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Review

High throughput molecular diagnostics in bladder cancer – on the brink of clinical utility

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ABSTRACT

An enormous body of high-throughput genome-wide data, in particular gene expression data, has been gathered from roughly all human cancer forms in the past 10 years. This has widely increased our understanding of the cancer disease and its molecular changes and pathways, with a large contribution from studies of cancer cell lines and functional genomics. In the last three years, the focus has been moved to clinical outcome parameters as recurrence, progression, metastasis and treatment response. The huge variability of molecular changes and poor availability of samples have hampered progress in the field of epithelial cancer (carcinoma). However, independent validation of molecular profiles across high-throughput platforms, methods, laboratories and cancer populations has recently been successfully performed for several carcinomas, including bladder cancer. Application of advanced bioinformatics to identify interrelated pathways has revealed common signatures predictive of molecular subgroups, improving histopathological diagnosis, and ultimately outcome prediction. With breast cancer leading the field, colorectal, bladder and renal cell carcinomas well on their way, and many others soon to join, the era of clinical applications of high-throughput molecular methods in cancer lies closely ahead. This review illustrates in detail the perspectives for the management of bladder cancer.

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1. Molecular features of cancer development

Cancer development proceeds through a limited number of genetic alterations in regulatory circuits that control normal cellular proliferation and differentiation. These alterations lead to an overruling of anticancer barriers of the cell (Hanahan and Weinberg, 2000). Self-sufficiency in growth signals is acquired through activation of molecular “speeders” (oncogenes), complemented by insensitivity to antigrowth

signals achieved by e.g. disruption of the pRb (tumor suppressor) pathway. This leads to growth advantage. An acquired defect in DNA damage control mechanisms (e.g. the p53 tumor suppressor), overrules the G2/M-checkpoint of the cell cycle, and leads to unrestrained proliferation (Bartkova et al., 2005). With the loss of cell cycle control, DNA-repair in the G2-phase of the cell cycle becomes insufficient, giving way for genomic defects to accumulate (genomic instability). Interestingly, specific defects, e.g. DNA base pairing errors

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(point mutations) and DNA promoter-hypermethylation (e.g. of the p16 tumor suppressor), prevail in early stages, probably reflecting the repair mechanism first to become affected (Fox et al., 2006). This may indicate that tumors become “addicted” to certain molecular pathways early in their development (Baylin and Ohm, 2006). As the development proceeds, large scale DNA damage can be seen. This includes DNA copy number changes because of DNA double-strand breaks (due to unscheduled replication/stalled replication forks (Bartkova et al., 2005)), up to losses and gains of whole chromosome arms and chromosomes as well as aneuploidy through defects in chromosome segregation (Lai et al., 2007).

Pro-growth-signaling promotes cellular de-differentiation through downregulation of transcription factors that direct the cell towards terminal differentiation (post-mitotic state). The malignant development process requires furthermore the escape of programmed cell death/apoptosis by disruption of death signaling (e.g. FAS-pathway, p53-pathway), upregulation of anti-apoptotic signaling (e.g. Bcl-2 oncogene, survivin), or indirectly via stimulation of pro-growth signaling (e.g. PI3K-Akt/PKB-pathway). Further hallmarks of tumor development depend on interaction with the tumor environment and include altered patterns of cellular migration and adhesion, escape of host immune response, invasion and epithelial-mesenchymal transition, and angiogenesis.

Although the hallmarks of malignancy appear to be the same for different tumors, the pathways that cells take on their way of becoming malignant are highly variable, resembling a classical Darwinian evolutionary process (Hanahan and Weinberg, 2000). The result is a vast complexity of possible molecular alterations, even in histopathologically similar tumors. Basically, however, these changes have two principal dimensions: Different stages of malignant development, and different molecular pathways that cells take on their way (Fig. 1, illustrates the situation in bladder cancer).

2. Clinical challenges for cancer diagnostics

In spite of the enormous gain of insight in the molecular processes leading to cancer development in the recent past, there has been no significant improvement in the diagnosis and treatment of carcinomas (epithelial cancers). This is owed to several factors, including the lack of early symptoms and the requirement of invasive diagnostic methods (discomfort to patients), which both delay diagnosis. Improved early detection requires screening tools, which have to be appropriate in terms of sensitivity, simplicity, availability of analyte (e.g. body fluids), patient discomfort, and costs.

Most important, however, is the complexity of possible molecular alterations and the resulting divergence of biological behaviour even in neoplasms of similar appearance. The individual clinical courses are difficult to predict. The case of bladder cancer is no exception. The choice of the correct individual treatment is a clinical challenge, and has to consider the relationship between treatment toxicity/side-effects, patient-related factors like high age and comorbidity, as well as biological properties of the carcinoma in question. For example, low-grade non-muscle invasive bladder cancer may be handled by non-radical means for a quite long time,

avoiding complications and side-effects of radical treatment, especially in the elderly. High-grade bladder cancer, however, may show rapid progression and early spread, and even immediate radical treatment may come too late. The correct timing of radical treatment is particularly important. The main challenge is to find and treat the disease while it is still localized. However, our aptitude to avoid overtreatment, which at best does no good, but may also be harmful, dangerous and costly, is not satisfactory at present. As in most carcinomas, surgery is the preferred radical treatment option in bladder cancer; however, some cancers respond well to radiation or chemotherapy; their identification is a further challenge. Follow-up challenges include the detection of minimal residual disease, including micrometastases, in order to apply adjuvant therapy when appropriate. A recent challenge is the rapidly evolving field of targeted cancer therapeutics. Careful selection of treatment candidates is required to improve cost-effectiveness, in a scenario with desperate patients and aggressive commercial product promotion.

Consequently, there is a great need for improved diagnosis in carcinoma, including molecular diagnosis. Besides the early detection of cancer and the monitoring of residual disease after treatment, the challenge is a functional assessment of the aggressiveness of the disease, including state of cell-cycle control, ability to invade (correct staging), to spread (recurrence, metastasis), and its response to various therapies.

3. Clinical impact of high-throughput molecular diagnostics

Due to the complexity and diversity of the molecular changes, the diagnostic power of single biomarkers seems limited. However, appropriate marker panels may together increase the predictive values. High-throughput molecular methods provide complex “profiles” of the cancer in question, rather than alterations of single specific marker molecules. This approach has enhanced our understanding of cancer development enormously in the recent years, with a large contribution of cell culture studies as well as functional and animal model studies. Although model data generally are not representative for the clinical cancer population, they facilitate the characterization of molecular pathways. Tumors of different molecular pathways, but also of different stages of development, may have different malignant potential. High-throughput tools will eventually unravel both the molecular pathways and the stages of cancer development (Fig. 1). The results will identify relevant pathways, specify their relationship to molecular subgroups and clinical outcome parameters, and guide the development of robust diagnostic biomarker panels (Bild et al., 2006; Nielsen et al., 2004). In essence is all molecular diagnosis that adds information to clinical and histopathological evaluation, potentially clinically useful.

High-throughput analysis of genomic (DNA-) changes illustrates increasing genomic instability as the malignant development progresses. Chromosomal instability is considered one of the main reasons for the huge variability of molecular changes observed in cancer (Lai et al., 2007; Nakao et al., 2004). More important from a clinical point of view, however, is the observation that patterns of genomic alterations are

non-random, particularly in early stages. This may identify molecular subtypes of cancer, and supplement histopathological diagnosis (Trautmann et al., 2006). Patterns of chromosomal changes may probably serve as markers of molecular pathways (Rennstam et al., 2003). Notably, these patterns seem to impact gene expression profiles (Dehan et al., 2007; Paterson et al., 2007; Vauhkonen et al., 2006). Genome-wide DNA copy number changes and allelic imbalance have been analyzed in high-throughput fashion by microarray-based comparative genomic hybridisation (CGH) and single nucleotide polymorphism (SNP) microarrays with a resolution of up to 500,000 probes, resulting in an average distance between markers of 0.9 M basepairs (“sub-megabase-resolution”). Microarray-based DNA-analyses are robust and reproduced with high accuracy. However, the resolution may still be too low to detect all relevant changes (Purdie et al., 2007).

Transcriptional (gene expression-, mRNA-) profiling using microarrays has been widely used to predict aggressive behavior, metastases, response to certain treatment forms and, first of all, survival in cancer. From a clinical point of view, most studies suffer from small sample numbers (given the variability of molecular changes), and lack of validation (using an independent set of samples). A further clinical drawback is that most validated profiles seem to be related to tumor stages or previously characterized histopathological subtypes with different prognosis (Ntzani and Ioannidis, 2003; Dupuy and Simon, 2007; Sun and Yang, 2006; Reddy and Balk, 2006; Nevins et al., 2003; Gruvberger-Saal et al., 2006). Since molecular diagnosis is hardly going to replace histopathological diagnosis, the results should be corrected for known risk factors to determine the independent diagnostic value of gene expression profiling. Finally, both diagnostic methods should be integrated to achieve the optimal results. Nevertheless, with the increasing body of data and improved biostatistical methods, we begin to see large-scale studies of successful validation of gene-expression profiles as classifiers for clinical relevant outcome parameters (Table 1). Some of these studies were able to validate their profiles across different sampling methods, array platforms and cancer populations. These results imply indeed hope for the near future.

4. Identification and validation of clinical relevant profiles

Molecular profiles are usually obtained by hierarchical clustering. This is a multistep mathematical process where for every step the two samples or genes with the closest correlation of all markers are clustered together. If there is significant

correlation between clusters of samples and clinical outcome parameters, a molecular profile predictive of outcome may be developed using supervised clustering of a set of tumors with known clinical outcome (training-set). A molecular classifier can be constructed using advanced biostatistical methods, reducing the number of markers to a set with optimal prediction in the training-set. The molecular classifier should be validated by applying it to an independent set of tumors (test-set). Validation across different analysis platforms, methods, laboratories and populations strengthens the result (Warnat et al., 2005). The quality and length of clinical follow-up has to be considered as a bias source in predictive profiles (Michiels et al., 2005).

The validation of a molecular profile/classifier is an essential part for the establishment of its clinical utility. Successful validation is dependent on whether the training-set was representative for the cancer type in question, which requires a large sample size, given the biological divergence. If the number of variables (markers) tested exceeds the number of samples analyzed, common statistical methods do not perform well. To reduce the number of variables, markers with insignificant changes across all samples are usually filtered out before analysis. However, the general problem remains that potentially significant changes are blurred by the noise of the biological diversity. To improve results, the mutual dependency of the markers should be employed. Chromosomal markers (microsatellites, BAC-clones, SNPs) are mapped, which enables segment analysis of chromosomal changes (Sorensen et al., 2007). Gene expression data are interrelated through molecular pathways (Korn et al., 2005; Pospisil et al., 2006). Notably, different molecular classifiers constructed in comparable populations with similar outcome parameters hardly show common markers. Nevertheless, mutual successful validation is possible (Fan et al., 2006; Blaveri et al., 2005a). This illustrates the usefulness of the signatures, although their individual markers are specific for the conditions and the samples used under their construction. Clustering of gene expression data in pathways enhances significance, reduces the number of variables significantly and facilitates validation of data obtained on different platforms, including proteomic data (Subramanian et al., 2005; Kemming et al., 2006; Sorlie et al., 2006).

The molecular signatures should finally be converted and validated using “gold standard” methods. Genomic marker panels may be validated by multiplexed PCR assays (Sorlie et al., 2006; Killian et al., 2007; Hughes et al., 2006; Perreard et al., 2006). Validation in a high-sample-throughput fashion is achievable by tissue microarrays, applicable to both protein expression changes (immunohistochemistry) and genomic

Figure 1 – Impact of molecular pathways on molecular cancer diagnosis, example bladder cancer. A: Molecular pathways and malignant development are different dimensions impacting on molecular alterations. If tumors following a certain pathway usually become clinically apparent in an early stage of malignant development (yellow dots), molecular features of this pathway will be associated with a good prognosis. In contrast, “blue” tumors will be associated with a worse prognosis. These patterns are, however, of little help to predict the individual risk of tumors at the same stage of malignant development (red box). Note that pathway characteristics merge and diverge as the disease progresses. The challenge is to distinguish factors leading to “downward” development from those broadening the field. B: illustrates the potential impact of molecular pathway determination as a supplement to histopathology. Papillary structures in preinvasive stages (stage Ta) are easily identified. However, images are not as distinguished in superficial invasive tumors with high grade of dysplasia. Moreover, high-grade papillary tumors may follow the “CIS-type” pathway. Identification of a tumor as e.g. “papillary” (=following the papillary pathway) may allow for relevant stratification of molecular alterations into those related to the pathway and those related to malignant development.

Table 1 – Independently validated large-scale gene expression profiling studies showing improvement of cancer outcome prediction independent of clinicopathological risk factors

Reference	Cancer	No. studied samples (training)	No. samples for independent validation	Validation method	Outcome variables
Hu et al. (2006)	Breast	105	311	External (two different array platforms) and internal (1300 gene profile)	Molecular subtypes, survival. Corrected for systemic therapy
Lin et al. (2007)	Colorectal	55	149	External cross-validation (two different microarray platforms)	Recurrence (local and systemic)
Zhao et al. (2006)	Renal cell	88	89	Internal (259 gene profile)	Survival
Sotiriou et al. (2006)	Breast	69	597	Internal and external (97 gene profile)	Molecular grading, recurrence, survival
Dyrskjöt et al. (2007)	Bladder	79	404	Internal and external (45 gene profile)	Molecular subtypes, stage, progression, survival
Naderi et al. (2007)	Breast	135	715	External (70 gene profile)	Survival

Internal validation means that the test-set is sampled from the same population as the training-set, and analyzed under identical conditions (sampling method, laboratory procedure, array type etc.). External validation means that the test- set is recruited from a different population.

changes (in-situ hybridisation). The tools and methods are at hand, and we actually see the dawn of the era of clinical applications of high-throughput molecular methods in the diagnosis of carcinoma.

In the following, recent advances in the field of high-throughput clinical diagnostics in bladder cancer are discussed in detail. An excellent review concerning the situation in breast cancer (which is “leading the field”) was published recently (Sotiriou and Piccart, 2007).

5. Bladder carcinoma: clinical challenges of the disease

Bladder cancer is among the five most common malignancies in industrialized countries. In etiology environmental exposures, especially tobacco smoking, prevail over inherited factors. Transitiocellular carcinoma (TCC) is the most frequent histological type (95% in the western world). Squamous cell carcinoma (SCC) forms a substantial fraction in areas of endemic *schistosoma* infections in Africa. Other histological types are rare. SCC and about 25% of TCC show a solid, invasive growth pattern. In contrast, about 75% of TCC form exophytic, papillary lesions. These phenotypes can be distinguished both macroscopically and microscopically; however, mixed forms exist.

Most papillary tumors are already diagnosed at early non-invasive or superficially invasive stages, presumably because they are fragile and tend to bleed, thus becoming symptomatic. Superficial lesions can be completely resected using a transurethral approach, i.e. by organ-sparing surgery. Characteristically, the disease tends to recur in the bladder at sites distant from the primary lesion. Most recurrent tumors are found to be of the same clonal origin, but also subjected to clonal evolution. The majority of the cases (75%) follow a benign course with no disease progression. However, the disease is at all stages capable of rapid tumor progression and metastasis within few months. These features of early diagnosis, frequent clonal recurrences and potentially rapid progression

offer a unique opportunity for the study of carcinoma development.

The most important risk factors for progression are the presence and depth of invasion in the suburothelial connective tissue, and high grade of cellular dysplasia. Of significance is the simultaneous histopathological evaluation of invasion and the grade of dysplasia, which provides prognostic clues regarding the malignant potential. Although histopathology is fairly accurate, molecular diagnostics could be capable of improving the diagnosis of malignant potential. If high malignant potential (=about 25% risk of progression) is presumed, the re-resection of the tumor site within 6 weeks and mapping of the urothelium using selected-site biopsies or fluorescence guided photodynamic diagnosis (PDD) is currently state-of-the-art, to ensure complete tumor removal. Furthermore, more aggressive intravesical chemotherapy and closer surveillance is recommended. Some departments even perform radical cystectomy at this point. However, the individual risk of progression is difficult to predict.

Patients with recurrent high grade or superficially invasive tumors have a particular high risk of progression (>50% lifetime risk). It is currently not completely understood how these recurrences arise; possible explanations include intraluminal seed and intraurothelial migration of tumor cells. However, it is evident that cellular alterations already can be found in non-tumor-bearing mucosa of patients with bladder tumor (“field disease”) (Muto et al., 2000). In some cases, high grade field disease can be demonstrated as flat dysplastic intraurothelial lesions, named *carcinoma in situ* (CIS). These lesions are considered as precursors of invasive growth, because they have a high risk of progression (50%) and are frequently (50%) associated with invasive tumors. Pre-invasive field disease can be treated by instillations of *Bacille Calmette Guérin* (BCG) in the bladder, which stimulates host immune response against tumor cells. The treatment is effective in 70%. However, it is difficult to predict the individual treatment response, and the “therapeutic window” is narrow: valuable time may be spoiled while the disease progresses. Consequently, the early diagnosis of high-risk residual tumor cells in the bladder after resection of a superficial bladder tumor,

including field disease, is most important. Voided urine is readily available as an appropriate analyte. Cytologic evaluation of urine is the gold standard, but suffers from low sensitivity, notably in low to moderate dysplastic disease. This is also the major concern regarding the novel, mostly protein-based urine assays. A panel of urine-based molecular markers could probably improve the results. The pattern of recurrence (indicating field disease) could be accessible by molecular methods as well. Although not completely understood, functional cellular capabilities to implant or migrate inside the urothelium could be of clinical significance. Evidence of such molecular changes could direct treatment towards more intensive recurrence prophylaxis (repeated or sustained intravesical instillations). Independent molecular signatures predictive of BCG response may also be present (Lebret et al., 2007).

Primary muscle-invasive bladder cancer (stage T2 and worse) is characterized by a solid invasive growth pattern. Clinically silent and rarely diagnosed at early stages, some of these tumors may have developed during several years, and may have slower and less aggressive growth patterns compared to progressing papillary tumors (stage T2 and worse). A retrospective study reporting a better overall prognosis of primary invasive tumors supported this view (Schrier et al., 2004). Molecular signatures might facilitate the identification of tumors with relatively low malignant potential, in particular metastasis potential. Long-term local tumor control with bladder-sparing resections of primary invasive tumors has been reported (Wolf et al., 1987; Danesi et al., 2004) and may represent an alternative to radical treatment, particularly in the elderly. Radical treatment includes surgery (radical cystectomy = complete removal of bladder and adjacent organs including iliac lymph nodes, and urinary diversion), or external beam radiation therapy. These are extensive procedures with a substantial risk of severe complications, treatment-related morbidity and reduced quality of life. Many patients of the elderly bladder cancer population are not generally fit for these procedures. Others already show metastases at diagnosis and are usually out of reach of surgery and radiation as well. High-dose systemic cisplatin-based chemotherapy may be attempted in selected cases to cure the disease. Otherwise the patients are offered palliative transurethral resections and cisplatin-based chemotherapy to relieve symptoms from pain, hematuria, obstruction or metastases. The response prediction to radiotherapy and/or certain chemotherapy modalities is of prominent clinical significance, and an obvious issue for molecular diagnosis. Novel molecular targeted therapeutics are urgently needed; however, validating the effectiveness of such substances, although underway, has not yet left the preclinical stage (Mittra et al., 2006).

6. Molecular subgroups and outcome prediction in bladder cancer

Various groups have studied molecular markers for identifying superficial bladder tumors compared to more advanced cases using gene expression profiling (Table 2). Dyrskjot et al. (2003) identified a 32 gene expression signature from 40 tumor samples for classifying tumors as stage Ta, T1 or T2–

4, and validated the signature using 68 independent test samples successfully. Blaveri et al. (2005b) reported a similar signature. Although there was little overlap between the two signatures, Blaveri et al. successfully validated their signature using data from the Dyrskjot et al. study and vice versa. Notably, in the first study tumors diagnosed as superficial using histopathology, but classified as invasive (stage T2–4) using the molecular classifier, had a significant higher probability of subsequent disease progression, indicating independent predictive potential of the molecular signature (Dyrskjot et al., 2003). This promising result was further assessed in a subsequent study. A 45-gene molecular classifier was constructed using 29 superficial tumors with and without later progression. The classifier was tested on a series of 74 independent tumors using a customized gene-expression microarray system. The classification identified low-risk tumors with no future progression with high precision (0.95). The positive predictive value (prediction of progression) was only 0.3; however, a favourable course following resection of a high-risk tumor may reflect complete resection rather than lack of malignant potential (Dyrskjot et al., 2005). This underscores the importance of diagnosing residual tumor cells/malignant field disease. Fitting this, the Dyrskjot et al. (2003) study also found positive correlation between the gene expression profiles of superficial tumors with concomitant CIS and muscle invasive tumors. Similar results were reported by other groups (Sanchez-Carbajo et al., 2003; Wild et al., 2005). The authors constructed and successfully validated a diagnostic signature of concomitant CIS (Dyrskjot et al., 2004). Interestingly, the signature was also present in apparently normal urothelium in bladders with CIS and invasive tumor elsewhere. A possible correlation between expression profiles of CIS and progression was also suggested in the study by Wild et al. (2005). Dyrskjot et al. (2007) successfully validated all three aforementioned classifiers in a multi-center-study with more than 400 bladder tumors from 5 different countries using the customized microarray system. A higher risk of progression was predictable using the progression classifier, and this was independent of stage and grade at diagnosis, indicating clinical usefulness. Notably, a positive correlation between the classification results of the CIS and progression classifiers was confirmed, regarding the prediction of both CIS and progression. The two classifiers mutually improved classification results when analyzed together. The prospective validation of an improved risk-classifier using a multiplex RT-PCR system for routine use is now ongoing.

Distinct molecular pathways of development for papillary tumors and tumors with concomitant CIS are well established (recently reviewed in Knowles, 2006). Unraveling these pathways may further improve classification results (Fig. 1). Papillary tumors are characterized by activating mutations or upregulation of the fibroblast growth factor receptor 3 (FGFR3) gene (Billerey et al., 2001). The gene has tyrosine kinase activity and acts as a molecular speeder (oncogene) (Bernard-Pierrot et al., 2006). Mutations have influence on and characterize gene expression profiles in superficial bladder cancer (Zieger et al., 2005; Lindgren et al., 2006). FGFR3 mutations and the occurrence of CIS are mutually exclusive in early stages (Billerey et al., 2001), and the same has been observed using gene expression profiles (Zieger et al., 2005). Chromosomal

Table 2 – Recent high-throughput gene expression profiling studies of bladder cancer using clinical samples and with clinical outcome

Expression signatures	Reference	No. patients	Stage Ta	Stage Tis	Stage T1	Stage T2–4	Independent validation	Other validation
Stage	Dyrskjøl et al. (2003)	40	14	5	11	10	68 patients + 386 patients (Dyrskjøl et al., 2007) Failed (Dyrskjøl et al., 2007; Lindgren et al., 2006; Schultz et al., 2006)	
Recurrence		31	31					
Mol. subtypes, recurrence	Lindgren et al. (2006)	75	57		18			
Stage, metastasis	Modlich et al. (2004)	34	14		2	20		2 array types, RT-PCR (9 genes)
Stage, papillary/CIS	Sanchez-Carbayo et al. (2003)	15	4	3		8		
Papillary/solid, progression	Wild et al. (2005)	67	46	3	10	8		RT-PCR (1 gene), TMA (1 gene)
Stage, squamous/ TCC, survival	Blaveri et al., 2005b)	80	17		10	53	External dataset (Dyrskjøl et al., 2003) 10 patients + 150 patients (Dyrskjøl et al., 2007) 74 patients + 294 patients (Dyrskjøl et al., 2007)	TMA (3 genes)
Papillary/CIS	Dyrskjøl et al. (2004)	41	15	13		13		
Progression, high/low risk	Dyrskjøl et al. (2005)	29	20	1		8		
Stage, node-metastasis, survival	Sanchez-Carbayo et al. (2006)	105	33			72		TMA (1 gene)
Stage, metastasis, survival	Kim et al. (2005)	19	5	8	1	13		RT-PCR (20 genes), TMA (6 genes)
Chemotherapy response	Takata et al. (2005)	18				18	9 patients + 22 patients (Takata et al., 2007)	RT-PCR (14 genes)
Chemotherapy response	Als et al. (2007)	30				30		TMA (2 genes)

RT-PCR, reverse-transcriptase (quantitative) polymerase chain reaction. TMA, immunohistochemistry using tissue microarray.

aberrations characterizing CIS-related tumors were reported as well (Zieger et al., 2005). Integration of histopathological phenotype (papillary growth pattern/concomitant CIS), FGFR3 mutation analysis, (a panel of) chromosomal alterations, and gene expression profiles may identify the molecular pathway with high specificity and probably allow further substratification. This would probably improve the classification results of marker panels predicting outcome (Fig. 1). One form this could take would be nomograms as previously constructed for clinicopathological parameters. These nomograms provide a continuous risk score, thereby adding information to the physician when selecting follow-up and treatment strategy.

7. Molecular diagnosis of recurrence and early residual disease detection

The prediction of recurrence was attempted using gene expression profiles as well (Dyrskjot et al., 2003). However, this profile could not be validated in the multicenter validation study (Dyrskjot et al. (2007), and neither in other independent studies (Wild et al., 2005; Lindgren et al., 2006; Schultz et al., 2006). Lindgren et al. (2006) developed an independent profile with no overlap with the profile published by Dyrskjot et al. However, as the authors discuss, an evaluation of common deregulated functional pathways may pave the way towards a more reliable classification. Much effort has been spent to predict the response of BCG-treatment, including evaluation of molecular markers like urinary cytokines and overexpression of fibronectin by tumor cells (Patard et al., 2003). However, to date no high-throughput analyses assessing this issue have been published.

The early detection of residual tumor cells in the urine with high sensitivity might be possible with a combined analysis of several tumor markers using high-throughput proteomics (Munro et al., 2006; Vlahou et al., 2001; Liu et al., 2005). In the study of Vlahou et al. (2001) using SELDI-TOF ("ProteinChip®") technology, the overall detection of bladder tumors was possible with 72–87% sensitivity and 63–72% specificity, comparable to cytology, and was independently validated. There was a potential improvement in the detection of low-grade tumors. Prior identification of the molecular subtype of the bladder tumor disease in question may probably improve the results of subsequent urine analysis. DNA alterations of urinary tumor cells may detect tumor recurrences successfully using microsatellite analysis with sensitivity up to 85%. In a high-throughput approach using 1.5K SNP microarrays, Hoque et al. (2003a) could detect allelic imbalance in all urine samples from 31 patients with bladder cancer, opposed to no allelic imbalances detected in 9 normal control subjects and in four of five patients with hematuria. However, almost exclusively high grade bladder cancers were examined.

8. Predicting outcome of invasive bladder cancer

Histological subtypes of invasive bladder cancer (Squamous cell carcinoma, CIS-type, papillary type) can readily be identified by gene expression profiling (Blaveri et al., 2005b; Dyrskjot

et al., 2003; Wild et al., 2005). Only few studies regarding muscle invasive cancers separately evaluated associations between gene expression profiles and prognosis beyond histopathology (Table 2). In a small study, Modlich et al. (2004) profiled 20 cystectomy specimens. Eleven of these had metastases or local recurrence with subsequent death of disease. Hierarchical clustering using a dataset of 1185 cancer-related genes revealed two principal clusters that contained 4 of 9 and 7 of 10 tumors with unfavourable disease courses, respectively. Blaveri et al. (2005b) profiled 47 muscle-invasive bladder cancers using cDNA microarrays. Unsupervised clustering generated three principal clusters, of which one contained tumors with very short overall-survival. A 24-gene classifier for outcome prediction (threshold: 18 months overall survival) with 78% success in internal validation was constructed. Sanchez-Carbayo et al. (2006) profiled 72 muscle-invasive bladder cancers. 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 which showed 90% accuracy in cross-validation. One of the genes (Synuclein) was independently validated to be associated with survival at the protein level using a tissue microarray with tissue cores from 294 patients ($p = 0.002$). Unfortunately, no independent validation of the latter two signatures has been performed yet, although comparable outcome parameters render them suitable for mutual external validation. In an array-CGH analysis of global chromosome copy-number changes in 55 muscle invasive cancers, a high grade of chromosomal instability was associated with poor prognosis (Blaveri et al., 2005a). Interestingly, the average grade of chromosomal instability in muscle invasive tumors was lower than in superficially invasive (stage T1) tumors. This contrasts previous findings of equal or higher degrees of instability in stage T2–4 tumors following superficial tumors (Koed et al., 2005; Hoque et al., 2003b). These data provide evidence for a distinct molecular subgroup among the primary invasive cancers, which is chromosomal stable. The data suggest that these tumors apparently have a comparatively good prognosis. This issue deserves further clarification.

9. Diagnosis of metastatic potential

Most patients with invasive bladder cancer die from systemic spread (metastases) rather than local progression, and the event of metastasis is consequently linked to poor prognosis (Sanchez-Carbayo et al., 2006). Although the classification of metastatic potential has not yet been established in bladder cancer, some studies have used high-throughput analyses in their search for markers of metastasis. The small study of Modlich et al. (2004) is not independently validated. Kim et al. (2005) validated the predictive potential of the expression of two genes (CDH1 and TOP2A), derived from microarray profiling, on a tissue microarray of 251 bladder cancers of all stages and grades with immunohistochemistry. Although the result was highly significant, it was not adjusted for clinicopathological parameters such as stage and grade. Using gene expression profiling of a human bladder cancer metastasis model, Gildea et al. (2002) identified a metastasis

suppressor gene, *RhoGDI2* (GeneID 397), in human carcinoma. The group validated this interesting finding using a bladder cancer tissue microarray and immunohistochemistry. Reduced *RhoGDI2* expression was independently correlated with reduced cancer-specific survival in multivariate analyses ($p = 0.03$) (Theodorescu et al., 2004). This is in line with the association of the Rho/ROCK pathway with progression reported in several carcinomas, including bladder cancer. The finding awaits further validation.

10. Predicting response to chemotherapy

Gene expression profiles predictive of chemotherapy response have been published in several neoplasms including breast (Ayers et al., 2004), gastrointestinal tract (Jensen et al., 2006) and ovary (Dressman et al., 2007). In a small study of muscle invasive (>T2) bladder cancer, Takata et al. (2005) investigated the response to neoadjuvant (prior to surgical treatment) chemotherapy using cDNA microarrays. Response was defined as downstaging of the tumor (<pT2), according to the pathological diagnosis of the following cystectomy specimen. 14 tumors were used to identify a signature of 14 predictive genes, which was validated using additional 9 tumors. RT-PCR validation showed good correlation with the microarray data. The group further validated their data using 22 new tumors, showing a PPV of 100% and a NPV of 72%; furthermore, the results were associated with recurrence-free and overall survival (Takata et al., 2007). These results are encouraging; however, the sample size is still small. The results should be further validated in larger materials. Recently, Als et al. (2007) examined 30 advanced bladder cancers out of reach for surgery (stage T4b, or N2+, or M1) treated with Cisplatin-based chemotherapy with the intent of cure. Using gene-expression profiling and crude survival as outcome variable, the authors identified two well-annotated prognostic genes (*emmprin*/BSG and *survivin*/BIRC5), whose prognostic potential could be successfully validated in an independent retrospective material (124 tumors) using IHC. The prognostic value was most pronounced in the group of locally advanced cancer (no metastases), but notably independent of the presence of metastases, which otherwise is the most significant prognostic factor for this group of patients. The prospective evaluation of this promising result is now ongoing.

11. Conclusion and perspectives

A growing body of data suggests the clinical utility of high-throughput molecular diagnostics to improve diagnosis and ultimately outcome prediction in various forms of epithelial cancer. The future lies in the integration of all diagnostic measures, including clinical, histopathological, immunohistochemical and molecular diagnosis. This will yield differentiated insight into molecular pathways, subgroups, and stages of malignant development. High-throughput tools facilitate this process, ultimately leading to reproducible marker panels. This process has already begun for some of the most frequent cancer forms, and will hopefully be extended to others in the near future. However, careful validation in large

groups of cancer patients with thorough follow-up is still required for these methods to become introduced into clinical routine.

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