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Commentary

What is a Bladder Cancer Molecular Subtype? – Counterpoint

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Abstract. In an accompanying paper, Mattias Höglund discusses on what is a bladder cancer molecular subtype. He emphasizes the need to consider the aim of tumor classification, which is obviously critical to the approach. He also focuses on considering primarily the identity features of the neoplastic cells. Here, we provide a counterpoint. While largely agreeing with his views, we underline that other parameters that may vary in a spatial or temporal scale, and the tumor microenvironment, can also provide relevant information to render tumor classifications clinically useful. Furthermore, tumor heterogeneity and evolution during the disease course - natural or under therapeutic pressure - should be considered.

Keywords: Bladder cancer, molecular subtypes, tumor classification, genomics, tumor microenvironment, prediction of outcome

The "Commentary" article by Mattias Höglund [1], an expert and pioneer in the field of bladder cancer molecular classification [2–4], is certainly instructive and useful. His historical perspective points to the fact that humans have long aimed at classifying "the world around them" in order to better understand it.

Many of the points he raises are important, including the clear distinction between classifications primarily oriented to clinical applications and those oriented to dissect the biology of the tumor. The former are largely based on immunohistochemistry to characterize tumor cells and the latter use so far mainly bulk whole transcriptome data to assign tumors to molecular subtypes without discriminating which cell populations express the subtype-associated gene sets. As he points out, these two types of classifications do not always match. He asserts that molecular classification should be based on the phenotype of the tumor cells themselves, and not include the microenvironment, to define "intrinsic" subtypes (based on features of the cancer cells). He also argues that the molecular classification should also not depend on "state-related" tumor cell features such as proliferation signatures because proliferation is a continuous parameter that can change across time. Similarly, he proposes to

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avoid the use of tumor microenvironmental features to classify tumors because the extent of intratumoral stromal/inflammatory cell infiltration can be heterogeneous and therefore subject to sampling bias, and it can also change across time and in response to therapeutic pressure. Finally, he defends the idea that classification should encompass both non-muscle-invasive (NMIBC) and muscle-invasive (MIBC) tumors.

All these points merit further thought and many of them are relevant beyond bladder cancer. However, a few of them acquire particular importance in the context of this tumor. More specifically, bladder tumors display wide inter-tumor molecular heterogeneity [5], muscle invasion has a much greater impact on patient management than in other carcinomas, and sampling bias may also be a greater problem. In addition, the impact of intra-tumor heterogeneity is starting to be unraveled [6, 7], and bladder cancer has one of the highest rates of mutations in genes coding for chromatin regulators [8] – pointing to the relevance of epigenetic analyses and "lineage plasticity" – The ongoing revolution in bladder cancer therapy demands urgently better tools for precision medicine.

We would like to discuss some of the ideas that Mattias Hoglund raises as they have important consequences for future bladder cancer research.

FEATURE(S) TO USE FOR MOLECULAR CLASSIFICATION

The earliest efforts to classify tumors at the molecular level were based on transcriptome profiling because they were the first large-scale datasets that became available, thanks to cDNA arrays and later oligo arrays. This, combined with the fact that the transcriptome is more easily interpretable than other available omics, explains why most molecular classifications performed to date are based on the protein-coding transcriptome. However, many other types of data can be used now, including the non-coding transcriptome, somatic genomic alterations (including mutations and copy number changes), the epigenome (DNA methylome and chromatin accessibility), the proteome and post-translational modifications (such as the phosphoproteome), and the metabolome. The TCGA integrated many of these features to produce "clusters of clusters" to subtype bladder cancers and other solid tumors. Other features, such as variation in germline DNA, including mutations in tumor suppressor genes, could also be considered [9, 10]. The greater difficulties in data generation, biological interpretation (e.g., the non-coding transcriptome and the DNA methylome), and cost (e.g., the metabolome and chromatin accessibility) have made transcriptomic classifications more popular. However, all the other molecular features will provide additional information. The different layers of data may reinforce existing subtypes, introduce further subdivisions, and – in some cases – highlight similarities between tumors that are currently ascribed to different transcriptomic molecular subtypes. They could also point to genes and pathways that are highly relevant for bladder cancer tumorigenesis but are difficult to capture using the transcriptome alone [11]. Thanks to recent advances [12, 13], we envision that the proteome – which is readily interpretable – will become an important data layer in the short term [11, 14].

Importantly, the clinical relevance of each data type will mandate which classifiers become standard beyond biology. In lung cancer, for example, DNA analyses have major impact in patient management and therefore dominate the genomic taxonomy. By contrast, the management of patients with breast or colorectal cancer responds to a combination of DNA-based, transcriptomic, and protein expression features.

CLASSIFYING NMIBC AND MIBC: TOGETHER OR SEPARATELY?

In most cancers, the key event required for tumor progression/dissemination is the invasion of the basement membrane. In bladder cancer, an additional key event is the invasion of the muscularis propria *which has a major impact on management.* Invasion of the bladder wall can be viewed as a continuous (progressively invading) or discontinuous phenomenon, whereby additional properties must be acquired by the tumor cells, or even possibly in certain cases only by a phenotype switch (i.e., epithelial-to-mesenchymal transition) [15]. The rather unique binary classification of bladder tumors according to muscle-invasion has strong clinical prognostic value and currently serves as the basis for deciding whether a patient needs definitive therapy (radical cystectomy or trimodal therapy). Therefore, one can argue – with good reason – that NMIBC and MIBC are distinct disease entities. One can go even further by studying Ta and T1 tumors separately [16, 17].

In contrast, to acquire a global view of bladder cancer progression, the joint analysis of NMIBC and MIBC is a tempting way to go. For example, this strategy has recently been applied to the molecular classification of upper tract urothelial tumors [18]. Several issues have hampered this effort in bladder cancer. One is that a large fraction of MIBC are diagnosed *de novo*, i.e., in patients without a clinical history of NMIBC. This implies that most of the precursors of MIBC are understudied. A second issue is that the tissue micro-environment (TME) of NMIBC and MIBC are substantially different. Therefore, in simple unsupervised analyses, one of the first divisions that will become apparent will be the division between MIBC and NMIBC. To circumvent this problem, the group of Mattias Höglund has pioneered the approach of focusing on tumor cells [1].

CLINICALLY ORIENTED VS. BIOLOGICALLY ORIENTED CLASSIFICATIONS

As stressed by Mattias Höglund, the aim of classifying is key to the outcome: one can perform an unsupervised classification mainly based on the biology of the tumors, with a subsequent view to the relevance to the diagnosis and response to treatment. Another way to go is to focus on a classification aimed at predicting such clinical parameters using supervised analyses. The clinically oriented classification is, by its construction, highly dependent of the treatments used, whereas the first one is not. The often-protracted clinical course of patients with bladder cancer introduces major confounding factors in the classifications, especially when considering incident and prevalent tumors together. However, the clinically oriented and the biologically oriented classifications need not be considered as mutually exclusive. Indeed, diagnostic or prognostic markers can be specific for a given biological subtype (for example, *CDKN2A* loss has been reported to be a marker of tumor progression in *FGFR3*-mutated NMIBC) and a biological subtype could be, in itself, a clinical marker (for example, the basal subtype is associated with poor prognosis) [19, 20].

MOLECULAR CLASSIFICATIONS AND THE TME

Focusing on the tumor cells enables to jointly study NMIBC and MIBC and may avoid sampling biases. However, recent single-cell RNAseq data indicate that all bladder cancers contain mixtures of tumor epithelial cells that can be assigned to different molecular subtypes [21]. These studies have also revealed that cells within the TME are also highly heterogeneous. The diversity of the TME, with CAFs, nerve cells, T-cells, macrophages, and other hematopoietic cells that can also be assigned to different molecular subtypes [22] adds complexity but may also allow for refinement in classifiers. Overall, it is unclear how tumor cell and stromal cell heterogeneity will affect our ability to develop robust prognostic and predictive biomarkers. With that said, bulk gene expression signatures remain among the best predictive biomarkers for immune checkpoint inhibitors – cytotoxic T cell and interferon signatures are associated with benefit, whereas TGF-beta signatures are associated with resistance [23]. There are different types of TME, each with different dialogues with tumor cells; therefore, considering the TME in the molecular classification can be of great importance. The stromal content could result from sampling bias, in which case one could question the existence of the stroma-rich subtype of the consensus classification [24] and the largely overlapping "P53-like" subtype of the MDA classification [25] as well as the "luminal-infiltrated" subtype of the TCGA classification [8]. On the other hand, it is also conceivable that the stroma reflects features that are dictated by the tumor cells, indeed reflecting crucial aspects of the tumor biology that may impact on specific tumor – stroma paracrine interactions and specific clinical features.

MOLECULAR SUBTYPES AND CELL PROLIFERATION

As with the stroma, proliferation can be viewed as a continuous feature. However, different types of proliferation can exist, even in normal cells, such as symmetric or asymmetric division. In bladder cancer, mutations, fusions, and copy number changes in genes involved in the cell cycle (e.g., *CCND1*, *CCNE1*, *CDKN2A*, *RB1*) are frequent relevant events. Tumors enriched in early vs. late cell cycle genes have been reported [3, 26]. Therefore, proliferation is not only a quantitative trait (percentage of cells cycling) but also a qualitative trait and both types of information may be incorporated in the molecular classification.

SUBTYPE, TUMOR EVOLUTION, AND INTRA-TUMOR HETEROGENEITY

In a given bladder cancer patient, the different tumors diagnosed either synchronously or metachronously generally originate from a single somatic cell (clonal origin of tumors). During tumor evolution, different subclones arise [27]. Although the different subclones often belong to the same molecular subtype, differences can be observed. In the most used mouse model of bladder cancer, induced by BBN [28, 29] a switch to the basal subtype is frequent, starting at an early progression stage [15]. In humans, a similar phenotype switch could occur at the CIS stage [30, 31] and different subtypes can be observed in the same tumor (estimated to be present in 7 to 15% of MIBC cases) [6, 21, 32]. This subtype heterogeneity must be also considered in terms of clinical outcomes, including chemotherapy response [6]. How to best approach this point from a technical standpoint in clinical practice remains an important task. Artificial intelligence-based image analyses and multiparameter *in situ* approaches (as subtype cannot be determined by a set of antibodies alone) are some of the available options. In this regard, single cell and spatial transcriptomics will make significant contributions [21, 33, 34]. Issues related to reproducibility, standardization, and cost need to be considered.

HOW MANY SUBTYPES?: FROM STRATIFIED TO PERSONALIZED MEDICINE

Claude Bernard already pointed out "there are no diseases, there are patients ", in agreement with the fact that each tumor results from the myriad of possible combinations of mutations and genotypes. Therefore, as indicated by Mattias Höglund, ultimately, the number of subtypes could be as high as the number of tumors (personalized medicine). In bladder cancer, the simplest division would be to differentiate between luminal and non-luminal tumors. Further subdivisions of luminal and non-luminal tumors can be considered: among the non-luminal neoplasms, the basal/squamous tumors and the neuroendocrine-like tumors have distinct molecular and clinical features; among the luminal tumors, a subgroup enriched in *FGFR3* mutations emerges in many molecular classification systems [24]. As the number of samples analyzed with omics data increases, the finer the subdivision into subtypes can be.

Subtypes are important for the identification of altered genes and pathways specific to a group of tumors and for stratification-based medicine. The future goal of precision medicine is to tailor treatments to individual patients and, therefore, to specifically target the different tumor subclones, the genes and pathways that are altered, and to predict their evolution under treatment pressure. The rapid development of new technologies should make these achievable goals in the foreseeable future: the implementation of liquid biopsy, bioinformatics tools, and artificial intelligence are examples thereof. In the meantime, molecular classification continues to play a valuable role and the establishment of consensus agreement has been a key step to facilitate communication among the scientific community [24, 35]. Through experience with several other tumor types, including breast [36–38] and brain cancers [39], we now know that it is feasible and clinically useful to group tumors into molecular subtypes. A major challenge is to leverage the knowledge acquired in bladder cancer to prospectively test their value in the management of patients [40]. Clinical trials based on molecular subtyping have already been initiated [41].

We might conclude this counterpoint commentary by saying that, "All classifications are wrong (or not completely right) but some are useful", paraphrasing statistician George Box [42].

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