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Molecular Classification of Bladder Cancer

Gottfrid Sjödahl

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DOCTORAL DISSERTATION
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December 12th at 13.00 .

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Molecular Classification of Bladder Cancer

Gottfrid Sjödahl

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Department of Oncology, Clinical Sciences

Lund University, 2013

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1. David Lindgren, Attila Frigyesi, Sigurdur Gudjonsson, **Gottfrid Sjödahl**, Christer Hallden, Gunilla Chebil, Srinivas Veerla, Tobias Ryden, Wiking Månsson, Fredrik Liedberg, Mattias Höglund. Combined gene expression and genomic profiling define two intrinsic molecular subtypes of urothelial carcinoma and gene signatures for molecular grading and outcome. Cancer Res. 2010 70(9):3463-72.

2. **Gottfrid Sjödahl**, Martin Lauss, Kristina Lövgren, Gunilla Chebil, Sigurdur Gudjonsson, Srinivas Veerla, Oliver Patschan, Mattias Aine, Mårten Fernö, Markus Ringnér, Wiking Månsson, Fredrik Liedberg, David Lindgren, Mattias Höglund. A molecular taxonomy for urothelial carcinoma. Clin Cancer Res. 2012 18(12):3377-86.

3. **Gottfrid Sjödahl**, Kristina Lövgren, Martin Lauss, Oliver Patschan, Sigurdur Gudjonsson, Gunilla Chebil, Mattias Aine, Pontus Eriksson, Wiking Månsson, David Lindgren, Mårten Fernö, Fredrik Liedberg, Mattias Höglund. Towards a molecular pathological classification of urothelial carcinoma. Am J Pathol 2013 183(3):681-91.

4. Oliver Patschan[1], **Gottfrid Sjödahl**[1], Gunilla Chebil, Kristina Lövgren, Martin Lauss, Sigurdur Gudjonsson, Petter Kollberg, Pontus Eriksson, Mattias Aine, Wiking Månsson, Mårten Fernö, Fredrik Liedberg, Mattias Höglund. Molecular sub-classification of T1 urothelial carcinoma identifies high-risk subtypes. Submitted

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[1] These authors contributed equally to the manuscript.

List of additional papers not included in the thesis

1. **Gottfrid Sjödahl**, Martin Lauss, Sigurdur Gudjonsson, Fredrik Liedberg, Christer Halldén, Gunilla Chebil, Wiking Månsson, Mattias Höglund, David Lindgren. A systematic study of gene mutations in urothelial carcinoma; inactivating mutations in TSC2 and PIK3R1. PLoS One. 2011 Apr 14;6(4):e18583

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3. Fredrik Liedberg, Martin Lauss, Oliver Patschan, mattias Aine, Gunilla Chebil, Margareta Cwikiel, Helgi Engilbertsson, Sigurdur Gudjonsson, Petter Kollberg, **Gottfrid Sjödahl**, Wiking Månsson, David Lindgren, Mattias Höglund; Lund Bladder Cancer Group. The importance of being grade 3: WHO 1999 versus WHO 2004 pathologic grading. Eur Urol. 2012 Oct;62(4):620-3

4. Martin Lauss, Mattias Aine, **Gottfrid Sjödahl**, Srinivas Veerla, Oliver Patschan, Sigurdur Gudjonsson, Gunilla Chebil, Kristina Lövgren, Mårten Fernö, Wiking Månsson, Fredrik Liedberg, Markus Ringnér, David Lindgren, Mattias Höglund. DNA methylation analyses of urothelial carcinoma reveal distinct epigenetic subtypes and an association between gene copy number and methylation status. Epigenetics. 2012 Aug;7(8):858-67.

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List of abbreviations

aCGH	Array Comparative Genome Hybridization
ANOVA	Analysis of Variance
BCG	Bacillus Calmette-Guerin
CIS	Carcinoma in Situ
CMV	Cisplatin, Metotrexate, and Vinblastine
COSMIC	Catalog of Somatic Mutations in Cancer
CUETO	Club Urológico Español de Tratamiento Oncológico
EAU	European Association of Urology
ECM	Extracellular Matrix
EORTC	European Organization for Research and Treatment of Cancer
FDR	False Discovery Rate
FFPE	Formalin-Fixed, Paraffin Embedded
GC	Gemcitabine and Cisplatin/Carboplatin
HCA	Hierarchical Clustering Analysis
IHC	Immunohistochemistry
ISUP	International Society of Urological Pathology
LOH	Loss of Heterozygosity
LVI	Lymphovascular Invasion
MIUC	Muscle-invasive Urothelial Cancer
MS	Molecular Subtype
MVAC	Metotrexate, Vinblastine, Adriamycin, and Cisplatin
NMIUC	Non muscle-invasive urothelial cancer
PUNLMP	Papillary Urothelial Neoplasm of Low Malignant Potential
QTC	Quality Threshold Clustering
ROC	Reciever operator characteristics
SCC	Squamous cell carcinoma
TCGA	The cancer genome atlas
TMA	Tissue microarray
TUR-B	Trans-urethral resection of the bladder
UC	Urothelial cancer
WHO	World health organization

Abstract

Decisions in the treatment of bladder cancer today are based on clinical and pathological risk variables such as tumor stage and tumor grade. The importance of these conventional risk variables is well documented since more than 10 years, and they are used routinely in the clinics. Over the last ten years, cancer research has seen a gradual transition towards personalized medicine, i.e. the exploitation of specific molecular properties in the treatment of tumors. The starting point for personalized medicine is a taxonomy of the tumor type, where genome, transcriptome, and/or proteome data is used to define molecular subtypes that make sense from biological and clinical viewpoints.

The overall aim of the work presented in this thesis is to define the major gene expression subtypes of bladder tumors. The gene expression based subtypes should be viewed as a framework which can be refined either by the integration of genomic, epigenetic, or proteomic data or by the analysis of larger patient cohorts so that the subtypes can be described in greater detail. An exhaustive tumor classification should be based on biological similarity between tumors, and not only group together tumors with similar clinical risk profile. This will increase the probability that the taxonomy is relevant in the evaluation of novel therapies that function by altering pathways or transcriptional programs. In paper 1 we define the two major subtypes of bladder cancer, termed molecular subtype 1 and 2 (MS1 and MS2). In paper 2, MS1 and MS2 are subdivided into five major subtypes named Urobasal A, Urobasal B, Genomically Unstable, SCC-like, and Infiltrated, named after their dominating molecular characteristics. The subtypes were identified in an unsupervised manner and were identified also in external data sets, showing their general applicability.

Secondary to the aim of tumor classification is the evaluation of the potential prognostic value of the described subtypes. To allow for clinical comparisons, tumor classification should be possible using immunohistochemistry (IHC) on archived material. In paper 3 we make use of the same set of tumors as in paper 2 and device a simplified classifier based on IHC and histology. This classifier identifies the five subtypes with the exception of Urobasal B which could not be reliably distinguished from the related Urobasal A subtype. The molecular pathological classifier defined in paper 3 thus has room for improvement and will need to evolve as the true molecular subtypes are refined.

Up to this point we have shown that the subtypes differ in prognosis, but we could not determine whether this was independent of differences observed in stage and grade. In paper 4 we use an independent population based cohort of T1 tumors to retrospectively estimate the prognostic value of the molecular subtypes. The IHC/histology classifier defined in paper 3 is applied, and the molecular subtypes

are compared to a current clinical risk stratification model in multivariate analyses. The results show that the subtypes contain as much prognostic information as the current clinical model, and that the best risk stratification is achieved by combining the subtypes with clinical data and an estimate of CD3+ lymphocyte infiltration.

Background

The urinary bladder

The urinary bladder as an organ functions to collect and excrete the urine. The urine is the major clearance route for toxic chemicals and metabolic byproducts, making impermeability to such substances an essential function of the bladder.

The urinary bladder consists of several layers with specialized roles within the organ. Closest to the bladder lumen is the urothelium, a stratified epithelium that serves as the physical barrier to the urine. The human urothelium is approximately five to seven cells thick and consists of basal, intermediate, and luminal cells (Figure 1, inset). The urothelium is specialized in going through expansion and rapid contraction during micturition. An example of this is the specialized nature of the most luminal cells, termed umbrella cells, which are large, often multinucleated cells with the capability of rapidly changing the area of the apical plasma membrane by dynamic fusion/budding of intracellular vesicles. The urothelial cell layers are continuously renewed by means of asymmetrical division of basally located progenitor cells and shedding of differentiated intermediate and luminal cells. The fraction of mitotic cells in the urothelium is low[1,2] (around 0.1 %), and the cellular turnover rate is slow (around 200 days)[3] compared to other epithelia. During metaplastic or inflammatory conditions the proliferation and turnover rate of the urothelium increases, and both conditions are associated with increased risk of developing urothelial tumors. Over the course of a lifetime the urothelial progenitor cells are exposed to oxidative stress resulting in accumulation of DNA damage and increased cancer risk.

Apart from the urothelium, the bladder mucosa consists of a stromal layer made up of elastic tissue, blood vessels and smooth muscle fibers (Figure 1, inset). The mucosa, in turn, rests on the detrusor muscle and perivesical fat which makes up and surrounds the bladder wall (Figure 1).

The kidneys are connected to the bladder through the ureters, which are lined by a similar urothelium. The bladder is emptied though the urethra, which is also lined by a urothelium that becomes a squamous epithelium in the distal urethra. The bladder neck, located close to the urethral orifice is more prone to squamous metaplasia, a process that can be triggered by urinary tract infections and is more commonly observed in women than in men.[4] Another type of histological change seen in the urothelium is glandular metaplasia, in which the urothelium assumes features of glandular cells. This premalignant condition is often observed with Von Brunn's nests, which are non-malignant nests of urothelial cells within the bladder mucosa. Cytological or pathological evaluation may be necessary to differentiate metaplastic or inflammatory conditions from non-papillary urothelial

cancer or carcinoma in situ (CIS), a high grade intraepithelial lesion which indicates high risk for developing overt tumor.[5]

Bladder cancer

Urothelial cancer (UC) is the most common cancer type found in the urinary bladder. It affects males more frequently than females, and it presents either as a papillary growing tumor, as a flat lesion as in CIS or as a solid tumor growing into the bladder wall. The papillary growth pattern is usually associated with a lower tumor stage (invasive depth) and grade (cellular features) in pathological evaluation as compared to the solid invasive tumors. The current consensus system for pathological evaluation of urothelial cancer is outlined in Table 1 for stage, and in Table 2 for grade. The most superficial tumors are papillary neoplasms of low malignant potential (PUNLMPs) or UC of stage Ta, growing without invasion.

Tumors of stage T1 are stroma-invasive, meaning that tumors cells have crossed the basal membrane separating the urothelium from the stroma. Stages T2-T4 represent tumors that have invaded the muscularis propria (T2), the perivesicular fat (T3), or other organs (T4). Each of the invasive stages (pT1-pT4) have additional sub-stages delineating the extent of invasion at that stage level. Invasive tumors are also pathologically and clinically classified by lymph node involvement and/or presence of distant metastases.

Tumor stage

Tumor stage is closely related to patient outcome and it is the main prognostic basis for treatment selection. Today the most crucial decisions depend on whether the tumor is of stage Ta or stage T1, treated by local resection and therapeutic instillations in the bladder, or if the tumor is muscle-invasive (≥ stage T2), requiring radical cystectomy or radiotherapy often in combination with systemic chemotherapy regimens. For this reason the correct pathological stage evaluation is of critical importance in the current treatment of bladder cancer. Unfortunately, reports have shown that inter-observer variability between pathologists is high, and that misclassification of pT1 and pT2 tumors occur due to failure to distinguish between the muscle bundles within the muscosa and those of the muscularis propria.[6-8] Studies have shown that second opinion pathology frequently result in changes of both diagnosis and treatment of bladder cancer.[9,10] Correct stage evaluation is also dependent on the quality of the tissue sample; as many as 50% of non-muscle-invasive tumors do not have any muscle in the resected tissue (unpublished data 2010). In the case of stage T1 tumors, this means that muscle-invasive disease cannot be excluded. Pathology will then only confirm stage T1 disease, which may result in under treatment of some T2 tumors.

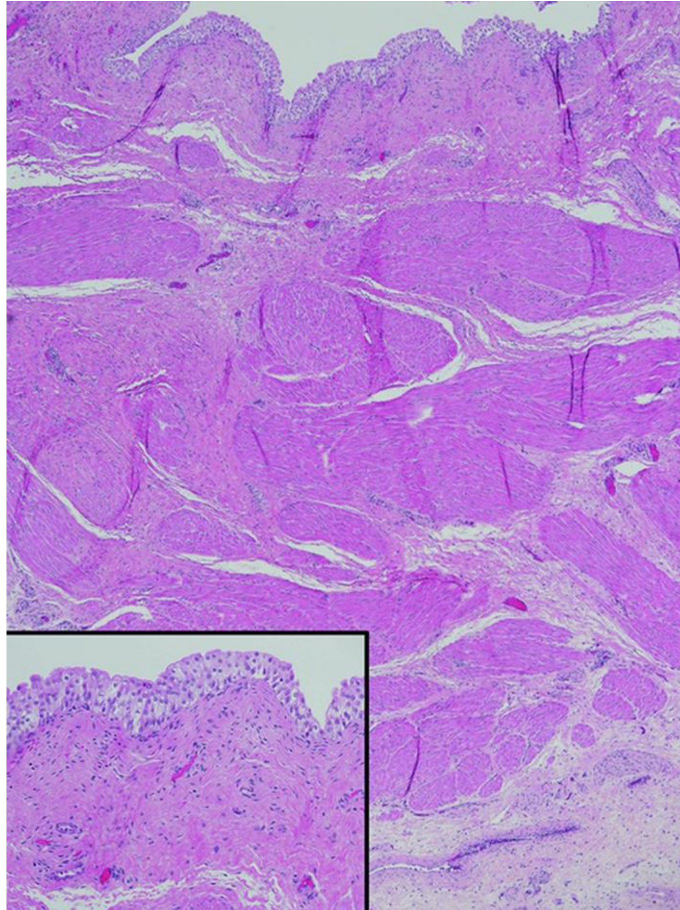

Figure 1. Full thickness view of the normal bladder wall

Large bundles of detrusor muscle make up most of the thickness of the bladder wall. The inset is showing the normal urothelium and underlying connective tissue. (H&E, 4x; inset, x20. Adapted with kind permission from Springer Science and Business Media).[11]

Even if the TUR-B and tissue preparation are perfect, randomness in tissue sectioning is another potential source of uncertainty. Operating urologists do not always fractionate the specimen obtained from the tumor base, increasing the difficulties for the pathologist to determine whether invasion is present or not. Thus, the muscle-invading cells that would prompt the pathologist to upstage a pT1 tumor to pT2 may not be found depending on how the tissue is partitioned into smaller pieces and sectioned under the microscope. Each tumor thus has a true

stage, which is clinically very important, for which both detection and interpretation has room for improvement.

Tumor grade

Unfortunately, two parallel systems exist for the evaluation of pathological grade. In 1973, the WHO consensus system was published describing a three tier grading system for UC (G1- G3).[12] In this grading system the grade 1 category is reserved for tumors with lowest, and grade 3 for tumors with the highest degree of abnormality at the cellular level. The wording of the definitions in the WHO 1973 system led to an excessive amount of cases classified as G2. This drawback of the 1973 system was addressed in the 1999 update of the WHO grading system. The main changes in the updated version is that the definitions of G1 and G3 are less strict allowing not only tumors of the highest or lowest grade to be of grade 1 or grade 3. In addition, the category of papillary neoplasm of low malignant potential (PUNLMP) is introduced. PUNLMPs represent tumors of the lowest grade that are smaller in size than regular TaG1 tumors.

In 1998 WHO/ISUP first proposed an alternative grading system that was updated in 2004. The major difference of this system compared to the WHO 1973/99 system is the use of only two grade categories in addition to PUNLMP, namely "high grade" and "low grade". Classifications according to the WHO 1973/99 can be translated into the new system. As can be seen in Table 2, high grade tumors according to WHO/ISUP 2004 may overlap with both WHO 1999 G2 and G3 tumors. The current guidelines of the European Association of Urology (EAU) recommend the use of both WHO 1973 and WHO/ISUP 2004,[13] whereas the Swedish national healthcare program of 2013 recommends the use of the WHO 1999 and WHO/ISUP 2004 systems.

Table 1. Pathological stages of urothelial cancer [14]

Tumor stage	Description
Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Ta	Noninvasive papillary carcinoma
Tis	Carcinoma in situ: "flat tumor"
T1	Invades subepithelial connective tissue
T2	Invades muscularis propria
T2a	Invades superficial muscularis propria (inner half)
T2b	Invades deep muscularis propria (outer half)
T3	Invades perivesical tissue
T3a	Microscopically
T3b	Macroscopically (extravesical mass)
T4	Invades surrounding tissue/organ
T4a	Invades prostate, uterus, vagina
T4b	Invades pelvic wall, abdominal wall

It is reasonable to believe that the proponents of the WHO/ISUP 2004 system considered the distinction between WHO 1973/1999 G2 and G3 to be an arbitrary one, a surprising standpoint since the prognostic importance of the G2/G3 distinction had been made convincingly clear before 2004.[15-17] On the other hand, it had also been suggested that reproducibility may be increased by switching to a two tier system.[18] Indeed, studies have reported reduced inter-observer variability within the WHO/ISUP 2004 system, although the degree of agreement (κ) was still moderate for both systems.[19] Regarding prognosis and tumor biology, the opinions of the scientific community on the WHO/ISUP 2004 system have ranged from favorable [20-22] to skeptical [23,24] or outright critical,[25-27] with some authors arguing that the reasons for a system change, i.e. reproducibility issues and the vaguely defined G2 group, had already been addressed in the WHO 1999 update.

Histologic variants of bladder cancer

More than 95 % of bladder tumors are of epithelial origin[12] and the vast majority of these are of urothelial histology. Tumors with variant histology are found at lower frequencies. The two major types of non-urothelial tumors found in the bladder are adenocarcinomas and squamous cell carcinomas of the bladder. The occurrence of such different histologies within the urothelium indicates a strong plasticity of urothelial stem/progenitor cells.

Primary adenocarcinoma of the bladder is either the result of a progressive glandular metaplasia of the urothelium or arising from remnants of the urachus, an embryonic tissue with a simple epithelial lining. Primary adenocarcinoma of the bladder is very rare and most adenocarcinomas are actually secondary tumors originating from colon, prostate or female genital organs.[28]

Squamous cell carcinoma (SCC) of the bladder represents only a few percent of bladder tumors in the western world. Its main cause worldwide is related to infection of the parasite *Schistosoma haematobium*.

Table 2. Comparison of the WHO 1973/99 and the WHO/ISUP 1998/2004 pathological grading systems for papillary urothelial cancer [12,29,30]

WHO 1973/1999		WHO/ISUP 1998/2004
Papilloma	→	Papilloma
Grade 1	→	Low malignant potential
	↘	
Grade 2	→	Low grade papillary UC
	↘	
Grade 3	→	High grade papillary UC

Infection with *S. Haematobium* is a major health problem in African countries,[31] where in some populations, primary SCC is more prevalent than conventional urothelial carcinoma. In contrast to urothelial carcinoma, SCC of the bladder has a male:female ratio close to 1:1.[32]

Small cell carcinoma of the bladder is a rare tumor type which has a clinical profile similar to aggressive urothelial carcinoma. Weather small cell carcinoma of the bladder originates from urothelial stem/progenitor cells, from neuroendocrine cells in the bladder, or from another cell type is under debate.

Additionally, several types of mesenchymal tumors may arise in the bladder, including those arising from blood vessels, nerves, muscle, and fibroblasts. The clinical and pathological details of these tumors vary greatly and their only common denominator is that they are very rare.

Incidence, mortality, risk factors, and cost of UC

Several studies have estimated the incidence of bladder cancer in different parts of the world. In the United states, a recent study estimated that the incidence of bladder cancer of any stage has increased from 21 in 1973 to 26/100,000 person years in 2009.[33] In Sweden, the corresponding rate for 2011 is 27, with an approximate 2500 new cases per year.[34] The historical increase in incidence is likely affected by improvement in diagnosis and data collection. Bladder cancer incidence in the rest of the world is lower, which is due to differences in population age distributions and non-Caucasian ethnicity being a protective factor for UC development.[35] Bladder cancer is more common in white than in black individuals, and more common in men than in women, whereas prognosis is worse in black individuals and women partly owing to higher tumor stage at presentation.[35]

Tobacco smoking is by far the most important environmental risk factor for bladder cancer; current smokers have a threefold increased risk and previous smokers a twofold increased risk of developing bladder cancer. Approximately half of the deaths of bladder cancer can be attributed to smoking.[36]

In 2006, the European Union average mortality rate for bladder cancer was 5.5 for men and 1.3/100,000 deaths for women.[37] Environmental risk factors other than smoking include workplace related chemical exposure in the paint, metal and petroleum industries.[38] The impact of hereditary factors on the risk of developing bladder cancer is low compared to other tumor types. In Sweden, inherited factors account for only 7% of the causation of bladder cancer compared to 20-30% for tumors of the cervix, breast, testis, and endocrine organs.[39]

The lifetime cost of bladder cancer per patient is the highest of all tumor types. Surveillance and treatment of recurrent tumors represents 60% of the total costs of

UC management.[40,41] In spite of the expenses associated with bladder cancer care, research funding is lagging behind. A recent study identified bladder cancer as one of four tumor types for which research is funded at levels far below other tumor types that impose similar societal burden.[42]

Molecular alterations in UC

Several molecular alterations are known to be involved in the development and progression of bladder cancer. In the late 1990's, mutations of the fibroblast growth factor receptor 3 (*FGFR3*) gene were found at high frequency in UC.[43] These mutations were most frequently found in superficial tumors with a good prognosis.[44] In addition to *FGFR3*, oncogenes that are activated by point mutation include *HRAS*, *KRAS*, PI3-kinase catalytic subunit (*PIK3CA*) and beta-catenin (*CTNNB1*).[45-47] Similar to what was observed for *FGFR3*, *PIK3CA* mutations are primarily found in tumors of low stage and grade. These mutations all affect key signaling proteins in mitogenic pathways and they all result in increased levels of the Cyclin D1 protein that drives G1/S-phase transition of the cell cycle. The Cyclin D1 gene (*CCND1*) itself is amplified in UC,[48] which combined with the fact that mitogenic pathways converge on *CCND1* overexpression provides strong evidence for CCND1 as a key driver of early stage tumors. Unlike what is seen for *FGFR3* and *PIK3CA*, activating mutations in the *RAS*-family genes and *CTNNB1* are not strongly linked to low stage and grade, suggesting that mutations in these genes may have pleiotropic effects in addition to activating *CCND1*.

Tumor suppressor genes inactivated by mutation/deletion in bladder cancer include *TP53*, *RB1*, *CDKN2A*, *PTEN*, and *TSC1*.[46,49] As was seen for the genes with activating mutations, these genes all have the potential to directly (*TP53*, *RB1*, *CDKN2A*) or through signaling pathways (*PTEN*, *TSC1*) affect cell cycle progression. In addition to controlling proliferation, these genes have several other tumor suppressive functions, meaning that their inactivation are critical for the development of aggressive tumor behavior. For example, loss of *PTEN* is associated with epithelial to mesenchymal transition in UC,[50] whereas loss of *TP53* and *CDKN2A* is associated with activation of DNA damage response[51] and induction of senescence,[52] respectively. As a complement to mutation, most of these cancer genes can be activated or inactivated by genomic amplification or loss. Among the most common genomic events in UC is loss of entire arms of chromosome 9. This event is unique, because it is the only genomic rearrangement associated with early tumors of low stage and grade. It is likely that loss of chromosome 9 confers a growth advantage to papillary growing UC. Some oncogenes are preferentially amplified, rather than activated by mutation; e.g. amplification of the *E2F3* oncogene located on the p-arm of chromosome 6 represents yet another means for tumor cells to deregulate the balance of transcription factors governing cell-cycle progression.[53]

Recently, next generation sequencing technology made a large contribution to our knowledge of mutations in UC. The exomes of nine MI tumors were sequenced and all somatically mutated genes were investigated in an additional 88 cases.[54] The study revealed high mutation frequencies of several genes involved in chromatin remodeling such as *UTX*, *ARID1A*, *CREBBP*, and *EP300*. More than half of the tumors carried a mutation in at least one chromatin remodeling gene. Mutations in these pathways have been found in several other tumor types, and are likely to have profound effects on tumor development by broadly affecting the accessibility of the chromatin to transcription factors. Another type of molecular alteration identified through the use of next generation sequencing is the recent identification of somatic variants in the promoter sequence of the telomerase gene (*TERT*).[55] These variants affect potential binding sites of activating transcription factors driving expression of the gene. These promoter mutations are found at very high frequency in bladder cancer,[56,57] and likely represent a common way for these tumors to circumvent the problem of telomere shortening.

A recent study that classified the genomic alterations of UC based on whether they are actionable, i.e. if the alteration can be therapeutically targeted, concluded that more than half of UC cases have such alterations.[58] Although this is a very promising result, it is possible that the success of targeted therapy is dependent on the context of the molecular alteration. A clear example of this comes from breast cancer research, where high proliferation index measured by Ki-67 staining has prognostic value in estrogen receptor (ER) positive tumors only.[59] In ER negative tumors, proliferation index has little value, and for the ER negative subtypes, the level of immune related genes is important for prognosis.[60] It is possible that the response to targeted therapy may show similar subtype dependencies. It is also conceivable that the effects of early events in tumorigenesis may be redundant in a later state of tumor development. In such cases, a targeted therapy may be more likely to succeed if the analysis is made knowing the molecular subtypes of the tumors.

Proposed models for UC development

To understand the molecular data that has accumulated over the last decades, it is necessary to understand UC development. Several models exist and are compatible with molecular data. The most cited model states that UC develops along one of two pathways.[61] Tumors of the first pathway start out as NMI with papillary growth, driven by mitogenic mutations in *FGFR3*, *RAS*, or *PIK3CA*. The patient may experience several recurrences, and eventually one tumor acquires a genetic change such as *RB1* loss or *TP53* mutation, that enables uncontrolled growth and invasion.

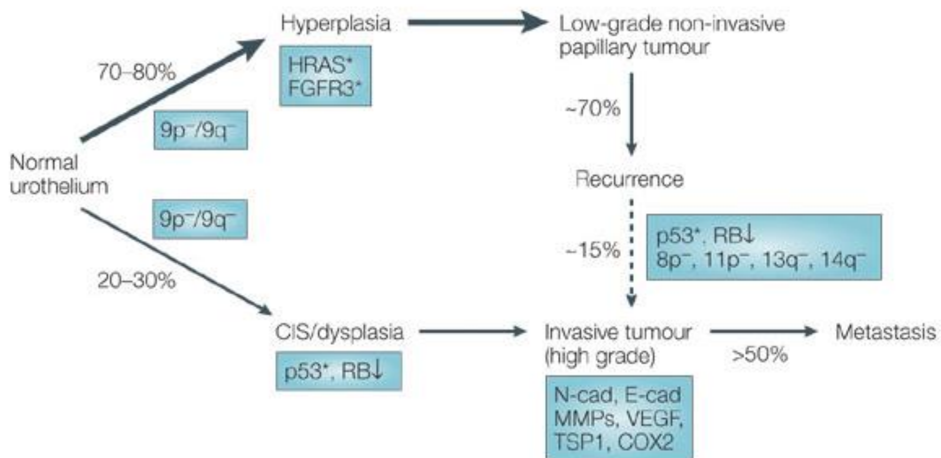

Figure 2. Two-pathway model of UC development

Schematic image describing the most cited model of UC development. Adapted from Wu 2005.[61] Reprinted with permission of Nature Publishing Group.

The second pathway originates with *RB1* or *TP53* inactivation in areas of CIS and genomic instability. These cells later on acquire an invasive phenotype through altered interactions with the tumor microenvironment. This model is in agreement with the clinically observed differences between the papillary and flat growth patterns seen in NMIUC and MIUC/CIS, respectively. The model is also favored by research describing the interactions between cancer genes in mouse models.[62] However, the model does not explain that some tumors may have papillary growth pattern, several recurrences and genomic instability or *TP53* mutation.[63,64] Similarly, a significant portion of de-novo invasive tumors do not have *RB1* or *TP53* inactivation but do have genomic instability.[65] These observations instead suggested the existence of molecular phenotypic states that may be arrived at through different mechanisms, e.g., *FGFR3* mutation usually gives rise to the less aggressive subtype, but occasionally non-progressed papillary tumors with *TP53* mutation have the same non-aggressive phenotype. Another important finding that the two-pathway model does not address is the discovery of incompatible genomic changes in papillary recurrent tumors, suggesting that recurrent papillary tumors also originate from a heterogeneous pool of progenitor cells.[66] It is now known that in 20-30% of cases, recurrent tumors do not share *FGFR3* mutation status with the primary lesion.[67] Furthermore, studies of cystectomy specimens have shown that patches of morphologically normal urothelium may share *TP53* mutations[68] and loss of heterozygosity (LOH) events seen in advanced tumors.[69]

These results favor the field-cancerization hypothesis first suggested in the 1950's,[70] in which epithelial fields acquire genetic changes and the heterogeneity of these fields affect the probability of tumor development.[71] In this model, CIS represents a readout of a urothelium with high degree of genetic heterogeneity.[72]

The urothelial progenitor cells reside in the basal cell layer and each one maintains one clonal patch of urothelium.[73,74] The basal cells have a phenotype that differs in many ways from the more differentiated intermediate and umbrella cells. It has been noted that in some tumors, the majority of cells share characteristics with the basal, progenitor like cells, whereas in others most tumor cells share characteristics with intermediate or differentiated cells. The pathological differentiation state of tumor cells is however not related to the urothelial differentiation state that the tumor corresponds to; e.g. grade 3 tumors with low degree of differentiation frequently do not express keratin 5 (KRT5), a markers of basal urothelial cells, but may overexpress keratin 20 (KRT20) and other markers of differentiation.[75] In spite of this, attempts have been made to classify and understand tumor development based on urothelial differentiation markers.[76,77] In these studies, tumor cells expressing basal markers grew better when grafted into immunodeficient mice and were associated with a worse prognosis. Although the results are interesting, these studies are based on the assumption that the urothelial differentiation states are relevant to tumor biology. This may not always be the case; i.e. the most basal marker used, keratin 14 (KRT14), is overexpressed in squamous epithelia regardless of differentiation state,[78,79] and is simply not expressed in most high grade UC. The results do not exclude the possibility that KRT14 merely identifies cells of a squamous phenotype, which when they form part of tumors are known to convey a poor prognosis.[80]

Prognostic factors in UC

Several clinical and molecular biomarkers have been investigated for association to outcome in UC, either as single markers, or as part of signatures or nomograms. For UC, three types of prognostic markers exist; those that predict recurrence of NMIUC, those that predict progression of NMIUC, and those that predict survival of MIUC. Most molecular markers are proteins that are measured by quantitative-PCR or IHC. Several other types of molecular markers exist, including LOH and genomic copy number markers,[81] methylation markers[82] and clinical risk factors.

The EORTC and CUETO risk tables

The EORTC risk tables are based on results of a large meta-analysis that used clinical variables to predict the outcome of patients with NMIUC.[83] Tumor stage, tumor grade, concomitant CIS, tumor size, multifocality and history of prior recurrences were all independently associated with high risk for progression. Based on the predictive model, each variable was given a weight depending on its

independent predictive value. By weighting the clinical data for a newly diagnosed NMI tumor, a risk score can be calculated, which can then be used to estimate the patient's risk for progression. This risk stratification is used both by clinicians and by researchers wanting to summarize the prognostic information contained in clinical data.

Five years after the publication of the EORTC risk tables, the Club Urologico Español de Tratamiento Oncologico (CUETO) collaborative research group published a meta-analysis in order to validate the EORTC model.[84] The results show that progression could not be predicted as well as in the original study. Progression status after five years was predicted with an accuracy of 68%, compared to 75% in the original study. This is explained by some important differences in the study populations. Firstly, the CUETO study contained more high-risk patients with respect to all the variables included in the risk score. This is not a problem in itself, since the risk predictor is applied individually to each case. Instead, the differences observed could be explained by the fact that the more high-risk CUETO cases were all treated with Bacillus Calmette-Guerin (BCG). In the EORTC study, the patients were at a lower average risk and the patients were given BCG only in one out of seven of the included studies. In the remaining studies intravesical chemotherapy had been given, which has a good effect against early recurrences, but is less effective in preventing progression. The conclusion was that more effectively treated cases with initially higher risk were stratified with a model based on lower risk patients treated thereafter.

This resulted in an overestimation of the progression risk when the EORTC risk score was applied to the CUETO cases. Additional follow-up studies have shown that not only the EORTC, but also the CUETO model overestimates the risk of both progression and recurrence in high-risk cases.[85] These findings underscore that difference in patient population and treatment can affect the usefulness of these risk models.

Molecular prognostic markers

A large number of publications have shown that single molecular markers are associated to recurrence and progression of NMIUC (summarized in Table 3). Most of the markers were significant also in a multivariate model that included at least tumor stage and tumor grade, and sometimes several other clinical variables that are known after pathological examination of TUR-B material. In spite of the large amount of positive data, none of these prognostic markers are used for clinical decision making. This may be due to variability in cohort selection. Some markers are investigated in cohorts that are mostly T1G3 cases, whereas others are made up of more than half Ta cases of grade 1 or 2. The extent to which patients in the cohorts have received treatment is another potential source of sample bias, since the use of BCG and chemotherapy for NMIUC varies between different

hospitals and times of sample taking. If such differences in study cohorts are too large, study results may not be comparable one to another, or to real life situations.

Furthermore, several clinical/pathological variables and endpoints are used in slightly different ways in these studies. For example, the definition of progression may include Ta to T1 progression and/or NMI cases that are cystectomized due to multiple recurrences in addition to progression to metastasis or muscle-invasion. In recognition of this problem a standardized definition of progression was recently proposed.[86] Yet another problem is that most of the published markers have not been independently validated. A fraction of these results may be chance findings due to selective data reporting or publication bias. Until the studies have been properly reproduced, they are not likely to enter routine clinical use. For survival analyses of MIUC the statistical power of population based cohorts poses a problem, seeing as more than half of the diagnosed patients may not receive radical cystectomy.[87] The outcome of patients treated with curative intent and patients receiving palliative treatment cannot easily be compared.

In addition to single molecular markers several investigations have aimed at combining markers into marker panels[88,89] or gene signatures[90-95] with prognostic value. In addition attempts have been made to use markers to replace pathological grade by molecular grading, in order to improve prognostic value and reproducibility.[96,97] Although marker combinations are less prone to give chance findings, they represent a higher risk of data overfitting, and seldom work efficiently when applied to external data sets.[98]

Treatment of NMIUC

The main treatment modality for NMIUC is transurethral resection of the bladder (TUR-B). Intravesical instillations such as BCG or chemotherapy may be given to prevent tumor recurrence and progression.

Bacillus Calmette-Guerin (BCG)

BCG treatment consists of several, usually 6-12 intravesical instillations of a solution containing *Mycobacterium bovis*. Following TUR-B, it is administred weekly during the induction treatment, and with longer intervals during maintenance treatment, where many different protocols have been described. Recently a large prospective study investigated different maintenance schedules and doses,[99] but to date, there is no consensus about the optimal instillation schedule. BCG is believed to function as a general immune stimulating agent, increasing anti-tumor immunity in the bladder mucosa. Several studies have shown that BCG is effective to reduce the risk of recurrence and progression for NMIUC.[100,101]

Table 3. Published molecular markers for recurrence and progression of NMIUC.

Marker	High/low	Endpoint	Method	Univar. P	Multivar. P	References
CCND1	low	recurrence	IHC	0.003	0.03	Sgambato. Int J Cancer. 2002. 10;97(5):671-8.
CD24	high	recurrence	IHC	<0.001	-	Liu. Oncol Lett. 2013. 6(1):96-100.
CD44v6	high	recurrence	IHC	0.003	0.006	Klatte. J Urol. 2010. 183(6):2403-8.
CDH1	abnormal	recurrence	IHC	0.005	0.02	Mahnken. Oncol Rep. 2005. 14(4):1065-70.
CDKN1A	low	recurrence	IHC	0.005	0.03	Behnsawy. Urol Oncol. 2011. 29(5):495-501.
CDKN1B	low	recurrence	IHC	0.001-0.006	0.05 - 0.005	Sgambato. Br J Cancer. 2001. 84(9):1242-51.
CDKN2A	low	recurrence	IHC	0.01	0.007	Yurakh. Eur Urol. 2006. 50(3):506-15.
COX2	low	recurrence	IHC	<0.001-0.05	<0.001, NS	Tadin. Diagn Path. 2012. Czachorowski. PLoS
ESR2	low	recurrence	IHC	0.021	NS	Han. World J Urol. 2012. 30(6):861-7.
FGFR3	low	recurrence	IHC	0.04	-	Barbisan. Cancer. 2008. 1;112(3):636-44.
HSP70	high	recurrence	IHC	0.001	0.003	Yu. Histopathology. 2013. 62(5):788-98.
KRT20	abnormal	recurrence	IHC	0.001	0.01	Bertz. Eur Urol. 2012. May 19.
LGALS3	low	recurrence	IHC	0.03	0.01	Kramer. Oncol Rep. 2008. 20(6):1403-8.
MCM2	high	recurrence	IHC	<0.001	<0.001	Burger. Br J Cancer. 2007. 4;96(11):1711-5.
MKI67	high	recurrence	IHC	0.003 - 0.04	0.0005	Quintero. J Clin Pathol. 2006. Barbisan. Cancer.
PAK1	high	recurrence	IHC	<0.01	0.008	Ito. J Urol. 2007. 178(3 Pt 1):1073-9.
RBX1	high	recurrence	IHC	<0.001	0.04	Wang. J Surg Oncol. 2013. 107(7):758-61.
SNAI1	high	recurrence	IHC	0.028	0.04	Bruyere. Urol Oncol. 2010. 28(6):591-6.
SOX2	high	recurrence	IHC	0.001	0.029	Ruan. Med Oncol. 2013. 30(1):445.
SPINK1	low	recurrence	IHC	0.02	NS	Patschan. World J Urol. 2012. 30(6):785-94.
TIMP2	high	recurrence	IHC	<0.01	0.04	Hara. J Urol. 2001. 165(5):1769-72.
TP53	abnormal	recurrence	IHC	-	0.004 - 0.06	Reviewed in: Malats. Lancet Oncol. 2005.
TYMP	high	recurrence	IHC	0.021	0.02	Nonomura. Int J Clin Oncol. 2006. 11(4):297-

Marker	High/low	Endpoint	Method	Univar. P	Multivar. P	References
AIB1	high	progression	IHC	0.003	0.009	Tong. Br J Cancer. 2013. 16;108(7):1470-9.
ANXA10	low	progression	IHC	0.0001	0.003	Munksgaard. Br J Cancer. 2011. 105:1379 – 1387.
AQP3	low	progression	IHC	0.02	0.03	Otto. BMC Cancer. 2012. 12:459.
BIRC5	high	progression	IHC	0.002	0.01	Fristrup. Am J Pathol. 2012.180:1824–1834.
C16orf74	low	progression	qPCR	<0.001	0.001	Kim. PLoS ONE. 2010. 5(12): e15260.
CCND1	low	progression	IHC	<0.001-0.005	0.05	Yurakh Eur Urol. 2006. Fristrup. Am J Pathol. 2013.
CD9	low	progression	IHC	<0.001	0.007	Mhawech. Cancer. 2003. 15;98(8):1649-57.
CDKN2A	loss	progression	IHC	0.018	0.009	Krüger. Eur Urol. 2005. 47(4):463-7.
CTAG2	high	progression	qPCR	0.001	0.02	Dyrskjøt. Br J Cancer. 2012. 107, 116–122
CTSE	low	progression	IHC	0.002	0.02	Fristrup. Am J Pathol. 2012. 180:1824–1834.
EGFR	high	progression	IHC	<0.0001-0.01	0.0004	Mellon. J Urol. 1995. Liukkonen. Eur Urol. 1999.
ERBB4	high	progression	IHC	0.001	-	Puerta-Gil. Am J Pathol. 2012. 180(5):1808-15.
ESR2	high	progression	IHC	0.002	-	Miyamoto. BJU Int. 2012. 109(11):1716-26.
GATA3	high	progression	IHC	0.05	0.05	Miyamoto. Hum Pathol. 2012. 43, 2033–2040
HMGA2	high	progression	IHC	0.004	0.006	Yang. EJSO. 2011. 37:265e271
HMOX1	high	progression	qPCR	0.001	0.001	Yim. JURO. 2011. 185:701-705
HSP27	low	progression	IHC	<0.001	<0.001	Yu. Histopathology. 2013. 62(5):788-98.
HYAL1	high	progression	IHC	0.001	<0.001	Kramer. Eur Urol. 2010. 57:86-94
KPNA2	high	progression	IHC	<0.001	0.001	Jensen. Eur Urol. 2011. 59:841-848
MCM7	high	progression	IHC	<0.001	NS	Fristrup. Am J Pathol. 2013. 182(2):339-49.
MKI67	high	progression	IHC	<0.01-0.002	0.0005-0.002	Liukkonen. Eur Urol. 1999. Bertz . Eur Urol. 2012.
PFKFB4	high	progression	IHC	0.003	0.01	Yun. Urol Oncol. 2012. 30(6):893-9.
PLK1	high	progression	IHC	0.003	NS	Fristrup. Am J Pathol. 2012. 180:1824–1834.
RBX1	high	progression	IHC	0.005	0.03	Wang. J Surg Oncol. 2013. 107(7):758-61.
S100A8	high	progression	qPCR	0.001	0.004	Ha. Kor J Urol. 2010. 51:15-20
SERPINB5	low	progression	IHC	0.001	NS	Fristrup. Am J Pathol. 2012. 180:1824–1834.
TOP2A	high	progression	IHC	0.04	0.05	Kim. Urology. 2010. 75(6):1516.e9-13.
TP53	abnormal	progression	IHC	-	0.06 - 0.0005	Reviewed in: Malats. Lancet Oncol. 2005.
TRIM29	low	progression	IHC	<0.001	0.02	Fristrup. Am J Pathol. 2013. 182(2):339-49.
UBE2C	high	progression	IHC	<0.001	<0.001	Fristrup. Am J Pathol. 2013. 182(2):339-49.

BCG treatment is associated with a number of side effects, including fever, bladder inflammation and voiding complications, which is why it is primarily used for high-risk NMIUC patients.

Intravesical chemotherapy

Several single instillation chemotherapeutic agents such as adriamycin, gemcitabine, mitomycin, doxorubicin or epirubicin have been tested as alternative to BCG for high-risk NMIUC. Single instillation chemotherapy has also been tested for its usefulness in reducing the risk for recurrence in low-risk NMIUC.[102] The overall results indicate that many chemotherapy-regimens may prevent recurrence,[103] but not progression in high-risk patients.[104,105] There may be a role for intravesical chemotherapy as second-line therapy for patients that do not respond to BCG treatment.[106]

Treatment of MIUC

Radical cystectomy

Radical cystectomy is the current standard treatment for MIUC and is sometimes used also for high-risk NMIUC. The procedure removes the entire bladder and regional lymph nodes and is considered a treatment with curative intent. The five-year recurrence-free rate for organ confined, node negative disease treated by cystectomy alone is 73-85%. The corresponding figures for non-organ-confined and node positive disease are 56-70% and 33-35%, respectively.[107-109]

Radiotherapy

As a bladder sparing alternative to cystectomy, especially in patients unfit for surgery, radiotherapy can be used for MIUC. Adding chemotherapy to radiotherapy after as much of the tumor as possible is removed by TUR-B improves cure rates.[110] Treatment will be shifted to salvage cystectomy only if the patient has recurrence. The outcome after chemoradiotherapy has not been compared to that of radical cystectomy in the required randomized setting. Studies comparing the two modalities are prone to selection bias and tumor stage may be underestimated in radiotherapy treated patients where the TUR-B was incomplete.

Chemotherapy

Chemotherapy for MIUC may be given neoadjuvant or adjuvant to cystectomy, or in combination with radiotherapy. In the neoadjuvant setting, chemotherapy has been shown to increase survival enough to be of value despite the usual increase in adverse side effects. The investigated regimens usually consist of either GC (gemcitabine and cisplatin/carboplatin), or MVAC (methotrexate, vinblastine, adriamycin, and cisplatin). Both regimens have shown modest survival benefits (6-

7% improvement in 3-year survival rate) and serious but tolerable side-effects.[111-112] Comparative studies have found no significant difference between GC and MVAC.[113-114] Although the best studies recommend the use of neoadjuvant chemotherapy it is only given in a minority of cases due to tolerability issues and a lack of response predictive factors.[115] It is likely that the usage of neoadjuvant chemotherapy would increase if it was recommended only for a convincingly selected group of high-risk patients.

Targeted therapy

Targeted therapies have been developed and introduced into clinical use for many tumor types, but not for bladder cancer. Many targets have been tested in preclinical studies using UC cell lines or xenograft mouse models. For most of these studies, however, the expression of the target protein itself is not investigated, and as shown in paper 2 of this thesis, many potential drug targets are differentially expressed between molecular subtypes and tumor stages. Additionally, the activity of a potential protein target is likely to depend not only on its expression but also on post-translational modification status, levels of substrates and products of enzymatic reactions and the genetic and epigenetic states of the tumor cells. Regardless of these caveats, a trial and error approach may be successful if a proper stratification is performed. For example, most targeted therapies have been tested as neoadjuvant agents to improve survival after cystectomy. The effects of these drugs need to be tested in the appropriate systems, i.e. high grade cell lines for a target that is active in MIUC. Confirmation that the target remains expressed in micrometastatic cells might also be necessary since that is not always the case.[116] Conversely, for targets in mitogenic pathways active early in tumorigenesis, non-invasive model systems could be used. In the following sections, some of the most studied therapeutic targets in UC are discussed.

The *FGFR3* gene is activated by mutation or overexpression in nearly all NMI UC cases. Several studies have shown that knock-down of *FGFR3* expression in cell lines carrying mutated *FGFR3* but not in normal urothelial cells decreases proliferation and clonogenicity.[117-119] The effects of *FGFR3* knockdown on tumor growth was confirmed in a xenograft mouse model using the RT112 cell line that is known to be highly dependent on FGFR3 signaling.[119] The authors suggested the use of an in-house monoclonal antibody blocking both wild-type and mutant FGFR3. Another study showed specific effectiveness of a small molecule inhibitor (PD173074) in a xenograft model using *FGFR3* mutated cell lines.[120] A recent study investigating the effect of two small molecule inhibitors on a myeloma cell line with FGFR3 mutation showed an initial effect followed by a secondary mutation conferring acquired restistance to both inhibitors.[121]

In non-small cell lung cancer (NSCLC), response to the EGFR inhibitor gefitinib is dependent on the presence of an activating mutation.[122] In UC, the *EGFR* gene is not mutated[123] but rather overexpressed in a subset of primarily MI tumors. Consequently, anti EGFR antibody and inhibitor testing has been performed in combination with chemotherapy for MIUC treated with cystectomy.[124-127] The results have been disappointing with no improvement in patient outcome. A preclinical study using gefitinib on bladder cell lines has shown a highly variable effect, which may be attributed to a dependency on E-cadherin expression.[128]

The *ERBB2* gene is amplified in around 10% of high grade UC,[129,130] whereas the frequency of protein overexpression is around 50% in the same group.[131] Reports have shown that virtually all *ERBB2* overexpressing primary tumors show overexpression also in subsequent lymph node and distant metastases. On the other hand, half of the ERBB2 negative primary tumors also showed overexpression in metastases,[116] meaning that one cannot predict with good specificity the ERBB2 status of a metastasis based on the primary tumor. A large clinical study investigating the effect of trastuzumab in combination with chemotherapy for metastatic UC showed a high response rate (70%) for patients with *ERBB2* overexpressing tumors.[131] Although the results of this study are encouraging, a randomized trial of neoadjuvant chemotherapy plus trastuzumab versus chemotherapy alone for patients undergoing cystectomy for MIUC overexpressing *ERBB2* in the TUR-B specimen would be needed to establish the value of trastuzumab in treatment of UC.

In UC, the mTOR kinase is activated by several molecular mechanisms. The *MTOR* gene itself is only rarely activated by mutation, whereas genes regulating the signaling pathways that lead to mTOR activation are frequently mutated or show deregulated expression. Clinical studies of the inhibitor everolimus have only been performed in the metastatic setting with tolerability as primary endpoint.[132,133] Both studies show partial responses and a number of cases with tumors regression. Interestingly, in one of these studies cancer exome panel sequencing was recently performed for 14 patients showing that the patients with inactivating *TSC1* mutations were all among the responders.[134] As of today there has been no trial for tumors carrying *TSC1* mutation, randomizing patients to standard treatment plus everolimus versus standard treatment alone.

High throughput molecular techniques

DNA sequencing

Establishing the DNA sequence of tumor tissue is one of the most straightforward ways to obtain data from a tumor. According to the catalog of somatic mutations in cancer (COSMIC) database, the average number of somatic variants carried by

tumors with known whole genome sequence is around 150.[135] Only a fraction of these somatic variants are thought to be causally involved in tumorigenesis. The traditional sequencing method, Sanger sequencing, relies on PCR amplification of genomic regions of interest. In Sanger sequencing a certain ratio of deoxyNTPs to chain terminating dideoxyNTPs is used which stops the elongation reaction. DNA fragments are then detected based on their size difference and the identity of the chain terminating nucleotide.

Over the course of the last ten years, next generation sequencing methods have dramatically increased the power of DNA sequencing. Briefly, this method involves producing a library of DNA fragments from the entire sample, or a captured selection of sequences, e.g. exome sequences. The ends of each fragment are ligated to known primer sequences. A parallel sequencing reaction then takes place on a surface covered with immobilized primers. The DNA fragments of the library will be clonally amplified in cycles with extension of a single nucleotide per cycle. In each cycle, a fluorescence scanner records the identity of the incorporated nucleotide in each clonal amplification reaction. Each amplified fragment results in a sequence read that is aligned to a reference genome. Specific software suites have been developed for the tasks of alignment and detection of sequence variants in this type of data.

Early studies using Sanger sequencing identified a number of cancer genes that are commonly mutated in many tumor types including bladder cancer. Previous knowledge of the importance of genes such as *TP53*, *RB1*, *CDKN2A*, *TSC1*, *PIK3CA*, *FGFR3* and members of the *RAS* gene family led to the identification of recurring mutations in these genes. Very few studies have been published that make use of massively parallel sequencing, although the number of studies using this technology is likely to increase dramatically. The cancer genome atlas (TCGA) project is currently gathering high throughput data on a variety of different tumor types. In august 2013, next generation sequencing data has already been made publicly available for close to 250 MIUC and data for an additional 200 cases is on the way. This project will once and for all reveal the genes, mutation types, frequencies, and context of mutations in MIUC. The TCGA project unfortunately does not plan to provide any data on NMIUC.

Array comparative genome hybridization

The most commonly used high-throughput technique to obtain DNA copy number data is array comparative genome hybridization (aCGH). This technique relies on fluorescent labeling (Cyanine 3) of the DNA of a tumor sample. The labeled sample is mixed with DNA of normal copy number labeled with a different fluorophore (Cyanine 5) and hybridized to arrays containing thousands to millions of spots of printed DNA probes. After hybridization, the ratio of fluorescent light emitted from each spot will correspond to the tumor to normal ratio of the DNA complementary to the probe sequence. One of the limitations of aCGH is that it

does not give information on the chromosomal context of the probed DNA sequences; i.e. it is not possible to determine how an amplified region is structured, or whether it is located on the correct chromosome, the wrong chromosome, or in extrachromosomal DNA.

Gene expression microarrays

Gene expression microarrays are used to simultaneously measure the relative levels of all the mRNA molecules in a sample. In short, the mRNA molecules are converted to cDNA and fluorescently labeled. For single channel platforms, such as Illumina HT-12, each sample is labeled with only one dye, and only one sample is hybridized to each microarray. The intensity detected at each spot on the array corresponds to the relative abundance of the mRNA transcript that the probe sequence was directed towards. There are several strengths and weaknesses of gene expression microarrays. For example, due to technical bias (variation in array production, as well as mRNA amplification, labeling and hybridization efficiency), some target-probe interactions are more efficient than others. This means that a higher raw intensity from one spot does not mean the transcript is more abundant in the sample. For this reason, this type of microarray experiment must include many samples. Only then, under the assumption that each target-probe interaction is subjected to the same technical bias in all samples, can transcript levels be compared relatively between samples. Before this is possible, several data processing steps need to be performed, including data normalization. Quantile normalization means that each probe on the array is ranked by intensity then reassigned a value based on the probe intensity distribution on all arrays. This process removes the effect of global differences between samples/arrays and diminishes differences due to technical bias. When a data set is quantile normalized is assumed that all samples have the same distribution of transcript levels. This assumption may not hold true if the samples are biologically very different. In the case of a cohort of tumor samples, this drawback is usually accepted and the assumption is made.

The main strength of gene expression microarrays is the wealth of powerful analysis methods that can be used on data from a well-designed and well-performed experiment. Unsupervised techniques such as hierarchical clustering and factor analysis can be used to assess similarity between samples and association to sample annotation variables. Given a grouping variable, such as molecular subtype, tissue type, or patient outcome, an ANOVA-, a t-test, or a linear model can give the gene/genes with strongest association to the grouping variable. Genes with similar expression profile across samples can be identified by clustering of genes, and functional annotations can be given to gene clusters.

The major pitfalls of array based gene expression analysis are batch effect problems, biological inference based on data from only one or few probes, and faulty estimations of statistical significance in multidimensional data. Below, each

of these three pitfalls will be briefly discussed and our strategies to avoid them will be outlined.

Batch effects in gene expression microarray experiments

A batch effect is a type of technical bias that cannot be corrected by standard normalization methods. The problem occurs when samples are treated batch wise, which usually happens on several occasions from retrieval of the tumor sample to hybridization. Examples of sources of batch effects can be hospital of origin, RNA extraction and storage methods, labeling batch and hybridization slide. To combat batch effects it is essential to know about all these sources and to use proper randomization in the experimental design. The worst case scenario would be that all MI samples are extracted using one extraction method and all NMI samples using a different method. In that case one could never separate stage associated gene expression from the potential technical bias. If the batches are large (like two 96-well labeling plates) randomization is usually sufficient, but for smaller batches (labeling in 8 tube strips) the known variables expected to broadly affect gene expression should be varied as much as possible within the batch to assure that as much of the biological variation as possible is independent of the batch variable. If all batches are known and care has been taken in the experimental design, any potential batch effects can be identified and hopefully dealt with. Lauss et al.[136] have developed a method based on calculating the association between batch annotations and the principal components of the data. Important variables such as labeling batch are often strongly associated to the second or even the first principal component of a data set, but usually not the same component that is most strongly associated to biological variation. This method also includes ways to correct the identified batch effects and to check that they are gone after correction. The simplest way of dealing with a batch effects is to set the mean of each probe in all batches to the mean of one reference batch. This will distort the data if variables associated to biological variation are not evenly distributed among batches.

Biological inference from microarray data

One of the major weaknesses of gene expression microarray experiments is that each single measurement, i.e. each probe, is not calibrated to a known reference and does not have appropriate controls. Single probes might also cross hybridize to transcribed ortholog or unknown sequences. These problems are generally handled by using the mean or median probe value for several probes of a gene or a signature of genes, attenuating the effects of single bad probes. As a result one should not draw too strong conclusions based on results from a single, or a few probes.

Statistical significance in multidimensional data

As mentioned, one of the strengths of gene expression microarray experiments is that it allows testing association of each variable to sample annotations. This is usually done with an ANOVA or a t-test. For single variable testing, the usual null hypothesis is that this variable is not associated to the sample annotation. Here, the more common situation is that the statistical test is used as an exploratory tool and that the test statistic and significance is used as a ranking of how associated the variable is to a sample annotation. If the data set contains several thousand variables, one would a priori expect a quite large number of variables to show a significant association, and some correction for multiple testing must be used. There are several ways this can be done. One of the most crude and stringent methods is the Bonferroni correction in which the significance level of each test is set to 0.05 divided by the number of tests. This correction method usually underestimates the number of significant results, and using the false discovery rate (FDR) is a better correction strategy.[137] The FDR is a complementary measure to the p-value which indicates the proportion of the significant findings that is false. Knowing the FDR allows the adjustment of significance level so that both the type I and type II errors can be minimized. This method is especially well suited for analyses on microarray data where conclusions should not be based on only one tested variable regardless of statistical method.

Immunohistochemistry using tissue microarrays

IHC is a laboratory technique in which primary antibodies specific for biomolecules are applied to a tissue sample. Primary antibody binding is then visualized using a secondary antibody conjugated to a peroxidase that catalyzes the production of a dark brown color from the substrate 3,3'-Diaminobenzidine. IHC has been used for decades, whereas it was only in the late 90's that the concept of tissue microarrays (TMAs) was first described.[138] A TMA is a collection of cylindrical tissue cores embedded in a single paraffin block. The possibility of simultaneous staining of several different samples increases the throughput of IHC and decreases the technical variation between staining of different samples. Compared to gene expression microarrays, quantitative PCR, or mass spectrometry, IHC using TMAs is a relatively cheap and simple procedure.

The end result of this technique is the image of a stained TMA section. Although image analysis software that can quantify pixel intensity of scanned images exist, most experiments are still evaluated manually. Potential sources of non-biological variation in an IHC experiment can be either technical, such as unspecific binding of antibodies, or incomplete blocking of endogenous peroxidase activity, or they can be related to evaluation bias. The most common situation is that the extent and intensity of the staining is evaluated on a semi-quantitative scale, usually 0-3 or 0-5, where cut-off points are determined for each experiment. Within the

experiment, a higher value represents a stronger staining. The data can then be analyzed much in the same way as a microarray experiment, only with fewer and discrete variables. Since the evaluation is a subjective process it is essential that the identity of the samples on the TMA is not known to the observer until evaluations are complete.

Aims of the thesis

The overarching aim of this thesis is to produce a classification system for UC that stratifies tumors based on their biological differences. It is our hope that stratification using this system, or an extension of it, will contribute to future translational research findings that could not be discovered by means of standard clinical/pathological examination.

Specific aims of the included papers

Paper 1

This paper had two specific aims. The first one was to use to elucidate the major gene expression patterns in bladder cancer and relate these patterns to tumor stage and grade. The second aim was to analyze the relationship between inactivating *TP53* mutation and genomic instability, which is a key question in the understanding of UC development.

Paper 2

The specific aims of paper 2 were to define the molecular subtypes of UC and describe the molecular findings associated with the subtypes.

Paper 3

This papers major aim was to investigate the possibility to determine molecular subtype based on IHC data and evaluate IHC classification using the gene expression subtypes as a gold standard. A secondary aim was to describe which molecular findings associated with the subtypes that show cell type specific expression patterns.

Paper 4

The aim of this paper was to describe the prognostic utility of subtype classification using IHC on primary T1 tumors. An additional aim was to describe the relationship between molecular subtype, the EORTC risk model, and levels of immune infiltration.

Tumor material and patient cohorts

Papers 1-3 included in this thesis have used overlapping but not completely identical cohorts of tumors. The patient cohorts in papers 1-3 were retrospectively selected based on clinical and pathological reports from a large material collected from year 2000 until the present. Paper 1 has 144 samples, of which 81 overlap with paper 2. Paper 2 has 308 samples, including all the 237 samples of paper 3. Paper 4 is based on a completely independent population based cohort of primary T1 tumors identified in the Swedish Bladder Cancer Registry. The inclusion criteria used in paper 2 are potentially important, since the identified subtypes would be affected by a possible selection bias. Since inclusion based on the true population frequencies of stage and grade categories would lead to a low representation of MI tumors, we decided to include approximately equal numbers of the three major stage groups Ta, T1 and MI. Additionally we included only one tumor per patient, and excluded cases of non-UC histology or of poor RNA quality. In this selection process, the number of MI tumors became a limiting factor and in the end only 92 MI tumors were included. This also meant that for patients with more than one tumor available (recurring tumours), the MI tumor was always selected, in order to satisfy the criteria of equal numbers of the stage groups. Unfortunately, this implied an indirect exclusion of some superficial tumors with subsequent MI tumors, making the cohort suboptimal for analysis using recurrence or progression of NMIUC as an endpoint. On the other hand, the selected cohort included the highest possible number of MI tumors, capturing the full diversity of the stages of UC. To support the general relevance of the taxonomy, the outcome of NMI and MI tumors in the cohort of papers 1-3 is similar to what is observed for UC in general.

Tumor classification based on gene expression profiles (paper 1-2)

The strategy to identify gene expression subgroups was the same in paper 1 and in paper 2. The full data set was filtered (50%) to contain only the probes that varied across the data. Based on this filtered data set, a hierarchical clustering analysis (HCA) was performed. Using a bootstrap approach, the co-clustering frequencies were calculated for each sample. Based on the matrix of co-clustering frequencies it was evaluated if the separation of groups was consistent in bootstrapped HCA. If this was the case, the division of the groups was accepted, and the procedure repeated on the groups to identify further subgroups. When the co-clustering frequencies indicated that samples no longer consistently formed groups in bootstrap HCA, no further subgroup divisions could be made. In paper 1, a cohort of 144 cases was analyzed and this approach led to the division of the data set into two groups of approximately equal size.

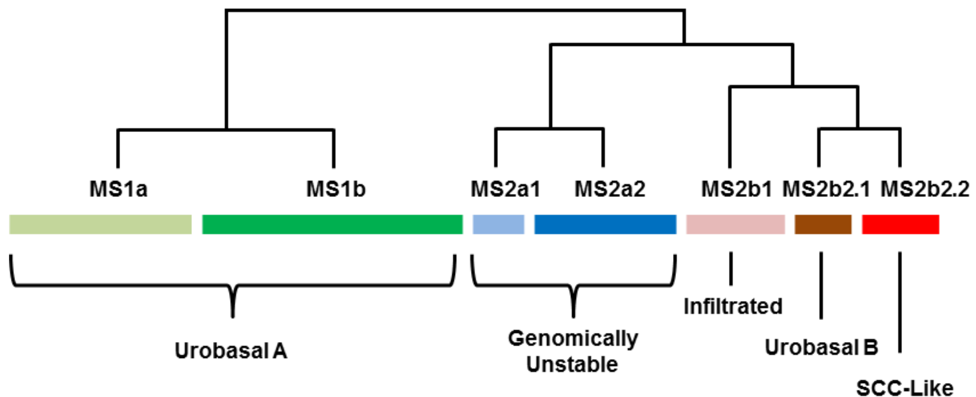

Figure 3. Overview of the hierarchical structure of the molecular subtypes of UC

Scematic figure of the hierarchical relationship of the 7 bootsrap HCA groups of paper 2 (colored bars). On the bottom is indicated the 5 molecular subtypes arrived at after merging of MS1a/b and MS2a1/a2.

In paper 2, the larger cohort of 308 cases allowed identification of seven well defined clusters of tumors. After a thorough biological analysis of the groups, four of the clusters were merged to two, reducing the final number of subtypes to five (Figure 3). This was done because the two pairs of HCA groups that were merged (MS1a/MS1b, and MS2a1/MS2a2) only differed in proliferation- and immune-related signatures, respectively. These signatures show a continuous distribution across the respective subtypes and a division would have been based on a quantitative rather than qualitative difference between the groups. The large variation of proliferatve and immunological potential of the Urobasal A and genomically unstable subtypes, respectively, may be of biological importance and is something to keep in mind in future analyses.

The large amount of genes related to proliferation and immune response caused another curious effect on the analysis. The Urobasal B (Uro B) subtype, which is biologically similar to Urobasal A (Uro A), clustered in the MS2 branch of the subtypes because it has significantly higher average proliferation and immune response than Uro A. This brings up an interesting aspect of the method: Processes involving regulation of many genes will have a stronger influence on subtype determination than processes involving regulation of few genes. Seen from a critical point of view, this indirect weighting of processes is rather arbitrarily determined by the representation of genes in the genome and on the array. On the other hand, as we can only tentatively determine the relative importance of

biological processes anyway, some sort of unbiased gene weighting is necessary. Allowing each gene to have the same impact on the clustering is by far the most straightforward and commonly used method.

To be certain that the subtypes are true entities of UC, we performed the entire process on three published data sets.[139-141] We arrived at 3-5 groups in each of these data sets, and we were able to show that each identified group corresponded to one of the subtypes in enrichment analyses (See Supplementary material, paper 2). In fact, from a simple look at a HCA ordered heatmap of the 500 genes with highest association to subtypes, the identities of the subtypes in the validation data sets were obvious.

For future subtype classification of unknown samples, a nearest-centroid classifier containing almost 1,000 genes with the highest association to molecular subtype will be used. Since whole-genome gene expression data will be necessary for classification, we do not see a need to provide a classifier using fewer genes at this stage.

Biological description of the molecular subtypes of UC (papers 1-4)

The analysis of biological patterns showing significant differences between subtypes was carried out in the following way. A quality threshold clustering (QTC) of the genes was used as an unsupervised way to identify clusters of genes with similar expression across all samples.[142] This resulted in the identification of 23 clusters (QTCs) that contained at least 20 genes with internal correlations of $r > 0.5$. Each cluster was analyzed, both by manually deducing a common functionality, and by unsupervised enrichment analysis against known databases such as the KEGG pathway annotation database (http://genome.jp/kegg) and the MsigDB gene signature database.[143] Most of the clusters were in some way associated to processes important for tumor biology. In addition, supervised analysis, e.g., of all cell-adhesion genes or all receptor tyrosine-kinase genes, led to the identification of subtype-specific expression of genes or groups of genes with known biological functions. In the evaluation of IHC data in paper 3 and 4, the score of a marker was tested for its differential expression by use of the Mann-Whitney U test. Additional categorical information such as the tissue stratification, or aberrant subcellular expression of a marker was analyzed using Fisher’s exact test. Other categorical data such as mutation data was handled in the same way.

In each of the four papers in this thesis, most of the effort is put into describing the results of these biological analyses of the subtypes. Because the results and discussion sections of the papers cover the biological description of the subtypes in detail, here it will be summarized in Figure 4.

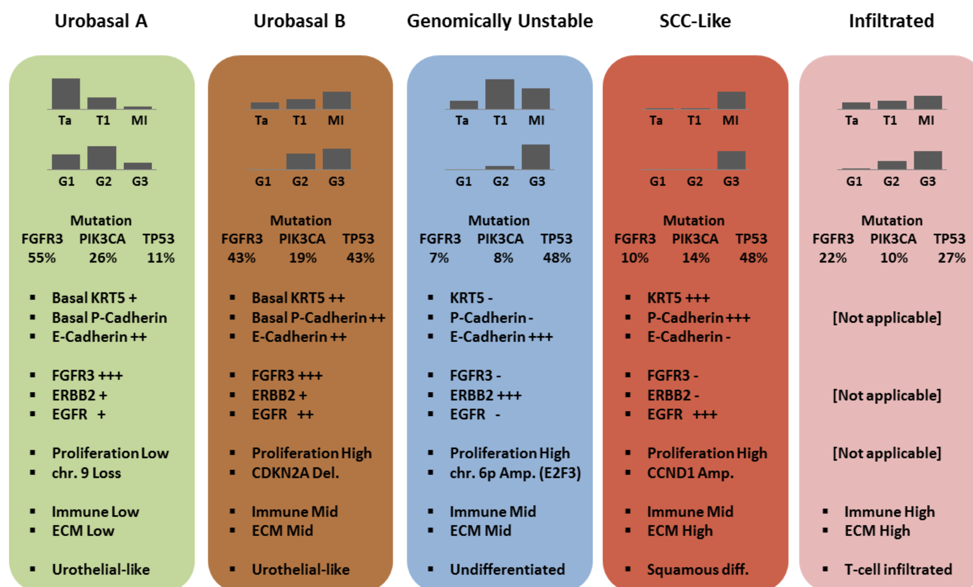

Figure 4. Biological characteristics of the molecular subtypes of UC.

Barplots show fractions of tumor stages and tumor grades within each subtype. Strength of expression is indicated with +, ++, and +++. Degree of immune and ECM component indicated by Low, Mid, or High. For the infiltrated subtype, the tumor cell intrinsic traits are similar to one of the UroB, GU or SCCL subtypes.

Translation of the molecular subtypes to an IHC classifier (paper 3)

The translation of the molecular subtypes to IHC is an important step to improve their usefulness. Since a TMA is a limited resource, and a section has to be taken for each staining, the markers used were chosen with care. Ideally, the top discriminating markers for each subtype would be selected for IHC analysis. The number of proteins for which high quality IHC-FFPE suitable antibodies exist on the market is low and limited to quite well known proteins. Thus, we tested only a few markers that did not pass quality control, and many of the markers had been used in our pathology lab before. Nonetheless, the functions of the marker proteins were selected to cover many important aspects of biology, making sure that the most relevant processes can be described also at the IHC level.

The process of developing the IHC classifier started out by considering data from 20 IHC markers along with pathological variables such as stage, grade, invasive growth pattern, urothelial-like growth pattern and presence of squamous or glandular differentiation. Each variable was ranked by its association to molecular

subtype as determined from the gene expression data. Since the hierarchical structure of the molecular subtypes is known, the IHC classifier predicts molecular subtype in a series of stepwise decisions, mimicking the structure of the original classifier. In each step, the variables that best predicted true subtype in a logistic regression model were included, and cut-offs were determined by optimal receiver operator characteristics (ROC).

The final classifier uses tumor grade (WHO 1999), urothelial-like growth pattern, Cyclin B1 (CCNB1) and Keratin 5 (KRT5) staining. In the first step, if the tumor has two or three out of low grade (G1-G2), low CCNB1, and urothelial-like growth pattern the tumor is classified as Urobasal. If not, the KRT5 staining decides whether it's classified as Genomically unstable (GU) or SCC-like (SCCL), with KRT5 high scoring cases assigned to the SCCL subtype. This classifier correctly predicted subtype with an accuracy of 0.88 as determined by internal leave-one-out cross validation.

The IHC classifier only assigns samples to one of three subtypes, whereas five subtypes were originally defined. The reason for this is that the infiltrated subtype is defined by its high levels of infiltrating immunological cells. When analyzed by IHC, however, protein expression is evaluated in the tumor cells revealing the true molecular subtype of the tumor cells. Immune cell infiltration is not an intrinsic property of the molecular subtypes and should ideally be included as a complementary analysis. Prediction of the Uro B subtype was not possible as no variable was found that could discriminate the Uro A and Uro B subtypes. One reason for this may be that only 20 Uro B tumors were identified. Although Uro B is a distinct subtype as determined from the gene expression data, it could not be robustly identified when limited to the comparably small number of variables in the IHC data.

Prognostic utility of molecular subtypes for stage T1 tumors (paper 4)

In the final paper of this thesis, the molecular subtypes are applied for the first time to samples that have not been used to define them. A cohort of primary T1 tumors with long-term follow-up were subtype classified using the IHC classifier and analyzed using tumor progression as endpoint. In order to compare the results with current clinical standards, the risk factors of the EORTC risk model were collected for most of the cases. To dissect the interaction of tumor subtype and infiltrating cells, the density of CD3+ cells was also evaluated.

We could show that the tumors classified as Urobasal showed significantly higher expression of Urobasal markers than GU or SCCL cases, and vice versa. Survival analysis revealed that GU and SCCL cases had significantly shorter time to progression than those classified as Urobasal. The comparison of GU/SCCL to the

EORTC high risk group revealed that while both groups were significantly enriched for progressing cases, GU/SCCL identified a larger number of cases than EORTC high risk. The result suggests that if only EORTC high risk tumors are considered at risk, a large number of progression events will be missed. Considering all information, the best risk-stratification was obtained when using the GU/SCCL vs. Urobasal stratification and adding high EORTC risk or high density of infiltrating CD3+ cells to identify the GU/SCCL cases at highest risk of progression.

Discussion

Throughout the process of establishing molecular subtypes of bladder cancer it has been striking how clearly the subtypes separate on nearly all variables investigated. Of the genes with detectable expression, 60% showed a significant association with the subtypes in ANOVA tests. Although pathological stage and grade remains the stratification system of choice for clinical research, the molecular subtypes can be useful as complementary information. As we show in paper 4 for T1 tumors, there is a need to go beyond stage and grade in clinical stratification of UC. As with all genes, the expression levels of most biomarkers are associated to subtype, which consequently may be a major confounder in this type of studies. I believe that studies on prognostic biomarkers in UC would be more informative if the marker association to, not only stage and grade, but also molecular subtype was taken into account.

For preclinical studies on molecular mechanisms involved in tumorigenesis, the relevance of a molecular stratification system is perhaps more direct. Most studies of this nature classify the studied samples only by stage and grade which is often not satisfactory. Stage and grade are even used to stratify cell lines, where it may be particularly important to know the molecular phenotype rather than the original tumors stage and grade.

As an example of this situation, approximately half of stage T1 tumors are positive for expression of the transcription factor *TP63*, and half of T1 tumors overexpress *ERBB2*. Stratifying only by means of tumor stage and grade, one would probably conclude that T1 tumors are heterogeneous with respect to these important cancer genes. With the knowledge of the molecular subtypes of the samples, one would instead observe that nearly all the *TP63* expressing cases are of the Urobasal subtypes whereas the majority of the *ERBB2* overexpressing cases are of the GU subtype. Associations between molecular findings can thus be compared within the relevant groups. The importance of having a correct stratification system in research also increases when processes are context dependent, which we cannot exclude that they might be. There are even results favoring context dependent processes. For example, as mentioned in the supplementary material of paper 2, Urobasal cases with *FGFR3* mutation overexpressed *FGFR3* along with a signature of 16 co-regulated genes. On the other hand, the rare GU or SCCL cases with *FGFR3* mutation did not express the FGFR3 signature, indicating that the effects of *FGFR3* mutation may be context dependent (Figure 5). To what extent molecular events have different effects in tumors of different subtypes remains to be seen; the more common the subtype dependent effects are, the more important the subtypes will be to both clinical and preclinical research.

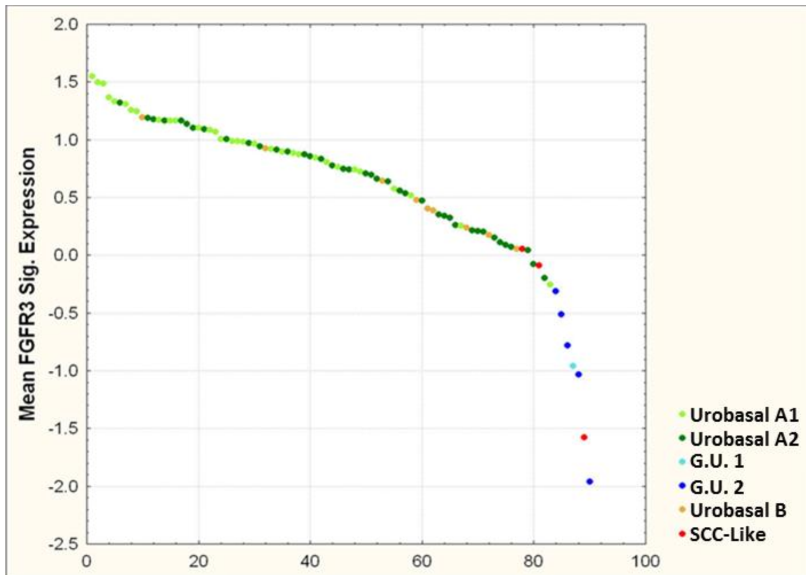

Figure 5. FGFR3 signature expression in *FGFR3* mutated cases; an example of subtype dependent effect of a molecular event.

Expression plot showing mean relative expression of the FGFR3 QTC gene signature (see Supplementary material paper 2) in samples carrying an activating *FGFR3* mutation. Samples are represented by a dots ordered by mean signature expression, and the colors represent the molecular subtype. GU/SCCL cases express low levels of the FGFR3 signature even though they have an activating *FGFR3* mutation.

A number of steps have to be overcome before molecular subtype classification can be easily performed in research labs and clinics. The simultaneous analysis of the expression of hundreds of genes required for using the centroid classifier from paper 2 is still too costly to be routinely used. If a q-PCR panel or a targeted RNA-seq panel would be used, a study would be needed to show the biological and statistical accuracy of classification.

For studies on FFPE tissue, we have shown that the IHC classifier can be useful. Compared to gene expression classification, the IHC classifier has some drawbacks. It does make some errors in prediction, although the accuracy of 0.88 in internal cross validation is high. Most notably, the Uro B subtype cannot be reliably identified, somewhat reducing the value of the IHC classifier.

Future perspectives

The whole process from discovery of the molecular subtypes to their refinement and translation into an IHC classifier was developed by our single research group. It is of great importance that the same tumor types are observed and described by other labs, verifying the findings both at the gene expression and protein levels. The IHC classifier makes use of both tumor grade and a set of histological criteria indicating a urothelial-like appearance. These evaluations are likely to suffer from the same reproducibility issues as conventional pathology, although the evaluation of CCNB1 and KRT5 immunostaining will probably be more stable, once the cut-offs are calibrated to account for technical and observer bias.

An important next step will be to determine the effects of the molecular subtype on the outcome of patients with MIUC that have been treated with radical cystectomy. Will it help us to be able to subdivide these advanced tumors into mainly the GU, SCCL, and to some degree Urobasal subtypes? In the patient cohorts used in study 2-3, half of the MIUC cases had not been treated with cystectomy, making survival analysis difficult. We are now planning to address these issues in a study where only cystectomy treated MIUC patients are included. The study will also test the hypothesis that response to neoadjuvant chemotherapy may be associated to molecular subtype.

Meanwhile, a wealth of data produced by the cancer genome atlas (TCGA) and other collaborations of cancer researchers is likely to be published within a year or two, hopefully adding new depth to our understanding of the subtypes of advanced UC. Similarly, with the increase in available genomic and epigenetic data, the potential roles of methylation and mutation associated phenotypes can add new dimensions to the molecular subtypes.

In addition to tumor stage and grade, it is clear that some kind of molecular taxonomy of UC is needed to put future research findings in a biological context. Conversely, the implementation and gradual improvement of a molecular taxonomy will also be dependent on how much it is used in future research.

Populärvetenskaplig sammanfattning

Cancer i urinblåsan är ett stort hälsoproblem i Sverige och världen. Det är den femte vanligaste cancerformen i Sverige och är den som belastar sjukvården med högst kostnader per patient. Ungefär tre fjärdedelar av de drabbade är män och patienterna är oftast kring 60-80 år gamla. Den behandling vi idag kan erbjuda en person som drabbas av blåscancer har inte förändrats på mer än tio år. De framsteg vi har sett inom forskningen kring cancer i andra delar av kroppen i form av nya behandlingsformer, kartläggningar av riskfaktorer och genetiska förändringar har inom blåscancerforskningen varit få.

Det främsta målet för det arbete som presenteras i denna avhandling är att åstadkomma en kartläggninig av de tumörer som uppstår i urinblåsan. Även tumörer som ser likadana ut vid operationstillfället har många mikroskopiska och biokemiska skillnader. Det råder en tro bland cancerforskare att det är dessa skillnader som gör det svårt att hitta effektiva behandlingar mot cancer. Vår hypotes är att en rättfram men avancerad beskrivning av de viktigaste molekylära skillnaderna kan leda till en gruppering av tumörer som kan ha en bättre eller sämre prognos, och därför bör få eller inte få en viss behandling. Det är vår förhoppning att detta klassificeringssystem även kommer vara till stor hjälp för framtida blåscancerforskning.

I det första arbetet lades grunden till klassificeringssystemet. Genom att analysera vilka gener som är aktiva i olika tumörer fann vi två huvudtyper av blåscancer. Den ena typen var mera godartad, medan den andra var mera elakartad. Vi beskrev även i detalj vilka förändringar i arvsmassan som sker i tumörer av de olika typerna.

Det andra arbetet byggde på det första genom att använda samma metodik men inkluderade prov från fler tumörer. På så vis kunde vi göra en förfinad klassificering. Vi kom till slutsatsen att det finns fem subtyper av blåscancer som utvecklar aggressiv tillväxt olika fort och på helt skilda sätt. I detta arbetet visade vi också att många av de läkemedel som testats mot blåscancer riktar sig mot molekyler som endast finns i någon/några av de fem huvudsakliga subtyperna.

I det tredje arbetet försökte vi förbättra och göra vårt klassificeringssystem mera användbart genom att byta metodik. I de två första arbetena användes genuttrycksanalys, som är en förhållandevis dyr och komplicerad analysmetod. I detta tredje arbete användes istället immunhistokemi, d.v.s. infärgning av vävnadssnitt från tumören. Detta ger en delvis förenklad bild av vad vi tidigare observerat, men ger även ny information om subtyperna som vi inte tidigare kände till. Slutligen utarbetade vi en modell för att med den nya, enklare metodiken kunna avgöra vilken av subtyperna en tumör tillhör. Anledningen till att vi bytte metod var dels för att möjliggöra analys av stora historiska biobanker med sparad

tumörvävnad, och dels för att underlätta att vårt klassificeringssystem skall kunna användas för diagnostik i dagens sjukvård.

I det sista arbetet tillämpade vi vår subgruppsindelning på 167 fall av stadium T1 blåscancer. Blåscancer i detta stadium är ett problem för sjukvården eftersom de i genomsnitt inte är aggressiva nog för att man skall vilja operera ut hela urinblåsan, men är aggressiva nog för att ibland utvecklas till en dödlig sjukdom. I detta arbetet utredde vi om vår klassificering fungerar bättre eller sämre än de riskfaktorer man använder idag för att avgöra om en tumör är farlig eller ofarlig. Slutsatsen är att både vår indelning och de rådande riskfaktorerna är värdefulla för att avgöra behandling. För att så bra som möjligt kunna avgöra om det är en potentiellt dödlig tumör skall man använda subtypsklassificering tillsammans med rådande riskfaktorer och en bedömning av patientens immunologiska svar mot tumören.

Framöver finns även planer på att tillämpa klassificeringen på tumörer av högre stadium där patienten fått urinblålsan utopererad. Förhoppningen är att indelningen i subtyper hjälper oss utröna vilka patienter som gagnas av kemoterapi och för vilka patienter kemoterapi skulle innebära en överbehandling med onödiga biverkningar.

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To anyone who shared the floor with me at BMC: You made it a great place to be.

To Frida, I want to tell you that you make me happy. I could write this book all over again (no problem) as long as we come home to each other at the end of the day.

Finally and most importantly, I would not be here if not for the constant encouragement of my mother.

Speciellt tack till dig mor!

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