

Translational research in bladder cancer

From molecular pathogenesis to useful tissue biomarkers

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Abbreviations: BCG, bacillus calmette-guerin; CIS, carcinoma in situ; DSS, disease-specific survival; EGFR, epidermal growth factor receptor; FISH, fluorescence in situ hybridization; LOH, loss of heterozygosity; MAPK, mitogen activated protein kinase; MMPs, matrix metalloproteinases; MVD, microvessel density; NMI, non-muscle-invasive; RC, radical cystectomy; TMA, tissue microarray; TUR, transurethral resection; UCB, urothelial carcinoma of the bladder; VEGF, vascular endothelial growth factor

Current risk stratification using clinical and pathological parameters in patients with urothelial carcinoma of the bladder (UCB) is insufficient. Additional information on the tumor biology can be derived from molecular biomarkers assessed in surgical UCB specimens and the predictive accuracy of oncologic end points may be improved. Biomarkers may also be used to predict the response to novel multimodal therapeutic approaches. Insights from the molecular pathogenesis of UCB can help identify potential biomarkers. A variety of candidate markers for future clinical use have been identified. However, results from comprehensive validation trials and prospective studies are still lacking. This precludes an application of molecular biomarkers in clinical practice at the moment. In this article, we review a selection of tissue biomarkers that are linked to important steps in molecular pathogenesis of UCB. We focused on biomarkers already tested in patient populations with potential clinical utility.

Introduction

Urothelial carcinoma of the bladder (UCB) is the seventh most prevalent type of cancer worldwide, with an estimated 70,980 new cases and 14,330 deaths in 2009 in the United States.¹ The relative 5-year survival rate is highly variable depending on the different UCB stages. Overall, the 5-year survival rate is 82%, ranging from below 10% in patients with metastatic disease to over 90% for localized UCB.²

UCB is classified into two groups according to the pathologic stage and grade. Up to 75% of patients are diagnosed with non-muscle-invasive (NMI) UCB. It is confined to the urothelium or the suburothelial connective tissue. Most of these patients can be managed by transurethral resection (TUR) and intravesical therapy. NMI UCB is associated with a high local recurrence

rate of 50–70% and 10–15% of patients will progress to muscle-invasive disease over a 5-year period.³ The frequent recurrences of NMI UCB represent a therapeutic challenge and a significant health economic problem. There is a distinct difference in the behavior of low grade and high grade NMI UCB. Low grade tumors frequently recur and rarely progress, whereas high grade UCB, including carcinoma in situ (CIS), has a propensity for progression, local invasion and metastasis. Muscle invasive tumors are responsible for approximately 25% of UCB and the major cause of morbidity and disease-specific mortality. In these cases, radical cystectomy (RC) with lymphadenectomy is the standard therapy. However, disease recurrence is a common event and the 5-year disease-specific survival (DSS) after radical surgery remains 50–60%.^{4,5} Patients with locally invasive UCB (pathologic stages T1-T3a) but without lymph node involvement (status pN0) recur in up to 25% of all cases at 5-years, with most dying of their disease.^{4,5}

A failure to identify patients at high risk for recurrence and disease-specific mortality is a major problem in the management of UCB. Despite recently improved assessment of individual risk calculation utilizing clinical and pathological parameters, predictive accuracies for recurrence and progression of NMI UCB are still limited to a maximum of 66 and 75%, respectively.³ In order to identify high risk patients and to predict outcomes of UCB, clinicians most commonly use the American Joint Committee on Cancer TNM staging system. It provides general outcome estimates based on conventional pathological criteria. The TNM classification is clearly limited in its ability to identify patients at high risk for UCB-specific mortality. Nomograms, including a number of clinical and pathologic features, have been developed for patients undergoing RC in order to improve prediction of the clinical course following RC.^{6–8} They provide more individualized information on the likelihood of disease recurrence and mortality, exceeding the predictive power of the TNM classification to almost 80%.^{6–8} At this time, nomograms are sparsely used in patient counseling and clinical decision-making. Even though they represent the most accurate prognostic tools available for UCB treated by RC at the moment, there is still significant room for improvement. A major limitation of current TNM criteria and nomograms is that tumors of similar

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stage and grade still have a significantly different biology and metastatic potential.

Identification of high-risk patients may allow for better utilization of multimodal therapy. For example, neoadjuvant platinum-based combination chemotherapy can significantly improve survival at 5 years and there is also evidence suggesting a survival benefit from adjuvant chemotherapy.⁹⁻¹⁵ However, chemotherapy is only sparsely used in the treatment of UCB for several reasons,¹⁵ including the expected cure by RC alone according to TNM criteria as well as concerns about toxicity. An inadequate selection based on TNM criteria might be the reason for the significant variability of outcomes in published chemotherapy trials for UCB. The value of adjuvant chemotherapy might be underestimated since trials included many patients at lower risk for recurrence and thus a lower likelihood of benefit. Identification of only high-risk patients may improve utilization because of current hesitation to use it in patients who are elderly or with significant comorbidities. The selective application of chemotherapy based on accurate risk prediction may allow for more individualized and less radical management in selected patients.

Molecular Biomarkers

Information on molecular alterations and differences in tumor biology has the potential of improving the characterization of an individual's tumor and optimizing the accuracy of predictive models. Furthermore, besides improved staging, biomarkers can identify patients who will respond to chemotherapy or potentially be used to identify targets for novel therapeutic interventions. Thus, a more individualized treatment could be implemented. Tissue biomarkers for UCB are characteristic molecules produced by the tumor that are detectable and measurable in UCB specimens.¹⁶ Protein expression profiles are the most commonly used biomarkers, as they can be directly and efficiently assessed using immunohistochemical techniques, for example on tissue microarrays (TMAs). The incorporation of molecular markers into clinical practice requires a systematic approach comparable to the development of a new drug.¹⁷ After initial development and validation of an assay, the biomarker is tested in a small group of patients to determine its ability to discriminate between health and disease or predict a predetermined outcome. Multiple internal and external validation studies need to be performed during the next steps. The final step of biomarker development is prospective validation and, if retrospective findings are confirmed, one would proceed with a randomized trial to show that use of the assay generates better clinical decision-making than current standards.

There is evidence that molecular biomarkers can assist in identifying tumor-specific expression profiles that indicate tumor aggressiveness in TUR or RC specimens.¹⁸⁻²³ When integrated into current prognostic models, biomarkers may prove helpful during patient counseling and treatment decision-making.²¹ Improved methodologies, such as gene- and protein-expression analysis using TMA techniques, have accelerated the discovery of molecular alterations involved in the complex biological behavior of UCB. A variety of key molecules have been associated with disease recurrence and progression of UCB, thus representing

candidate markers that are likely to improve prognostic accuracy.² Given the lack of large prospective validation studies, there is not yet a role in the clinical routine management of patients with UCB. However, molecular biomarkers offer great potential in improving patient care and many markers are currently under investigation in different phases of preclinical development.

The two main applications for markers in the management of UCB are prediction of patients at high risk for disease recurrence and prediction of response to neoadjuvant and adjuvant therapies. Many markers are predictors of recurrence and progression in small retrospective single-center studies.²⁴ However, the most important goal for clinicians is identifying the means to translate information regarding molecular biomarker status into improved patient care. Therefore, it is crucial to assess the added information to conventional predictors of outcome such as TNM staging.¹⁷ Molecular biomarkers for UCB identify some of the inherent characteristics in urothelial molecular pathogenesis. They can be used to predict different events. An important field of biomarker use is to estimate which therapies might be of benefit for an individual patient. An increasing availability of novel targeted agents offer the potential to select a specific therapy. The use of a drug based on a specific altered pathway may improve the response to treatment. In order to identify potential biomarkers, it is important to consider the molecular pathogenesis and tumor biology of UCB.

Molecular Pathogenesis of Bladder Cancer

The molecular pathogenesis of UCB is incompletely understood. It involves a multistep carcinogenesis process, with a variety of distinct biological and functional characteristics.²⁵ Diverse molecular alterations are responsible for the heterogeneous course of UCB even within identical clinical and pathologic staging groups. Primarily, dysregulated cellular processes in UCB include gene and cell cycle regulation, altered microRNA expression, apoptosis, cell growth and signal transduction.²⁵⁻²⁷ Moreover, during tumor progression and development of micro-metastatic disease, epithelial-to-mesenchymal transition by cancer cells occurs and factors promoting tumor cell migration, local invasion and (lymph)angiogenesis are involved.^{25,28}

Unlike most epithelial tumors passing through a linear pathway of malignant transformation, the molecular pathogenesis of UCB is characterized by at least two separate "cancer pathways".²⁹ These separate pathways lead to the development of non-invasive low grade tumors that appear papillary or to more aggressive high grade neoplasms appearing either papillary or sessile.²⁵ The loss of heterozygosity (LOH) of chromosome 9 is present at an early stage in the molecular pathogenesis of UCB.³⁰ It has been suggested that a preconditioning effect of this chromosomal aberration within the urothelium, leading to either less aggressive papillary tumors or aggressive high grade UCB.³¹ Molecular alterations of non-invasive low grade UCB are mainly characterized by mutations of the oncogenes HRAS, FGFR3 and PIK3CA.^{25,32-35} Conversely, invasive high grade UCB is frequently characterized by mutations (e.g., LOH) in tumor suppressor genes, mainly TP53 and the retinoblastoma (RB1) gene.^{36,37}

Genetic Markers

Whereas the detection of chromosomal abnormalities of UCB cells in urine is already used in clinical practice with the fluorescence in situ hybridization (FISH) (UroVysion®) assay, tissue markers for chromosomal abnormalities have not yet been sufficiently evaluated. UCB shows characteristic genetic changes at different levels. The main chromosomal changes in UCB affect the loss of chromosome 9q and loss of RB1 as well as amplifications of 6p22.^{38,39} Using FISH, Hartmann et al. found that Chromosome 9 LOH is not specific for low grade UCB, as it was also detectable in CIS lesions.³¹ The prognostic value of numerical aberrations of chromosomes 3, 7, 17 and of the 9p21 gene locus has been investigated by Kruger et al. in 71 pTa UCB specimens using the UroVysion® assay on paraffin sections.⁴⁰ In a multivariable Cox Regression analysis, pentasomy/higher polysomy 17 and loss of 9p21 were identified as predictors of UCB recurrence. They concluded that these numerical chromosomal aberrations may serve as a new tool to assess UCB recurrence in pTa tumors. Similarly, Kawachi et al. recently presented a novel 9p21 index derived from studies using dual-color FISH for 9p21 and chromosome 9 centromere in bladder washing cytology samples obtained from 61 patients with UCB treated by TUR.⁴¹ Patients with a low 9p21 index had significantly reduced recurrence-free survival rates compared to patients with a high 9p21 index. These findings require further validation. Recently, results from another novel marker assay study were published.⁴² The authors used array-based comparative genomic hybridization for genome-wide screening in 41 patients with NMI UCB. A loss of 8p23.3 correlated significantly with higher grade and advanced tumor stage. In patients with loss of 8p23.3, recurrence-free and progression-free survival rates were significantly reduced. Unfortunately, no multivariable analysis was done to assess the independent prognostic value of loss of 8p23.3.

Besides chromosomal aberrations, multiple genes have been identified as dysregulated or mutated in UCB. Recent studies suggested a role of CpG island promoter hypermethylation, leading to transcriptional inactivation of UCB-related genes and consequently proteins.^{43,44} Dysregulated genes in UCB include FGFR3, PIK3CA, HRAS, NRAS, TP53 and CDKN2A. The most frequent mutations are activating mutations in FGFR3 and inactivating mutations in TP53. TP53 mutations are frequently present in invasive high grade UCB, confirming the two-pathway pathogenesis hypothesis in UCB. As mentioned before, FGFR3 mutations are associated with papillary low-grade tumors. FGFR3 mutations have been evaluated as to their prognostic significance. One large study in NMI UCB found that FGFR3 mutations were associated with increased recurrence but only in patients with pTaG1 and there was no association with progression or survival.⁴⁵ Another study confirmed these results in stage pT1 UCB. Mhaweche-Fauceglia et al. demonstrated that FGFR3 expression was a predictor for time to recurrence in T1a/T1b tumors. However, only grade and depth of invasion were independent predictors of UCB progression.⁴⁶ A study by van Rhijn et al. in 286 patients with UCB found that a combination of FGFR3 and MIB-1 status was an independent predictor

of recurrence, progression and disease-specific survival.⁴⁷ FGFR3 mutation was associated with a favourable clinical course. By contrast, Burger et al. recently reported that FGFR3 status discriminated patients who progressed from those who did not in a subgroup of patients with high-grade UCB.⁴⁸ The authors prospectively assessed the prognostic value of 3 biomarkers (FGFR3, CK20 and Ki-67) in 221 patients with UCB using immunohistochemistry and SNaPshot mutation detection. Despite a relatively small subgroup analysis, FGFR3 status and tumor grade were independent predictors of progression. Clearly, these contradictory data regarding the prognostic value of FGFR3 need evaluation in larger cohorts with standardized methods.

An overexpression of activated HRAS induces the molecular pathogenesis of UCB and HRAS mutations are associated with papillary NMI UCB.^{25,49} In order to provide a molecular grading of UCB, a recent gene expression and genomic profiling study evaluated HRAS mutations as part of a panel of markers, including FGFR3, PIK3CA, KRAS, HRAS, NRAS, TP53, CDKN2A and TSC1.⁵⁰ Lindgren et al. identified characteristic gene signatures of two distinct molecular subtypes of NMI UCB. The signatures precisely distinguished low grade from high grade tumors in validation studies. The authors also identified gene expression signatures providing independent prognostic value regarding metastasis-free and disease-specific survival. Interestingly, TP53 and FGFR3 mutations had only a moderate effect on the division into low risk and high risk subtypes NMI UCB.⁵⁰ This finding confirms that a panel of (genetic) markers may provide significantly more accurate information on the clinical course of UCB, which is a highly heterogeneous entity.

In a quantitative, pathway-specific approach, Birkhahn et al. recently identified a gene marker panel to predict outcomes in patients with NMI UCB.⁵¹ The authors studied the expression of 24 genes, which are known to be deregulated in UCB, by real-time polymerase chain reaction on tumor specimens of 48 patients with distinct oncological outcomes. All patients with low HRAS expression developed UCB progression. The authors showed that HRAS, VEGFR2 and VEGF identified progression with 81% sensitivity and 94% specificity. This gene marker panel was also identified as an independent predictor of progression by multivariable analysis. CCND3 and HRAS were robust predictors of recurrence and progression, respectively. The study by Birkhahn et al. showed that an assessment of gene expression predicted oncological outcomes more accurately than standard clinicopathologic features. However, despite these promising findings, only an initial phase of marker development has been performed and the results need to be further validated.

Prediction of response to therapy in NMI UCB is crucial for clinical decision-making regarding early RC vs. bladder-preserving approaches. Intravesical BCG immunotherapy is an effective treatment but there is a significant risk of progression in patients who fail to respond. Kim et al. recently addressed this issue, aiming to identify a gene marker panel predicting the response to BCG in NMI UCB using microarray gene expression profiling in 80 patients with primary UCB.⁵² The authors first identified differentially expressed genes in responders and nonresponders. In the next step, they validated these results using real-time

reverse-transcriptase PCR. After validation, 24 genes remained as “predictive gene signatures”. The signatures were independent predictors of recurrence and progression. These findings are promising and a validation in larger cohorts is necessary.

Cell Cycle Markers

Biomarkers indicating a defect in cell cycle regulation are among the most intensively studied markers in UCB and include p53, p21, pRB, p27 and the cyclins D1 and E1.^{20-23,53-62} The cell cycle is controlled by the p53 and RB signalling pathways. Extracellular growth signals regulate diverse processes and are transduced via the Ras-mitogen activated protein kinase (MAPK) pathway. Cell cycle markers characterize multiple different aberrations in cellular processes and are a predominant characteristic of malignant UCB cells.

The first report on the prognostic significance of p53 expression status in UCB showed that, at time of RC in patients with organ confined node-negative disease, p53 predicts a significantly increased risk of recurrence and death, independent of tumor grade, stage and lymph-node status.⁵⁷ Many confirmatory studies have suggested that an altered p53 status predicts a poor outcome in patients with UCB, both for patients treated with TUR and RC.^{22,23,54,56,57,61-64} However, controversy still remains regarding the prognostic value of p53.⁶⁵⁻⁶⁹ A meta-analysis of studies using p53 in bladder cancer yielded 117 studies with over 10,000 patients.⁶² This meta-analysis found mixed benefit to p53. In fact, p53 independently predicted recurrence, progression and mortality in only 27% (9 of 34), 50% (12 of 24) and 29% (10 of 35), respectively. In studies that used Cox models, the overall risk of recurrence was 1.6 (95% CI 1.2–2.1), progression was 3.1 (1.9–4.9) and mortality was 1.4 (1.2–1.7). The inconclusive and variable results from different studies targeting comparable groups of patients with regard to tumor stage might be due to different methodologies and evaluation criteria. Despite recent well-designed studies showing a concise stage-related increase in the proportion of altered p53 and powerful prediction for UCB recurrence and survival; other series, including the only prospective studies on p53 status in NMI UCB, have failed to show any independent prognostic value.⁷⁰⁻⁷² The lack of association between an altered p53 protein status and patient outcomes may also be explained by a discordance between p53 nuclear overexpression and actual TP53 gene mutation.^{25,45,73} Despite some contradictory studies, an adjuvant chemotherapy study utilizing p53 status to randomize patients to chemotherapy or observation has been performed. Patients were included within 10 weeks of RC (for details see: www.uscnorris.com/p53/).⁷⁴ This clinical trial will provide more evidence on the utility of p53 expression status in identifying patients at high risk for recurrence.

Regarding the other cell cycle regulatory molecules, both p21 and p27 were independent predictors of UCB recurrence and DSS in immunohistochemical studies of patients who underwent RC.^{22,23} The product of the *p21* gene, the p21 protein, is a downstream effector of p53. It inhibits the activity of cyclin-dependent kinase-2 or -4 complexes, and thus functions as a regulator of cell cycle progression at G₁. p21 expression status has shown prognostic value in predicting disease recurrence not only in RC specimens of patients with more advanced UCB, but

also in NMI UCB.²⁰ A large study of p21 and p53 expression in 242 patients showed independent predictive value of p21 status regarding UCB recurrence and DSS following RC.⁶¹

The biological and prognostic benefit of cyclins in UCB is controversial. Cyclins have a role in malignant urothelial transformation during molecular pathogenesis. Cyclin E1 has a regulatory function, determining rates of cell cycle transition from the G₁ to the S phase. Cyclin D1 and D3 have been reported to provide independent prognostic value for progression-free survival in patients with NMI UCB.⁷⁵ However, data are inconsistent and a recent study found no significant association with UCB progression, presumably due to inactivation of Cyclin D3 by the functionally associated transcription factor, E2F1.⁵¹ A different study found that tissue expression of cyclin D1 or E1 did not add significant prognostic value to standard features in patients with NMI UCB.⁷⁶ Regarding more advanced UCB, a study in RC specimens from 226 patients found that low cyclin E1 expression was significantly associated with UCB-specific mortality.⁷⁷

The retinoblastoma gene is a prototypical tumor-suppressor gene. It encodes a nuclear protein (pRB) that acts as a cell cycle control checkpoint at the G₁ phase.⁷⁸ Inactivating mutations of the RB gene are associated with high grade UCB.² Some retrospective evaluations of pRb expression in RC specimens identified altered pRb expression as independent predictors of oncological outcomes.^{23,54,55,79}

The prognostic value of pRb and p53 expression was recently studied by Cormio et al. in a cohort of 27 patients with high grade NMI UCB treated by TUR and intravesical instillations with bacillus-Calmette-Guerin (BCG).⁸⁰ Altered expression of pRb and p53 was associated with progression. Thus, the expression status of these markers may be useful in identifying patients who will respond to BCG treatment. A prospective validation of these results in a larger cohort of patients is required.

Apoptosis Markers

Apoptotic markers in UCB include caspase-3, Bcl-2, Bax and Survivin.^{19,81-83} Activation of caspases is required to initiate the physiological cell-death program. Caspase-3 as a pro-apoptotic protein and was associated with aggressive UCB.¹⁹ Survivin is a member of the ‘inhibitor of apoptosis’ gene family. It controls mitotic progression and induces changes in gene expression that are associated with tumor cell invasion.⁸⁴ In a cohort of 222 patients treated with RC, the loss of caspase-3 and Survivin have shown independent prognostic value regarding UCB-specific mortality.⁸³ Karam et al. evaluated the combined effects of apoptosis markers on oncological outcomes after RC.¹⁹ An altered expression of caspase-3 was an independent predictor of UCB recurrence and DSS. Moreover, a cooperative effect of Bcl-2, caspase-3, p53 and Survivin expression was observed on outcomes after RC. Significantly reduced DSS rates were found in patients with a higher number of altered apoptotic markers (e.g., 89% at 7 years when no marker expression was altered vs. 8% when all four were altered).¹⁹ The study by Karam et al. also showed that Bcl-2 expression alone was associated with unfavourable outcome. Interestingly, higher Bcl-2 expression was associated with reduced effectiveness of chemotherapy in patients

with invasive UCB. Bcl-2-positive tumors had significantly lower response rates than Bcl-2-negative UCB.⁸⁵ These findings confirm results from preclinical studies in which bcl-2-mRNA was targeted and downregulated.⁸⁶ After further validation, Bcl-2 expression status in invasive UCB may be used to estimate chemo- or radio-sensitivity.

Cell Growth and Signal Transduction Markers

Dysregulated cell growth is one of the main characteristics of human cancers, including UCB. Ki-67, a nuclear protein expressed by rapidly proliferating cells, can be detected by immunohistochemistry. The nuclear Ki-67 antigen expression represents the cell-growth fraction and correlates with biological aggressiveness of malignancies.⁸⁷ An overexpression of Ki-67 was significantly associated with aggressive pathological features of UCB in patients treated by RC.⁸⁸ It served as an independent predictor of disease recurrence and DSS in NMI and more advanced UCB.^{69,88} These findings were confirmed in a multicenter study of patients who underwent cystectomy.⁸⁹

Another molecule associated with cell proliferation represents Galectin-3. It belongs to a family of carbohydrate-binding proteins with a role not only in proliferation but also in the adhesion and apoptosis of solid tumors. A recent study showed that Galectin-3 gene and protein expression levels were associated with reduced survival in patients with high grade UCB.⁹⁰

Functional alterations in signal transduction, including activation of the Ras-MAPK pathway in papillary low grade tumors have been observed in UCB.^{2,35} In invasive UCB, overexpression of receptor tyrosine kinases, e.g., members of the epidermal growth factor receptor (EGFR) family, has been associated with poor oncological outcome.⁹¹⁻⁹⁴ The human epidermal growth factor receptor 2 (HER-2) is a transmembrane tyrosine kinase receptor in the EGFR family. HER-2 plays a fundamental role in cell growth, survival and migration and abnormal activation of HER-2 has been proposed to lead to oncogenic transformation.² The binding of EGF causes activation of HER-2 and induction of the RAS-MAPK pathway. In breast cancer, for example, the hormone receptor status has been guiding therapy for years and the expression of receptors such as HER-2 is used to identify patients who may benefit from targeted therapies.⁹⁵ HER-2 represents also an attractive target in UCB.⁹⁶ Although data on the prognostic significance of HER-2 expression and other members of the EGFR family is lacking, there seems to be a subgroup of patients who overexpress a specific receptor and may thus benefit from targeted therapy.^{91,92,94,97,98} Based on survival benefits in breast cancer patients treated with chemotherapy plus the monoclonal antibody trastuzumab targeting HER-2, this approach has recently been tested in UCB in a clinical phase II trial. Patients with advanced disease were screened for HER-2 overexpression and a combination therapy consisting of trastuzumab, paclitaxel, carboplatin and gemcitabine was applied. The response rate was 70% and the median survival time was 14 months, which represent encouraging findings.⁹⁸ Further studies will be necessary to determine if expression of certain markers will provide information regarding response to targeted therapies.

Migration and Invasion Markers

Loss of intercellular adhesion, local migration and invasion of cancer cells through extracellular matrix can result in micrometastases.^{99,100} Distant recurrence of disease as a result of micrometastases are the main cause of mortality and early metastases are not detectable with current diagnostic tools.⁴ E-cadherin is a transmembrane glycoprotein and plays an important role in cell adhesion via regulation of cytoskeletal filaments. A “cadherin switch” is involved in the initial stages of cancer progression in which epithelial-to-mesenchymal transition occurs.¹⁰¹ In a small cohort of patients with CIS, loss of E-cadherin expression predicted disease recurrence, progression and DSS.¹⁰² These findings have recently been corroborated in larger cohorts.^{101,103}

During the process of local migration and invasion, degradation of the extracellular matrix is essential. Herein, matrix metalloproteinases (MMPs) are involved. MMPs are a group of proteolytic enzymes degrading the extracellular matrix of the basement membrane, the surrounding stroma, cytokines and membrane-bound receptors.¹⁰⁴ An overexpression of MMPs may induce cancer cell migration, invasion and metastases. The regulation of MMP activity is complex and the role of the tissue inhibitors of metalloproteinases is not well understood. While serum and plasma levels of different MMPs seem to have a prognostic role in predicting UCB recurrence,¹⁰⁵⁻¹⁰⁷ their usefulness as molecular tissue biomarkers is controversial. It has been reported that elevated MMP-2 levels provide independent prognostic value in patients with UCB, whereas MMP-9 expression had no significant predictive value.¹⁰⁸⁻¹¹⁰ Results from larger cohorts and validation trials regarding prognostic information of MMP expression are lacking. In a recent study, however, another member of the MMP family, MMP-7 was identified as a promising prognostic biomarker using gene expression analyses. Szarvas et al. found that high MMP-7 expression levels in UCB, as well as high serum concentrations, are independent predictors of metastasis-free survival and DSS.¹¹¹ The authors concluded that MMP-7 in serum and/or TUR specimens may identify patients who are likely to benefit from early aggressive therapy.

Another molecule involved in local cell migration and invasion is the hepatocyte growth factor (HGF). Overexpression of its specific receptor, HGF receptor HGFR/c-Met, has correlated with carcinogenesis, cancer cell invasion, metastasis and survival in different malignancies.^{112,113} The prognostic significance of HGFR/c-Met was evaluated in TUR and RC specimens of 133 patients with non-metastatic UCB.¹¹⁴ The authors found that the presence of phosphorylated HGFR/c-Met pY1349 was an independent predictor of metastasis-free survival and DSS on multi-variable analysis.

The Rho/ROCK pathway is an alternative, activating signal for local invasion and formation of metastasis. The expression of RhoGDI2, a metastasis suppressor gene, has been studied by Theodorescu et al.¹¹⁵ In a small cohort of 51 patients who underwent RC, RhoGDI2 expression was an independent predictor of DSS after adjusting for the effects of tumor grade and stage. The results from larger validation trials of migration and invasion markers are awaited.

Angiogenesis Markers

Tumors require oxygen, nutrients and growth factors to survive and flourish. As a consequence, tumors utilize substances to attract new blood vessels in a process termed angiogenesis. The development of novel substances targeting growth factors involved in angiogenesis, e.g., vascular endothelial growth factor (VEGF), has resulted in a growing interest in angiogenic markers and targets in UCB. Initially, the potential significance of angiogenesis in UCB was evaluated by morphologic studies on microvessel density.¹¹⁶ Bochner et al. investigated the predictive value of tumor microvessel density (MVD) in 164 patients with invasive UCB. Using multivariable analysis, the authors found that tumor angiogenesis was an independent predictor of UCB recurrence and DSS. However, a more recent study could not confirm these findings, possibly due to the use of a different methodology.¹¹⁷ Growth factors that have been associated with induction of angiogenesis have also been evaluated as prognostic biomarkers but data on the utility of the VEGF family is controversial. One large study by Herrmann et al. included 286 patients with UCB who were treated by RC.¹¹⁸ They tested the prognostic value of the angiogenic and lymphangiogenic markers VEGF-C, VEGF-D and Flt-4 in TMAs. The only independent prognostic parameters identified by multivariable analysis were tumor stage and the presence of lymph node metastasis. In a cohort of 204 patients treated with RC, Sharjati et al. found that VEGF expression was associated with UCB recurrence and DSS on univariable analysis.¹¹⁷ However, an independent predictive value was not present. By contrast, Crew et al. found in 55 patients with NMI UCB that VEGF mRNA expression was an independent prognostic factor for early recurrence in multivariable analysis.¹¹⁹ Another recent study suggested prognostic relevance when overexpression of VEGF and its specific receptor, VEGFR2, is detected, which was similar to a previous study.^{51,120} Birkhahn et al. performed quantitative gene expression analyses in pTa NMI UCB. They found that VEGF and VEGFR2 were part of a gene panel predicting progression with high accuracy, suggesting biological relevance of the VEGF cascade in UCB.⁵¹ The assessment of VEGF expression needs standardization regarding different members of the VEGF family and their specific receptors as well as standardization of methods to assess expression status.

Thrombospondin-1 (TSP-1) is an extracellular matrix glycoprotein. It is an inhibitor of angiogenesis and may allow for increased angiogenic potential in case of altered expression. A study evaluating TSP-1 expression in 163 patients who underwent RC found that decreased TSP-1 expression was an independent predictor of UCB recurrence and overall survival.¹²¹ These findings confirmed the results of a previous study by Grossfeld et al. who identified altered TSP-1 expression as an independent predictor of UCB recurrence overall survival in 163 patients treated with RC.¹²² Moreover, Shariat et al. recently demonstrated in the aforementioned study that downregulation of TSP-1 and an upregulation of basic fibroblast growth factor were independent predictors of clinical outcomes in 204 patients with UCB.¹¹⁷

For the prediction of DSS, only TSP-1 was a useful biomarker. Interestingly and in contrast with previous reports,¹²² the expression TSP-1 did not correlate with p53 expression but did with the cell cycle regulators, p21 and p27. These observations make TSP-1 an interesting molecule for further investigation and as a potential therapeutic target. Novel approaches to restore p53 expression and consequently re-establish TSP-1 expression have been described but need further preclinical evaluation.¹²³

The Future of Molecular Biomarker Research

Urothelial carcinoma of the bladder is a heterogeneous disease and the molecular pathogenesis is a complex multistep process. Therefore, it seems unlikely that a single molecular biomarker adequately reflects the tumor biology. Clinical decision making needs to be based on accurate and robust evidence. Several studies have found that combining markers improves predictive accuracy over one marker abnormality.^{19,22,23,37,54,56,59,124} Our group found that a marker panel (cyclin E1, p53, p21, p27, pRB) performed better than individual markers and improved prediction of recurrence and mortality when modelled along with conventional clinicopathological features.²¹ The study included 191 patients with pTa-3 N0 M0 UCB treated with RC. We found that the addition of altered biomarkers (cyclin E1, p53, pRB, p21 and p27) to a nomogram based on TNM staging significantly increased the predictive accuracy for UCB-specific mortality to 86.9%. A larger, multicenter validation confirmed the results.¹²⁵ Currently, at the University of Texas Southwestern Medical Center, we have a prospective protocol using a marker panel (p53, p21, p27, cyclin E and Ki-67) in all patients with high-grade or invasive disease (\geq pT1). Prospective studies are an essential final step before biomarker use can be recommended in clinical practice.

In order to accelerate the clinical implementation of molecular biomarkers, standardized methods, both on technological and statistical levels, are required to assess the accuracy of prognostic marker panels. A universal definition of test positivity and clearly defined clinical end points are highly desirable. Moreover, a critical appraisal of the various methods used to evaluate biomarkers is necessary. It is important to note that immunohistochemical results may not correlate with mutation status assessed by more sophisticated methods.

In conclusion, current staging systems have limited ability to predict oncological end points in patients with UCB. Characteristic biomarkers derived from the molecular pathogenesis of UCB need to be considered to identify patients at high risk for disease recurrence and mortality. Moreover, the selection of patients who may benefit from multimodal and targeted therapeutic approaches can be improved through optimized risk stratification and prediction of response to therapy with biomarkers. None of the molecular biomarkers has yet successfully entered clinical practice in UCB patients mainly due to insufficient validation and lack of prospective data. More prospective trials validating prognostic markers and evaluating neoadjuvant and adjuvant chemotherapy as well as targeted therapies are required to promote clinical use of molecular biomarkers for UCB.

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