

Bladder Cancers

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PRESENTATION

This long, somewhat tedious review would like to retrace the progress made in recent years by genetic progress and those made as a result in the understanding of tumor pathology. This path has led to a better definition of the subtypes of bladder carcinomas. Since the 2000s, methods for identifying genetic abnormalities in tumors have developed considerably qualitatively and quantitatively. Thanks to computers and high-throughput sequencing techniques, we can know, in a few hours or a few days, information that previously required weeks or months of investigation. The aim of this review is not only to list the genes involved in bladder tumours as it would be difficult to be exhaustive. The objective is to establish that it is possible, through reasoning on the transcriptome and the proteome, to give phenotypic expression a value established on its direct relationship with certain genetic abnormalities. A better knowledge of all these elements should allow pathologists to better coordinate with molecular biologists. The prior establishment of a tumor phenotype should make it possible to focus genetic investigations on smaller panels. After the description of FGFR3 mutations in 1999 and since the description of the genetic abnormalities that contributed to the establishment of the Madrid consensus in 2015 and the elements known today, we are witnessing a better understanding and a progressive simplification of the links with the phenotype. A new formulation emerges, next-generation immunohistochemistry, which draws a parallel with next-generation genetic sequencing. In this approach to genome, proteome, transcriptome relationships, it is the team from the Swedish University of LUND that has contributed the most to illuminating the classification of bladder tumors, with diagnostic, prognostic and predictive implications. The main articles analysed in this review came from this working group.

Introduction

Bladder cancer is one of the most common and deadliest cancers with approximately 550K new cases and 200K deaths recorded in 2018 worldwide. Some advances in treatments have occurred over the years, but there is still a need to develop more selective and personalized (targeted) therapeutics. The vast majority of bladder cancers are urothelial type, i.e. presumed to have developed from transitional epithelial cells in the bladder mucosa. The 2016 WHO classification of urothelial tumors is partly superimposed on a concept of a dual pathway of carcinogenesis [1-3], but this concept has since undergone new developments. These developments should help to specify the algorithms favouring multidisciplinary therapeutic options [4]. The type of bladder cancer known as transitional has therefore long been pathologically separated, according to its severity, into two main types of lesions: non-invasive bladder cancer of the muscular plane (detrusor) of the bladder wall (NMIBC) (75%) and invasive bladder cancer of the detrusor muscle (MIBC) (25%). NMIBCs include tumours that have not ruptured and crossed the basement membrane of the bladder mucosa (TNM pTa classification), high-grade tumours that invade the submucosa but not the underlying muscle (TNM pT1), and carcinoma in situ (TNM pTis), a high-grade lesion at high risk of progression to MIBC. pTa tumours frequently recur, but progression to muscle invasion is uncommon (10% to 15%) and has a good prognosis. On the other hand, patients with MIBC at the time of diagnosis (pT2 or higher) have a poor prognosis (<50% survival at 5 years). pT1 tumours represent a clinically concerning and molecularly heterogeneous group. Some of its features are related to both the NMIBC and the MIBC. More than 90% of bladder cancers are urothelial carcinomas (UC), which share differentiating characteristics with the normal lining of the bladder. These are the cancers that have been the most studied [5]. Rare epithelial variants include squamous cell carcinoma, adenocarcinoma, and small cell carcinoma. As no significant molecular analysis of rare cases was available in the first or later studies, they will not be discussed in this review.

This review takes up the elements that contributed to the Madrid consensus conference in 2015, with an update of the data largely gathered in review articles such as that of Carolyn D. Hurst and Margaret A. Knowles in: Principles and Practice of Oncology. Primer of Molecular Biology in Cancer. Vincent T. DeVita; Theodore S. Lawrence; Steven A. Rosenberg Editors. Finally, extensions of molecular data on immunohistochemical characteristics will be taken into account on the basis of recent publications resulting from the LUND (Sweden University) classification [6]. We will therefore follow a path that will take into account the diversity and complexity related to the different initial methodological approaches and follow the simplifying effects of the 2015 consensus with their recent extensions and their implications on clinical practice. It should be noted that one of the main unresolved issues to date is the lack of distinctive signs to characterize pT1 tumors that will become deeply infiltrative, those that will have a limited potential for superficial or submucosal extension. Separating tu-

mors conceptually into two groups with respect to muscle invasion is a paradigm based solely on prognosis and not on the identification of a mechanism specific to infiltration, which is established well before muscle invasion. To acquire this invasiveness, a tumor must gradually change its genetic status to be able to change its environment. It is the complex and progressive genotypic and phenotypic signs related to this infiltration capacity that we seek to identify.

The Madrid Consensus 2015: Search for Genotypic and Phenotypic Correlations

D'après S.P. Lerner et al. / A Consensus Bladder Cancer Molecular Taxonomy Bladder Cancer, vol. 2, no. 1, pp. 37-47, 2016. The wealth of information resulting from the genomics revolution has provided an opportunity for a dismemberment of tumor phenotypes based on histology alone. This results in the identification of a large number of molecular phenotypes. Summarizing these large datasets defining tumor subtypes is a major challenge in 2015 to formulate a new molecular taxonomy of bladder cancer. This pioneering effort, which began in the 2000s in the field of breast cancer [7], has been expanded to include virtually all types of tumors in recent years through massive genome sequencing. This effort was conducted with The Cancer Genome Atlas (TCGA) and the Genome Consortium International Cancer (ICGC), Urothelial bladder cancer (UBC) in the years leading up to the search for consensus [1]. The advances of these groups have led to several molecular subclassification proposals that announce the promise of a clinical application with selective consequences for patient management.

In urothelial bladder cancer (UBC), the Lund group (Sweden University) was the first to use the expression profile of tumors covering a broad spectrum of the disease, to propose a molecular classification of taxonomy correlated with phenotype [8]. Subsequent studies, on biology or on the basis of multidisciplinary platforms, quickly yielded remarkably consistent evidence, supporting the existence of bladder cancer subphenotypes. Most of this research has been conducted on muscle-invasive bladder cancer, which explains some of the difficulties. As a result, at the same time, this work has revealed unmet needs about tumor status in situations where there is no muscle invasion (NMI). Analysis by the integrated Pan-Cancer multidata (TCGA) platform of 12 different cancer types [9] also allowed to place molecular subphenotypes of bladder cancer in a broader context of tissue-specific cell differentiation. Issues related to the problem of differences in genomic technologies, bioinformatics strategies, genome analysis tools, nomenclature, biological basis of classifications related to clinical traits (among others) have been raised by the independent molecular taxonomy proposals, hence the need to build consensus. A consensus meeting was intended to facilitate progress in this area and stimulate collaboration. The meeting held at the Spanish National Cancer Research Center CNIO (Madrid, Spain) on March 24, 2015 brought together all the groups that proposed molecular subtyping of bladder cancers based on a genomic study carried out in recent years.

The main objectives were to:

1. Discuss the selection of samples and the methods used to justify the different molecular taxonomies.
2. Discuss the overlap between different molecular taxonomies.
3. Develop a cooperation strategy to optimize the declared classifications.
4. Join efforts to validate optimized classification in prospective studies.

The following summary of presentations provides an overview of the development of the consensus agreement. It should be noted that some groups initially launched molecular investigations without associating them with the precise context of histopathology and immunohistochemical phenotype. This probably contributed to delaying the legibility of the results obtained. The heterogeneity of tissues taken from an infiltrating tumor is related to a representation of the tumor and its environment. It is therefore difficult to attribute precisely what is due to each stromal, tumor and inflammatory component. We will take up this ambiguity in the discussion later. The different projects with their particularities and their contribution to the identification of tumor groups will be briefly mentioned below. Methodological details and comprehensive data are provided for each project in the bibliography [10-30].

The TCGA Project

S. Lerner and K. Hoadley [5] presented the work of the TCGA (bladder cancer project). The initial TCGA report concerned the genomic analysis of the first 131 patients [1]. Chemotherapy-free invasive urothelial cancers were analyzed for somatic mutations, DNA variant copy number (CNV), mRNA and microRNA expression, their proteins and phosphoproteins (reverse phase protein arrays, RPPAs), and DNA methylation. Other histological types were excluded from the study. Genetic information was integrated with comprehensive clinical and pathological data. This invasive tumor cohort carries one of the highest somatic mutation rates with a mean of 7.7 and a median somatic mutation rate of 5.5 per megabase, similar to that of adenocarcinoma and squamous cell carcinoma of the lung and also melanoma. The result identified 32 significantly mutated genes (SMGs) involved in multiple pathways, which concern cell cycle regulation (93% of tumors), chromatin remodeling (76%), DNA damage response, transcription factors, receptor tyrosine kinases (RTK)/RAS/PI3K (72%) and regulation of signaling pathways. Four epigenetically significantly mutated genes (SMGs) (ARID1A, MLL2, KDM6A and EP300) are present in nearly a quarter of tumors. A third of the tumors were characterized for DNA hypermethylation specific to the cancer process [1]. The combination of copy number variation (CNV) with somatic mutations shows that 69% of tumors harbor one or more therapeutic targets, which gives new hope.

Molecular Taxonomy and Immunohistochemistry Studies (LUND Sweden)

Mr Hoglund and his group presented a summary of the work carried out in Lund since 2012. A first attempt at classification by broad exploration of genome expression was due to Lindgren et al. on invasive tumors, including NMI [7]. This analysis of 144 samples identified two main molecular subtypes named MS1 and MS2. The split between MS1 and MS2 divides samples into grade 1 or 2 (MS1) and grade 3 (MS2) (WHO 1999), and between T1 (MS1) and \geq T2 (MS2) tumors. T1 is roughly evenly distributed between the two subtypes. The MS1 and MS2 categories differ significantly in terms of the number of genomic alterations, FGFR3 and TP53 mutations, and survival. To further the analysis, Sjö Dahl et al. [10] extended the study to include 100 T_a, 100 T₁, and 100 tumors \geq T₂ which allowed the MS1 cases to be subdivided into two groups (MS1A and MS1B) and the MS2 subtype into five groups (MS2a1, MS2a2, MS2b1 and MS2b2.1 and MS2b2.2). In-depth biological interpretation of gene expression data identified biological, immune, cell cycle, keratin expression, and signature tyrosine kinases (RTKs), which determined the structuring of the data. In addition, a signature associated with the expression of FGFR3 is derived from it.

Based on histology, genomic signatures, FGFR3, PIK3CA, and TP53 mutations, three major bladder tumor (UBC) subtypes were defined: urobasal (Uro) (MS1A, MS1B, and MS2b2.1), genomically unstable (GU) (MS2a1 and MS2a2), and SCC as (SCCL) (MS2b2.2). In addition, an “infiltrating” group has been recognized in which non-tumor inflammatory transcripts dominate the expression profiles. A subset of urobasal tumors (MS2b2.1) showed a “proliferative phenotype” with high cell cycle activity and basal cytokeratin expression. This group largely corresponded to invasive tumors and was called urobasal B which infiltrates the muscle in almost all cases to distinguish it from urobasal A. An important finding is that molecular subtypes dominate disease stages. For example, all four subtypes (Uroa, UroB, GU and SCCL) were detected in T₁ tumors and no fundamental differences were observed between invasive and NMI GU cases [9].

Lindgren et al. extended these analyses by combining gene expression data with CGH array data for 146 cases [9]. This revealed for a small number of bladder cancers simple genomic alterations, usually the loss of chromosome 9 and gains of 1q, whereas genetically unstable (GU) and squamous cell (SCCL) tumors showed complex changes with frequent focal genomic alterations, namely 6p22 (E2F3/SOX4) amplifications. Through an integrated approach, two major genomic circuits have been found to participate in bladder cancers. A CCND1 FGFR3 circuit operating in Uro tumors and an E2F3/RB1 circuit in GU tumors. For the SCCL subtype, no specific circuit could be established. In addition, homozygous CDKN2A deletions (9p21) were found to represent a progression event among Uro tumors. To validate gene expression results using immunohistochemistry (IHC), a 20-protein detection antibody panel was used to provide additional support for Uroa, UroB, GU, and SCCL subtypes [10].

Urobasal tumors commonly express basal CK 5 and P-CAD with TP63+ basal cell islands; GU tumors were CK5-, P-CAD- and TP63- but E-CAD+ and ERBB2+; and SCCL tumors were CK5+ and P-CAD+ throughout the tumor. The IHC, on the “infiltrated” group, showed that it was composed of either GU or SCCL tumor cells, with an infiltrate of immune cells. The previously described genomic circuits could therefore be evaluated by IHC to identify Uro and GU cases. More recently, the immunohistochemistry-based classification system described by Sjodahl, et al. [10] was applied to 165 T1 tumors, showing that molecular subtypes (Uro vs. GU and SCCL) had a major impact on progression rates, supporting the clinical value of the taxonomy [11]

Thus a classification with six distinct entities was obtained:

1. SCCL / UroB,
2. A GU group,
3. A urobasal group,
4. Two groups with slightly different profiles from the “infiltrators” category, and 6 a new variant provisionally classified as “small cell/neuroendocrine-like”. The subtypes have been validated by genomic alterations (chromosomal aberrations) and genetic mutations [1-5]. Because the Lund group ranks by associating invasive and noninvasive bladder carcinomas as well as some of the closely related subtypes in pure invasive carcinomas, they conclude that gene expression phenotypes converge on progression. However, subtype-specific gene expression signatures are already present in invasive tumors.

University of North Carolina Classification

B. Kim’s strategy is focused on the analysis of invasive tumors. Using consensus clustering (K2 consensus), a robust classification (16) of tumors enriched into subtypes was achieved: - basal phenotype (CK5/6 and CD44) and - phenotype luminal (PPARG, Gata3, CK20 et UPK2). A signature of 47 genes (BASE 47) was generated by microarray prediction analysis (PAM) which was associated with three independent patient series. The basal subtype revealed similarities to the basal subtype of breast cancers, as demonstrated by the PAM50 signature to the UBC datasets. This group contains a “Claudine low” subgroup as defined in breast cancer, with epithelial-mesenchymal transformation (EMT). Patients whose tumors are of the “Claudine-bas” type have a course similar to the “basal” category. Using the GSEA pathway, an association related to inflammatory cell infiltration and immune checkpoints was observed in the basal subgroup and, more specifically, in Claudine-bas tumors. The basal subgroup defined by RNA is also selectively enriched in alterations in the suppressor gene RB while the luminal a subtype is enriched in FGFR3 and TSC1 mutations. On the other hand, there is no enrichment of the alterations of the TP53 pathway. The basal group is significantly more represented among women [16].

Baylor College of Medicine Houston Classification (Tumor Differentiation)

K. Chan presented an update on his group’s recent work on subtyping bladder cancer based on urothelial cell differentiation (2, 3, 17). CK14/Thy1/CD44 positive cells, (stem cell population) giving rise to better differentiated CK/CK17/CD44-positive cells that can acquire the CK 8/18 phenotype and then the terminal expression of differentiation into luminal cells, uroplakins and CK20. Knowledge about the normal urothelial differentiation program was therefore used to classify tumors. A cohort of patients with a poor prognosis is identified by the CK14+ (basal subtype) group of basal tumors that are resistant to Cisplatin-based neoadjuvant chemotherapy in a small cohort of patients [18].

The Anderson MD Subtypes

To identify intrinsic subtypes, D. McConkey [24] and the group at MDA modeled their approach after the pioneers of breast cancer subtyping studies, Peru et al. [21,22]. They generated profiling data from broad expressions of mRNA genomes from a cohort of 142 rapidly frozen invasive and NMI tumors using UBC Illumina arrays [20]. The results revealed the presence of three distinct groups; further analyses of the significantly differentially expressed genes defining each group, revealed that they were enriched with biomarkers already implicated by the Peru group and other characteristics of basal-like (CK5, CK14, CDH3, CD44) and luminal (CK20, CD24, FOXA1, GATA3, ERBB2, ERBB3) intrinsic markers of breast cancer subtypes [20,23,24]. One of the subtypes was characterized by wild-type P53 gene expression designated “p53like” [20]. Tumors in the p53-like subtype were essentially resistant to neoadjuvant chemotherapy based on Cisplatin [20].

CIT Classification (Identity Card for “Basal-Like” Tumors)

A group of French groups supported by the League against Cancer. F. Radvanyi et al. of the CIT consortium (Institute Curie, Henri Mondor and Foch hospitals, Institut Gustave Roussy, CEPH, La Ligue Contre le Cancer) focused on the subgroup of “basal-like” tumors because it was the largest homogeneous group identified by various unsupervised methods of invasive bladder cancer from transcriptome data [19]. In a joint analysis of 7 invasive tumor datasets, the more advanced cases represented among the basal-like tumors, survival analyses showed the worst outcome for this group of patients, regardless of stage, grade, nodal status, and metastatic. The survival curves of patients with basal-like tumors were very different from those of other patients with bladder cancer, with many deaths within a year. In terms of DNA alterations, this group was characterized by an increase in EGFR copies or FHIT deletions, and P53 mutations; at the transcriptome level, an enrichment of the activation of the EGFR pathway has been identified. Radvanyi also discussed the different

strategies for identifying the basal-like subgroup, comparing a 40-signature-based transcriptomic assay and IHC based on two antibodies recognizing CK5 and CK6 as a positive marker of the subgroup and FOXA1 as a negative marker [19]. In their studies, an 85% agreement was observed between the two techniques.

Discoveries in the Fight Against “Basality”

As mentioned, all classifications include a set of tumors expressing basal or SCCL markers. F. X. Real examined the relevance of various classifications in search of putative transcription factors/networks that might be involved in the activation of different molecular subphenotypes. He has performed important work pointing out genes involved in urothelial differentiation, including FOXA, GATA, PPARG, ELF3 and IRF1 [26-28]. FOXA1 and GATA3 which are more represented among tumors in TCGA groups I and II and virtually absent from groups III and IV. STAT3 and DNp63a have been proposed as regulators of the basal phenotype.

Definition of “Intrinsic Subtypes” of Bladder Cancer

The term “intrinsic” was first applied to the molecular classification of breast cancers. The work by Charles Perou’s group demonstrated that a tumor subtype generally remains stable regardless of where and when a tumor was sampled [21]. This leads to the conclu-

sion that belonging to a subtype is an “intrinsic” property of a type of breast cancer. The original definition of “intrinsic” refers to properties of tumor cells. Although the MDA group consistently observed their three subtypes in several independent datasets, this does not necessarily indicate that all subtypes are “intrinsic”. Direct measurement of group stability using a “silhouette” score analysis showed that most “p53-like” tumors in the cohort identified by MDA were unstable, while “basal” and “luminal” tumors were stable [20]. In addition, analysis of tumors paired before and after neoadjuvant chemotherapy showed that most “luminal” tumors acquired “p53-like” characteristics after treatment [20]. This observation was confirmed in each cohort of neoadjuvant chemotherapy analyzed (n = 5) [23]. A comparative analysis of tumors and lymph node metastases (n=33) indicated that basal tumors almost never changed their subtypes, whereas luminal and p53-like tumors had significantly more “switching” subtypes. Therefore, membership in the luminal subtype appears to be a basic or intrinsic property of the tumor, but p53-like tumors can enter or exit the subtype as a result of environmental stimuli (a kind of plasticity). Finally, a definition of “intrinsic subtypes” of bladder cancer must be agreed in perspective during the 2015 consensus (Figures 1 & 2) (Tables 1 & 2).

Table 1: Subtypes of urothelial tumors identifiable by the IHC phenotype in 2016.

Type de cancer de vessie	Type moléculaire	Phénotype à explorer
		Type luminal (papillaire), uroPk,
Luminal	Groupe I hyper muté	ER+, HER 2+, Gata 3 +, CK 20 +/-, CK 8/18 +, Mib-1, E-Cadh +
	Groupe II ER +, HER 2 +	P 53 (?)
	Groupe III + Marqueurs des cellules souches. CD 44 + *,	CK5-6 +, CK 14 +, Gata 3-,
Basal		CD 44 *, Mib-1, P 53 (?)
	Réponse immunitaire élevée, et marqueurs malpighiens.	CD 45 (Tils), CD 4, CD 8.
Basal sous-groupe		P 40, P63, Mib-1, P 53 (?)

Table 2: Ebauche de classification phénotypique en 2015-2016.

Phénotypes établis	Sous-groupes
Luminal (CK 8 CK 18 +)	
Basal CK 14 +)	Hypermuté type MSI, P53 - WT, « P 53 like »P53 mutée, inflammatoire. EGFR, HER2.
Squameux (épidermoïde)	

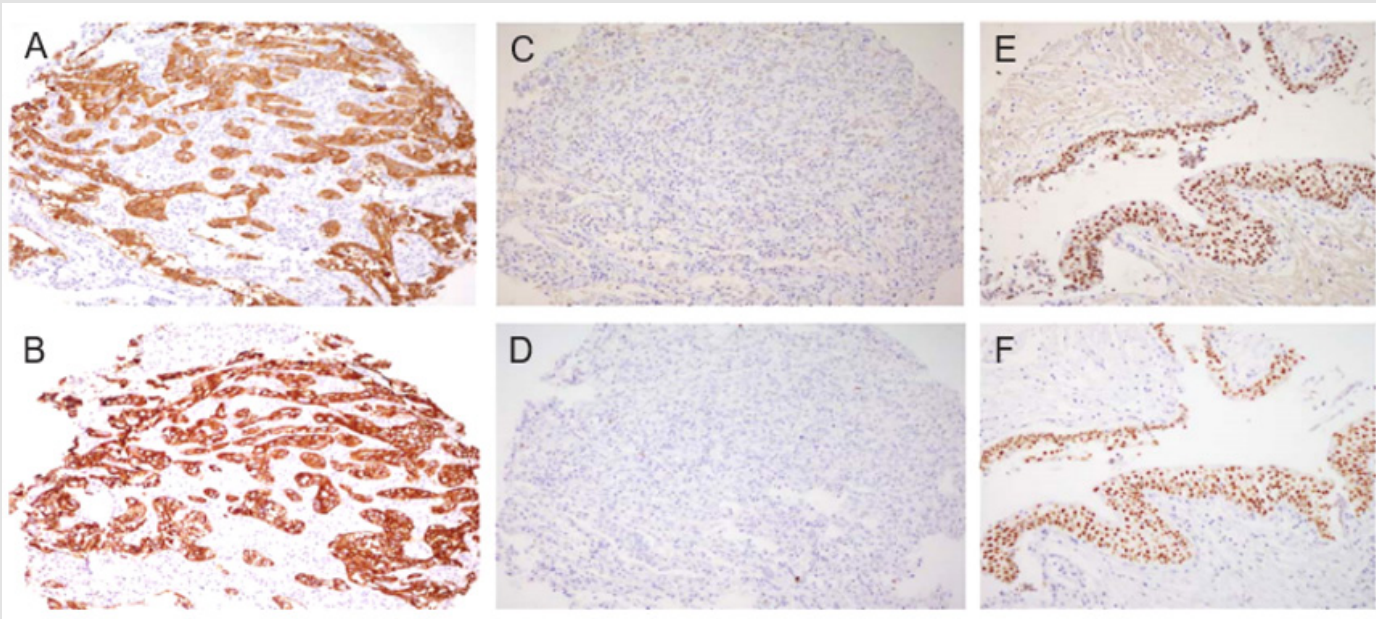


Figure 1: Basal-type bladder carcinomas are characterized by high expression of CK 5-6 (A), CK 14 (B) low expression or undetectable FOXA1 (C) and GATA 3 (D). On the other hand, normal urothelial cells strongly express FOXA1 (E) and GATA 3 (F). According to S.P. Lerner, et al. (2016).

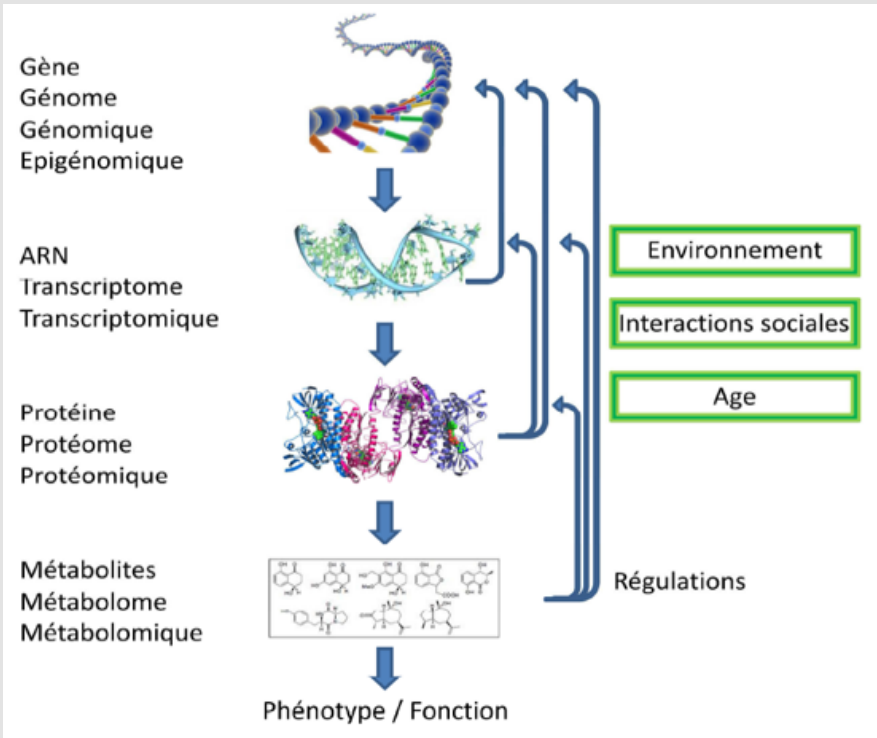


Figure 2 : The “omics”.

LE Consensus

It therefore seems necessary, given these different approaches, to reach a consensus on the subtypes of bladder cancer. There is a need to use these data to classify tumors in order to improve their management, on differences in their evolution, and in response to therapy. This last aspect concerns both the standard and the new therapies targeted towards precision medicine. The descriptions of the subtypes established since 2015 clearly indicate that all the groups involved in this definition of invasive bladder cancers identify a subset of tumors characterized by the expression of the typical (phenotypic) markers of the basal cells of the stratified urothelial epithelium and more particularly CK 5/6 and CK 14. Low levels of expression (RNA) of FOXA1 and GATA3 and proteins also characterize this tumor subtype. In addition, several bladder cancer working groups, as well as groups interested in other types of tumors, have shown that there are good markers for detecting the proteins encoded by these genes on paraffin-embedded tissue sections. Therefore, the group came to the conclusion of a consensus that a subgroup of invasive bladder cancers can be identified as CK5/6 + CK14 + FOXA1- GATA3- IHC outlook for a state of play in 2015. Basal-like bladder carcinomas are characterized by high expression of CK 5-6 (A), CK 14 (B) low or undetectable expression of FOXA1 (C) and GATA 3 (D). In contrast, normal urothelial cells strongly express FOXA1 (E) and GATA 3 (F). Adapted from S.P. Lerner et al. (2016). Comparison of bladder cancer classifications reported to the "BASQ" (Basal-squamouscar-like) consensus group. In the red background, the subtypes that are enriched in this group. Sub-classes of tumors in other colors (p53-like, TCGA II, Infiltrator) that include samples that would be included in the BASQ group and others that would not. Tumors in these three categories also express typical markers of urothelial differentiation to varying degrees. In red, the consensus definition of the subtype "BASQ". Each established intrinsic phenotype can diversify into a subgroup, responding to particular traits. Some are exclusive of each other: hypermutated / p53 mutated, some are non-exclusive: hypermutated / inflammatory. Phenotypic CK losses or gains are more or less pronounced or complete. Some phenotypes are EGFR, HER2 therapeutic targets.

After 2015

New concepts and new therapeutic approaches. High-throughput tumor genome sequencing. Molecular genetics has undergone a major revolution in recent years. This revolution is the consequence of technological advances applied to the sequencing of genomes in general, including that of humans. To know the complete sequence of nucleotide bases in a genome is to know the information necessary for normal life and its pathological variants. The implementation of the "Genome Programs" is linked to the extraordinary developments in sequencing techniques.

Historical

DNA sequencing made considerable progress in 1975. Two fun-

damentally different methods have been developed, one by the team of Walter Gilbert (Maxam and Gilbert 1977) in the United States, and the other by that of Frederick Sanger (Sanger, Nicklen et al. 1977) in Great Britain. These two methods are based on diametrically opposed principles: the Sanger approach is a selective enzymatic synthesis method, while Maxam and Gilbert's approach is a selective chemical degradation method. Gilbert and Sanger were awarded the Nobel Prize in Chemistry in 1980.

Next-Generation Sequencing (NGS)

The beginning of the 21st century is marked by the advent of the new generation of sequencing techniques. These new techniques are the result of advances resulting from the evolution of knowledge in physics, computer science, chemistry, nanotechnologies and biotechnologies. These innovations lead to a reduction in the cost and time required for partial or complete genome sequencing, based on the parallel grouping of miniaturized reactions. High-throughput sequencing (NGS) enables the rapid sequencing of thousands or even millions of DNA or RNA molecules simultaneously, by determining the unique and specific order of nucleic acid bases. This tool allows the sequencing of several genes and several individuals simultaneously, by comparing the patient's sequence to a reference sequence. NGS allows the analysis of large regions of interest, which was not possible with "classical" sequencing (Sanger method). Used since the 1980s, its application was limited to "gene-by-gene" sequencing approaches. NGS has enabled the development of new mutational analysis strategies, three main ones of which are currently in use: Analysis of "gene lists" or "gene panels": this is the simultaneous analysis of the sequence of a certain number of genes of interest (usually one or more dozens). As with Sanger sequencing, the analysis is usually focused on the coding regions of genes and the flanking intronic regions of exons, where deleterious mutations are located.

The "gene panel" approach is currently the most widely used in diagnostics, particularly for analysing lists of genes known to be involved in a type of cancer or a type of genetic disease. Some very large "gene panels" are marketed. They contain the majority of the genes involved in human pathology (more than 6,000) in the OMIM (Online Mendelian Inheritance in Man, www.omim.org) database. These "super-panels" are also called "clinical exomes." Whole Exome Analysis (WES): This approach consists of simultaneously analyzing all the coding sequences (and flanking intron regions) of all the genes in the genome (about 20,000), corresponding to about 1% of the genome, i.e. about 180,000 exons and 30 million base pairs. Whole Genome Analysis (WGS), i.e. 3 billion bases, includes both coding and non-coding sequences. It should be noted that some laboratories have chosen to set up "broad" sequencing strategies (OMIM panel, exome, or even genome for some pioneering Anglo-Saxon laboratories), and then to carry out a restricted filtering on the list of genes of interest, which allows a "opening" of the computer analysis filter to a larger list in the event of an inconclusive initial result. The choice of strategy currently

takes into account the capacities of the sequencers available to the laboratories, in connection with the costs of the analysis. The direct consequence of the increase in sequencing capabilities has been the considerable increase in the mutational data to be interpreted. For example, the analysis of an individual's exome generates an average of 25,000 variants compared to the reference sequence of the human genome.

The objective is to identify the deleterious mutation(s) responsible for the pathology presented by the patient. The challenge is to collect as much information as possible for each of the sequence variants identified and to carry out an interpretation at two levels: judging the pathogenic nature or not of the variants, and then their link with the pathology concerned. This essential step of the analysis, called "annotation", makes it possible to compile a wide variety of information (type of variant, heterozygous/homozygous/hemizygous status, description and frequency data from mutational databases, bioinformatics data predictive of pathogenicity, family segregation data, etc.), which will allow sorting filters to be applied. Despite the use of filters, the conclusion is not always obvious, and the validation of the involvement of a gene mutation(s) in the patient's pathology is largely optimized by a joint discussion of the results between geneticists and clinicians.

DNA or RNA

The DNA method is used to identify point mutations as well as small insertions, duplications, and deletions. NGS is particularly useful when several genes of interest need to be tested, which is the case in tumors. This method is not quantitative. In addition, large deletions and duplications as well as other chromosomal rearrangements are not always detected by this technique due to DNA fragmentation in formalin-fixed tumors. The DNA method shows a high sensitivity with a detection limit of 1% for hotspot mutations and 5% for other mutations. In a follow-up context, a lower detection limit may be applied (up to 0.1%). The RNA method is used to identify fusion genes ob-

tained when a sequence is fused with other sequences of the same or a different gene. However, the method is not quantitative. In addition, the analysis only detects rearrangements included in the panel used and cannot detect rearrangements with other genes not included. All these technical advances have made it possible to redefine the main categories of genomic alterations, to identify new tumor escape systems, and to propose new therapeutic approaches. These advances have benefited all forms of tumors, and in particular the most common ones.

Follow-Up to the Madrid Consensus

Therefore, since 2015, molecular alterations have been identified in key candidate genes concerning bladder tumor pathology. The differential frequency of alterations in these genes in NMIBC and MIBC initially supported a two-pathway pathogen model [7]. Recent advances in high-throughput genome technologies have enabled high-resolution analyses of DNA, RNA and proteins on a large number of tumours. The knowledge updated since 2015 on the molecular landscape of bladder cancer makes it possible to define the criteria for better surveillance of the disease. This molecular landscape heralds a shift towards personalized medicine and the development of new targeted or specific therapies. An important point would be to define for tumors that do not infiltrate the muscle (pTa and pT1) predictive phenotypic signals of transition to deep infiltrating mode.

Mutational Landscape

Studies of candidate genes have identified key genes involved in bladder cancer, including FGFR3, PIK3CA, CDKN2A, TP53, TSC1, RB1, STAG2, and the RAS gene family. Next-generation sequencing (NGS) studies have confirmed the presence of frequent alterations in well-characterized cases, and have also revealed other oncogenes and tumor suppressor genes (Table 3) [31]. These studies have provided insight into the mutational processes that shape the genomes of bladder tumors.

Table 3: From Knowles MA, Hurst CD. Molecular biology of bladder cancer: new insights into pathogenesis and clinical diversity. *Nat Rev Cancer* 2015; 1525–41 ; [31].

- a) The genes mutated at $\geq 10\%$ in the studies of Hurst, et al. [35] and The Cancer Genome Atlas (TCGA) 2017 [41] are presented.
- b) Data from Hurst et al [35]. The sample cohort includes 79 TaG1/TaG2 tumors and 3 TaG3 tumors. Exome sequencing of 24 tumors and targeted sequencing of 58 tumors was performed. The frequency of mutations is shown in parentheses when only exome sequencing data are available.
- c) Data from Nordentoft et al. [36] Exome sequencing was performed on 20 TaG1/TaG2 tumors.
- d) Data from Pietzak et al. [51] Targeted sequencing of 55 Ta tumors (23 grade 1/2; 32 grade 3) and 38 T1 tumors was performed.
- e) Data by Guo et al. [46] Exome sequencing was performed on 32 T1 tumors.
- f) Data from the 2017 TCGA. (41) Exome sequencing was performed on 412 MIBCs.

Frequently Mutated Genes in NMIBC and MIBC						
Gene ^a	Hurst, et al. [2017] ^b	Nordentoft, et al. [2017] ^c	Pietzak, et al. [2017] ^d	Pietzak, et al. [2017] ^d	Guo, et al. [2013] ^e	TCGA 2017 ^f
	Ta(%)	Ta (%)	Ta(%)	TI(%)	TI(%)	MIBC(%)
FGFR	79	40	66	30	25	14
PIK3CA	54	25	36	22	6	22
KDM6A	52	65	50	43	50	26
STAG2	37	25	24	22	25	14
KMT2D	30	15	31	26	0	28
ARIDIA	18	35	25	27	6	25
EP300	18	25	20	8	16	15
CREBBP	15	20	23	19	12	12
KMT2C	15	20	16	5	3	18
RHOB	13	0	ND	ND	0	11
HRAS	12	10	2	8	16	9
KMT@A	11	0	9	11	9	11
TSC1	11	5	5	22	12	8
BRCA2	10	0	11	11	0	7
COL11A1	10	0	ND	ND	0	5
RDM10	10	20	22	5	0	9
TP53	4	5	11	35	25	48
FAT1	(2)	10	13	17	0	12
KRAS	2	0	11	8	6	4
ATM	(1)	5	13	19	3	14
CDKN1A	(1)	0	11	13	0	9
ELF3	(1)	25	ND	ND	12	12
ERCC2	(1)	0	21	13	6	9
ERBB2	(0)	0	11	19	3	12
ERBB3	(0)	0	9	19	3	10
RB1	(0)	5	0	5	9	17

Reminder: NMIBC, non-muscle-invasive bladder cancer; MIBC, muscle-invasive bladder cancer; ND, the genes were not covered by the target capture design.

Mutation Rates, Mutational Signatures and Mutational Processes

It should be remembered that the 2014 TCGA study (reported above) involved 131 chemotherapy-free MIBCs. It showed a high rate of somatic mutations (mean and median somatic mutation rates of 7.7 and 5.5 mutations per megabase [Mb], respectively) [32], similar to those reported for non-small cell lung cancer and melanoma [33,34]. This was confirmed in the expanded TCGA study of 412 tumors where mean and median mutation rates of 8.2 and 5.8 mutations per Mb were recorded, respectively [34]. In the NMIBC, mu-

tation rates are much lower, with studies reporting 2.4 (mean) and 1.64 (median) overall mutations per Mb in Ta tumours [5] and 1.8 non-synonymous mutations per Mb (median) in a cohort of stage Ta and T1 tumours [35]. Beyond the overall “burden” of mutation, the pattern of base changes (the mutational signature) can provide insight into the underlying processes. To date, there are 30 such signatures in the COSMIC (Catalogue of Somatic Mutations in Cancer) database. (<http://cancer.sanger.ac.uk/cosmic/signatures>). Bladder cancer is strongly linked to smoking, but genome sequencing studies have not detected the most common signature linked to smoking. The latter is characterized by C > A transversions (COSMIC 4 signature).

Instead, studies suggest that APOBEC activity and deficiencies in the nucleotide excision repair pathway (NER) may contribute to the mutation spectra seen in bladder cancer. The APOBEC signature is one of the most important mutation signatures in cancer, it is present in more than half of human tumors, is called the 2/13 signature, and derives from the activity of cytosine deaminases APOBEC3A (A3A) and APOBEC3B (A3B), which preferentially deaminate cytosines immediately preceded by a thymine.

A signature characterized by C > T and C > G mutations at TCW motifs, where W is A or T, has been attributed to the activity of the APOBEC family of cytosine deaminases [36]. Several studies have found that mutational loads in NMIBC and MIBC are primarily induced by APOBEC-mediated mutagenesis [32,35,37-41]. APOBEC3B expression is correlated with APOBEC mutagenesis in MIBC and in NMIBC, APOBEC3A and APOBEC3B expression was associated with a specific expression subtype [39]. In a recent study in primary pTa tumors, APOBEC3H expression was implicated and showed an association with a copy number subtype [37]. ERCC2 is a DNA helicase, which plays a central role in the NER pathway. Kim et al. [42] reported that ERCC2 mutation status was significantly associated with a mutational signature characterized by a broad spectrum of base changes. Examination of three independent cohorts composed mainly of MIBC revealed a strong association between ERCC2 somatic mutations and this signature, suggesting that this is due to the loss of normal NER function. The presence of the signature was associated with exposure to tobacco, which creates DNA additions that are usually repaired by the NER pathway.

In the 2017 TCGA study, matrix factorization analysis of single nucleotide variants (SNVs) classified into 96 basic substitution types in the context of trinucleotides was used to identify processes contributing to the high mutation rate [41]. This revealed five mutation signatures including two variants of the APOBEC mutagenesis signature (APOBEC-a and APOBEC-b) that accounted for 67% of all SNVs. Among the other signatures, one was characterized by C > T transitions at the CpG motifs, probably resulting from the spontaneous deamination of 5-methylcytosine. A POLE signature in a hypermutated sample that carried a functional mutation in POLE (DNA polymerase epsilon) mutation that is expected to affect its proofreading activity and ERCC2 signature [42]. Unsupervised pooled mutation signature analysis identified four clusters (MSig1 to MSig4). Patients with MSig1 cancers are characterized by a high APOBEC signature mutation burden, a high mutation burden that presumes a high neoantigen load, with the highest probability of 5-year survival (75%). Patients with MSig2 cancers, who had the lowest mutation rate, had the lowest probability of 5-year survival (22%). The MSig4 samples showed enrichment of ERCC2 signature mutations and ERCC2 mutations.

FGFR3, PIK3CA and RAS Genes

The identification of FGFR3 (fibroblast growth factor receptor 3) mutations in bladder tumors in 1999 sparked major interest in this

receptor. Over the next 20 years, much was learned about the mutational profiles of bladder cancers, the associated phenotypes, and the potential of this mutated protein as a therapeutic target. Based on mutation and expression data, it is estimated that > 80% of non-muscle-invasive bladder cancers (NMIBCs) and ~ 40% of muscle-invasive bladder cancers (MIBCs) have upregulated FGFR3 signaling, and these frequencies are likely to be even higher if alternative receptor splicing, ligand expression, and changes in regulatory mechanisms are considered. Significant efforts by the pharmaceutical industry have led to the development of a range of agents targeting FGFR3 and other FGF receptors. Several of these have entered clinical trials, some have shown very encouraging early results in advanced bladder cancer. Recent reviews have summarized treatments and related clinical trials in this area. Thus, activating point mutations of (FGFR3) are present in ≥ 70% of Ta cases [43]. These are located in the codons at hotspots of exons 7, 10 and 15 and activate the receptor. The frequency of mutations is lower in T1 NMIBC (10% to 45%) and MIBC (about 15%) (Table 3) [43]. In high-grade T1 (T1G3) tumors, FGFR3 and TP53 mutations exhibit an independent distribution, unlike the situation in Ta tumors where these mutations are virtually mutually exclusive [44,45].

Mutations are also found in urothelial papilloma, a likely precursor to superficial NMIB carcinoma [43]. Increased expression of the mutant protein FGFR3 is common in these tumors. Although only 15% of MIBCs have an FGFR3 mutation, protein expression is upregulated in 40% to 50% of non-mutant MIBCs [43]. An alternative mechanism for FGFR3 activation in a subset of cases (2 % to 5 %) is chromosomal translocation that generates fusion proteins [41,46,47]. In cultured normal human urothelial cells, expression of mutant FGFR3 leads to activation of the RAS-MAPK pathway and PLCγ, leading to cell proliferation and suggesting a possible contribution of FGFR3 activation to urothelial hyperplasia in vivo [43]. The phosphatidylinositol 3-kinase (PI3K) pathway plays a central role in tyrosine kinase receptor signaling. Activating mutations in the catalytic subunit p110α (PIK3CA) are common for low-grade TNM pTa classifications (about 40% to 50%), compared to pT1, NMIBC and MIBC (about 20%) (Table 3) [48]. E542K and E545K missense mutations in the helical domain are the most common (22% and 60%, respectively), and the H1047R kinase mutation, which is the most common mutation in other cancers, is less common. A recent NGS-based study of the primary stage pTa NMIBC reported that 17 of the 48 PIK3CA mutations detected, some of which confirmed gain of function, were not found in these three major hotspot codons [37].

This justifies caution when using tests that only detect mutations in these hotspots, in particular in a clinical trial. Activating hotspot mutations in the RAS gene family occur most often in HRAS or KRAS and, unlike FGFR3 and PIK3CA mutations, are not associated with either NMIBC or MIBC (mutations in approximately 10% of the total) (Table 3). The PIK3CA mutation usually coexists with the FGFR3 or RAS mutation in the NMIBC [35,49]. However, the RAS and FGFR3

mutations are mutually exclusive, [50] possibly reflecting the fact that both activate the RAS-MAPK pathway. A 2017 study reported mutually exclusive alterations in FGFR3 and receptor tyrosine kinase ERBB2 in 57% of high-grade UC (Ta, T1, CIS) [51].

Telomerase Reverse Transcriptase Promoter

Mutations in the telomerase reverse transcriptase promoter (TERT) represent the most common genomic alteration identified in urothelial carcinomas to date. They occur at a high frequency (60% to 80%) at all stages and grades [52,53]. The high frequency of mutations suggests that it is an early event and a requirement in all pathways of urothelial tumorigenesis. Interestingly, the frequency of these mutations in early diseases (in young subjects) would be much lower (46%), perhaps suggesting different mechanisms of tumorigenesis in young patients. The mutations are mainly found in hotspot zones -124bp[G>A] and -146 bp [G > A] compared to the starting site of ATG translation, which has favored the design of robust detection methods. The ease with which these mutations can be detected in urinary sediments [52,53] is likely to make a major contribution to the development of non-invasive urine tests for the detection of bladder tumors of all grades and stages in disease diagnosis and surveillance settings. These mutations create binding sites for ETS/TCF transcription factors and are believed to increase transcriptional activity. [54]. The effect of mutation on the likelihood of disease recurrence has been shown to be altered by the presence of a common polymorphism (rs2853669) in a pre-existing ETS/TCF binding site in the promoter region, with mutations in the absence of the variant allele being associated with increased recurrence in NMIBC.26 [55].

TP53, RB1 et CDKN2A

As in other aggressive cancers, the tumor suppressor genes TP53, RB1 and CDKN2A are involved in MIBC. Pathways controlled by p53 and RB1 regulate cell cycle progression and stress responses. The TCGA study reported alterations in the p53/cell cycle pathway, including TP53 mutation, MDM2 amplification or overexpression, RB1 mu-

tation or deletion, and CDKN2A mutation or deletion, in 89% of MIBC [41]. TP53 is the most commonly mutated gene in MIBC (about 50%) [11]. Mutations are very uncommon in low-grade Ta tumors (about 1%), but occur at a higher frequency in T1 tumors (Table 3) [56]. The detection of a mutation or accumulation of the p53 protein is associated with a poor prognosis. IHC detection of p53 with an increased half-life identifies many mutant p53 proteins has been commonly used as a surrogate marker for mutation. But some TP53 mutations (about 20%) produce unstable or truncated proteins that cannot be detected in this way. Thus, overexpression of the p53 protein is not a useful prognostic marker. Two meta-analyses indicate only a small association between p53 positivity and a poor prognosis [57,58]. However, examining both the protein expression and the TP53 mutation provides more useful prognostic information [59]. The RB pathway regulates cell cycle progression from the G1 phase to the S phase.

Suppression of 13q14 and loss of expression of the RB1 protein are common in the MIBC [31]. Loss of p16 expression is inversely related to positive expression of RB1, and high-level expression of p16 results from negative feedback in tumors with loss of RB1. Thus, loss of expression and high-level expression of p16 are associated with dysregulation of the RB pathway, and these are unfavorable prognostic biomarkers found in > 50% of MIBC.1 Interestingly, in MIBC with FGFR3 mutation, a high frequency of homozygous CDKN2A (HD) deletion has been reported, which could identify a pathway of progression of non-invasive FGFR3 mutant tumors towards muscle invasion via loss of CDKN2A [41,60,61]. Amplification and overexpression of E2F3, which is normally repressed by RB1, is associated with the loss of RB1 or p16 MIBC proteins [62]. The RB and p53 pathways, are connected by p16 and p14ARF due to multiple feedback mechanisms, Simultaneous inactivation of both pathways is expected to have a greater impact than inactivation of either pathway alone. This is confirmed by obtaining greater predictive power in studies using simultaneous analyses of multiple changes that deregulate the G1 checkpoint [63] (Figure 3).

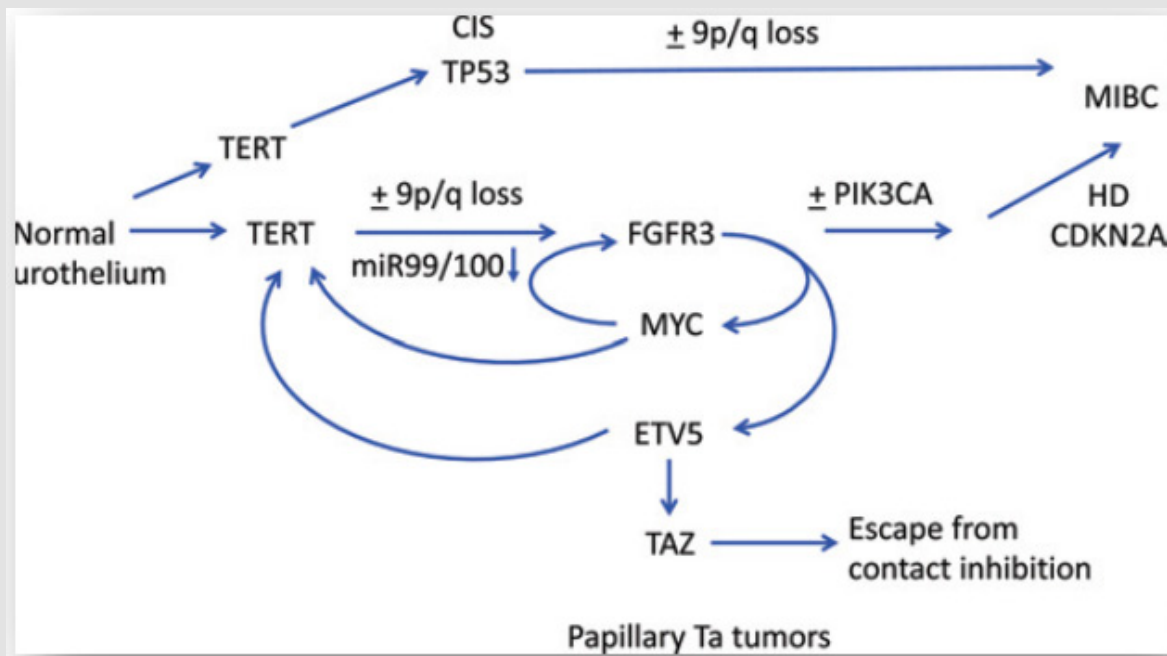


Figure 3 : Hypothetical pathways of pathogenesis of non-invasive and invasive bladder lesions. The right arrows indicate the likely occurrence of selective events during NMIBC and MIBC development, on dysplasia, CIS, and NMIBC and MIBC data. FGFR3, PIK3CA: activation mutation. TERT: point mutation of the promoter. TP53: Inactivation mutation. HD: homozygous deletion. The curved arrows indicate an interactive regulation of the expression of FGFR3, TERT, MYC, ETV5 and TAZ. From Margaret A. Knowles: FGFR3 – a Central Player in Bladder Cancer Pathogenesis? Bladder Cancer 6 (2020) 403–423.

Genes Involved in Chromatin Modification and Architecture (CM)

One of the key findings of genome sequencing studies in bladder cancer is the high frequency of mutations in chromatin-modifying (CM) genes, including histone demethylase (KDM6A), histone methyltransferases (KMT2A, KMT2C, KMT2D), histone acetyltransferases (CREBBP, EP300), SWI/SNF complex genes (ARID1A, ARID4A), and polycomb group genes (ASXL1, ASXL2) (Table 3). Mutations of this type were reported in the first exome sequencing study of bladder carcinoma [64] and subsequent studies have demonstrated that such mutations are common in tumors of all stages and grades and are more common in the NMIBC [36,37,51]. Many mutations in these genes are inactivating (small deletions/insertions, nonsense, essential splicing site). ARID1A, CREBBP and KDM6A are also targets in large deletions, suggesting that all of these genes have a tumor-suppressing function [41]. KDM6A, a histone demethylase that catalyzes the demethylation of tri-/dimethylated histone H3 lysine 27 (H3K27me_{2/3}), is the most frequently mutated CM gene in NMIBC (35, 36, 51). With mutations occurring at a higher frequency (38% to 65%) than in MIBC (26%) (Table 3) KDM6A associates with KMT2C/D in a COMPASS-like complex, which acts to maintain gene expression. KMT2C/D are methyl transferases that write the H3K4me₃ brand name associated with active promoters and the H3K4me brand name associated with activators. The predicted effect of the inactivation of these genes is therefore

to silence transcription via effects on both promoters and amplifiers.

Loss of KDM6A results in enrichment of the PRC2-regulated signaling pathway and, therefore, PRC2-enhanced or null KDM6A cells are susceptible to EZH2 inhibition [65]. Studies have implicated the loss of KDM6A function as an early event of tumorigenesis [36,66]. KDM6A (Xp11.3) has been reported to show more mutations in noninvasive tumors in females than males, possibly indicating sex differences in the epigenetic landscape of the normal bladder [35]. Interrogation of exome sequencing data did not reveal a similar association in MIBC. Inactivating mutations in ARID1A are associated with high-grade bladder cancer (Table 3) [67]. Patients with tumors with ARID1A mutations have significantly shorter recurrence-free survival after bacillus Calmette-Guérin (BCG) induction therapy [51]. Further studies are needed to elucidate whether ARID1A mutations can predict response to BCG or whether they simply identify a subset of patients with poor prognosis. A recent study of clear cell carcinoma of the ovary (OCCC) showed that treatment with an EZH2 methyltransferase inhibitor (GSK126) is synthetically lethal in the mutant ARID1A OCCC [68]. Such treatment could represent a valid approach in urothelial carcinoma with inactivated ARID1A.

STAG2

Inactivating mutations in the STAG2 (Xq25) cohesin complex were identified in NMIBC and MIBC by single-gene analyses [40,41]

or whole exome sequencing (Table 3) [37,46,48]. Cohesion plays an important role in ensuring precise chromosome segregation during mitosis and in some types of cancer, the STAG2 mutation is associated with aneuploidy [69]. However, other studies on bladder cancer have reported conflicting results. Some have reported that these mutations are more common in NMIBCs with stable genomes [37,67,70] while others have associated the loss of STAG2 with aneuploidy [46,71]. The relationship between STAG2 mutation status and prognosis is also ambiguous, with two studies reporting a poorer prognosis for patients with STAG2 mutant tumours [46,72] and another indicating that STAG2 loss was associated with a better prognosis for patients with NMIBC or MIBC (68). This may reflect differences in the stages and grades of tumor cohorts used in individual studies and/or an alternative role of cohesin in bladder cancer [73]. For example, in addition to its role in chromosome segregation, cohesin plays a role in anchoring the base of chromatin loops to facilitate long-distance chromatin interactions that regulate transcription. As STAG2 mutations are often associated with mutations in other chromatin rearrangement genes, the loss of function may contribute to a general pattern that tends to silence genes.

DNA Damage Repair (DDR) Alterations

DNA-targeting agents play an important role in the treatment of NMIBC and MIBC. Mitomycin C is an alkylating agent that is administered intravesically following transurethral resection of NMIBC. First-line systemic chemotherapy for MIBC involves the use of platinum-based DNA-targeting agents, such as cisplatin administered in an adjuvant or neoadjuvant setting. The therapeutic efficacy of these drugs exploits deficiencies in DNA repair pathways. There is a strong interest in exploring alterations in these pathways and identifying markers that can help guide therapy. Somatic mutations in DNA damage repair (DDR) genes, including ATM, ATR, ERCC2, ERCC4, BRCA1, BRCA2, CHEK2, PALB2, POLE, FANCA, FANCC, FANCD2, FANCM, and MSH6 have been reported in NMIBC and MIBC (Table 3) [35,41,51,68]. In the 2017 TCGA study, the most frequently mutated DDR genes identified were ATM (14%) and ERCC2 (9%) (Table 3). Recurrent somatic mutations in ERCC2 have been reported in several studies [42,46,51,74] and are associated with better outcomes in patients treated with cisplatin [74]. A recent study using a targeted capture-based NGS assay, MSK-IMPACT, identified a high frequency (30%) of DDR gene alterations, including ERCC2, in the high-grade NMIBC [51].

These alterations were associated with a higher mutational load and a higher neoantigen load, suggesting that treatment with BCG or immune checkpoint inhibitors may be promising therapeutic approaches in these patients. The expression levels of the components of the DDR pathway have been extensively studied in relation to outcomes, prognosis, and response to treatment. For example, low expression of the ERCC1 NER pathway component has been associated with better overall survival in patients with metastatic urothelial carcinoma treated with cisplatin-based chemotherapy [75]. Similarly,

high levels of MRE11A, a protein in the homologous recombination pathway involved in the repair of double-strand breaks, were associated with better outcomes in patients treated with radiotherapy [76].

Structural Alterations in the Genome

Structural alterations in the bladder tumor genome include allelic loss, DNA copy number gains and losses, and rearrangements. The genomes of NMIBC and MIBC are very different. NMIBCs, especially pTa tumors, are usually diploid or quasi-diploid and have very few copy number alterations [35]. On the contrary, MIBCs can be highly aneuploid and exhibit many genomic alterations [41,45]. It should be noted that some pT1 tumors exhibit MIBC profiles, suggesting that these tumors are able to cross the basement membrane and be aggressive. However, other pT1 tumors show remarkable similarity in their copy number profiles to pTa tumors, suggesting that there are distinct biological subgroups [45]. The most common genomic alteration in NMIBC and MIBC is loss of heterozygosity (LOH) or loss of chromosome 9 copy number. More than 50% of UCs of all grades and stages have a loss of chromosome 9. One critical region on 9p contains CDKN2A (9p21), which encodes the two cell cycle regulators, p16 and p14ARF (31). The p16 gene, let's remember, is a negative regulator of the RB pathway and p14ARF is a negative regulator of the p53 pathway. TSC1 is the best-validated 9q tumor suppressor gene, with biallelic inactivation in approximately 12% to 16% of cases [47,48].

TSC1 in complex with TSC2 downregulates the rapamycin target mammalian branch (mTOR) of the PI3K pathway. High-level DNA amplification is uncommon in NMIBC. It is mainly associated with high-grade and pT1 tumors with gains of 20q [45,77]. Other alterations reported for NMIBC pTa include losses of 10q, 11p, 11q, 17p, 19p, and 19q and gains of 20q [45,78]. On the contrary, MIBC genomes are very complex, with many alterations and rearrangements of copy number [41,45,79]. High-level amplifications are common, with candidate regions containing key genes involved in bladder cancer, including CCND1, CCNE1, E2F3, EGFR, ERBB2, FGFR3, MDM2, MYCL1, PPARG, and YWHAZ [11,15,33,50]. The most common region of homozygous deletion (HD) reported is 9p21 containing CDKN2A. Other key regions of HD include 10q23 containing PTEN, a key PI3K channel regulator, and 13q14 (RB1). Recurrent focal deletions at 14q24 containing RAD51B are also reported [41].

Genome doubling (tetraploidy) in combination with increased tolerance to chromosomal aberrations is proposed to explain the accelerated evolution of the cancer genome [80]. This event has been reported in bladder cancer [41,81]. The 2017 TCGA study reported that TP53 mutations were enriched in tetraploid tumors, suggesting the responsibility for P53 loss in this process [41,81]. A long series of unpublished personal measurements, carried out with a modified Feulgen DNA quantification technique, to monitor bladder tumors in cytology, have allowed us to observe that many urothelial tumors are likely to progress to tetraploidy (reduplication) and then to descend to a triploid intermediate state after having lost in aneuploidy many

sequences or chromosomal fragments that are useless to the aneuploidy, their progression and aggravation. (B. Dutillaux, Y. Remvikos and G. Leseq). Several gene fusions have been reported in bladder cancer. The most common is an intrachromosomal FGFR3-TACC3 fusion [41,46,47]. All fusions identified to date show a loss of the final exon of FGFR3 with frequent fusion in the frame to transform the coiled acid containing protein 3 (TACC3). These fusion proteins are highly activated and transforming oncogenes [47]. Other FGFR3 mergers include FGFR3-BAIAP2L117.53 and FGFR3-TNIP2 [51]. FGFR3 fusions have primarily been reported in MIBC, but have also been found in two NMIBC-derived cell lines and a low-grade pTa tumor [51]. The 2017 TCGA study reported mergers involving PPARG (TSEN2-PPARG, MKRN2-PPARG), with PPARG expression being higher in samples with fusions than without fusion [41]. The majority retained the DNA and ligand binding domains of PPARG, suggesting that they are functional. Two other studies described the activation of PPARG in bladder cancer cells by PPARG amplification or mutation or RXRA S427F mutation, suggesting that PPARG may represent a therapeutic target candidate in these tumors [82,83].

Heterogeneity and Clonal Evolution

Multifocality and/or the development of multiple recurrent tumors in the same patient is a common feature of bladder urothelial tumors. Although some patients develop more than one molecularly distinct tumor (oligoclonal disease) [84] in most cases, tumors in the same patient are related (monoclonal) [85]. Several NMIBC studies have sequenced individual tumors, synchronous multifocal tumors, primary and recurrent tumors from the same patient, and samples collected before and after disease progression [36,38,40,85]. Higher within-patient variation in tumor mutation spectrum and frequency of APOBEC-related mutations has been reported in patients with progressive disease, implying that APOBEC activity in these tumors was a subsequent tumor-specific event [40]. Monoclonality was also confirmed in this study. Nordentoft et al [36] analyzed paired samples from patients with progressive disease and showed that, although non-invasive and invasive tumors share several identical mutations indicating a common origin, progressing tumors also showed large divergence [36].

Recent genome sequencing studies have revealed a lot of information regarding intratumoral heterogeneity (ITH), with clonal diversity strongly associated with higher stage and grade, with a much higher level of heterogeneity in metastasis [86]. Multiregional analysis of cystectomy samples from patients with multifocal or unifocal disease also revealed higher spatial heterogeneity in multifocal lesions [87]. Analysis of adjacent “normal cells” in this study detected more mutations in the samples in patients with multifocal disease than in those with single-vision disease. Some interpret it (botanical hypothesis) as a “radicular” intraepithelial migration or seeding from vegetative tumors. The presence of genomic alterations in the morphologically normal urothelium in tumor-bearing bladders has been widely reported [88,89]. When the normal cell population uniformly contains

alterations, this has also been interpreted as a clonal expansion of the altered cells in the urothelium to generate “fields” of abnormal cells within which tumor development occurs as a result of the acquisition of additional modifications. Intratumoral heterogeneity was also assessed in 2016 [90] in 16 matched sets of primary and advanced tumors prospectively collected before and after chemotherapy. Intra-patient mutational heterogeneity in samples collected after chemotherapy is evident, with most mutations not shared with samples collected before treatment. Caution is therefore necessary when using primary specimens to guide the treatment of metastatic disease. Molecular subtyping therefore plays an important role in providing a prognosis but also in predicting the response to treatment in patients with bladder cancer.

Current subtyping studies are mainly based on the study of DNA or RNA from tumor tissues or cells. However, traditional sequencing technology is based on total tumor tissue rather than a cell-by-cell study; However, intratumoral heterogeneity can be an important factor affecting the accuracy of subtyping. Warrick et al. [91] conducted a study of 309 bladder cancer markers again in 2019. They found that 83 of them had intratumoral variation. Then, these 83 markers were subtyped with the Lund system. More than one-third (39%) had molecular heterogeneity. Finally, these 83 samples were divided into “urothelial-like”, genomically unstable, baso-squamous, mesenchymal, and neuroendocrine. The basosquamous subtype shows the greatest variability; Approximately 78% of these tumors simultaneously exhibit the genomically unstable or “urothelial like” profile. It is therefore necessary to take note of the frequency of heterogeneity in bladder cancer, which is related to variation and complexity in molecular subtypes. This also affects prognosis and response to treatment. It is therefore necessary to pay particular attention to it.

Molecular Subtypes and Phenotypes Attempt at Integration

Bladder tumors of similar grade and stage have divergent clinical behavior. In particular, pT1 tumors have a high molecular and clinical diversity. Until recently, we have seen that molecular characteristics have failed to explain or predict this divergence. The two tumor groups (NMIBC and MIBC) that have dominated the bladder cancer literature for so long are clearly insufficient. We have seen in the above that recent studies based on DNA and RNA (transcriptome) of the whole genome have undertaken to unravel this complexity, revealing several subgroups. These subgroups, it should be remembered, are independent of conventional groupings based on grade and stage, hence the interest in identifying them to guide treatments. The following sections describe the main results of these studies to date. Some have already been cited as a contribution to the 2015 Madrid Consensus

Subtypes Identified by DNA Abnormalities

DNA copy number and mutation status identified several tumor subgroups in the conventional grade and stage groups [45]. But the

expression-based subtypes and classification signatures are not yet fully defined in 2012. Analysis of copy number data from 49 high-grade pT1 tumors initially identified three clusters, one of which was associated with disease progression [45]. This study also separated 58 pTa tumors into two copy number groups. Later in 2017 in a larger tumor panel (n=140), the existence of two major genomic subtypes of primary pTa tumors with different copy numbers was confirmed [37]. Genomic subtype 1 (GS1) contained little or no copy number al-

terations, whereas genomic subtype 2 (GS2) was characterized by a higher level of genomic instability, particularly the loss of 9q (including the TSC1 genomic region). Whole-exome analysis and targeted sequencing revealed that GS2 tumors have a higher mutation rate, enrichment of APOBEC-related signatures, and more mutations in TSC1. Consistent with the loss of one or both copies of TSC1 (a regulator of mTORC1 activity), GS2 tumors had upregulated mTORC1 signaling (Figures 4 & 5).

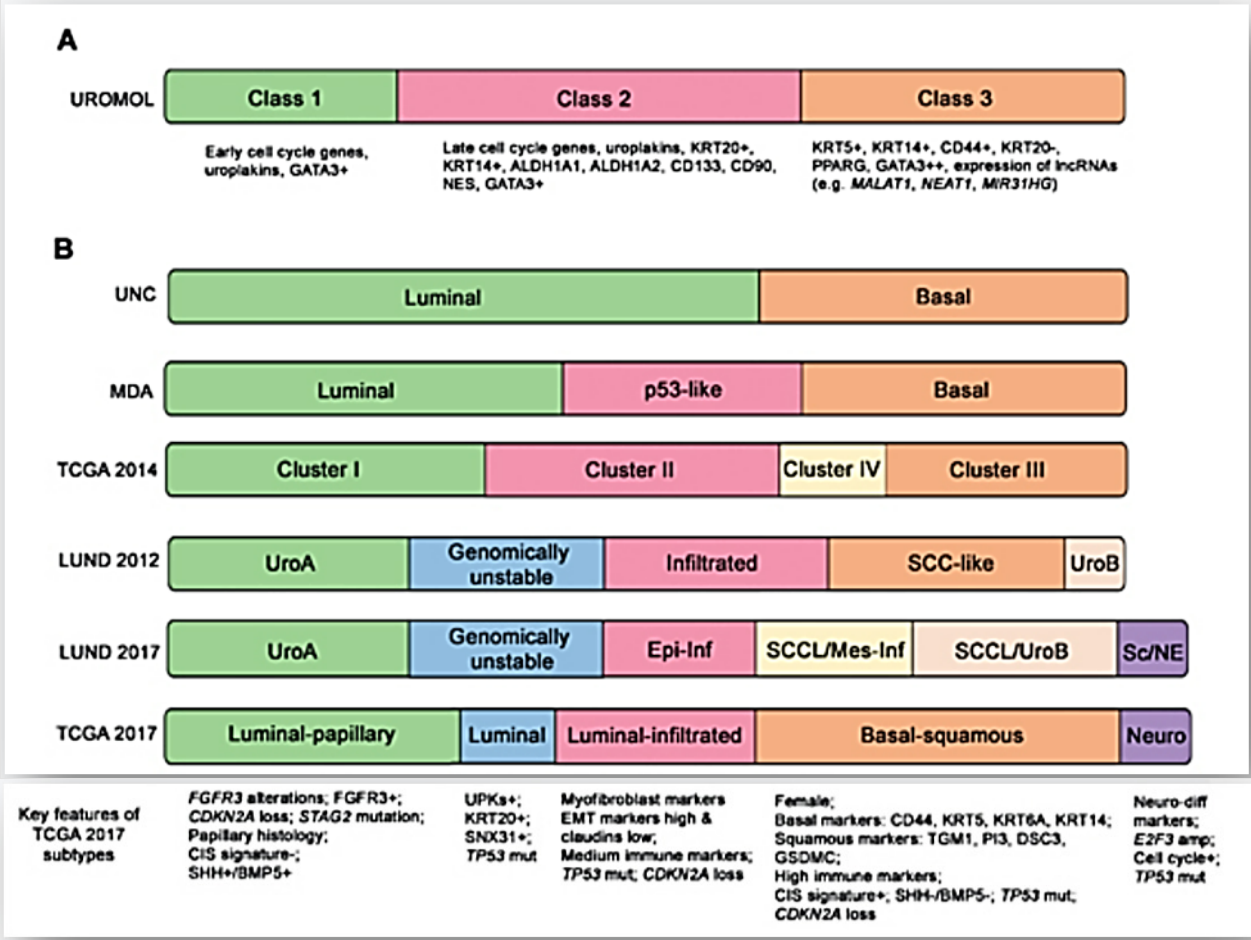


Figure 4 : Molecular subtypes of bladder cancer. after Carolyn D. Hurst and Margaret A. Knowles Three subtypes of non-muscular bladder cancer (NMIBC) messenger RNA (mRNA) expression (class 1, class 2 and class 3) were defined by the UROMOL study [39]. The main molecular characteristics of each subtype are presented. Overview and proposed overlap of mRNA expression subtypes defined in six studies conducted by four groups: MD Anderson Cancer Center (MDA), [99] University of North Carolina (UNC), [100] Lund University (LUND), [96,104], and The Cancer Genome Atlas (TCGA) [32,41]. The LUND studies included both NMIBC and muscle-invasive bladder cancer (MIBC), while the MDA, UNC, and TCGA studies included MIBC only. The newly described neuronal (TCGA) and small cell/neuroendocrine (Sc/NE) (LUND) subtypes are shown on the right side of the figure. The overlap of this subtype with the other subtypes described is not yet fully defined. The main molecular characteristics of the five subtypes recently described in the TCGA 2017 study are presented in the bottom panel. UroA, urobasal A; SCC, squamous cell carcinoma; UroB, urobasal B; Epi-Inf, infiltrated epithelial; Mes-Inf, mesenchymal infiltrator; SCCL, squamous cell carcinoma type.

Univ. North Caroline	Basal		
MDA Collège Andersson Houston Texas USA	BASAL		P53Like
TGCA (Genome Atlas)	III	IV	II
Lund Sweden	SCC like		Infiltr
CIT France & Col. BAYLOR Houston Texas USA	BASAL-like		
Consensus Madrid 2015	BASQ (basal squamous like) CK 5/6+/CK 14+ FOXA1- GATA 3-		

Dr Guy Lesec d'après S.P. Lerner et al.

Figure 5: Comparison of bladder cancer classifications reported to the “BASQ” (Basal-squamouscar-like) consensus group. In the red background, the subtypes that are enriched in this group. Subclasses of tumors in other colors (p53-like, TCGA II, Infiltrator) that include samples that would be included in the BASQ group and others that would not. Tumors in these three categories also express typical markers of urothelial differentiation to varying degrees. In red, the consensus definition of the subtype “BASQ”.

Transcriptome-Based Subtypes

Initial evaluation of messenger RNA (mRNA) expression profiles of urothelial carcinomas of all grades and stages by the Lund group identified two major molecular subtypes (MS1 and MS2), separated primarily, but not entirely, by grade and stage with pT1 tumors distributed with relative equality between the two subtypes [92,93]. The same group then reported a new molecular taxonomy of urothelial carcinomas based on transcriptional profiling of 308 bladder tumors of all stages and grades [94]. Five main subtypes have been identified: urobasal A (UroA), urobasal B (UroB), genogically unstable (GU), squamous cell carcinoma (SCCL) and “infiltrated” (Figure 4). The tumors in the latter group were highly infiltrated with non-tumor cells, whereas the definition of the other groups reflected tumor cell-specific criteria. Clear differences in the expression of cell cycle regulators, keratins, receptor tyrosine kinases, and cell adhesion molecules were evident. The UroA and UroB subtypes expressed elevated levels of FGFR3, CCND1, and TP63; GU tumors had low levels of expression of these proteins but expressed high levels of ERBB2 and E-cadherin, and SCCL tumors expressed P-cadherin and high levels of KRT5, KRT14, and proteins involved in keratinization. These subtypes have distinct clinical outcomes. UroA has a good prognosis, GU has an intermediate prognosis, and SCCL and UroB have the worst prognosis.

UroB tumors shared epithelial features with UroA tumors, including the FGFR3 mutation, but they also had the TP53 mutation and were often invasive. Tumors classified as pT1 appeared uniformly distributed among molecular subtypes. Lund’s group then reported immunohistochemical markers that could distinguish these subtypes [94].

In the first part of this review we cited the RNA profiling studies of MIBC reported for the Madrid conference by three groups (MD Anderson Cancer Center [MDA], University of North Carolina [UNC], TCGA). These studies have all identified two major subtypes that show considerable overlap with the Lund subtypes (Figure 4) [32,95,96]. These “luminal” and “basal” subtypes respectively express markers of urothelial differentiation and normal basal cells of the urothelium, They show a similarity emphasized by several authors with the basal and luminal subtypes of breast cancer [96,97]. Basal tumors typically express high levels of KRT5, KRT6, KRT14, and CD44, and luminal tumors are characterized by high expression of FGFR3, uroplakin, and transcription factors PPARG, GATA3, and FOXA1. The previously cited MDA group also described a “p53-like” subset of luminal MIBC characterized by the expression of luminal markers and genes expressed by cancer-associated fibroblasts, which corresponds to the infiltrated subtype described in Lund’s study. The first TCGA study of 131 MIBCs identified four clusters (I to IV), enriched with luminal (I and II) and basal (III and IV) markers. Clusters I and II corresponded to the lu-

linal and “p53-like” subtypes, respectively, described in the MDA study. Group III overlapped with the basal subtype, with group IV being similar to the “claudine low” breast cancer subtype [97]. These expression subtypes showed relationships with outcome and therapeutic response. The TCGA reported on the most comprehensive “omics” study to date [41]. It confirms the overlap between the basal and luminal subtypes and it refines and adds to the current consensus. Five subtypes based on mRNA expression (luminal, luminal-papillary, luminal-infiltrated, basal-squamous and a new “neuronal” subtype) have been identified (Figure 4). The majority of tumors in the luminal subtypes express high levels of uroplakins (UPK1a and UPK2) and markers of urothelial differentiation (FOXA1, GATA3, PPARG).

The luminopapillary subtype consists mainly of tumors with papillary architecture, stage less than T2 and of high purity. They are characterized by FGFR3 overexpression, enriched for FGFR3 mutations and amplification and FGFR3-TACC3 fusions. A low CIS expression signature score [98] and high expression of genes involved in Sonic Hedgehog signaling (SHH and BMP5) are also characteristic. Based on these observations, it was suggested that tumors in this group may have developed from the precursor NMIBC. Uroplakins are highest in the luminal subtype, as are genes expressed in differentiated superficial (umbrella) urothelial cells (KRT20, SNX31). The luminal-infiltrated subtype is less pure than the other luminal subtypes, with lymphocytic infiltrates and expressing high levels of signature genes of the smooth muscle and myofibroblast type. This subtype shares characteristics with the MDA subtype ‘p53-like’ which has been associated with chemoresistance [99]. The majority of infiltrated liminal tumors were classified as Group II in the previous TCGA study and have increased expression of immune checkpoint markers, receptor (PD1) and its ligand 1 (PD-L1). Patients with cluster II subtype tumors have been reported to respond best to anti-PD-L1 therapy, atezolizumab [99]. The basosquamous subtype expresses high levels of basal and stem cell markers (CD44, KRT5, KRT6A, KRT14) and differentiation markers (TGM1, DSC3, PI3). This subtype was more common in females and a large proportion of tumours had scaly histology. Enrichment of TP53 mutations, a strong CIS signature, and low expression of the Sonic Hedgehog signature gene led to the suggestion that these tumors may have developed from basal cells and CIS lesions. Immune marker expression is highest in this subtype, reflecting the relatively low purity of the samples.

A newly described “neuronal” subtype is characterized by high expression of neuronal differentiation and development genes and typical neuroendocrine markers, although the majority of tumors do not exhibit a so-called small cell histological or even neuroid type. Alterations in genes affecting the p53/cell cycle pathway, including TP53 and RB1 mutations and E2F3 amplifications, are common in this subtype, which is also characterized by the lowest survival. A similar subtype (small cell/neuroendocrine type) and a refinement of their molecular classification system have been described since the Madrid Consensus in 2017 by the Lund group [100]. Fewer studies have described mRNA-based subtypes of NMIBCs [35,39,98]. The low-grade

Ta tumors included in Lund’s study were classified primarily as UroA, expressing high levels of urothelial differentiation markers, FGFR3 signature genes, early cell cycle genes, and cell adhesion genes.

On the contrary, pT1 and high-grade tumors were found to be very heterogeneous, being classified into UroA subtypes, genomically unstable (GU) or infiltrated [96]. The UROMOL study reported transcriptome profiling of 460 NMIBCs of all stages and grades, including CIS and 16 MIBC [39]. Three molecular subtypes (Class 1, Class 2 and Class 3) were defined. Class 1 contained mainly low-grade pTa tumors with the best prognosis and overlapped with the Lund UroA subtype. The other two classes were associated with tumors that had the highest risk scores from the European Organization for Research and Treatment of Cancer (EORTC) and contained more T1 tumors, high-grade tumors, and CIS. Class 2 also contained the majority of MIBC samples and tumors from patients with progression events.

These tumors expressed high levels of uroplakins, characteristic of luminal cells, but also expressed high levels of late cell cycle genes, stem cell markers, epithelial-mesenchymal transition (EMT) markers, progression signatures, and CIS. Thus, class 2 could represent tumors that cause luminal MIBC tumors. The class 3 tumors had not only some luminal features (GATA3+ mutation, FGFR3 mutation), but also basal MIBC features (CD44+, KRT5+, KRT14+, KRT15+, KRT20-, PPARG-). This class could represent a dormant tumor state as it is also characterized by the expression of many long noncoding RNAs, some of which have been shown to be upregulated in oncogene-induced senescence [39]. In the study by Hurst et al. [63], profiling of the expression of pTa tumors at the primary stage confirmed the overall luminal status of GS1 and GS2 samples and showed alignment mainly with the UroA subgroup [96].

Therapeutic Opportunities and Future Prospects

The overall therapeutic efficacy of standard of care treatments for NMIBC and MIBC is relatively low, and until recently, little progress had been made in identifying new therapeutic approaches. Patients with NMIBC have a very high recurrence rate (approximately 70%) and 10% to 15% progress to MIBC despite intravesical chemotherapy or BCG therapy. As Julita [8] points out, new approaches to localized therapy are urgently needed to reduce their need for long-term cystoscopic monitoring and its associated costs. Platinum-based chemotherapy has long been recognized as the gold standard treatment for metastatic bladder cancer, but only about 40% of patients respond to it and relapses are common. Recently, checkpoint-targeted immunotherapy has been approved for second-line treatment in patients who have failed first-line chemotherapy. While impressive and sustained responses have been reported, overall response rates are modest and robust predictive biomarkers are currently lacking. This review shows that over the past 5 years, molecular profiling studies using whole genome technologies have significantly improved our knowledge of the molecular landscape of bladder cancer. These revealed clinically exploitable alterations (activating mutations, amplifications, fusions) and discovered molecular signatures with predictive relevance.

The high frequencies of CM gene alterations indicate that chromatin modification is a key factor in bladder cancer. The reversible nature of epigenetic modifications highlights a potential therapeutic opportunity in patients with such alterations. Molecules targeting epigenetic alterations are being developed and several are in clinical trials. These include DNA methyltransferase inhibitors (e.g., 5-aza-2'-deoxycytidine), histone deacetylase inhibitors (e.g., vorinostat, romidepsin, mocetinostat), and histone methyl transferase inhibitors (e.g., tazemetostat). A Phase II trial (NCT02236195) to evaluate the efficacy of mocetinostat (a histone deacetylase inhibitor) in patients with late-stage urothelial carcinoma with gene deletions or inactivating mutations in the histone acetyltransferase (HAT) genes CREBBP and/or EP300 has recently been completed and results are pending. However, a recent study showed that some mutations in CREBBP or EP300 did not abrogate HAT activity, highlighting the need to fully understand the functional impact of variants detected in these genes. This study also developed a gene expression signature associated with loss of HAT activity that could be used to stratify patients [101]. Many canonical pathways that can be targeted with available drugs are impaired in bladder cancer.

Given that alterations in the p53/cell cycle pathway occur in 89% of MIBCs [41], targeting cell cycle components may represent a therapeutic option. For example, palbociclib, a selective inhibitor of cyclin-dependent kinases, CDK4 and CDK6, has been approved for use in some breast cancer patients and may be suitable as a second-line treatment in advanced bladder cancer with alterations in cell cycle regulators such as RB1 and CDKN2A. Frequent mutations and copy number changes in the ERBB family of receptor tyrosine kinases (ERBB2, ERBB3 and EGFR) are potential targets for treatment with tyrosine kinase inhibitors. For example, in platinum-refractory metastatic urothelial carcinoma, alterations in ERBB2 and ERBB3 have been associated with a response to treatment with the tyrosine kinase inhibitor afatinib [101]. Similarly, FGFR3 is considered a good therapeutic target and several inhibitors are currently in clinical trials. Good responses of FGFR3 mutant bladder cancers to the selective tyrosine kinase inhibitor FGFR1-3 BGJ398 have been reported [102]. Alterations of the PI3K/AKT/mTOR pathway, such as PIK3CA mutation and mTOR activation, may also represent exploitable targets. Indeed, durable responses to everolimus have been reported in patients with MTOR or TSC1 mutations [103,104].

The use of molecular markers shows promise in predicting patients' response to chemotherapy before surgery. Alterations in DNA repair genes show some association with the response to chemotherapy. For example, mutations in ERCC2 are associated with better outcomes in patients treated with cisplatin [74]. Prospective identification of these genes in clinical trial samples should help identify robust markers of chemosensitivity. A clinical trial (Southwest Oncology Group [SWOG] tested the effectiveness of a gene expression profiling (CoXEN)-based algorithm to predict a patient's response to neoadjuvant chemotherapy (NAC). Recently described mRNA subtypes are

also promising for predicting response to therapy [41]. Patients with basosquamous MIBC have the worst prognosis, and when treated with cystectomy alone, they have significantly shorter specific and overall survival. However, when receiving adjuvant cisplatin-based therapy, patients with basal-like tumors have better outcomes than those with luminal or p53-type tumors [99]. This superior response of basal tumors has been confirmed recently [105]. Cisplatin-based combination therapies (e.g., etoposide-cisplatin) are currently used for the treatment of neuroendocrine tumors in other tissues and may be appropriate for the newly identified bladder neuronal subtype [41,104].

Recent data suggest that tumors of the luminopapillary subtype identified in the 2017 TCGA study show a poor response to cisplatin-based NAC [106]. This subtype has better overall survival and is characterized by FGFR3 alterations (activating mutations, FGFR3-TACC3 fusions, amplifications), suggesting that treatment with FGFR inhibitors may be a valid approach. The infiltrated luminal subtype of the 2017 TCGA is expected to be resistant to cisplatin-based chemotherapy as it shares features with the 2014 TCGA Group II and the p53-type subtype identified by Choi et al. [32,99]. However, treatment with checkpoint inhibitors may be a valid approach in patients such as the group II subtype that has previously been shown to respond well to atezolizumab treatment [103]. Basosquamous tumors express high levels of immune markers, but basal subtypes III and IV show a reduced response to checkpoint inhibitors compared to group II, possibly suggesting that other immunosuppressive factors exist in the basal subtype [103].

Tumors with the MSig1 mutation signature may benefit from immunotherapy. These tumors have a high mutational load, a high APO-BEC signature mutational load, and a high predictable neoantigen load. The probability of 5-year survival in these patients is very high (75%) compared to the cluster with the lowest mutational burden (22%). This improvement in survival could be linked to a greater host immune response [41]. It will be important to examine these features in future and ongoing clinical trials, particularly those using immune checkpoint therapy. In NMIBC, especially patients with pTa-stage disease, the use of systemic therapies is unlikely to be appropriate. However, for patients with high-risk NMIBC, particularly BCG-refractory disease, some systemic therapies may be considered. They include immune checkpoint inhibitors and certain targeted therapies (e.g., FGFR or ERBB family inhibitors). Ultimately, a better understanding of the molecular characteristics of NMIBC may lead to the reformulation of drugs for local treatment and the development of new therapeutic approaches in this context. Genome-wide profiling has generated a wealth of data and suggests exciting potential therapeutic advances for the treatment of bladder cancer. However, clinical application will require careful validation through retrospective analysis of samples from previous studies and through carefully designed prospective studies. To increase predictive power, they must take into account not only changes in the therapeutic target, but also the overall molecular

landscape in which the target operates. The ultimate goal will be the development of robust markers, suitable for routine application in a clinical setting.

Interest of Molecular Subtyping for Tumors that do not Infiltrate the Muscle

Research is still needed to identify genes and explore pathways from NMIBC to MIBC. Gottfrid Sjö Dahl’s team [107] followed 357 cancer patients (UBC) and analyzed the genes of 73 patients who evolved from a NMIBC lesion to a MIBC. They found that even though FGFR3, PIK3CA or TERT were the most common mutations in NMIBC, they were not associated with progression, whereas TP53 was a common mutation in advanced UBC and strongly associated with highly invasive subtypes. This suggests that mutated TP53 may be a key factor in NMIBC progression [108]. Van Kessel [109] analyzed the high-risk status of GATA2 and the FGFR3 mutation status of the NMIBC. He found that the NMIBC profile with GATA2 methylation associated with wild-type FGFR3 was more likely to evolve to the MIBC [110]. While it is unclear which genes lead to the transformation of NMIBC into MIBC, the molecular approach provides important clues. Molecular subtyping is also useful for monitoring and managing recurrence and progression of NMIBC, especially high-risk NMIBC. High-risk NMIBC was then classified into good, moderate and poor subtypes, which increased the accuracy of its prediction of progression risk [110]. Therefore, molecular subtyping has the potential to be considered in future guidelines for the assessment of NMIBC. Molecular subtyping of NMIBC is developing slowly at present, there are still few reports in the literature but studies are continuing.

Interest of Molecular Subtyping for Muscle-Infiltrating Tumors

During progression, not only do the intrinsic characteristics of bladder tumors change, but changes in the tumor microenvironment (TME) must also be taken into account. Early MIBC classification systems from 2012 onwards tended to focus on the molecular classification of the tumor cells themselves, such as the luminal and basal subtypes. With the progressive understanding of tumor cell alterations, and their environment, subtyping efforts have focused more on heterogeneity, extracellular matrix (ECM) and immune infiltration, with the aim of promoting the development of new targeted therapies and immunotherapy. After the classification resulting from TCGA 2017, Kamoun established and verified in 2019 a classification system that proposes to divide MIBC into lumino-papillary, luminal NOS, luminal unstable (GU), stroma-rich, basal/squamous and neuroid-like [111]. This classification system takes into account not only the heterogeneity of tumor cells but also the influence of infiltrated cells (immune cells and stromal cells) on the clinical characteristics and prognosis of tumors. As a result, the new subtyping systems will partially retain the classic subtypes and offer new subtypes.

The prognosis of each subtype is different, and the response to neoadjuvant chemotherapy is also different. Subtypes with mature luminal differentiation have a better prognosis, while poorly differentiated basal and neuronal subtypes have a poor prognosis. Although the prognosis of the luminopapillary subtype is good, it is not susceptible to neoadjuvant chemotherapy (NAC) [110]. Although basal/scaly and LumNOS (luminal not specified) are with a poor prognosis, they can benefit from NAC. Molecular subtyping therefore makes it possible to select patients with specific subtypes sensitive to NAC, which should help avoid the toxicity of unnecessary chemotherapy. Moreover, based on the complete analysis of the genome, transcriptome, and non-coding RNA, molecular subtyping provides the information to choose a precise personalized treatment. In addition, studies indicate [110] that EMT and immune infiltration play an important role in adjuvant chemotherapy response and prognosis.

Effects of Chemotherapy on Molecular Typing

NAC affects the biological signature of a tumor, and the traditional molecular subtyping system is no longer the same before and after NAC treatment. Seiler’s research team [110] examined genes from 133 pre- and post-NAC MIBCs, of which 116 were pre- and post-NAC paired samples. By analyzing the gene expression signature of cisplatin-resistant, bladder cancer, patients were divided into four main subtypes: CC1-basal, CC2-luminal, CC3-immune, and CC4-scar-like. NAC does not affect the phenotype of the CC1-basal and CC2-luminal subtypes, which possess a pre-NAC basal and luminal phenotype. Basal and luminal markers are lost in the CC3 immune subtype which is characterized by the highest level of activated immunity after NAC. After NAC, the CC4-scar subtype strongly expresses “scarring” genes, its prognosis is the best. Liu et al. [112] performed a comprehensive analysis of pre- and post-NAC MIBC exon sequencing to find that chemotherapy does not increase the overall mutation burden of tumors but increases mutations in some subclonal tumors. After chemotherapy, these subclonal MIBCs are associated with poor survival and genes involved in the cell cycle and regulation of immune checkpoints are significantly altered. Cisplatin-based chemotherapy affects the genetic signature of bladder cancer, therefore, studying altered genes before and after chemotherapy and studying the effects of these changes can provide important information for studying the mechanisms of chemotherapy resistance (Table 4) (Figure 6).

Table 4: What Place Should Be Given to Pathology and Phenotype.

Pathological characteristics
First recurrence before or after 1 year.
Diamètre des récidives < 3 cm>
TNM : Ta ou T1.
Grade : G1-G3
CIS associé : oui non.

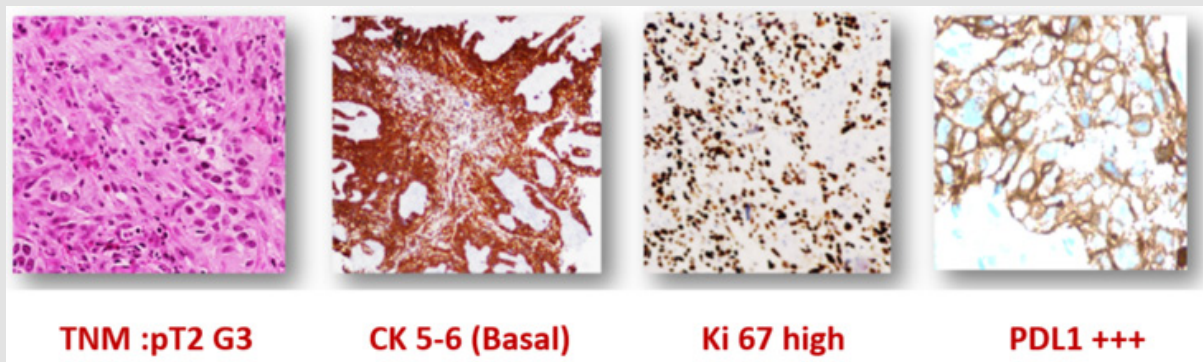


Figure 6: High-grade urothelial carcinoma that deeply infiltrates the muscle. (Dr. Guy Leseec).

The traditional classification system for bladder cancer is based primarily on disease parameters. However, it is now established that under a similar TNM classification and pathological classification, the recurrence and progression of bladder cancer varies considerably between individuals, which affects the optimization of follow-up and the treatment schedule. For example, according to the EORTC Software System Calculation (<https://www.eortc.be/tools/>) grade 3 primary bladder cancer pTa is at high risk with a 5-year recurrence rate of 46% and a 5-year risk of progression rate of 6%, indicating that 94% of patients will not progress to the invasive muscle phase (MIBC) within 5 years. Several questions therefore arise:

First question: why are all patients followed and treated with the same program given that similar pathological parameters characterize tumors with different evolutionary profiles?

For a few patients classified as low-risk, the tumor actually demonstrates a capacity for early invasive evolution and sometimes even metastatic evolution, justifying a second question: can conservative treatment be applied to all low-risk patients?. Some high-risk patients do not respond to BCG (Bacille Calmette-Guérin), while others are sensitive to BCG [113,114]. The traditional classification system cannot predict this response. This makes it difficult for physicians to choose a BCG treatment program, radical cystectomy, or another treatment based on this prediction system. Some tumors are less likely to metastasize and require only local resection, while others that are deeply invasive require radical cystectomy and/or other adjuvant therapy. But, there is still no effective way to distinguish these categories. In addition, traditional classification can predict the risk of recurrence and progression of NMIBC, but not the risk of MIBC. After radical cystectomy, some patients with muscle-invasive bladder cancer (MIBC) benefit from neoadjuvant chemotherapy (NAC), while others are resistant [115-117], which is not predicted by the traditional classification system.

The pathological parameters of the tumour cannot fully reflect the “intrinsic characteristics” of bladder cancer. It is therefore difficult for

doctors to ensure individual and precise follow-up in the treatment of bladder cancer on the basis of the criteria derived from the pathology alone. With the rapid development of sequencing, mass spectrometry and other techniques based on genomics and transcriptome [118], epigenetics [119], proteomics [120], and other omics [121,122] will provide a new direction for the accuracy of bladder cancer diagnosis and treatment. A good classification system must meet the following criteria:

1. It must provide details on the risk of recurrence and progression of bladder cancer and allow for individualized follow-up and a possible choice of conservative treatment. It must guide surgical algorithms, adjuvant therapies and monitoring schedules.
2. It must help with therapeutic choices. It must be able to accurately identify candidates for tolerance to chemotherapy, targeted therapy, immunotherapy and help develop individualized adjuvant treatments.
3. With the help of molecular biology it should not only provide important information to predict the recurrence and prognosis of bladder cancer but also provide effective information for the study of molecular mechanisms, such as tumor development, progression. It should make it possible to predict tolerance to chemotherapy and immunotherapy and also to predict their possible benefit.
4. It should also help in the development of diagnostics and new molecular treatments. The development of molecular subtyping based on genomics and transcriptomes offers this new way to understanding bladder cancer.

Subtypes, even pejorative ones, to which the right treatment is opposed, can therefore be accompanied by a favourable prognosis. The typical example is the HER2 receptor for breast cancer. Transcriptomic and proteomic profiling, as we have seen, makes it possible to classify bladder cancers into luminal and basal molecular subtypes, but

there are still prognostic and predictive associations that are being studied and sometimes controversial. The complexity of published subtyping algorithms is a major barrier to understanding their biology and validation or reluctance for their clinical use. A recent paper coordinating the efforts of Canadian and Swedish researchers [112] validates compact algorithms based on Lund's taxonomy, which separates luminal subtypes into urothelial-like (Uro) and genogically unstable (GU). It characterizes and exploits phenotypic expression data from two cohorts of MIBC muscle-infiltrating bladder cancers ($n = 193$, $n = 76$) and proposes efficient subtyping models with a decision tree. The published algorithm uses routine testing (GATA3, KRT5, p16) and it classifies basal/luminal subtypes and basal/Uro/GU subtypes with accuracies of 86% to 95% and 67% to 86%, respectively. KRT14 and RB1 are less frequently used in routine pathology practice, but they are the simplest and most accurate models for basal/luminal and basal/Uro/GU discrimination, with accuracies of 93% to 96% and 85% to 86%, respectively. More complex models with up to eight antibodies did not perform better than simpler models with two or three antibodies.

The authors conclude that simple immunohistochemistry classifiers can accurately identify luminal (Uro, GU) and basal subtypes and are attractive options for clinical implementation. What are the pathological and phenotypic criteria that can coordinate treatment choices and decide which tumors are the focus of further genetic investigations? The pathology defines robust criteria related to tumor morphology and its phenotype. The vegetative or infiltrating nature of the tumor can be easily assessed and its evolutionary nature reinforced by simple and accessible phenotypic markers that are easily observed. (Proliferation coefficient, nuclear grade, epithelial isolation in infiltration, lymphatic or vascular invasion, level of infiltration, fusiform metaplasia (EMT) or squamous, etc.). Some level of confusion was initially related by the definition of two groups of tumors, one infiltrating the muscle and the second not infiltrating the muscle. A tumor must simply become infiltrating and infiltrate the submucosa before infiltrating the muscle. It is easy to imagine that in this form of progression, according to a Darwinian model, the tumor must acquire new characteristics and develop new advantages that gradually distance it from the normal initial genetic status. The hypothesis that considers a tumor to be the result of a stable genetic clone from the beginning to the end of its evolution is now recognized as a conceptual error resulting from experimental models, more specifically in hematology.

This does not only concern bladder tumors but all solid tumors. A fact that can be easily established by morphology is the presence of multifocal tumors of a homogeneous or heterogeneous character. The "botanical" hypothesis put forward by some authors as a form of multifocal tumor implantation linked to exfoliation is a figment of the imagination. The more multifocal a tumor is and its phenotypic is clonal, the more it evokes an ancient mutation common to the precursors of the bladder mucosa with a greater probability of reflecting an

early genetic or even constitutional abnormality. The heterogeneous multiclonal nature of tumors can be explained by a common genetic or non-genetic predisposition, somatic or not, modified by less specific subsequent events such as those related to the occupational inhalation of chemicals or derivatives related to smoking. There is a difference between tumors affecting men and women and we should also find explanations in relation to endocrine particularities.

Reasoning on phenotypic profiles is sometimes complex and must be linked to a good interpretation of the phenomena of phenotypic loss or gain. Some molecules such as the p53 protein will be overexpressed in case of mutations. For bladder tumors, it is also known that it can be overexpressed in the normal state in a reactive mode. Finally, for the p53 protein, it is known that the associated strong overexpression of aneuploidy images or the absence of total expression correspond to mutated forms. Other types of reasoning can be applied to the expression of the PTEN phenotype insofar as the PTEN mutations accompanied by a loss of phenotypic expression are exclusive of PIK3CA mutations, because they are located on the same signaling pathway. The double mutation is as unlikely as it is useless in terms of a selective advantage. We can also reason about the alternative expression of p16 and RB knowing that a high expression of the p16 protein is significant of an alteration of RB and vice versa, which becomes very useful to identify tumors with unstable genomes.

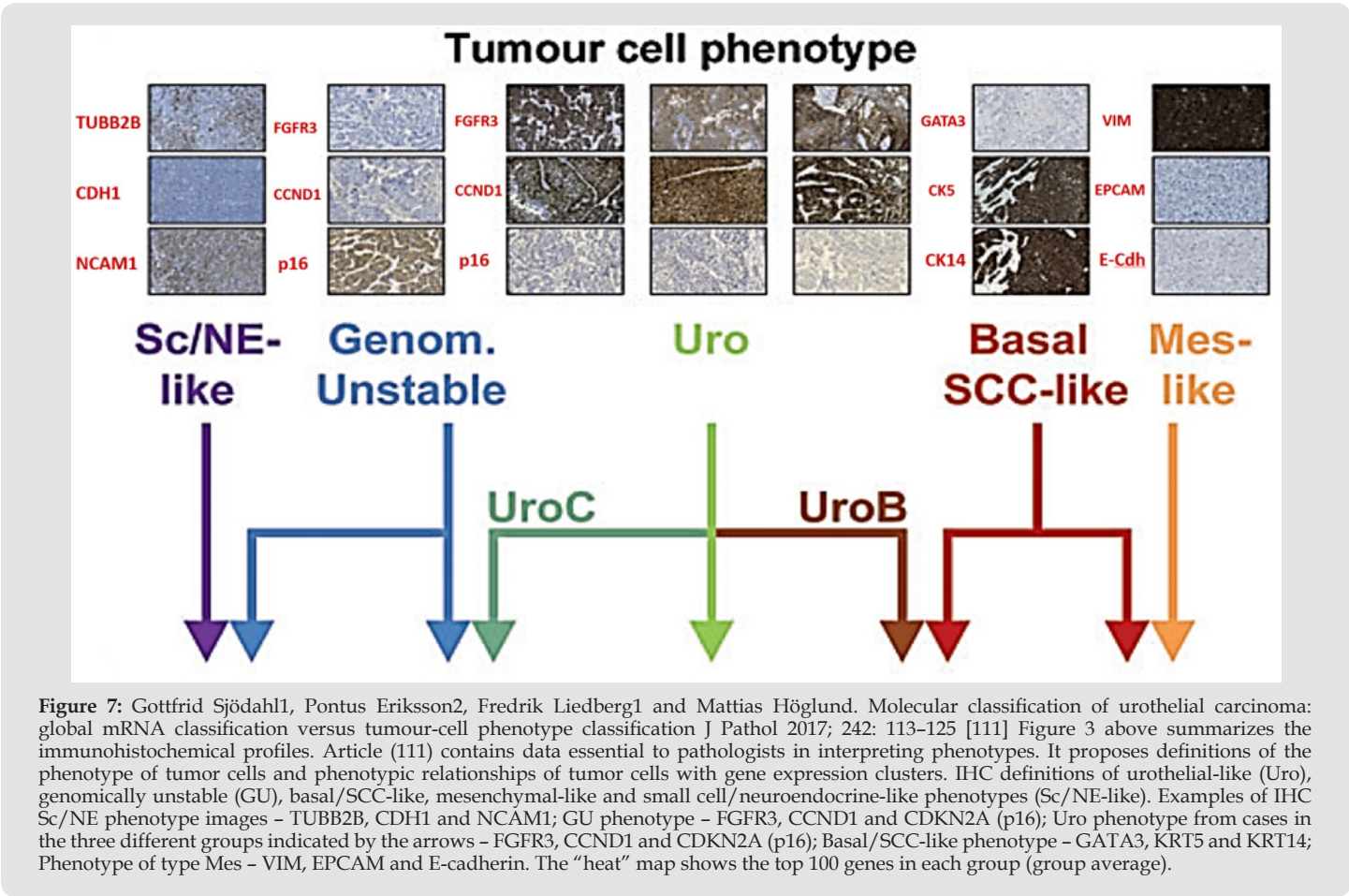
In addition, we now have markers signaling checkpoint inhibition, PDL1 immunity. Pathologists have gradually learned to interpret these signals more precisely, especially when the expression clearly concerns tumor cells and not only lymphocyte cells. In pathology on endoscopic tumor resections, we can therefore propose a more complete phenotypic study. In the context of pathology, it will make it possible to give prognostic classification criteria, predictive criteria and criteria to specify whether tumor identification is sufficient to guide therapeutic choices or whether it should be supplemented by genomic or omics studies in general. As mentioned in the preamble, the phenotypic pathway is the approach followed by the LUND group to propose a simplified phenotypic classification correlated with mRNA expression profiles.

Two articles, one from 2017 following the Madrid consensus, the other from 2022 which updates the phenotypic pathway, formulate the evolution and summarize this work [111,112]. A consensus article was signed by authors from different groups in 2020 [123]. The 2017 Lund paper [111] emphasizes that global analysis of mRNA expression is effective for phenotypic profiling of tumors. It was used to initially define the phenotype of molecular subtypes for major tumor types. But most tumors, as has been pointed out since 2019 by the Swedes [124], are communities between tumor and non-tumor cells. This problem is particularly important for the analysis of advanced invasive diseases. Infiltrating tumors are known to induce major changes and responses in both the tumor and the surrounding tissues. Immunohistochemistry is a way of distinguishing the share that belongs to tumor cells and stroma. Identifying the phenotypes of

bladder cancer tumour cells and comparing phenotypic classification with classification by global gene expression analysis was the goal of the LUND group.

In the LUND article, advanced bladder cancers (n=307) from cystectomy parts were investigated by gene expression analysis and immunohistochemistry with antibodies targeting 28 proteins. According to the systematic analysis of gene and protein expression data, focusing on key molecular processes, the authors describe 5 phenotypes characterizing advanced urothelial carcinoma tumor cells: urothelial type, genomically unstable, basal/SCC type, mesenchymal type, and small cell/neuroendocrine type. Molecular pathological definitions for each subtype are proposed. Tumors expressing

urothelial differentiation factors exhibit inconsistent and abnormal protein expression of terminal markers of differentiation, suggesting pseudo-differentiation. In global mRNA analyses, cancers of different phenotypes may cluster (converge), and cases with identical tumor cell phenotypes may diverge. This divergence/convergence suggests that important commonalities related to the invasive process may exist between muscle-invasive tumors regardless of the specific phenotype of tumor cells. As a result, there is systematic disagreement in the classification of subtypes determined by global mRNA profiling and by tumor cell level. The authors suggest that a combination of pathology (tumor cell phenotype) and global mRNA profiling (background) are needed for adequate classification of muscle plane invasive bladder cancer subtypes (Figures 7 & 8).



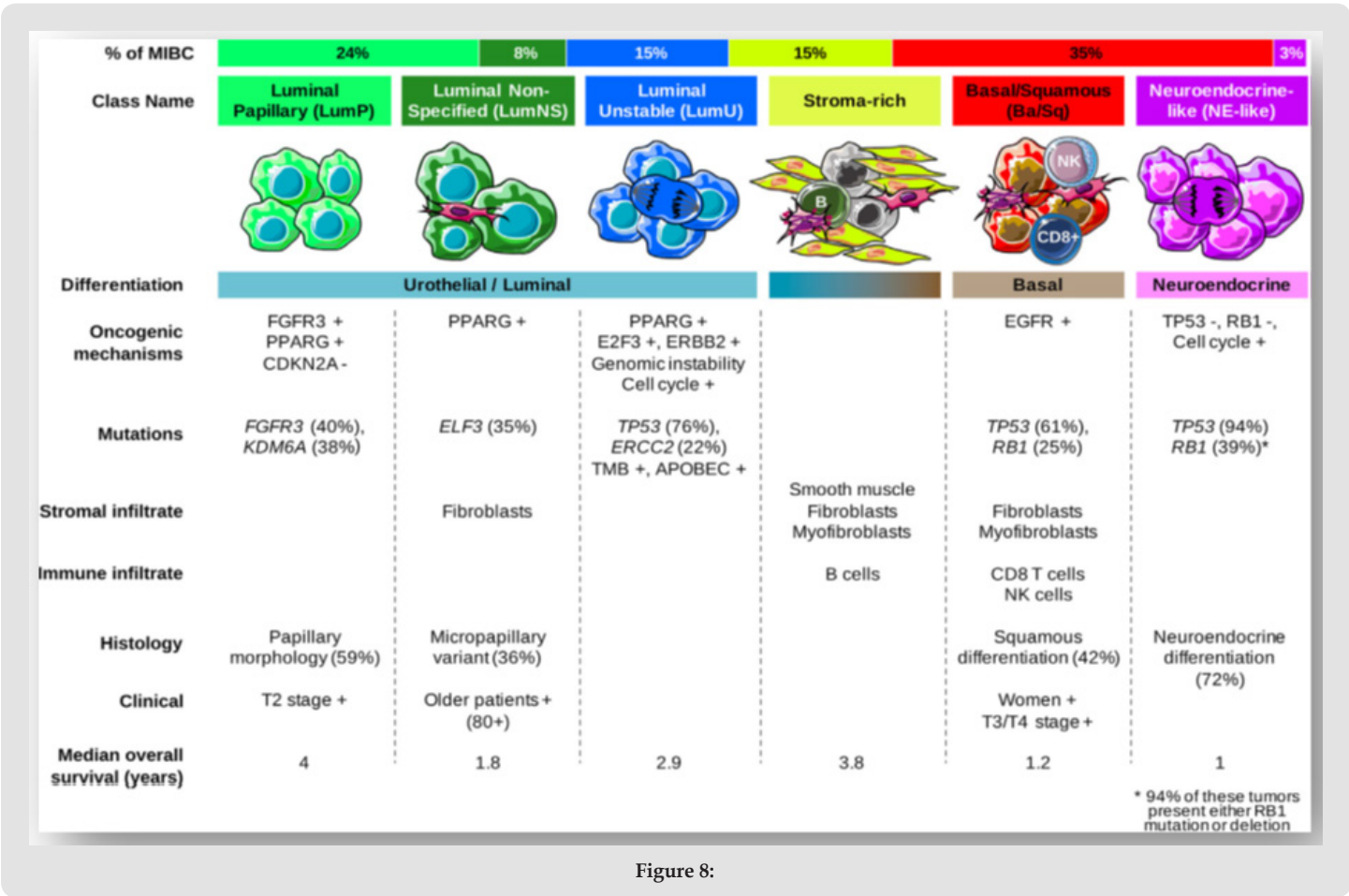


Figure 8:

Summary of the key features of the 2020 consensus classes. From top to bottom, the following characteristics are presented: proportion of consensus classes in the 1750 tumor samples; consensual class names; schematic graphical representation of tumor cells and their microenvironments (immune cells, fibroblasts, and smooth muscle cells); differentiation-based color scale showing features associated with consensus classes, including luminal to basal gradient and neuroendocrine differentiation; and a table presenting dominant features such as oncogenic mechanisms, mutations, stromal infiltrate, immune infiltrate, histology, clinical features, and median overall survival. The 2022 paper, the most recent, again comes from the Lund group [112]. He points out that transcriptomic and proteomic profiling classifies bladder cancers into luminal and basal molecular subtypes, with associations of prognostic and predictive values.

However, the complexity of published subtyping algorithms, which we have reviewed, remains a major obstacle to understanding their biology and validating or refuting their clinical use. The paper

proposes to optimize compact algorithms from Lund's taxonomy, to separate luminal subtypes into urothelial (Uro) and genogically unstable (GU) types. Immunohistochemical expression data from two cohorts of muscle-invasive bladder cancers (n = 193, n = 76) are characterized and allow to propose efficient models of decision trees. An algorithm using routine tests (GATA3, KRT5, p16) can classify the basal/luminal and basal/Uro/GU subtypes with an accuracy of 86% to 95% and 67% to 86%, respectively. The KRT14 and RB1 phenotypes are less frequently used in the practice of pathology, but they represent the simplest and most accurate models for basal/luminal and basal/Uro/GU discrimination, with 93%–96% and 85%–86% accuracy, respectively. More complex models using up to eight antibodies did not perform better than simpler models with two or three antibodies. The authors conclude that simple immunohistochemistry classifiers can accurately identify luminal (Uro, GU) and basal subtypes and represent attractive options for clinical implementation. (J Histochem Cytochem 70: 357–375, 2022) (Table 5).

Table 5.

Subtypes	Phenotype subject to expression score calculation
URO/UROB	Cycline D1+, FGFR3+, RB1+, P16-, GATA3+
Génome instable	Cycline D1 -, FGFR3-, RB-, P16+.
EMT like MES	VIM +, ZEB2, BERP4 (Ep Cam) -, E-Cad -.
Basal/Squamous/SCC	KRT5+, KRT14+, GATA3-, FOXA1-
Small cell/NE-like	TURB2B+, EPCAM+, E-Cad -, GATA3-
Sore d’expression	For details and score calculation, pathologists should refer to the article.

In Conclusion: on the Need for an Integrated Pathology

Routine histopathology has made it possible, after the numerous historical works of the Mostofi group (AFIP), to identify lesional processes and to distinguish tumor proliferations whose definition is based solely on morphological criteria. In particular, these are all categories of non-infiltrating vegetative tumors whose spectrum ranges from benignity, to tumors with a low potential for malignancy and to tumors that are genuinely malignant but predisposed to maintain an exophytic or superficial development in the more or less long term. All these descriptive criteria are known and used in practice on a daily basis by pathologists. However, these are now supposed to provide more precision as soon as these tumors are of high grade, whether they are vegetative or infiltrative, to guide therapists to make relevant treatment choices, those that will limit the recurrence or extension of these tumors. Similarly, for tumors diagnosed at an advanced stage, i.e. at the time they infiltrate the muscle (pT2), pathologists will have to contribute to the identification of phenotypic characteristics with a prognostic and predictive function. We have seen that the presence of particular phenotypes makes it possible to identify certain types of mutations and that as such they are exclusive of other mutations.

The good knowledge of its exclusions or association allows pathologists requesting genetic investigations of the NGS type to make more targeted proposals.

It is clear from all the data available today that we can extend concepts established on other types of tumors to bladder tumors. Today, tumors are recognized as evidence of their proliferative activity, their genetic instability, and their ability to evade the control of the immune system. Certain tumor phenotypes also recognize particular capacities of the tumor genome to lose its stability or its ability to repair DNA alterations of the double-stranded type. (HRD).In the subtypes of bladder tumor currently identified, it is important not to remain too rigid on phenotypic characteristics alone, insofar as an immune reaction specific to the subject can modify the prognosis of an infiltrating tumor subtype and justify a particular treatment. The same is true of the nature of genetic instability. The latter, if it is responsible for a hypermutated or ultramutated neoantigenicity, implies an appropriate choice of treatment. It will therefore be important in the future to learn how to use phenotypic markers in a relevant way, especially those that allow the tumor to evade immune recognition, more specifically in the mesenchymal or infiltrated subtype, and this from the early stages of infiltration. This should be the spirit of this new generation immunohistochemistry [125-129] (Figure 9).

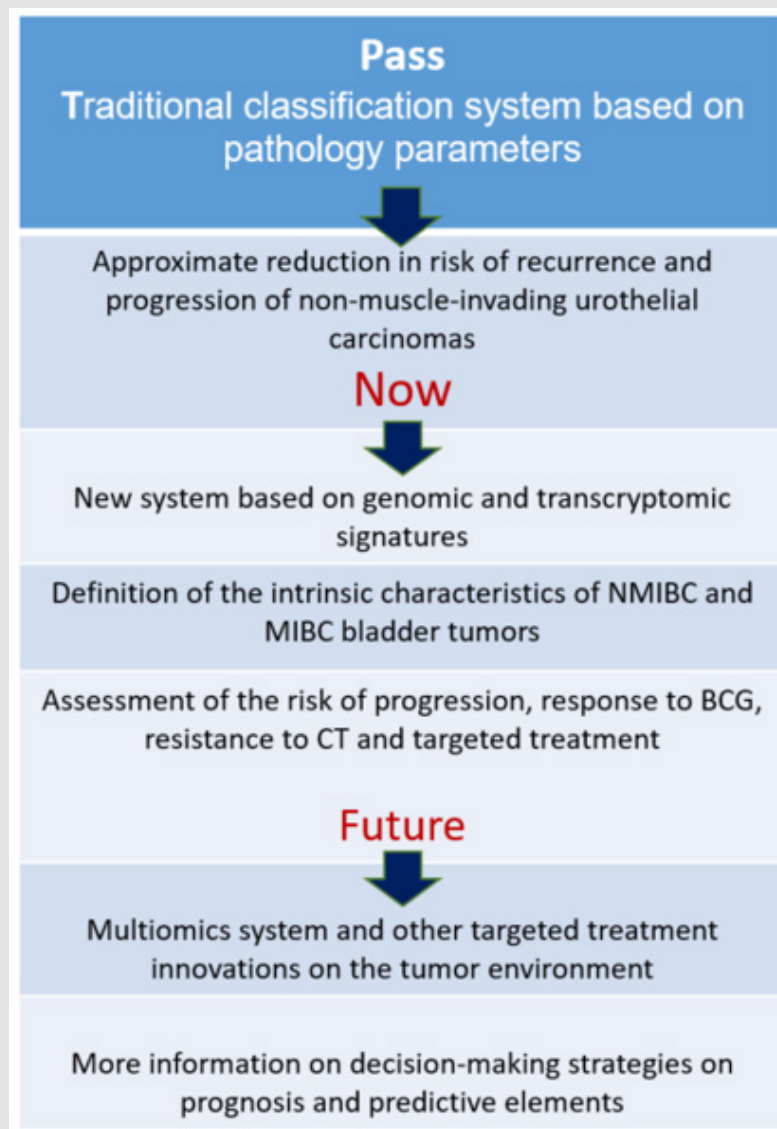


Figure 9: Traditional classification and new subtyping systems for bladder cancer. NMIBC, non-muscle-invasive bladder cancer; MIBC muscle infiltrating cancer. BCG, Bacillus Calmette-Guérin; CT chemotherapy, TT treatments.

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