A novel 5x multiplex immunohistochemical staining reveals PSMA as a helpful marker in prostate cancer with low p504s expression

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DOI: https://doi.org/10.1016/j.prp.2021.153667

Posted at the Zurich Open Repository and Archive, University of Zurich
ZORA URL: https://doi.org/10.5167/uzh-211117
Journal Article
Published Version

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Originally published at: Rüschoff, Jan H; Stratton, Steven; Roberts, Esteban; Clark, Samantha; Sebastiao, Noemi; Fankhauser, Christian D; Eberli, Daniel; Moch, Holger; Wild, Peter J; Rupp, Niels J (2021). A novel 5x multiplex immunohistochemical staining reveals PSMA as a helpful marker in prostate cancer with low p504s expression. Pathology, Research and Practice, 228:153667.
DOI: https://doi.org/10.1016/j.prp.2021.153667
A novel 5x multiplex immunohistochemical staining reveals PSMA as a helpful marker in prostate cancer with low p504s expression.

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ARTICLE INFO
Keywords:
Prostatic neoplasms
Staining and labeling
ERG3864
Ki-67 antigen

ABSTRACT

The ability to combine multiple immunohistochemical (IHC) markers within a single tissue section facilitates the evaluation and detection of co-expressions, while saving tissue. A newly developed 5x multiplex (MPX) IHC staining of five different IHC markers (Basal cell cocktail (34βE12 + p63), p504s (SP116), ERG (EPR3864), Ki-67 (30/uni2212.texminus9), PSMA (EP192)) was applied on whole sections of n = 37 radical prostatectomies (RPE) including normal and cancerous tissue. Four different colors including brown, magenta, yellow and teal coded for different stainings, whereas magenta was used twice for nuclear Ki-67 and cytosolic / membranous PSMA. The staining of multiplex IHC was compared to single stains of ERG, PSMA and p504s. The proper staining of the basal cell cocktail and Ki-67 could be assessed by internal positive controls in the multiplex staining. The proportion of PSMA and p504s expression revealed a significant correlation between multiplex and single stains (p < 0.01) as well as a concordant staining pattern for ERG (n = 14 prostate cancers were identified ERG positive with both methods). Our proof of concept study demonstrates a robust staining pattern of all five different antibodies with this newly developed 5x MPX IHC. This approach facilitates the recognition of prostate cancer, in particular by adding PSMA in cases with low p504s expression.

1. Introduction

Prostate cancer (PCa) is one of the most common cancers in men and the second leading cause of male cancer mortality in western countries [1].

Examination of formalin-fixed, paraffin-embedded (FFPE) prostate needle biopsies (NBX), transurethral resections of the prostate (TUR-P) and radical prostatectomies (RPE) are routinely investigated in histopathology laboratories. To corroborate the reliable diagnosis of PCa, immunohistochemical (IHC) stainings are widely applied. Among the most commonly used antibodies for PCa work-up are multiplex basal cell cocktails (usually containing CKHMW [34βE12], p63 and p504s [AMACR]) as well as single marker IHCs such as PSMA and ERG. CKHMW [34βE12] and p63 are useful markers of prostatic basal cells in benign glands and prostatic intraepithelial neoplasia (PIN), staining cytoplasm and nuclei, respectively. Invasive PCa in contrast, typically lacks a basal cell layer [2], however intraductal carcinoma typically shows a retained basal cell layer [3], α-methylacyl coenzyme A racemase (AMACR), also known as p504s, has been shown to be frequently expressed in prostatic adenocarcinoma. Additionally, PIN has been found to express p504s, whereas it is nearly undetectable in benign glands [4].

Prostate-specific membrane antigen (PSMA) is a 100 kDa type II transmembrane protein [5] and often upregulated in prostate carcinoma. Furthermore, increased PSMA expression in prostate cancer is associated with higher tumor grade [6,7], disease progression [8] and
has been shown to be a prognostic factor on prostate biopsies [9].

ERG IHC is a reliable surrogate marker for the detection of TMPRSS2-ERG gene fusions [10–12], which is a recurrent and highly specific finding in up to 40–50% of prostate carcinomas [13–15]. Despite technical challenges, combining these markers in multiplexed IHC approaches (e.g. PIN-4™ Cocktail) are increasingly used and further developed. The advantage is the ability to combine multiple IHC markers within a single tissue section. This enables the evaluation and detection of co-expressions also when having only a very limited amount of tissue available, e.g. in core biopsies.

The aim of the present study was to evaluate and validate a newly developed 5x multiplex (MPX) IHC staining, consisting of five markers to define and characterize prostate cancer on a single tissue slide.

2. Materials and methods

2.1. Tissue samples

All 37 formalin-fixed, paraffin-embedded (FFPE) RPE samples originated form patients operated on between 2016 and 2017 and were retrieved from the Department of Pathology and Molecular Pathology, University Hospital Zurich. Written informed consent was obtained from all patients for further use of the tissue and analysis of the corresponding data, approved by the local ethics committee (Kantonale Ethikkommission Zürich; ID numbers: 06/08, 40/08, 2016–02231, 2016–02586). Hematoxylin and eosin stained (H&E) slides of all specimens were re-evaluated by two experienced genitourinary pathologists (NJR, JHR). Tumor stage and Gleason score / Gleason score prognostic grade group (Table 1) were assigned according to the 8th Edition of UICC TNM Classification of malignant tumors [16], and World Health Organization Classification of the Urinary System and Male Genital Organs 2016 / International Society of Urological Pathology criteria [17,18].

All methods were performed in accordance with the relevant guidelines and regulations.

2.2. Multiplex immunohistochemistry

Fully automated multiplexed detection was performed on a DISCOVERY ULTRA system (Roche Tissue Diagnostics, Tucson, AZ). Antibodies were used against basal cell cocktail (BCC) (34E12 + p63), p504s (SP116), ERG (EPR3864), Ki-67 (30-9) and PSMA (EP192) where Ki-67 and PSMA were combined and deposited as a cocktail. Detection was accomplished using the DISCOVERY RUG Chromomap DAB kit (Cat. No. 760–159), DISCOVERY RUG Purple kit (Cat. No. 760–229) and DISCOVERY RUG Yellow kit (Cat. No. 760–239). The teal color was generated using a Ventana research chromogenic substrate and DISCOVERY UltraMap anti-Ms Alk Phos (Cat. No. 760–4312). The DISCOVERY Universal Procedure was used to create a protocol for the 5x MPX IHC. The antibody/chromogen assignments for the multiplex and single stains are detailed in Table 1.

Each marker of the multiplex staining was evaluated separately. ERG expression (yellow) was evaluated in tumor cells using a two-tiered system (positive/negative), whereas endothelial cells and lymphocytes served as an internal positive control. p504s (teal) and membranous PSMA (magenta) was evaluated by estimating the area of tumor covered by staining (% positive tumor area in increments of 5%). For PSMA expression, a three-tiered semiquantitative scoring system (weak (1 +), moderate (2 +) and strong (3 +)) was applied. BCC (brown) and Ki-67 (nuclear magenta) were assessed by internal positive controls (i.e. benign glands and lymphocytes, respectively). Slides were digitalized (NanoZoomer NDP digital slide scanner C9600–12) and viewed using Hamamatsu NDP.view 2.6.8 Software. Evaluation was done by two experienced genitourinary pathologists (NJR, JHR) with a consensus-based decision.

2.3. Single-marker immunohistochemistry

Immunohistochemical single stainings for PSMA (3E6, Dako, 1:25), p504s (13H4, Biologo, 1:30), ERG (EPR3864, Roche, prediluted) were performed on a BenchMark ULTRA staining system, as described previously [11] (Table 2). Evaluation of all three stainings was done analogous to the multiplex staining described in the previous section. Evaluation was done separately and blinded to the results of the MPX staining.

2.4. Statistical analysis

Linear regression was used to compare the percent positive PSMA and p504 (AMACR) tumor area evaluated by multiplex and single marker staining. A P-value lower than 0.05 was considered to indicate statistical significance. Statistical analysis was performed using SPSS® version 25.0.0.0 software (IBM®, Armonk, NY, USA). Graphs were generated using GraphPad Prism v8.

3. Results

3.1. Multiplex immunohistochemistry

The 5x multiplex staining was coded by four different colors as described in Table 2. PSMA and Ki-67 showed a magenta cytosolic / membranous and nuclear stain, ERG a yellow nuclear, basal cell cocktail a combined brown membranous and nuclear and p504s a teal cytosolic stain (Fig. 1A-B).

3.2. PSMA expression

PSMA expression was noted in all 37 (100%) prostate adenocarcinoma specimen (Fig. 1A-B) with a range from weak to strong membranous expression (1 +–3 +). The cytosolic PSMA expression was not evaluated because of a frequent overlay with the cytoplasmic p504s staining and the nuclear Ki-67 staining using the same color. Heterogeneous PSMA expression could be observed in 28 of 37 cases (76%). Of these, all 28 cases (100%) showed PSMA negative areas (from 10% to 80%).

3.3. p504s expression

Cytoplasmic tumoral p504s expression was seen in 36 of 37 (97%) cases (Fig. 1A-B). Heterogeneous expression could be observed in 22 of 37 cases (59%). 14 of 37 cases (38%), showed a diffuse expression in 100% of the tumor cells, whereas the heterogeneous pattern of the 22 cases showed an expression between 10% and 90% in the tumor cells. Seven out of 37 cases (19%) showed an expression of < 50% in the tumor cells.

3.4. ERG expression

Nuclear ERG expression (Fig. 1A-B) was found in 14 of 37 cases (38%). In all negative cases (n = 23, 62%), a positive staining in

Table 1

Clinicopathological cohort characteristics (n = 37).

| Age at diagnosis (years) | 63.86 ± 6.32 |
|--------------------------|--------------|
| T-Stadium                |              |
| pT2a (n = 2)             | 5.4%         |
| pT2b (n = 2)             | 5.4%         |
| pT2c (n = 22)            | 59.5%        |
| pT3a (n = 6)             | 16.2%        |
| pT3b (n = 5)             | 13.5%        |
| Prognostic Grade Group   |              |
| Group 2: Gleason 3 + 4 (n = 6) | 16.2% |
| Group 3: Gleason 4 + 3 (n = 13) | 35.1% |
| Group 4: Gleason 4 + 4 (n = 9) | 24.3% |
| Group 5: Gleason 4 + 5, 5 + 4 (n = 9) | 24.3% |
endothelial cells and lymphocytes could be observed as an internal control.

3.5. Basal cell cocktail and Ki-67 expression

For the Basal cell cocktail, basal cells of benign glands and for Ki-67 proliferating lymphocytes showed a consistent positive internal staining in all multiplex IHC stained samples ($n = 37$, 100%; Fig. 1A-B).

3.6. Single-marker immunohistochemistry

The single-marker immunohistochemical stainings showed a brown membranous, nuclear or cytoplasmic expression, dependent on the antibody used.

3.7. PSMA expression

PSMA expression was noted in all 37 (100%) prostate adenocarcinoma specimen (Fig. 1C) with a range from medium to strong membranous expression (2–3+). At least weak additional cytosolic PSMA expression was observed in all cases. Heterogeneous PSMA expression could be observed in 22 of 37 cases (59%). Of these 22 cases, 15 cases (68%) showed PSMA negative areas from 5% up to 80%.

3.8. p504s expression

Cytoplasmic tumoral p504s expression was seen in all 37 (100%) specimen (Fig. 1D). Heterogeneous expression could be observed in 29 of 37 cases (78%). Eight cases (22%) showed a diffuse expression in 100% of tumor cells, whereas the heterogeneous pattern of the 29 cases showed an expression between 10% and 90% in tumor cells. Six out of 37 cases (18%) showed an expression of < 50% in tumor cells.

3.9. ERG expression

Nuclear ERG expression was found in 14 of 37 cases (38%; Fig. 1E). In all of the ERG-negative cases, a positive internal control consisting of endothelial cells and lymphocytes could be observed ($n = 23$, 62%).

3.10. Correlation of PSMA, p504s (AMACR) and ERG expression evaluated by multiplex and single marker immunohistochemistry

The proportion of tumoral PSMA and p504s (AMACR) expression assessed in the multiplex and single marker immunohistochemistry was correlated respectively. A significant correlation (each $p < 0.01$) for both markers ($R^2 = 0.5386$ for PSMA and $R^2 = 0.8452$ for p504s) could be detected (Fig. 2). Seven cases showed a discrepancy of ≥ 30% tumoral PSMA expression between the multiplex and single staining. In all of these cases, the more extensive PSMA expression was evaluated in the

Table 2

| Antibodies          | Clone   | Dilution | Source          | Color          | Staining pattern          | Type  |
|---------------------|---------|----------|-----------------|----------------|---------------------------|-------|
| Basal cell cocktail | 34/E12  | predilute| Ventana/Roche   | brown          | membranous/nuclear        | MPX   |
| p504s               | SP116   | predilute| Ventana/Roche   | teal           | cytosolic                 | MPX   |
| ERG                 | EPR3864 | predilute| Ventana/Roche   | yellow         | nuclear                   | MPX   |
| Ki-67               | 30–9    | predilute| Ventana/Roche   | magenta        | nuclear                   | MPX   |
| PSMA                | EP192   | 1:15 in Ki-67 | Ventana/Roche | magenta        | cytosolic membranous      | MPX   |
| p504s               | 1H14    | 1:30     | Biologo         | brown          | cytosolic                 | Single|
| ERG                 | EPR3864 | prediluted| Ventana/Roche   | brown          | nuclear                   | Single|
| PSMA                | 3E6     | 1:25     | Dako            | brown          | cytosolic membranous      | Single|

Fig. 1: High magnification of a representative 5x multiplex staining. (A) The brown basal cell cocktail shows nuclear / membranous staining of the basal cells in the benign glands (brown arrowhead middle). Colored in magenta, the membranous PSMA expression (arrowhead upper middle) and nuclear Ki-67 expression (arrowhead middle left) are visualized. The yellow color shows nuclear ERG expression in the tumor cells (arrowhead upper right) and a positive internal control in endothelial cells (arrowhead middle left). The teal color illustrates cytosolic p504s expression (arrowhead lower right). (B) shows representative hematoxylin & eosin staining of the malignant (upper half) and benign glands (lower middle) in this specimen. In (C) the single PSMA staining is depicted showing enhanced expression in the carcinoma complexes. (D) shows cytoplasmic expression in the between benign glands infiltrating carcinoma cells (middle), whereas (E) depicts nuclear ERG expression in the aforementioned carcinoma cells. Scale bar = 250 µm.
single staining. Furthermore, all of these cases showed a diffuse p504s expression of at least 80% (Fig. 3 A-D). The expression patterns for p504s were similar on both stainings with only minor differences (mean difference 8.65% ± 1.63%). The single p504s negative case on the MPX staining showed only 10% p504s expression on the single staining. All cases with an expression of p504s in < 50% of the tumor cells had diffuse PSMA expression of at least 70% up to 100% (Fig. 4 A-D). ERG expression was detected by both IHCs in all 14 cases.

4. Discussion

Multiplex immunohistochemical stainings have emerged as an increasingly used tool in routine histopathology diagnostics. In the present proof of concept study, we evaluated a newly developed 5x multiplex IHC staining for prostate carcinoma. We corroborated a robust staining of all five multiplex markers (PSMA, ERG, Basal cell cocktail, p504s, Ki-67), when compared to single-marker IHC stainings and MPX internal controls. In the last several years, different multiplex IHC stainings for diagnostics have been developed. These assays facilitate the acquisition of more information from a single tissue sample, as well as spatial arrangements and co-localizations of proteins of interest within the tissue architecture. Some examples encompass e.g. the PIN-4™-Cocktail for prostate adenocarcinoma or the ADH-5 for breast carcinoma. The PIN-4™-Cocktail includes p504s, p63 and CKHKMW (manufacturer’s data).

The present 5x multiplex staining adds PSMA, ERG and Ki-67. PSMA and ERG can thereby be used for an easier recognition of carcinomatous patterns. As we depicted in Fig. 2, some prostate adenocarcinomas show
only scarce expression (<50%) of p504s (~20% of the cases in our cohort), so adding PSMA in this 5x approach may substantially help to recognize the malignant glands. All of the latter mentioned cases in our cohort showed a diffuse PSMA expression of 70–100% in tumor cells. The detection of ~40% ERG positive cases in our cohort is in line with previous data [12,14].

We observed a significant correlation between the tumoral PSMA expression in the multiplex and single stain. However, there were certain cases with obviously lower percentages of expression in the multiplex compared to the single staining. This can be explained in two ways: First, due to technical reasons we did not use the same antibody in both approaches, which could explain some differences. The PSMA 3E6 clone, used in the single staining, detects the 57–134 amino acid region of the extracellular PSMA domain, whereas the multiplexed EP192 antibody detects an extended range from the 1–150 amino acid region at the N-terminus (each manufacturer’s data). Second, one limitation of the multiplex staining is an overlay of different staining patterns. p504s shows a cytoplasmic staining, which can interfere with cytoplasmic PSMA staining and also if strongly expressed might veil membranous PSMA. All seven cases, which showed a discrepancy in tumoral PSMA expression (between single and multiplex staining) of at least 30% revealed a very diffuse p504s expression of at least 80% (Fig. 3). Despite the use of different clones, p504s expression showed a high concordance between both staining methods. This is consistent with results of a previously published abstract [19]. Interestingly, the cases with scarce p504s expression (<50%) showed a diffuse membranous PSMA expression (Fig. 4). There is a clear advantage combining these markers in order to capture at least one of these markers typically overexpressed in adenocarcinoma to help confirm the diagnosis. While this could be done using two separate single stains, the amount of specimen available from current prostate cancer biopsy techniques is limited. Therefore, the need to use further markers for defining adenocarcinoma while saving tissue supports the use of multiplexed IHC. We did not stain for basal cells or Ki-67 separately because benign tissue on each slide was considered sufficient to validate the basal cell loss in the tumorous tissue. Furthermore, as Ki-67 is known to be very heterogeneously expressed even within same slide [20], a comparative quantitative approach would probably not be meaningful. Ki-67 was added as a potential future prognostic marker [21], similar to breast cancer [22], but will certainly need another validation approach.

In comparison to newer multiplex approaches, the number of antibodies which can be used by enzyme-linked multiplex IHC assays is limited [23]. However, this style of assay is easily applicable in a daily routine clinical setting, since enzyme-linked IHC is common practice in pathology laboratories and multiplexed IHC is fully automatable.

5. Conclusion

In the present study, we evaluated a novel 5x multiplex IHC for prostate cancer which showed a robust simultaneous staining of all antibodies used. These results make this IHC staining a powerful tool facilitating the recognition of prostate cancer, in particular by
combining PSMA and p504s.

Ethics approval

Written informed consent was obtained from all patients for further use of the tissue and analysis of the corresponding data, approved by the local ethics committee (PB_2016–02586, KEK-StV-Nr: 40/08 and KEK-ZH-Nr. 06/08, BASEC 2016–02231).

Declarations

Funding: There was institutional research funding from F. Hoffmann - La Roche AG.

Conflicts of interest/Competing interests

SS, ER, SC, NS are employed by Ventana Medical Systems, Inc. NJR discloses an advisory board function including receipt of honoraria from F. Hoffmann - La Roche AG. HM has served in advisory board and received honoraria from F. Hoffmann - La Roche AG. JHR, PJW, DE, CDF declare no conflict of interest.

Availability of data and material

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

Code availability

Not applicable.

Additional declarations for articles in life sciences journals that report the results of studies involving humans and/or animals. Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

CRediT authorship contribution statement

Study concept and design: PJW, NJR, HM, Data collection: JHR, NJR, Data analysis and interpretation: JHR, NJR, Statistical analysis: JHR, NJR, Manuscript writing: JHR, NJR, SS, Critical revision of the manuscript for important intellectual content: JHR, SS, ER, SC, NS, CDF, DE, HM, PJW, NJR, Supervision: NJR, PJW, HM.

Acknowledgements

We thank Susanne Dettwiler, Fabiola Prutek and Christiane Mittmann for tissue processing and immunohistochemical stainings.

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