Applied Cancer Research

RESEARCH ARTICLE Open Access

PTEN loss in Gleason grade 7 prostate tumors exhibits intratumoral heterogeneity and is associated with unfavorable pathological features

C. G. Picanço-Albuquerque[1†], T. Vidotto[1†], C. S. Pereira[1], F. P. Saggioro[2], T. Jamaspishvili[3,4], M. Koti[5], D. M. Berman[3,4], J. A. Squire[2,3*] and R. B. Reis[6]

Abstract

Background: PTEN loss is observed in 20–30% of prostate cancers and is associated with a poor outcome, but clinical details of the impact of this biomarker are unclear for intermediate grade tumors.

Methods: We investigated 43 radical prostatectomy-derived grade 7 prostate tumors from the Clinics Hospital of Ribeirão Preto. Tissue microarray (TMA) blocks were constructed and *PTEN* copy number status was determined for all patients through fluorescence in situ hybridization (FISH). To determine the presence of PTEN protein loss in our study cohort, we performed immunohistochemistry (IHC) in TMA sections. We then developed an automated algorithm in HALO™ to identify regions of PTEN protein loss in whole prostate scanned sections from ten patients with known *PTEN* deletion status by FISH. Clinical analyses were conducted to determine the associations between PTEN loss and patient outcome. All statistical analyses were conducted in R v3.4.3 with *P*-values below 0.05 being considered statistically significant.

Results: In this study of 43 grade 7 tumors, we found *PTEN* deletions by FISH in 18.9% of tumors, and PTEN protein loss by IHC in 16.3% of tumors. Both techniques were highly concordant and complementary. Clinical analysis demonstrated that *PTEN* deletion by FISH was significantly associated with positive margin invasion ($P = 0.04$) and Gleason score upgrade ($P = 0.001$). Digital image analysis of ten representative tumors demonstrated distinct intratumoral heterogeneity for PTEN protein loss in four tumors.

Conclusions: This study shows that PTEN loss in Gleason grade 7 tumors can be heterogeneous and that a systematic analysis of this biomarker using a combination of FISH, IHC, and digital imaging may identify patients with a greater risk of poor outcome.

Keywords: PTEN, Digital pathology, Prostate cancer, Intratumoral heterogeneity, Fluorescence in situ hybridization, Immunohistochemistry

* Correspondence: jsquireinsp@gmail.com
†C. G. Picanço-Albuquerque and T. Vidotto contributed equally to this work.
[2]Department of Pathology and Legal Medicine, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil
[3]Department of Pathology and Molecular Medicine, Queen's University, Kingston, ON, Canada
Full list of author information is available at the end of the article

Introduction

Prostate Cancer (PCa) is the most common solid tumor in men and is the third more common cancer type in the world [1]. Phosphatase and tensin homolog (*PTEN*) is located in 10q23.31 and is the most frequent somatically mutated tumor suppressor gene in a variety of human malignancies (reviewed in [2]). Currently, immunohistochemistry and fluorescence in situ hybridization (FISH) assays of *PTEN* loss in PCa [3–5] have shown high specificity to identify tumors that carry the poor prognostic biomarker.

PTEN inactivation in prostate tumors is found in 20–30% of patients and is associated with adverse outcome [6]. Most importantly, *PTEN* was found to be an independent prognostic indicator of PCa-specific death in both conservatively treated and surgically treated patients and may be employed in the clinical setting as a biomarker for disease stratification (reviewed in [7]). Although PTEN deficiency carries an unfavorable prognosis, the extent of heterogeneity of loss of the PTEN protein has not been objectively studied by imaging methods.

This study was designed to characterize *PTEN* deletions and protein loss in Gleason grade 7 (comprising Gleason score 3 + 4 and 4 + 3) to provide more details of the use of loss of *PTEN* as a biomarker for improved stratification of PCa with intermediate risk. Moreover, we performed whole slide digital image analysis to precisely determine how much intratumoral heterogeneity of PTEN protein loss was present in this study group of Gleason grade 7 tumors.

Methods

Cohort description

We evaluated 43 representative PCa cases collected after radical prostatectomy between 2009 and 2010 for this cohort study. Samples were fixed in formalin, embedded in paraffin and were obtained from the archive of the Pathology Service from the Clinics Hospital of Ribeirão Preto (HCRP). From this cohort, 35 tumors were classified as Gleason score 7 (3 + 4) and eight tumors were classified as Gleason score 7 (4 + 3). Gleason score upgrade refers to patients that presented higher Gleason score from radical prostatectomy specimens when compared to the initial Gleason score from preoperative needle core biopsy. Biochemical recurrence was defined as PSA levels > 0.2 ng/mL during the entire patient follow-up of this cohort. Clinical details of the studied cohort are shown in Additional file 1: Table S1.

TMA construction, immunohistochemistry, and FISH analyzes

For the construction of the TMA, we used the Manual Tissue Arrayer (MTA-1 – Beecher Instruments, Silver Spring, MD, USA) to obtain four 1 mm diameter tumor cores per patient. An initial analysis of whole prostate H&E sections was performed by a pathologist (F. P. S.) to identify two regions with the most prevalent Gleason pattern, one region of the second most prevalent Gleason pattern, and one benign adjacent region for each patient. Then, 1 mm needle cores were obtained to build a tissue microarray (TMA). The histological preparations were made as described previously [4]. Four-Color *PTEN* FISH probes (PTEN del-TECT Four Color FISH probe, Biocare Medical, CA, USA) were used following recently published guidelines [8]. *PTEN* FISH results were analyzed and interpreted as previously reported [4].

For immunohistochemistry, we used the anti-PTEN antibody D4.3 XP® (Cell Signaling, MA, USA) diluted 1:50 in the TMA samples. More details in the methods can be found elsewhere [9]. The immunohistochemistry data were analyzed by two independent pathologists (F. P. S. and T. J.) based on a previously validated dichotomous scoring system [10]. Briefly, TMA cores having intermingling of tumor with PTEN intact with tumor that has PTEN protein loss were classified as PTEN loss when more than 10% of the neoplastic glands had PTEN negative staining. Benign glands and stromal cells presenting positive PTEN staining were used as internal controls. TMA cores showing heterogeneous patterns of PTEN protein expression were classified using the 10% cut off mentioned above. Moreover, TMA cores lacking internal positive controls or with weak IHC staining were classified as inconclusive and were removed from further analyses. Patients were classified as having PTEN loss when at least one TMA core had PTEN protein loss.

Digital pathological analyses of PTEN protein expression

Since PTEN protein expression in PCa is often heterogeneous [11], our study was designed to characterize the presence of focal regions of PTEN protein loss in whole prostate glands. We selected ten patient tumors in which *PTEN* status had previously been determined by FISH based solely on analysis of TMA tumor cores. Based on the results of *PTEN* deletion status in the sampled TMA cores, we chose a total of ten cases from our cohort, of which three cases had *PTEN* homozygous deletions, three cases had *PTEN* hemizygous deletions, and four samples were *PTEN* intact. We obtained whole tumor sections from all selected ten cases and we performed IHC staining with anti-PTEN antibody following the protocol described above. Whole sections were then scanned using the Aperio Biosystems and analyzed with Aperio Scanscope (Leica Biosystems Inc., ON, Canada). The pathologist in our study (TJ) annotated all tumor glands of each section while excluding regions enriched with stromal cells. The annotated tumor regions were then analyzed using an automated algorithm developed in HALO™ (Indica Labs, NM, USA) for PTEN staining

of prostate tumors (see Additional file 1: Supplementary Methods for more details).

Results

Comparison between FISH and IHC for PTEN loss

The analysis of *PTEN* copy number using FISH was conclusive for 95% (41/43) of the patients. Two cases remained inconclusive due to poor quality probe hybridization. For the IHC, the results were conclusive for 90% (39/43) of the patients. FISH analysis of the TMA showed that *PTEN* deletion was present in 18.9% (8/41), with *PTEN* hemizygous deletions in 11.6% (5/41) and *PTEN* homozygous deletions in 7.3% (3/41) of tumors. Moreover, by IHC, we detected PTEN protein loss in 16.3% (7/39) of the patient tumors (Additional file 1: Table S2). Representative images from tumors with PTEN protein loss and *PTEN* deletions are shown in Additional file 1: Figure S1.

The comparison between FISH and IHC showed that both assays were highly concordant, with 90% (27/30) showing protein intact and undeleted *PTEN* by FISH (Additional file 1: Table S3).

Intratumoral heterogeneity of PTEN protein loss

To determine the extent of intratumoral heterogeneity of PTEN protein loss we performed image analysis on ten whole sections in which *PTEN* status had been previously characterized by FISH and IHC in tumor cores. We found that tumors with *PTEN* homozygous deletions had regions with complete loss of PTEN protein expression that was evident in the regions from which the pathologist had previously removed the core for TMA construction (Fig. 1a-c). We found that the three tumors with *PTEN* homozygous deletions have less positively stained cells and more negatively stained cells when compared with tumors with either *PTEN* hemizygous deletions or with *PTEN* intact ($P = 0.01$) (Table 1). These data suggest that digital imaging is able to detect quantitative difference in protein expression.

It can be seen that two (see Fig. 1b and c) of the three tumors with homozygous deletions demonstrate spatially distinct regions of intratumoral heterogeneity for PTEN loss by digital imaging. These likely reflect variation in expression levels of PTEN resulting from clonal regions of tumor that retained a functional copy of the gene. In contrast, the homozygously deleted case shown in Fig. 1a has uniform loss of expression throughout the regions of tumor.

From the three whole sections that presented *PTEN* hemizygous deletions (Fig. 1d-f), two cases presented a tumor region that had glands with uniform moderate and strong PTEN protein expression in regions containing tumor (Fig. 1d and e). In the third case with a *PTEN* hemizygous deletion, we observed intratumoral heterogeneity by image analysis evident as two distinct regions of PTEN protein loss surrounded by an extensive region that had retained PTEN protein expression in the tumor (Fig. 1f).

From the four samples that were classified as *PTEN* intact by FISH (Fig. 1g-j) all tumors had moderate to strong protein expression from regions cored for TMA analysis. However, in one tumor, we observed a complete PTEN protein loss (negative staining) in two small distant regions, both > 5 mm away from the sampled core region (arrows in Fig. 1h). Moreover, as illustrated in Fig. 1h the tumor region adjacent to core 1 was primarily characterized as intraductal carcinoma of the prostate. This case also presented a distant region adjacent to core 2 that had glands with PTEN loss surrounded by PTEN intact glands (moderate and strong staining).

Prognostic impact of PTEN gene and protein loss

We evaluated the effect of PTEN loss by FISH, IHC and both techniques in clinical features of the patients with PCa (Table 2). *PTEN* loss by FISH was associated with aggressive pathological features, such as significant presence of positive surgical margins ($P = 0.04$) and a trend towards extraprostatic extension ($P = 0.09$) (Table 2). We also found that 71% (5/7) of the cases with Gleason score 7(4 + 3) had *PTEN* deletions ($P = 0.001$). Concordantly, 57% (4/7) of patients that had Gleason score upgrade to 4 + 3 had *PTEN* deletions ($P = 0.02$). Furthermore, Log Rank analysis showed no significant association between *PTEN* deletions by FISH and PTEN loss by IHC and biochemical recurrence ($P = 0.65$ and $P = 0.20$, respectively) (Additional file 1: Figure S2).

Discussion

Deciding the best treatment of newly diagnosed grade group 2/3 PCa is still challenging for urologists. In our study, we observed that *PTEN* deletion was detected in 18.9% for the patients of the HCRP cohort. Picanço-Albuquerque et al. (2016) showed that 17.2% of the Gleason 7 patients harbored *PTEN* deletions by FISH. PTEN protein analysis by IHC showed that 16.3% of the patients harbored protein loss, which is highly concordant for another Gleason score 7 cohort study by IHC (18.3%) [9].

Our findings demonstrate that *PTEN* deletions in grade group 2/3 PCa are associated with invasive pathological features and with Gleason score upgrade. A study conducted with 260 Gleason score 3 + 4 = 7 biopsies showed PTEN-deficient tumors are more likely to have non-organ confined disease at radical prostatectomy [12]. Furthermore, several reports have shown significant associations between *PTEN* deletions found in needle core biopsies and Gleason score upgrade [4, 13] and other invasive features found at the time of surgery [14].

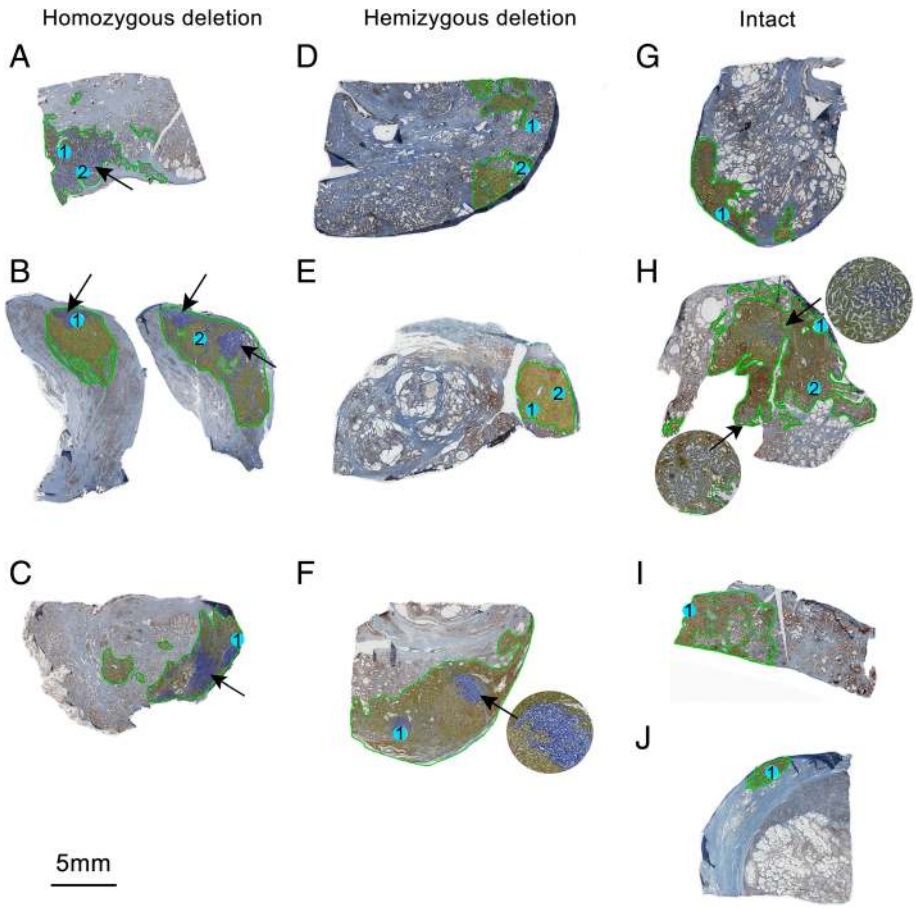

Fig. 1 Digital scans of whole sections of prostate cancer with known *PTEN* gene status. Each tumor had previously had the status of *PTEN* status determined using FISH and IHC analysis of TMA cores (small solid turquoise circles). In each panel, areas containing tumor are outlined with a solid green line. Magenta, yellow and blue pseudocoloring refer to high, moderate, and weak expression levels of the PTEN protein. **a** Gleason 3 + 4 tumor with a large area exhibiting uniformly weak PTEN protein levels (black arrow) and homozygous *PTEN* deletions were found on both cores 1 and 2 by FISH. **b** Gleason 3 + 4 tumor in which core 1 had homozygous *PTEN* deletions by FISH. This core was taken from an area with weak PTEN protein levels (black arrow) that was surrounded by a large area with moderate expression (yellow). Core 2 of this tumor from the contralateral lobe also has moderate PTEN protein levels (black arrow) and both *PTEN* genes were intact by FISH. Two regions (black arrows) either side of core 2 have weak levels of PTEN protein. **c** Gleason 3 + 4 tumor in which core 1 had a homozygous *PTEN* deletions with weak PTEN protein (black arrow) present in about 50% of the encircled area containing tumor. A small patch of tumor to the left also has moderate levels of PTEN protein. **d** Gleason 4 + 3 tumor in which both *PTEN* genes were intact by FISH in core 1 and protein levels were high. Core 2 was had a hemizygous *PTEN* deletion with weak PTEN protein levels. **e** Gleason 4 + 3 tumor with hemizygous *PTEN* gene deletions in both cores 1 and 2 and protein levels were moderate-high in the entire area of tumor. **f** Gleason 4 + 3 tumor with hemizygous *PTEN* gene deletions in core 1 and protein levels were moderate-weak close to the core. A distinct area of weak protein is evident above this region (enlarged circular inset). **g** Gleason 3 + 4 tumor with both *PTEN* gene intact in core 1 and protein levels were moderate in all areas containing tumor. **h** Gleason 3 + 4 tumor with both *PTEN* gene intact in cores 1 and 2 and protein levels were moderate in most of the areas containing tumor. However, two distinct regions arrowed and enlarged as circular insets exhibit weak intratumoral PTEN levels. The tumor region that presents PTEN loss adjacent to core 1 was classified as intraductal carcinoma. Another tumor region adjacent to core 2 shows glands with PTEN loss surrounded by PTEN intact glands (moderate and strong immunostaining) was classified as usual acinar adenocarcinoma (Gleason pattern 3). **i** Gleason 3 + 4 tumor with both *PTEN* gene intact in core 1 and protein levels were moderate in all areas containing tumor. **j** Gleason 3 + 4 tumor with both *PTEN* gene intact in core 1 and protein levels were moderate in all areas containing tumor

Digital image analysis of PTEN protein using ten whole sections identified distinct regions of intratumoral heterogeneity of PTEN protein loss in four of the ten tumors studied. Interestingly, we found that the presence of homozygous deletions of *PTEN* in a representative TMA cores was sufficient to predict that non-sampled region of the tumor will also show PTEN protein loss. In addition, we found that 25% (1/4) of the cases that were *PTEN* intact by FISH also had regions in the whole gland that presented with complete PTEN protein loss. We also found that one tumor with hemizygous *PTEN* gene deletion exhibited distinct regions with complete protein loss, suggesting that a second somatic inactivating mutation or epigenetic loss of function of the remaining intact *PTEN* took place during progression [15].

Table 1 Statistical analysis of ten whole sections stained for PTEN protein

	Homozygous	Hemizygous	Intact	*P*
Cell count	300,079	214,232	164,235	0.43
Area (μm^2)	55,822,500	42,726,067	41,546,478	0.86
Average % + ve cells	57.87	90.20	85.63	0.01*
Average % -ve cells	42.13	9.80	14.38	0.01*

Average values are shown for each group. One-way ANOVA test was employed to determine the associations between *PTEN* copy number by FISH and PTEN protein loss by immunohistochemistry. *$P < 0.05$

Conclusions

Our study demonstrates that PTEN loss in Gleason grade 7 tumors may be heterogeneous and that a systematic analysis of deletion or protein loss of this tumor suppressor gene using a combination of FISH, IHC, and digital imaging can help identify those patient tumors more likely to have a worse prognosis. Our findings also suggest that defining *PTEN* copy number status by FISH in TMA cores may be useful in predicting when whole pathological sections of PCa specimens have PTEN protein loss. Further investigation using the described approach should be carried out in larger cohorts to precisely determine how widespread PTEN inactivation is in PCa.

Table 2 Comparison between the PTEN gene and protein evaluation methods for clinical endpoints of the HCRP cohort

	FISH ($n = 41$)				IHC ($n = 39$)		
	Homo	Hemi	Intact	*P*	Loss	Intact	*P*
Age	62	66	62	0.50	62	62.5	0.77
Time to Biochemical Recurrence[a]	45	87	83.5	0.37	82	84	0.52
Preoperative PSA	15.5	12.4	7.08	0.47	10.9	7.57	0.46
Gleason Score							
3 + 4	2	1	30	0.001*	5	28	0.29
4 + 3	1	4	3		2	4	
Pathological Stage							
2	1	5	27	0.09	4	28	0.08
3	2	0	6		3	4	
Positive Surgical Margins							
No	0	4	23	0.04*	3	22	0.66
Yes	3	1	10		4	10	
Perineural Invasion							
No	2	1	12	0.42	5	10	0.08
Yes	1	4	21		2	22	
Extraprostatic Extension							
No	1	5	27	0.09	4	28	0.08
Yes	2	0	6		3	4	
Vesicle Invasion							
No	2	5	30	0.32	6	30	0.45
Yes	1	0	3		1	2	
Biochemical Recurrence[a]							
No	2	4	25	0.76	5	26	0.24
Yes	1	1	5		2	3	
Gleason Score Upgrade							
No	1	2	15	0.02*	2	16	0.40
3 + 4	1	0	15		3	12	
4 + 3	1	3	3		2	4	

Median per group for time to biochemical recurrence is shown in months. Median of preoperative PSA levels are shown in ng/mL. Median of age at surgery is indicated in years. Kruskal-Wallis test was used to compare continuous variables for FISH data and Mann-Whitney test for IHC data. Categorical variables were compared through Fisher's exact test. *$P < 0.05$. [a]Data available for 40 patients. *FISH* fluorescence in situ hybridization, *IHC* immunohistochemistry, *Homo* Homozygous deletion, *Hemi* Hemizygous deletion

Additional file

Additional file 1: Additional file containing supplementary methods, two figures and three tables. (PDF 666 kb)

Abbreviations

FISH: Fluorescence in situ hybridization; HCRP: Clinics Hospital of Ribeirão Preto; IHC: Immunohistochemistry; PCa: Prostate cancer; PSA: Prostate-specific antigen; PTEN: Phosphatase and tensin homolog; TMA: Tissue microarray

Acknowledgments

We acknowledge the São Paulo Research Foundation (FAPESP) for funding JAS and TV and the National Council for Scientific and Technological Development (CNPq) for funding CGPA. The authors also would like to thank Lee Boudreau for helping with immunohistochemistry staining and Shakeel Virk for scanning the stained TMAs on the Aperio Scanscope and developing HALO algorithm for protein analysis.

Funding

This research was supported by São Paulo Research Foundation (FAPESP) grants no. 2015/09111–5 (to JAS) and no. 2015/22785–5 and 2017/08614–9 (to TV). CGPA was funded by CNPq.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CGPA, TV, DB, RBR, and JAS designed the study. CGPA and CSP performed FISH and IHC experiments. TV performed statistical analysis. TJ and TV performed the digital scoring analysis. FPS and TJ performed pathological analyses. CGPA, TV, and JAS wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethical Committee for Research from the Clinics Hospital of Ribeirão Preto. A consent form was signed by all the patients that provided pathological specimens for this study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

Author details

[1]Department of Genetics, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil. [2]Department of Pathology and Legal Medicine, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil. [3]Department of Pathology and Molecular Medicine, Queen's University, Kingston, ON, Canada. [4]Division of Cancer Biology and Genetics, Queen's Cancer Research Institute, Queen's University, Kingston, ON, Canada. [5]Department of Biomedical and Molecular Sciences, Queen' University, Kingston, ON, Canada. [6]Division of Urology, Department of Surgery and Anatomy, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil.

Received: 20 November 2018 Accepted: 19 December 2018
Published online: 24 January 2019

References

1. Howlader N, Noone A, Krapcho M, Miller D, Bishop K. SEER Cancer Statistics Review, 1975–2013. Bethesda: National Cancer Institute; 2016.
2. Wise HM, Hermida MA, Leslie NR. Prostate cancer, PI3K, PTEN and prognosis. Clin Sci. 2017;131:197–210.
3. Yoshimoto M, Cunha IW, Coudry RA, Fonseca FP, Torres CH, Soares FA, et al. FISH analysis of 107 prostate cancers shows that PTEN genomic deletion is associated with poor clinical outcome. Br J Cancer. 2007;97:678–85.
4. Picanço-Albuquerque CG, Morais CL, Carvalho FLF, Peskoe SB, Hicks JL, Ludkovski O, et al. In prostate cancer needle biopsies, detections of PTEN loss by fluorescence in situ hybridization (FISH) and by immunohistochemistry (IHC) are concordant and show consistent association with upgrading. Virchows Arch. 2016;468:607–17.
5. Hamid A, Gray KP, Huang Y, Bowden M, Loda M, Pomerantz M, et al. Association of low PTEN expression by fluorescence immunohistochemistry (F-IHC) and lethal disease in men with surgically-treated prostate cancer (PrCa). J Clin Oncol. 2018;36:15.
6. Ahearn TU, Pettersson A, Ebot EM, Gerke T, Graff RE, Morais CL, et al. A prospective investigation of PTEN loss and ERG expression in lethal prostate Cancer. J Natl Cancer Inst. 2016;108:1–9.
7. Jamaspishvili T, Berman DM, Ross AE, Scher HI, De Marzo AM, Squire JA, et al. Clinical implications of PTEN loss in prostate cancer. Nat Rev Urol. 2018; 15:222–34.
8. Yoshimoto M, Ludkovski O, Good J, Pereira C, Gooding RJ, McGowan-Jordan J, et al. Use of multicolor fluorescence in situ hybridization to detect deletions in clinical tissue sections. Lab Investig. 2018;98:403–13.
9. Lotan TL, Carvalho FL, Peskoe SB, Hicks JL, Good J, Fedor HL, et al. PTEN loss is associated with upgrading of prostate cancer from biopsy to radical prostatectomy. Mod Pathol. 2014;28:128–37.
10. Lotan TL, Gurel B, Sutcliffe S, Esopi D, Liu W, Xu J, et al. PTEN protein loss by immunostaining: analytic validation and prognostic indicator for a high risk surgical cohort of prostate cancer patients. Clin Cancer Res. 2011;17:6563–73.
11. Yoshimoto M, Ding K, Sweet JM, Ludkovski O, Trottier G, Song KS, et al. PTEN losses exhibit heterogeneity in multifocal prostatic adenocarcinoma and are associated with higher Gleason grade. Mod Pathol. 2013;26:435–47.
12. Guedes LB, Tosoian JJ, Hicks J, Ross AE, Lotan TL. PTEN loss in Gleason score 3 + 4 = 7 prostate biopsies is associated with nonorgan confined disease at radical prostatectomy. J Urol. 2017;197:1054–9.
13. Lotan TL, Carvalho FL, Peskoe SB, Hicks JL, Good J, Fedor HL, et al. PTEN loss is associated with upgrading of prostate cancer from biopsy to radical prostatectomy. Mod Pathol. 2014;28:1–10.
14. Lokman U, Erickson AM, Vasarainen H, Rannikko AS, Mirtti T. PTEN loss but not ERG expression in diagnostic biopsies is associated with increased risk of progression and adverse surgical findings in men with prostate Cancer on active surveillance. Eur Urol Focus. 2017:1–7. Epub ahead of print.
15. Lee Y-R, Chen M, Pandolfi PP. The functions and regulation of the PTEN tumour suppressor: new modes and prospects. Nat Rev Mol Cell Biol. 2018;19:547–62.