Tissue protection immunohistochemistry – an innovative method to improve diagnosis accuracy in prostate cancer

Imunohistochimia cu protecție tisulară - o metodă inovativă de a îmbunătăți acuratețea diagnostică în cancerul de prostată

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Abstract:

Prostate biopsy is the standard procedure used in the diagnosis of prostate cancer. Immunostains, additional to standard stains, are sometimes necessary for correct interpretation and classification of atypical, minute lesions. However, immunohistochemistry becomes noncontributory in the situation of loss of suspected foci after repeated sections from the paraffin block. This situation, which is not at all infrequent in prostate pathology, led to the design of special techniques that make immunostaining possible. We further present the results of an innovative technique of tissue protection immunohistochemistry which allows the immunostaining on an initially H&E-stained slide. Unlike other methods described before, this technique does not require H&E destaining prior to session and did not involve saving intervening unstained sections for possible immunohistochemical examination. The great advantage of tissue protection immunohistochemistry is that it allows simultaneous examination of H&E and immunostaining on the same slide. It only requires that the original H&E-stained sections to be placed on charged slides.

Keywords: *technique, prostate biopsy, diagnosis, immunohistochemistry*

Rezumat:

Puncția biopsie prostatică reprezintă tehnica standard utilizată în diagnosticul cancerului de prostată. Adițional colorației standard, colorațiile suplimentare IHC sunt uneori necesare pentru interpretarea şi încadrarea corectă a leziunilor atipice limitate cantitativ. IHC devine, însă, inutilă în situația epuizării focarului suspect de malignitate la secțiuni repetate din blocul de parafină. Această situație deloc rară în patologia prostatică a condus la conceperea unor tehnici speciale prin care marcajul IHC să poată fi efectuat. Prezentăm

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în continuare rezultatele unei tehnici inovatoare de IHC cu protecţie tisulară care permite imunomarcajul unei lame iniţial colorate uzual. Spre deosebire de alte metode descrise anterior, această tehnică nu necesită decolorarea prealabilă a secţiunii tisulare şi nici nu implică salvarea de secţiuni intermediare necolorate. Marele avantaj al IHC cu protecţie tisulară este faptul că permite examinarea concomitentă a colorației IHC şi a colorației uzuale pe aceeaşi lamă. Singura condiţie necesară este aceea ca secţiunile colorate H&E să fie montate de la început pe lame incărcate.

Cuvinte cheie: *tehnică, biopsie prostatică, diagnostic, imunohistochimie.*

Introduction

According to the American Cancer Society's latest statistics, prostate cancer is the most frequent malignancy and the second cause of death by neoplasia among males in USA [1]. Usually the diagnosis of prostate cancer is established by microscopic examination of prostate biopsy (PB) specimens, performed in patients with an elevated level of prostate specific antigen (PSA). The significant increase of the number of biopsies performed in the last 10-15 years confronted the pathologists with the need to establish an accurate diagnosis on very small tissue specimens. In such cases, when the "suspicious" focus is reduced to only a few atypical glands, often located on the edge of the specimen, immunohistochemistry (IHC) is mandatory. The most useful markers are those for basal cells (the most used being high molecular weight cytokeratin) in conjunction with markers of malignancy (alpha-methylacyl-CoA racemase also known as AMACR/P504S). These markers are a really helpful tool in specifying the benign versus malignant nature of the lesion. However, IHC becomes useless when the suspected lesion is not present anymore on deeper sections from the paraffin block. We present the results of an original method of tissue protection immunohistochemistry (TPI) which allows the examination of both H&E and immunohistochemical stained sections on the same slide. The procedure does not require previous H&E destaining or the saving of intervening unstained sections. The only condition is to place the initial sections on adhesive slides.

Material and method

Prostate biopsy specimens were fixed in 10% formalin buffered solution, routinely processed into paraffin blocks, then cut at 3-4 μm thickness. In addition, sections were placed on Dako Silanized Slides, Code 3003, and then H&E-stained and examined at microscope. The cases suitable for immunostaining were selected after identification of the minute suspicious lesions. The immunohistochemical study used the following primary antibodies: HMWCK (5 cases), AMACR/P504S (5 cases), p63/AMACR cocktail (5 cases) (table 1).

The TPI protocol, first described by Kubier & Miller in 2002 [2] and reproduced with the permission of the authors, included the following steps:

1. Marking of the sections that included small suspicious lesion with a diamond pencil on the opposite side of the slide.

2. Removal of the cover slip through incubation in xylene.

3. Covering the unmarked section with liquid mount medium, leaving uncovered the section requiring immunostaining.

4. Placing the slides on a horizontal plane and incubating them overnight in the 56^0C incubating oven, to form a solid film that covers the "protected" H&E section.

5. Immunostaining, using the routine technique in our laboratory, as follows:

- *For single antibody immunostaining:*
  - Blocking the endogenous peroxidase activity with 0.3% hydrogen peroxide for 10 minutes.
  - Antigen unmasking using HIER meth-

od (Heat Induced Epitopes Retrieval - this step also allowed destaining of the uncovered section).
  - Incubation with primary antibody for 10 minutes (prediluted) or 30 minutes (concentrate).
  - Incubation with secondary HRP-conjugated antibody for 20 minutes.
  - Immersion with 3, 3' - diaminobenzidine tetrahydrochloride (DAB) solution for 5-15 minutes.
  - Nuclear counterstaining with hematoxylin.
  - Dehydration of sections through successive baths of alcohol.
  - Cover slip mounting.
- *For antibody cocktail (P504S/AMACR + p63) immunostaining:*
  - Blocking the endogenous peroxidase activity with 0.3% hydrogen peroxide for 10 minutes.
  - Antigen unmasking using HIER method (Heat Induced Epitopes Retrieval – this step also allowed destaining of uncovered section).
  - Slides incubation with AMACR/p63 cocktail for 30 minutes.
  - Application of secondary antibody (HRP-conjugated) for 30 minutes.
  - Immersion with 3, 3' - diaminobenzidine tetrahydrochloride (DAB) solution for 5-15 minutes.
  - Nuclear counterstaining with hematoxylin.
  - Dehydration of the sections.
  - Cover slip mounting.

Results

At the end of TPI procedure, the slides contained the H&E-stained section and also the immunostained one (*Figure 1*). All 15 cases that were processed in this manner were concomitantly "routinely" immunostained and the results were carefully examined by two pathologists. The comparison between the TPI immunostains and the usually performed immunostains showed very similar signal intensity in those cases where single primary antibodies (HMW-CK, AMACR) were used (*Figure 2*). In the particular situation of AMACR/p63 cocktail immunostaining, 3 of 5 cases showed a slight

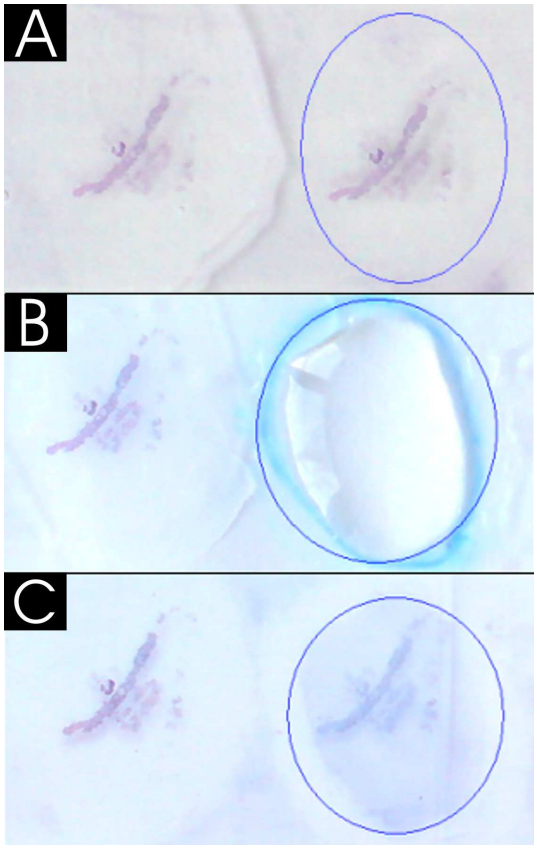

Figure 1. Gross appearance of the slide during different steps of TPI processed on a prostate biopsy specimen.
A. The liquid cover glass medium protects the left section during TPI, leaving uncovered the right one which requires immunostaining. B. The right section is completely destained following the HIER method, unlike the left one which is unaltered, protected by the mounting medium. C. Final gross appearance of TPI stained slide. It is obvious that the protected H&E-stained section is unaltered, while the right section is immunostained and counterstained with hematoxylin (it has a bluish color).

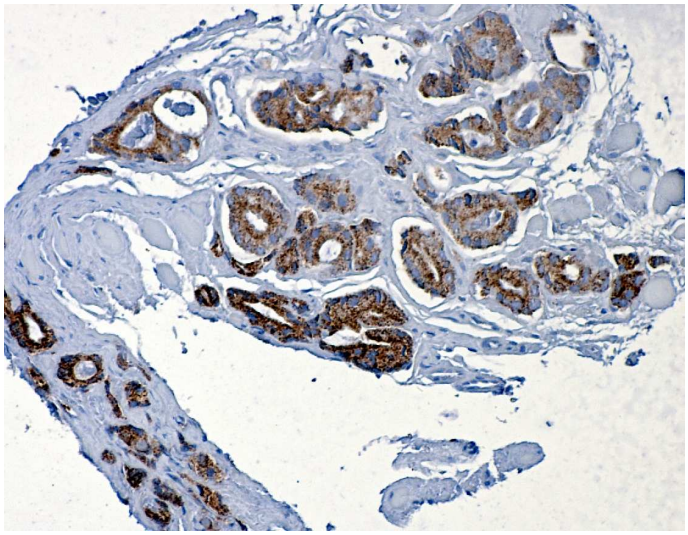

Figure 2. Microscopic appearance of tissue protection immunohistochemical AMACR stain demonstrating intense positive, granular, circumferential signal in the cytoplasm of malignant cells in the single suspect focus located on the edge of a PB section.

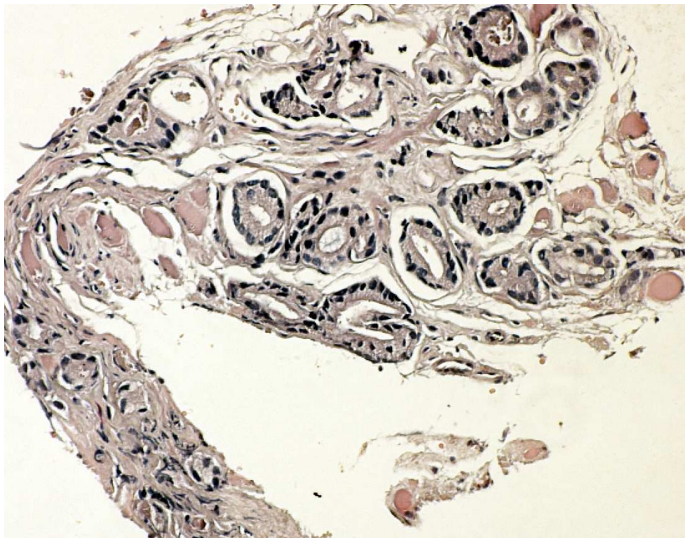

Figure 3. H&E-stained section preserved during TPI – microscopic appearance.

chromogen spreading from the basal cells nuclei marked with p63 into the cytoplasm of secretory cells, but only in a focal manner. Nevertheless, the sections were still satisfactory for interpretation. As a matter of fact, this was an inconvenient that was also present in the "routinely" immunostained slides. The discrimination between DAB spreading and true AMACR signal in secretory cells was made possible by evaluating the pattern of staining: very pale, focal, not homogenous, rather basally distributed in the case of chromogen diffusion, but granular, circumferential, more intense apically, homogenous/heterogeneous in true AMACR reaction. The immunostaining results were interpreted in the context of the morphological criteria, as we are usually doing.

The H&E-stained sections were optimal for interpretation as well. Some of them were perhaps slightly paler then before TPI, but still allowed an easy interpretation (*Figure 3).*

We had no difficulties concerning the tissue adhesion during TPI procedure using silanized slides.

Discussions

The particularly high incidence of prostate cancer has led to the implementation of very active screening methods, consisting in determining the level of serum PSA and digital rectal examination (DRE). At present PB is the only available method to diagnose prostate cancer [3,13]. The last 15 years were known as "PSA screening era" in the field of prostate pathology [4-6,7-9] and brought significant changes in PB sampling, interpretation and reporting. The PB sampling technique itself has evolved from 1-2 cores/specimen to 6, then 10-12 cores/specimen [4,9,10]. Consequently, more often the pathologists face "the challenge" to diagnose prostate cancer on very small biopsy specimens, which contains only minute foci suspicious for malignancy detected following a high serum PSA level [3,4,6,8-13]. This condition first led to the definition of new entities and secondly, to the increased use of IHC techniques to establish an accurate diagnosis.

The newly defined entities includes *atypical small acinar proliferation suggestive but not diagnostic of malignancy* (ASAP) and *minimal/limited/minute carcinoma*. Discrimination of these entities is very important because it entails different therapeutic attitudes, with great impact on patients' lives: repeating the biopsy in 3 to 6 months if the diagnosis is an ASAP lesion [3-6,12,13,15], or radical prostatectomy associated with hormone and/or radiotherapy, depending on stage, if minimal cancer is ascertained [5,15]. Therefore establishing an accurate histopathological diagnosis becomes mandatory.

The precise definition of the minimal cancer is still non-standardized and in the opinion of various authors it may represent either a focus of prostate adenocarcinoma of less than 1 mm or less than 5 % of the biopsy area, or a focus containing a minimum number of 4-5 atypical glands [3,5,8,11].

Although significant efforts have been made in the standardization of prostate cancer morphological diagnosis, including the definition of major and minor criteria [4-6,8,10,11,14], the cases that require additional IHC techniques are not rare [4,6,11-13]. The IHC mainly concerns the absence of basal cell layer revealed with specific antibodies (the most used being HMWCK/34ßE12) [3-6,11,13] in conjunction with overexpression of AMACR/P504S protein, a marker of malignancy in prostate cancer [3-5,11-13]. Besides classical methods that use a single primary antibody, antibody cocktails (AMACR/p63) showed enhanced diagnostic utility by simultaneous marking of two different antigens [5,11]. The particularity of PB specimens, including their small size and the limited area of the suspicious lesions caught through an active screening, predispose to loss of the suspicious areas after repeated sections from the paraffin blocks [5,10,13]. Various techniques have been described to eliminate that risk. Thus, Green and Epstein [16] compared the usefulness of saving intervening unstained sections with new sections made from the paraffin blocks in a group of 94 prostate biopsies. They performed HMWCK immunostaining in both conditions and for 31 cases, the lesion identified on the intermediate sections was not preserved in subsequent sections. Thus, a precise diagnostic could be established in 31 of the 94 biopsies (32.98%), due to saving of intervening sections. On the same topic Hameed & Humphrey [17] recently published a new paper supporting the practice of saving unstained sections of prostate biopsy specimens for potential IHC evaluation.

Another approach belongs to Dardik and Epstein [18] who marked with HMWCK 105 prostate biopsy specimens with minute atypical lesions that were initially H&E-stained and then destained. Working in this manner, they established a definite benign or malignant diagnosis in 58% of the cases. The major encountered problem was that the lesion fell off the slide during processing in 9% of the cases, and in other 19 percent the immunostain failed. Using charged slides was not a solution for this problem.

Tissue transfer technique was initially described by Miller & Kubier [19] as a purpose of additional immunohistochemical staining on smear cytology unaccompanied by tissue blocks. Hameed and Humphrey [20] have customized this method for prostate needle biopsies, performing tissue transfer from H&E-stained slides on silanized slides, regarding p63/AMACR cocktail immunostainig. The results were encouraging, with minimal tissue loss (only 2 of 71 sections [3%] fell off the slides during processing). The success of this method is limited by the H&E-stained "slide aging", this technique being especially recommended for sections not older than 1 month. The latter showed a staining intensity very similar to that seen in immunostained sections recently recut from the block with the same p63/AMACR antibodies cocktail.

The TPI technique that we tested in our laboratory was also described by Miller & Kubier [2] and involves immunostaining of an initially H&E-stained slide, without prior destain-

Table 1. Characteristics of primary antibodies used in our study

Antibody	Clone	Provider	Pretreatment	Dilution
AMACR/P504S	13H4	Dako	HIER - 99°C, Dako Target Retrieval, pH 9	RTU/ 1:200
HMWCK	34βE12	Dako	HIER - 99°C, Dako Target Retrieval, pH6	RTU
AMACR/p63 cocktail	AMACR– polyclonal, p63 - clone 4A4	Abcam	HIER - 99°C, Dako Target Retrieval, pH 6	1/80

ing. The method requires the use of charged (plus or lysine coated) slides that provide a better adhesion, minimizing the risk of losing the section during HIER technique. TPI is distinguished by ease of use, high reproducibility and good price/efficiency ratio. There is no need to save intervening unstained sections, does not require prior slides destaining, it does not imply high costs. It only requires a single adhesive slide, which allows simultaneous optimal examination of the H&E-stained section and of the immunostained one. First tested for HMWCK immunostaining, and investigated upon in our laboratory for other primary antibodies (AMACR, AMACR/p63 cocktail), the technique is suitable for any primary antibody on any type of small biopsy. In the field of prostate pathology, the great benefit of this method is that it substantially reduces the number of equivocal diagnoses on biopsy specimens, with concomitant decrease of the number of repeated biopsies, cost and discomfort associated with them as well. Its usefulness is even more substantial in our laboratory, as we process biopsy specimens using a spring-loaded 16 Gauge (versus 18 Gauge needle biopsy used by other centers outside the country). TPI remains a valuable method in the field of other pathology, suitable for various types of biopsies: breast, kidney, etc.

As a conclusion, TPI is an innovative, less expensive, reliable and easily reproducible method allowing an accurate diagnosis of one of the most important public health problem of our society, the prostate cancer.

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Conflicts of interest**:** None declared.

List of abbreviations:

PB - prostate biopsy
PSA - prostate specific antigen
HMWCK - high molecular weight cytokeratin
AMACR/P504S - alpha-methylacyl-CoA racemase
IHC - immunohistochemistry
TPI - tissue protection immunohistochemistry
DRE - digital rectal examination
ASAP - atypical small acinar proliferation suggestive but not diagnostic of malignancy
DAB - 3, 3' - diaminobenzidine tetrahydrochloride
HIER - heat induced epitopes retrieval
HRP - horseradish peroxidase

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