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Recent advances in high-throughput molecular marker identification for superficial and invasive bladder cancers

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1. ABSTRACT

Bladder cancer is the fifth most common neoplasm in industrialized countries. Due to frequent recurrences of the superficial form of this disease, bladder cancer ranks as one of the most common cancers. Despite the description of a large number of tumor markers for bladder cancers, none have individually contributed to the management of the disease. However, the development of high-throughput techniques for simultaneous assessment of a large number of markers has allowed classification of tumors into clinically relevant molecular subgroups beyond those possible by pathological classification. Here, we review the recent advances in high-throughput molecular marker identification for superficial and invasive bladder cancers.

2. INTRODUCTION

2.1. Bladder cancer

Bladder cancer is, in terms of incidence, the fifth most common neoplasm in industrialised countries, accounting for about 6% of all newly diagnosed malignancies in men, and about 2% in women. In addition to gender, other risk factors include age, smoking and occupational exposure to carcinogens. Bladder cancer is one of the most prevalent neoplasms. The prevalence is three to eight times higher than the incidence and, consequently, bladder cancer is a major burden for the health care systems. The overall cause-specific five-year survival rate is about 65%.

In the industrialised world, the far most common histological form of bladder cancer is transitional cell

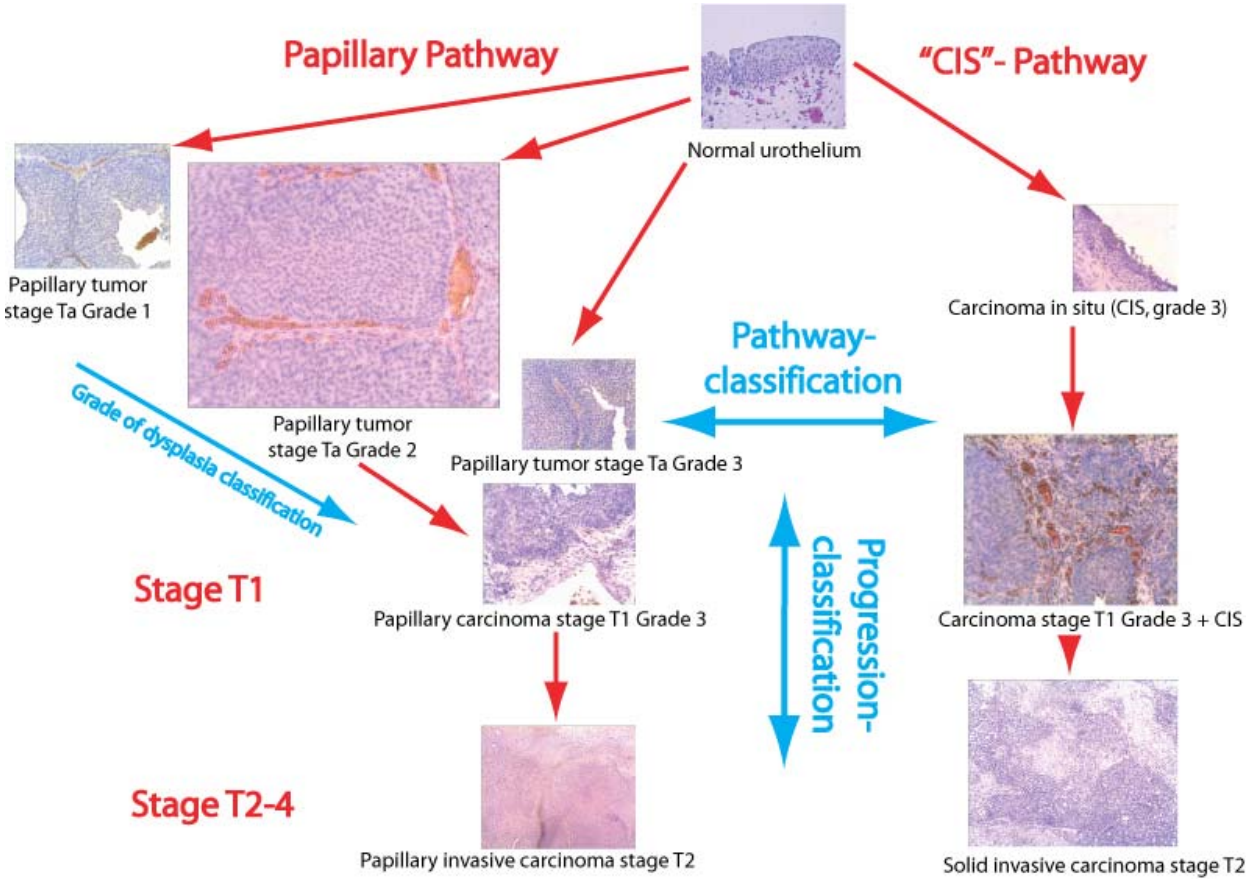

Figure 1. The Papillary- and CIS-pathways in bladder cancer development. The size of the histological figures approximately reflects the proportion of the respective tumor stages at primary diagnosis. To compare molecular profiles without stratification means comparison across stages, grades of dysplasia, molecular pathways of development and biological potential of progression. High-throughput molecular diagnosis has the potential to complement clinicopathological diagnosis by optimized stratification for molecular pathways AND biological potential in one step. This will pave the way towards an individualized treatment approach.

carcinoma (TCC). In regions where *schistosoma* infections are endemic, squamous cell carcinomas account for a significant fraction of bladder cancer; elsewhere they are less frequent (app. 5%). Squamous cell carcinomas almost exclusively have an invasive course. In contrast, TCC tend to occur in two principal forms: papillary superficial tumors and solid invasive cancers (Figure 1). Over the last decade, it has become increasingly clear that these two forms differ principally in terms of appearance, molecular changes and development, management, and prognosis.

2.2. Papillary superficial tumors

The most frequent of all newly diagnosed bladder cancers are superficial papillary tumors. This type is often diagnosed in an early and non-invasive stage (stage Ta), with mild forms of cellular dysplasia (low-grade). The prognosis of these tumors is reasonably good: under conservative (bladder-sparing) treatment only approximately 10% become fatal even after up to 20 years follow-up (1, 2). However, more than 60% of the Ta tumors recur, making this tumor type responsible for the high prevalence rate. About 40% of the patients experience multiple recurrences over many years. The frequency of recurrences has a significant impact on the patients' quality of life. Consequently, an attempt to prevent recurrences is frequently made after transurethral (endoscopic) resection of the tumors by intravesical instillations of *Bacille Calmette-Guerin* (BCG), which non-specifically stimulate the patient's immune response against the tumor cells. Alternatively, various chemotherapeutic agents may be used. Although the treatment is effective in most cases, long-term recurrences may occur (3). In selected cases of patients with very frequent recurrences, high grade lesions and failure of BCG treatment, surgical removal of the whole bladder (cystectomy) may be considered.

Invasion of the suburothelial connective tissue, the *lamina propria* (stage T1) occurs in the course of about 25%, depending on the grade of dysplasia (2, 4). The probability of progression for papillary stage T1 cancers is not known. However, it is most likely as high as for their non-papillary counterparts, namely about 30% (5). Some studies indicate that muscle invasive cancers with a papillary non-invasive history tend to be very aggressive,

and despite close surveillance and rapid radical treatment, mortality rates are high (6).

2.3. Solid, invasive cancers

Solid tumors are often high grade and usually diagnosed in an invasive stage (T1 or worse). In more than half of the cases, muscle invasion is diagnosed upon presentation (stage T2 or worse). They are believed to arise from flat, highly dysplastic but non-invasive lesions called *carcinoma in situ* (CIS, stage Tis). However, few symptoms are associated with CIS and consequently CIS is seldom diagnosed as the primary lesion; concomitant CIS is more common, and may be found in up to 40% of stage T1 cancers (7) and in 50% of the muscle invasive stages (8).

CIS is treated by BCG instillations (repeated or sustained if necessary). The treatment is effective in about 70% of the cases, and may also be given adjuvant after transurethral resection of a stage T1 tumor (9, 10). In case of failure, cystectomy is the preferred treatment of choice. With BCG treatment about 30% of stage T1 tumors of the CIS-type will progress to mucle invasion under a conservative (bladder sparing) regimen (7); without BCG, about 50% will progress (11). Large tumors and tumors with multiple recurrences or widespread CIS have a very high risk of progression, and many of these cases are primarily offered radical treatment (12).

Muscle invasive bladder cancer is a fatal disease if left untreated. Although endoscopic and partial bladder resections may cure the disease in selected cases, the standard treatment today is the complete removal of the bladder and adjacent organs (radical cystectomy) with pelvic lymph node dissection and urinary diversion. This is an extensive surgical procedure with high postoperative morbidity and risk of complications. External beam radiotherapy is an alternative radical treatment option, especially in case of co-morbidity that speaks against extensive surgery. Unfortunately, a substantial fraction of muscle invasive bladder cancers have distant metastases already at this stage, and this is critical for the long term prognosis. Metastatic disease is treated by cisplatin-based systemic chemotherapy. Five year cause specific survival rates are stage dependent and rank from 40% (stage T2) to less than 10% (stage T4).

2.4. Molecular diagnosis of bladder cancer

Being a rapidly evolving field, a large number of molecular tumor markers for bladder cancer have recently been published. However, the encouraging results are mitigated by small sample sizes, little overlap between studies, insufficient validation and the lack of adjustment for other clinical variables. These issues were discussed in a recent paper, and strict criteria for prognostic tumor marker studies have been recommended (13). The molecular features of bladder cancer have proved to be very complex and consequently the design of prognostic marker studies should be carefully planned. In contrast, histology has the advantage of a fairly good integration of molecularly very different cancers by their phenotype. However, there is considerable inter- and intra-observer variability. In consequence, the individual course of a cancer still cannot be predicted with sufficient safety. The challenge of molecular diagnosis is the integration of the various molecular features and the subdivision of bladder cancer beyond histological phenotype into clinical relevant molecular subgroups (Figure 1). The evolution of molecular high-throughput techniques allowing a large number of different molecular features to be assessed at the same time has opened up for the comprehensive investigation of these subgroups. Here we review the clinical challenges in bladder cancer diagnosis, describe the high-throughput molecular techniques currently in use for bladder cancer research, and give an overview over recent advances in the field of superficial and invasive disease.

3. CHALLENGES IN THE CLINICAL MANAGEMENT OF BLADDER CANCER

3.1. Superficial disease

Superficial stages account for about 75% of all new diagnosed bladder cancers. The therapeutical approach will usually attempt to spare the bladder, i.e. transurethral (endoscopic) resections and if necessary BCG instillations (12). As such, the clinicopathological staging of non-invasive tumors is usually correct. Most tumors are of the papillary type (stage Ta). If tumor cells in the *lamina propria* is suspected, the grade of dysplasia may help to distinguish between more benign or more malignant forms. This may give cause for closer surveillance, but hardly any change in therapeutic regimen. The frequency of recurrences forms the major clinical challenge.

In stage T1, the tumor cells have invaded beyond the basement membrane. There may be papillary and more solid, CIS-derived forms and the histopathological differentiation between these forms may be troublesome. If the tumor is deeply invasive in the connective tissue (so called stage T1b) it may be a further challenge to determine whether or not the tumor has become muscle invasive. The diagnosis depends on the quality of the resected specimen and the risk of incomplete resection is imminent too. A molecular signature indicating the aggressiveness of the tumor could guide a more radical treatment approach, or could – on the other hand – spare the patient from an extended, mutilating surgical procedure.

3.1.1. Grade of dysplasia

There is a significant correlation between high grade of dysplasia and invasion, which illustrates the process of malignant development. However, this process is not as delimited as the grades in the various classification systems may suggest. Moreover, the grade may vary within a single tumor. The pathological differentiation between grades is somewhat subjective and the reproducibility is low, especially if the specimen is insufficiently resected or artificially changed. Molecular grading as a supplement has the potential of adding valuable information about the malignant nature of a tumor (14).

3.1.2. Multiplicity and recurrence

Both papillary and CIS-derived cancer forms may behave as field change diseases. This means that in principle the whole urothelial lining is prone to malignant

transformation. This can be demonstrated by selected site biopsies and multiplicity. Multifocal carcinogenesis has been postulated; however, various studies have shown that in most cases recurrent bladder tumors are of clonal origin (15-18). Therefore, transitional carcinoma cells probably may also implant or migrate to other sites of the urothelium. A molecular assay that captures functional abilities of tumor cells to implant and migrate *in vivo* would yield valuable, prognostic information.

The diagnosis of residual viable tumor cells in the bladder after the transurethral resection of a superficial bladder tumor is of crucial importance. This applies not only to malignant disease, but also to the more conservative management of the innumerable cases of more benign, non-invasive papillary bladder tumors. The gold standard is still cytology, combined with repeated cystoscopic control assessments. However, especially in low-grade disease, the sensitivity of urine cytology is too low; this is also the major concern regarding the novel, mostly protein-based urine assays for detecting specific tumor markers (19). Other options include PCR based assays detecting specific gene mutations or epigenetic patterns, and FISH-based assays for chromosomal copy number changes. Improved molecular urine assays may provide additional help and are increasingly incorporated in clinical routine (20-22).

3.1.3. Progression of disease / prognosis

Diagnostic features like stage, grade, multiplicity, and recurrence are used to predict disease progression to muscle invasive stages. Stage T1G3 tumors, recurrent tumors with a history of T1G3 tumors, or CIS lesions are at high risk of progression. A clinical useful molecular assay should add prognostic information to these risk factors. A classification of the malignant potential of these "superficial" tumors based on molecular properties of the resected material seems reasonable. Critical for adding prognostic information is a relevant subdivision into molecular pathways of development (23). Separate risk assessments and further subdivision will, over time and with increasing experience and number of patients, improve the predictive value. However, the molecular properties of bladder tumors may change over time in pursuance of their evolution, and the risk of disease progression may be difficult to predict with certainty long time ahead. In case of recurrent tumors, repeated molecular assessments are most likely required.

3.1.4. Treatment response

Current treatment modalities include BCG instillations and intravesical chemotherapy, e.g. mitomycin C (24). These non-specific treatments are not without toxicity and discomfort for the patient, and there is a risk of serious adverse effects, especially in the elderly patients. Current methods are unable to predict treatment response, although risk groups may be specified (12). New alternative treatment options, e.g. photodynamic therapy (25) and interferon alpha and retinoids as supplements are under evaluation.

3.2. Muscle invasive cancer

About 25% of all primary bladder cancers are diagnosed as muscle invasive. The majority is of the solid / CIS-type, but mixed forms exist. Moreover, tumor cells of different histological differentiation (e.g. squamous cell, adenocarcinoma) occur. The probability of distant metastases increases with disease stage from about 15% (stage T1) to more than 90% (stage T4b). If cure is intended treatment options include radical surgery or radiation therapy. In retrospective studies, radical cystectomy is found superior to radiation in terms of local cancer control (26). However, not all patients are suitable for an extended surgical approach. In addition, locally advanced (stage T4b) or metastatic (stage N2 or M1) cancers are out of surgical reach, but may be cured by chemotherapy. Consequently, in many patients the aim of treatment is more or less palliative, where radiotherapy, chemotherapy and surgery (in reduced doses) are options to lessen tumor burden, treat complications (bleeding, pain, and obstruction) and enhance quality of life. Molecular signatures indicating a more or less aggressive nature of the disease, ability to metastasize, response to radiation therapy and different chemotherapy regimens will definitely improve diagnostic and therapeutic results. The aim is to individualize the treatment by choosing optimal treatment options according to biological properties of the cancer, the age and general condition of the patient, and individual expectations and needs in a holistic view.

4. TECHNOLOGICAL OVERVIEW OF HIGH-THROUGHPUT PLATFORMS

The completion of the Human Genome Project (27-29) and advances in microarray technology and robotics have equipped scientists with powerful new tools for performing systematic genome searches for chromosomal alterations and for disease specific polymorphisms (SNP arrays, CGH array). In addition, with the use of expression arrays it is now possible, genome-wide, to identify genes that are differentially expressed in different tissues or in tissues under different external influences (30). Technological advances in large scale protein profiling are also emerging in the form of tissue microarrays (TMA) and protein (or antibody) arrays. Several other high throughput methods exist; however, here we focus on the methods that have been used for bladder cancer research.

4.1. Platforms for identification of chromosomal alterations and variations

4.1.1. Array based comparative genomic hybridization

Array based comparative genomic hybridization (CGH) is a technique that detects and maps changes in the copy number of DNA sequences (31). In CGH, DNA from a test sample (e.g. from a tumor) and reference (genomic DNA from a healthy/normal individual) are differentially labeled and hybridized to a representation of the genome. The fluorescence ratios of the test and reference hybridization signals are determined at different positions (the array elements) along the genome and provide information on the relative copy number of sequences in the test genome compared to the reference genome. Array CGH provides a number of advantages over the use of metaphase chromosomes, including higher resolution, dynamic range, direct mapping of the copy number changes

to the genome sequence and especially high-throughput output.

4.1.2. SNP arrays

SNPs are single nucleotide polymorphisms with a unique physical location within the genome. The demand for high throughput methods for the parallel analysis of thousands of SNPs in a single experiment has produced a variety of DNA chip systems (32). In SNP arrays each oligonucleotide acts as an allele specific probe and the DNA sequences including the SNPs of interest are amplified by PCR and labeled, and then hybridized to the array. A variety of spotting technologies are being developed, such as pin-based fluid, piezo inkjet fluid-transfer system, and other methods including the use of photolithography for light-directed synthesis of large numbers oligonucleotides on solid surfaces as developed by Affymetrix. The Affymetrix SNP array now offers the possibility to analyze 100.000 SNPs in a single GeneChip experiment. By analyzing both test sample (e.g. from tumor) and corresponding reference (e.g. from blood leukocytes) in parallel, SNP arrays have also been used for high-throughput loss-of-heterozygosity analysis. In addition, using the dynamic range of the signal intensities and advanced bioinformatics, it has become possible to analyze chromosome copy number changes simultaneously.

4.2. Platforms for gene expression profiling

Many different DNA microarray techniques exist for profiling gene expression levels. In gene expression microarray assays, labeled transcripts isolated from biological samples are hybridized to the DNA microarray probes for determination of the transcript abundance or relative expression. In conventional protocols, total RNA from the biological sample is extracted and reverse transcribed into cDNA. In vitro transcription of the cDNA into cRNA is then carried out with incorporation of modified nucleotides for later coupling with fluorescent molecules. The labeled target is hybridized to the DNA microarray slide carrying oligonucleotide or cDNA probes. Following hybridization, scanning of array determines the probe intensities, which reflects the gene expression levels in the samples investigated. The probes used for expression microarray studies are either oligonucleotides or PCR products amplified from cDNA clones. Oligonucleotide probes have the advantage that it is possible to design probes with no cross-hybridization to other transcripts and, furthermore, to cover each gene using several probes. Both oligonucleotide and PCR probes are spotted directly onto a glass surface although it is also possible to synthesize oligonucleotide probes in situ on the glass surface (Affymetrix GeneChip® technology, Affymetrix, CA, USA). The Affymetrix GeneChip system now offers the opportunity to profile the entire human genome using a single GeneChip. Furthermore, the newest GeneChip Exon arrays makes it possible to obtain information about possible splice variants by probing all exons in the genome (5.4 million probes) and all possible genomic events using GeneChip Tiling arrays with probes tiled throughout the genomic sequence.

4.3. Platforms for protein expression profiling

4.3.1. Tissue microarrays

High throughput protein profiling by tissue microarrays (TMA) has successfully been used in several studies. Using this approach it is possible to analyze the expression level of a single protein in large series of tissue sections in one run. TMAs are produced by relocating tissue from conventional histological paraffin blocks into a single block. Ultimately this block contains hundreds or thousands of tissue cores from different patients. Each core biopsy can be as small as 0.6 mm in diameter. Sections from the TMA are then stained using antibodies against the protein of interest. Constructing TMAs is a labor intensive procedure and requires access to both histological paraffin blocks and corresponding clinical annotation. The technology is powerful because of the large number of samples it is possible to investigate in a single experiment. Furthermore, scoring of IHC staining from a single TMA experiment makes comparisons between several tissues much more reliable than traditional IHC on tissue sections. One concern regarding TMA has been how good the small biopsies in a TMA represent large heterogeneous tissues samples. Several studies have addressed this issue and found that adding 2-3 biopsies from each donor block will minimize the risk of using unrepresentative biopsies (33).

4.3.2. Antibody arrays

Another technology used for protein profiling is antibody arrays. Antibody array platforms contain from tens to hundreds of antibodies arrayed on a solid surface for binding labeled proteins. Many variants of antibody array technology have been developed. Basically two main types exist – namely "label-based" assays and "sandwich" assays. In label based assays the proteins are labeled with a tag for detection after specific antibody capture. The label based assay makes it possible to use two samples labeled with different detection molecules for normalization purposes as is done in the two color DNA microarray systems. In sandwich assays unlabelled proteins are captured by the immobilized antibodies on the array. Subsequently, a second labeled antibody detects the captured protein (34). The technology is far more difficult to handle than gene expression profiling and proteins with high homology may be difficult to distinguish from each other using this technology. Furthermore, small changes in pH may alter the protein conformation in such a way that antibody binding is difficult. Finally, the number of available antibodies against the proteins of interest may be limited and generation of antibodies is very labor intensive.

5. DIAGNOSIS AND PROGNOSIS IN SUPERFICIAL BLADDER CANCER

5.1. Disease stage

Molecular markers for identifying superficial tumors compared to more advanced cases have been studied intensively using gene expression profiling (Table 1). Dyrskjot *et al* identified a 32 gene expression signature from 40 tumor samples for classifying tumors as stage Ta, T1 or T2-4 (35). The gene expression signature was successfully validated using 68 independent test samples. Several research groups have subsequently reported similar results. In spite of the limited overlap between the published signatures, Blaveri *et al* validated their signature using the tumors from the Dyrskjot *et al* study and *vice versa (36)*. These results underline the robustness and

Table 1. Recent high-throughput gene expression profiling studies of human bladder carcinoma

Expression Signatures	No of Patients	Disease Stage				Validation		Ref
		Ta	Tis	T1	T2-4	Internal	Independent	
Stage	39	8 + 2[1]		1	6 + 2[1]			63
Stage	40	14	5	11	10	LOOCV	68 patients	35
Recurrence	31	31				LOOCV		35
Stage	34	14			20	2 array types		48
Stage, Papillary/CIS	15	4	3		8	SVM		37
Papillary/solid, Progression	67	46	3	10	8	LOOCV		38
Stage, Squamous/TCC, Prognosis	80	17		10	53	PAM	External dataset	36
Papillary/CIS	41	15	13		13	LOOCV	10 patients	44
Progression, High/low risk	29	20	1	8		LOOCV	74 patients	47
Stage, Survival	105	33			72	SVM		49

CIS: carcinoma *in situ*, Ref: reference, [1] Pools from several patients, LOOCV: leave one out cross-validation, SVM: support vector machine, PAM: prediction analysis for microarrays.

potential of gene expression profiling in bladder cancer diagnosis. Notably, in the study by Dyrskjot *et al* superficial tumors that were classified as a higher stage using the molecular classifier were found to have a significant higher likelihood of subsequent disease progression to a higher disease stage ($P<0.005$). Furthermore, the study showed that the gene expression profiles of Ta tumors with surrounding CIS resembled the gene expression profiles for the muscle invasive cases. Similar results were subsequently reported by other groups (37, 38). These data formed the basis for the investigation of underlying molecular subgroups beyond clinicopathological phenotype, using high-throughput methods.

5.2. Grade of dysplasia

To date only one study has investigated the global molecular differences between high and low grade dysplasia. Aaboe *et al* used gene expression profiling to identify a panel of 86 genes that distinguished low grade tumors from high grade Ta tumors (39). The genes identified were related to regulation of cell cycle (CDK4), cell growth (MAC30), and transcription (ILF2, FNBP3, and JUND). The study did not include independent validation of the reported gene-set. The authors also used hierarchical cluster analysis to illustrate the global differences between Ta PUNLMP tumors and Ta high grade tumors. The cluster analysis separated the two tumor groups significantly ($p=0.009$) based on 4144 genes.

5.3. Recurrence

Detection of recurrent bladder tumors has been studied by Hoque *et al* using 1.5K HuSNP GeneChip from Affymetrix (40). Urine samples from 31 patients with bladder cancer displayed on average allelic imbalances in 39 SNPs. The number of allelic imbalances was less for Ta, T1 and CIS compared to T2-4 tumors. Allelic imbalances were not detected in 9 normal control subjects and in four of five patients with hematuria.

Markers for predicting the likelihood of future tumor recurrences based on molecular alterations in previous tumors have also been studied using genome-wide methods. Dyrskjot *et al* identified a 26 gene expression signature for predicting disease recurrence in TaG2 tumors using Affymetrix GeneChips with probes for approximately 5000 genes (35). The 26 gene signature classified the tumors with a significant correlation to disease recurrence status in internal cross-validation tests ($p<0.006$, 75% correct classification). However, no independent validation of the signature has yet been performed. In future studies of recurrence markers it may be worthwhile to stratify for polymorphisms in e.g. nucleotide excision repair genes or in inflammatory genes as specific polymorphisms are related to elevated risk of disease recurrence (41, 42).

5.4. Papillary versus CIS type

The development of bladder tumors through (at least) two distinct genetic pathways – the papillary and the CIS pathway – is well established (43). In an array based gene expression profiling study, Dyrskjot *et al* demonstrated large gene expression differences between Ta tumors with surrounding CIS compared to Ta tumors without any surrounding CIS (44). A 16 gene expression signature was delineated and tested successfully in internal cross-validation as well as in independent samples of CIS and normal bladder mucosa. Using the 16 gene signature it was possible to discriminate between the CIS lesions taken from cystectomies and normal urothelium from healthy individuals with no bladder cancer history. Interestingly, the signature was also found to be present in normal urothelium adjacent to the CIS lesions. In another study of gene expression differences between CIS and papillary tumors Sanchez-Carbayo *et al* used a cDNA microarray with probes for 17842 genes. In this study hierarchical cluster analysis separated the CIS lesions from superficial papillary tumors (37).

In contrast to CIS, the papillary tumor type is frequently associated with activating mutations of the FGFR3 gene (45). Zieger *et al* recently reported different patterns in chromosomal changes between papillary FGFR3 mutated tumors and the tumors with concomitant CIS (5). Furthermore, fifty tumors analyzed for FGFR3 mutations were also analyzed using the gene expression profiles of 76 genes previously identified as CIS related in the study by Dyrskjot *et al* (44). FGFR3 mutated tumors were found highly correlated with the "no CIS" profile, and *vice versa* ($p=0.0008$). The major impact of the FGFR3 mutations on the global gene expression of superficial bladder cancer was confirmed in another recent study (46).

5.5. Progression / prognosis

Prediction of disease progression from superficial to invasive stage would be of great benefit in the clinical

management of patients diagnosed with early stage bladder tumors. In one study, a 45-gene molecular classifier was developed by comparing 29 superficial tumors (13 without later progression and 16 with later progression) using custom Affymetrix GeneChip arrays (47). The 45-gene classifier was tested on a series of 74 independent tumors using a two-color oligonucleotide array platform with only the genes of interest. The classification results showed a positive correlation to disease outcome ($P<0.03$) with a positive predictive value of 0.3 and a negative predictive value of 0.95. The low positive predictive value may be explained by the fact the patients were continuously treated with TURB and BCG. In another study of progression prediction the authors used 42 Ta tumors where 8 showed later progression to invasive bladder cancer and 8 showed later CIS lesions (38). Both events were used to delineate a gene-set optimal for prediction progression. Using a cross-validation test the predictor correctly classified 33 of the samples, resulting in a sensitivity of 86% and a specificity of 71%. No independent test set validation results have yet been reported for this gene-set. The consensus gene-set of 11 genes resulting from the most commonly used genes in cross-validation loops show no overlap with the 45-gene set signature from Dyrskjot *et al* (47).

In the search for markers predictive of disease progression it may be necessary to stratify the comparisons to only include tumors belonging to the same molecular pathway (CIS versus papillary) in order to increase the accuracy of the gene signatures. Furthermore, it will probably also strengthen the studies if only progression from T1 to muscle invasive stage were considered. Dyrskjot *et al* (47) used tumors progressing from Ta to T1 and from T1 to muscle invasive. Wild *et al* (38) included tumors progressing from Ta to muscle invasive stage and progression to CIS.

6. DIAGNOSIS AND TREATMENT OF MUSCLE INVASIVE BLADDER CANCER

6.1. Prognosis

Recent studies assessing muscle invasive cancer are listed (Table 1). Muscle invasive cancer can be distinguished from superficial forms by gene expression signatures. Moreover, histological subtypes (Squamous cell carcinoma, CIS-type, papillary type) can be identified by gene expression profiling. Some studies considered muscle invasive cancers separately to evaluate associations between gene expression profiles and prognosis beyond histopathology. Modlich *et al* examined 20 specimens obtained during cystectomy of wich 11 had metastases or local recurrence with subsequent death of disease. Hierarchical clustering using a dataset of 1185 cancer-related genes showed two subgroups that contained 4 of 9 and 7 of 10 tumors with unfavourable disease courses, respectively (48). Sanchez-Carbayo *et al* profiled 72 muscle-invasive bladder cancers using Affymetrix U133A microarrays. Hierarchical clustering (together with superficial tumors) generated three main clusters, of which one contained many tumors with adverse prognosis. The authors developed a "poor survival signature" consisting of 100 known genes. The signature showed 90% accuracy in cross-validation using a support vector machine algorithm. One of the genes from the signature (Synuclein) was found significantly associated with survival at the protein level using a tissue microarray with tissue cores from 294 patients ($p=0.002$) (49). Blaveri *et al* profiled 47 muscle invasive bladder cancers using cDNA microarrays. Unsupervised clustering generated three principal clusters, and one contained primarily tumors with very short overall-survival. Supervised clustering lead to a 24-gene classifier for outcome prediction (threshold: 18 months overall survival) with 78% success using prediction analysis of microarrays algorithm (PAM) (36). The same group performed array-CGH analysis of global chromosome copy-number changes in 55 muscle invasive cancers (50). A high grade of chromosomal instability was associated with poor prognosis. Interestingly, the average grade of chromosomal instability in muscle invasive tumors was lower than in stage T1 tumors. As previously suggested (51, 52) there may exist a distinct molecular subgroup of chromosomal stable, primary invasive cancers with comparatively good prognosis.

6.2. Metastases

Metastases can be local (pelvic lymph nodes, stage N+) or distant (stage M1), the former (stage N1) eventually manageable by surgery. Many distant metastases are overseen by conventional diagnostics because they are too small (micrometastases). A molecular method that reliably diagnoses the ability of a cancer to metastasize will most likely improve treatment results by the use of (neo-) adjuvant chemotherapy on one hand; it may also, on the other hand, limit the extent of surgery to what is necessary, perhaps in a palliative setting. Molecular signatures predictive of metastatic potential have been reported for many cancers (53). Recently, general "metastasis signatures" for solid tumors have been developed (54, 55). These signatures still lack validation in large-scale studies. For bladder cancer, data hitherto are very sparse. The study by Modlich at el. has limited patient numbers (48). Sanchez-Carbayo *et al* published a 100 gene signature of lymph-node status at cystectomy, together with overall survival data (49). Kim *et al* evaluated the predictive potential of the expression of two genes (CDH1 and TOP2A), derived from cDNA microarray studies, on a tissue microarray of 251 bladder cancers of all stages and grades with IHC (56). Although the result was highly significant, it was not adjusted for other clinicopathological parameters such as stage and grade. In conclusion, to date, no valid data exist that support a specific potential of high-throughput molecular analyses to predict metastases in invasive bladder cancer. Further studies are urgently awaited.

The question of residual tumor tissue / occult metastases may become an area for antibody-microarrays, which analyze tumor protein markers in serum or urine in a high-throughput fashion. Sanchez-Carbayo *et al* reported diagnostic sensitivity and specificity of 89.2% and 96.5%, respectively, in a series of 37 bladder cancers of all stages and grades and 58 healthy controls, using antibody arrays with antibodies against 254 and 144 well-annotated tumor markers. Moreover, cluster analysis successfully separated the tumor samples according to overall survival (57).

6.3. Treatment response

A number of studies indicates that molecular properties of muscle invasive bladder cancer may significantly influence the response to radiotherapy and chemotherapy, including cell-cycle regulators, apoptosis mediators and DNA repair genes, assayed by IHC and apoptosis assays. Polymorphisms in several DNA repair genes have gained attention as prognostic markers and in response to bladder carcinogens (58). These have been associated with treatment response in colon cancer (59). Gene expression profiles predictive of chemotherapy response have been published in several neoplasms including esophagus, breast, and ostoesarcoma. In a small study of muscle invasive bladder cancer, the response to neoadjuvant (in advance to surgical treatment) chemotherapy was investigated using cDNA microarrays (60). 14 tumors were used to identify a signature of 14 predictive genes, which was validated using an additional 9 tumors. RT-PCR results showed good correlation to the microarray data, warranting further validation in a larger series. To our knowledge, no further studies have yet addressed this issue.

6.4. Targeted therapy

Targeted gene therapy has not yet been established in bladder cancer in clinical routine. Target genes currently under evaluation include receptor tyrosine kinases, members of the Ras/MAPK and the Akt/PKB pathways, Mdm2, Bcl-2, and the restoration of TP53. High-throughput molecular profiling, in connection with pathway analysis / artificial networks, may identify further targets in "key pathways", driving oncogenesis and tumor development (37, 61). Salz *et al* published a gene expression signature predictive of aggressive behaviour, generated from a transgenic mouse model of Survivin (BIRC5)(62). Signalling networks associated with aggressive behaviour were assessed in a recent work (49). Further sub-classification of bladder cancer by assaying "key-pathways" using high-throughput methods would provide a strong support for individualised targeted gene therapy.

7. CONCLUSIONS AND PERSPECTIVES

High throughput molecular methods have shown the potential to differentiate bladder cancer into sub-groups with different clinical outcome. However, to be clinically useful, the molecular methods have to add significant diagnostic information to the established clinicopathological methods, rather than merely confirming them. Thorough data analysis has now substantiated the molecular profile of histopathologically well-characterised pathways of bladder cancer development. This has opened up the possibiliy of molecular sub-classification in order to dissect biological properties and clinical potentials of individual bladder tumors.

Future research should aim at large scale validation studies of the identified molecular signatures. In addition, technology transfer to e.g. qPCR would be preferable, as this technology can be handled in most clinical settings, in contrast to the more advanced microarray technology. In the future, larger series of tumors should be profiled in order to identify more accurate signatures. Furthermore, classifier accuracy will probably increase if information from several cell regulation layers (mutations, methylation patterns, gene expression patterns, gene splice patterns, protein expression, and microRNA expression) are included together with clinical and histopatological information in classification algorithms.

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