High throughput processing and analysis of prostate core biopsies for research applications[1]

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Abstract

Introduction: Lack of sufficient prostatic tissue samples has traditionally hampered prostate cancer research. Existing initiatives aimed at creating high quality, well characterized fresh or formalin-fixed, paraffin embedded tissue banks are mainly based on radical prostatectomy specimens. Research projects based on "surplus" tissue slides resulting from prostate biopsies would be highly desirable but until very recently no histological technique proved practicable and/or cost-effective.

Materials and Methods: A multiplex grooved matrix (Biopsy Chip) was used for aligning the specimens by the urologist who collected core biopsies with an ultrasound-guided biopsy gun from 30 consecutive patients suspected of prostate cancer. Haematoxylin-eosin staining was performed at four levels, 50 micrometers apart. For every level, four additional sections were saved for special stains. Congo Red, Toluidine Blue at pH 1.99, 2.5 and 3.2, Hucker-Twort Gram stain, Periodic Acid-Schiff and Tarpley's techniques were employed for selective staining of amyloid, mast cells, eosinophils, bacteria and mucosubstances, respectively. Aggregate length of cores on slides, sample distribution statistics, presence of corpora amylacea, bacteria, mast cells and eosinophils were examined by three independent observers.

Results: The biopsies did not show curling during processing, remained properly oriented, and maintained intact tissue relationships even when the cores were fragmented. The aggregate length of the cores on slides was 130.6 ± 24.7 mm (mean ± SD, min: 79 mm, max: 170 mm). Serial sectioning was greatly facilitated by the matrix employed and at least 50% of the bioptic material was saved in the paraffin block. Reporting of the histopathological findings was made in a quantitative fashion, and spatial representations of the neoplastic tissue were recorded. Mast cells, eosinophils, Corpora Amylacea, intraluminal mucin, calculi and intracytoplasmatic granules were identified in areas adjacent to prostate cancer, a lot more frequently than in benign hyperplasia or in cancerous acini.

Conclusions: The multiplex method of harvesting, processing and reporting of prostate biopsies is an easily applicable, cost-effective method, provides tumor location information and creates consistent duplicate arrays for analysis and research purposes. Unlike other methods, it can be used efficiently for parallel quantitative analysis of various biopsy samples.

Keywords: Biopsy Chip, Corpora Amylacea, histochemistry, imunohistochemistry, prostate cancer

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Introduction

In recent years, transrectal ultrasound-guided (TRUS) systematic needle biopsy [1] has emerged as a the gold standard in prostate cancer diagnosis, to such an extent that statistical performance values (sensitivity, specificity, positive and negative predictive values) of all other diagnostic tests, like digital rectal examination (DRE) or prostate-specific antigen (PSA) are computed according to the outcome of biopsy examination [2]. The emerging consensus, based on clinical trials, ex vivo biopsy approaches, as well as various computer simulations and mathematical models, is to take at least 10 biopsy cores, focusing the biopsies laterally at the base, mid-gland, and apex of the prostate, with mid-lobar biopsy cores at the base and apex [3], and adjusting the number of cores taken according to prostate volume and age of the patient [2]. Some investigators advocate even more aggressive biopsy schemes, with more than 12 cores [4], up to a saturation biopsy (≥ 20 cores), especially on repeat biopsies, reporting even higher cancer detection rates [5].

Despite the obvious need for multiple biopsy cores per patient, due to high expenses and limited resources the number of sections was reduced to the minimum required for an acceptable precision of the diagnosis and/or the maximum resources available within an institution. The current paradigm was that this methodology is the best one can do. Recently, our group introduced into clinical practice a new technique, based on a patterned grooved matrix for the simultaneous processing and analysis of multiple biopsies [6,7]. Since its introduction, the Biopsy Chip was used for the collection, analysis of over 2000 patients in a number of Romanian hospitals. Preliminary data on its impact showed that this technique was easy to implement, the learning curve was fast and the total costs in consumables and manpower (from the biopsy procedure to the final pathology report) per individual core were significantly reduced (from 4.75 to 3.07 RON and from 11.30 to 3.89 minutes) [8]. The yield of biopsies on slides increased (from 52.5% to 66.4 %). The volume of diagnostic slides and paraffin blocks to be archived (for 10, respectively 30 years) was reduced with almost one order of magnitude while the procedure for retrieval of paraffin blocks and tissue slides was significantly simplified. One important finding was that the multiplex technique is making available 3-4 times more tissue for research applications.

Interestingly, the need for ancillary tests for arriving at a definitive diagnostic (i.e. immunohistochemistry) was reduced from 8.93% to 5.32%. This surprising outcome presented us with the opportunity of using the surplus slides (unstained slides kept for a number of weeks and traditionally discarded after the definitive diagnostic is confirmed) for research applications. However, since after a few months of storage, most of the antigens would oxidize and as a result immunohistochemistry assays become impracticable [9,10] only a few select histochemical stains and DNA-based molecular techniques [11] might be of interest for the researcher. Traditionally, the major criteria used to establish the diagnosis, based on the microscopic appearance of slides stained using haematoxylin and eosin are: infiltrative glandular growth pattern, the absence of basal cells and nuclear atypia in the form of nucleomegaly and nucleolomegaly [12]. In difficult cases, basal cell absence may be confirmed by immunohistochemical stains for high molecular weight cytokeratins (marked with antibody 34βE12) or p63, which are basal cell markers. Minor criteria include intraluminal wispy blue mucin, pink amorphous secretions, mitotic figures, intraluminal crystalloids, adjacent high grade prostatic intraepithelial neoplasia, amphophilic cytoplasm and nuclear hyperchromasia.

Other topographical or histochemical staining methods are rarely employed, not only because it would be too expensive, but mainly because of the limited amount of tissue available.

Materials and methods

Inclusion criteria for participants in this study were based on clinical (positive DRE) or biochemical (PSA ≥ 2.5 ng/ml) suspicion of prostate cancer. Informed consent forms in agreement with FP7 guidelines (Directive 2001/20/EC, the recommendations of the Ethics Review Sector of the European Commission and of WHO ERC Ethics Review Committee) were signed by each patient attending the trial. An authorization was issued by the Ethics Committee of "Dr. Carol Davila" Central Military Emergency University Hospital.

Prostate samples were harvested via systematic TRUS-guided biopsy using a Bard® MAGNUM® biopsy gun (Bard Biopsy Systems Inc., Tempe, AZ, USA) and 18G x 20 cm core tissue biopsy needles. Local anesthesia was achieved by intrarectal application of 2% lidocaine gel, without periprostatic nerve block. In selected cases antibiotic prophylaxis was used, consisting in single dose fluoroquinolone derivatives or amoxicillin administered before the procedure. Up to 12 biopsy

cores per patient were harvested and placed in a Biopsy Chip™ matrix as follows:

1. the biopsy core was exposed at the tip of the needle (using the appropriate method according to the type of biopsy gun employed);
2. the biopsy core was deposited in a groove of the matrix by a gentle in-axis rotation of the needle;
3. the loaded matrix was labeled and placed between two foam biopsy pads in a standard histology cassette, immersed in neutral buffered formalin, and sent to the pathology department.

The individual cores were placed at specified positions and in the same orientation with respect to the entry point of the biopsy needle. The location of each core was marked with a number on a prostate diagram map for each biopsy protocol sheet, allowing unambiguous and easy location of the tumor and estimation of its size and extension. Processing of biopsy-loaded matrices was performed in a standardized fashion, and consisted in: fixation for 24 ± 2 hours with 0.2 M phosphate-buffered 4% formaldehyde (pH 7.5), dehydration in graded ethanols, clearing through transitional solvents, infiltration and embedding in paraffin. Semi-serial sections at 4-5 μm were mounted on positively charged glass slides. Every fifth section was stained with hematoxylin-eosin (HE). When needed, immunohistochemistry (IHC) was performed with PIN cocktail containing mouse anti-p63 and rabbit anti-P504S (Alpha-Methylacyl-CoA Racemase or AMACR) primary antibodies at dilution 1:100 (CO001K-05 - Zytomed Systems, Berlin, Germany) and visualized with Envision Flex™/HRP and DAB+ (DAKO, Glostrup, Denmark).

The main outcome measures on the diagnostic slides were the aggregated lengths of biopsy core fragments on slides at different levels and of the remaining tissue in the paraffin blocks, the time and material expenses for histopathology and immunohistochemistry using our new approach compared to the classical procedures.

The surplus slides from a number of select cases (benign hyperplasia, low grade and high grade prostate cancer) as well as from a radical prostatectomy specimen were stained with various histochemical stains, as follows: Highman's Congo Red (1946) [13, p271]; Gram-Twort stain (Twort 1924, Ollet 1947), [13, p313]; Periodic Acid-Schiff (modified McManus, 1946), [13, p171]; Acidified Toluidine Blue at pH 1.99, 2.5 and 3.2 [14]; Modified Congo Red-Acidified Toluidine Blue Stain [15].

For image acquisition we used:

Microscop: Leica DMRBE, Type 301-371.011;

Photo Camera: Leica DFC295, Twain Version 7.6.0.0, FxLib 5.1.0.10570, Firmware 1.0.11;

Acquisition Software: LAS (Leica Application Suite) Version 4.1.0 (Build: 1264).

Results

For each patient 22 semi-serial sections were cut, and the remaining material was left in the paraffin block. The average length (± SD) of processed biopsy cores was 13 ± 4 mm (n = 299). The average aggregate length of biopsy fragments per patient (± SD) was: 131.9 ± 25.3 mm for 10 cores (n = 27), 130.0 ± 9.1 mm for 12 cores (n = 2), and 99 mm for 6 cores (n = 1).

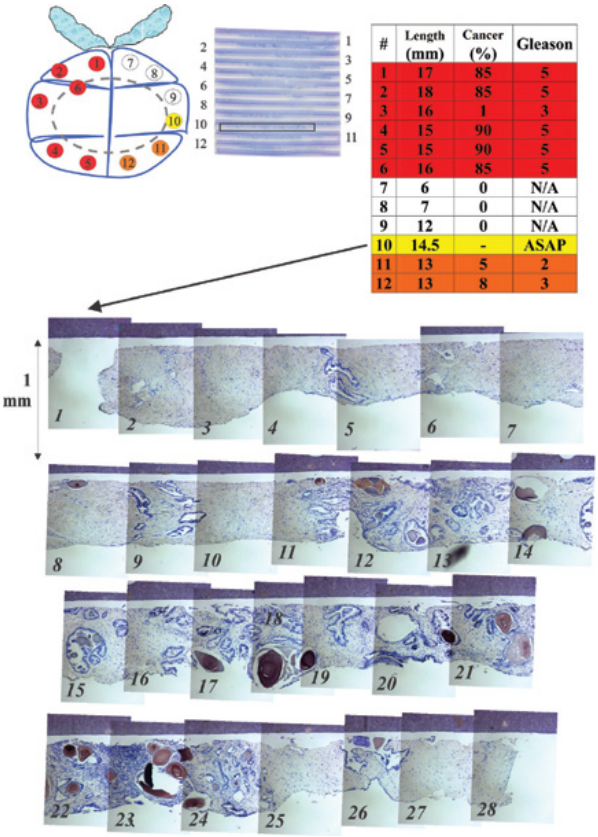
From the 30 patients included in this study, 3 presented benign prostate hyperplasia (BPH), 4 atypical small acinar proliferation (ASAP), 2 intraepithelial neoplasia (PIN), 1 ASAP and low grade PIN, 17 prostate adenocarcinoma, 1 nonspecific nodular chronic inflammatory disease, and 2 were found free of prostate disease. The average length of cancer regions per patient (± SD) was 26.0 ± 26.8 mm.

As expected, few mast cells were found in non-cancerous patients and benign areas of cancerous prostates, but they increased in number around neoplastic foci. Highest density of mast cells was observed around areas of well-differentiated adenocarcinoma. Areas of poorly differentiated adenocarcinoma were essentially devoid of mast cells. Specific histochemical staining for mast cells was best achieved with Toluidine Blue at acidic pH (0.5 to 1.99). While this acidic pH resulted in very specific (metachromatic) staining of mast cells, the euchromatic staining of other cellular elements (i.e. nuclei) was somehow too faint. Less acidic pH (2.5 or 3.2) achieved much better optical contrast of the stained slides while the specificity of the metachromatic staining of mast cells was not affected (Fig. 2, C,D).

Highman's Congo Red staining revealed the presence of numerous intraluminal corpora amylacea (Fig. 2, A) as well as scattered eosinophils (Fig. 2, A,H,J) mainly adjacent to neoplastic acini.

Tarpley's technique allowed simultaneous detection of both mast cells and eosinphils. Additionally, corpora amylacea could be easily recognized, either in the lumen of some acini or sometimes detached from the biopsy or even „floating" in close proximity to the biopsy. It is quite apparent that the matrix surrounding the biopsy prevented the fragmentation and eventual loss of corpora amylacea during histological processing, as

well as the fragmentation of the biopsies themselves. A complete biopsy core was reconstructed from multiple microscopic fields at x400 magnification from a sections stained with Tarpley's Congo Red-Toluidine Blue stain (Fig.1). Higher densities of corpora amylacea as well as of intraluminal mucin and calculi were found in areas adjacent to cancerous foci, when compared with benign areas as well as high grade adenocarcinoma.



#	Length (mm)	Cancer (%)	Gleason
1	17	85	5
2	18	85	5
3	16	1	3
4	15	90	5
5	15	90	5
6	16	85	5
7	6	0	N/A
8	7	0	N/A
9	12	0	N/A
10	14.5	-	ASAP
11	13	5	2
12	13	8	3

Fig. 1.A microscopic image of a TRUS prostate biopsy (nr. 10 from the adjacent diagram and whole slide scan) reconstructed from 28 microscopic fields at x400 magnification from a sections stained with Tarpley's Congo Red-Toluidine Blue stain.

An unexpected finding was the detection of congophilic/metachromatic intracytoplasmatic granules in the epithelial lining of some glandular acini. The density of these granules seems to be higher in areas adjacent to cancerous acini and absent in both benign and highly undifferentiated acini (Fig. 2, A,C,D,E,I). Staining with Gram-Twort stain for identifying bacteria failed to stain most of the granules, with few exceptions (Fig. 2, E).

Periodic Acid Schiff's staining identified both mast cells and some intraluminal mucin but the optical contrast was negatively affected by the high intensity background staining (data not shown).

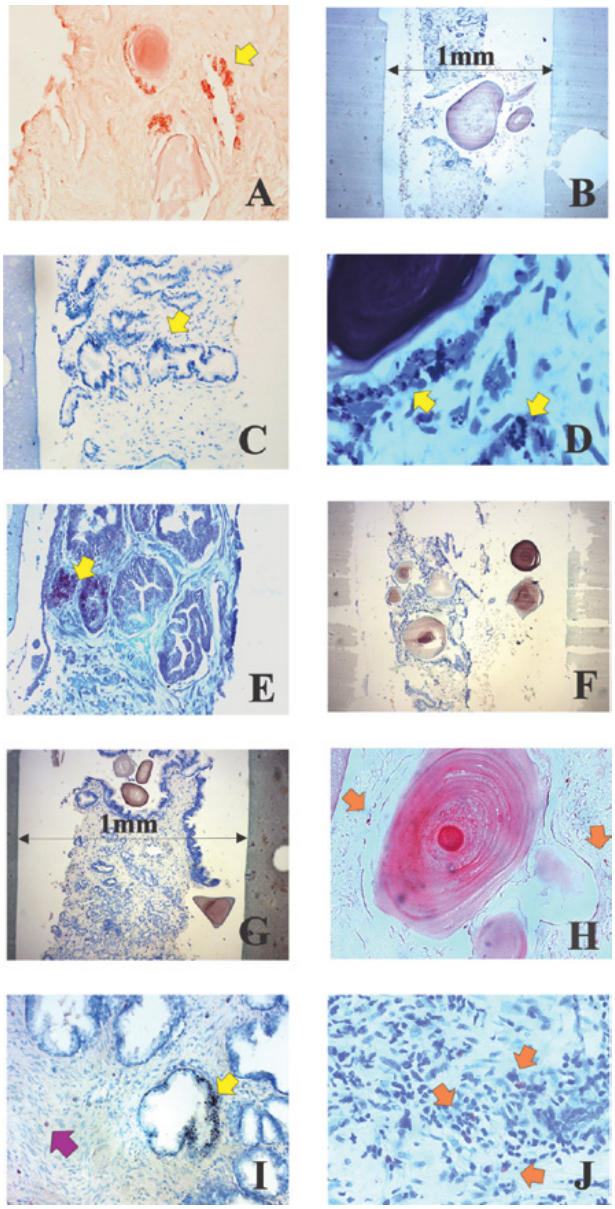


Fig. 2.Histochemical staining of a prostate biopsy from an area with atypical small acinar proliferation (ASAP) (biopsy nr 10 depicted in Fig.1.). A: Highman's Congo Red; B-D: Toluidine Blue pH 2.5; E: Gram-Twort stain for bacteria; F-J: Tarpley's Congo Red-Toluidine Blue stain. Yellow arrows: congophilic/metachromatic granules of unknown origin, Purple arrows: mast cells, Orange arrows: eosinophils.

Discussion

It was long known that solid tumors, including prostate cancer, are commonly infiltrated by a high number of innate and adaptive immune cells [16] even if it is still unclear whether aggressive disease caused increased inflammation or inflammation caused aggressive disease. While in allergies or parasitic infections the role of mast cells (MCs) and eosinophils is more or less ob-

vious, with regards to cancer data are still lacking consistence. The proinflammatory mediators seem to exert a protective role in some tumors, while in others MCs may be stimulating the neovascularity, tissue remodeling, and modulation of the host immune response [17]. In prostate cancer no conclusive data on MCs function are available and the complex roles of these cells remain poorly understood. Conflicting results are also reported with regards to the potential implication of tissue eosinophils in tissue remodeling and cell turnover during both homeostasis and disease. In tumors, eosinophils are associated with necrotic areas, and there is evidence for the cytotoxic effect of eosinophils on tumor cells both in vitro and in vivo, suggesting a protective effect. Whether this finding is a direct correlation or merely an associated phenomenon requires further studies [18].

While our data are still preliminary, there seems to be a strong association between inflammatory cells, prostatic corpora amylacea and prostatic acini bordering adenocarcinoma. Previous studies, including rodent models of spontaneous prostate cancer [19] failed so far to clarify the role corpora amylacea might play in prostate carcinogenesis [20]. However, it is also quite clear that this ambiguity is generated to a large extent by a lack of reliable histological techniques as well as by the paucity of representative tissue samples available for research studies [21]. The intriguing finding of congophilic/metachromatic granules in prostatic acini bordering neoplastic foci needs further study. A thorough review of the literature found only one reference describing similar findings [22]. However, the „Congo red positive dots" described in this paper were looked for only in benign glands since all prostatic adenocarcinomas were excluded from this study.

Conclusion

The techniques employed in this study suggest that multiplexing the core biopsies harvested during TRUS is making the process of serial sectioning fairly easy and decreases significantly the material, time and manpower expenses for prostate core biopsy processing and analysis. Obtaining a large number of surplus slides without prohibitive costs is making available to the researcher a new and potentially rewarding resource, until now untapped. Using this new method has no negative impact on various histochemical and inumohistochemical technics tested.

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