERG (21q22.2) (avian v-ets erythroblastosis virus E26 oncogene homolog) and TMPRSS2 (21q22.3) (transmembrane protease serine 2) is the most common recurrent genetic rearrangement in prostate cancer. The TMPRSS2-ERG fusion gene in prostate cancer was first reported by Tomlins et al in 2005 (5). The frequency of the fusion gene varies among study groups, from approximately 15.3% (6) to 72% (7) in the western population to approximately 20.9% (8) to 28% (9) in the Korean and Japanese populations. Various detection methods for the TMPRSS2-ERG fusion gene have been described

in previous studies.

TMPRSS2-ERG fusion results in androgen-induced over-expression of ERG, a gene that encodes erythroblast transformation-specific (ETS) transcription factors (5). The most common ERG fusion partner is TMPRSS2, although rare cases of other ERG fusion partners such as solute carrier family 45, member 3 (SLC45A3) and n-myc downstream regulated 1 (NDRG1) have been reported (10, 11).

Reverse transcription polymerase chain reaction (RT-PCR) and quantitative reverse transcription PCR have been used as the principle methods for the quantitative detection of genetic rearrangements (12), although fluorescence in situ hybridization (FISH) is considered the standard and confirmative molecular method for identifying gene fusions in single tumor cells and for quantitative assessment of gene fusions (13-16). Park et al. reported an efficient method for detecting the TMPRSS2-ERG fusion gene using the ERG-specific antibody EPR-3864 in formalin-fixed paraffin-embedded (FFPE) tissue (17). Detecting the TMPRSS2-ERG fusion gene using immunohistochemical staining (IHC) with antibodies against ERG is a relatively simple,

low-cost method that employs a routine laboratory procedure. The sensitivity and specificity of this approach was verified with FISH and other molecular laboratory methods in various studies; therefore, ERG IHC is regarded as a validated technique for detecting the TMPRSS2-ERG fusion gene (18-20).

Several studies that investigated the relationship between the TMPRSS2-ERG fusion gene and clinicopathological factors of prostate cancer, including clinical outcomes, in various groups (21-23) have reported inconsistent results. Although recent studies demonstrated that the ERG fusion gene is associated with a low Gleason score (15, 24, 25).

ERG and TMPRSS2 are located in close proximity to one another on chromosome 21. Loss of the chromosomal region between the TMPRSS2 and ERG genes (an interstitial deletion) is frequently associated with the TMPRSS2-ERG gene fusion and is associated with a more advanced tumor stage (21). Attard et al. later reported various ERG FISH patterns, including interstitial deletion and split signal (sometimes referred to as break apart) (26), and some previous studies reported the prognostic value of ERG FISH patterns (22, 26, 27).

Recently, multiple studies reported an association between the heterogeneity of TMPRSS2-ERG fusion gene rearrangement patterns and ERG expression (22, 27-29). Mehra et al. revealed that different tumor foci in multifocal prostate cancers frequently exhibited different patterns of TMPRSS2 rearrangement (22). Minner et al. reported that intrafocal heterogeneity in ERG expression, defined as heterogeneous ERG expression within one tumor focus, was observed in 69 out of 103 ERG-positive cases evaluated by IHC (29). However, some of these studies were associated with limitations. Mehra et al. observed only interfocal heterogeneity, i.e., ERG heterogeneity in different tumor foci, but did not observe ERG heterogeneity within a single tumor focus (22). In contrast, Minner et al. observed intrafocal heterogeneity in ERG expression but did not identify a correlation between specific gene rearrangement patterns and heterogeneity in ERG expression, nor did they identify the clinicopathological significance of heterogeneous ERG expression (29).

We hypothesized that heterogeneous ERG expression identified by IHC might be associated with different ERG gene fusion patterns identified by FISH. We also aimed to elucidate the

clinicopathological significance of the heterogeneity in the expression pattern of the ERG fusion gene.

Material and method

1. Patient information and tissue microarray construction

A total of 475 prostate cancer cases were screened in this study. Group 2 included samples from 303 patients who had undergone a radical prostatectomy at Seoul National University Hospital from 1999 to 2006 that had been used for ERG IHC in a previous study (9). Group 1 included 172 patients who had undergone a radical prostatectomy at Seoul National University Hospital in 2012. Information regarding age, Gleason score, primary Gleason grade, tumor volume (%), pT staging, preoperative PSA level and biochemical recurrence was obtained from patient medical records, pathologic records and the analysis of clinical slides. The Gleason score and primary Gleason grade were assigned according to the criteria recommended by the 2005 International Society of Urological Pathology (ISUP) Consensus Conference (30). Biochemical recurrence was defined as a postoperative prostate specific antigen (PSA) level greater than 0.2 ng/ml in 2 consecutive measurements.

A representative tumor block was immunostained using an ERG antibody, and each ERG IHC slide was reviewed to evaluate the ERG signal and staining intensity. ERG immunostaining intensity was scored from 0 to 3+. Among the 475 cases screened, 114 cases were ERG-positive according to IHC, and 10 cases from Group 2 were excluded due to insufficient tissue available for the tissue microarray (TMA) construction. A total of 104 ERG positive cases were ultimately included in this study. For the construction of the TMAs, 2 to 6 representative cores (1 mm in diameter) from a single tumor focus were used. In samples with heterogeneous ERG immunostaining intensity, areas of different intensity were separated for TMA construction (Figure 1). A total of 428 cores were constructed into 4 TMA blocks using a trephine apparatus (Superbiochips Laboratories, Seoul, Korea) (31).

2. ERG immunohistochemical stainings (IHC).

Sections mounted on superfrost slides were deparaffinized. IHC was conducted using the Ventana Benchmark XT automated staining system (Ventana Medical Systems, Tucson, AZ, USA) using

Ventana reagents. We used the anti-ERG rabbit monoclonal antibody (RTU, clone EPR3864, Ventana, Tucson, AZ, USA) (17, 20). Heat-induced epitope retrieval was performed with anti-ERG antibody. The slides were incubated with primary antibodies for 16 minutes at room temperature. For IHC using the anti-ERG rabbit monoclonal antibody (rabbit ERG-MAb), a secondary antibody (Ultraview anti-Rabbit HRP; Ventana Medical Systems, Tucson, AZ, USA) was applied for 16 minutes at room temperature. Secondary antibody detection was performed using the Ultraview DAB detection kit (Ventana Medical Systems). Slides were counterstained with Hematoxylin II for 4 minutes followed by Bluing Reagent (Ventana Medical Systems) for 4 minutes at 37 ℃. ERG staining in the nuclei of endothelial cells served as the endogenous positive control, as previously described (10). The intensity of ERG expression was scored as negative (0; no staining), weak (1+; only visible at high magnification), moderate (2+; visible at low magnification) and strong (3+; striking at low magnification).

3. ERG dual color break apart FISH.

FISH was performed as previously described (32, 33) using a dual color probe (Zytolight® SPEC ERG Dual Color FISH probes, ZytoVision GmbH, Bremerhaven, Germany). Briefly, 2 differentially labeled probes that provided coverage of the neighboring telomeric and centromeric regions of each locus were designed. The following telomeric/centromeric BAC clones were used to design the break-apart assays for assessing chromosomal rearrangement status (i.e., rearrangement vs. no rearrangement): target sequences mapping to 21q22.2, distal to the ERG breakpoint region (green signal); and target sequences mapping to 21q22.13-q22.2, proximal to the ERG breakpoint region (orange signal). Briefly, 2-μm sections were deparaffinized and dehydrated, and the slides were incubated in 0.2 N HCl at room temperature for 20 minutes and subsequently incubated in 8% sodium thiocyanate at 80 ℃ for 30 minutes. The slides were treated with 1% pepsin at 37 ℃ for 20 minutes. The probe set was applied to the slides, and a coverslip was added and sealed with rubber cement. The slides were incubated in ThermoBrite (Abbott Molecular, Des Plaines, IL) at 75 ℃ for 5 minutes to denature the probe and subsequently incubated at 37 ℃

for 16 hours to allow for hybridization. After the post-hybridization washing, the samples were counterstained with 4,6-diamidino-2-phenylindole (DAPI). The samples were analyzed using a 1000x oil immersion lens on an Olympus BX-51TRE microscope (Olympus, Tokyo, Japan) equipped with DAPI, green, orange and triple-pass (DAPI/Green/Orange) filters (Abbott-Vysis). A minimum of 100 tumor nuclei per case were assessed. A positive FISH result was defined as the presence of a break apart or interstitial deletion in at least 50% of the nuclei of epithelial cells. The break apart was defined as the combination of 1 green and orange signal, and 1 separated green and orange signal. The interstitial deletion was defined as one combined signal and one orange signal with the loss of a green signal (8). If both interstitial deletion and break-apart co-existed in a single core, we classified the sample according to the pattern observed in more than 50% of nuclei.

4. Determination of heterogeneity.

Heterogeneity of ERG IHC was defined as the presence of both ERG-positive and -negative cores or of 1+ and 3+ cores in the

same tumor focus. Heterogeneity of ERG FISH was defined as the presence of both positive and negative cores or of break apart and interstitial deletion cores in the same tumor focus (Figure 2).

5. Statistics

All statistical analyses were conducted using SPSS v. 23.0 (IBM Inc., Chicago, IL). Fisher extract test was used for identifying significant correlations, and Kaplan-Meier survival analysis was used to evaluate biochemical recurrence-free survival analysis

6. Institutional review board (IRB) permission

IRB of this study was deliberated and approved by the Center for Human Research Protection of Seoul National University Hospital / Biomedical Research Institute. (IRB approval number: 1212-054-450).

Results

1. Clinicopathologic characteristics and ERG IHC.

In Group 1, samples from 172 patients were evaluated by ERG IHC. The mean age of the patients was 66 years, with a standard deviation of 6.9 years. The mean value of the initial PSA level was 11.1 ng/ml, and 40 cases (23.2%) were confirmed to be ERG-positive by IHC. In Group 2, samples from 303 cases were evaluated by ERG IHC. The mean age was 65.7 years with a standard deviation 6.4 years. The mean value of the initial PSA level was 11.2 ng/ml, and 74 cases (24.4%) were confirmed to be ERG-positive by ERG IHC (9). ERG protein overexpression was related to a low Gleason grade only and to the Gleason score in Group 1 and 2 (Table 1 and 2). Other clinicopathological parameters were not associated with ERG expression levels.

ERG heterogeneity was observed in 47 of the 104 ERG-positive cases (45.2%). Among the 47 cases, 16 exhibited both positive and negative foci, and the remaining 31 cases exhibited both 1+ and 3+ foci.

2. Correlation between ERG IHC and ERG FISH in TMA cores

A total of 428 cores were constructed from Group 1 and 2. The ERG FISH results associated with 11 cores could not be interpreted due to a lack of sufficient tissue. Ultimately, 417 cores were analyzed and used to investigate the correlation between the results of ERG IHC and ERG FISH (Table 3). All of the ERG-positive cores exhibited ERG break apart (positive) in the FISH analysis. Discrepancies between the ERG IHC and FISH results were observed in 14 out of 39 ERG-negative cores (35.9%), i.e., these cores exhibited positive FISH results. ERG FISH patterns (break apart or interstitial deletion) were not associated with ERG IHC intensity and demonstrated a relatively even distribution (Table 3). The distribution of TMA cores according to IHC intensity, FISH pattern and Gleason score is described in Table 4, and representative images are presented in Figure 3.

3. ERG FISH heterogeneity.

All 104 IHC positive cases were confirmed to be ERG FISH positive. Seventy cases exhibited homogeneous ERG FISH patterns with either break aparts (n=37) or interstitial deletions (n=33). The remaining 34 cases exhibited heterogeneous FISH patterns, including 22 cases with both break aparts and interstitial deletions and 12 cases with both FISH positive and negative cores within a single tumor focus (Figure 4).

ERG FISH heterogeneity was not associated with clinicopathological parameters (Table 5). ERG FISH patterns (break apart and interstitial deletion) were also not associated with clinicopathological parameters (Table 6).

The cases exhibiting ERG heterogeneity in either the IHC or FISH experiments did not experience significantly different biochemical recurrence-free survival periods (Figure 5 and 6).

Table 1. Correlation with clinicopathological factors and ERG IHC results in Group 1

Parameter	ERG IHC (n=168*)		*p* value
	Negative	**Positive**	
Age (years)			
< 66 (n=67)	49 (73.1%)	18 (26.9%)	0.449
≥ 66 (n=101)	79 (78.2%)	22 (21.8%)	
Initial PSA level (ng/ml)			
< 10 (n=96)	64 (66.7%)	32 (33.3%)	0.076
≥ 10 (n=72)	64 (88.9%)	8 (11.1%)	
Tumor volume (%)			
< 10% (n=59)	39 (66.1%)	20 (33.9%)	0.083
≥ 10% (n=96)	78 (81.3%)	18 (18.7%)	
Primary Gleason grade (pattern)			
3 (n=121)	87 (71.9%)	34 (28.1%)	**0.036**
4 & 5 (n=47)	41 (87.2%)	6 (12.8%)	
Gleason score (sum of primary and second Gleason grade)			
≤ 7 (n=158)	118 (74.7%)	40 (25.3%)	0.068
> 7 (n=10)	10 (100%)	0 (0%)	
pT-stage (AJCC 7th edition)			
pT2 (n=100)	70 (70%)	30 (30%)	0.056
pT3 (n=68)	58 (85.3%)	10 (14.7%)	

IHC: immunohistochemical staining, PSA: prostate specific antigen, AJCC: American Joint Committee on Cancer.

* In 172 cases, 4 cases were excluded in this analysis due to preoperative hormonal therapy.

Table 2. Correlation with clinicopathological factors and ERG IHC results in Group 2 from previous study (9)

Parameter	ERG IHC (n=303)		*p* value
	Negative	**Positive***	
Age (years)			
< 66 (n=147)	109 (74.1%)	38 (25.9%)	0.595
≥ 66 (n=156)	120 (76.9%)	36 (23.1%)	
Initial PSA level (ng/ml)			
< 10 (n=191)	140 (73.3%)	51 (26.7%)	0.268
≥ 10 (n=112)	89 (79.5%)	23 (20.5%)	
Tumor volume (%)			
< 10% (n=100)	78 (78%)	22 (22%)	0.570
≥ 10% (n=203)	151 (74.4%)	52 (25.6%)	
Primary Gleason grade (pattern)			
3 (n=218)	155 (71.1%)	63 (29.9%)	**0.004**
4 & 5 (n=85)	74 (87.1%)	11 (12.9%)	
Gleason score (sum of primary and second Gleason grade)			
≤ 7 (n=253)	184 (72.7%)	69 (27.3%)	**0.011**
> 7 (n=50)	45 (90%)	5 (10%)	
pT-stage (AJCC 7th edition)			
pT2 (n=165)	125 (75.8%)	40 (24.2%)	0.521
pT3 (n=138)	104 (75.4%)	34 (24.6%)	

IHC: immunohistochemical staining, PSA: prostate specific antigen, AJCC: American Joint Committee on Cancer.

Table 3. Tissue microarray core analysis focused on IHC intensity variation and FISH pattern

IHC	Total core number	
Negative	**39**	
(0)	FISH Negative	25(64.1%)
	FISH Positive	**14 (35.9%)**
Weak positive (1+)	115	
(1+)	FISH Break apart	FISH Interstitial deletion
	66 (57.4%)	49 (42.6%)
Moderate positive (2+)	103	
(2+)	FISH Break apart	FISH Interstitial deletion
	56 (54.4%)	47 (45.6%)
Strong positive (3+)	160	
(3+)	FISH Break apart	FISH Interstitial deletion
	81 (50.6%)	79 (49.4%)
	417*	

FISH: fluorescence in situ hybridization.

*Total core number was 428, but 11 cores are not available for FISH because of limitation of tumor tissue.

Table 4. Tissue microarray core analysis: immunohistochemical staining (IHC) , FISH and Gleason score

	Total core number = 428			
FISH result	**Negative**	**Break apart**	**Interstitial deletion**	**NA**
	25	213	179	11
IHC result	**Negative**	**1+**	**2+**	**3+**
	39	115	103	160
Gleason score	**6**	**7**		**8**
	276	124		28

FISH: fluorescence in situ hybridization, IHC: immunohistochemical staining, NA: not available due to lack of tissue, 1+: weak positive, 2+: moderate positive, 3+: strong positive.

Table 5. Correlation between clinicopathological factors and ERG FISH heterogeneity

Parameter	ERG FISH Heterogeneity (n=104)		*p* value
	Homogeneous (N=70)	**Heterogeneous (N=34)**	
Age (years)			
< 66 (n=56)	41 (73.2%)	15 (26.8%)	0.165
≥ 66 (n=48)	29 (60.4%)	19 (39.6%)	
Initial PSA level (ng/ml)			
< 10 (n=75)	47 (62.7%)	28 (37.3%)	0.105
≥ 10 (n=29)	23 (79.3%)	6 (20.7%)	
Tumor volume (%)			
< 10% (n=38)	27 (71.1%)	11 (28.9%)	0.537
≥ 10% (n=66)	43 (65.2%)	23 (34.8%)	
Primary Gleason grade (pattern)			
3 (n=79)	53 (67.1%)	26 (32.9%)	0.933
4 & 5 (n=25)	17 (68%)	8 (32%)	
Gleason score (sum of primary and second Gleason grade)			
≤ 7 (n=96)	63 (65.6%)	33 (34.4%)	0.205
> 7 (n=8)	7 (87.5%)	1 (12.5%)	
pT-stage (AJCC 7th edition)			
pT2 (n=64)	41 (64.1%)	23 (35.9%)	0.372
pT3 (n=40)	29 (72.5%)	11 (27.5%)	

FISH: fluorescence in situ hybridization, PSA: prostate specific antigen, AJCC : American Joint Committee on Cancer.

Table 6. Correlation between clinicopathological factors and ERG FISH pattern

Parameter	ERG FISH Pattern in homogeneous ERG FISH result (n=70)		*p* value
	Break apart (n= 37)	**Interstitial deletion (n=33)**	
Age (years)			
< 66 (n=41)	23 (56.9%)	18 (43.1%)	0.518
≥ 66 (n=29)	14 (48.3%)	15 (51.7%)	
Initial PSA level (ng/ml)			
< 10 (n=47)	28 (59.6%)	19 (40.4%)	0.108
≥ 10 (n=23)	9 (39.1%)	14 (60.9 %)	
Tumor volume (%)			
< 10% (n=27)	15 (55.6%)	12 (44.4%)	0.720
≥ 10% (n=43)	22 (51.2%)	21 (48.8%)	
Primary Gleason grade (pattern)			
3 (n=53)	27 (50.9%)	26 (49.1%)	0.571
4 & 5 (n=17)	10 (58.8%)	7 (41.2%)	
Gleason score (sum of primary and second Gleason grade)			
≤ 7 (n=63)	31 (49.2%)	32 (50.8%)	0.066
> 7 (n=7)	6 (85.7%)	1 (14.3%)	
pT-stage (AJCC 7th edition)			
pT2 (n=41)	18 (43.9%)	23 (56.1%)	0.074
pT3 (n=29)	19 (65.5%)	10 (34.5%)	

FISH: fluorescence in situ hybridization, PSA: prostate specific antigen, AJCC : American Joint Committee on Cancer.

Figure 1. TMA construction by multiple cores from ERG IHC in full section

After representative full section ERG IHC, heterogeneity of ERG expression was reviewed. And multiple cores were extracted from heterogeneous or homogeneous area of tumor with ERG expression.

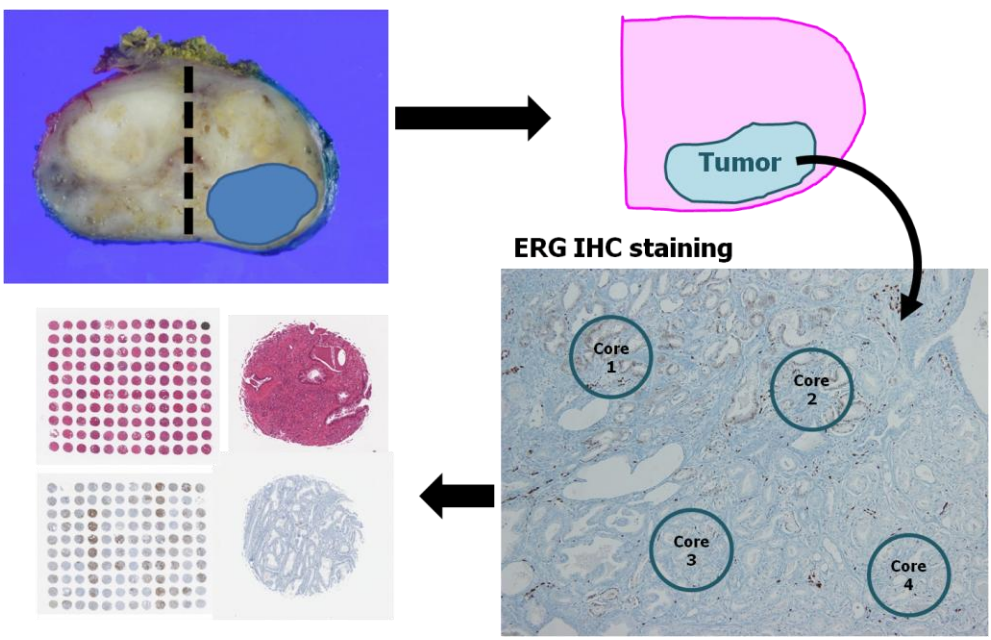
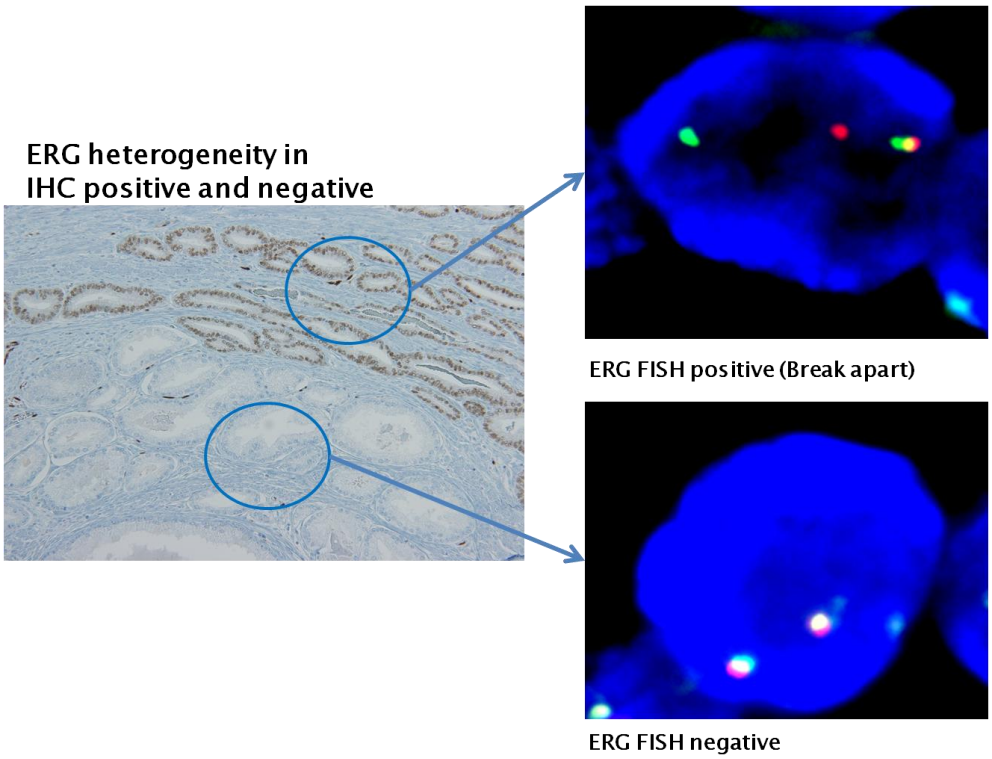
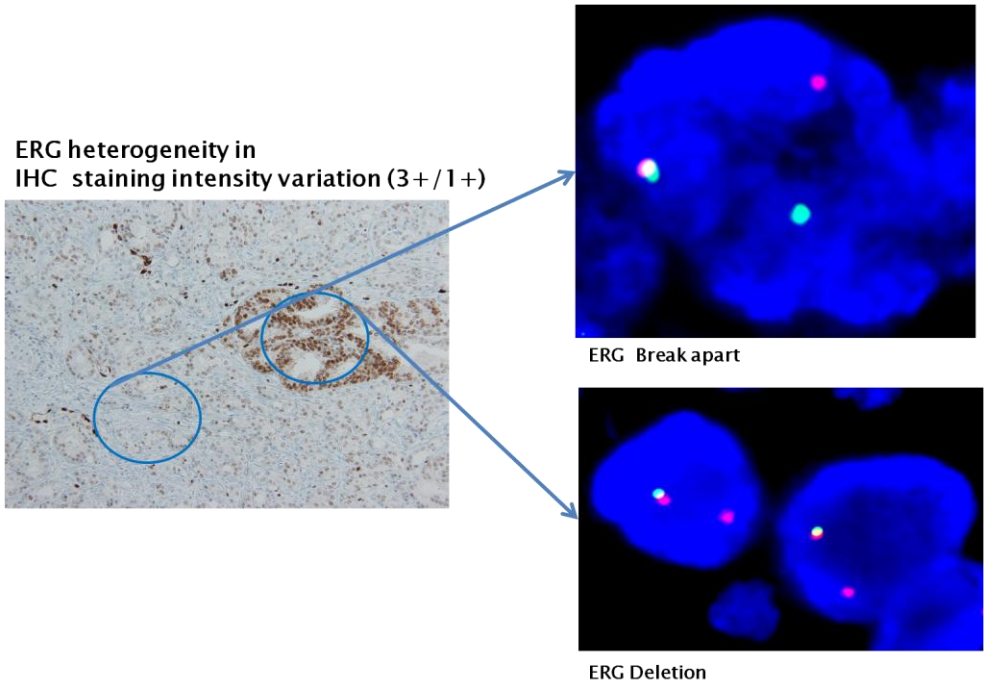


Figure 2. ERG heterogeneity determined as positive and negative (A) and ERG IHC intensity variation and mixed pattern in ERG FISH (B)



(A)


ERG heterogeneity in
IHC staining intensity variation (3+/1+)
ERG Break apart
ERG Deletion

(B)

Figure 3. ERG IHC intensity (A−D), Gleason score (E−G) and FISH result (H−J) of prostate cancer

A: ERG negative, B: ERG weak positive(1+), C: ERG moderate positive (2+), D: ERG strong positive (3+), E: Gleason score 6(3+3), F: Gleason score 7(3+4), G: Gleason score 8(4+3), H: TMPRSS2−ERG FISH negative (fusion signal), I: TMPRSS2−ERG FISH positive (break apart signal), J: TMPRSS2−ERG FISH positive (interstitial deletion signal)

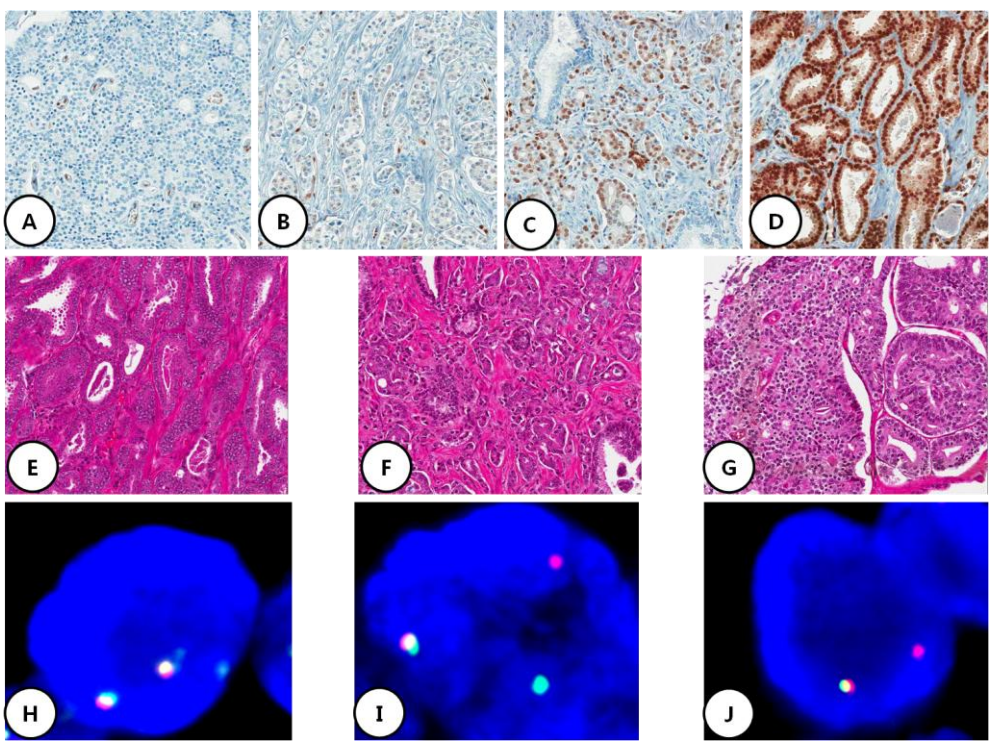


Figure 4. Flowchart of data evaluation in ERG FISH results of 104 ERG IHC positive cases

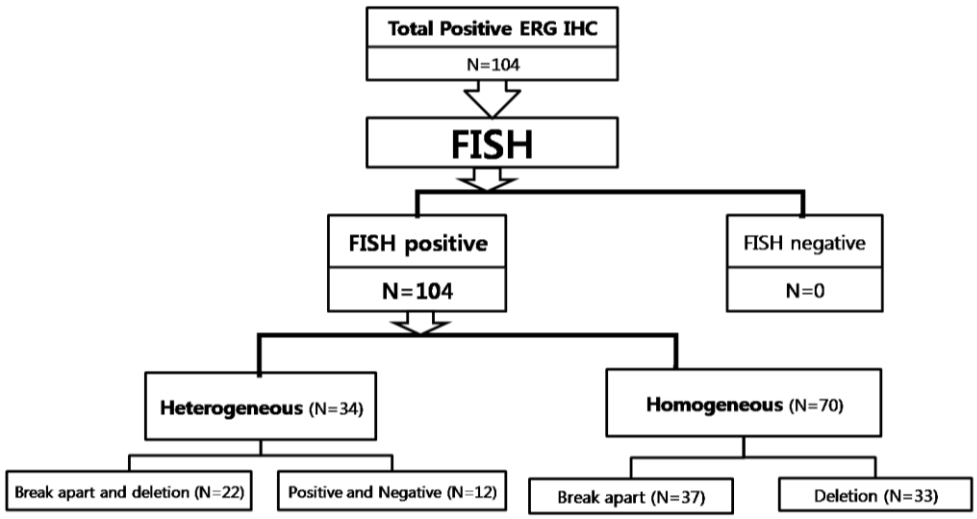
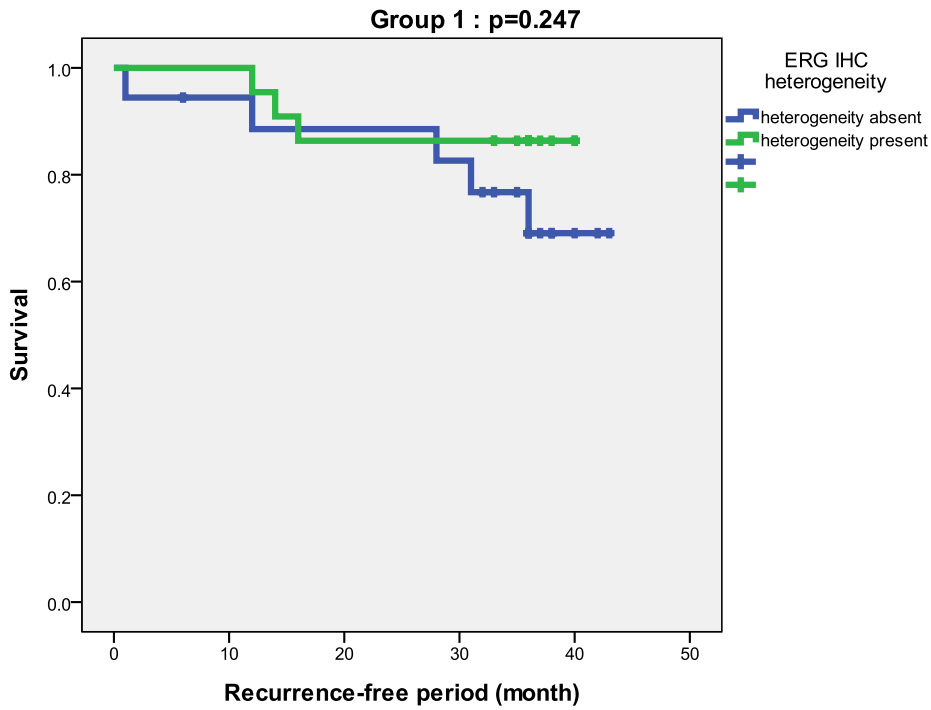
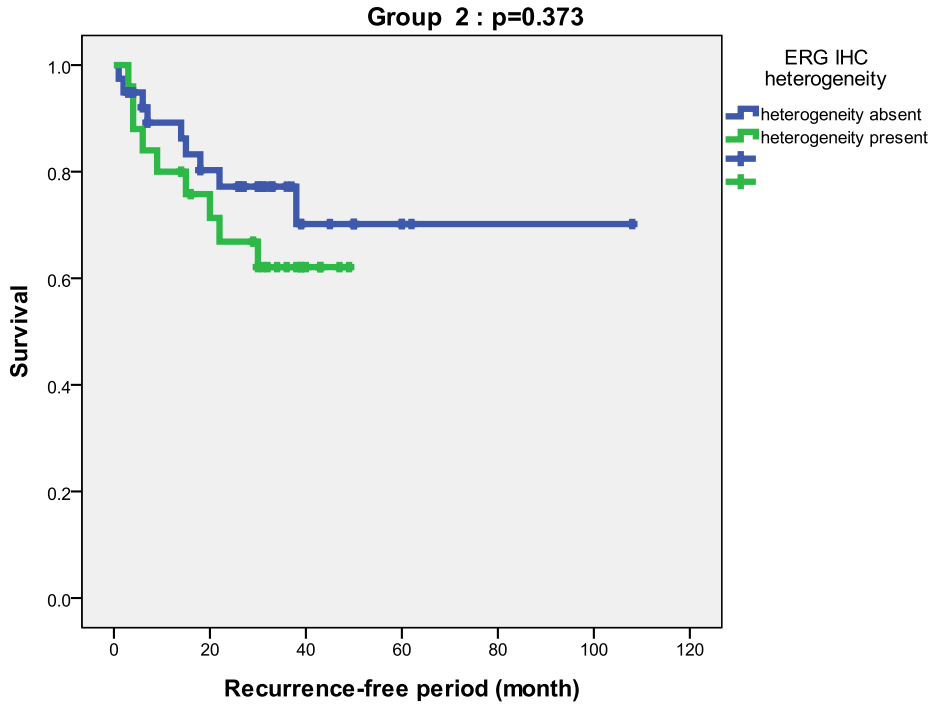


Figure 5. Kaplan-Meier curves of biochemical recurrence in Group 1 (A) and 2 (B) according to ERG IHC heterogeneity

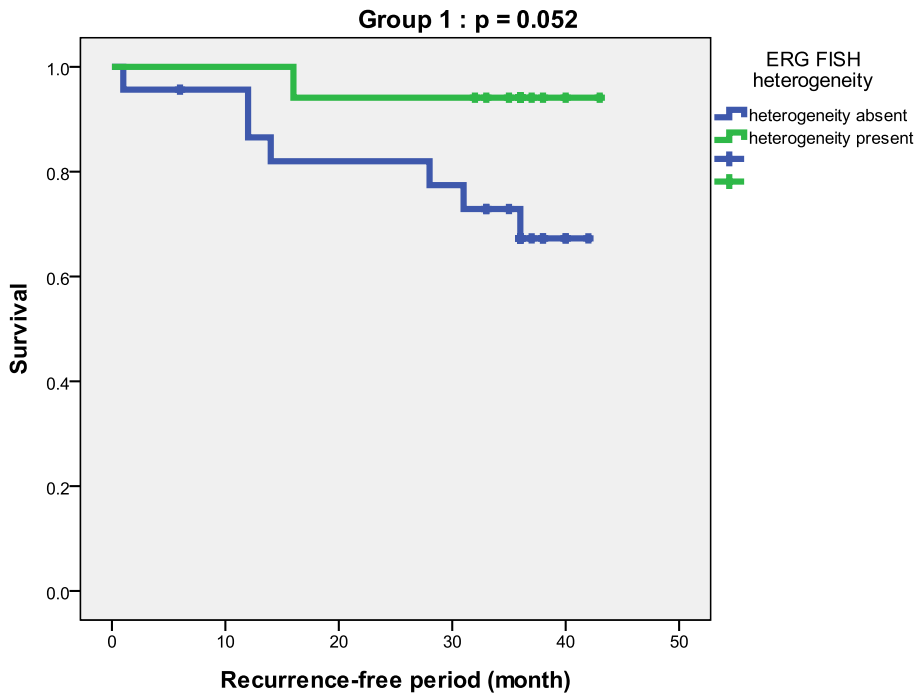


(A)

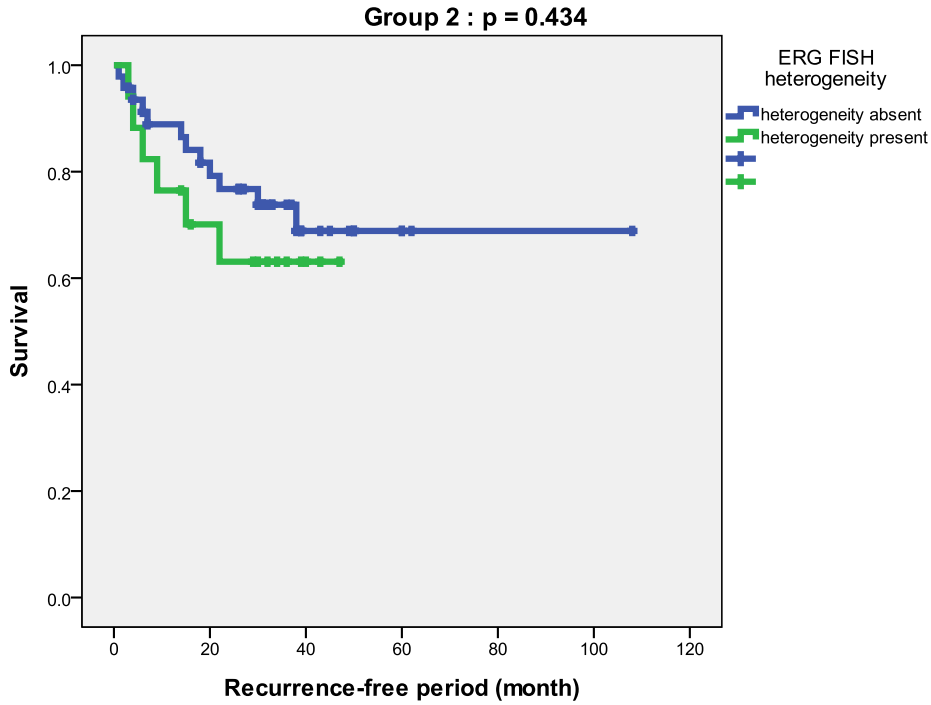

Group 2 : p=0.373
ERG IHC heterogeneity
heterogeneity absent
heterogeneity present
Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
120
Recurrence-free period (month)

(B)

Figure 6. Kaplan-Meier curves of biochemical recurrence in Group 1 (A) and 2 (B) according to ERG FISH heterogeneity

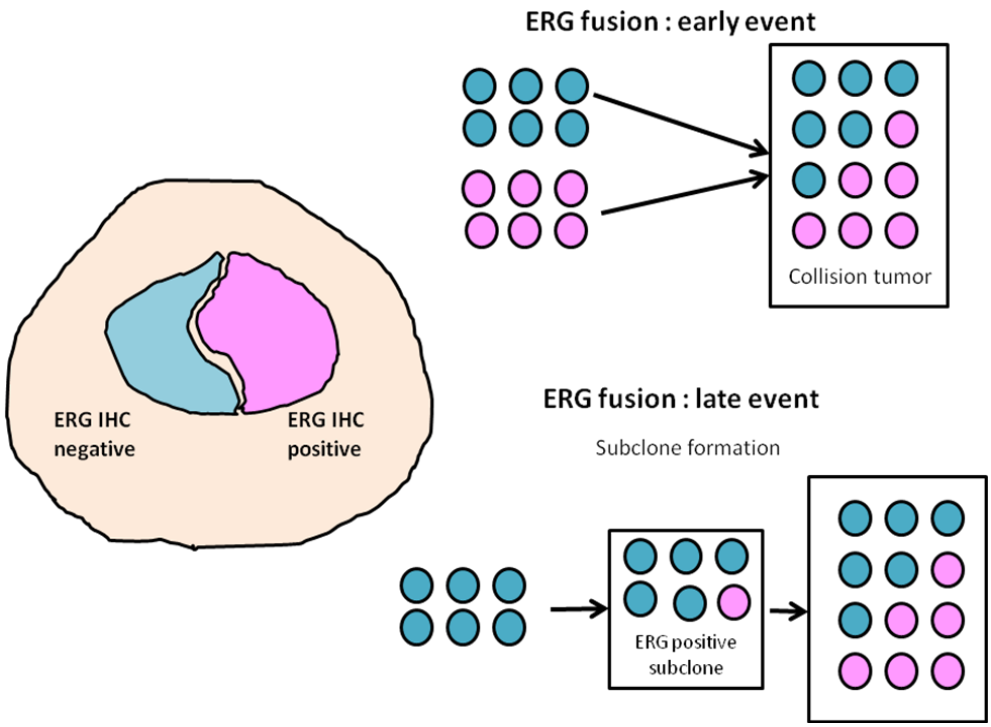


(A)

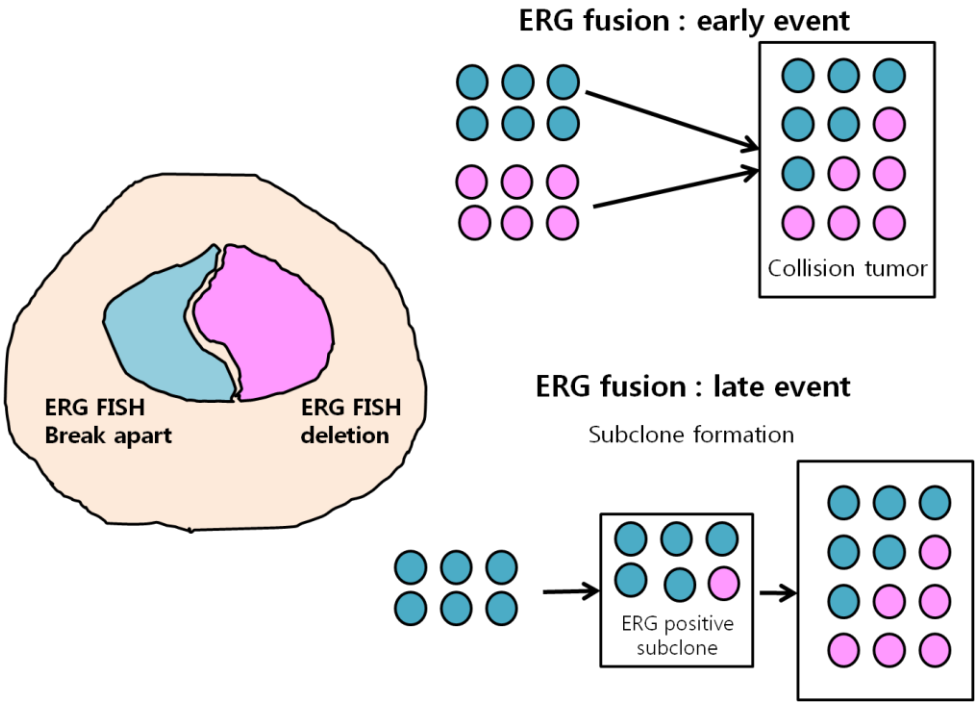

Group 2 : p = 0.434
ERG FISH heterogeneity
heterogeneity absent
heterogeneity present
Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
120
Recurrence-free period (month)

(B)

Figure 7. Heterogenity of ERG fusion status (A) and ERG FISH pattern (B) in prostate cancer. Both collision tumor and subclonal formation are considered as the interpretation of heterogeneity of ERG FISH patterns.



(A)


ERG fusion : early event
Collision tumor
ERG FISH
Break apart
ERG FISH
deletion
ERG fusion : late event
Subclone formation
ERG positive
subclone

(B)

Discussion

In this study, we studied the heterogeneity of EGR expression and gene fusion patterns in prostate cancer. 24% of cases were determined to be ERG-positive by IHC, a frequency lower than that reported in the overall western population (approximately 50%). A similar frequency was reported in the Japanese population (20.1%) (34). ERG status as measured by IHC was associated with a lower Gleason score and Gleason grade in this study. It has previously been established that the ERG fusion gene fusion is associated with low Gleason pattern (8).

In the TMA core analysis, a subset of cores exhibited negative ERG IHC and positive ERG FISH results (14 cores out of 39 ERG IHC negative cores, 35.9%). This result indicates that ERG IHC can potentially generate false negatives, and this should be taken into account when interpreting ERG IHC.

Regarding ERG expression heterogeneity, Minner et al. reported that 74 ERG-positive cases out of 103 cases (72%) evaluated by IHC exhibited ERG expression heterogeneity (29). In this study, we

observed ERG expression heterogeneity in 45.2% of ERG-positive cases evaluated by IHC, a frequency lower than that reported in a previous study (29).

We also evaluated the differences of FISH patterns in regions with heterogeneous ERG expression. Our results demonstrated that the intensity of ERG IHC was not associated with ERG FISH patterns (break apart and interstitial deletion). All ERG IHC positive cores exhibited the ERG fusion gene in FISH analysis. These results suggest that ERG IHC is correlated with ERG gene fusion status; however, the staining intensity observed in IHC and the specific ERG fusion gene patterns has no influence on ERG protein expression.

In this study, we analyzed ERG FISH patterns in multiple areas of the same tumor focus and found that 34 out of 104 FISH positive cases (32.7%) exhibited different FISH patterns in the same tumor focus. This if the first study to report this result, and our findings revealed that a single tumor focus can be associated with intrafocal heterogeneity of ERG gene fusion patterns (break apart and interstitial deletion). Mehra et al. also studied the heterogeneity of the TMPRSS2 fusion gene using FISH analysis (22). They reported

a high frequency (70%) of interfocal heterogeneity for the TMPRSS2 rearrangement, and they reported that individual tumor cells within a single tumor focus exhibited homogeneous TMPRSS2 rearrangement patterns (22). In contrast, we observed intrafocal heterogeneity in ERG fusion gene patterns. The explanation for this discrepancy is not entirely clear, although there were a few differences between the previous study by Mehra et al. and the present study that are worth noting. Our study used an ERG break apart FISH probe, whereas Mehra et al. used a TMPRSS2 FISH probe. In addition, we constructed all TMA cores from one tumor focus from each case, whereas Mehra et al. selected TMA cores from different tumor foci. Finally, we focused our attention on intrafocal heterogeneity rather than interfocal heterogeneity.

We found that the intrafocal heterogeneity of ERG FISH patterns included positive/negative heterogeneity and break apart/interstitial deletion heterogeneity. These results can potentially be explained by 1 of 2 mechanisms. One possibility is that 2 different tumors with different ERG fusion patterns collide to form a single tumor (the collision tumor) with a heterogeneous ERG gene fusion pattern. Another possibility is that the existence of subclones with different

ERG fusion gene patterns (Figure 7). Previous studies reported that the ERG fusion gene was observed in high grade prostate intraepithelial neoplasm (HGPIN). Mosquera et al. reported that the ERG fusion gene in HGPIN is analogous to the ERG fusion gene in prostate cancer (35). Tomlins et al. evaluated an in vivo and in vitro modeling system using transgenic mice expressing ERG to investigate the development of HGPIN and the mechanism of cellular invasion. They suggested that the ERG fusion gene mediates the invasion process in HGPIN and prostate cancer (36). These previous results support the hypothesis that the ERG fusion gene is an early genetic aberration in tumorigenesis and support the collision tumor mechanism rather than subclone mechanism.

A relationship between ERG heterogeneity and clinicopathological characteristics, such as biochemical recurrence, has not been reported in previous studies. In our study, a comparison of cases with heterogeneous FISH patterns (including mixed positive-negative results and mixed break apart and interstitial deletion FISH patterns) and cases with homogeneous FISH pattern did not reveal any significant differences in clinicopathological parameters between the 2 Groups. The frequency of biochemical recurrence

was also not significantly different between heterogeneous and homogeneous IHC and FISH groups. This result suggests that ERG heterogeneity has no clinical significance.

In the group with a homogeneous ERG FISH pattern (70 cases), 37 cases exhibited a break apart pattern and 33 exhibited an interstitial deletion pattern. The proportion of cases with break apart and interstitial deletion was fairly similar. In addition, clinicopathological factors are not correlated with different ERG FISH patterns. Attard et al. reported that interstitial deletions with polysomy were associated with a poor prognosis compared with other FISH patterns (37). In contrast, we did not identify any clinical significance of ERG FISH patterns.

Conclusion

Heterogeneity of ERG expression was observed in 32.7% of the cases evaluated by IHC and FISH. However, clinicopathological factors, including age, initial PSA level, pathological staging, tumor volume, Gleason score, primary Gleason grade and biochemical recurrence were not significantly correlated with ERG heterogeneity.

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Abstract

The relationship between ERG protein expression heterogeneity and TMPRSS2-ERG fusion gene patterns in prostate cancer

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Prostate cancer is considered to be a heterogeneous tumor with various morphologic features and biologic behaviors. The TMPRSS2-ERG fusion gene is the most frequently observed genetic aberration observed in prostate cancer. The aim of this study is to determine the correlation of ERG protein expression heterogeneity and TMPRSS2-ERG fusion gene patterns in prostate cancer.

We analyzed 2 groups of prostate cancer samples. The first group

was composed of 172 radical prostatectomy specimens that were performed at Seoul National University Hospital in 2012, and the second group was composed of 303 radical prostatectomy specimens that were performed at Seoul National University Hospital from 1999-2006. We constructed tissue microarray blocks composed of 2 to 6 representative samples from each ERG-positive case to use for immunohistochemistry. Using the tissue microarray block, ERG immunohistochemical staining (IHC) and ERG fluorescence in situ hybridization (FISH) were conducted to evaluate the heterogeneity of ERG protein expression and ERG fusion gene patterns, respectively, in a single tumor focus. In addition, we analyzed the clinicopathological significance of ERG heterogeneity in prostate cancer.

A total of 104 ERG-positive cases were identified among the 475 prostate cancer cases evaluated by IHC, and presence of the ERG fusion gene was confirmed by FISH in all 104 cases. Heterogeneous ERG FISH patterns were observed in 34 cases (32.7%), including 22 cases with a mixed FISH pattern (break apart and interstitial deletion) and 12 cases of a mixed positive-negative FISH pattern. Clinicopathological factors, including age, initial prostate-specific

antigen (PSA) level, Gleason score, primary Gleason grade, tumor volume and pathological staging were not significantly associated with ERG FISH heterogeneity. Biochemical recurrence was not associated with heterogeneity in either ERG IHC or ERG FISH results.

In summary, ERG heterogeneity was observed in 32.7% of cases with the ERG fusion gene. However, clinicopathological factors and biochemical recurrence were not significantly correlated with ERG heterogeneity.