Molecular diagnostics in uro-oncology

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Forward: Molecular diagnostics in uro-oncology

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Foreword: Molecular diagnostics in uro-oncology

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Research on molecular diagnostics in uro-oncology goes along with the development of complex emerging techniques, ranging from the application of next generation sequencing platforms to archival pathology specimens, cytological samples, liquid biopsies, and to patient-derived tumor models. The identification of effective biomarkers has become a major focus, mainly due to the necessity of selecting potentially responsive patients and to improve their outcomes, as well as to reduce the toxicity and costs related to ineffective treatments. The diagnostic pathologist has to integrate information from pathological evaluation with the data from different sources to achieve a final diagnosis, prognosis and prediction [1].

The current special issue on 'Molecular diagnostics of urological cancers' for Expert Review of Molecular Diagnostics covers a wide range of novelties in the field of research on molecular diagnostics in uro-oncology and, in particular, identification of effective biomarkers in genitourinary tumors (Table 1) [2-15]. The wide range of novelties reported in this special issue has led us to a series of considerations and questions related to a better understanding of the current and future role of effective molecular biomarkers in uro-oncology:

1. Multiplexed quantitation in tissue sections

2. Liquid biopsy and genitourinary tumors

3. Urine-based molecular diagnostics of prostate cancer

4. Tumor mutation burden (TMB) in tissue samples

5. Information fusion.

1. **Multiplexed quantitation in tissue sections:** *An integrative approach to guiding (immune)therapy*

For every new drug in development, appropriate biomarker assays that can be applied to formalin-fixed paraffin-embedded tissue samples are developed and then tested for correlation with clinical responses and efficacy. Any tissue-based biomarker that is predictive of a clinical response to a specific (immune)therapy will sooner or later become a part of routine clinical practice [16].

The main problem for a contemporary diagnostician derives from the probability that there are several and multiple biomarkers to test as well as for multiple analytical assays to perform to obtain the best and most comprehensive profile - "To capture and understand increasingly complicated data from biomarkers, multiplexed methods of analysis coupled to dedicated bioinformatics analyses are likely to be integrated into clinical practice" [16].

Multiplexed phenotyping assays applicable to formalin-fixed paraffin-embedded samples are in the early phases of deployment and under very active development. These include multiplexed quantitative immunofluorescence. First of all, this relies on immunostaining formalin-fixed paraffin-embedded tissue with several primary antibodies and an equal number of fluorescently conjugated secondary antibodies applied sequentially. It also

includes imaging the stained tissue sections with a multispectral imager or with a microscope with specific and defined spectral filters [16].

Image processing and analysis software is then applied to construct a cell-derived image of the tissue in which the expression of each biomarker is quantitated [17] (Figure 1). A disadvantage of the approach is the complexity of the multiplexed immunostaining compared to traditional immunohistochemistry in the routine [16-17]. For instance, by the use of multiplexed IHC with high-resolution whole-slide tissue imaging and analysis is possible to attain an automatic classification of epithelial cells and glands (benign vs. neoplastic) in prostate cancer with concurrent analysis of androgen receptor (AR) and alpha-methylacyl-CoA (AMACR) expression at cell-level resolution [17].

According to Lovitch and Rodig [16], this approach allows for an objective evaluation of "(a) biomarker expression in individual cells; (b) cell phenotype, according to multiple biomarkers; (c) cell number, according to phenotype; and (d) the geometric relationship among cells of identical or distinct phenotypes".

2. **Liquid biopsy and genitourinary tumors:** *How do tumor cells and their components reach the blood stream?*

Liquid biopsy offers a promising perspective for cancer diagnosis and monitoring, with several advantages compared to traditional diagnostic tissue-based procedures. Indeed, it allows to better track tumors and mutations over a period of time. Quick and minimal risk

technique, minimal invasiveness, less expensive procedure are among the advantages of liquid biopsy compared to tissue. However, the sensitivity and the concordance between genomic alterations obtained by sequencing tumor tissue and ctDNA are usually very low. The liquid biopsy of circulating tumor cells (CTCs) has been validated as a useful prognostic tool in a variety of genitourinary tumors. This is based not only on the ability of CTCs to be a mirror of tumor heterogeneity but also on the possibility to combine the genetic and transcriptomic status of single CTCs with epigenome analyses [18]. Epigenome analysis of the promoter of three genes regulating epithelial-to-mesenchymal transition (EMT) was performed in single CTCs isolated from blood samples of metastatic castration-resistant prostate cancer patients. Higher level of methylation at the promoter of microRNA-200 family was found in prostate CTCs pointing out a tumor-specific activation of EMT-associated genes during metastatic spreading [19].

The question is how the tumor cells and their constituents can reach the blood stream from their original location in the tissue. One way could be related to blood and/or lymphatic vessels located in the tumor stroma or tumor vascular invasion. Via the lymphatic way, at a certain point, tumour cells may reach the blood stream, allowing circulating Tumor DNA (ctDNA), and more in general cell-free DNA (cfDNA), to become detectable [20-21].

Another scenario is represented by the fact the tumor induces not only angiogenesis in the way it has traditionally been depicted or shown, but also the formation of sinusoidal spaces where tumor cells may protrude into the lumen, however, covered with an intact

endothelial barrier (Figure 2). "Cell components, including ctDNA, are shed by the tumor itself into the blood stream, through or filtered by the endothelial barrier" [21].

3. Urine-based molecular diagnostics and retrograde colonization of benign prostate glands with malignant cancer cells

Voided urine is increasingly adopted for the diagnostic, prognostic and predictive evaluation of genitourinary tumors, in particular of prostate cancer, by measuring cancer-associated proteins RNA transcripts and methylation [22-23]. A risk score based on the mRNA profiling in urine, combined with traditional clinical risk factors, has been validated to identify patients with high-grade PCa (Gleason score ≥7) on prostate biopsy [23].

The question is "*How do prostate malignant cells and their components reach the urine?*". It is widely accepted among genitourinary pathologists that intraductal carcinoma of the prostate, a lesion characterized by the presence of highly atypical malignant cells with a cribriform pattern or solid architecture "that may show acinar/glandular expansion and is surrounded by basal cells", represents invasion of established invasive prostate cancer into normal glandular structures [24-25]. In addition to this, invasive prostate cancer can histologically mimic high grade prostatic intraepithelial neoplasia through retrograde colonization of benign ducts and acini with malignant cells [24] (Figure 3).

Since the prostate glands drain into the urethra, genetic material from prostate cancer, that has spread within preexisting ducts and acini, could be more easily detected in voided urine than from the tumors infiltrating the prostatic stroma [21].

4. Tumor mutational burden in tissue samples

Biomarkers able to predict which patients are most likely to benefit from immune checkpoint inhibitors are urgently needed for the clinical management of cancer patient. Tumor mutational burden (TMB) is considered as a potential predictive biomarker for immunotherapy with checkpoint inhibitors in non-small cell lung cancer (NSCLC) and other tumor types. This assumption is based on the hypothesis that a higher TMB is associated with a great number of immune reactive neo-antigens that are necessary to elicit the immunity system against tumor cells. Moreover, increased TMB correlates with better outcomes in non-small cell lung cancer, colorectal cancer, bladder cancer and melanoma [26]. However, the assessment of TMB requires a whole exome sequence (WES) analysis that is an expensive technique with a long turnaround time, not feasible in a routine scenario. Targeted NGS panels developed also for bladder cancer [27] might be a valid compromise to assess TMB. Compared to the evaluation of PD-L1 immunohistochemistry subject to interpretation variability and antibody choice, TMB has the advantage of being a quantitative measure that can be standardized.

5. Information fusion and genitourinary tumors

The process of merging knowledge and multiple data from disparate sources is what in nonmedical fields is defined as *multi-criteria decision making and information fusion* [28-29]. An information fusion approach is frequently adopted in those fields, including robotics, where a huge amount of data has to be interpreted as well as contextualized as high-level information, "such as multiple concurrent criteria considered in autonomous multi-criteria decision making" [29]. The resulting information as well as diagnostic approaches and therapeutic decisions, when applied to genitourinary tumors, not only examined with large format histology and whole slide imaging [30], but also and above all with multiple biomarkers derived from tissue, blood and urine samples, is more accurate with less uncertainty than when the disparate sources are evaluated individually or/and separately [31]. However, there are still some concerns about the application of these new tools, first of all the validation, software design, storage/back-up and costs.

Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties. No writing assistance was utilized in the production of this manuscript.

References

1. Montironi R, Santoni M, Cimadamore A, Lopez-Beltran A, Cheng L. Editorial: Emerging Biomarkers in Genitourinary Tumors. Front Oncol. 2019;9:326.

2. Kerr PS, Freedland SJ, Williams SB. The current status of molecular biomarkers in patients with metastatic urothelial carcinoma of the bladder. Expert Rev Mol Diagn. 2019 Sep 9:1-3. doi: 10.1080/14737159.2019.1665509.

3. Drake RR, Angel PM, Wu J, Pachynski RK, Ippolito JE. How else can we approach prostate cancer biomarker discovery? Expert Rev Mol Diagn. 2019 Sep 10:1-3. doi: 10.1080/14737159.2019.1665507.

4. Falzarano SM, Feely MM. How can biomarkers assist the prognosis of urologic malignancies? Expert Rev Mol Diagn. 2019 Sep 9:1-3. doi: 10.1080/14737159.2019.1665506.

5. Franceschini T, Giunchi F, Montironi R, Scarpelli M, Lopez-Beltran A, Cheng L, Fiorentino M. Liquid biopsies in urological cancers: what we need to know before starting using them. Expert Rev Mol Diagn. 2019 Sep 11:1-5. doi: 10.1080/14737159.2019.1665508.

6. Cimadamore A, Massari F, Santoni M, Mollica V, Di Nunno V, Cheng L, Lopez-Beltran A, Scarpelli M, Montironi R, Moch H. Molecular characterization and diagnostic criteria of renal cell carcinoma with emphasis on liquid biopsies. Expert Rev Mol Diagn. 2019 Sep 11:1-10. doi: 10.1080/14737159.2019.

7. Zeuschner P, Linxweiler J, Junker K. Non-coding RNAs as biomarkers in liquid biopsies with a special emphasis on extracellular vesicles in urological malignancies. Expert Rev Mol Diagn. 2019 Sep 18:1-17. doi: 10.1080/14737159.2019.1665998.

8. Rebuzzi SE, Perrone F, Bersanelli M, Bregni G, Milella M, Buti S. Prognostic and predictive molecular biomarkers in metastatic renal cell carcinoma patients treated with immune checkpoint inhibitors: a systematic review. Expert Rev Mol Diagn. 2019 Oct 24:1-17. doi: 10.1080/14737159.2019.1680286. [Epub ahead of print]

9. Kouba E, Lopez-Beltran A, Montironi R, Massari F, Huang K, Santoni M, Chovanec M, Cheng M, Scarpelli M, Zhang J, Cimadamore A, Cheng L. Liquid biopsy in the clinical management of bladder cancer: current status and future developments. Expert Rev Mol Diagn. 2019 Oct 17:1-10. doi: 10.1080/14737159.2019.1680284.

10. Iacovelli R, Ciccarese C, Schinzari G, Rossi E, Maiorano B, Astore S, D'Angelo T, Cannella A, Teberino M, Pirozzoli M, Pierconti F, Martini M, Tortora, G. Biomarkers of response to advanced prostate cancer therapy. Expert Rev Mol Diagn. (in press).

11. Lopez-Beltran A, Cheng L, Gevaert T, Blanca A, Cimadamore A, Santoni M, Massari F, Scarpelli M, Raspollini MR, Montironi R. Current and emerging bladder cancer biomarkers with an emphasis on urine biomarkers. Expert Rev Mol Diagn. 2019 Dec 11:1-13. doi: 10.1080/14737159.2020.1699791.

12. Boerrigter E, Groen LN, Van Erp NP, Verhaegh GW, Schalken JA. Clinical utility of emerging biomarkers in prostate cancer liquid biopsies. Expert Rev Mol Diagn. 2019 Oct 11:1-12. doi: 10.1080/14737159.2019.1675515.

13. Mollica V, Di Nunno V, Santoni M, Cimadamore A, Scarpelli M, Lopez-Beltran A, Cheng L, Mariani C, Battelli N, Montironi R, Massari F. An evaluation of current prostate cancer diagnostic approaches with emphasis on liquid biopsies and prostate cancer. Expert Rev Mol Diagn. 2019 Oct 29:1-11. doi: 10.1080/14737159.2019.1684265.

14. Fisher RR, Pleskow HM, Bedingfield K, Miyamoto DT. Noncanonical Wnt as a prognostic marker in prostate cancer: "you can't always get what you Wnt". Expert Rev Mol Diagn. 2019 Dec 12:1-10. doi: 10.1080/14737159.2020.1702522.

15. Chovanec M, Kalavska K, Mego M, Cheng L. Liquid biopsy in germ cell tumors: biology and clinical management. Expert Rev Mol Diagn. 2019 Oct 29:1-8. doi: 10.1080/14737159.2019.1685383.

16. Lovitch SB, Rodig SJ. The Role of Surgical Pathology in Guiding Cancer Immunotherapy. Annu Rev Pathol. 2016;11:313-41.

17. Blom S, Paavolainen L, Bychkov D, et al. Systems pathology by multiplexed immunohistochemistry and whole-slide digital image analysis. Sci Rep, 2017; 7:15580.

18. Cimadamore A, Gasparrini S, Massari F, et al. Emerging Molecular Technologies in Renal Cell Carcinoma: Liquid Biopsy. Cancers (Basel). 2019;11(2). pii: E196.

19. Pixberg CF, Raba K, Müller F,et al. Analysis of DNA methylation in single circulating tumor cells. Oncogene. 2017;36(23):3223-3231.

20. Vandekerkhove G, Struss WJ, Annala M, et al. Circulating Tumor DNA Abundance and Potential Utility in De Novo Metastatic Prostate Cancer. Eur Urol. 2019;75(4):667-675.

21. Montironi R, Cheng L, Scarpelli M, et al. Re: Gillian Vandekerkhove, Werner J. Struss, Matti Annala, et al. Circulating Tumor DNA Abundance and Potential Utility in De Novo Metastatic Prostate Cancer. Eur Urol 2019;75:667–75. Eur Urol (2019), https://doi.org/10.1016/j.eururo.2019.05.035 in press

22. Di Nunno V, Gatto L, Santoni M, et al. Recent Advances in Liquid Biopsy in Patients With Castration Resistant Prostate Cancer. Front Oncol. 2018; 8: 397.

23. Van Neste L, Hendriks RJ, Dijkstra S, et al. Detection of High-grade Prostate Cancer Using a Urinary Molecular Biomarker-Based Risk Score. Eur Urol. 2016;70(5):740-748.

24. Haffner MC, Weier C, Xu MM, et al. Molecular evidence that invasive adenocarcinoma can mimic prostatic intraepithelial neoplasia (PIN) and intraductal carcinoma through retrograde glandular colonization. J Pathol. 2016;238(1):31-41.

25. Gasparrini S, Cimadamore A, Mazzucchelli R, et al. Pathology and molecular updates in tumors of the prostate: towards a personalized approach. Expert Rev Mol Diagn. 2017;17(8):781-789.

26. Goodman AM, Kato S, Bazhenova L, et al. Tumor Mutational Burden as an Independent Predictor of Response to Immunotherapy in Diverse Cancers. Mol Cancer Ther. 2017; 16(11):2598-2608.

27. Rosenberg JE, Hoffman-Censits J, Powles T, et al. Atezolizumab in patients with locally advanced and metastatic urothelial carcinoma who have progressed following treatment with platinum-based chemotherapy: a single-arm, multicentre, phase 2 trial. Lancet. 2016; 387(10031):1909-20.

28. Goldenberg SL, Nir G, Salcudean SE. A new era: artificial intelligence and machine learning in prostate cancer. Nat Rev Urol. 2019. doi: 10.1038/s41585-019-0193-3. [Epub ahead of print]

29. Montironi MA. From image analysis in pathology to robotics and artificial intelligence. Anal Quant Cytopathol Histol. 2016;38(5): 268–269.

30. Montironi R, Cimadamore A, Massari F, et al. Whole Slide Imaging of Large Format Histology in Prostate Pathology: Potential for Information Fusion. Arch Pathol Lab Med.2017;141(11):1460-1461.

31. Cimadamore A, Scarpelli M, Santoni M, et al. Genitourinary Tumors: Update on Molecular Biomarkers for Diagnosis, Prognosis and Prediction of Response to Therapy. Curr Drug Metab. 2019;20(4):305-312.

Table 1. Novelties in the field of research on molecular diagnostics in uro-oncology

| First author (Reference No.)	Topic
Kerr PS (2)	The current status of molecular biomarkers in patients with metastatic urothelial carcinoma of the bladder
Drake RR (3)	How else can we approach prostate cancer biomarker discovery?
Falzarano SM (4)	How can biomarkers assist the prognosis of urologic malignancies?
Franceschini T (5)	Liquid biopsies in urological cancers: what we need to know before starting using them
Cimadamore A. (6)	Molecular characterization and diagnostic criteria of renal cell carcinoma with emphasis on liquid biopsies
Zeuschner P. (7)	Non-coding RNAs as biomarkers in liquid biopsies with a special emphasis on extracellular vesicles in urological malignancies
Rebuzzi SE (8)	Prognostic and predictive molecular biomarkers in metastatic renal cell carcinoma patients treated with immune checkpoint inhibitors: a systematic review
Kouba E (9)	Liquid biopsy in the clinical management of bladder cancer: current status and future developments
Iacovelli R (10)	Biomarkers of response to advanced prostate cancer therapy
Lopez-Beltran A. (11)	Current and emerging bladder cancer biomarkers with an emphasis on urine biomarkers.
Boerrigter E (12)	Clinical utility of emerging biomarkers in prostate cancer liquid biopsies
Mollica V (13)	An evaluation of current prostate cancer diagnostic approaches with emphasis on liquid biopsies and prostate cancer
Fisher RR (14)	Noncanonical Wnt as a prognostic marker in prostate cancer: "you can't always get what you Wnt".
Chovanec M (15)	Liquid biopsy in germ cell tumors: biology and clinical management.

Figures
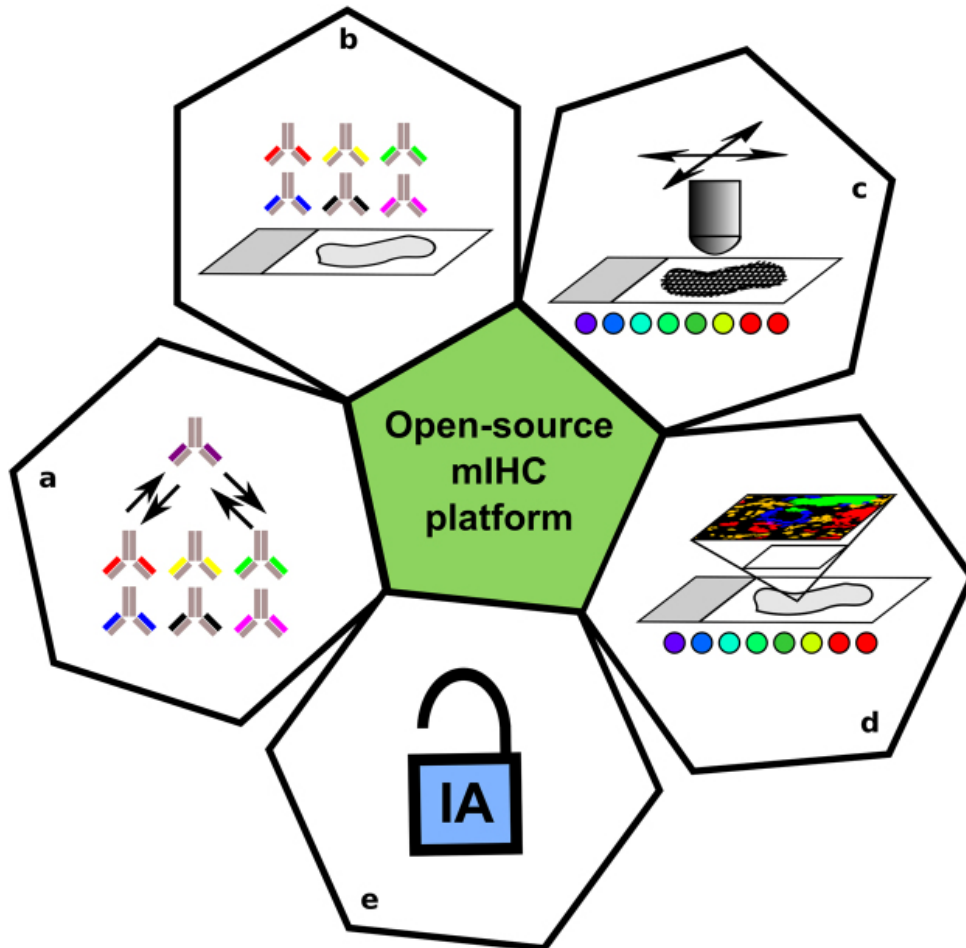
Figure 1. Multiplexed quantitative immunofluorescence to assess cellular immunity and the expression of ligands in the tumor microenvironment on formalin-fixed paraffin-embedded tissue. Key aspects described in Multiplexed quantitative immunofluorescence platform combining (a) rapid implementation of new antibodies for targets of interest, (b) >5-plex mIHC assay design, (c) high-resolution whole-slide image acquisition, and (d,e) open-source high-resolution whole-slide image analysis. (For additional details, see Reference No. 3. http://creativecommons.org/licenses/by/4.0/.).
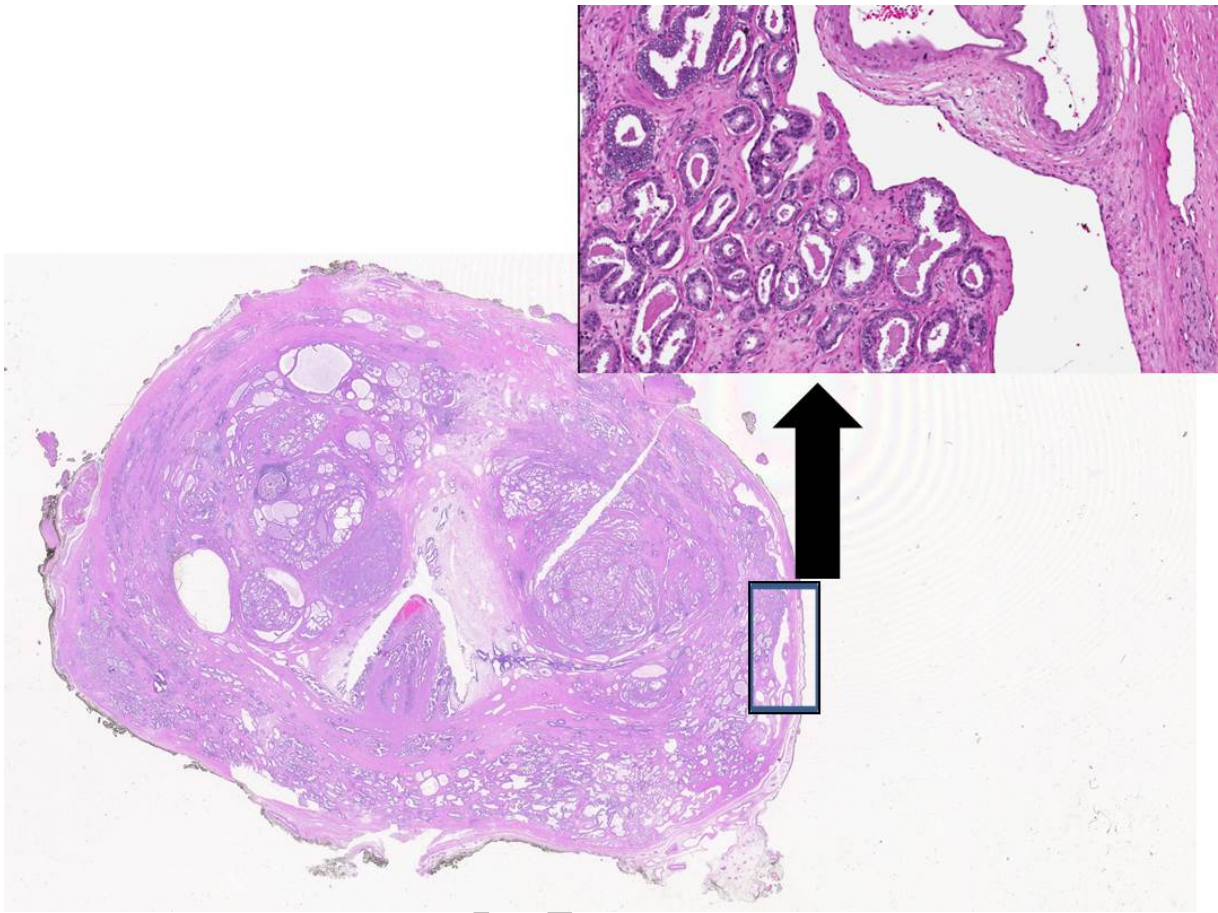
Figure 2. Slit-like vessels with tumor cells protruding into the lumen (Enlarged).
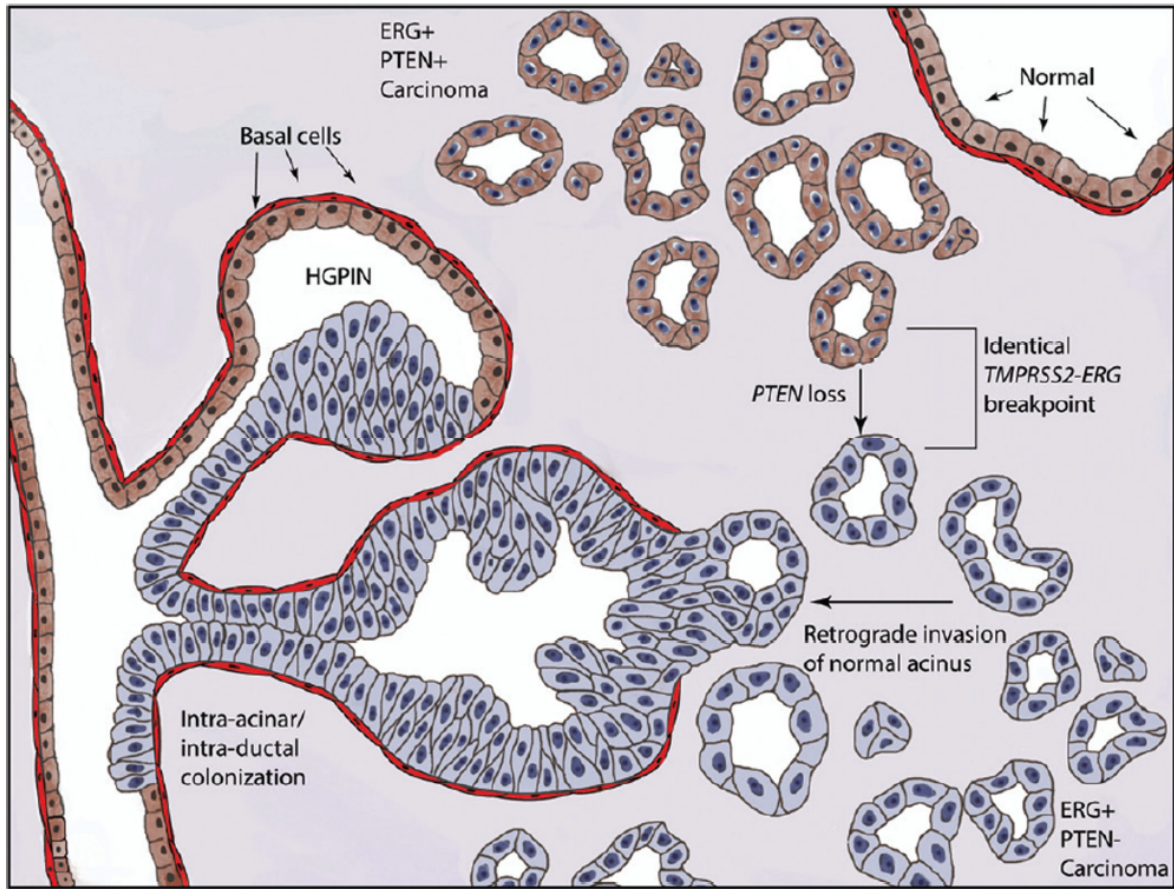
Figure 3. Model of retrograde glandular colonization (For additional details, see Reference No. 24. Reproduced with permission from the Publisher).