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Commentary

What is a Bladder Cancer Molecular Subtype?

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Abstract.
BACKGROUND: Several molecular classification systems for bladder cancer have been proposed, but due to differences on how to define molecular subtypes, controversies and misunderstandings have arisen.
OBJECTIVE: To discuss different aspects of the molecular classification of bladder cancer and to point to the consequences of using different conceptual approaches. To question some underlying assumptions when defining molecular subtypes.
METHODS: To critically reflect on some of the principles and methods used when defining molecular subtypes.
RESULTS: Depending on underlying assumptions and aims for the definitions of subtypes, different types of molecular subtypes will be arrived at.
CONCLUSION: The underlying assumptions and their consequences must be better clarified when defining molecular subtypes.

INTRODUCTION

A major step towards a better treatment for bladder cancer patients has been the search for molecular subspecies that better correspond either to prognosis or to treatment response. In addition, molecular studies have also been aimed at increasing the biological understanding of the tumor type as such. Thus, the search for molecular subtypes has served two purposes, one clinical and one biological. One of the most powerful tools with which to explore the molecular heterogeneity of bladder cancer has been the gene-expression profiling of tumor biopsies. Gene-expression profiling has the advantage of being data rich, objective, and quantitative. Up to now, several classification schemes for bladder cancer have been proposed, but in most cases, each group has used their own criteria and methods to define molecular subtypes. The TCGA classification system uses the clustering of gene-expression data obtained from whole biopsies and is limited to muscle invasive tumors [1]. The Consensus classification system merges various published and classified data to arrive at a core of well-defined subtypes, the majority being originally determined by gene-expression analyses of biopsies from muscle invasive tumors [2]. The Lindskrog et al. [3] system is based on the hierarchical clustering of global gene expression of TURB samples, but is limited to non-muscle invasive cases. Hurst et al. [4] analyzed non-muscle invasive tumors by global gene-expression analyses, but then produced separate systems for Ta and T1 tumors, respectively. The Lund Taxonomy, on the other hand, is based on a combination of gene-expression analyses and cancer cell phenotypes as determined by immunohistochemistry, and applies to both non-muscle invasive and muscle invasive tumors [5]. In all of these cases, genomic and mutation data has been used to support the classification schemes, but rarely to change them. Seiler et al. [6] use a different approach, as their aim was to design a system that gave the best information on response to treatment. These authors combined signatures/classes from previously published systems to arrive at a system

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optimized for clinical (treatment) purposes. Due to differences in the above approaches, controversies and misunderstandings have arisen regarding what a molecular subtype represents, how many exist and, in particular, what is meant by an intrinsic molecular subtype. In this paper, I make use of experiences and concepts from the historical period when classification systems as such were developed and discussed. During this period, detailed and highly relevant analyses were made of the different classes of features by which objects may be characterized. The text thus starts with a historical summary. I continue to point to the problem of how to define a molecular subtype for bladder cancer using classical definitions. I then address the biological level at which a subtype may be defined, followed by a critical analysis of infiltration and proliferation signatures as possible class defining features. I challenge the necessity to have different systems for non-muscle invasive and muscle invasive tumors and also address the fact that any given molecular subtype will show intra-subtype heterogeneity. The aim is thus to critically evaluate some of the principles and underlying assumptions used to define bladder cancer molecular subtypes. Many of the controversies in the field have been caused by a lack of transparency with regard to what authors are actually referring to when they use the term "molecular subtype".

TAXONOMY PRINCIPLES, A HISTORICAL PERSPECTIVE

The organization or categorization of objects in discrete sets is usually called a taxonomy. Taxonomies often have a hierarchical structure whereby low level groups are organized in higher order categories. The best-known taxonomy is probably the Linnaean system, which organizes all living things into a hierarchical order, first presented in *Systema Naturae* in 1735. The smallest unit in this system is the *species,* with groups of similar species organized into a *genus*, and then into higher *taxa*. For flowering plants, the species is defined by features of their reproductive system such as *pistils* and *stamens,* and higher taxa by the number of pistils and stamens. The most crucial defining element in such a system is the smallest unit of objects: the *species*. The definition of species was, however, not clear among the contemporaries of Linnaeus, and neither was it clear which organizing principle was to be used to determine genus and higher taxa, accordingly several parallel systems were discussed and in use. The main botanical text for the one and a half millennia preceding Linnaeus was the *Materia Medica,* compiled in 50 AD by Dioscorides, who was considered to be the supreme authority on plants. In his classification, plants were organized according to their usefulness for man, such as medicinal use, or to provide spices, oils, resins, or fruits. Both of these systems, the Linnaean and that of Dioscorides, are equally valid, but their purposes are different. Linnaeus' aim was to identify the natural order of nature created by a higher divinity (God), whereas the Dioscoridean classification was based on the usefulness of the plants for humans. Another issue was whether classification should be top-down or bottom-up. The prevailing principle for almost two millennia before Linnaeus was the top-down classification principle based on "logical" divisions, of which the Aristotelian approach dominated, heavily elaborated during the scholastic period (14th century). Scholastic classification includes variables such as *essence,* a necessary and defining characteristic*, genus,* a defining characteristic shared by other groups of objects, *accidentals*, an attribute that may or may not be present in a group of objects, and *differentia*, the part of the essence that distinguishes one species from another. Classification systems organized objects in fixed categories and an object could not move from one category (species) to another. In this context, any observed change in character is not a change in *essence*, but a change in the set of *accidentals* present. Although no direct reference to specific and older classification systems is made in the contemporary literature of tumor classification, many of these older principles and concepts reappear with new names/significances, sometimes mixed up and in the wrong context. The current use of the concept "intrinsic" is quite similar to the archaic *essence*. Controversies may develop about what should be included as a feature or not, what a subtype (*species*) is and is not, and how many subtypes (*species*) exist or are needed. Many of these controversies originate from the initial aim of the classification, which could either be a biological classification with the aim of defining distinct subtypes (*species*) of tumors: the Linnaean perspective, or to classify tumors by how they should be acted upon for best treatment: the Dioscoridean (clinical) perspective. Two such systems may not necessarily overlap, however, both are equally valid. What is important though, is that the criteria used to create one type of classification system cannot

be used to invalidate a second type. For instance, a biologically relevant subtype with no ascribed clinical consequence should not be ignored, it still exists, and may in fact be of value in future clinical situations.

HOW TO DEFINE A SUBTYPE

Generally speaking, a category or a subtype, is a group of objects that share features, are similar, and differ from other objects at the group level. Another way to express this is that the formation of two groups is motivated if variations within the group are smaller than the variation between groups; the basis for an ANOVA analysis. These principles may seem simple, but there are major problems in determining "similarity", as well as which features should be included in the comparison. In the simplest case, the presence/absence of a single feature may determine class assignment. One such instance is chronic myeloid leukemia (CML), defined by the t(9;22)(q34;q11) translocation creating the *BCR/ABL* fusion gene. Once discovered, the presence of this fusion gene defined the disease and determined treatment. Later, the definition had to consider additional fusion variants, but they all included the *ABL* gene. Patients with CML often have this aberration as the sole change i.e. the change is necessary and sufficient. Thus, the definition of CML can be reduced to a single genomic event representing both *essence* and *differentia*. In inherited forms of cancer, the disease is inherited through one or a few paralogous genes. In the case of breast cancer, the presence of *BRCA1*/*BRCA2* mutations is a defining feature of "BRCA tumors" and is thus a necessary property, but not a sufficient one, as additional genomic changes are needed to produce a tumor. In the case of urothelial carcinoma (UC), the situation becomes increasingly complex/fuzzier, as no necessary or sufficient changes seem to exist. For instance, *FGFR3* gene mutations are frequent in Luminal papillary tumors (LumP, TCGA classification [1]; LumP, Consensus classification [2]; Urothelial-like A (UroA), Lund Taxonomy [7]), but are not seen in all Luminal papillary tumors, and can thus not be used as a single classification variable, i.e., not as an *essence*. Furthermore, *TP53* mutations are frequently seen in Luminal Unstable tumors (LumU, Consensus classification; Genomically Unstable (GU), Lund Taxonomy) but cannot be used as a differentiating feature between LumP/UroA and LumU/GU tumors, as some of the former also show *TP53* mutations. Thus, it is probably impossible to find single variables which definitely assign a given tumor to a UC subtype or make it different from another subtype; *essence* and *differentia* do not exist in this case. Hence, simple, logical (monothetic) definitions for subtypes are not an option. Instead, classification has to be polythetic and rely on what is commonly known as *family resemblance*, that is, the sharing of some of several characteristics frequently occurring in one group or class but not in another, in which no feature is essential for membership of that group or class. Even if this sounds simple and straightforward it nevertheless creates some problems. To be able to define which characteristics the family resemblance should be based on, an idea of what makes up a family/class/subtype in the first place is necessary; an *a priori* idea is needed of what a family/class/subtype is. For a pathologist, the morphology and growth patterns of cancer cells may guide classification; for a molecular biologist, clustering based on genome-wide gene expression (mRNA) profiles may guide classification; and for a clinician, the pattern of response to a given treatment may guide classification.

CANCER CELL PHENOTYPE VERSUS BULK BIOPSY CLASSIFICATION

Tumors may exhibit different levels of infiltration of non-tumor cells without essential *cancer cell* characteristics being altered. Such infiltration may create major discrepancies between tumor classifications based on immunohistochemistry, in which the actual cancer cells are characterized, and gene expression classification based on bulk biopsies, in which the resulting profile is the sum of the cell types and cell states present in the sample. Consequently, it may not always be correct to refer to a *cancer-cell specific* subtype if this is based on genome-wide mRNA-expression data only. In the literature, some investigators, typically pathologists using immunohistochemistry, refer to the cancer cells proper with the term "subtype", whereas others refer to "tumor samples that form a cluster based on gene expression profiling". As bladder-cancer tumor samples are transurethral resected samples in most investigations, what is actually being classified, or forming a cluster, is *types of biopsies,* i.e. a sum of cell types and cell states. This has led to mixed nomenclatures, in which some "subtypes" are defined by the nature of the cancer cells only, whereas other "sub-

types" are infiltrated versions of the same cancer cell subtype. These phenomena have been named *convergence* and *divergence* at the gene-expression level [7], meaning that two different *cancer cell subtypes*, defined by the immunohistochemistry of the cancer cells, become increasingly similar at the genome-wide gene-expression level when infiltrated and may thus form a single cluster, or that a given *cancer cell subtype* may form two clusters, one "not infiltrated" and one "infiltrated". As the level of infiltration may vary between cancer-cell subtypes as such, subtypes may, by themselves, be more or less defined by infiltration-related signatures when applying gene-expression profiling alone, and hence the actual classification principles will not be uniform. Just as infiltration may result in convergence and divergence at the gene-expression level, so may proliferation; tumors of different cancer-cell types, but with very high proliferation, tend to cluster together. Conversely, large sets of cases of the same cancer cell type often separate in "low" and "high" proliferation "subtypes" or "classes". Hence, classification based on features of the cancer cells proper will give a different result to that obtained when using whole biopsies or TURB samples in a case of bladder cancer.

INFILTRATION AND PROLIFERATION GENE SIGNATURES AS CLASS-DEFINING FEATURES

In most cases, both infiltration and cell-cycle activity show a continuous variation that forms monomodal distributions with no "natural" thresholds for "high" or "low" respectively. Such distributions may be divided into any number of "types", in which all solutions are of equal weight. Furthermore, infiltration gene signatures do not originate from the cancer cells proper, and are thus not a cancer-cell intrinsic property. Proliferation, on the other hand, may be considered a transitional feature, meaning that a given cell type may enter the cell-cycle and consequently change many of its molecular features, only to resume its original molecular profile when cell division is completed. It is common for statistical tests between any two gene-expression clusters to identify gene signatures for infiltration and proliferation as the most significantly different gene signatures; both infiltration and proliferation produce large and distinct gene-expression signatures. One may thus question whether infiltration and proliferation are intrinsic properties of cancer-cell classes or merely cluster-generating variables. Again, one needs to have an *a priori* idea of what a class or a subtype is. Should a tumor class or subtype be defined by features of the actual cancer cells only, or by the sum of different cell types and cell states in a biopsy. In one sense, infiltration and proliferation behave as *accidentals;* they may or may not be present. Irrespective of this, standard clustering of genome-wide gene-expression data will automatically be heavily affected by infiltration and proliferation signatures.

DIFFERENT SYSTEMS FOR NON-MUSCLE INVASIVE AND MUSCLE INVASIVE TUMORS?

The prevailing idea in the bladder cancer community is that non-muscle invasive (NMI) and muscle invasive (MI) tumors should be treated as different entities, and that separate classification systems are needed. Hence, very few have derived classification systems based on cohorts containing both NMI and MI tumors. This is understandable from a clinical perspective; NMI and MI tumors are treated very differently, and sometimes by different sections of the hospital system. Consequently, the NMI/MI distinction has been around for a long time and has become the "gold standard" by which to approach urothelial carcinomas. However, from a biological perspective there is no reason to assume that a T1 tumor, of say subtype A, invading the lamina propria, will completely change its crucial biological features (*essence*) to become another subtype, say B, when it eventually invades the muscle layer and becomes a T2. Instead, the cancer cells will probably acquire new features (*accidentals)* which make the tumor more invasive without necessarily changing its molecular identity. To clarify this issue, cohorts employed to determine molecular subtypes should be mixed NMI and MI cohorts. Only then can the question of whether an NMI subtype may also appear as a MI version, or vice-versa, be resolved. Hence, from a Linnaean viewpoint, there might not be any essential difference at the level of cancer-cell phenotype between NMI and MI subtypes; pathological stage behaves here as an *accidental*. On the other hand, from a Dioscoridean point of view, there is a major difference, and one should act differently upon the given information. But this difference in action is primarily related to pathological stage and not to the molecular subtype *per se*; a patient with a $\geq$T2 tumor will always have a worse prognosis, and will

be treated differently to a patient with a T1 tumor, even if the tumors are of the same molecular subtype.

WHAT DEGREE AND TYPE OF INTRA-SUBTYPE VARIABILITY SHOULD BE ACCEPTED?

It is common that the number of detectable "clusters" increases with cohort size. This often leads to further subdivisions of previously-determined molecular subtypes. Even if further subdivision may be indicated, a critical question is to what extent intra-subtype variation can be accepted, and what type of variability should motivate further subdivisions. In principle, as many "subtypes" could be produced as there are individual tumors, as no two tumors are identical at all levels. This issue becomes even more pressing when additional genomic layers of investigation are added. Apart from gene expression, genomic alterations, and mutation data, there have also been efforts to include information from biological layers such as the methylome, from epigenetics, noncoding RNAs, and proteomics, and recently also single-cell analysis and spatial transcriptomics, when defining molecular subtypes. The general pattern after adding such additional layers has been more of an increasing divergence than a coalescence to more well-defined subtypes, i.e. an increase in the number of genomic layers analyzed results in an increase in diversity. However, this does not mean that some of these levels will not identify clinically relevant signatures. Hence, one can always claim "heterogeneity", but this then becomes a truism; classes of molecular subtypes will always show some level of intra-subtype variation. Consequently, the issue of further subdivision relates back to how molecular subtypes are defined in the first place. What type of variation should motivate a further subdivision? How many features are needed and how much can they vary within a subtype? Are the additional subtypes linked to a higher-order class, such as the LundTax Urothelial-like A, B, and C, which are all Urothelial-like (the *genus* level), or are they to be considered as new higher-order subtypes (the *essence, species* level)? In initial studies, the LundTax Genomically Unstable and the later defined Small cell/Neuroendocrine-like (Sc/NE) were grouped together due to convergence, but today they are seen as two distinct subtypes (Neuronal, TCGA classification; Neuroendocrine-like, Consensus classification). As argued above, neither infiltration nor proliferation are good criteria for further subdivision. This does not mean that infiltration and proliferation are unimportant from a clinical perspective, on the contrary, but estimates of these variables may be derived as separate indices from the gene expression data. Can a further subdivision be biologically motivated only – the Linnaean approach – or should it also be of clinical relevance – the Dioscoridean approach? One could argue that only discontinuous variation of key biological features should be used for further sub classification, but then how discontinuous, how many, and what is a key biological feature?

FINAL COMMENTS

Given the large variability among urothelial carcinomas, a variability that may involve several biological levels such as histopathological, gene-expression, genomic and gene-mutation levels, one may question whether there are any "natural" divisions of types or subtypes. This does not, however, mean that "anything goes", but rather that each investigator has to be clear about and critically analytical of the underlying assumptions and consequences of their particular decisions. Much of the controversy in the field is caused by a lack of transparency with regard to what investigators actually mean by a molecular subtype. Often the methods employed to sample and organize the data are taken as optimal tools to achieve the aims – what the algorithm says is a group, is also a relevant group – but then any applied method includes assumptions of the underlying structure. One critical question is whether one should allow for different systems depending on the methods used, e.g. one system for gene-expression profiling, one for immunohistochemistry of the cancer cells, and one for histology, or whether one should demand a strong link between molecular subtypes defined at different biological levels, or alternatively, determine a hierarchical order of their significance. In some instances, it is believed that a proper biological classification will automatically lead to clinically-relevant classes and that a "correct" classification system solves both problems. There is no logical reason to believe that this will be case. A feature which, for example, increases the risk for progression may very well be present independent of any biologically-defined molecular subtype. Another issue is whether a molecular subtype should refer to a type of biopsy or a type of cancer-cell phenotype. As transurethral-resected biopsies include variable numbers of non-tumor cells by nature, classification

at the biopsy level will automatically be affected by the level of infiltration, and then the term "intrinsic" becomes problematic. The exclusion of infiltration and proliferation as class-defining features does not mean that they are of no clinical value, quite the opposite, but it is a reasonable scientific approach to sort out variables which together produce specific complex outcomes, such as progression, to be able to perform proper analyses. Only then will it be possible to conclude which factors contribute to a given outcome, and under which conditions. One could argue that different classification systems should be produced and that the future will show which are of most value. In this case, classification becomes operational and depends on what is meant by "value", more "biologically relevant" or more "clinically useful"? Linnaeus and Dioscorides again. From a birds-eye perspective, controversy within the urothelial-cancer community on how to classify urothelial carcinoma is analogous to the debates regarding the classification of plants in the 18th century. We could learn from history.

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AUTHOR CONTRIBUTION

Höglund drafted and finalized the manuscript.

CONFLICTS OF INTEREST

Mattias Höglund has no conflict of interest to report.

REFERENCES

[1] Robertson AG, Kim J, Al-Ahmadie H, Bellmunt J, Guo G, Cherniack AD, Hinoue T, Laird PW, Hoadley KA, Akbani R, Castro MAA, Gibb EA, Kanchi RS, Gordenin DA, Shukla SA, Sanchez-Vega F, Hansel DE, Czerniak BA, Reuter VE, Su X, de Sa Carvalho B, Chagas VS, Mungall KL, Sadeghi S, Pedamallu CS, Lu Y, Klimczak LJ, Zhang J, Choo C, Ojesina AI, Bullman S, Leraas KM, Lichtenberg TM, Wu CJ, Schultz N, Getz G, Meyerson M, Mills GB, McConkey DJ; TCGA Research Network, Weinstein JN, Kwiatkowski DJ, Lerner SP. Comprehensive molecular characterization of muscle-invasive bladder cancer. Cell. 2017;171:540-56.

[2] Kamoun A, de Reyniès A, Allory Y, Sjödahl G, Robertson AG, Seiler R, Hoadley KA, Groeneveld CS, Al-Ahmadie H, Choi W, Castro MAA, Fontugne J, Eriksson P, Mo Q, Kardos J, Zlotta A, Hartmann A, Dinney CP, Bellmunt J, Powles T, Malats N, Chan KS, Kim WY, McConkey DJ, Black PC, Dyrskjøt L, Höglund M, Lerner SP, Real FX, Radvanyi F; Bladder Cancer Molecular Taxonomy Group. A consensus molecular classification of muscle-invasive bladder cancer. Eur Urol. 2020;77:420-33.

[3] Lindskrog SV, Prip F, Lamy P, Taber A, Groeneveld CS, Birkenkamp-Demtröder K, Jensen JB, Strandgaard T, Nordentoft I, Christensen E, Sokac M, Birkbak NJ, Maretty L, Hermann GG, Petersen AC, Weyerer V, Grimm MO, Horstmann M, Sjödahl G, Höglund M, Steiniche T, Mogensen K, de Reyniès A, Nawroth R, Jordan B, Lin X, Dragicevic D, Ward DG, Goel A, Hurst CD, Raman JD, Warrick JI, Segersten U, Sikic D, van Kessel KEM, Maurer T, Meeks JJ, DeGraff DJ, Bryan RT, Knowles MA, Simic T, Hartmann A, Zwarthoff EC, Malmström PU, Malats N, Real FX, Dyrskjøt L. An integrated multi-omics analysis identifies prognostic molecular subtypes of non-muscle-invasive bladder cancer. Nat Commun. 2021;12:2301.

[4] Hurst CD, Cheng G, Platt FM, Castro MAA, Marzouka NS, Eriksson P, Black EVI, Alder O, Lawson ARJ, Lindskrog SV, Burns JE, Jain S, Roulson JA, Brown JC, Koster J, Robertson AG, Martincorena I, Dyrskjøt L, Höglund M, Knowles MA. Stage-stratified molecular profiling of non-muscle-invasive bladder cancer enhances biological, clinical, and therapeutic insight. Cell Rep Med. 2021;2(12):100472.

[5] Höglund M, Bernardo C, Sjödahl G, Eriksson P, Axelson H, Liedberg F. The Lund taxonomy for bladder cancer classification - from gene expression clustering to cancer cell molecular phenotypes, and back again. J Pathol. 2023. Epub ahead of print.

[6] Seiler R, Ashab HAD, Erho N, van Rhijn BWG, Winters B, Douglas J, Van Kessel KE, Fransen van de Putte EE, Sommerlad M, Wang NQ, Choeurng V, Gibb EA, Palmer-Aronsten B, Lam LL, Buerki C, Davicioni E, Sjödahl G, Kardos J, Hoadley KA, Lerner SP, McConkey DJ, Choi W, Kim WY, Kiss B, Thalmann GN, Todenhöfer T, Crabb SJ, North S, Zwarthoff EC, Boormans JL, Wright J, Dall'Era M, van der Heijden MS, Black PC. Impact of molecular subtypes in muscle-invasive bladder cancer on predicting response and survival after neoadjuvant chemotherapy. Eur Urol. 2017;72:544-54.

[7] Sjödahl G, Eriksson P, Liedberg F, Höglund M. Molecular classification of urothelial carcinoma: Global mRNA classification versus tumour-cell phenotype classification. J Pathol. 2017;242:113-25.