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UROTHELIAL CARCINOMAS OF THE URINARY TRACT
MOLECULAR ANALYSES FOR CLINICAL APPLICATIONS

Sigrun I. Langbein

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UROTHELIAL CARCINOMAS OF THE URINARY TRACT
MOLECULAR ANALYSES FOR CLINICAL APPLICATIONS

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CHAPTER 1

GENERAL INTRODUCTION AND OUTLINE

General introduction

More than 357,000 new cases and 145,000 deaths caused by urothelial carcinoma (UC) are estimated to occur worldwide.[1] The majority of patients (85%) are age 55 years or older at the time of diagnosis, and with the burden of increasing lifespan, an increasing incidence of urothelial tumours can be expected.
UCs reflect a heterogeneous group, varying in tumour location, recurrence, and progression rates, and finally, in disease-specific survival. Low-grade Ta and T1 tumours progress completely differently compared to muscle-invasive T2-T4 tumours, reflecting underlying genetic alterations. While on one the hand non-muscle-invasive Ta tumours are not life threatening but frequently recurring, on the other hand, muscle-invasive tumours (≤pT2) are a serious threat and lethal in about 50% of patients. Unfortunately, there has not been a substantial decline in the mortality rate for patients with advanced urothelial tumours over the last decades. Progression and high recurrence rates, aspects of a lifelong tumour risk, and the necessity of controlling the complete urinary tract, demand complex surveillance and extensive diagnostic and therapeutic strategies, leading to the highest cost per patient ($96,000 to $187,000 in the US) from diagnosis to death among all cancers.[2]
The visual diagnosis and imaging of tumours of the urothelial tract has impressively advanced. Instead of the prototype "Light Conductor" (Phillip Bozzini, 1806)[3-5], a funnel-shaped metal tube with an internally positioned candle, nowadays we use flexible videoscopes with integrated simultaneous multicolor chips.
Nevertheless, urologists depend on invasive diagnostics in detection and surveillance of UCs. The great aim, a non-invasive tool for the diagnosis of urothelial tumours, has not yet been achieved. Voided urine cytology is the most common adjunct in tumour diagnosis, even if it has a moderate overall sensitivity and its value is extremely dependent on the investigator's expertise.[6-8]
Therefore, more sensitive and specific biomarkers with low intra-assay and inter-assay variability are needed. New and advanced molecular approaches are expected to broaden the detection rate of marker molecules and therapeutic strategies. Because urothelial tumours are characterized by various and inconsistent genetic and enzymatic alterations, a single-molecule approach might currently be insufficient. Profiling approaches such as FISH (fluorescence *in situ* hybridization), SELDI-TOF MS (surface-enhanced laser desorption ionization-time of flight mass spectroscopy), and microarrays allow combinations of putative biomarkers to be simultaneously

assessed, which may be more informative given the heterogeneity of the disease.[9-12] To improve disease management, we should use the full capability of modern techniques in medical research and retransfer the results to clinical routine.

Locally advanced or metastasized diseases have little chance of curative treatment even with radical surgical management and chemotherapy. Attempts to use adjuvant and neo-adjuvant chemotherapy fail in many patients in the face of long-standing tumour control. Adjusting toxic substances and their application regimes has led to noticeable improvements in side effects but has not resulted in a significant extension of tumour-specific survival. Therefore, on the one hand, a better selection of patients profiting from therapies is needed, and on the other hand, new strategies in cancer treatment are essential. The detection of carcinogenic molecular pathways allows insights into tumourigenesis and tumour-specific networks, which promotes improved diagnostics and therapies, such as the new generation of antibodies or multi-kinase inhibitors. Recognition of important pathways - for example, the lectin network or aerobic glycolysis in tumour metabolism - has opened the way to new approaches in cancer-specific treatment.[13-18]

This manuscript enumerates major problems in the clinical management of urothelial cancer and demonstrates the impact of molecular approaches brought into clinical practice in trying to overcome these long-standing limitations.

Outline

The first part of this manuscript emphasises the challenges in the clinical treatment of urothelial cancer, such as high recurrence rates and unpredictable progression courses of non-muscle-invasive tumours. Experimental and clinical data suggest multifactorial causes of tumour recurrence with one possible reason being the treatment (transurethral resection) itself. Transurethral resection (TUR) is the gold standard treatment of bladder cancer worldwide but lacks a standardization of instruments and resection techniques, making comparison among studies difficult. We analyzed in ***Chapter 2*** if various resection techniques have a notably different effect on recurrence and progression rates. Published data have impressively demonstrated that patients with progressive superficial tumours do have a bad prognosis even after undergoing radical operative treatment. In general, muscle-invasive tumours have a tumour-specific mortality of almost 50%. To improve patient survival, adjuvant chemotherapy was administered. The

widely used cisplatin-based regimen in combination with methotrexate, vinblastine, and epirubicin (M-VEC) resulted in good response rates but in moderate benefit for patient's survival. Furthermore, the M-VEC regimen caused severe side effects, often resulting in discontinuation of the therapy. In ***Chapter 3***, a randomized, multicenter, phase III trial is presented, which evaluates the efficacy of cisplatin in combination with methotrexate (CM) versus M-VEC concerning progression-free survival and toxic side effects.
The second part of this work focuses on molecular approaches to better understand carcinogenesis and to improve diagnostics and therapies of urothelial tumours.
Cancer is viewed as a disease resulting from cancer-causing genes that deregulate cellular proliferation, apoptosis, and biochemical pathways, resulting in abnormal tumour cell metabolism. To identify potential tumour suppressor genes in urothelial carcinoma, specific regions on chromosome 2 were evaluated using microsatellite analysis. The region at 2q21.2 was investigated in detail, and deletions at the LRP1B gene were highly significantly correlated with high grade carcinomas, as described in ***Chapter 4***. Typically, urothelial tumours are characterized by various genetic alterations, which reflect two different types of tumours, the genetically stable and the genetically unstable tumours. Fluorescence *in situ* hybridization (FISH) is a technique that uses fluorescently labelled DNA probes to detect numerical or structural chromosomal abnormalities in tumour cells. In addition to the detection of bladder malignancies, this approach might offer a broad spectrum of other diagnostic possibilities, such as the specification of genetically unstable tumours, the diagnosis of upper urinary tract tumours, and even locally advanced kidney cancers. An extensive literature review and a detailed description of this technique addressing all of the pros and cons in the diagnosis of urothelial cancer are given in ***Chapter 5***.
Biomedical research has advanced rapidly in recent years with the sequencing of the human genome. However, this progress in genomic research does not directly transfer to routine clinical use. Because relevant genetic alterations typically lead to changes in the proteome and proteins are main actors in physiological and pathological processes, comprehensive protein profiling technologies, such as 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and ProteinChip®, have engendered great interest. The identification of target proteins or protein expression patterns seems promising for improved diagnosis, therapy, and surveillance. In ***Chapter 6***, we evaluated modern protein profiling techniques in urothelial tumours, and in ***Chapter 7***, we described in detail the use of protein chip platforms

in combination with complementary analytical methods for serum analysis. Serum is a preferred medium for biomarkers because it can be obtained with low levels of invasiveness, it is available in sufficient quantity, and it is a source with enormous information content. Until now, serum data about protein expression patterns in patients with urothelial carcinomas have been lacking but seem promising, especially in respect to potential biomarkers of invasive disease.

As described above (Chapter 6), we detected potential biomarkers using proteomic approaches and found galectin-3 to be overexpressed in high-stage and -grade tumours. Galectins are a family of carbohydrate-binding proteins and play a pivotal role in tumourigenesis by regulating apoptosis, angiogenesis, tumour immune escape, and tumour metabolism. Much attention has focused on galectin-1 and -3, but similar advances are lacking in the elucidation of the molecular aspects of other galectins. No detailed data have been published about the importance of the galectins in urothelial cancer. In ***Chapter 8***, a comprehensive expression study was performed to evaluate if other galectins beyond the family members -1 and -3 are present and linked in UC. Galectin-3 has been extensively investigated for its antiapoptotic activity. Various mechanisms by which galectin-3 protects cells from apoptosis have been proposed, one being the activation of the PI3K/Akt pathway. This pathway promotes malignant transformation, enhances aerobic glycolysis, and renders cells dependent on glycolysis for survival. Signalling through the insulin receptor activates PI3K and Akt, resulting in stimulation of glucose uptake, glycolysis, and inhibition of β-oxidation of fatty acids, the most fundamental alterations in tumour-specific metabolism. The increased dependence of cancer cells on the glycolytic pathway for ATP generation (transketolase-dependent pentose phosphate pathway) enhanced our study of cancer-specific deregulations and their possible impact on therapeutic strategies, as described in ***Chapter 9***.

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CHAPTER 2

PERSISTENCE, RECURRENCE, AND PROGRESSION RATES OF SUPERFICIAL BLADDER TUMOURS AFTER RESECTION USING THE DIFFERENTIATED TECHNIQUE

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Abstract

Objective

To investigate whether the differentiated resection technique for excising superficial bladder cancer leads to higher recurrence and progression rates as compared with regular resection.

Subjects and Methods

We evaluated 163 patients, 66 undergoing a differentiated and 97 a regular resection. All patients underwent a routine second resection within 6-10 weeks. Recurrence and progression rates, as well as tumour persistence on second resection were analysed.

Results

Patients with differentiated resections of bladder tumours did not have higher tumour recurrence and progression rates. Also, these patients had a significantly higher percentage of tumour-free second resections ($p=0.03$).

Conclusion

The differentiated resection technique for excising superficial bladder cancer has no negative influence on recurrence and progression rates, but it leads to reduced tumour persistence.

Introduction

The major problems in superficial bladder cancer are the high recurrence (50% to 70%) and progression (10% to 50%) rates. Transurethral resection is the first choice of treatment; unfortunately the resection itself has never been standardized. The use of differing resection techniques, taking random biopsies, or performing second resections complicate the comparison of institutional results. Whether or not recurrent tumours originate from tumour cell implantation, field effect or incomplete resection is debated. In routine second resections, residual tumour tissue has been found in 30% - 76% of the cases.[1] A high percentage of synchronous and metachronous bladder tumours result from a single original tumour. This has been demonstrated by microsatellite analysis.[2] It has not been determined whether primary resection results in spreading of these tumours or whether the tumours have been seeded before treatment. It is difficult to ascertain whether resection itself has an impact on recurrence or progression.
Experimental data strongly indicate that cell implantation into the traumatized bladder wall plays an important role in tumour recurrence and pro-

gression. Uncoated extracellular matrix especially attracts tumour cells, providing ideal conditions for cell implantation in vitro.[3] Deep and extensive transurethral resections, as with the differentiated resection technique, may lead to increased recurrence and progression rates. Therefore, the aim of this study was to compare whether the differentiated resection technique for excising superficial bladder cancer leads to higher recurrence and progression rates than regular resections.

Subjects and Methods

Patients with primary and recurrent superficial bladder cancer treated with video-guided transurethral resection between 1993 and 2000 were included in this study. Inclusion criteria were routine second resections within 6-10 weeks and completely documented follow ups. 163 patients, 134 male and 29 female, met the inclusion criteria: 66 patients were resected using the differentiated technique described by Bressel *et al*[4], with separate deep resection of the tumour base and tumour surroundings; 97 patients were resected using a non-differentiated technique with complete resection of all visible tumour. All first tumour resections were carried out by urologists in training, supervised by senior urologists, while the routine second resections were performed by senior urologists. Senior urologists determined the method of resection and treated their respective patients with their preferred technique.

Visible tumours, including detrusor muscle were removed completely during transurethral resection; for histological staging detrusor muscle had to be included. Routine second resection was performed within 6-10 weeks. Patients with unresectable and muscle-invasive tumours were excluded from this evaluation.

The minimum follow-up period was 48 months. The histological profile of the tumours determined the further treatment as recommended by the European Association of Urology (EAU) guidelines. Mitomycin C was administered to 34 and 38 patients in the differentiated and in the non-differentiated resection groups, respectively, with an average of 11 and 14 instillations, respectively. Bacillus Calmette-Guérin therapy was administered in 3 and 6 patients with a mean of 5 and 8 applications in the differentiated and in the non-differentiated resection groups, respectively. Repeat cystoscopy was done every 3 months for the first 24 months and every 6 months thereafter.

Tumour recurrence and progression rates in the differentiated resection group, with resection of visible tumours including surrounding tissues

and extra resection of the tumour base, were correlated with those in the non-differentiated resection group, where only visible tumour tissue was removed. Analysis of tumour persistence in routine second resection as well as overall recurrence and progression, influence of concomitant carcinoma in situ, early recurrences (24 months), and multifocality was carried out. Statistical analysis was performed with SAS-software (version 8.2). Chi-square and Fisher`s exact test were used. The significance level was determined at $\alpha = 0.05$.

Results

The mean patient age was 67±9 years. The clinical stage and grade distribution at first and second resection are given in Table 2.1. Altogether, 244 transurethral resections for bladder tumours and 221 for second resections were performed. No significant difference was noted in between the groups concerning histological staging and grading ($p = 0.8$), low versus high grade ($p = 0.5$) or in application of intravesical chemotherapy ($p = 0.6$). All 163 patients showed transitional cell carcinoma.

Table 2.1. Tumour stages and grades at first and routine second transurethral resection of the bladder (TURB) using the differentiated resection (DR) and the non-differentiated resection (non-DR) techniques; 100 second TURBs showed no evidence of tumour persistence.

	First TURB(%)				Positive second TURB(%)			
Stage	**DR**	**%**	**Non-DR**	**%**	**DR**	**%**	**Non-DR**	**%**
Ta	30	(46)	46	(47)	15	(68)	25	(61)
T1	36	(54)	50	(52)	4	(18)	9	(22)
CIS	0		1	(1)	3	(14)	5	(12)
>T1	0		0		0		2	(5)
Grade								
G1	12	(18)	22	(22.5)	7	(32)	15	(36.5)
G2	49	(74)	53	(55)	10	(45)	15	(36.5)
G3	5	(8)	22	(22.5)	5	(23)	11	(27)
Total	66		97		22		41	

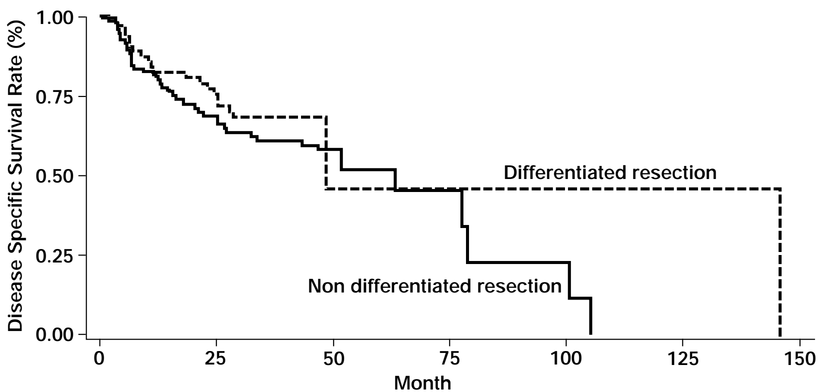

Figure 2.1. Kaplan-Meier plot with recurrence rates in patients treated with differentiated and non-differentiated resection techniques.

After 24 months, 14 of the 66 patients (21%) in the differentiated resection group and 21 of the 97 patients (22%) in the non-differentiated resection group had tumour recurrences (p=0.27). After 48 months, 18 of the 66 (27%) patients and 28 of the 97 patients (29%) had tumour recurrences in the differentiated and non-differentiated resection groups, respectively (p=0.33). Progression from superficial to muscle-infiltrating tumours occurred in 2 (3%) of the differentiated and in 5 (5%) of the non-differentiated resection patients. Recurrence and progression rates did not show any significant difference between the groups (p=1.0; Fig. 2.1). Of the 163 patients, 59 (36%) presented multiple tumours at first resection. One unifocal tumour (1%) and 6 multifocal tumours (10%) showed muscle-invasive growth patterns within 48 months. Statistically, there was a significant correlation between progression and multifocal growth pattern (p=0.005). In univariate analysis, concomitant carcinoma in situ was not a significant risk factor for progression. However, multifocality, carcinoma in situ and early recurrence combined were statistically significant (p<0.05) parameters for the development of progression. In the differentiated resection group, 33% of the patients had a positive second resection, as had 42% in the regular resection group (p=0.03).

Discussion

Differentiated resection was first described by Bressel *et al.*[4], suggesting lower tumour persistence with resection of tumour base and tumour sur-

roundings. Studies on tumour persistence found that the proportion of residual tissue in routine second resections increased with tumor stage: 27% and 53%, in Ta and T1 tumours, respectively.[5] Flamm and Steiner[6], however, reported a lower tumour persistence rate of 10% in differentiated resected tumours, which is in accordance with our finding: 9% less tumour persistence in patients in whom the differentiated resection technique was applied. Considering that tumour tissue left behind will lead to new cancer growth, complete tumour elimination is of great importance. Still, the differentiated resection technique is not standard, because it may cause more severe urothelial defects and cancer cell implantation, leading to increasing recurrence and progression rates.[7-10]

Whether or not deep transurethral resection enhances tumour progression is still under debate. El-Abbady *et al.*[11] demonstrated unusual patterns of tumour expansion in cystectomy specimens after previous transurethral resections. When transurethral resection had been performed, bladders tissues showed clusters of malignant cells in between muscle fibres and perivesical fat. They concluded that tumour cells were brought into the surrounding tissue when deep resections were performed. Cystectomy specimens from patients who had not undergone a previous transurethral resection did not show cancer cells in adjacent tissues. Therefore, higher progression rates may be expected in differentiated resections with deep resection of the tumour ground. The progression rate in this study, however, did not show statistically significant differences between both groups. There was no evidence that differentiated resections led to aggressive growth by releasing tumour cells into deeper muscle layers. Indeed, the resection technique with a separate elimination of the tumour base and surroundings showed a much higher rate of tumour-free second resections, indicating a higher rate of tumour elimination at the first treatment.

Experimental data suggest possible influences of transurethral resection on cell adhesion and migration. The tumour cell implantation is enhanced by intravesical trauma due to enlarged areas of extracellular matrix.[3, 12] This implantation theory is supported by the fact that most metachronous and synchronous tumours are of the same clonal origin.[2, 13] Whether cells had seeded before surgery or were disseminated during the resection cannot be determined.

The challenge is to verify the implantation theory and to transfer in vitro data to clinical applications. The current study can be a step in estimating the advantage or disadvantage of differentiated resection of bladder tumours. Although there are many aspects influencing recurrence and progression, it is clinically relevant to demonstrate that the differentiated

resection method is not associated with perceived disadvantages, such as increased tumour recurrence or progression.

Attempts to lower recurrence rates by using laser resection seemed promising, but subsequent studies could not confirm better outcomes. Beisland and Seland[14] achieved a significant reduction in recurrence rates by performing laser resection, implying that less urothelial trauma and fewer free-floating tumour cells result in a lower recurrence rate. Sakkas *et al.*[15] demonstrated a decrease in local recurrence rate only, but the overall recurrence was 45%.

This study did not show any significant differences between the two techniques employed. The data showed equivalent results with respect to recurrence and progression rates in both groups. Further prospective randomized studies are needed to provide comparative data using various resection techniques, including laser resections, and the benefits of instillation therapies. Since early instillation therapy significantly reduces recurrences within the first 24 months, perioperative cell seeding can be better addressed.[16] Mitomycin or equivalent chemotherapeutic agents are proposed for lowering recurrence rates by destroying floating tumour cells rather than influencing already seeded but invisible tumours, as chemoresection does.

Conclusions

Overall, the findings in this study show that both differentiated and non differentiated operating techniques provide equivalent outcomes with respect to recurrence and progression rates. Extensive tumour resection with the differentiated technique does not influence recurrence and progression rates, but it is an effective tool for reducing the risk for tumour persistence and facilitates more precise staging. The combination of differentiated resection and early instillation therapy for superficial bladder cancer is recommended.

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CHAPTER 3

ADJUVANT CISPLATIN PLUS METHOTREXATE VERSUS METHOTREXATE, VINBLASTINE, EPIRUBICIN, AND CISPLATIN IN LOCALLY ADVANCED BLADDER CANCER: *RESULTS OF A RANDOMIZED, MULTICENTER PHASE III TRIAL*

(AUO-AB 05/95**)**

J. Lehmann, M. Retz, C. Wiemers, J. Beck, J. Thüroff, C. Weining, P.Albers, D. Frohneberg, T. Becker, P. Funke, P. Walz, S. Langbein, F. Reiher, M. Schiller, K. Miller, S. Roth, T. Kälble, D. Sternberg, S. Wellek, M. Stöckle

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Abstract

Purpose

Radical cystectomy as standard treatment of muscle-invasive urothelial carcinoma of the urinary bladder cures less than 50% of patients with locally advanced bladder cancer. We compared two adjuvant combination chemotherapies in patients with stage pT3a-pT4a, and/or pathologic node-positive transitional cell carcinoma of the bladder after radical cystectomy.

Patients And Methods

A total of 327 patients were randomly assigned to either adjuvant systemic chemotherapy with three cycles of cisplatin 70mg/qm^2 on day 1 and methotrexate 40mg/qm^2 days 8 and 15 of a 21-day cycle (CM) or three cycles of methotrexate 30 mg/qm^2 days 1, 15, and 22, vinblastine 3mg/qm^2 days 2, 15, and 22, epirubicin 45mg/qm^2 day 2, and cisplatin 70mg/qm^2 on day 2 of a 28-day cycle M-VEC).

Results

The hazard ratio for progression-free survival as the primary endpoint was 1.13 (90% CI, 0.86 to 1.48) for 163 CM patients compared with 164 M-VEC patients whose right-hand limit remained below the upper bound compatible with the noninferiority hypothesis ($\alpha = .0403$). The 5-year progression-free, tumorspecific, and overall survival rates (point estimates$\pm$SE) for CM versus M-VEC were 46.3$\pm$4.6 v 48.8$\pm$4.5%, 52.0%$\pm$4.6% v 52.3$\pm$4.8%, and 46.1%$\pm$4.3% v 45.1%$\pm$4.6%, respectively. WHO grade 3 and 4 leukopenia occurred in 7.0% of patients treated with CM and 22.2% of patients treated with M-VEC ($P < .0001$).

Conclusion

CM cannot be considered inferior to M-VEC with regard to progression-free survival of patients with locally advanced bladder cancer after radical cystectomy. Moreover, patients receiving adjuvant CM combination therapy experienced significantly less grade 3 and 4 leukopenia than patients treated with M-VEC.

Introduction

Radical cystectomy is recommended as curative treatment for locally advanced bladder cancer including tumor stages pT3 and pT4a, and/or involvement of regional lymph nodes. However, more than half of these patients experience relapse, with distant metastasis being the predominant form of disease recurrence. Therefore, combination chemotherapy has been investigated as neoadjuvant or adjuvant adjunctive treatment in numerous trials. A recent meta-analysis on neoadjuvant chemotherapy for

invasive bladder cancer described a modest but clear improvement in survival, encouraging the use of platinum-based combination therapy.[1] For the adjuvant approach, three randomized trials have suggested a relapse-free survival improvement for patients receiving combination chemotherapy after radical cystectomy compared with patients undergoing surgery alone.[2-4]

The combination regimen of methotrexate, vinblastine, doxorubicin, and cisplatin (M-VAC), which was first reported as a palliative approach for advanced metastatic urothelial carcinoma in 1985[5], has succeeded as a gold standard treatment in randomized trials.[6,7] The first randomized trial comparing adjuvant M-VAC or methotrexate, vinblastine, epirubicin, and cisplatin (M-VEC) to radical cystectomy alone found a significant progression-free survival improvement of 50% for the adjuvant group 40 months after surgery.[3] Despite the efficacy of M-VAC or M-VEC combination therapy, severe hematologic toxicity and non-hematologic toxicity and a treatment-related mortility rate of up to 4% have caused concern.

The present study was initiated in 1994 as a noninferiority trial of a reduced chemotherapy regimen consisting of cisplatin plus methotrexate (CM) compared with conventional M-VEC, with the intention of finding a less toxic but effective regimen for the adjuvant treatment of patients with resected locally advanced bladder cancer with no substantial loss of efficacy.

Patients And Methods

The study protocol was reviewed and approved by the committee of the "Arbeitsgemeinschaft Urologische Onkologie" of the German Cancer Society as well as local ethics committees of participating centers.

Eligibility

Patients eligible for the randomisation process had to meet the following inclusion criteria. Disease status after radical cystectomy must be histologically confirmed stage pT3a-4a and/or pathologic node-positive transitional-cell carcinoma of the bladder (1997 TNM-classification[8]). Squamous cell carcinoma and/or adenocarcinoma components were allowed if transitional-cell carcinoma was present. Radical cystectomy denoted the removal of the entire bladder, prostate, and seminal vesicles in men and removal of anterior pelvic organs in the female, including, if indicated, a portion of the anterior vagina. The method of urinary diversion was left to the discretion of the investigator. Bilateral pelvic lymph node dissection was a prerequisite for correct staging and included a full dissection of the lymph nodes bordered by the internal iliac arteries, external iliac arteries, and the pelvic floor bilaterally including obturator nodes. Any evi-

dence of macroscopic or microsopic incomplete resection (tumor-positive margin of the specimen or grossly enlarged unresected lymph nodes) was considered an exclusion criteria. Distant metastasis was excluded by preoperative staging, including at least chest x-ray and abdominal ultrasound. Furthermore, patients between 18 and 70 years old were included if they had a Karnofsky performance score of at least 80 with adequate renal and liver function as well as bone marrow reserve (measured creatinine clearance $\geq$70mL/min, serum creatinine $\leq$1.3mg/dL, bilirubin $<$1.5mg/dL and ALT or AST$<$60U/L, WBC count $\geq 3.0 \times 10^9$/L, platelets $\geq 150 \times 10^9$/L, and hemoglobin $\geq$10g/dL). Local intravesical adjuvant chemotherapy or immunotherapy before radical cystectomy was allowed. Furthermore, a written informed consent according to institutional and federal guidelines had to be signed by the patient. Patients were ineligible if the time interval between radical cystectomy and the first day of chemotherapy exceeded 4 months and if they had used any investigational agent in the months before enrollment onto the study.

Treatment plan

The CM regimen consisted of cisplatin 70mg per square meter of body-surface area (mg/qm^2) day 1 and methotrexate 40mg/qm^2 on days 8 and 15 every 21 days for a maximum of three cycles. M-VEC (methotrexate 30 mg/qm^2 on days 1, 15, and 22; vinblastin 3 mg/qm^2 on days 2, 15 and 22; epirubicin 45mg/qm^2 on day 2, and cisplatin 70mg/qm^2 on day 2) was administered every 28 days for a maximum of three cycles.

The following dose adjustments were planned. Methotrexate was omitted if the leukocyte count was less than 2.5×10^9/L or the thrombocyte count was less than 100×10^9/L. The beginning of a new cycle was delayed until the leukocyte count was greater than 3.0×10^9/L and the thrombocyte count was greater than 10×10^9/L. Doses of cisplatin, and methotrexate were reduced by 50% if measured creatinine clearance was 50 to 70mL/min, or the drugs were omitted if the creatinine clearance was less than 50mL/min. Patients were to receive full supportive care. Use of growth factors was recommended only in case of leukopenic fever, dose omissions for methotrexate and vinblastine as a result of leukopenia, or delay of a consecutive cycle as a result of leukopenia. Hematologic and non-hematologic toxicity was graded according to the WHO grading system. Study drug therapy was discontinued if there was evidence of progressive disease under therapy, if the attending physician considered a change of therapy to be in the best interest of the patient, if the patient requested discontinuation, or if the drug exhibited unacceptable toxicity.

Follow-up of patients

Follow-up of both treatment groups occurred at 3-month intervals for 2 years, then at 6-month intervals for 3 years, and yearly thereafter. Follow-up consisted of physical examination, abdominal ultrasound and chest radiography, and computed tomography scanning or bone scanning as clinically indicated. The site and date of the first relapse, as well as the date and the reason in case of death, were recorded. Relapse was defined as the detection of at least one suspicious lesion that could not be identified by histologic, clinical, or laboratory criteria as an independent secondary malignancy. In case of relapse, it was left to the discretion of the patient and the attending physician whether further therapy, such as second-line chemotherapy, radiation, or surgical treatment, was initiated.

Statistical methods

The primary basis for the statistical planning and analysis of the trial was the noninferiority version of the log-rank test for equivalence of two survivor functions[9] satisfying an ordinary proportional hazards model in the sense of Cox.[10] The test was applied with an equivalence margin of $\delta = .15$, which gives the maximum tolerable difference of progression-free survival probabilities to be expected under both treatments for the same time since cystectomy. Practical implementation of the procedure was performed by estimating the regression coefficient β of a proportional hazards model, with the indicator of treatment group CM as the only covariate, and comparing the right-hand limit of a 90% CI for the hazard ratio $(HR) = e^{\beta}$ to 1.5077 as a critical upper bound. For the purpose of checking the adequacy of the proportional hazards assumption, the procedure implemented by the S-plus (Staistical Science, Seattle, WA) function cox.zph was used (for a detailed description of the statistical rationale behind it see Therneau and Grambsch[11]). Because the p-value obtained from this test turned out to be as large as .45, there was no reason to question the appropriateness of the model.
However, estimation of median progression-free survival time was based on a fully parametric model because both observed survivor functions dropped below 50% so late that the number of patients still at risk was too small for providing a sufficiently accurate estimate of the conditional probability of surviving the interval containing the observed median. Of the standard parametric models, the log-normal was the model that showed the best fit to the data. Accordingly, confidence limits for the median progression-free survival time were computed by transforming those for the expected value of the distribution of log T.

In addition to progression-free survival time, which had been chosen as the primary endpoint for the confirmatory analysis of this trial, the same techniques were also applied to the data obtained for the length of survival and time until death from the specific tumor. All eligible patients who received any adjuvant treatment were included in the analysis of possible toxic effects. The incidences of WHO grade 3 and 4 toxicities were compared using Fisher's exact test for homogeneity of two binomial distributions.

Results

Between January 1, 1994, and September 30, 2000, 335 patients were registered onto the trial (Fig. 3.1). Eight patients (2.4%) were deemed ineligible because inclusion criteria were not met (age, renal function, metastatic disease at time of radical cystectomy, secondary malignancy, and withdrawal of consent). The remaining 327 patients were equally assigned to adjuvant systemic chemotherapy with CM or M-VEC by randomization after having signed informed consent.

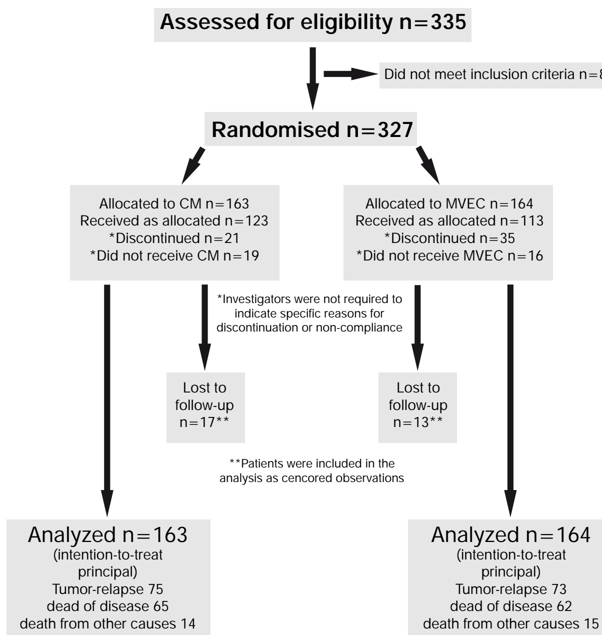


Figure 3.1. Consort diagram.

Table 3.1. Patient demographics.

	CM n (%)	M-VEC n (%)	total n (%)
	163	164	327
T category			
pTis/pT1 pN+	7 (4.3)	4 (2.4)	11 (3.4)
pT2 pN+	14 (8.6)	29 (17.7)	43 (13.1)
pT3 pN0	58 (35.6)	61 (37.2)	119 (36.4)
pT3 pN+	56 (34.3)	44 (26.8)	100 (30.6)
pT4a pN0	13 (8.0)	10 (6.1)	23 (7.0)
PT4a pN+	15 (9.2)	16 (9.8)	31 (9.5)
Nodal status			
pN0	71 (43.6)	71 (43.3)	142 (43.4)
pN+	92 (56.4)	93 (56.7)	185 (56.6)
1 ln	43 (46.7)	38 (40.9)	81 (43.8)
2-5 ln	37 (40.2)	48 (51.6)	85 (45.9)
>5 ln	12 (13.1)	7 (7.5)	19 (10.3)
median pos ln	2	2	2
Age			
≤50	26 (16.0)	24 (14.6)	50 (15.3)
51-60	61 (37.4)	61 (37.2)	122 (37.3)
61-70	76 (46.6)	79 (48.2)	155 (47.4)
median	60.2 (95% CI 58.5-61.7)	60.7 (95% CI 59.6-61.7)	60.5 (95% CI 59.6-61.4)
Gender			
male	123 (75.5)	134 (81.7)	257 (78.6)
female	40 (24.5)	30 (18.3)	70 (21.4)

Abbreviations: CM, cisplatin plus methotrexate; M-VEC, methotrexate, vinblastine, epirubicin and cisplatin.

Demographic factors were similar between the two groups (Table 3.1). Median age was 60.5 years, and the ratio of males to females was almost 4:1. More than half of the patient group (56.6 %) had tumor-positive regional lymph nodes. The median follow-up time for patients living free of relapse at the time of analysis was 42 months.

Table 3.2. Administration of chemotherapy.

	CM nPat	M-VEC nPat
Total	**163 (%)**	**164 (%)**
Pts receiving chemotherapy	**144** (88.3)	148 (90.2)
3 cycles	123 (75.5)	113 (68.9)
2 cycles	14 (8.6)	17 (10.4)
1 cycle	7 (4.3)	18 (11.0)
Pts not receiving chemotherapy	19 (11.7)	16 (9.8)
Administered cycles	404	391
Administered cycles (per cycle 1/2/3)		
Cisplatin	404 (145/137/122)	372 (142/123/107)
MTX	756 (270/253/233)	1022 (386/346/290)
Epirubicin	-	376 (145/123/108)
Vinblastin	-	384 (145/129/110)
Pts eligible for doseintensity	144	145

Abbreviations: CM, cisplatin plus methotrexate; M-VEC, methotrexate, vinblastine, epirubicin and cisplatin.

Treatment

The median time from surgery to chemotherapy was 56 days in both arms. Of the 163 patients assigned to receive adjuvant CM, 123 (75.5%) completed three cycles as planned (Table 3.2); 12.9% (21 of 163 patients) discontinued treatment early because of toxic effects or for other reasons. Nineteen patients (11.7%) declined treatment, and four patients (2.5%) died during the course of treatment. One patient died of cardiac failure (day 18 of a third cycle), two patients experienced severe bone marrow toxicity after two cycles and one patient developed lethal pneumonia after one cycle.

Of the 164 patients assigned to the M-VEC arm, 113 (68.9%) completed three cycles; 21.4% (35 of 164 patients) stopped treatment early. Sixteen patients (9.8%) declined treatment, and one patient (0.6%) died during treatment with M-VEC as a result of cardiac dysfunction. All patients are analyzed as belonging to the treatment arm assigned by randomization, adopting the intention-to-treat principle.

Surgical procedures

The only surgery-related requirements for eligibility were radical cystectomy with curative intent and en bloc resection of the tumor with negative

margins, as well as absence of metastatic or unresected transitional-cell-carcinoma. However, because patients were usually identified postoperatively, specific surgical as well as histopathological procedures could not be required.

Toxicity

Hematologic toxic effects predominated. The most common hematologic toxic effect was leukopenia, with a significantly higher rate in patients treated with M-VEC compared with CM (22.2% *v* 7.0%, respectively). Except for a significant difference in grade 3 alopecia in favor of CM (1.9% in CM *v* 24.7% in M-VEC), no difference was observed for other hematologic or non-hematologic factors. Four patients (2.5%) died as a result of a toxic effect attributed to CM chemotherapy, and one patient (0.6%) died as a result of therapy in the M-VEC arm. This difference of therapy-related mortality was not statistically significant (Fisher's exact test, P=.371).

Table 3.3. Toxicity.

	CM		M-VEC		Fisher-test (Tox 3/4)
WHO toxicity grade	3	4	3	4	
hematologic					
anemia	6/94 (6.4%)	3/94 (3.2%)	10/100 (10.0%)	5/100 (5.0%)	p=.282
leukopenia	9/142 (6.3%)	1/142 (0.7%)	31/144 (21.5%)	1/144 (0.7%)	p<.0001
thrombocytopenia	3/140 (2.1%)	3/140 (2.1%)	1/144 (0.7%)	1/144 (0.7%)	p=.282
renal function	-	-	-	-	-
liver function	1/101 (1.0%)	-	2/137 (1.4%)	-	p=.99
Fever 38.0°C	15/123 (12.2%)		12/129 (9.3%)		p=.543
Leukopenic fever (Leuko<1.0 x 10^9/L, ≥38°C)	1		-		-
Chemotherapy related death	4 (2.5%)		1 (0.6%)		p=.371
non-hematologic					
nausea/vomiting	12/127 (9.4%)	2/127 (1.6%)	13/121 (10.1%)	2/121 (1.6%)	p=.848
alopecia	2/107 (1.9%)	-	27/109 (24.7%)	-	p<.0001
Infection	1/110 (0,9%)	1/110 (0.9%)	4/112 (3.6%)	1/112 (0.9%)	p=.448

Abbreviations: CM, cisplatin plus methotrexate; M-VEC, methotrexate, vinblastine, epirubicin and cisplatin.

The toxic effects classified as WHO grade 3 or higher that occurred among treated patients are listed in Table 3.3.

Progression-free survival

The median follow-up period for patients living free of progression was greater than 3 years. Tumor relapse was reported in 46.0% of the patients (75 of 163 patients) in the CM group and 44.5% of patients (73 of 164 patients) in the M-VEC group. The median duration of progression-free survival was 43.4 months in the CM arm and 49.7 months in the M-VEC arm by intent-to-treat analysis (Table 3.4). The 5-year progression-free survival rates were 46.3% in the CM treatment group and 48.8% in the M-VEC group (Fig. 3.2). The HR for disease progression in the CM group, as compared with the M-VEC group, was 1.13 (90% CI, 0.86 to 1.48; P=.0403). In the subgroup analyses of patients with lymph node positive disease, the median progression-free survival was 36.2 months (90% CI, 27.5 to 47.7 months) in the CM group and 32.4 months (90% CI, 24.8 to 42.3 months) in the M-VEC group. The 5-year progression-free survival rates of patients

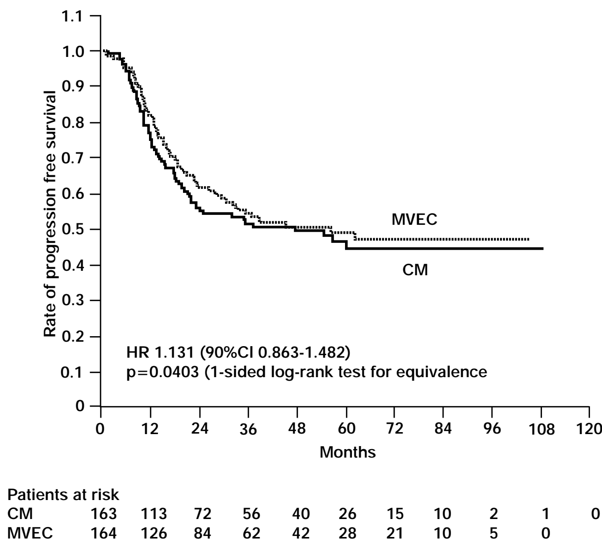
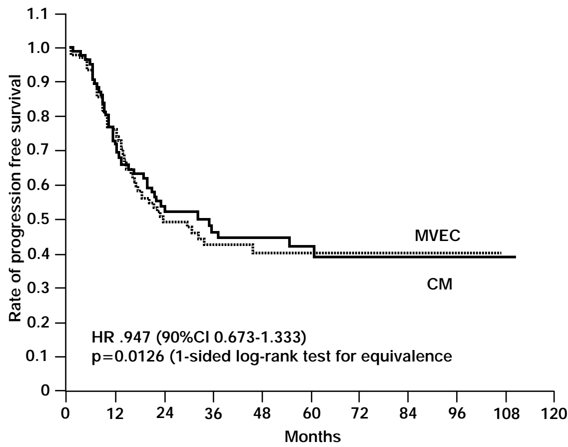
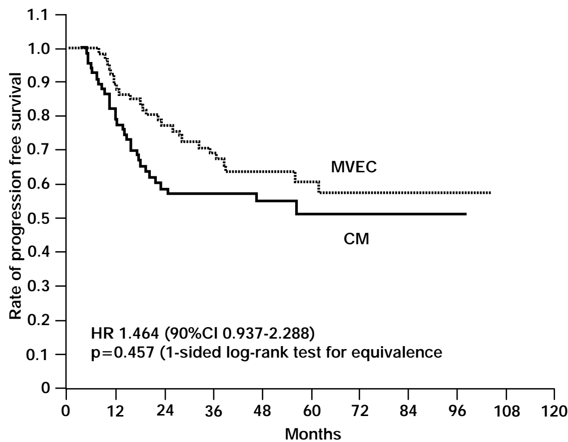


Figure 3.2. Progression-free survival among all eligible patients, according to treatment-group assignments. Abbreviations: HR, hazard ratio; CM, cisplatin plus methotrexate; M-VEC, methotrexate, vinblastine, epirubicin and cisplatin.



Patients at risk											
CM	92	60	36	35	18	14	9	5	1	1	0
MVEC	93	66	25	22	14	8	8	5	3	0	

Figure 3.3. Progression-free survival among all eligible patients with lymph node positive disease, according to treatment-group assignments. Abbreviations: CM, cisplatin plus methotrexate; M-VEC, methotrexate, vinblastine, epirubicin and cisplatin.



Patients at risk										
CM	71	53	36	31	22	12	6	5	1	0
MVEC	71	60	49	40	28	20	13	5	2	0

Figure 3.4. Progression-free survival among all eligible patients with lymph node negative disease, according to treatment-group assignments. Abbreviations: CM, cisplatin plus methotrexate; M-VEC, methotrexate, vinblastine, epirubicin and cisplatin.

Table 3.4a. Progression-free, Tumor-Specific, and overall survival analyses.

	CM (n=163)		M-VEC (n=164)
* Median time to progression [months (90%CI)]	43.4 (34.6-54.4)		49.7 (39.6-62.4)
Proprtional hazards regression			
Parameter estimate		.1232	
Standard error		.1648	
Hazard ratio (90%CI); p-value†		1.131 (.863-1.482), p=.0403	
Censored observations	54.0 % (n=88)		55.5 % (n=91)
	Estimated probability ± S.E. (%) of remaining event-free		
1-year progressionfree survival	76.4 ± 3.5		82.0 ± 3.1
2-year progressionfree survival	55.8 ± 4.1		61.7 ± 4.0
3-year progressionfree survival	51.5 ± 4.3		54.4 ± 4.2
5-year progressionfree survival	46.3 ± 4.6		48.8 ± 4.5
pN0 (n=142)	**CM (n=71)**		**M-VEC (n=71)**
* Median time to progression [months (90%CI)]	52.0 (36.5-74.2)		87.7 (59.8-128.6)
Proprtional hazards regression			
Parameter estimate		.3814	
Standard error		.2712	
Hazard ratio (90%CI); p-value†		1.464 (.937-2.288) p=.4571	
Censored observations	57.7 % (n=41)		64.8 % (n=46)
	Estimated probability ± S.E. (%) of remaining event-free		
1-year progressionfree survival	80.7 ± 4.8		89.6 ± 3.7
2-year progressionfree survival	58.7 ± 6.1		77.4 ± 5.1
3-year progressionfree survival	57.1 ± 6.1		69.1 ± 5.8
5-year progressionfree survival	51.5 ± 6.8		60.8 ± 6.5
pN+ (n=185)	**CM (n=92)**		**M-VEC (n=93)**
* Median time to progression [months (90%CI)]	36.2 (27.5-47.7)		32.4 (24.8-42.3)
Proprtional hazards regression			
Parameter estimate		-.0544	
Standard error		.2078	
Hazard ratio (90%CI); p-value†		0.947 (.673-1.333) p=.0126	
Censored observations	51.1 % (n=47)		48.4 % (n=45)
	Estimated probability ± S.E. (%) of remaining event-free		
1-year progressionfree survival	72.9 ± 4.8		76.2 ± 4.5)
2-year progressionfree survival	53.4 ± 5.6		49.2 ± 5.5)
3-year progressionfree survival	46.4 ± 5.9		42.6 ± 5.7)
5-year progressionfree survival	41.9 ± 6.1		40.1 ± 5.9)

* Parametric estimates based on an accelerated failure-time model with log-normal survival distribution.
† Log-rank test for one-sided equivalence (noninferiority).
Abbreviations: CM, cisplatin plus methotrexate; M-VEC, methotrexate, vinblastine, epirubicin and cisplatin.

Table 3.4b. Progression-free, Tumorspecific and overall survival analyses.

	CM (n=163)		M-VEC (n=164)
* Median tumorspecific survival [months (90%CI)]	57.3 (46.1-71.2)		63.3 (50.9-78.9)
Proprtional hazards regression			
Parameter estimate		.1232	
Standard error		.1776	
Hazard ratio (90%CI); p-value †		1.131 (.845-1.515); p=.0528	
Censored observations	60.1 % (n=98)		62.2 % (n=102)
	Estimated probability ± S.E. (%) of remaining event-free		
1-year tumorspecific survival	88.2 ± 2.6		92.3 ± 2.1
2-year tumorspecific survival	71.9 ± 3.8		71.5 ± 3.7
3-year tumorspecific survival	58.2 ± 4.3		63.3 ± 4.1
5-year tumorspecific survival	52.0 ± 4.6		52.3 ± 4.8
* Median overall survival [months (90%CI)]	47.1 (38.1-58.3)		51.8 (41.9-64.1)
Proprtional hazards regression			
Parameter estimate		.0979	
Standard error		.1602	
Hazard ratio (90%CI); p-value †		1.103 (.877-1.435); p=.0255	
Censored observations	51.5 % (n=84)		53.0 % (n=87)
	Estimated probability ± S.E. (%) of remaining event-free		
1-year overall survival	83.8 ± 2.9		88.3 ± 2.5
2-year overall survival	66.4 ± 3.8		67.1 ± 3.8
3-year overall survival	51.7 ± 4.2		56.4 ± 4.1
5-year overall survival	46.1 ± 4.3		45.1 ± 4.6

* Parametric estimates based on an accelerated failure-time model with log-normal survival distribution.
† Log-rank test for one-sided equivalence (noninferiority).
Abbreviations: CM, cisplatin plus methotrexate; M-VEC, methotrexate, vinblastine, epirubicin and cisplatin.

with lymph node-positive disease were 41.9% in the CM group and 40.1% in the M-VEC group (Fig. 3.3). The HR for disease progression in the CM group, as compared with the M-VEC group, was 0.95 (90% CI, 0.67 to 1.33; P=.0126).

In the analyses of patients with tumor extension beyond the bladder wall but without lymph node involvement the median progression-free survival was 52.0 months (90% CI, 36.5 to 74.2 months) in the CM arm and 87.7 months (90% CI, 59.8 to 128.6 months) in the M-VEC arm. The 5-year progression-free survival rates were 51.5% in the CM group and 60.8% in the

M-VEC group (Fig. 3.4). The HR for disease progression in the CM group, as compared with M-VEC group, was 1.46 (90% CI, 0.94 to 2.29; P=.4571).

Tumorspecific and overall survival

Sixty-five patients in the CM treatment group (39.9%), and 62 patients in the M-VEC treatment group (37.8%) died of tumor disease during the follow-up period.

The median duration of tumorspecific survival was 57.3 months in the CM group and 63.3 months in the M-VEC group (Fig. 3.5). The HR for tumor-related death in the CM group, as compared with the M-VEC group, was 1.13 (90% CI, 0.84 to 1.51; P=.0528).

The 5-year rates of tumor-specific survival were 52.0% in the CM group and 52.3% in the M-VEC group (Table 3.4, Fig 3.5). In the CM and M-VEC arms, 14 patients (8.6%) and 15 patients (9.1%) died as a result of causes not related to tumor, respectively. The median duration of overall survival was 47.1 month in the CM group and 51.8 months in the M-VEC group (Table 3.4, Fig. 3.6). The HR for death irrespective of causes in the CM

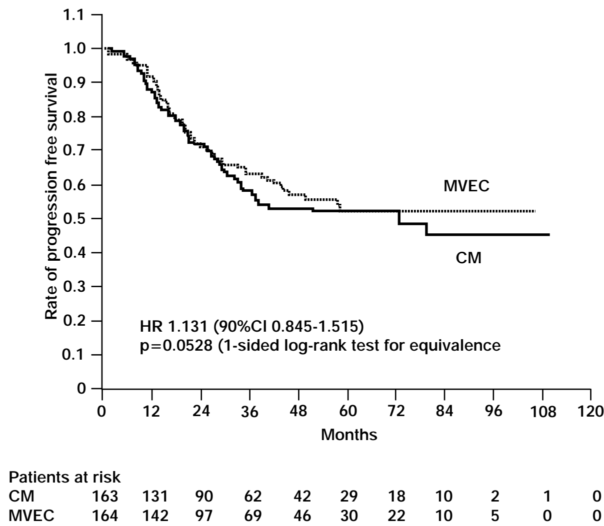


Figure 3.5. Tumorspecific survival among all eligible patients, according to treatment-group assignments. Abbreviations: CM, cisplatin plus methotrexate; M-VEC, methotrexate, vinblastine, epirubicin and cisplatin.

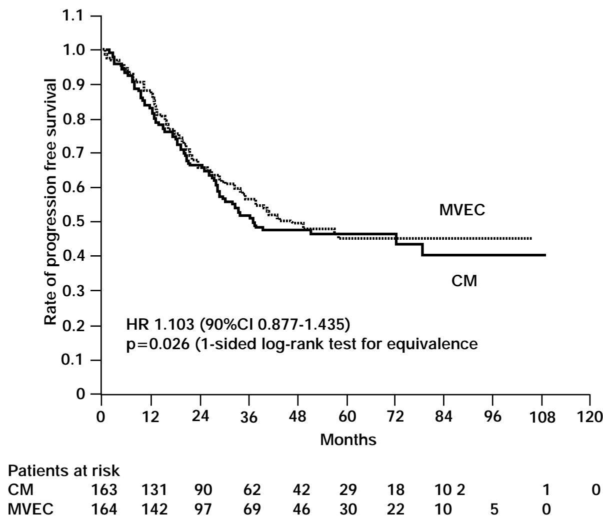


Figure 3.6. Overall survival among all eligible patients, according to treatment-group assignments. Abbreviations: HR Hazard ratio; CM, cisplatin plus methotrexate; M-VEC, methotrexate, vinblastine, epirubicin and cisplatin

group, as compared with the M-VEC group, was 1.10 (90% CI, 0.88 to 1.44; P=.0255). The 5-year rates of overall survival were 46.1% in the CM group and 45.1% in the M-VEC group.

Site of Tumor Relapse

Classification of first relapses with regard to site resulted as follows (Table 3.5). Local recurrence only occurred in 12 patients in the CM group (7.3%) and seven patients in the M-VEC group (4.3%). Lymphatic relapse only, which was typically encountered as retroperitoneal lymph node disease, was reported in 4.3% of CM patients (seven of 163 patients) and 6.1% of M-VEC patients (10 of 164 patients). Visceral metastases (without bone metastases) were found in 20.2% in the CM group (33 of 163 patients) and 15.9% of patients in the M-VEC group (26 of 164 patients); at least 12.3% of CM patients (20 of 163 patients) and 15.2% of M-VEC patients (25 of 164 patients) developed bone metastases.

Table 3.5. Sites of Relapse.

	No. of Relapses*		
Sites of Relapse*	***CM (n=75)***	***M-VEC (n=73)***	***Total (n=148)***
Local	18	12	30
Nodal	29	20	49
Visceral	33	26	59
Osseous	20	25	45

Abbreviations: CM, cisplatin plus methotrexate; M-VEC, methotrexate, vinblastine, epirubicin, and cisplatin.

* Because patients could have relapse at multiple sites, the total number of relapses is greater than the number of patients in each group who experienced relapses.

** Sites of relapse were classified as local if tumor was detected in the region of the excised bladder, as nodal if tumor was detected in regional or distant lymph nodes, as osseous if metastasis was present in bone, and as visceral if metastasis was present in other organs.

Discussion

The frequent occurrence of tumor relapses after radical cystectomy for locally advanced bladder cancer, the favorable results of three former adjuvant trials[2-4], and the limited tolerability of adjuvant M-VEC or M-VAC combination chemotherapy provided the rationale for the present study. After introducing effective M-VAC combination chemotherapy in urothelial cancer in 1985[5], two successive, randomized, phase III trials demonstrated the superiority of the M-VAC regimen in advanced urothelial cancer compared to single-agent cisplatin[7, 12] or with cisplatin, cyclophosphamide, and doxirubicin combination therapy.[6] Because of the pronounced hematologic and non-hematologic toxicity with M-VAC or M-VEC combination therapy, other potentially less toxic combination therapies, such as CM have been investigated in advanced urothelial cancer.[13,14] Neoadjuvant administration of chemotherapy has been exceedingly investigated compared with the adjuvant approach for patients with a high risk of relapse undergoing definite treatment by surgery or radiation. Although the majority of individual neoadjuvant studies was not able to demonstrate a significant survival advantage for pre-emptive chemotherapy compared with definitive treatment alone, a recent meta-analysis based on individual patient data from 10 randomized neoadjuvant trials was able to demonstrate a significant 13% reduction in the risk of death (HR of 0.87; 95% CI, 0.78 to 0.98, P=.016), which was equivalent to a 5% absolute overall survival benefit at 5 years from 45% to 50%.[1] This meta-analysis also stated that

a significant advantage exists using platinum-based combination chemotherapy compared with single-agent platinum. The most recent neoadjuvant study, which was not included in the former meta-analysis, achieved a survival advantage with borderline significance for 154 patients assigned to three cycles of neoadjuvant M-VAC combination therapy versus 153 patients randomly assigned to surgery alone (median survival, 77 months in the neoadjuavant arm *v* 46 months in the surgery-only arm; P=.06).[15]
Whether neoadjuvant or adjuvant application of systemic chemotherapy for locally advanced bladder cancer is the preferable treatment modality remains a matter of debate.
To date, only one study has investigated the neoadjuvant and adjuvant treatment strategy directly.[16] Similar to our experience, 40% of patients in this trial with histologically proven regional lymph node metastasis experienced long-term progression-free survival in both treatment arms.
In contrast to neoadjuvant administration of chemotherapy, proponents of adjuvant chemotherapy for locally advanced bladder cancer emphasize optimal patient selection based on exact assessment of histopathological tumor stage. This allows the selection of patients with the highest risk for relapse, and only minimal occult tumor burden after radical cystectomy needs to be treated. Subsequently, improvement of recurrence-free survival by adjuvant combination chemotherapy was corroborated by three individual randomized controlled studies from Germany and the United States for the adjuvant administration of combination chemotherapy after radical cystectomy.[2-4]
Skinner *et al.* randomly assigned 91 locally advanced bladder cancer patients to radical cystectomy plus four cycles of adjuvant chemotherapy (predominantly cisplatin, cyclophosphamide, and doxorubicin combination therapy) versus surgery alone.[2] The trial was stopped because of an interim analysis that demonstrated a significant disease-free survival advantage for the adjuvant treatment arm 5 years after cystectomy (51% for adjuvant chemotherapy *v* 34% for surgery alone, P<0.011). Stöckle *et al.*[3] terminated their study after an interim analysis of 49 patients which demonstrated a significant disease-free survival difference of 50% at 3.5 years in favor of 26 patients receiving adjuvant treatment with M-VAC or M-VEC compared with the control group (63% for adjuvant chemotherapy *v* 13% for cystectomy alone, P=.0005).
A third randomized trial with a favorable result for adjuvant combination chemotherapy performed at Stanford University compared adjuvant cisplatin, methotrexate, and vinblastine combination chemotherapy to cystectomy alone.[4] In contrast to the former two studies, deferred chemother-

apy on progression was suggested by the protocol for patients assigned to the nonadjuvant arm. This study was also closed before accrual of the preplanned number of patients because of a significant difference in progression-free survival in favor of patients receiving adjuvant chemotherapy. Median time to progression for the 25 patients assigned to the adjuvant treatment arm was 37 months *v* 12 months in the cystectomy-alone group ($P < .01$), whereas overall survival was not significantly different. Criticisms of all three former trials have been summarized by Sylvester and Sternberg[17] and address small sample size, early stopping of patient entry and premature closure, statistical analyses, reporting of results and drawing conclusions.

Adjuvant chemotherapy trials for invasive bladder cancer that did not find a significant difference for patients receiving adjuvant chemotherapy compared with a surgery-only group have been reported from Switzerland[18], Italy[19], and Germany.[20] This may be attributed to the exclusion of lymph node-positive patients[19] and to the administering of only single-agent cisplatin as adjuvant treatment to a predominantly pT2pN0 population.[18]

Only limited information on the degree of toxicity has been revealed from any of these adjuvant trials. Reports on palliative M-VAC or M-VEC combination chemotherapy causing grade 3 to 4 myelosuppression in more than half of the patients, a 25% incidence of nadir sepsis, and drug-related deaths in at least 3% of patients[21] prompted investigators to search for more tolerable but still active regimens. Efficacy of CM combination therapy as implemented for the current trial had been reported previously for 53 patients with advanced urothelial cancer.[13] Although the majority of patients had dose omissions or delays, an overall response rate of 46% was noted. The standard M-VEC treatment arm of the current trial incorporated epirubicin instead of doxorubicin, as in the original M-VAC schema, because cardiotoxicity was of concern at the time of drafting the protocol.

Because of the nature of the current trial testing for noninferior efficacy of the reduced combination (CM) compared with the full combination (M-VEC), the results may only lend support to but not prove the superiority of adjuvant combination chemotherapy compared with surgery alone. Remarkably, the 5-year progression-free survival rate of greater than 40% for lymph node-positive disease after radical cystectomy plus adjuvant combination chemotherapy (CM or M-VEC) represents an average standard of care of 40 uro-oncologic centers in Germany. Retrospective studies of patients with lymph node-positive bladder cancer receiving adjuvant chemotherapy after radical cystectomy versus surgery alone reported significant 5-year overall survival advantages of 43% *v* 17% ($P = .03$)[22] and 45% *v* 21% ($P = .031$)[23] for the adjuvant group, respectively. A similar sur-

vival comparison from the Mayo Clinic demonstrated a 5-year tumor-specific survival advantage of 55% *v* 32% ($P = .005$) in favor of the adjuvant chemotherapy group compared with surgery alone, respectively,[24] notwithstanding the limitation that any retrospective result may be biased by patient selection. Finally, the recurrence-free survival rate of 41% at 5 years for all lymph node-positive patients ($n = 185$) in our trial is slightly higher than 39% rate that was recently reported from another North American center of excellence administering adjuvant chemotherapy to 139 patients with lymph node-positive disease.[25]

When assessing the possible effects of baseline factors (eg, age, sex, tumor stage, blood cell counts, renal and liver function, and size of recruiting center) on the risk of progression using multivariate Cox regression analysis, the only significant predictor was the number of tumor positive lymph nodes (data not shown). The current trial did not control for either the number of lymph nodes removed during radical cystectomy or the extent of pelvic lymphadenectomy because patients were usually identified postoperatively to be eligible for this trial. Therefore, a specific surgical and histopathological protocol could not be required. Some authors have suggested a survival improvement for patients undergoing extensive lymphadenectomy with cystectomy in a retrospective survey,[25-27] whereas others have seen a significant survival advantage with extensive lymphadenectomy only in patients with organ confined (≤pT2) bladder cancer.[28] Future trials on adjuvant chemotherapy for locally advanced bladder cancer may focus on this issue with more precision.

As previously reported for the palliative indication of combination chemotherapy in unresectable advanced urothelial cancer, the gold-standard status of M-VAC therapy has declined.[29] Results of a large, multinational, phase III trial comparing M-VAC with gemcitabine plus cisplatin combination therapy showed similar response rates and comparable overall survival in 405 patients. Although patient numbers and study design of this trial did not allow for the testing of significant noninferiority, gemcitabine plus cisplatin has become a well-established regimen for the treatment of advanced bladder cancer because of significantly less clinically relevant toxicity and better cost effectiveness.

The implications of our results must be assessed with a view to the equivalence margin of 0.15, which had been specified in the protocol for the difference between the respective survivor functions at any point of time. Thus, the major statistical hypothesis that could be established by means of our data does not rule out the possibility that the full combination therapy comprising all four drugs is more efficient than the reduced

chemotherapy regimen. But we can be certain that this advantage, which is supported by the positive signs of the point estimates obtained from the total samples, fails to be of significant clinical relevance. For the adjuvant indication of combination chemotherapy with curative intent in resected locally advanced bladder cancer, the current report provides evidence that efficacy of the less toxic regimen CM cannot be considered substantially inferior to the efficacy of M-VEC.

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Appendix

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Secretariat - M.Stöckle (principal investigator), S.Wellek (biostatistician), J.Lehmann, M.Retz, C.Wiemers (data managers). Investigators (the number of patients enrolled is shown in parentheses): C.Wiemers, D.Sternberg, L.Franzaring, M.Stöckle, J.Thüroff, J.Beck, C.Huber, Johannes-Gutenberg-Universität, Mainz (37); C.Weining, H.J.Piechota, L.Hertle, Universitätsklinikum Münster (32); G.Steiner, P.Albers, S.C.Müller, Universitätsklinikum Bonn (28); C.Vierneisel, A.Göll, M.Beitzinger, D.Frohneberg, Städtisches Klinikum, Karlsruhe (26); T.Becker, T.Kälble, H.Riedmiller, Philipps-Universität, Marburg (22); H.J.Knopf, G.Engelhardt, P.-J.Funke, Ev.-Jung-Stilling Krankenhaus, Siegen (21); J. Roloff, T.Schönfeld, P.Walz, Klinikum Lüdenscheid (14); C.Wiemers, M.Retz, J.Lehmann, A.Bannowsky, M.Stöckle, Christian-Albrechts-Universität, Kiel (13); S.Langbein, F.Reiher, E.P.Allhoff, Otto-von-Guericke-Universität, Magdeburg (13); M.Schiller, D.Sternberg, G.Hutschenreiter, Evangel. und Johanniter Klinikum, Oberhausen (13); M.Staehler, M.Müller, K.Miller, Universitätsklinikum Benjamin-Franklin, Berlin (12); U.Gertenbach, U.Kaldenbach, J.Thüroff, S.Roth, Universität Witten-Herdecke, HELIOS Klinikum Wuppertal (11); S.Tedsen, A.Böhle, D.Jocham, Universität Lübeck (7); W.Kieser, H.Riedmiller, Julius-Maximilian-Universität (6); G.M.Wacker-Backhaus, G.Ostendorf, C.Ehlert, H.Becker, Marienkrankenhaus, Hamburg (6); L.Weissbach Krankenhaus am Urban, Berlin (6); C.Charvalakis, L.Lampante, Städtisches Krankenhaus Kemper Hof, Koblenz (6); V.Poulakis, E.Becht, S.Al-Batran, A.Knuth, E.Jaeger, Krankenhaus Nordwest (6); R.Groh, R.Horsch, Kreiskrankenhaus Offenburg (5); N.Vennemann, R.G.Anding,

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CHAPTER 4

ALTERATION OF THE LRP1B GENE REGION IS ASSOCIATED WITH HIGH GRADE OF UROTHELIAL CANCER

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Abstract

We have delineated regions of interest at chromosome 2q21.2, 2q36.3, and 2q37.1 by deletion mapping of 114 urothelial cancers (UC). Altogether, 17%, 18%, and 63% of the G1, G2, and G3 tumors displayed loss of heterozygosity at chromosome 2q, respectively. The region at 2q21.2 was narrowed down to the LRP1B gene (NT_005129.6). Hemi- and homozygous deletion at the LRP1B gene region was seen in 31 of 114 UCs. Only 8% of the UCs with G1 and none with G2 tumors showed loss of heterozygosity at the LRP1B gene, whereas 49% of the G3 UCs had allelic loss at this region. RT-PCR analysis of the LRP1B gene showed the lack of expression of several exons in 2 of 9 cases analyzed. Our analysis suggests that the LRP1B gene is a candidate tumor suppressor gene in UCs.

Introduction

Cancer of the urinary bladder is one of the most common tumors in the Western world. The majority of urothelial cancers (UC) are diagnosed as noninvasive tumors (Ta), whereas 20% to 25% of the cases show an invasive growth (T1-4) at the time of first presentation. From the clinical point of view, the question arises whether these two major groups of tumors are distinct entities or correspond to different stages of progression of a single tumor entity. Pioneering cytogenetic analyses before the chromosome banding era have suggested that the number of gross karyotype alterations predicts the clinical course of UCs, for example, recurrency and progression.[1, 2] Later, several studies showed that allelic changes at specific chromosomal regions and alterations of tumor suppressor genes, such as *PTEN*, *RB*, and *TP53* correlate with stage and grade of bladder cancers.[3]
Comparative genomic hybridisation (CGH) studies also suggested quantitative differences of genetic changes, including DNA losses at chromosome 2q22-33, 2q32-qter, and 2q34-qter regions, between the noninvasive and invasive bladder cancers.[4, 5, 6] Loss of heterozygosity (LOH) at chromosome 2q is also associated with aggressive growth of head and neck and non-small cell lung carcinomas.[7, 8] Recently, Liu *et al.*[9] identified a putative tumor suppressor gene *LRP1B* from the chromosome 2q21.2 region that was found to be homozygously deleted in several cancer cell lines, including the bladder cancer cell line VM-CUB-2.
To delineate putative tumor suppressor gene regions, we analysed 114 UCs for 20 microsatellite loci at the chromosome 2q including those from the *LRP1B* region. We identified three distinct regions of LOH in 40% of tumors and found a correlation between LOH at chromosome 2q and tumor grade.

Materials and Methods

Tumor Samples and DNA Extraction

Fresh tumor tissues were obtained by transurethral resection or radical cystectomy at the Departments of Urology, Philipps-University Marburg and Mannheim Clinic, Ruprecht-Karls-University Heidelberg. All tumors were histologically reevaluated according to the World Health Organisation Classification[10] by two of the authors (GK, ALB). This study comprised 54, 20, and 57 tumors of pathologic Stage Ta, T1, and T2-4, respectively. Grades 1, 2, and 3 were identified in 35, 22, and 57 cases, respectively (Table 4.1). A part of the tumor tissue was immediately snap-frozen in liquid nitrogen and stored at -80°C. A frozen tumor sample was placed on a plastic Petri dish, covered with 1ml TE9 buffer, and allowed to thaw. The tumor cells were then carefully scraped off the transurethral resection samples and pushed out from cystectomy specimens under an inverted microscope by a pathologist (GK) experienced in this technique. By this method, the contamination with normal cells was reduced to a minimum. Afterwards, the tumor cells were resuspended in 2 to 5ml TE9 buffer with 1% SDS and 0.2mg/ml proteinase K and were incubated for 8 to 12 hours at 55°C. DNA was extracted by phenol-chloroform and dissolved in TE buffer after ethanol precipitation. Normal control DNA was extracted from blood lymphocytes by the same method.

Microsatellite Analysis

Microsatellite markers used in this study are shown in Figure 4.1. The sequences and location of the markers were obtained from the Genome Database (http://gdbwww.gdb.org/gdb/gdbtop.html) and from the Whitehead Institute for Biomedical Research (http://www-genome.wi.mit.edu/). Primer sequences of the tetranucleotid repeat tetra30 were described by Liu *et al.*[9] The precise location of microsatellites at and around the *LRP1B* gene was obtained from the sequence segments NT_029242.1 and NT_005129.6, the latter containing the *LRP1B* gene (http://ncbi.nlm.nih.gov). Matched normal/tumor DNA samples were amplified in 10-μl reactions with 50 ng genomic DNA, 50mM KCl, 10mM Tris-HCl, pH 8.3, 1.5mM $MgCl_2$, 200 μM each dNTP, 5pmol Cy5-labeled forward primer, 5pmol reverse primer, and 0.5U *Taq* DNA polymerase (Gibco BRL, Eggenstein, Germany). After 2 minutes of denaturation at 94°C, the PCR mixes were subjected to the following conditions: 40 seconds at 94°C, 30 seconds at 55°C and 40 seconds at 72°C for 28 cycles, with a delayed last elongation step for 10 minutes at 72°C in a PTC200 thermal cycler (MJ Research, Watertown, Massachusetts). Before loading, 20 μl stop

solution of 50mM EDTA and 5mg/ml Dextran Blue 2000 in 100% deionized formamide were added and the samples were denaturated at 95°C for 2 minutes and immediately cooled on ice for 1 minute. Analysis was carried out on an automated DNA analysis system (ALFexpressII, Amersham/ Pharmacia Biotech, Freiburg, Germany). The 6% denaturing polyacrylamide gels (acrylamide:bisacrylamide=19:1) were run at 400V, 55mA, 30W in 1xTBE buffer at a constant gel temperature of 55°C. The collected raw data were evaluated using the Fragment Manager (FM 1.2) software (Amersham/Pharmacia Biotech).
Allelic changes were evaluated according to our score system. Briefly, the complete lack of signal at one allele indicates that each tumor cell has lost one allele and that the tumor DNA is not contaminated with normal DNA. This change is scored as 4. The reduction of signal of one allele to approximately 50% of the corresponding allele in normal tissue was scored as 2, which may correspond to the loss of one allele in approximately 50% of the tumor cell population or to a trisomy, eg, duplication of one allele resulting in a 1:2 allelic ratio. Retention of the normal allelic status in the tumor cells was scored as 0. Allelic status between 0 and 2 is scored as 1, whereas status between 2 and 4 is scored as 3. Because minimal differences between signals at alleles in normal and tumor tissues may occur due to PCR conditions, we did not evaluated a single score 1 as an allelic imbalance. In cases where most loci were scored 4 along the entire chromosome, the remaining few score 3s were changed to score 4. Thus, at the final evaluation we only used scores 4, 2 and 0. We detected score 4 at least once in each tumor, thereby asserting the purity of the analyzed tumor samples (not shown).

RT-PCR Analysis of the Genes LRP1B and CUL3

Total RNA was extracted from urothelial carcinomas of the bladder and normal bladder epithelium after their homogenisation with Trizol (Life Technologies, Rockville, Maryland) solution according to the manufacturer's instruction. The integrity of the RNA was checked on a 1% formaldehyde/agarose gel. One μg of total RNA was reverse transcribed for 2 hours at 42°C in 10-μl volumen using 200 units of MMLV-RT (Promega, Mannheim, Germany). After heat-inactivating the enzyme for 5 minutes at 94°C, 10 μl RNAse-free dH_2O were added to the cDNA solutions. Two microliters of cDNA were used as template for each subsequent 20 μl PCR-reaction. Exon primers 1, 8, 10, and 29 of the LRP1B gene and the control LRP1 primers were used, as described by Liu *et al*[9]. The primers for CUL3 were: CAA CAT CCC ACC AGC ACC AAG (CUL3-F) and

ACT GCT GAG TTA CCT CCG CTA C (CUL3-R). The PCR program consisted of an initial denaturation step for 2 minutes at 94°C, and then by 40 cycles of 1 min at 94°C, 1 min at 68°C, and 2 min at 72°C, finishing with 10 min at 72°C. For exon 29 the annealing temperature was reduced to 64°C and for CUL3 to 60°C. Ten microliters of the PCR products were run on a 1% agarose gel and stained with ethidium bromide.

CGH

DNA of a healthy male donor (reference DNA) was labelled with digoxigenin-11-dUTP, and DNA from 18 UCs were labeled with biotin-16-dUTP (Roche Diagnostics, Mannheim, Germany) using standard nick translation protocols.[11] CGH analysis was performed as described previously with minor modifications.[12] Image acquisition, processing, and evaluation were performed using a Leica DM RXA RF8 epifluorescence microscope (Leica, Bensheim, Germany) equipped with a Sensys CCD camera (Photometrics, Tucson, Arizona, Kodak KAF 1400 chip) controlled by the Leica Q-FISH software (Leica Microsystems Imaging Solutions, Cambridge, United

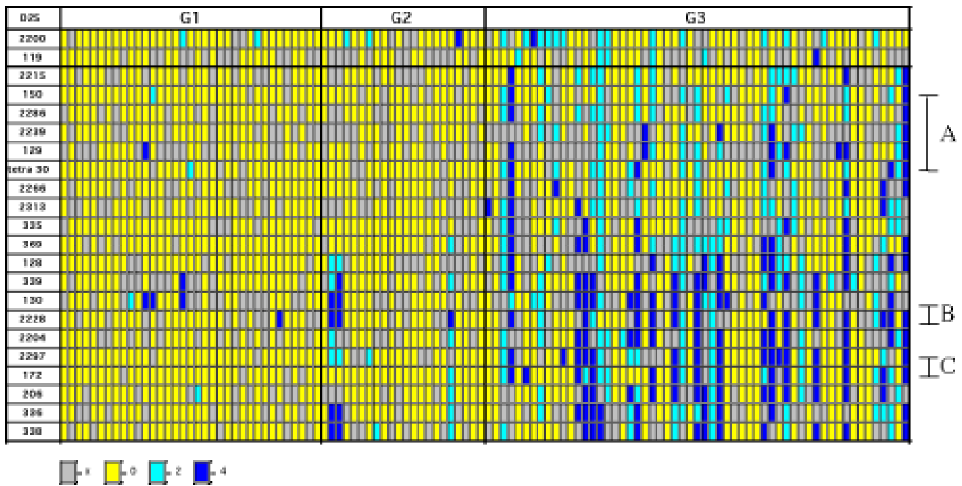

Figure 4.1. Summary of microsatellite analysis of 114 bladder cancers. Microsatellite loci are listed on the left side. Retention of heterozygosity is marked with yellow, allelic imbalance with light blue, loss of heterozygosity with dark blue and noninformative loci with grey. The three regions of interest (**A** to **C**) are shown on the right side. Only five of the G1 urothelial tumors showed allelic changes at 2q, whereas loss of heterozygosity at these regions occurred at high frequency in G3 cancers.

Kingdom). Three-color images, green for the tumor DNA, red for the reference DNA, and blue for the DAPI counterstain were acquired from 10 metaphases per sample. Images were processed using the Leica Q-CGH software. The threshold values for detection of genomic imbalances were 0.75 for losses and 1.25 for gains, respectively.

Results

Three Target Regions of Allelic Loss at Chromosome 2q

CGH analysis of 18 Grade 3 (G3) UCs of this series revealed a gain at chromosome 2p in 7 cases and loss of DNA at chromosome 2q in 11 cases (one example is shown in Fig. 4.2). Therefore, we evaluated score 2 at chromosome 2p as a duplication of one allele, but at chromosome 2q as a loss of heterozygosity (LOH) occurring in mosaic form. Allelic changes at chromosome 2p were detected in 23 (20%) of 114 UCs. The deletion mapping delineated three regions of interest at chromosome 2q (Fig. 4.1). Region A was narrowed down between loci D2S150 and tetra30 encompassing loci D2S2286, D2S2239, and D2S129, which are localized to the 1,230 kb genomic region of the *LRP1B* gene (NT_005129.6). Region B is localized to an approximately 1.5-Mb genomic segment between loci D2S130 (NT_005126.5) and D2S2228 (NT_005106.6), including the *CUL3* gene. Region C is mapped between loci D2S2297 (NT_005403.6) and D2S172 (NT_026242.3), which spans approximately a 1.8-Mb genomic segment. Altogether, LOH involving at least one of these regions was seen in 46 (40 %) of the 114 bladder cancers.

LOH at 2q is Associated with High Grade of UCs

Alterations at regions A, B and C occurred with increasing frequency in tumors from Stage Ta through T1 to T2-4 (Table 4.1). The correlation between stage and LOH was similar for all regions (data not shown). Altogether, LOH at chromosome 2q occurred in 20%, 60%, and 57% of Ta, T1, and T2-4 UCs, respectively.

Evaluation of the genetic alterations at region A regarding the grade of tumors showed a cut off between G1-G2 versus G3 tumors. Only three of 35 G1 UCs and none of the 22 G2 tumors showed LOH at the *LRP1B* gene. In contrast, 28 of 57 G3 tumors showed allelic loss at this region. No LOH was found at region C and D in G1 tumors, whereas G2 and G3 UCs showed LOH in 14% to 18% and 44% to 49% of the cases, respectively. Altogether, 17% and 18% of the G1 and G2 tumors displayed LOH at chromosome 2q, compared with 63% LOH in G3 UCs (Fig. 4.1; Table 4.1).

Table 4.1. Loss of Heterozygosity at Chromosome 2q and Stage or Grade of Urothelial Cancer.

	Tumor stage			
Grade	pTa	pT1	pT2-4	Total[a]
1	6/34	-/1	-	6/35(17)
2	3/17	1/4	-/1	4/22(18)
3	2/3	11/15	23/39	36/57(63)
Total*	11/54(20)	12/20(60)	23/40(57)	46/114(40)

[a] Numbers in brackets indicate the percentage of allelic changes.

Involvement of the LRP1B Gene

LOH including the *LRP1B* gene region was seen in 31 of 114 UCs, including 21 cases with partial deletions occurring exclusively at the *LRP1B* gene. The 91 exons of the *LRPB1* gene are distributed along a 1,230kb genomic region between position 229kb and 1,459kb in the sequence segment NT_005129.6. Loci D2S2286, D2S2239, and D2S129 are mapped to positions 604kb, 966kb, and 1,172kb, respectively, therefore being in the *LRPB1* genomic region. The loci tetra30 and D2S2266 are mapped to posi-

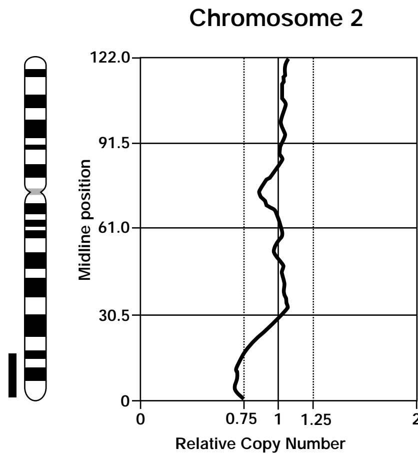

Figure 4.2. Comparative genomic hybridisation analysis of a urothelial carcinoma showing DNA loss at chromosome 2q33-qter.

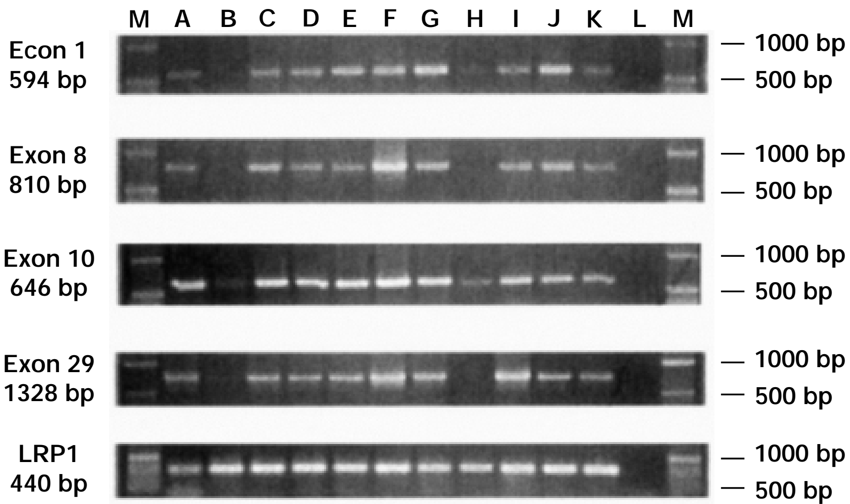

Figure 4.3. RT-PCR analysis of the 5′-end, the middle part, and 3′-end of the coding sequences of the LRP1B gene in normal urothels (J, K) and urothelial cancers (A to I). Note the lack of signal in tumors B and H.

tion 1,797kb and 1,837kb, respectively, which are outside the *LRP1B* region. Locus D2S2313 is localized approximately 400kb distally (NT_005102.6) and locus D2S150 is mapped about 270 kb proximally to the *LRP1B* gene region (NT_029242.1). The LOH data indicate clearly that a hemizygous deletion affects the *LRP1B* gene region in 27 cases. Allelic imbalance was detected at all informative loci along the chromosome 2q excepting of loci D2S2286, D2S2239, and tetra30 in one, one, and two UCs, respectively. These data indicate a possible homozygous deletion at these loci. The results of the RT-PCR analysis in primary UC tissues also indicate the involvement of *LRP1B* in UC. Although the *LRP1* gene showed a strong signal in all normal urothelial and tumor samples, the PCR product from *LRP1* exons 1, 8, 10, and 29 was missing in tumor 157 and exons 1, 8, and 29 in tumor 101 (Fig. 4.3). RT-PCR analysis showed expression of the *CUL3* gene in all normal and tumor tissues (not shown).

Discussion

We delineated three regions of LOH at chromosome 2q21.2, q36.3, and q37.1 by a detailed microsatellite analysis of 114 UCs. The region at chro-

mosome 2q21.2 was restricted to the *LRP1B* gene. Lisitsyn *et al* found homozygous deletion within this region in cell lines established from distinct types of tumors including bladder cancer.[13] Recently, these authors have detected homozygous deletion at one or more exons of the *LRP1B* gene in several lung cancer cell lines.[9] Our data showed that in UCs, a hemizygous deletion is the most common genetic alteration within the *LRP1B* gene region, but in some cases a homozygous deletion may occur. The RT-PCR analysis of the 5′-end, the middle part, and the 3′-end of the coding sequences of the *LRP1B* gene indicated the lack of transcripts in some UCs. Because the large number of exons of the *LRP1B* gene we did not sequence genomic DNA for mutation analysis. The genetic data and pathological parameters suggests that inactivation of the *LRP1B* gene is associated with high grade, invasive growing UCs.
Region B harbours the *CUL3* gene (a member of the cullin/Cdc53 protein family), and five hypothetical proteins (KIAA0694, FLJ20220, FLJ22746, FLJ12701, and KIAA1435). Overexpression of the CUL3 increases the ubiquitination of cyclin E, whereas deletion of the CUL3 leads to an accumulation of the cyclin E protein in cells and consequently to a high proportion of cells in the S-phase.[14] CUL3 is ubiquitously expressed in normal and UC cells and likely another gene might be the target of LOH at this region. Region C harbours the *SP140* gene (nuclear body protein Sp140), the hypothetical genes LOC2737, LOC92739, LOC116397, and LOC82459, as well as the hypothetical protein FLJ20701. No data are available on these genes in bladder cancer.
Previously, CGH analyses revealed chromosome 2q alterations in 3% to 9% of UCs with Stage Ta tumors[5, 15, 20], and in 17% to 36% of the T1 tumors.[5, 15, 16, 20] Based on CGH data, a major genetic difference between the noninvasive Ta and invasive T1-4 tumors, as well as a high degree of genetic instability in invasive tumors, was suggested[17]. Although we found a correlation between LOH at chromosome 2q and tumor staging, our study clearly shows that alteration of chromosome 2q is primarily associated with the grade of UCs. We found a cutoff between G1-G2 versus G3 UCs of the bladder. The higher frequency of LOH in invasive UCs (T1-4) is associated with the high proportion of G3 tumors in this group as shown in Table 4.1. Previously, we obtained a similar correlation between LOH and grade of bladder cancer by analysing the chromosome 8p23.3 and chromosome 5q regions.[18, 19]
The stage, eg, the invasive growth, of a malignant tumor relies on the interaction between tumor cells and their local environment, as well as on the host reaction against the tumor cells. The grading system reflects the proliferative activity of tumor cells themselves. Our allelotyping study sug-

gests that loss of chromosome 2q, especially at the *LRP1B* gene, is primarily associated with the malignant proliferation of urothelial cells. Identifying relevant genes from the target regions, such as the gene *LRP1B*, will be helpful in molecular grading of UCs.

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CHAPTER 5

FLUORESCENCE IN SITU HYBRIDIZATION
A MULTITARGET APPROACH IN DIAGNOSIS AND MANAGEMENT OF UROTHELIAL CANCER

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Abstract

Noninvasive tests for detecting genetic or molecular alterations in urine indicative of urothelial cancer are increasingly becoming the focus of urological cancer research. Since its approval by the US FDA in 2001, the fluorescence in situ hybridization test (Vysis® UroVysion™) has been widely evaluated. In general, published data demonstrate better sensitivity and equal or better specificity compared with routine cytology, which is still considered the ´gold standard´ in diagnosing and monitoring bladder tumors. However, the fluorescence *in situ* hybridization test seems to provide not only a useful tool in bladder cancer detection, but also in diagnosis of upper urinary tract tumors, surveillance and determining therapy effectiveness. This multitarget assay that detects four different chromosomal aberrations in tumor cells is a kind of objective molecular cytology and has proven advantages over routinely used cytology.

Introduction

Bladder cancer is the fourth most common malignancy among men, with an estimated 61,420 new cases and 13,060 deaths in the USA in 2006 alone.[1] More than 90% of bladder tumors are urothelial cell carcinomas (UCCs). The incidence of bladder tumors is increasing, and consequently, the financial burden of its diagnosis, treatment and follow-up. An analysis of US Medicare payments shows that bladder cancer entails a very high per-patient cost from diagnosis to death due to frequent treatment, intensive surveillance and long-term survival. In fact, owing to the lifelong need for monitoring and treatment for recurrent tumors, the cost per patient with bladder cancer is the highest among all cancers.[2]

Urothelial cancer is a heterogeneous disease with a variable natural history. The majority of tumors present initially as superficial non muscle-invasive tumors (Ta, T1, and carcinoma *in situ* [CIS]), and 20-30% present primarily as muscle-invasive (≥T2) tumors. While the so-called low-grade superficial tumors are not life-threatening, muscle-invasive tumors tend to progress and overall survival is only approximately 50% despite aggressive surgery and chemotherapeutic treatments. Standard of care in superficial bladder cancer involves transurethral tumor resection with the complete removal of all visible tumor tissue and adjuvant intravesical chemo- or immunoprevention in recommended cases. Once the pathologist confirms muscle-invasive tumor growth, lymphadenectomy and cystectomy or radiotherapy is recommended. In any case, a close follow-up of the patient is required as UCC is considered to be a panurothelial disease and also due to possible monoclonal spread of urothelial cancer cells. The probability of recurrence and progression

in superficial bladder cancer at 5 years ranges from 31 to 78% and from less than 1 to 45%, depending on grade and stage, respectively.[3]
A noninvasive diagnostic tool for urothelial cancer detection and surveillance, similar to prostate-specific-antigen in prostate cancer, is lacking. Follow-up and diagnosis of bladder tumors is still based on cystoscopy, which remains an invasive procedure and does not achieve the degree of diagnostic accuracy that urologists would like. Cystoscopy is usually combined with urine cytology, which has a median sensitivity of 13-75% and a specificity of 85-100%.[4] In a multicenter study, Grossman and colleagues recently reported an overall sensitivity of only 16% for urinary cytology to detect bladder cancer involving several institutions.[5] It seems that the sensitivity of cytology has decreased over the years and better tools for early tumor detection are needed.[6]
Even more challenging is the role of markers for diagnosis and follow-up of upper urinary tract (UUT) tumors, especially in times where endoscopic, organ-preserving approaches of low-grade and stage tumors in the ureter and renal pelvis become an appropriate treatment option. Considering the lack of highly specific and sensitive diagnostic methods in clinical routine use for UUT tumors, a urine test with these characteristics would certainly be helpful. Furthermore, there is a need for a urinary marker for surveillance of patients after receiving intravesical instillation therapy, or cystectomy and urinary diversion. These treatments change the typical cellular and molecular characteristics of tumor cells, compromising the diagnosis of recurrences.
Many approaches have been applied to improve noninvasive cancer detection methods and this includes the extensive search for new markers (e.g., DNA, RNA and protein approaches).[7-9] Unfortunately, all of these tests were unsuccessful in replacing cytology, which is still the most frequently used urine test, despite its poor sensitivity, especially in low-grade tumors. Several single urinary markers failed to show high sensitivity and high specificity at the same time (Table 5.1).[10] Due to the uncertainties of a single-marker model, hope has raised that multimarker models have a major contribution in urothelial tumor detection.
A promising example of a multimarker model is the fluorescence *in situ* hybridization (FISH) test (Vysis®, UroVysion™). It takes into consideration the multistep carcinogenic process that is involved in transforming urothelium into tumor tissue, including the different alterations in low- and high-grade tumors by detecting four different points of interests in urothelial tumor cells.

Markers for diagnosis & follow-up in urothelial tumors

The follow-up protocol after initial bladder tumor resection consists of cys

Table 5.1. Sensitivity and specificity of cytology and FDA approved urine tests in the diagnosis of urothelial bladder cancer.

	Author	Sensitivity% (95% CI)	Specificity% (95% CI)	n
Cytology	Grossman(2005)[5]	16(7.6-24)	99(98.7-99.7)	1331
	Halling(2000)[6]	58	98	265
	Placer(2002)[28]	64	86	86
	Pode(1999)[29]	39.8(31.8-49.3)	95.1(91.1-98.5)	240
BTA Stat	Friedrich(2003)[30]	67	78	103
	Halling(2002)[31]	78	74	265
	Pode(1999)[29]	82.8(75.7-88.7)	68.9(60.8-77.1)	250
	Sarosdy(1997)[32]	67(60-73)	95(90-98)*	220/167
	Wiener(1997)[33]	57	68	208
BTA TRAK	Ellis(1997)[34]	72	97*	216/212
	Heicappell(1999)[35]	72	51	298
	Mehnert(1999)[36]	62	54	244
	Thomas(1999)[37]	66(56-75)	69(60-77)	220
NMP22	Friedrich(2003)[30]	71	66	103
	Grossman(2005)[5]	56(44.1-66.7)	86(83.8-87.6)	1331
	Wiener(1997)[33]	48	70	208

*healthy volunteers.
BTA: Bladder Tumorantigen; CI: Confidence Interval; NMP: nuclear matrix protein.

toscopy and urine cytology, 3-monthly for the first 24 months, 6-monthly for the following 2 years and thereafter, in many cases for life, annually. Cystoscopy is a pivotal method for the detection of primary or recurrent bladder cancer but even with flexible instruments it remains invasive, expensive and uncomfortable for the patient. Moreover, in most cases cystoscopy is inadequate to accurately identify flat urothelial lesions, particularly carcinoma *in situ* (CIS), which is aggressive, unpredictable in behavior and associated with a high risk of tumor progression. A combination of fluorescence cystoscopy and intravesical 5-aminolevulinic acid has demonstrated that some malignant areas can be missed with standard cystoscopy.[11] Many urinary markers have been tested but in addition to low sensitivity, complicated analysis and expensive procedures have prevented routine use in clinical diagnostics. Others such as BCLA-4, survivin, telomerase, hyaluronic acid-hyaluronidase and DNAse X are still investigational.

Currently, there are five US FDA-approved urine tests: BTA Stat®, BTA-TRAK® (both Polymedco), FISH, ImmunoCyt™ (Diagnocure) and nuclear matrix protein (NMP)-22® BladderChek® (Matritech, Inc.).

Urine cytology

Urine cytology has, for unknown reasons, experienced a decrease in sensitivity and specificity within the last 15 years. Reports prior to 1990 described a sensitivity of 94% for grade 3 tumors, while recent analyses describe an average sensitivity in grade 3 tumors of only 60%.[6] In fact, a recently published multicenter study by Grossmann and colleagues showed an overall sensitivity of only 15.8% for urine cytology[5]. To miss the diagnosis of TaG1 tumors in approximately 85% by using urine cytology only might be acceptable owing to the low malignant potential of these tumors, but it seems hazardous to risk 40% false-negative results of high-grade carcinomas. However, publications regarding sensitivity and specifity of urine cytology vary quite frequently and it is certain that the results are directly correlated to the experience of the pathologist.[12] There may be a correlation between the low performance of cytology and the good performance of new ancillary tests for whatever reasons; a prospective multicenter study with analysts of different stages of experience might be useful.
In addition, the interpretation of urine cytology can be technically difficult due to factors such as inflammation, reactive changes due to therapy and subtle cytological abnormalities. Cytology relies on a subjective and operator-dependent interpretation of visible findings and there is a considerable inter- and intra-observer variability.[13] Any test that correlates pathological findings with molecular, observer-independent detection criteria would therefore be advantageous in clinical use.

Nuclear matrix protein

NMP-22 is a nuclear mitotic apparatus protein that is involved in the chromatin regulation and cell separation during replication. NMP-22 is thought to be released from the nuclei of tumor cells during apoptosis. The NMP-22 assay is a double monoclonal antibody (mAb) (one capture antibody and one reporter antibody) immunoassay for the quantitative measurement of NMP-22, which is highly immunogenic. It is performed on stabilized urine samples.

Bladder tumor antigen

The original bladder tumor antigen (BTA) test was a latex agglutination assay for the qualitative detection of basement membrane protein antigens in the urine. These basement membrane protein antigens are released into

the urine as the tumor degrades the basement membrane. The subsequently introduced qualitative point-of-care test BTA Stat and the quantitative BTA-TRAK detect human complement factor H-related protein.

ImmunoCyt

ImmunoCyt is a combination of cytology and an immunofluorescence assay. It uses three fluorescent mAbs targeting a sialylated carbohydrate epitope on a mucin protein (M344), a cell surface glycoprotein (19A211) and a surface cell antigen (LDQ10) specifically expressed by tumor cells.

Fluorescence in situ hybridisation

The FISH test (UroVysion) is a molecular, urine, multitarget assay that detects chromosomal abnormalities, based on frequently observed alterations in urothelial cancer. In 1998, a FISH assay that initially included ten probes was developed by Sokolova and colleagues at the Mayo Clinic (MD, USA).[14] Finally, the combination of four probes, chromosome enumeration probes (CEPs) 3, 7, 17 and locus-specific indicator 9p21 had the highest sensitivity for UC detection and were chosen to form the multicolor Vysis UroVysion test (Abbott/Vysis, IL, USA), which received US FDA approval in 2001.

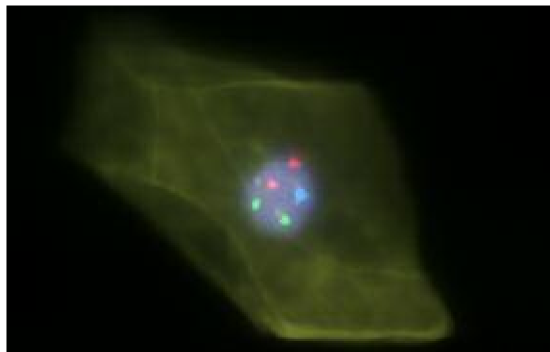

Figure 5.1. Normal Urothelial Cell. Multicolor and multiprobe fluorescence in situ hybridisation for the detection of bladder cancer in a voided urine specimen. Representative normal urothelial cell with two copies of chromosome 3 (spectrum red), chromosome 7 (spectrum green), and chromosome 17 (spectrum aqua), and both copies of the 9p21 locus (gold spectrum). Photo courtesy of Dr. N. Arens, Department of Pathology, University Hospital Mannheim, Germany.

How the fluorescence in situ hybridization test works

FISH is a technique that utilizes fluorescently labeled DNA probes to detect numerical or structural chromosomal abnormalities in tumor cells. The assay includes two different types of probes: CEPs and locus-specific indicators (LSIs). CEPs hybridize to the centromeres of the different chromosomes and provide useful information about aneusomy in tumor cells. LSIs provide specific information on genetic alterations of certain genes as they hybridize to specific loci.[15] Using the commercial multiprobe FISH test for urine analyses, four probes, CEP 3, CEP 7, CEP 17 and LSI 9p21, can be detected, which are labeled with red, green, aqua and gold fluorophores, respectively. To perform the assay with voided urine, at least 30-100ml of urine is necessary to obtain enough cells for the analysis. Washings from the UUT have only 3-10ml but contain more cells due to the lavage. The isolated cells are fixed on slides and pretreated. SpectraVysion® probe (Vysis) is added and heat denaturation is performed followed by overnight hybridization. After washing several times with different solutions at

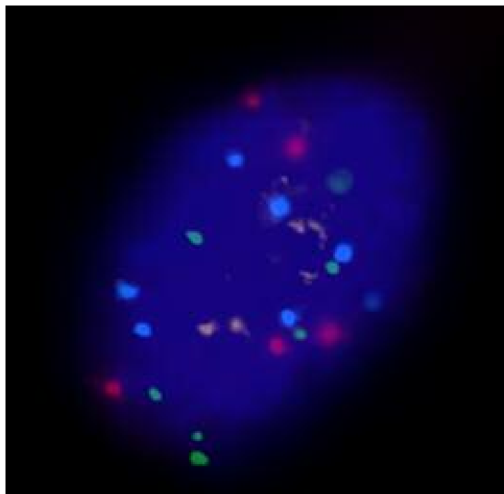

Figure 5.2. Urothelial tumor cell. Multicolor and multiprobe fluorescence in situ hybridisation for the detection of bladder cancer in a voided urine specimen. Representative urothelial carcinoma cell with different polysomies of chromosome 3 (spectrum red), chromosome 7 (spectrum green), and chromosome 17 (spectrum aqua). Both copies of the 9p21 locus (gold spectrum) have been lost. Photo courtesy of Dr. N. Arens, Department of Pathology, University Hospital Mannheim, Germany.

different temperatures, the slides are air dried and mounted.
The performance and the interpretation of the test vary from group to group.[6, 16, 17] Hybridization of the UroVysion probes is viewed using a fluorescence microscope equipped with appropriate excitation and emission filters allowing visualization of the multicolor signals (Fig. 5.1 & 5.2).
The most efficient method to evaluate the probe is to scan the slide for cells with morphologically abnormal features, such as nuclear enlargement, nuclear irregularity and abnormal chromatin staining. These cells are then viewed for chromosomal aberrations suggestive of malignancy.

Pitfalls in using fluorescence in situ hybridization to detect urothelial cancer

Interpretation of the FISH assay analyzing urine probes has changed and the initially used counting method, enumerating the number of copies in each FISH probe in 100 cells, has been replaced by the scanning method, where the slide is scanned for cells with nuclear abnormalities and signal copy numbers are determined in these cells.[14] Using the counting method, a case was considered positive if more than 7.6% of the 100 cells showed polysomy (gain of two or more different chromosomes in one cell). This method emerged as a time-consuming procedure. Sokolova and colleagues presented with the scanning method a faster method of analyzing FISH probes.[12] FISH-positive cells show in a very high percentage, nuclear abnormalities such as nuclear enlargement, irregular borders and inhomogeneous staining. Furthermore, it seems that polysomy is a more reliable indicator for cell malignancy than the gain of one single chromosome. According to the manufacturer's criteria, a case is considered positive if four or more cells out of a minimum of 25 morphologically abnormal cells demonstrate polysomy in two or more chromosomes (chromosome 3, 7, and 17) in the same cell or 12 or more morphologically abnormal cells demonstrate homozygous loss of 9p21. Interestingly, various decision criteria are still used for the determination of FISH positivity. Bollmann and colleagues defined 30 abnormal nuclei per slide with 10 or more cells showing 9p21 homozygous or heterozygous deletion, 10 or more cells showing polysomy of one chromosome or 5 or more cells showing polysomy of more than one chromosomes.[18] Tetrasomic chromosomal sets were not classified as malignant. Okamura and colleagues defined a probe as positive if more than 20 cells with polysomy of 2 or more chromosomes were found in 100 examined cells.[19] Degtyar and colleagues used the cut-off in a computerized imaging system if the percentage of nuclei with one (monosomy) or 3 or more signals (polysomy) per cell exceeded

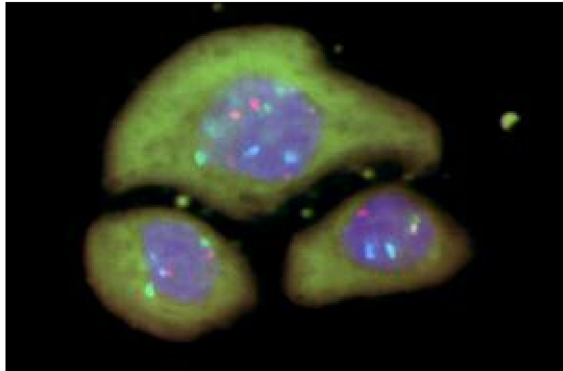

Figure 5.3. Umbrella cell. Multicolor and multiprobe fluorescence in situ hybridisation (FISH) for the detection of bladder cancer in a voided urine specimen. Umbrella cell demonstrating a typically tetraploid chromosomal pattern. Not all of the signals are visible on the 2D image, because it is not respecting the three-dimensionality of the cell. The tetraploid FISH pattern with four signals of each of the four probes seen as the only alteration in less than 5 cells in the urine samples is no sign of malignancy. Photo courtesy of Dr. N. Arens, Department of Pathology, University Hospital Mannheim, Germany.

10% in 50-500 examined cells. In this study, the tetrasomic FISH pattern was not excluded from the positive results.[20]
The tetrasomic FISH pattern described by Bubendorf and colleagues is defined as the presence of four copies of each colored DNA probe within one cell without other chromosomal alterations[16]. Since specimens with four or less tetrasomic cells have not been counted as tumor-positive, the cut-off of the number of aneusomic cells to define a FISH-positive specimen could be lowered without compromising the high specificity of the test.[16] This tetrasomic FISH pattern was detected in 29% of the control group (benign prostatic hyperplasia [BPH]) but in only 5% in pTa tumors and in 0% in pT1-pT4 tumors. The tetrasomic pattern is often found in umbrella cells (Fig. 5.3). By using these adjusted criteria, specificity was 96% and sensitivity 73% in nonmuscle-invasive and 100% in muscle-invasive tumors. Zellweger and colleagues published a prospective study demonstrating a nonsignificant correlation between FISH-positive results and recurrence rates in patients with a history of bladder cancer when applying the manufacturer's criteria. Adjusting the criteria, regarding probes with less than 10 cells with tetrasomic pattern and less than four cells with other aberrations as FISH-negative, a highly significant correlation

($p=0.006$) was detected.[21] The data from FISH analyses suggest that the criteria to define a FISH-positive result still need some fine-tuning. The published differences in specificity and sensitivity may be caused by varying criteria defining a positive and negative FISH result. Therefore, an important tool in determining the effect of various cut-offs on the sensitivity and specificity of an assay are receiver-operator characteristic (ROC) curves (in a ROC plot the sensitivity of an assay is plotted against its 1-specificity).

Voided urine versus bladder washings

The aim of performing a noninvasive urine test should be the use of voided urine rather than relatively invasive bladder washings. When basing on the cell amount, it might be difficult to gain enough cells in spontaneous voided urine. In a comparison of cytological interpretation between bladder washings and voided urine, the detection rate of pTa (71 vs 42%) and pT1 (88 vs 83%) tumors were better in bladder washings. In muscle-invasive tumors there was almost no difference in the detection rate between bladder washing and spontaneous voided urine cytology.[16] Degtyar and colleagues found that the method of exfoliated urothelial cell harvesting (voided urine or bladder washing) did not affect the sensitivity of cytology or FISH.[20] In any case, the amount of examined urine seems to be relevant and in volumes of 50-100ml the chance of sufficient cell count seems likely even in voided urine. Concerning low-grade superficial tumors, cytology and other cellular-based urine markers seem to work more precisely in bladder washings. Nevertheless, the amount of the examined urine should be standardized in future for a better comparison of test results. Furthermore, the discussion about which urine portion should be used is still ongoing; while some pathologists avoid using the first voided urine, other pathologists prefer the first morning urine.[12]

Efficacy of FISH in detecting bladder cancer

In comparative studies the FISH test outperformed urine cytology concerning sensitivity (Table 5.2 & 5.3). Depending on institutional experience, FISH was almost as good as or sometimes even better than conventional cytology concerning specificity (Table 5.4). In experienced hands both urine tests work excellent in high-grade urothelial tumors, while the detection rate of well-differentiated tumors is still not optimal. In low-grade cases, the FISH test appears to have slight advantages. The lower sensitivity of FISH for low-grade noninvasive papillary tumors is perhaps not surprising since these tumors are frequently diploid and have relatively few chromosomal aberrations.[22, 23]

Table 5.2. Sensitivity of cytology and FISH in the diagnosis of urothelial bladder cancer concerning tumor grade. Sensitivity % (n).

	G1		G2		G3	
	Cytology	FISH	Cytology	FISH	Cytology	FISH
Bubendorf(2001)[16]	14(21)	71(21)	41(29)	86(29)	76(17)	94(17)
Friedrich(2003)[30]	No data	57(7)	No data	63(28)	No data	83(11)
Halling(2000)[6]	27(11)	36(11)	54(24)	76(25)	71(34)	97(37)
Placer(2002)[28]	25(16)	53(15)	67(12)	83(12)	95(19)	100(19)
Sarosdy(2002)[25]	18(22)	55(22)	44(9)	78(9)	41(17)	94(18)
Skacel(2003)[24]	No data	83(23)	No data	80(35)	No data	96(24)

FISH: Fluorescense *in situ* hybridization.

Correlation of single FISH aberrations with recurrence & progression

It seems that not all FISH aberrations are equally important. Bollmann and colleagues recently presented a study correlating chromosomal patterns with tumor progression and recurrence using the FISH test. All progressive tumors were characterized by 9p21 deletion combined with high-frequency polysomies of chromosomes 3,7 and 17. None of the patients with only 9p21 deletion showed recurrence or progression during a mean follow-up of 41 months.[18] Similar results have been published by Skacel and colleagues; they detected loss of 9p21 as the only abnormality in low-grade/low-stage tumors.[24] A significant association between chromosome 9 and 17 aberrations and tumor invasion was found by Okamura and colleagues[19], while Degtyar and colleagues detected a correlation between polysomy of chromosomes 7 and 9 and high-grade tumors.[20] Bubendorf and colleagues described that CEP 17 probe detected 100% of T1-T4 tumors.[16] In general, results regarding 9p21 deletion in routine diagnostic FISH tests are contradictory concerning recurrence but not concerning progression. While loss of 9p21 has been correlated with an increased risk of tumor recurrence, there does not appear to be any correlation with progression.[18, 20, 21] In contrast, polysomy of any combination of the chromosomes 3, 7, 9 and 17 suggests the possibility of tumor progression (unstable tumors).[39] It will be useful to prospectively report the individual FISH pattern for each patient to correlate recurrence and progression data. In summary, it seems that FISH has the potential to detect urothelial tumors with gross chromosomal instability. In the future, this might be useful in a patient-tailored follow-up.

Table 5.3. Sensitivity in %(n) of cytology and FISH of urothelial bladder cancer of varying tumor stage. Sensitivity % (n=patients).

	pTa		pTis		pT1		pT2-pT4	
	Cytology	**FISH**	**Cytology**	**FISH**	**Cytology**	**FISH**	**Cytology**	**FISH**
Bubendorf(2001)[16]	24(45)	73(45)	No data	No data	50(12)	100(12)	85(13)	100(13)
Friedrich(2003)[30]	No data	62(21)	No data	No data	No data	67(18)	No data	83(6)
Halling(2000)[6]	47(36)	65(37)	78(18)	100(17)	*	*	60(15)	95(19)
Placer(2002)[28]	42(26)	64(25)	No data	No data	92(12)	100(12)	89(9)	100(9)
Sarosdy(2002)[25]	25(32)	66(32)	33(6)	100(7)	67(6)	83(6)	33(3)	100(3)
Skacel(2003)[24]	No data	83(64)	No data	100(3)	No data	83(6)	No data	100(9)

*pT1 was included in the invasive tumorgroup (pT1-pT4).

FISH: Fluorescense in situ hybridization.

Table 5.4. Overall specificity of cytology and FISH in the diagnosis of urothelial bladder cancer. Specificity % (n=patients).

Author	Cytology	FISH
Friedrich(2003)[30]	No data	89(55)
Halling(2000)[6]	98(49)	96(78)
Placer(2002)[28]	86(36)	85(34)
Sarosdy(2002)[25]	No data	95(275)
Skacel(2003)[24]	No data	97(29)
Varella-Garcia(2004)[38]	43(7)	86(7)

FISH: Fluorescense *in situ* hybridization.

Anticipatory positive FISH results

Published data have demonstrated that the FISH test can detect recurrences of UCC before it is clinically evident by cystoscopy.[6, 16, 24-26] These results were often categorized as false-positive, but nowadays the majority of these positive FISH results, without a direct clinical correlation, are known to predict very early tumor recurrence (anticipatory positive [AP] results). In a prospective study design from the Cleveland Clinic laboratory (OH, USA), 62% of patients with AP results developed recurrent tumors, compared with only 5% with negative FISH results.[27] Skacel and colleagues described nine patients with positive FISH results but negative biopsies. Over a period of 15 months, all of these patients developed recurrent transitional cell tumors.[24] AP findings in patients with urothelial carcinoma preceded cancer detection between 4 and 15 months. In general, these recurrent cancers tend to be high-grade tumors, a group which needs special interest during follow-up and may require a more aggressive therapy than patients with FISH-negative results.

Expert commentary

Management of nonmuscle-invasive bladder cancer is a dynamic process. Standardized diagnosis and therapy can be optimized by minimization of investigations applying subjective criteria. Intra- and interobserver variability in detecting urothelial tumors by cystoscopy or by cytology are well-known phenomena, yet there still exists no consensus classification for cytology. With the FISH test, urologists and pathologists have a tool of molecular cytology and a highly sensitive and specific noninvasive test not only for diagnosis but also for surveillance strategies. One advantage is the so-called multimarker strategy. Since one molecular marker fails to detect the

heterogeneous group of urothelial cancers in a highly specific and sensitive manner, the FISH test examines four different chromosomal alterations, most likely representing two different conditions in urothelial carcinogenesis with polysomy of the chromosomes 3, 7 and 17 (instable tumors) versus alteration of 9p21 (stable tumors). Furthermore, there are objective and reproductive criteria available to define a FISH probe as positive or negative. The ongoing discussions about the combination of cytology and FISH, using the UroVysion test only in uncertain cytology results, might be beneficial for institutions with a great expertise in cytology. Otherwise, it might lead to a double investigation resulting in extra time and costs but no increased benefit. The expertise to correctly evaluate routine cytology might be difficult to reach in every pathological institute and a test with objective decision criteria might be easier to apply in general, but in addition to cytology, FISH must be performed by a trained specialist. However, results of the FISH assay will correlate with the investigators experience in cytomorphology. Even if the cytological diagnosis of a high-grade (G3) bladder cancer appears straightforward and similar, a high-grade urothelial neoplasia seems very unlikely in case of negative cytology, the place for the FISH test still has to be defined. In general, an international standard with guidelines for the diagnosis of urothelial cancer in urine would be very helpful.
The UroVysion test might be more than a diagnostic tool for bladder cancer. Estimating the potential of this molecular approach, there might be a role in surveillance of patients with panurothelial disease and in detection of UUT tumors.
With all the available data on FISH testing in urothelial cancer, studies have been started to evaluate UroVysion in patient-tailored follow-up and FISH-adapted surveillance strategies. If the surveillance standard in superficial bladder cancer can be adjusted by using a urine test, the costs will be reduced.

Five-year view

FISH has been increasingly used over the last 5 years to detect bladder cancer and considering the expanded possibilities offered by this test will find its way even more into clinical practice. UroVysion test could improve the diagnosis of UUT tumors.
Over the last few years, endoscopic treatment of ureter and renal pelvis tumors has gained acceptance in the case of grade 1 and 2 tumors. The correct grading of the tumor remains a challenge, but adding a test (e.g., FISH) that may be able to profile the tumor as possibly nonrecurrent or highly suspicious for progress will make treatment decisions easier. Since the UroVysion test seems not to be influenced by instillation treatment and

chemotherapy, it will be interesting to evaluate its potential in the surveillance of urothelial tumors. There may be a place for the FISH test in the surveillance of patients after urinary diversion. Patients with a panurothelial disease, developing UCC in different areas of the urothelium, have to be considered at high risk for loosing both kidneys and the bladder. Detecting an ongoing process very early, for example, with AP FISH results, new strategies such as chemoprevention might be an option for organ-sparing treatment.
The UroVysion test seems to be helpful in controlling the therapeutic effects of instillation therapies in the upper and lower urinary tract.

Key issues

Classic follow-up modalities to detect recurrence after resection of a non-muscle-invasive bladder tumor are cystoscopy and urine cytology.
Neither cystoscopy nor cytology alone or in combination has optimal diagnostic accuracy in the screening for bladder cancer. This has led to several attempts to develop easy-to-use, noninvasive, less subjective and more accurate urinary markers for detecting and monitoring urothelial cancer. Until today single marker tests have failed to diagnose urothelial tumors with high sensitivity and specificity.
UroVysion™ is a commercial fluorescence *in situ* hybridization (FISH) assay that is used to identify malignant urothelial cells in the urine. It allows the detection of numerical and structural chromosomal aberrations, representing early and late molecular events. The UroVysion test offers an objective measurement of a cell's genetic profile and might be correctly termed molecular cytology.
Results of FISH tests compared with conventional cytology demonstrate a higher sensitivity and a similar specificity and it has an additional benefit by detecting the urothelial tumors with gross chromosomal instability.
Owing to the fact the FISH test is not influenced by instillation or urinary diversion therapies it could become a useful tool in surveillance strategies.

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CHAPTER 6

PROTEIN PROFILING OF BLADDER CANCER
USING THE 2D-PAGE AND SELDI-TOF-MS TECHNIQUE

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Abstract

Protein profiling is a promising tool for tumor characterization and the detection of tumor markers in bladder cancer. Techniques for 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and surface-enhanced laser desorption/ionization with time-of-flight mass spectrometry (SELDI-TOF-MS) have improved; both were evaluated using bladder tumor tissue. Normal urothelium and pTa G2, pT1 G3, and ≥pT3 G3 tissues were obtained from the operating room and, after macrodissection, subjected to 2D-PAGE and to SELDI-TOF-MS ProteinChip®. 2D-PAGE gels expressed significantly different protein patterns for pTa G2 and pT3 G3 tumors. pT1 G3 tumors showed expression profiles similar to those of the invasive tumors, with upregulation of galectin 3, gelsolin, villin 2, moesin, and annexin 6. Similarly, distinct protein peaks were detected for superficial and muscle-invasive urothelial cancers by SELDI-TOF-MS. Six of seven superficial pTa G2 tumors showed an intense peak at 6.7 and 10.1kD, while invasive carcinomas showed an intense peak near 9.5kD. No disturbing influence of surrounding tissue on the results was detected. It was shown that both techniques (2D-PAGE and ProteinChip) work well, and especially ProteinChip analysis seems promising for clinical application.

Introduction

The incidence of transitional cell carcinoma (TCC) of the bladder is increasing, and with it the financial burden of its diagnosis and treatment. An analysis of Medicare payments in the US shows that bladder cancer entails very high per-patient cost from diagnosis to death due to frequent treatment, intensive surveillance, and long survival.[1] The diagnosis of bladder cancer is established either by cystoscopy and transurethral biopsy or by resection, which both represent invasive procedures. Many approaches have been made to improve non-invasive cancer detection, and only recently some marginal successes have been reported.[2] Nevertheless, markers to distinguish recurrent, nonrecurrent, and progressive superficial bladder cancer at first diagnosis are still lacking.

Molecular markers offer a new and promising approach to cancer diagnosis. Attempts to correlate individual markers with the biological behavior of subsets of bladder cancers have been described in numerous reports.[e.g., 3-6] However, results are often contradictory. This has been particularly demonstrated for the p53 molecule, the most investigated prognostic marker in bladder cancer.[7] Due to the uncertainties of a single-marker-model, hopes have been raised that multi-marker models may make it possible to define sets of markers for the identification of tumor subgroups.

DNA microarrays have helped to identify gene clusters associated with

tumor stage and tumor-specific pathways.[8] On the one hand, the observation of alterations within a tumor in order to identify specific biological behavior seems promising; on the other hand, DNA arrays may not be ideal instruments for routine clinical use because of their complex technical requirements, limited reproducibility, and cost.[9] There is an ongoing demand for sophisticated techniques that will provide reliable molecular information for clinical purposes.

Recent developments in proteomic research make it possible to analyze proteins in serum, urine, tissues, and even tumor interstitial fluid.[10,11] Discrimination between protein expression profiles in benign and malignant tissue, as well as in primary and recurrent tumors, could lead to a risk-adjusted therapy and surveillance strategy for bladder cancer patients. The use of practical, easily-handled, and affordable research tools that can be used in clinical routine is often limited by the complex preparation of the probes and by time-consuming techniques. Urine has often been used in bladder cancer research, but composition varies enormously and detailed information about the biological behavior of the tumor is difficult to obtain.[12,13] Most information about the tumor will be found in the tumor itself. Bladder tumors are heterogeneous tissues; with microdissection, cell populations can be selected which do not necessarily represent tumor specific characteristics.[14, 15]

In order to evaluate the potential of proteomic analyses in bladder cancer we used two approaches, the 2D-PAGE and the SELDI-TOF-MS (ProteinChip®) techniques. To evaluate the potential of both methods for later use in clinical routine, we used tumor tissues that had been macrodissected.

Materials and Methods

Bladder-tumor biopsies were obtained during tissue removal for routine pathological examination. The tumor tissue was immediately snap-frozen in liquid nitrogen and kept at -80°C until further use. Tumor stage and grade were assigned according to the 1997 consensus classification of urothelial neoplasms of the urinary bladder (World Health Organization). For 2D-PAGE, we pooled and analyzed tissue from five pTa G2 tumors, three pT1 G3 tumors, three pT3 G3 tumors, and three normal urothelium samples from non-TCC cancer patients. For SELDI-TOF-MS, we separately analyzed seven pTa G2 and five ≥pT3 G3 transitional cell carcinomas. The study was approved by the scientific ethical committee, Faculty of Medicine, University of Heidelberg, Germany.

2D-PAGE

Deep-frozen cancer tissue was disrupted, solubilized, and prepared. Each probe was analyzed five times and the resulting gels were correlated digitally to create the average mastergels. Aliquots of 200 μg protein were applied to an immobilized pH-gradient gel for isoelectric focusing. Then SDS-polyacrylamide gel electrophoresis (PAGE) was performed using 12.5% polyacrylamide gels. Protein spots were visualized by 0.2% Coomassie Brilliant Blue (Roth Chemie, Karlsruhe, Germany). Postelectrophoretic fluorescent labeling was carried out with SYPRO ruby (Bio-Rad Laboratories, Hercules, CA, USA) and the resulting images were analyzed with standard software for 2D electrophoresis (ProteomWeaver 2.2, Definiens AG, Munich, Germany). This revealed areas that were differently expressed to a statistically relevant degree. After differently expressed proteins in the pT1 G3 group were excised from the gel, destaining, reduction, alkylation, and digestion were performed. Afterward, matrix-assisted laser desorption ionization/mass spectrometry was performed and known proteins were identified by searching human protein databases (National Center for Biotechnology Information, NCBI, www.ncbi.nlm.nih.gov). The 2D gel electrophoresis was performed in cooperation with Toplab GmbH, Martinsried, Germany.

SELDI-TOF-MS

Frozen samples were disrupted (20mM HEPES buffer, 1% Triton X-100) and centrifuged at 14*g*. The supernatant was mixed with an appropriate amount of binding buffer. Equal protein concentrations were applied on chips with varying chromatographic properties (strong anionic exchange, weak cationic exchange). Each ProteinChip array was placed on an orbital shaker and incubated for 30 minutes. The samples were then taken out and washed three times with binding buffer and then with deionized water in order to remove buffer salts. 0.6 μL of a saturated matrix solution of sinapinic acid in 50% (v/v) acetonitrile and 0.5% trifluoroacetic acid was applied to each spot and allowed to dry. Each ProteinChip array was next analyzed using the ProteinChip Reader (PBS-IIc, Ciphergen Biosystems, Inc., Guildford, UK). Time-of-flight spectra were generated using data-acquisition parameters optimized for sensitivity as well as peak resolution for a particular array type. All samples were scanned in the 4-20kD window and data were collected according to an automated data-collection protocol. After comparing results from different chips, the weak cationic exchange (WCX-2) ProteinChip was chosen for subsequent analyses. Samples were evaluated in triplicate to make sure that detected proteins during individual runs were

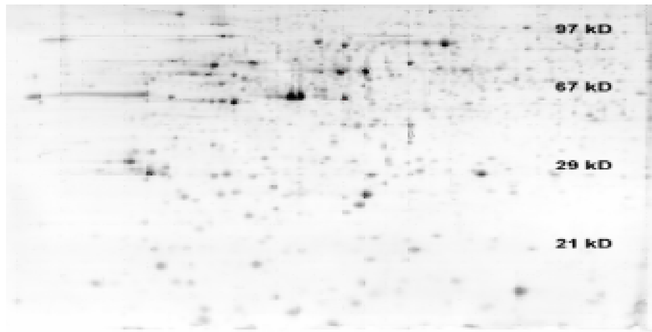

Figure 6.1. Mastergel of pTa G2 superficial bladder tumors. Proteins were separated by 2D-PAGE (IPG 4-7), T12% SYPRO ruby stain.

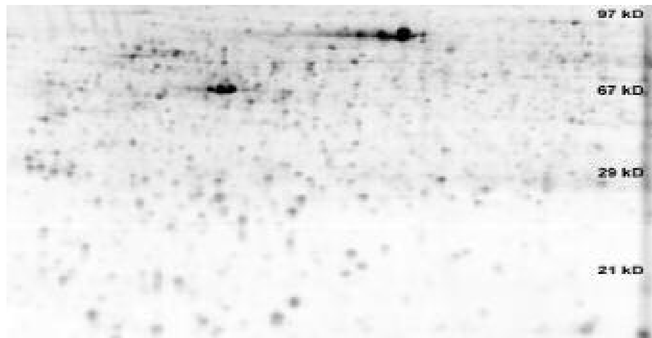

Figure 6.2. Mastergel of pT3 G3 muscle-invasive bladder tumors. Proteins were separated by 2D-PAGE (IPG 4-7), T12% SYPRO ruby stain.

Figure 6.3. Master gel of high-risk pT1 G3 tumors. Proteins were separated by 2D-PAGE (IPG 4-7), T12% SYPRO ruby stain.

reproducible. Data interpretation was enhanced by the use of the ProteinChip software version 3.1 (Ciphergen Biosystems, Inc.). Since this was a preliminary study employing a limited number of samples, we did not attempt to evaluate statistical significance.

Results

Normal urothelium, superficial pTa, high-risk pT1, and muscle-invasive tumor tissues were pooled and analyzed with the 2D gel electrophoresis technique. The master gels showed significantly different protein expression for pTa and ≥pT3 urothelial carcinomas (Figs. 6.1 and 6.2). Protein expression of high-risk bladder tumors (pT1 G3) was distinctly different from that of pTa and ≥pT3 tumors. However, the pT1 expression profile was somewhat similar to that of the muscle-invasive tumor group (≥pT2). We focused on pT1 G3 carcinomas and analyzed spots that showed a distinct differential expression (Fig. 6.3). Disregulated proteins of the pT1 G3 tumors are listed in Table 6.1.
Upregulated proteins in pT1 G3 like gelsolin, moesin, and villin 2 (ezrin) belong to the actin-related proteins, controlling the cytoskeletal motility. Annexin 6 is connected with the organization of membrane domains, particularly those of the cytoskeleton.
Protein analysis using the SELDI-TOF technique was easy to learn and perform. WCX-2 chips provided the most promising spectra and were used for further analysis. The instrument readout for each sample produced a

Table 6.1. Disregulated proteins in pT1 G3 bladder tumors.

NCBI ID	Upregulated	NCBI ID	Downregulated
113962	Annexin VI	4504517	Heat shock 27 kDa protein 1
4504165	Gelsolin	16041670	Unknown protein
12804047	Hypothetical protein FLJ10805	763431	Similar to human albumin, Swiss-prot Acc. No. P02768
27924135	TLK2	129379	Heat shock 60 kDa protein
4504983	Galectin 3	181573	Cytokeratin 8
4505257	Moesin	30311	Cytokeratin 18
21614499	Villin 2	27894337	Cytokeratin 20

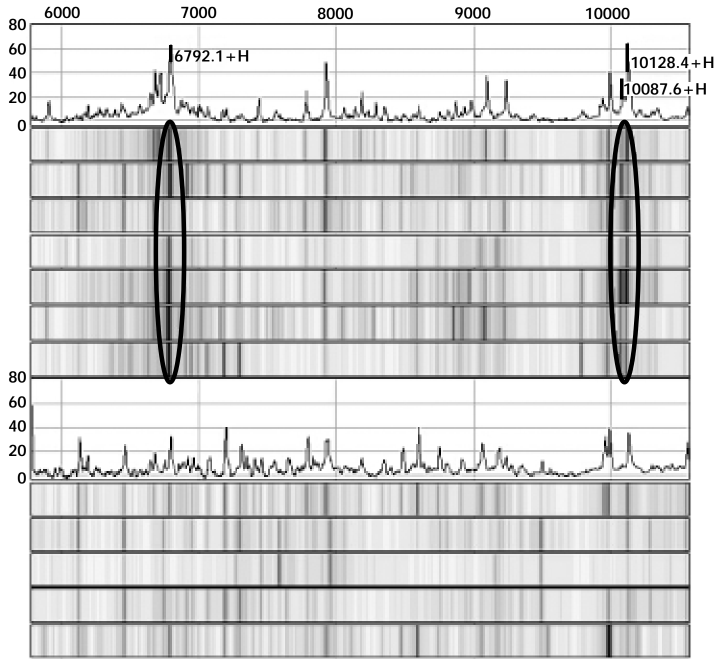

Figure 6.4. SELDI-TOF-MS protein expression pattern in Ta and T3 tumors, mass range 6–10.5kD, WXC chip. n represents tumor identification. Combined spectra and gel view.

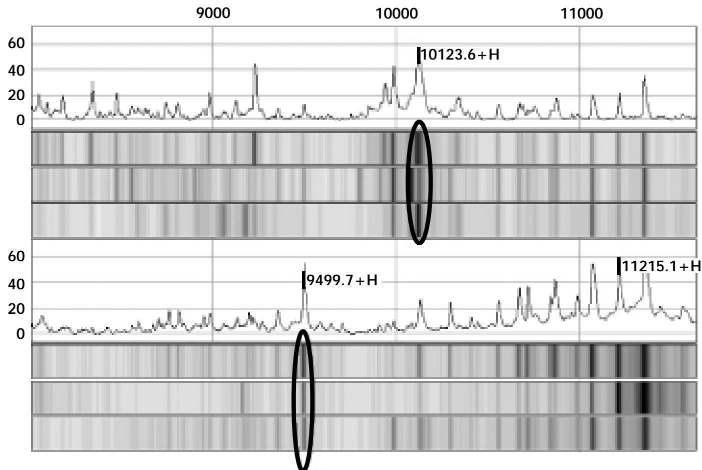

Figure 6.5. SELDI-TOF-MS protein expression pattern in Ta and T3 tumors, mass range 8–11.5kD, WCX chip. n represents tumor identification. Combined spectra and gel view.

spectrum of protein peaks (shown as spectra or gel view). The pattern of the spectrum was defined by the peak key mass/charge positions along the x axis and the proportionate abundance of the individual proteins along the y axis. Gel and spectra views demonstrate the differentially expressed protein peaks. We found that six out of seven superficial pTa G2 tumors showed intense peaks at 6.7 and 10.1kD (Fig. 6.4), while the invasive carcinomas all showed an intense peak near 9.5kD (Fig. 6.5).

Discussion

The search for tools that will help to predict recurrence and progression of bladder cancer is ongoing. Gene-expression profiling, microsatellite examinations, comparative genomic hybridization or fluorescence *in situ* hybridization have been used with some success but cannot pinpoint tumor-associated markers.[16,17] At the same time, technical innovations have taken place in proteomic research. 2D gel electrophoresis has developed as a standardized and widely-used technique, thus offering new chances for biomarker detection. Its advantages include a high resolving power and the ability to display several thousand proteins at once.

Our 2D gel electrophoresis data show a strong similarity in protein expression profile between pT1 G3 and muscle-invasive tumors, confirming the results of Wild *et al.*, who detected a similarity in gene expression profile between pT1 G3 and muscle invasive tumors.[15] We identified proteins like gelsolin, which is mainly involved in the organization of the cytoskeleton. Gelsolin is a major actin-regulatory protein and belongs to a group of filament-cutting proteins. It takes part in the regulation of cellular apoptotic processes by influencing caspase activities. Gelsolin expression was recently found to play an important role in urothelial carcinoma.[18] Two proteins of the ERM family - villin 2 (ezrin) and moesin - were also found to be upregulated. These actin-binding proteins act not only as a link between the actin-cytoskeleton and the plasma membrane but also as signal transducers in cytoskeletal transformation.[19] Complexity of cytoskeletal structure-modeling is not yet understood in detail but is certainly tightly connected to cell motility and metastasis.[20]

In this study we did not attempt microdissection of tissue samples, as this technique has its limitations in regard to tumor heterogeneity. Koed *et al.* used high-density single nucleotide polymorphism arrays with tissue microdissected from bladder cancer. Different tumours from the same patient were examined and it was statistically significant that Ta and T1 tumors had less allelic imbalances in common compared with T1 and T2

tumors. Only two out of three allelic imbalances were reproduced in T1 tumors when compared with Ta tumors, indicating a clonal evolution or a mixture of different clonal subpopulations within Ta tumors.[14] Cells gained by microdissection of bladder tumors might, therefore, not be representative for a given superficial tumor. Wild *et al.* did not detect differentially expressed genes, as expected, when they compared tissue from carcinoma *in situ* and normal urothelium.[15] They proposed, that the use of microdissection had led to the extraction of mainly normal urothelial cells within the carcinoma *in situ* probes. Therefore, the analysis might have shown similar genetic alterations between urothelium and carcinoma *in situ*.

We conclude, from both the literature and our own experience, that 2D-PAGE is an excellent method for the identification of differentially expressed proteins and functionally correlated protein groups in bladder cancer even without microdissection.

SELDI-TOF-MS analysis was fast, efficient, and reproducible as well as easy to learn and perform. Protein-chip technology also offers the advantage of processing large numbers of samples and allows the examination of almost any body liquid or tissue.[11] Efforts have recently been made to use protein chips for the identification of markers in urine samples in order to diagnose bladder cancer and to obtain information about possible tumor recurrence.[12,13] Liu *et al.* recently published a protein-profiling study using protein chips showing that discrimination between bladder-cancer patients and non-cancer patients was possible.[21] In their investigation, SELDI-TOF-MS protein profiling was used for pattern analysis of urine samples without identification of the proteins that actually caused the peaks. That is to say, it was shown to be sufficient to compare the protein profiling patterns of the urine samples without identifying all proteins. Detection of the patient's protein pattern was more helpful than identifying single proteins, in this case.[21] The diagnosis of protein profiling patterns, omitting the laborious identification of the specific proteins, is a potential advance over traditional diagnostic approaches.

Using proteomic profiling of tumor tissue, it might be possible to identify subsets of bladder cancers such as recurrent or progressive tumors. Our results show that superficial bladder tumors express a different pattern than invasive cancers. We did not examine high-risk tumors such as like pT1 G3 tumors using the SELDI-TOF-MS technique, but we believe that progressive urothelial carcinomas could be identified through specific pattern analysis. Proteomic chip technology as a diagnostic tool may identify recurrent and progressive tumors and so alter approaches to treatment and surveillance.

Our study demonstrates great potential for protein profiling, which pro-

cesses tumor tissue directly, in the diagnosis and treatment of bladder cancer. 2D gel electrophoresis is a sophisticated, mature technique and offers wide possibilities in protein identification, but seems inappropriate for routine clinical use at this time due to its complexity and low throughput. Chip-based arrays, in contrast, make it possible to characterize and classify bladder tumors with the help of protein-profiling patterns in order to define a patient's risk for tumor recurrence and progression. Further studies with larger number samples are warranted in order to identify specific protein-expression profiles of progressive and recurrent transitional cell tumors. The ProteinChip technology, in particular, seems likely to facilitate the introduction of this diagnostic tool into clinical routine and - more broadly - may develop toward a "bedside" test for bladder cancer patients.

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CHAPTER 7

IDENTIFICATION OF DISEASE BIOMARKERS
BY PROFILING OF SERUM PROTEINS USING SELDI-TOF MASS SPECTROMETRY

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Abstract

Proteins are the main actors in all physiological and pathological processes. Since the final structure of the protein does not depend on the DNA sequence or even the mRNA sequence alone, the search for direct approaches on the proteome has gained great interest. The most complex and probably the largest proteome is serum, making it clinically the most important.

ProteinChip technology in combination with modern mass spectrometry allows the complex search for biomarkers, molecular interactions, signalling pathways, and the identification of novel therapeutic compounds. Here we describe the surface-enhanced-laser-desorption-ionisation (SELDI) in combination with the time-of-flight (TOF) mass spectrometry for analysing serum samples (SELDI is a patented technique from Ciphergen, Fremont, USA). Aluminium-based arrays contain chemical or biological surfaces allowing capture of proteins, which interact with the surface. The bound proteins are laser desorbed and ionized for mass spectroscopy analysis. The differential mass spectral patterns reflect the protein expression bound on the chip surface and allow the comparison between various samples. Proteins of interest can be identified using peptide mass fingerprinting (PMF).

Introduction

The direct identification of biological endpoints is one of the major advantages of proteomic research. The primary sequence of proteins does not depend on the DNA or the mRNA sequence alone, various posttranslational modifications like acetylation, glycosylation, phosphorylation or other types of covalent alterations can diversify proteins.[1,2] Protein biomarkers are among the important tools for detection, diagnosis, treatment and monitoring diseases.[3-9] The estimated number of different human proteins is about 500,000 and it will be challenging to identify, to catalogue and to functionally assign these molecules. Serum is one of the preferred mediums when searching for markers because it is obtained with low levels of invasiveness, it is available in sufficient quantity and it is a source with great information content.[5, 6, 10-12] Serum is probably the most complex proteome, containing other tissue proteomes as subsets.[13] Profiling of human serum is ambitious because it contains large proportions of albumin (55%) and glycoproteins with an enormous heterogeneity. Twenty-two protein groups, such as albumin, immunoglobulins, haptoglobin, transferrins and lipoproteins, account for 99% of the protein mass in serum. Estimated, as many as 10,000 unique proteins are present within the human serum proteome that span a dynamic range of concentrations approximately of >10.[9, 13, 14] Therefore, the background matrix represents

a very complex milieu and proteins being observed in general analyses are, in general, of high abundance.
The ProteinChip® System was introduced with the intention of an easy to use, time- and resource saving method by Ciphergen Biosystems, Inc, Fremont, CA, USA (www.ciphergen.com). Based on the Surface Enhanced Laser Desorption/Ionization process (SELDI) it combines two methods: solid phase chromatography and time-of-flight mass spectrometry (TOF-MS).[10, 15, 16] This technology integrates the complex protein expression on one integrated platform and enables the study of complex serum protein profiles.[17] Chip arrays capture individual proteins out of a probe which are subsequently resolved by mass spectrometry (MS). Varying coatings of the chips allow the binding of different protein classes. ProteinChip arrays are available with different chromatographic properties, including hydrophobic, hydrophilic, anion exchange, cation exchange, and immobilized-metal affinity surfaces, or they are pre-activated for the coupling of capture molecules. Thus, each analysis is a sort of an on-spot fractionation step, which reduces the complexity of the serum sample. This kind of fractionating will in selected cases not be effective enough and the dynamic range of proteins and protein concentrations in human serum sometimes necessitates effective fractionating methods capable of separating high abundance from low abundance proteins. The sample analysis takes place in a SELDI-TOF MS and data are processed by complex bio-informatic software. Further detailed information and images about the SELDI-TOF MS technique can be found on the homepage of Ciphergen (www.ciphergen.com, www.bio-rad.com) and in the literature.[5, 15, 18, 19]

Material

Probes Serum samples are obtained in routinely used serum separator tubes.

Fractionating the serum

Serum may be used unfractionated or fractionated. Any method to fractionate the serum probe is fine. Expression Difference Mapping Kits (Ciphergen, Freemont, CA, USA) are especially designed for serum fractionating in combination with SELDI TOF analyses (procedures are described in detail in the Expression Difference Mapping™ Kit - Serum Fractionating, www.ciphergen.com, www.bio-rad.com), but any method to fractionate the serum is suitable.

Protein Chip Arrays

IMAC30-immobilized metal affinity capture array with a nitriloacetic acid (NTA) surface with an updated hydrophobic barrier coating.

CM10-weak cation exchange array with carboxylate functionality, with an updated hydrophobic barrier coating.

Q10-strong anion exchange array with quaternary amine functionality, with an updated hydrophobic barrier coating.

H50-bind proteins through reversed phase or hydrophobic interaction chromatography with an updated hydrophobic barrier coating.

H4-mimic reversed phase chromatography with C16 functionality.

NP20-mimic normal phase chromatography with silicate functionality.

Au-gold chips to be used directly for MALDI-based experiments.

Mainly used ProteinChip surfaces for serum diagnostic: Metal Affinity Capture (IMAC3/IMAC30) and Weak Cation Exchange (CM10/WCX2) Reversed Phase (H50).

Binding buffers for the different chip arrays

PBS buffer: 2.7mM potassium chloride, 120mM sodium chloride, and 10mM phosphate buffer maintained at a pH of 7.0.

100mM ammonium acetate pH 4.0 (with or without 0.1% Triton X-100) for CM10.

100mM Tris-HCl pH 9.0 (with or without 0.1% Triton X-100) for Q10.

100mM Na phosphate, 500mM NaCl pH 7.0 (activated with either 100mM copper sulphate or 100mM nickel sulphate hexahydrate) for IMAC30.

10% acetonitrile (AcN), 0.1% trifluoroacetic acid (TFA) or phosphate buffered saline (PBS) for H50.

Energy absorbing molecules (EAM)

50% saturated sinapinic acid (in 50% acetonnitrile and 0.5% trifluoroacetic acid) or 50% saturated α-Cyano-4-hydoxycinnamic acid (in 50% acetonitrile and 0.5% trifluoroacetic acid). Both materials from Ciphergen, Freemont, CA, USA.

Mass Spectrometry

SELDI-TOF MS: PBS-IIc ProteinChip Reader or ProteinChip System Series 4000 (Ciphergen, Freemont, CA, USA).

SELDI-QTOF: QStar Pulsar or QStarXL instrument (Applied Biosystems, Darmstadt, Germany) equipped with a PCI 1000 ProteinChip® Interface (Ciphergen, Fremont, USA).

Software

Analytical software package: ProteinChip software version 3.1 or later versions (Ciphergen, Fremont, USA).

Methods

SELDI ProteinChip® technology binds distinct groups of serum proteins on the chip surface after adding an energy absorbing matrix (EAM). The bound proteins are finally read in a SELDI-TOF-MS. Generally, serum is processed according to the technique of each particular institution and many different procedures have been described. Regardless of the method chosen, standardization, with careful attention to details, is imperative in protein research.

Samples Preparation for Storing

Standardized blood collection in a serum separator tube, allow to clot for 30 min at room temperature, centrifugate (10 min at 1000g), and aliquot the supernatant serum before flash freezing at 80°C (*Note 1*). It is also possible to aliquot the samples later (for example first thaw) but the quality of the probes deteriorates with each freeze-thaw cycle.

Using unfractionated serum

1. After thawing frozen samples on ice spin aliquots at 20 000*g* for 10 min at 4°C (*Note 2*).
2. Mix 20 μl of serum sample with 30 μL of PBS buffer and vortex samples 20 min. Dilute this samples with 50 μL of PBS buffer.
3. This 1: 5 diluted serum sample can be further diluted 1:8 to 1:10 into the respective binding buffer. Incubate for 40 minutes in a Bioprocessor under constant shaking (Ciphergen Biosystems, Fremont, Ca, USA).
4. Wash three times with 150 μL binding buffer for 5 minutes with shaking.

5. Wash twice with 150 μL distilled water for 1 minute to remove buffer salts.
7. Add twice 1 μL of 50 % saturated matrix solution 50% saturated sinapinic acid (in 50% acetonnitrile and 0.5% trifluoroacetic acid) or 50% saturated α-Cyano-4-hydoxycinnamic acid (in 50% acetonitrile and 0.5% trifluoroacetic acid) to the spot surface and allow to air dry in between.
8. The probes are now ready to be analysed if necessary store chips in a dark room at RT until processing.

Fractionating Serum Probes

If the serum is pre-fractionated, different techniques can be used.[19] Expression Difference Mapping Kits (Ciphergen, Freemont, CA, USA) are especially designed for serum fractionating in combination with SELDI TOF analyses (procedures are described in detail in the Expression Difference Mapping™ Kit - Serum Fractionating, www.bio-rad.com), but many methods to fractionate the serum are suitable.

Sample Application on the Chip

The minimum sample requirement is between 1 and 10 μg total protein per spot, the total sample volume is variable (between 50 μL -200 μL using a bioprocessor).
The serum samples can now be profiled with different array surfaces. The most often used and widely tested surfaces with serum are Metal Affinity Capture (IMAC3/IMAC30), Weak Cation Exchange (CM10/WCX2) and Reversed Phase (H50). To find the Chip which suits best the study design the different surfaces have to be pre-evaluated by testing or studying the literature.

1. Dilute the fractionated serum samples 1:5 -1:10 into the appropriate binding buffers. Add 50-150 μL to the bioprocessor and incubate 30 min under shaking.
2. Wash three times with 150 μL binding buffer for 5 minutes with shaking.
3. Wash twice with 150 μL distilled water for 1 minute to remove buffer salts.
5. Add twice 1 μL of 50% saturated matrix solution 50% saturated sinapinic acid (in 50 % acetonnitrile and 0.5% trifluoroacetic acid) or 50% saturated α-Cyano-4-hydoxycinnamic acid (in 50% acetonitrile and 0.5% trifluoroacetic acid) to the spot surface and allow to air dry in between.

The probes are now ready to be analysed if necessary store chips in a dark room at RT until processing.

Mass analyzer

The ProteinChip arrays are placed in the SELDI-TOF MS (PBS IIc ProteinChip reader or ProteinChip System 4000) and irradiated with a pulsed UV nitrogen laser. Hereby protons are transferred onto the peptides and proteins that are subsequently accelerated by electromagnetic fields through a flight tunnel. The time-of-flight corresponds inversely to the molecular mass. The resulting signals are converted to mass-to-charge (m/z) ratios based on the time each species takes to pass through the TOF mass analyzer.

1. Set the mass analysis range you want to evaluate.
2. Adjust the laser intensity and some other parameters before measurements.
3. Calibrate the instrument using molecular markers.

Analysis of TOF MS spectra

Complex software packages, basing on genetic algorithms, decision trees, and unified maximum separability algorithm are used for data analyses. The concept of an independent procedure, a supervised analysis with a training data set to differentiate between sample classes, is used. The results are visualized in a graph with the mass to charge ratio of the sample components on the x-axis and the corresponding signal intensities on the y-axis(*20*). Modern software allows a wide spectrum of different views and spectra for the best visualization of the data. Widely used is the ProteinChip Biomarker Wizard™, the EDM package, and the Biomarker Patterns Software (Ciphergen Biosystems, Fremont, CA, USA). More detailed information's can be found in the literature (*20*) or on the homepage of Ciphergen (www.ciphergen.com/www.bio-rad.com).

Protein Identification (Peptide Mass Fingerprinting)

Using the SELDI based technique identification of target proteins or suspected biomarkers is the last step. The detailed steps of protein purification and identification are beyond the scope of this manuscript. Briefly, each protein of interest needs to be purified or enriched for subsequent analysis. Different methods (mini-spin columns, microplate formatted chromatographic devices, preparative scale columns, and antibodies in combination

with pre-activated arrays) can be used. Finally, the enriched protein is proteolytically cleaved and the masses of the resulting peptide fragments are determined using MS. The obtained data can directly be submitted to protein databases, matching the measured masses with peptide fragments of known sequences (overview of protein databases: http://userpage.chemie.fu-berlin.de/biochemie/protprak.html).

Notes

1. Proteins are easily altered or destroyed, when thawed or frozen. Therefore, serum samples should not undergo more than 1 or 2 freeze-thaw cycles prior to the assay. The handling, including the freeze-thaw cycles should be well documented, occurring differences of the later results might be artefacts due to sample handling.
2. If serum is obtained and stored until use, prepare serum samples as soon as possible after collecting.

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CHAPTER 8

GENE-EXPRESSION SIGNATURE OF ADHESION/GROWTH-REGULATORY TISSUE LECTINS (GALECTINS) IN TRANSITIONAL CELL CANCER AND ITS PROGNOSTIC RELEVANCE

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Abstract

Aims

Lectins, and especially galectins, appear to be important in malignancy-associated processes. This study comprehensively analyzes the presence of galectins in urothelial tumours (UT).

Methods and Results

Non-cross-reactive antibodies against seven family members from the three subgroups (proto type: galectins-1, -2 and -7; chimera type: galectin-3; tandem-repeat type: galectins-4, -8 and -9) were used. Gene expression was monitored in specimens of normal urothelium, fresh tumour tissue and cell lines by real-time PCR. Presence and evidence for tumour-associated upregulation were revealed for galectins-1 and -3. This was less clear-cut for galectin-4 and -8. Galectin-7 was expressed in all cell lines; galectins-2 and -9 were detected at comparatively low levels. Galectin-2, -3 and -8 upregulation was observed in superficial tumours, but not in muscle-invasive tumours ($P<0.05$). Staining correlated with tumour grading for galectin-1, -2 and -8, and disease-dependent mortality correlated with galectin-2 and -8 expression. Binding sites were visualized using labelled galectins.

Conclusions

Our results demonstrate a complex expression pattern of the galectin network in urothelial carcinomas. Galectin-1, -2, -3 and -8 are both potential disease markers and also possible targets for bladder cancer therapy.

Introduction

The incidence of urothelial carcinoma, the fifth most common tumour in industrialized countries, is increasing and with it the financial burden of its diagnosis and treatment. An analysis of Medicare payments shows that bladder cancer entails very high per-patient costs from diagnosis to death due to frequent treatment and intensive surveillance. In fact, because of the lifelong need for monitoring and treatment for recurrent tumours, the cost per patient with bladder cancer is the highest among all cancer types.[1] Urothelial tumours are a heterogeneous group including low-grade superficial tumours (pTa, pT1 G1-2) with an intermediate risk of recurrences and a low risk of disease progression, high-grade superficial tumours (pTa, pT1 G3) with a high risk for tumour progression and recurrence, and muscle-invasive tumours (≥pT2) with an overall survival of only 50 %. While improved therapies generally prolonged life expectancy of many cancer patients during the last 15 years, no

extended survival was documented in patients with muscle-invasive urothelial cancer.
The potential for improving diagnostic procedures and therapeutic management of urothelial cancer motivates the search for new disease markers. Ideally, they should directly be involved in carcinogenesis. In this respect, the recent insights into the fundamental roles of cellular glycans as sugar-encoded signals controlling adhesion, growth or tissue invasion gives research a clear direction.[2] Distinct carbohydrate determinants are the targets of endogenous lectins, and this specific interaction leads to intracellular signalling which can then modulate adhesion and growth control.[3,4] Preferential docking sites in this process are the spatially accessible glycan epitopes at branch ends. Of note, they are the site of frequent substitutions introduced enzymatically to swiftly alter the physiological meaning of the respective sugar code word.[5] On the protein side, such β-galactoside termini are specifically bound by members of the galectin family. The ligand selection and post-binding effects of lectins underlie the same levels of specificity and efficiency as known from protein-protein interactions. Galectin-1, for instance, exerts its growth-regulatory role on carcinoma and neuroblastoma cells in culture via binding to sugar chains of the fibronectin receptor and of ganglioside GM_1, respectively, with impact on p27 expression in carcinoma cells.[6-8] Also, galectin-1 is involved in tissue invasion of glioblastoma cells.[9] In addition to the exquisitely specific interplay with cell surface glycans galectins are also engaged in intracellular recognition processes. Family members -1 and -3 are (among other proteins) binding partners of either oncogenic H-Ras or activated K-Ras. This association accounts for signal routing to Raf-1 at the expense of phosphoinositide 3-kinase or to pathways with attenuation of activity of extracellular signal-regulated kinase.[10,11] With relevance for urothelial cancer, overexpression of galectin-3 in J82 bladder carcinoma cells led to an Akt-dependent escape from TRAIL-induced apoptosis.[12] Thus, the proteins of this lectin class appear to be versatile effectors, and our current knowledge suggests that expression analysis of galectins in urothelial malignancies may yield insights into their role in this process.

Galectin presence in urothelial cancer was initially inferred by the application of neoglycoproteins, and further studies focused on two proteins of this family.[13-16] Galectins-1 and -3 were found to be present in bladder tumours, and strong positivity in high-grade tumours implied a role in disease progression.[17-19] Of note, expression profiling for galectins-2 and -8 in a large panel of human organs and tumours had previously revealed their

presence in bladder cancer, and proteome monitoring of bladder squamous cell carcinomas pinpointed galectin-7, a predictor of recurrence in hypopharyngeal tumours, as indicator of tumour differentiation.[20-23] When combined, these results underscore the importance of a study testing the mentioned galectins in parallel. Consequently, we herein report fingerprinting for seven human galectins, first by RT-PCR and then by immunohistochemistry using a panel of non-cross-reactive antibodies. Results on 61 cases were correlated to grading, staging and survival. In addition, we tested biotinylated galectins as probes. These experiments provide first evidence on galectin binding in urothelial cancer.

Materials and Methods

Galectin purification and labelling

Recombinant production of human galectins, their purification from extracts using affinity chromatography on lactosylated Sepharose 4B, obtained by ligand coupling to divinyl-sulfone-activated resin, as crucial step as well as quality controls by one- and two-dimensional gel electrophoresis and silver staining, gel filtration and mass spectrometry were performed as previously described.[24-26] The proteins were labelled by biotinylation using the N-hydroxysuccinimide ester of biotin under activity-preserving conditions, excess of reagent was removed by extensive dialysis, extent of labelling determined electrophoretically by high-resolution 2D gel electrophoresis and maintenance of carbohydrate-binding activity ascertained by solid-phase and cell-binding assays and galectin histochemistry using positive controls.[27-30]

Antibody production and quality controls

Polyclonal antibodies were raised in rabbits, immunoglobulin G fractions were purified by chromatographic fractionation of serum on protein A-Sepharose 4B resin (Amersham Biosciences, Freiburg, Germany), and the specificity of each preparation was rigorously checked by Western blotting and ELISA assays.[23,31-33] In cases of cross-reactivity between an antibody and another tested member of the galectin family affinity depletion on Sepharose 4B-immobilized lectin was carried out to remove the cross-reactive part of the respective immunoglobulin G fraction, as for example done for antibodies raised against galectin-2 to exclude minor activity against galectins-1 and -3 and against galectin-7 to exclude minor activity against galectins-1 and -4, respectively, followed by another round of quality controls.[22]

Reverse transcriptase-PCR

Three urothelium tumour cell lines (UM-UC 3, RT 112, HT 1197), the normal urothelium cell line UROtsa, normal urothelium from non-bladder cancer patients and urothelial tumours were analyzed. Cell lines were cultured by standard methods in E-MEM (HT 1197) or in D-MEM (UM-UC 3, RT 112, UROtsa) medium, respectively. All culture media contained L-glutamine (final concentration of 2mM) and were supplemented with 10% fetal calf serum. Bladder tumour tissue was obtained during tissue removal for routine pathological examination (approval by the scientific ethical committee of the University Hospital Mannheim, Faculty of Medicine, University of Heidelberg, Germany). Tumour tissue was immediately snap-frozen in liquid nitrogen and kept at -80°C until further use. pTa G1 (n=4) and ≥pT2 G3 (n=7) transitional cell tumours (TCC) of bladder and upper urinary tract were used for further analysis. RNA extraction and processing as well as primer design and RT-PCR analysis were performed as described previously.[33]

Lectin and immunohistochemical staining

A total of 61 cases with urothelial carcinoma and normal urothelium (no history of UC) were subjected to analysis using galectin-specific antibodies and biotinylated galectins. Tumour stage and grade were assigned according to the World Health Organization classification from 1973.
Routinely, 3µm-thick paraffin sections, cut freshly to minimize decay in tissue immunoreactivity, were deparaffinized, rehydrated, and washed, endogenous peroxidase activity was then blocked by incubation with solution containing 1% H_2O_2 in methanol for 30 min. After a heat-induced antigen-retrieval step non-specific protein-binding sites and any sites with affinity for biotin were saturated, and the sections were incubated overnight at 4°C with primary antibodies or labelled lectins at a concentration of 10µg/mL. After using a second-step reagent in immunohistochemistry the extent of bound markers was visualized in both protocols by avidin-biotin-peroxidase complex kit reagents (Vectastain Elite, Vector Labs, Burlingame, CA, USA) with diaminobenzidine in 0.03% H_2O_2 solution as chromogenic substrate. Slides were counterstained with Mayer's haemalaun. Control sections were treated with pre-immune serum to exclude antigen-independent reactions or with kit reagents only to assess any probe-independent staining.
Level of staining of tissue specimens was evaluated using a semi-quantitative scoring system. This system yields an average staining intensity score for tumour cells of the entire specimen that accounts for tumour het-

erogeneity. All fields of a given specimen were systematically examined, and staining intensity and distribution were assessed. Staining was assigned to one of four different groups formed on the basis of the percentage of stained tissue; lack of staining received score 0, 0-20% staining a score of 1, 21-50% staining a score of 2, 51-80% a score of 3, and >80% staining a score of 4. Sections were evaluated in a blinded manner by two independent observers.

Statistical analysis

Relevant data were entered into a MS-Excel database and checked to spot any input error. After conversion into the SAS software system (version 8.2; SAS Institute, Cary, NC, USA) subsequent statistical computations followed, using data on tumour staging and grading and the non-parametric approach (U-test, Kruskal-Wallis test). Survival analyses were performed by the Kaplan-Meier method along with the Logrank test in the case of stratification according to disease progression. The CORR procedure and Cox regression analysis were used to define the correlation between galectin parameters and invasive tumour growth. All tests were two-sided. A p-value of ≤ 0.05 was considered to be statistically significant.

Results

Galectin fingerprinting by RT-PCR

In order to characterize the galectin network in bladder cancer beyond galectins-1 and -3, we first tested cDNA preparations from four different cell lines, normal urothelial cells and 10 urothelial tumours (pTa G1, pT2 G3, and pT3 G3) using RT-PCR. As summarized in Table 8.1, galectins other than family members 1 and 3 are differentially expressed in urothelial cancer cell lines and tumour tissues. The genes for the tandem-repeat-type galectins-4, -8 and -9 (the two latter ones present in two forms differing in linker length) are frequently expressed. Interestingly, presence of galectin-7-specific mRNA could only be detected in cell lines and normal urothelium, whereas galectin-2 positivity was only seen in one tumour case. These results support our concept to investigate galectin presence in tumours with a scope encompassing all three subgroups. Having prepared non-cross-reactive antibody fractions against these galectins, we proceeded to answer the question on immunohistochemical localization of the galectins tested positive in RT-PCR analysis.

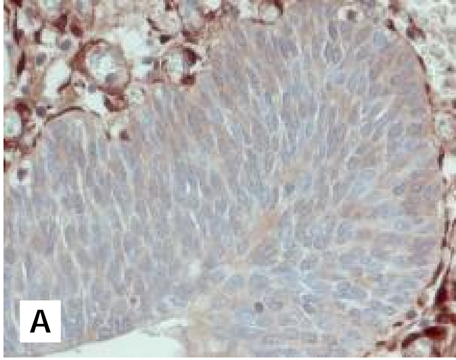
Table 8.1. Profiling of expression of galectin-specific mRNA in selected cell lines and tumour tissues determined by RT-PCR[a].

Cell lines		Gal-1	Gal-2	Gal-3	Gal-4	Gal-7	Gal-8[b]		Gal-9[b]	
							A	B	A	B
UM-UC 3		++	-	++	-	(+)	(+)	++	(+)	+
RT 112		++	-	++	-	++	(+)	++	++	(+)
HT 1197		++	-	++	(+)	(+)	++	+	++	+
UROtsa		++	-	++	(+)	++	(+)	++	++	-
normal urothelium		++	-	++	(+)	++	(+)	++	++	(+)
pTa G1	#22	87.2	-	85.0	29.1	-	-	3.6	11.4	-
	#72	68.2	-	119.0	-	-	-	12.7	-	-
	#76	62.0	-	85.2	15.0	-	-	12.7	-	-
pT2 G3	#05	114.3	-	106.3	-	-	3.5	5.0	-	-
	#07	81.1	-	93.3	-	-	6.7	6.9	8.4	1.5
pT3 G3	#98	76.4	-	74.1	28.5	-	-	5.8	17.9	-
	#41	82.7	-	76.0	17.3	-	-	-	7.1	5.2
	#93	82.2	-	93.2	-	-	-	-	20.9	6.8
	#10	79.3	8.8	88.7	59.5	-	6.7	6.9	30.6	12.6
	#12	91.2	-	135.4	27.1	-	3.5	-	-	-

[a] Semiquantitative grading was performed for specimen from cell lines, the β-actin loading control was used as standard for specimen from tumors.

[b] Amplification yielded the sequence of expected size and an isoform with extended linker length.

Figure 8.1. Morphological illustrations of immunhistochemically detected galectin expression in urothelium cancer. (A) Galectin-1 expression in urothelium (Gx400) demonstrating weak intracellular staining but intense staining of the lamina propria and the connective tissue. (B) Galectin-1 expression in a muscle-invasive pT2 G3 tumour (Gx200) with intense antigen expression intracellular in invasive cancer cells. (C) Galectin-3 with heterogeneous expression pattern in a high-risk pT1 G3 tumour depending on the degree of differentiation (Gx20). (D) Galectin-3 expression in a highly aggressive pT4 G3 urothel tumour (Gx400); strong staining intensity in tumour cell cytoplasm in contrast to weak reactivity in the connective tissue. (E) Galectin-4 expression in a pT1 G2 upper urinary tract tumour (Gx100). The tumour presents variable intensity of intracellular staining. (F) A muscle-invasive pT3 G3 tumour (Gx200) lacked presence of galectin-4 in contrast to the situation in the pT1 G2 tumour in panel E. (G) Galectin-7 expression in a pT4 G3 tumour was seen intracellularly, no nuclear staining was detected (Gx100). The surrounding tissue was negative. (H) Galectin-8 expression in a pT3 G3 muscle-invasive tumour (Gx200). Strong intracellular staining but no staining of nuclei and connective tissue was detected.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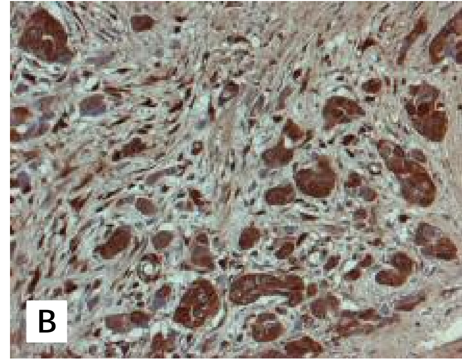
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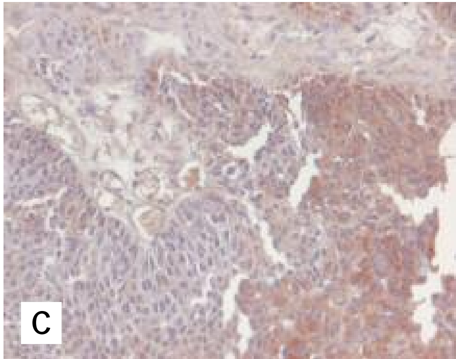
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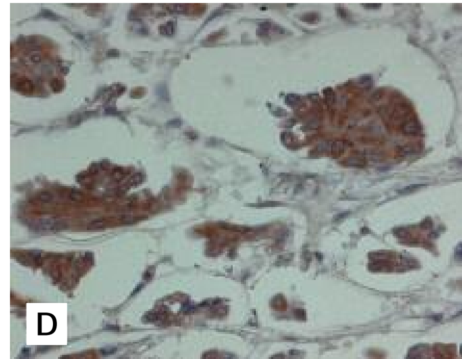
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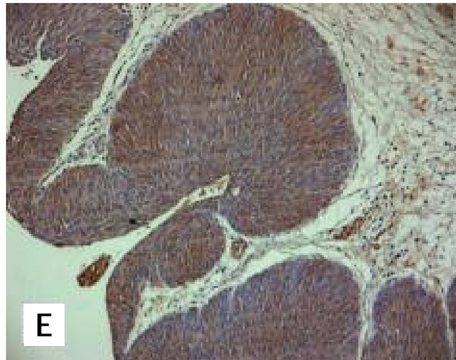
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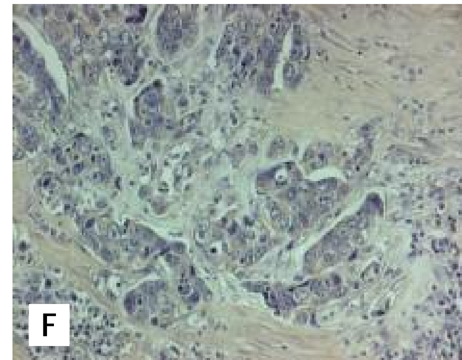
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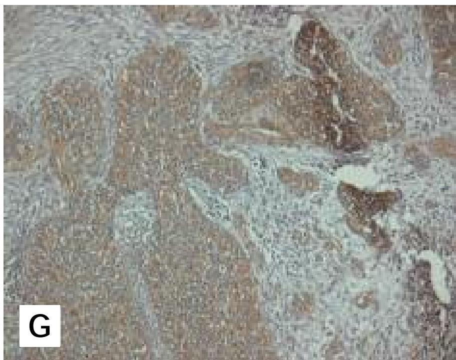
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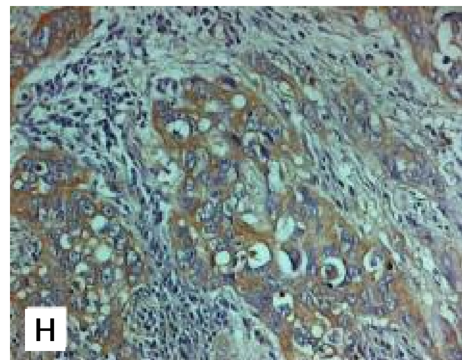
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Galectin fingerprinting by immunohistochemistry

Paraffin-embedded specimen of 61 urothelial carcinomas and of normal urothelium of the urinary bladder and the upper urinary tract were analyzed. The studied series included normal (n=6), pTa (n=11), pT1 (n=18), pT2 (n=11), pT3 (n=15), and pT4 (n=6) cases. Of the 61 patients with transitional cell carcinoma 11 were lymph-node positive at the time of surgical treatment. The follow-up period was 60 months.

Galectin-1 was abundantly present in connective tissue and muscle cells rather than in normal urothelium (Fig. 8.1A) or superficial transitional cell tumours. The staining was predominantly detected in the cytoplasm, almost no nuclear positivity was found. In contrast, intense staining reactions were seen in muscle-invasive high-grade tumours (Fig. 8.1B), whereas stroma tissue was conspicuously less reactive. In tumour cells, lectin presence was mainly seen in the cytoplasm.

Galectin-2 staining showed different intensity patterns in superficial and muscle-invasive urothelial tumours, with labellling on average being more intense in muscle-invasive tumours. This protein was almost exclusively present in the cytoplasm. Connective tissue was rather weakly positive, also an inherent quality control for the antibody (data not shown).

The distribution of galectin-3 was heterogeneous in urothelial tumours. Whereas low-grade low-stage tumours showed weak or no staining, high-risk pT1 G2 and G3 tumours presented areas with strong cytoplasmic positivity. Nuclear staining was not observed, the connective tissue was almost devoid of positivity. Figure 8.1C illustrates this typically heterogeneous staining pattern in a section of a pT1 G3 urothelial tumour. As shown in Figure 8.1D, strong galectin-3 staining in the cytoplasm of invasive urothelial tumour cells was detected. The majority of the muscle-invasive tumours harboured an intense cytoplasmic positivity.

Galectin-4 was weakly detectable in normal urothelium, muscle and connective tissue. Extent of its presence varied among urothelial carcinomas. The obtained results ranged from no staining to a strong reaction in pTa and in muscle-invasive tumours, with a tendency towards marked staining in superficial tumours. High-grade high-stage carcinomas were frequently negative (Fig. 8.1E-F). Nuclear staining was sporadically detected.

Galectin-7 staining revealed inter-individual differences in urothelial tumour tissue, whereas the majority of normal bladder urothelium demonstrated no or very weak positivity. Strong extent of staining was seen in two normal urothelium tissue specimens from the upper urinary tract; the respective kidneys had been removed due to chronic infections, which might explain this noted difference in staining features. Muscle invasive

tumours showed intense staining of the cytoplasm (Fig. 8.1G). Galectin-8 was detected at a moderate level in the cytoplasm of normal tissue and low-grade low-stage carcinomas, whereas it was strongly expressed in high-grade high-stage tumours (Fig. 8.1H) and in some cases of the pT1 G2/3 cohort.

Clinical correlations

The immunohistochemical profiles of galectin presence in the urothelial carcinomas were set into relation to tumour parameters of cases without muscle- (pTa, pT1) and those with muscle-invasion (≥pT2). As listed in Table 8.2, significant correlations to T staging were found with galectins-2 ($p=0.001$), -3 ($p=0.01$) and -8 ($p<0.0001$). The proportion of tumours expressing galectin-1 increased with advancing T stages, but failed to reach the level of statistical significance. Using the Spearman correlation coefficient the group of muscle-invasive tumours appeared to be characterized by presence of galectins-7 and -8 at a statistically significant level ($p=0.04$), the Cox regression analysis delineated a predictive positive correlation between galectins-1, -7 and -8 and muscle-infiltrating growth ($p=0.03$). Grading significantly correlated with data for galectins-1 ($p=0.01$), -2 ($p=0.03$), and -8 ($p=0.02$), whereas the results with galectin-3 failed to reach the level of statistical significance ($p=0.08$).
Files of clinical follow-up for at least five years were available for 57 patients (93 %); 24 patients died due to disease progression and four patients died due to other reasons. Presence of galectins-2 and -8 correlated significantly with disease-specific mortality ($p=0.02$), while data on galectins-1, -3, -4 and -7 failed to reach statistical significance (Table 8.2). The lymph-node-positive tumours were not separately analysed because of their small number. Having herewith detected correlations between the expression of distinct galectins and clinical parameters, it is likely that the interplay of certain lectins with their ligands can influence tumour biological aspects.

Table 8.2. Statistical evaluation of correlations between immunohistochemical galectin fingerprinting and clinical parameters. (*) $p<0.05$ statistically significant.

	p-value					
	Gal-1	***Gal-2***	***Gal-3***	***Gal-4***	***Gal-7***	***Gal-8***
[a] Staging (pTa/1 versus ≥pT2)	0.11	0.001*	0.01*	0.29	0.11	<0.0001*
[b]Grading (G1/2 versus G3)	0.01*	0.03*	0.08	0.30	0.40	0.02*
5 year survival	0.16	0.02*	0.23	0.84	0.94	0.02*

Evidently, lectin properties in this respect are non-identical, intimating different profiles of binding sites. We tested this hypothesis by applying labelled galectins as histochemical tools to map accessible binding sites.

Tumour reactivity with galectins

Sites with reactivity to galectin-1 were abundantly present in connective and muscle tissue of normal urothelium and in tumour-associated stroma. Cytoplasmic positivity for the labelled lectin was increased in invasive tumour cells (Fig. 8.2A, black arrow head), whereas nuclear staining was rarely found. Galectin-2-binding sites were observed at a moderate level in tumour and stroma cells (Fig. 8.2B). Biotinylated galectin-3 was reactive with tumour cells and tumour-associated stroma at a medium level. In a minority of muscle-invasive tumours no binding sites were detectable (Fig. 8.2C). Figure 8.2D illustrates intense membrane staining (black arrow head) with labelled galectin-4. Galectin-7-binding sites were rarely detectable in superficial bladder cancer, whereas a strong signal was seen intracellularly (black arrow head) in muscle-invasive tumours (Fig. 8.2E and F). These data revealed that sites specific for galectins can be detected by employing the tissue lectins as histochemical tools.

Discussion

Our data illustrated a remarkable intra-family diversity of galectin expression in urothelial cancer profiles. The agreement to a previous study reporting increased expression of galectins -1 and -3 in 38 tumour cases, the latter attributed to an early event in bladder carcinogenesis, can be considered as internal quality control.[17] Of note, nuclear localization of galectin-3, an independent predictive parameter of recurrence in squamous cell carcinoma of the lung[34], was not observed in this tumour class in our study. The overexpression of galectin-1 in high-grade tumours did not turn out to be a unique feature within this family. In addition to galectin-1 the expression of family members -2 and -8 was correlated to grading at a significant level. Setting further clinical parameters into correlation to galectin expression, staging also revealed a statistically valid connection. It was most pronounced in the case of galectin-8 and then decreasing but still reaching the level of significance for galectins-2 and -3. Cox regression analysis delineated a positive correlation between muscle-invasive growth and the presence of galectins-1, -7 and -8. Thus, statistically, there is a link between clinical parameters and galectin family members other than galectins-1 and -3.

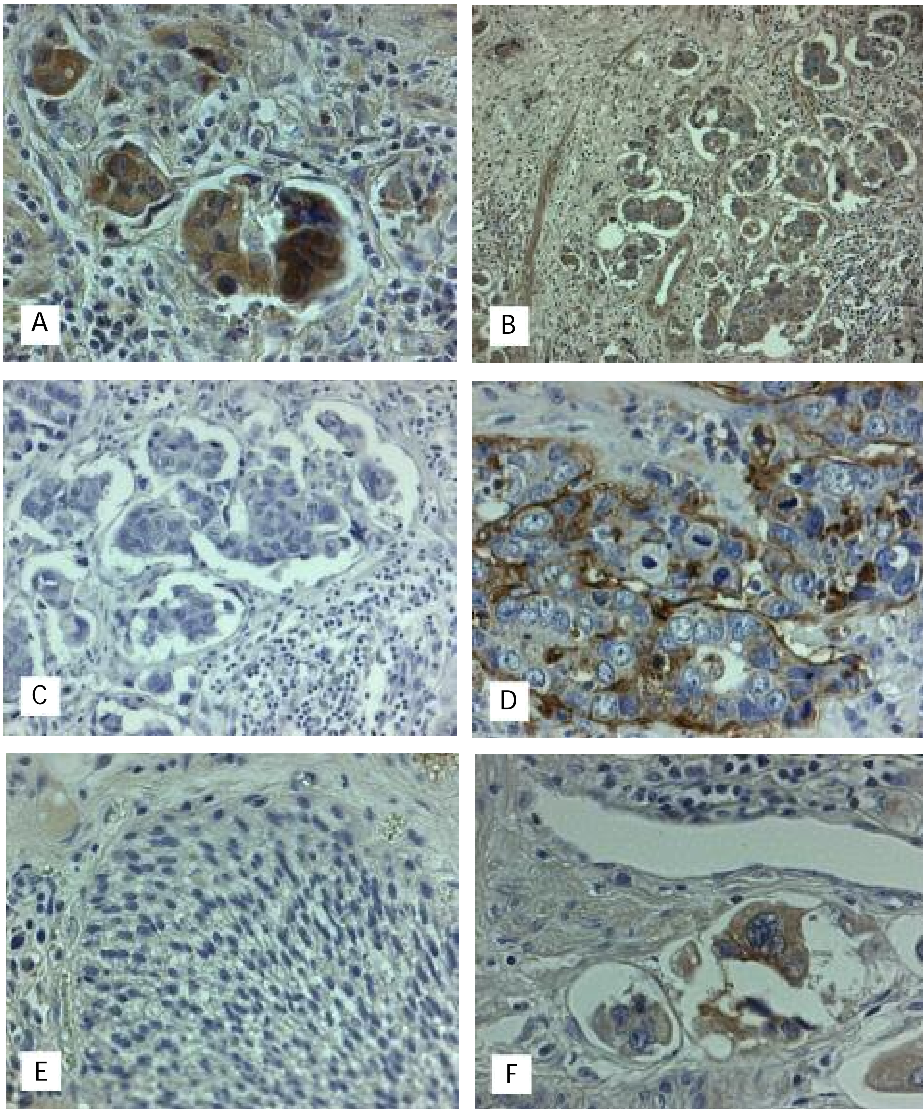

Figure 8.2. Morphological illustrations of lectin-histochemically detected galectin-binding sites in urothelial tumours. (A) Intracellular signal for galectin-1-binding in invasive tumour cells (Gx400). (B) Intracellular binding sites for galectin-2 in tumour cells (Gx200). (C) Lack of binding sites for galectin-3 was detected in this pT4 G3 tumour (Gx400). (D) Intense staining by labelled galectin-4 at tumour cell membranes and moderate intracellular binding were observed (Gx400). (E) No binding of labelled galectin-7 was detected in this pT1 G3 tumour (Gx200). (F) Intracellular binding of galectin-7 in tumour cells with high mitotic activity. No nuclear binding was detected (Gx400).

Two galectins with impact on disease-dependent mortality were singled out, i.e., the homodimeric proto-type galectin-2 and the tandem-repeat-type galectin-8. Because the other members of the two mentioned subgroups failed to yield a statistically significant correlation, it is reasonable to assume non-redundant functions. They are reflected in the distinct ligand profiles described so far for these two galectins, with α- and β-tubulins for galectin-2 and promatrix metalloproteinase-9/α_M-,α_3-,α_6- and β_1-integrins/serum fibronectin for galectin-8.[35-38] The interaction with integrins qualifies galectin-8 as a potent matricellular regulator of cell adhesion.[35] Because a relatively high labelling index had also been defined as negative prognostic factor in Dukes C and D colon tumours, this cellular activity may underlie the negative association with survival for these two carcinoma types.[39] A similar correlation between colon and urothelial carcinoma survival concerning tumour metabolism, using the transketolase-like-1 pathway in highly aggressive subtypes, was recently reported.[40] Because the fine-specificity of galectins towards natural glycans can differ and library approaches have been introduced to define selective inhibitors,[41-43] these results identify galectin-8 as a potential target for developing blocking compounds. Besides glycan ligands galectin-8 - and also galectin-2 - may interact with not yet characterized cytoplasmic ligand(s), as documented for galectins-1 and -3 and oncogenic H- or K-Ras, respectively.[10,11,44,45]
It is worth noting that when galectin-3 is introduced into bladder cancer cells, it activates Akt and confers resistance to TRAIL.[12] Phosphorylated galectin-3 (p-galectin-3) has been identified as a novel regulator of PTEN, which also regulates Akt.[46] The PI3K/Akt signalling pathway directly promotes malignant transformation and enhances aerobic glycolysis, which is a new and promising target in anticancer therapy. However, data from transfected cell cultures have to be analyzed very critically before these finding can be applied in a clinical setting. In light of the galectin histochemistry data presented here, the analysis of the spectrum of galectin binding partners is likely to turn biochemical insights into novel rational approaches for therapeutic interventions. In conclusion, our study reveals the complexity of the galectin network in urothelial cancer, the diagnostic potential of these observations and a perspective on the therapeutic implications.

Acknowledgements

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CHAPTER 9

EXPRESSION OF TRANSKETOLASE TKTL1 PREDICTS COLON AND UROTHELIAL CANCER PATIENT SURVIVAL: WARBURG EFFECT REINTERPRETED

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Abstract

Tumours ferment glucose to lactate even in the presence of oxygen (aerobic glycolysis; Warburg effect). The pentose phosphate pathway (PPP) allows glucose conversion to ribose for nucleic acid synthesis and glucose degradation to lactate. The nonoxidative part of the PPP is controlled by transketolase enzyme reactions. We have detected upregulation of a mutated transketolase transcript (TKTL1) in human malignancies, whereas transketolase (TKT) and transketolase-like-2 (TKTL2) transcripts were not upregulated. Strong TKTL1 protein expression was correlated to invasive colon and urothelial tumours and to poor patient outcome. TKTL1 encodes a transketolase with unusual enzymatic properties, which are likely to be caused by the internal deletion of conserved residues. We propose that TKTL1 upregulation in tumours leads to enhanced, oxygen-independent glucose usage and a lactate-based matrix degradation. As inhibition of transketolase enzyme reactions suppresses tumour growth and metastasis, TKTL1 could be the relevant target for novel anti-transketolase cancer therapies. We suggest an individualised cancer therapy based on the determination of metabolic changes in tumours that might enable the targeted inhibition of invasion and metastasis.

Introduction

Cancer is now viewed as a disease resulting from cancer-causing genes that deregulate cellular proliferation, differentiation, and death. Genetic alterations acquired by tumours also modify their biochemical pathways, resulting in abnormal metabolism. Warburg proposed a model of tumourigenesis involving altered energy production in tumours. He identified a particular metabolic pathway in carcinomas characterized by the anaerobic degradation of glucose even in the presence of oxygen (aerobic glycolysis) that leads to the production of large amounts of lactate (known as the Warburg effect).[1] The relevance of aerobic glycolysis to cancer cell biology remains controversial.[2, 3] However, the widespread clinical use of positron-emission tomography (PET) for the detection of aerobic glycolysis in tumours and recent findings have rekindled interest in Warburg's theory. Studies on the physiological changes in malignant conversion provided a metabolic signature for the different stages of tumourigenesis[4]; during tumourigenesis an increase in glucose uptake and lactate production have been detected. The fully transformed state is most dependent on aerobic glycolysis and least dependent on the mitochondrial machinery for ATP synthesis.[4]

Other important links between cancer-causing genes and glucose metabolism have been already identified. Activation of the oncogenic kinase Akt has been shown to stimulate glucose uptake and metabolism in

cancer cells and renders these cells susceptible to death in response to glucose withdrawal[5]. Such tumour cells have been shown to be dependent on glucose because the ability to induce fatty acid oxidation in response to glucose deprivation is impaired by activated Akt.[6] In addition, AMP-activated protein kinase (AMPK) has been identified as a link between glucose metabolism and the cell cycle, thereby implicating p53 as an essential component of metabolic cell-cycle control.[7] These findings suggest that tumourigenesis requires dearangements in known cancer-causing genes and altered energy production.

Despite this appreciation, the reason enhanced anaerobic glucose degradation occurs in tumours remains elusive. If carcinogenesis occurs by somatic evolution, then common components of the cancer phenotype result from active selection, and must, therefore, confer a significant growth advantage.[3] A prerequisite for the understanding of the altered glucose metabolism in tumours and its predicted selective growth advantage is the detailed analysis of glucose degrading pathways.

Two main pathways of glucose degradation have been identified. The observation in the 1930s that muscle extracts can catalyse the glycolysis of glucose to lactate led to the identification of the Embden-Meyerhof pathway. In this pathway, fructose-1,6-diphosphate is cleaved leading to pyruvate, which is reduced to lactate in the absence of oxygen. In addition to this pathway, glucose is also degraded by the pentose phosphate pathway (PPP).

The nonoxidative part of the PPP is controlled by thiamine- (vitamin B1) dependent transketolase enzyme reactions. Transketolase enzyme reactions of the nonoxidative part of the PPP enable oxygen-independent glucose degradation, and play a crucial role in nucleic acid ribose synthesis utilizing glucose carbons in tumour cells. More than 85% of ribose recovered from nucleic acids of certain tumour cells is generated directly or indirectly from the nonoxidative pathway of the PPP[8]. The importance of transketolases for tumour cell metabolism is underlined by the fact that the application of specific transketolase inhibitors to tumours induces a dramatic reduction in tumour cell proliferation.[9] In addition, the activation of transketolases by application of thiamine stimulates tumour growth[10]. Furthermore, several natural products have been reported to inhibit transketolase enzyme activity *in vitro*, and also to inhibit cell proliferation or suppress tumour growth in mouse models or cancer patients as a result of reduction of transketolase activity.[11-15]

To establish a transketolase-inhibitory anti-cancer therapy, a high throughput screening of library compounds using recombinant human transketolase (TKT) has been performed. This approach resulted in the identification

of two novel small-molecule inhibitors, which inhibit human TKT and suppress proliferation of cancer cell lines.[16]

So far, three human transketolase genes have been recognised, and the relative contributions of TKT, transketolase-like-1 (TKTL1), and transketolase-like-2 (TKTL2) to tumour-specific transketolase metabolism have not been investigated. Here, we provide evidence that TKTL1 mRNA and protein are specifically overexpressed in tumours, whereas TKT and TKTL2 expression are not upregulated. We demonstrate that TKTL1 protein is expressed in invasive tumours and predicts poor patient survival in colon and urothelial cancer. Our findings strongly indicate that overexpression of TKTL1 is responsible for the observed tumour-specific effects of transketolase enzyme reactions, and represents the basis for the observed inhibition of proliferation of cancer cells by anti-transketolase approaches.

Materials and Methods

Real-time PCR and Western blot

Real-time PCR-based transcript quantification and Western blot analysis have been described previously.[17]

Patients

In this study, 55 men and 15 women (median age of 60 ± 15 years) with colon adenocarcinoma were included. All patients underwent colectomy, and 30% of colon adenocarcinomas were diagnosed as non-invasive (pTis), whereas 70% of colon adenocarcinomas were invasive at the time of diagnosis. Eleven tumours were classified as pT1, 8 as pT2, 14 as pT3, and 16 as pT4, according to the UICC classification.

A total of 64 patients (median age 67.5 years) with urothelial carcinoma were enrolled in this study. Of these, 59 patients underwent treatment for urothelial carcinoma in which: 21 underwent transurethral resection for superficial bladder cancer; 22 underwent radical cystectomy; 16 underwent surgery for upper urinary tract carcinomas; and 5 underwent surgery for benign reasons. At the time of surgery, 14% (8 out of 59) of the patients had lymph node metastases. Overall, 28 tumours were classified as non-muscle invasive (pTa, pT1 and carcinoma *in situ*) and 31 were classified as muscle-invasive (^{3} pT2). Overall, 42% of the tumours showed no or only weak staining (staining score 0 or 1), 10% showed some staining (score 2), and 48% showed strong staining (score 3).

Patient samples

Surgical resection specimens were obtained from the Department of Surgery, University Hospital Mannheim, Faculty of Clinical Medicine of Ruprecht-Karls-University Heidelberg, Germany (approval by the local Ethics Committee), and the University of Palermo, Department of Surgical and Oncological Science, Surgery Pathophysiology Section. None of the patients received neoadjuvant radiotherapy or chemotherapy. From each patient, cancerous and normal tissue was available. For RNA extraction, the specimens taken during the operation were immediately snap-frozen in liquid nitrogen and subsequently stored at -80°C until use. For immunohistochemistry (IHC), the specimens were fixed in 3.4% buffered formalin for 24h and embedded in paraffin. Histological diagnosis was performed by three independent, experienced pathologists (A.z.H.; R.G.; G.S.).

Immunohistochemical staining

Three to 5 μm thick paraffin sections were analyzed by IHC. Dewaxed sections were heated for antigen unmasking in 10 mM sodium citrate (pH 6.0) in a microwave oven for 1 minute at 450 W followed by 5 minutes at 100 W. After rinsing in dH_2O, inhibition of endogenous peroxidase was performed with a 5 min incubation with 3% H_2O_2. Endogenous avidin-biotin was blocked by the use of a commercial biotin blocking system (DAKO) for 10 min. After two washes in Tris/saline buffer (TBS), slides were incubated with 1% goat serum for 30 min to block unspecific staining. Sections were subsequently exposed to mouse anti-TKTL1 (clone JFC12T10; mouse $IgG2_b$) antibody (15 μg ml^{-1}) or anti- Ser473 phospho-Akt (587F11; mouse $IgG2_b$; Cell Signaling Technology) overnight at 4°C. The monoclonal anti-TKTL1 antibody JFC12T10 has been described previously[17]. Slides were washed in TBS and incubated with biotinylated anti-mouse immunoglobulins for 30 min at room temperature and treated with streptavidin-peroxidase (DAKO). Staining was revealed using 3-amino-9-ethylcarbazole (AEC) substrate and counter-stained with haematoxylin (Fig. 9.2 M-T and Fig. 9.3).

Alternatively, primary antibodies were visualized with avidin-biotinylated horseradish peroxidase complex (ABC) and diaminobenzidine tetrahydrochloride (DAB) (Elite kit; Vector Laboratories), and counter-stained with Mayer's haematoxylin (Fig. 9.2 A-L).

For scoring of TKTL1 expression, a scale from 0 to 3 was defined as: score 0 indicates 0-20%, score 1 indicates 21-50%, score 2 indicates 51-80%, and score 3 indicates >80% of the tumour cells were stained for TKTL1.

Results

TKTL1, but not TKT or TKTL2, mRNA is overexpressed in carcinomas

Identification of genes selectively expressed or overexpressed in tumours is a crucial prerequisite for molecular diagnosis and treatment of cancer by addressing molecular targets. To identify such targets, we used the real-time PCR technique. When comparing the transcript levels from five colon cancer tissues to nontumour samples from the same patients, we initially detected a 35-fold overexpression of TKTL1 in one colon carcinoma sample. Since TKTL1 is one of three highly similar transketolases encoded by three separate genes (TKT, TKTL1, TKTL2[17, 18]), we designed primers to specifically discriminate expression of the three transketolase genes in human carcinomas.

Using these primers, a 79-fold overexpression of the TKTL1 gene was identified in one colon carcinoma tissue, whereas none of the tested colon carcinomas showed an overexpression of the TKT transcript. In contrast, the TKTL2 transcript was downregulated more than 10-fold in three out of five colon carcinomas (Table 9.1). To test whether overexpression of the TKTL1 transcript occurs in other tumour types, cDNA from five gastric and five lung adenocarcinomas and their corresponding normal tissues were analysed using the real-time PCR technique. Two of five gastric carcinomas and two of five lung adenocarcinomas had greater than 10-fold overexpression of TKTL1 (Fig. 9.1A), whereas TKT expression was unchanged in all tested carcinoma tissues (not shown). Similar to its downregulation in colon carcinomas, TKTL2 expression was downregulated more than 10-fold in two of five lung adenocarcinomas.

In the colon carcinomas depicted in Table 9.1, the total amount of transketolase transcripts in four of five carcinoma tissues (Table 9.1; T2-T5) with an overexpression of TKTL1 (Table 9.1; T5). Overexpression of TKT was lower than that in normal tissue, even in the colon carcinoma tissue

Table 9.1. Fold overexpression of transketolase transcripts in colon carcinomas (T1-5) in comparison to their corresponding normal tissues.

	T1	T2	T3	T4	T5
Transketolase wild type (TKT)	1	1	1	1	1
Transketolase-like-1 (TKTL1)	1	1	1	1	79
Transketolase-like-2 (TKTL2)	1	-9500	-3	-12	-375

Negative numbers indicate fold downregulation. Results are mean values of three independent experiments.

was not detected in any of the 54 carcinoma tissue samples tested. The only transketolase gene overexpressed in carcinoma tissue was the TKTL1 gene.

TKTL1 protein is overexpressed in human carcinomas

In order to determine TKTL1 protein expression levels in human carcinomas, we performed IHC on 1030 human carcinomas derived from 16 different epithelial tumour entities using a monoclonal antibody (JFC12T10) that specifically detects TKTL1.[17] A gastric carcinoma specimen that we found to have 1000-fold overexpression of TKTL1 mRNA when compared to the corresponding normal tissue showed a strong overexpression of the TKTL1 protein on Western blot level (Fig. 9.1B) as well as a strong TKTL1 immunoreactivity on paraffin sections (Fig. 9.2C-G). Mainly cytoplasmic expression was detected. TKTL1 expression was restricted to tumour cells, and the surrounding stromal tissue showed no staining. In the corresponding normal tissue, no staining was detected (Figs. 9.2A and B). Immunohistochemical analysis of two gastric carcinoma samples without overexpression of TKTL1

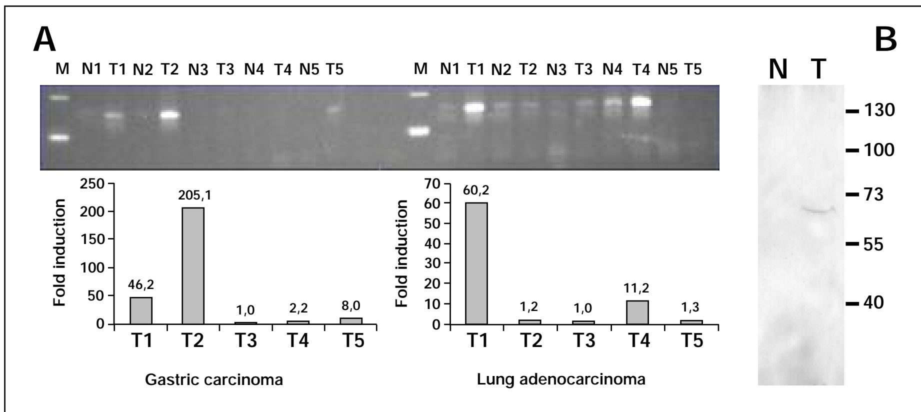

Figure 9.1. (A) Quantification of TKTL1 transcripts in gastric carcinoma and lung adenocarcinoma samples, and their corresponding normal tissues. 15 µl of the real-time PCR reaction was loaded onto a 3%-agarose gel to visualise the 150 bp TKTL1 amplification product. Expression differences between tumour and corresponding normal tissue were calculated, and are shown as fold induction in tumour sample relative to the corresponding normal sample. (B) TKTL1 protein expression in tumour and corresponding normal sample of a gastric carcinoma patient with a tumour-specific overexpression of TKTL1 on the transcript level was evaluated by Western blot with antibody JFC12T10. An over-expression of TKTL1 protein was observed in the tumour sample (T) compared to its corresponding normal tissue (N). Sizes of the protein marker are indicated in kDa.

transcript did not reveal TKTL1 protein expression. These results demonstrate a strong correlation of TKTL1 mRNA and protein expression.

In some cases of undifferentiated gastric carcinoma, strong nuclear expression was observed (Figs. 9.2H and I). Analysis of bladder carcinomas showed absence of TKTL1 reactivity in superficial, nonmuscle-invading tumours (Fig. 9.2J), whereas invasive tumours showed immunoreactivity (Figs. 9.2K and L). Non-small-cell lung carcinomas (NSCLC) (Fig. 9.2M), breast carcinomas (Fig. 9.2N), follicular thyroid carcinomas (FTC) (Fig. 9.2O), papillary thyroid carcinomas (PTC) (Fig. 9.2P), prostate carcinomas (Fig. 9.2Q), pancreas carcinomas (Fig. 9.2R), and undifferentiated thyroid (UTC), ovarian, cervix, rectal, and kidney carcinomas (not shown) also showed strong upregulation of TKTL1. Similar to bladder carcinomas, no or weak reactivity for TKTL1 was observed in noninvasive colon carcinomas (Fig. 9.2S), whereas in invasive tumours, strong TKTL1 staining was detected (Fig. 9.2T). All his-

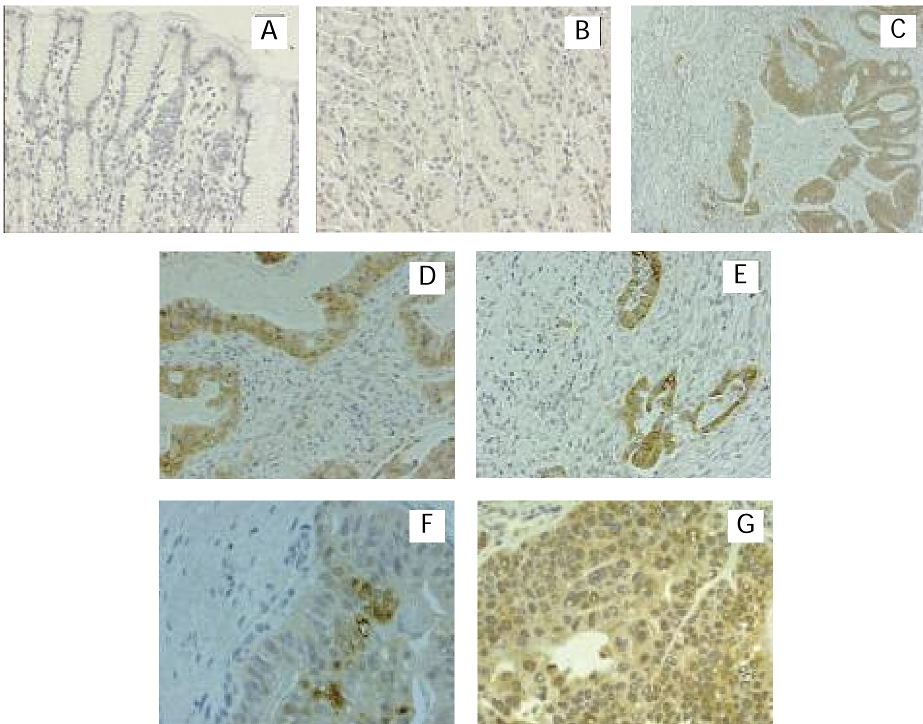

Figure 9.2A-G. Expression of TKTL1 in normal and carcinoma tissues. Specimens of a gastric carcinoma (**C-G**) and corresponding normal tissue (**A, B**); (**A, B**) No expression of TKTL1 in normal tissue. (**C-G**) Strong cytoplasmic expression in tumour tissue, but no expression in the surrounding stroma cells. Note the elevated expression within the inner region of the tumour (**F**).

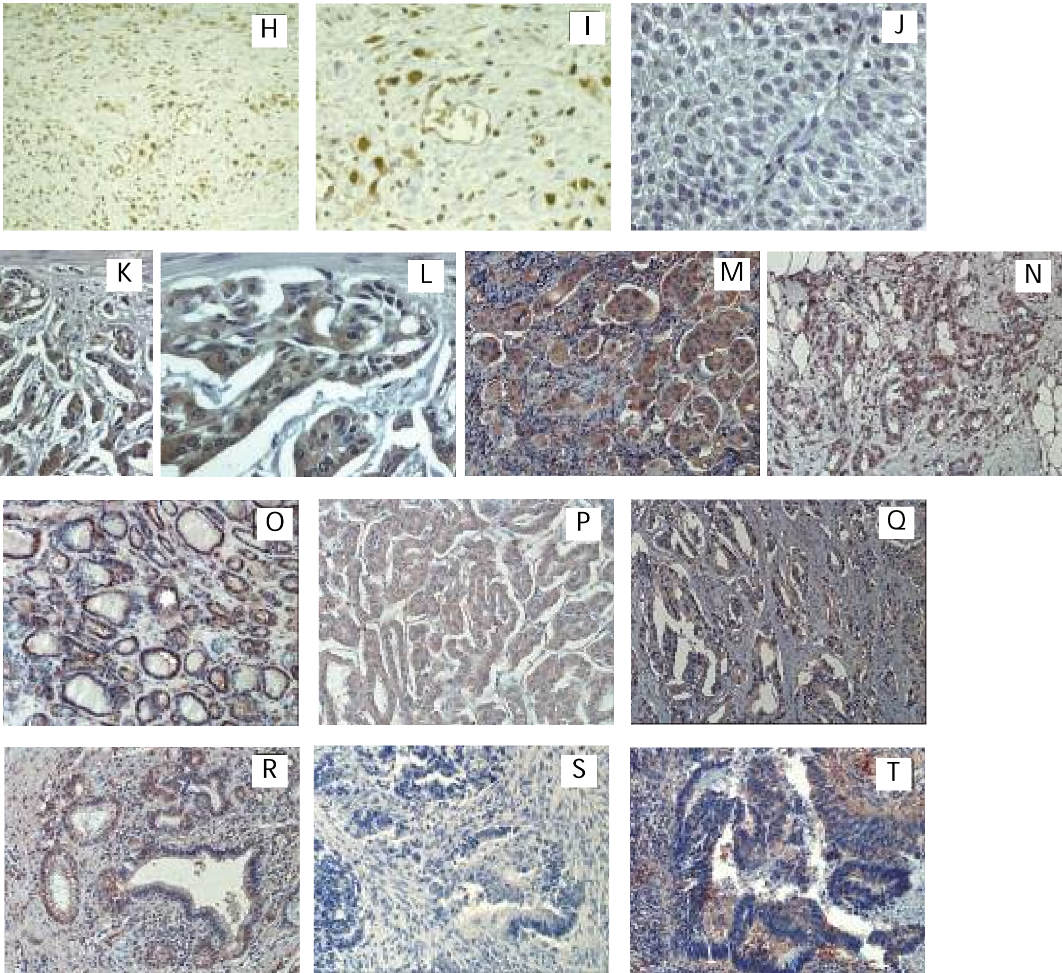

Figure 9.2H-T. Expression of TKTL1 in normal and carcinoma tissues. (H, I) Nuclear TKTL1 expression in a poorly differentiated gastric carcinoma. (J) No expression of TKTL1 in a superficial, Ta bladder carcinoma. (K, L) Strong TKTL1 cytoplasmic expression in an invasive, poorly differentiated bladder carcinoma. Strong TKTL1 upregulation in carcinomas of the lung (non-small cell lung carcinomas; M), breast (N), thyroid (follicular thyroid carcinoma (O), papillary thyroid carcinoma (P)), prostate (Q), and pancreas (R). No expression of TKTL1 in a non-invasive colon carcinoma (S), and strong expression in an invasive colon carcinoma (T). Anti-TKTL1 was revealed by diaminobenzidine tetrahydrochloride (DAB; brown staining) (A-L) and 3-amino-9-ethylcarbazole (AEC; red staining) (M-T).

tological variants of thyroid cancer (FTC, Fig. 9.2O; PTC, Fig. 9.2P; UTC, not shown) revealed abundant TKTL1 expression within the cytoplasm. Interestingly, the majority of nuclei were stained in FTC (Fig. 9.2O), whereas in PTC (Fig. 9.2P), and UTC, (not shown) only few nuclei were TKTL1-positive. Non-small-cell lung cancer cells were strongly positive for TKTL1 within the cytoplasm, whereas nuclear staining was absent (Fig. 9.2M).

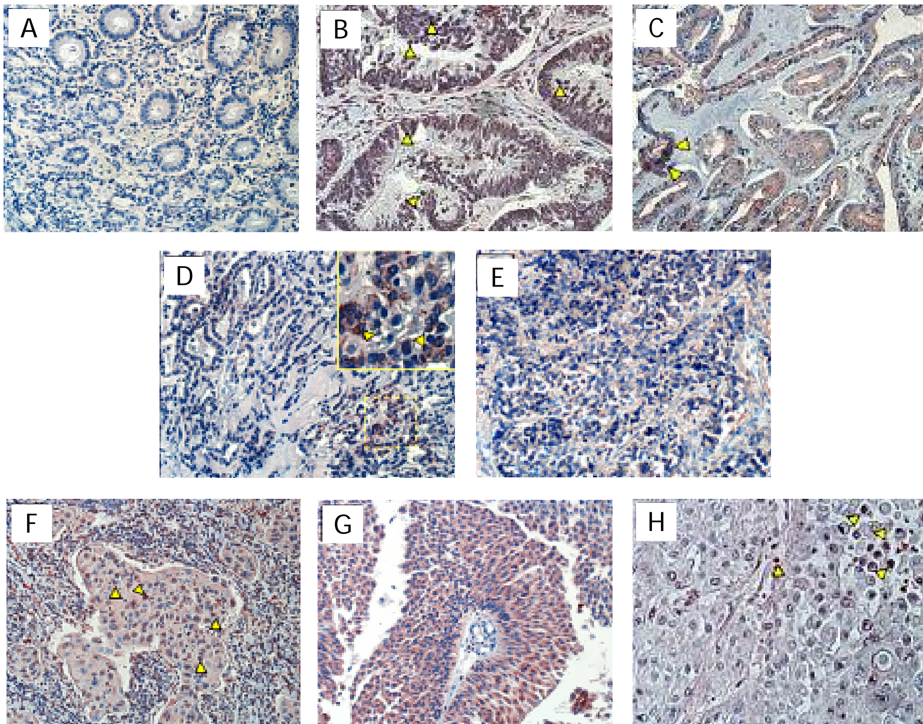

Figure 9.3. Upregulation of phosphorylated Akt (pAKT) in epithelial tumours. Immunohistochemical analysis of pAKT on paraffin-embedded sections from normal colon as negative control (**A**), colon cancer (**B**), papillary (PTC) (**C**), follicular (FTC) (**D**), undifferentiated thyroid carcinoma (UTC) (**E**), non-small cell lung cancer (NSCLC) (**F**), bladder cancer (**G**), and prostate cancer (**H**) (red staining). All the different types of cancer examined showed strong staining for pAkt (cytoplasmic, nuclear, or both cytoplasmic and nuclear), while normal tissues showed no or very weak staining (**A**). A mainly nuclear localization of pAkt was been detected in colon, lung, and prostate carcinomas (**B**, **F**, **H**; yellow arrowheads). In all the histological variants of thyroid and bladder cancers, strong cytoplasmic staining was detectable (**C-E**, **G**) and only few nuclei in PTC and FTC samples were positive for pAkt (**C**, **D**; yellow arrowheads).

Immunohistochemical localisation of TKTL1 protein and activated Akt

Recent studies have demonstrated that activated Akt exerts a direct influence on glucose metabolism leading to a dose-dependent stimulation of aerobic glycolysis[5], and to an inhibition of β-oxidation of fatty acids[6]. Therefore, we investigated whether tumours that overexpress the TKTL1 protein also have activated Akt (phospho-Akt; p-Akt) indicative of an activated glucose metabolism and an inhibited β-oxidation of fatty acids. We performed IHC to detect p-Akt in thyroid, lung, colon, bladder, and prostate cancer specimens (Fig. 9.3). All the different types of cancer examined showed strong staining for p-Akt in the cytoplasm, nucleus, and both the cytoplasm and nucleus, whereas normal tissues showed no or very weak staining (Fig. 9.3A). In lung, colon, and prostate carcinomas, mainly nuclear localization of p-Akt was seen (Figs. 9.3B, F and H; yellow arrowheads), whereas in all the histological variants of thyroid cancer, and

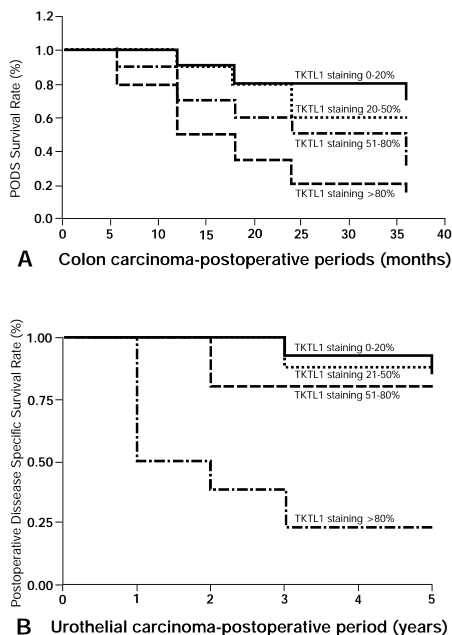

Figure 9.4. Kaplan-Meier plots demonstrating the significant correlation between TKTL1 staining intensity and survival in colon carcinoma (**A**), and in urothelial carcinoma (**B**). Scores indicate the fraction of tumour cells in each sample that stained for TKTL1 protein, as defined in the 'Materials and methods' section.

bladder cancer, strong cytoplasmic staining was seen (Figs. 9.3C-E, and G). Only few nuclei in PTC and FTC samples were positive for p-Akt (Figs. 9.3C and D, yellow arrowheads). Activated Akt was detected in 69% of carcinomas examined, whereas 83% of tested carcinoma specimens showed overexpression of TKTL1.

TKTL1 protein is overexpressed in invasive colon carcinomas and is associated with poor patient survival

In order to study the correlation of TKTL1 expression on clinical-pathological parameters, a retrospective survey of surgical samples from 70 patients (55 men and 15 women, median age of 60±15 years) with colon adenocarcinoma was performed. Non-neoplastic colon tissues examined did not reveal TKTL1 expression, and noninvasive colon cancer specimens were negative or barely positive for TKTL1 staining (Fig. 9.2S). In contrast, all invasive colon carcinomas showed TKTL1 expression (Fig. 9.2T), in particular, 20% were classified as weakly positive for TKTL1 (score 1+), 25% as positive (score 2+), and 55% as strongly positive (score 3+). A significant correlation between TKTL1 expression and survival was found (Fig. 9.4A).

TKTL1 protein is overexpressed in invasive urothelial carcinomas and predicts poor patient survival

To see if the above results could be extended to other tumour types, we performed a retrospective survey of urothelial carcinomas for expression of TKTL1. Of 64 surgical cases, 59 had malignancies of the urothelial tract. The correlation between tumour stage and TKTL1 protein staining intensity was 100% for score 0 in pTa superficial carcinomas, 76% score 0 or 1 and 24% score 2 or 3 in pT1 tumours. Of the muscle-invasive tumours (^{3}pT2) 3% had score 0,3% had score 1,13% had score 2, and 81% had score 3 TKTL1 staining. There was a statistically significant correlation between staging and staining intensity (Spearman correlation coefficient 0.836, confidence interval 0.76-0.92). The overall disease-specific 5-year survival rate was 47% (6 pTa, 14 pT1, 1 cis, 7 pT2). Patients dying because of tumour progression showed in 83% most intensive staining pattern of the tumours. Three patients were lost to follow-up and four patients died because of other reasons. Of 10 tumours initially found to be metastatic, nine showed strong overexpression of TKTL1 (grade 3). The correlation between staining intensity and patient survival was significant (P=0.001), and is shown in the Kaplan-Meier plot (Fig. 9.4B).

Discussion

In 16 epithelial tumour entities we tested, a subgroup of tumours was found to have upregulated TKTL1 protein. This demonstrates that TKTL1 upregulation is a general phenomenon in epithelial malignancies. To determine whether TKTL1 expression correlates with clinical outcomes of colon and urothelial cancer patients, we performed a retrospective survey of surgical samples and determined the survival of patients with tumours expressing or lacking TKTL1. We found that in colon carcinomas and urothelial carcinomas, expression of TKTL1 transketolase correlated with invasiveness of tumours and poor patient survival. As it is important to have diagnostic tests to distinguish between clinically aggressive and clinically indolent forms of cancer, our findings demonstrate that the expression of TKTL1 may indicate which tumours have invasive behaviour leading to poor patient survival.

Why does TKTL1 overexpression correlate with tumour invasion? Transketolase enzyme reactions control the nonoxidative part of the PPP. Using metabolic control analysis methods and oxythiamine, Comin-Anduix *et al* demonstrated that transketolase enzyme reactions determine cell proliferation in the Ehrlich's ascites tumour model[10]. The transketolase enzyme reactions and other reactions of the PPP allow glucose conversion to ribose for nucleic acid synthesis and generate NADPH, a reducing agent required for synthetic reactions. Both of these products of the PPP are required for growing tumour cells. In addition, the nonoxidative part of the PPP allows anaerobic glucose degradation; anaerobic conditions are often present in tumours and limit the growth of tumours. Even in premalignant lesions, which are often characterized as highly vascularised, near-zero partial pressures of oxygen are observed at distances of only 100 μm from blood vessels.[3] Tumour cells that upregulate transketolase enzyme reactions can use glucose as an energy source through the nonoxidative generation of ATP.[17]

If the changes on the path toward tumourigenic conversion are seen as a Darwinian selection process conferring a selective growth advantage, activation of a nonoxidative glucose degradation pathway is likely to contribute to the individual fitness of such a tumour cell if anaerobic conditions are present. Mutations in anaerobic glucose degrading pathways allowing such a selective growth advantage would represent additional mutations leading to a fully transformed tumour phenotype. By determining physiological changes in a cell-line model of tumourigenesis, a fully transformed cell line was most dependent on aerobic glycolysis and least dependent on the mitochondrial machinery for ATP synthesis.[4] During progression to full transformation, sensitivity to oligomycin, an inhibitor of mitochondrial ATP synthase, declined progressively. No

decrease in ATP levels upon treatment with oligomycin was observed in the fully transformed cell line, consistent with the predominant production of ATP by aerobic glycolysis.[4] The fully transformed cell-line was most sensitive to both 2-deoxyglucose and oxamic acid, an inhibitor of lactate dehydrogenase. The findings in this cell-line model of tumourigenesis confirm the Warburg effect and the important role of an anaerobic glucose degradation pathway for tumorigenesis.

Although the molecular and biochemical basis of this metabolic pathway has been elusive yet, both glucose usage as well as lactate production are clinically relevant. The glucose usage in tumours can be noninvasively visualized using PET with the glucose-analogue tracer, fluorodeoxyglucose (FdG). Furthermore, it has been shown that both glucose usage and lactate production represent markers indicating poor prognosis.[19-22] The important role of transketolase enzyme reactions for this glucose degrading and lactate producing pathway has been shown.[23, 24]

The PPP allows nonoxidative glucose degradation and enables a lactate-based matrix-degradation of the surrounding tissue.[25] A lactate-based acidification of the surrounding tissue may be supplemented by the production of H_2CO_3, if CO_2 is produced by the oxidative part of the PPP. Both lactate acid and H_2CO_3 production lead to excretion of protons and a decline in pH of the surrounding matrix. This tissue acidosis triggers a p53-mediated cell death of neighbouring healthy cells.[26, 27] Tumour cells survive due to mutations in p53 or some other components in the apoptosis pathways. An acid-mediated tumour invasion model including promotion of angiogenesis, proteolytic cleavage of matrix proteins, and inhibition of immune response has been proposed.[28]

Although aerobic glycolysis leads to the above-mentioned selective growth advantages, the most important question remains unresolved. What is the energetic basis of anaerobic glucose degradation? An analysis of the contribution of different fuels and metabolic pathways in proliferating MCF7 breast cancer cells has demonstrated that 65% of the total ATP turnover is from unidentified sources.[29] Ramanathan *et al* have shown that the fully transformed tumour cell-line does not show a decrease in ATP levels, even if mitochondrial ATP production is inhibited.[4] What is the source of ATP in fully transformed tumour cells when mitochondrial ATP production is not important?

While considering the above-listed advantages the nonoxidative PPP confers upon tumour cells, it is important to stress that the PPP is energetically as inefficient as the anaerobic glucose degradation via the Embden-Meyerhof pathway. When oxygen is absent, glucose degradation via both the Embden-

Meyerhof pathway and the PPP leads to lactate production. Muscles perform the final mitochondrial oxidative steps of oxidative phosphorylation only if oxygen is present (Pasteur effect[1]). Whereas tumour tissues do not.[1] An unappreciated finding Warburg made was that aerobic glycolysis occurs in healthy tissues like retina and testis.[1] Strikingly, those healthy tissues in which Warburg detected a high aerobic glycolysis were precisely the same tissues in which a high level of TKTL1 expression has been observed.[17] Why do tumour cells still continue to degrade glucose to lactate even in the presence of oxygen if this anaerobic pathway is energetically inefficient?
The energetic output of anaerobic glucose degradation, either based on the Embden-Meyerhof pathway or the PPP reactions as presented in text books, is worse than the energetic output of oxidative glucose degradation. Interestingly, the proposed reactions of the nonoxidative PPP are not firmly established; experimentally measured degree of ^{14}C isotope labelling and its distribution in carbon atoms of fructose-6-phosphate differs from that predicted by reaction sequences.[30-32] This discrepancy demonstrates the presence of other types of nonoxidative PPP reactions, although the molecular and biochemical basis of these reactions remain elusive.
During the evolution of higher vertebrates, mutations in the TKTL1 gene have led to an altered substrate specificity and altered enzymatic reaction kinetics. An enhanced, one-substrate reaction allowing the use of xylulose-5-phosphate as sole substrate has been detected.[17] A TKTL1-based lactobacillae-like glucose degradation pathway has been postulated to explain the observed glucose metabolism in tumours[17], and such a pathway would result in enhanced glucose usage, enhanced carbonic anhydrase enzyme activity, enhanced *de novo* fatty acid synthesis, inhibition of Embden-Meyerhof glycolysis, enhanced lactate production, and mitochondria-independent ATP generation. Such postulated metabolic changes indeed have been observed in tumours. Enhanced glucose usage[1, 19-21], upregulation of carbonic anhydrase IX[33], fatty acid synthase and *de novo* fatty acid synthesis upregulation[34], pyruvate kinase activity downregulation[35], enhanced lactate production[1, 22], and mitochondria-independent ATP generation[1, 4, 36] have been detected in tumours, and most of these metabolic changes are prognostic markers indicating poor patient survival.
As lactate and pyruvate regulate hypoxia-inducible gene expression independently of hypoxia by stimulation of hypoxia-inducible Factor 1 alpha[37], activation of the TKTL1 pathway leading to lactate and pyruvate may also contribute to angiogenesis under normoxic conditions.
The presence of the TKTL1 pathway in tumour cells would also explain why tumour cells with an inhibited mitochondrial respiration and a con-

comitant enhanced aerobic glycolysis are less sensitive to induction of apoptosis by common anticancer agents, but highly sensitive to an inhibition of aerobic glycolysis.[36] Furthermore, TKTL1-expressing tumours may derive an additional selective growth advantage if the synthesis of fatty acids is not reversed by β-oxidation of fatty acids. Recent studies have shown that this metabolic adaptation for an efficient growth is present in tumours. In tumours, activated Akt inhibits β-oxidation of fatty acids.[6] If glucose is available, such a tumour cell has a selective growth advantage because produced fatty acids are not degraded; however, this metabolic change leads to absolute glucose dependence and death, in response to glucose withdrawal.[5, 6] The immunohistochemical analysis of expression of TKTL1 and activated Akt presented here demonstrate that the majority of TKTL1 overexpressing tumours do have activated Akt and therefore not able to perform β-oxidation of fatty acids.

All the observed metabolic changes in tumours are consistent with a TKTL1-based, lactobacillae-like glucose metabolism in tumours. Which of the altered enzymatic activities leading to these metabolic changes can be exploited for future cancer therapies? The most effective way to inhibit tumour proliferation should be to block the generation of energy for tumour growth. Metabolic control analysis and inhibition of transketolase enzyme reactions have already shown that tumour proliferation can be inhibited by anti-transketolase approaches.[9, 10, 12-14, 16] These successful results have been attributed to TKT transketolase. The enzymatic properties of TKTL1[17], its upregulation in tumours, and the absence of upregulation of TKT and TKTL2 indicate that TKTL1 is the transketolase targeted by anti-transketolase drugs. The determination of TKTL1 expression in human malignancies may help to identify cancer patients that would benefit from an anti-transketolase cancer therapy. As a proportion of TKTL1 overexpressing tumours does not express activated Akt, cancer patients with such tumours may benefit from a concomitant inhibition of fatty acid β-oxidation by activation of Akt or inhibition of AMPK. Future TKTL1-based anticancer therapy may also be improved by substrate limitation through application of a ketogenic diet.[38]

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CHAPTER 10

DISCUSSION AND FUTURE PERSPECTIVES

„Of things some are in our power, and others are not"
(Epiktet, 50-135 n. Chr.).

Urothelial cancer is the fifth most common malignancy in Europe with more than 357,000 new cases and 145,000 deaths worldwide.[1] Bladder cancer, mainly urothelial cancer, is a tumour of superlatives in terms of costs, recurrences, and surveillance. The cost per bladder carcinoma patient from the time of diagnosis to death is the highest among all malignancies diagnosed in the U.S.[2] Similar to other neoplasms, urothelial cancer is a disease that increases in incidence with age. It is a heterogeneous group, embracing a spectrum of tumours with varying degrees of clinical behaviour. The majority of tumours are non-muscle-invasive, papillary, and of low grades, managed through transurethral resection and intravesical instillation therapies. Prognosis of these tumours is excellent with an estimated 5-year survival rate of 94%.[3] Nevertheless, urothelial cancer is troublesome: high recurrence rates, lifelong surveillance, invasive diagnostics, unpredictable progression, and perioperative morbidity vex patients and urologists.

Recurrence

The high recurrence rate of approximately 60% to 70% in non-muscle-invasive tumours is caused by early tumour cell seeding and panurothelial disposition, and possibly by transurethral tumour resection.[4-9] Transurethral tumour (TUR) resection is believed to be often incomplete with residual tumour being reported in up to 76%.[10, 11] Therefore, the quality of TUR is critical because it determines how successful the treatment outcome is. Besides incomplete resection, it has been assumed that during operative treatment, vital tumour cells adhere at denuded urothelium and grow into new tumours.[7, 8] This idea is based mainly on experimental and *ex vivo* studies; clinical studies are rare, and a prospective val-

idation seems difficult because such studies would hardly meet ethical requirements.
In our retrospective analysis, we evaluated the hypothesis of iatrogenically triggered tumour recurrences and progression, comparing both parameters in one group of patients undergoing a differentiated resection (larger areas of wounded urothelium) and in another group of patients undergoing non-differentiated resections (smaller areas of wounded urothelium). Our data did not demonstrate higher recurrence or progression rates in patients with enlarged urothelial defects, but there was a significantly lower tumour persistence rate at 6 to 10 weeks after the first operation in patients receiving differentiated resection.[12] Because recent reports favour a second resection to overcome the common problem of incomplete resections (41% to 76%) and to improve the efficacy of instillation therapies [6, 13, 14], our data show clear advantages with the differentiated resection: it will reduce tumour persistence and might reduce the numbers of second resections. Unfortunately, a tool for selecting patients with tumour persistence after transurethral resection is lacking. However, comparable to our results, large studies in patients undergoing tumour resection and random biopsies did not identify higher recurrence rates compared to patients undergoing TUR without biopsies.[15, 16] The clinical importance of tumour recurrences caused by implanted tumour cells in denuded urothelium remains unclear, but broad application of the differentiated resection technique seems to improve tumour treatment and will allow a better comparison of study results.
Limitations of current therapies and surveillance of urothelial cancer include the need for frequent invasive procedures of the urinary tract, such as cystoscopy, which has a moderate sensitivity and specificity. In addition, cytopathology is characterized by high interobserver variability and a low overall sensitivity.[17, 18] Assays for non-invasive detection of urothelial tumours have been introduced with varying success.[19, 20] One FDA-approved test is fluorescence *in situ* hybridization (FISH) for detecting chromosomal alterations in tumour cells associated with low- and high-grade carcinomas. This multitarget approach has an overall sensitivity of 71% to 85% with an almost 100% sensitivity and specificity for the clinically high-risk G3 tumours.[21,22] Remarkably, this method seems to provide much more information than just detecting bladder cancer in situ[23] the test is frequently found to be positive in urine with negative or atypical cytology findings (anticipatory positive results), predicting tumour recurrence 3 to 29 months in advance.[24] High sensitivity and specificity, differentiation between genetically stable and unstable tumours, and early information

about the chances of recurrence make the FISH test an exciting tool for surveillance and therapeutic strategies. Furthermore, recently published data have demonstrated the detection of locally advanced prostate and renal cancer in urine analysis using FISH.[24] If prospective studies confirm these results, it might be possible to differentiate between locally advanced renal and urothelial cancer of the kidney using the FISH tests with adjusted probes. Distinguishing between these two entities can be difficult preoperatively, but it has major impact on operative treatment; therefore, a urine test for better discrimination would be of great benefit.

Progression

In analyses of genetic alterations in urothelial carcinoma, subgroups have been identified, allowing distinction of two discrete but somewhat overlapping disease developments: primary non-muscle-invasive but often recurrent tumours (genetically stable) and muscle-invasive tumours with a poor prognosis (genetically unstable).[25] Genetic imbalances are found significantly more often in invasive, high-grade urothelial tumours than in low-grade and low-stage tumours.[26, 27] At present, we cannot objectively, precisely, or prospectively predict the clinical behaviour of urothelial tumours. About 20% to 50% of primarily non-muscle-invasive carcinomas will progress to a higher stage or grade, and in general, these patients have a worse prognosis. The correct treatment of the so-called high-risk tumours is still a dilemma; early cystectomy can be excellent for cancer control but will be an over-treatment in almost half of the patients. Conservative treatment, including instillation therapy, might risk delaying a curative approach in a potentially metastasizing cancer disease.
However, overall more than 50% of patients with initially curative operative treatment experience relapse. Cisplatin-based chemotherapy has been the standard treatment for metastasized urothelial tumours and has further been investigated as neoadjuvant and adjuvant treatment to improve patient survival. In both settings, chemotherapy provides a modest survival advantage with severe toxic side effects. Modified substances and application regimes have reduced toxicity and led to a higher acceptance.[28] Herr *et al.* recently reviewed data about the impact of chemotherapy in the period from 1985 to 2006 and described an overall survival benefit of 5% for neoadjuvant and varying results (from no benefit to significant benefit) for adjuvant treatment.[29] Unlike many other tumours, the death rate of urothelial carcinoma has not decreased in recent years. Alternative treatments like tumour-specific agents to improve efficacy and reduce toxicity are still lacking in the treatment for urothelial cancer.

Given the heterogeneity of tumour mechanisms and the limitations of analytical methods, it is likely that a variety of strategies will be needed and will be complementary. Advanced techniques, like 2D gel electrophoresis and protein chip platforms are promising approaches. They allow tumour characterisation for better diagnosis and stratification of patients, and for biomarker discovery for predicting and monitoring response to treatment and recurrence.[30, 31] Examining all genes and large numbers of proteins simultaneously facilitates a broad biological approach for the investigation of pathways, modules, and regulatory networks. The primary sequences of proteins do not depend only on the DNA or mRNA sequence because various posttranslational modifications diversify the polypeptides; for this reason, the direct identification of biological endpoints is among the important tools for detection, diagnosis, treatment, and monitoring of diseases. At present, the detection and therapy control of muscle-invasive urothelial cancer is based solely on imaging diagnostics, and biomarkers or profiles that allow fast and easy access to tumour-specific information would be of significant benefit. We evaluated the impact of proteome analyses in urothelial tumours and detected a differential protein expression pattern in low-grade pTa, pT1 G3, and ≥pT2 G3 tumours, and concluded that a better selection of patients needing radical and early treatment is possible.[32] The protein-expression profile of tumour tissue in general can identify subgroups within heterogeneous tumour groups and can determine patients after unsuccessful treatment.[31, 33-37] Upcoming protein chip studies evaluating large numbers of urothelial tumours can contribute to databases of expression patterns, which may provide a clustering in this heterogeneous group and might offer the possibility for detecting high-risk tumours requiring radical treatment.
Serum is one of the preferred media in the search for biomarkers and probably offers the most complex proteome information. Unfortunately, there has been little attempt to investigate serum proteins in diagnosis, prognosis, follow-up, and monitoring of therapies in muscle-invasive urothelial cancer. However, bladder cancer is being diagnosed currently at the same stage that prostate carcinoma was being diagnosed 20 years ago.[38] The application of a reliable available diagnostic test (PSA) has significantly improved prostate cancer detection and treatment, and a serum biomarker would have a similarly invaluable usefulness in muscle-invasive urothelial carcinoma. As described in Chapter 7, with the use of chip technology, serum protein profiling is promising and manageable in urothelial cancer.

Tumour Metabolism

Understanding the biological differences between normal and cancer cells is essential for the development of diagnostics and tumour-specific therapy. However, the mechanisms underlying carcinogenesis and disease progression are extremely complex, and even within a specific cancer type the cell population contains diverse genetic changes. These genetic alterations accumulate over time, correlating with genetic instability as the disease progresses; developing tumour-specific gene therapies seems in this respect rather complex. A strategy to achieve therapeutic selectivity and efficacy is to take advantage of prominent metabolic alterations in cancer cells, such as increased aerobic glycolysis and dependency on the glycolytic pathway for ATP generation, known as the Warburg effect.[39,40]
Our knowledge of the high glucose consumption and several-fold increase in glucose flux in cancer cells is already clinically exploited for tumour imaging through FDG-PET, which has confirmed that more than 90% of tumours demonstrate increased glucose uptake.[41] Use of PET has revealed a direct correlation between tumour aggressiveness and the rate of glucose consumption.[42] Details about the underlying mechanism of enhanced glucose turnover in tumour cells have remained elusive because the dominant anaerobic respiration using the non-oxidative pentose phosphate pathway (PPP) is less efficient than its aerobic counterpart by greater than an order of magnitude. Still, more than 85% of ribose recovered from nucleic acids is generated from the transketolase-dependent PPP, implicating the central position of this pathway in tumour cells.[43] Furthermore, correlating tumour-specific metabolic changes, such as upregulation of carbonic anhydrase IX, fatty acid synthase, activation of the kinase Akt (activation through galectin-3), enhanced lactate production, and independent ATP generation are directly or indirectly involved in glucose metabolism. Our study of the three different transketolase enzymes (TKT, TKTL1, and TKTL2) and their impact in tumours detected the unique overexpression of TKTL1, whereas TKT and TKTL2 were not overexpressed [44]. The detection of the transketolase-like 1 enzyme (TKTL1), its function, and its overexpression in aggressive tumour types for the first time elucidates the association of many of the known but previously unexplainable metabolic alterations. High concentrations of TKTL1 enable tumours to engage in an enhanced, oxygen-independent glucose usage and provide a remarkable growth advantage.[45] Because inhibition of transketolase enzyme reactions suppresses tumour growth and metastasis, the specific targeting of the thiamine-dependent TKTL1 enzyme offers new therapeutic options.[45-48] Furthermore, complementary therapeutic options, such as

influencing linked metabolic steps (galectin-3, hexokinase, G6PD, Akt, or the mTOR-pathway) seem promising in cancer therapy.[49-56]
These latest insights into tumour-specific metabolism break into a new area of cancer treatment. In addition to new molecular targets directly tackling the tumour, new therapeutic agents can control tumour acidosis, which is caused by high lactate levels and results in p53 activation and acid-induced cell death of the surrounding normal cells. This lactate-based matrix degradation is supposed to be the main step in the metastasizing process of malignancies. Furthermore, tumour acidosis causes drug resistance and enhances the tumor-specific immune escape.[57-59] Targeting underlying vulnerable metabolic alterations (the non-oxidative arm of the pentose phosphate pathway, galectin-3 expression) can help to overcome drug resistance.[51, 57]
Furthermore, substrate limitation seems an appropriate approach in supporting cancer therapy. Activated Akt is found to be significantly upregulated in progressing tumours, and it inhibits β-oxidation of fatty acids. If glucose is available, such tumour cells have a selective growth advantage because produced fatty acids are not degraded; at the same time, it causes an absolute glucose dependence and cell death in response to glucose withdrawal.[60, 61] Substrate limitation through application of a ketogenic diet can lead to tumour control in patients with metastasized tumours.[62, 63]
A direct transfer of our research data to clinical application has already resulted in the use of TKTL1 as a progression marker (Institute of Pathology, University of Freiburg and Institute of Pathology, Prof Ihling, Frankfurt am Main, Germany), in the ongoing development of an anti-TKTL1 small compound (Tavargenix GmbH, Würzburg, Germany), and in use of a TKTL1-adjusted diet (substrate limitation, Evomed GmbH, Darmstadt, Germany) for patients with progressing tumours under cytotoxic therapy (Department of Urology, AMC, University of Amsterdam, Netherlands).
Future significant improvements in clinical treatment of tumours will mainly be achieved if technical advance is combined with advances in basic molecular research. Innovative cooperation between highly specialized clinics and labs creates the platform for the urgently needed advances in diagnosis and treatment of urothelial cancer.

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CHAPTER 11

SUMMARY & SAMENVATTING

Summary

Impressive advances have been made in endoscopic imaging processes and operative strategies in the treatment of urothelial cancer. Unfortunately, approaches to the major problems of invasive diagnostic procedures, high recurrence rates, and unpredictable progression in non-muscle-invasive tumours as well as high mortality in muscle-invasive carcinomas have not shown great improvement. An overall survival benefit of 5% to 13% for patients receiving neoadjuvant or adjuvant combination chemotherapy is unsatisfactory. A better understanding of carcinogenesis and the application of molecular screening of the heterogeneous group of urothelial tumours are needed to provide selective diagnostics and patient-adapted surveillance and therapy.

The first part of this manuscript emphasises the challenges in clinical treatment of urothelial cancer. High recurrence rates of non-muscle-invasive tumours have multifactorial causes, with experimental data suggesting an impact of the transurethral tumor resection (TURT) itself. Our analysis of two different TUR techniques concerning recurrences, progression, and tumour persistence in non-muscle-invasive bladder tumours showed a clear advantage of the differentiated tumour resection with respect to significantly lower tumour persistence. A difference in recurrence and progression rates was not detected, which seems important because published data impressively demonstrated a worse outcome for patients with early recurrent or progressive tumours (***Chapter 2***).

Systemic urothelial disease has a poor prognosis with a long-term survival of less than 10%. Administration of adjuvant multi-agent chemotherapy aims to improve the course for the patients but severe toxicity is inherent in this approach. The use of modified combinations of chemotherapeutic substances, such as cisplatin/methotrexate, or more recently cisplatin/gemcitabine has led to fewer side effects and a better acceptance. Significant extension of the overall survival was not achieved (***Chapter 3***).

Molecular progression markers or marker profiles are needed for better patient selection and improved tumour-specific therapy. Cancer is viewed

as a disease resulting from cancer-causing genes that deregulate cellular proliferation, apoptosis, and biochemical pathways, resulting in abnormal tumour cell metabolism. Using genomic analyses, we identified a potential tumour suppressor gene at 2q21.2. The region was investigated in detail and deletions at the LRP1B gene were more frequently detected in high-grade carcinomas (***Chapter 4***). Ligands of the low density lipoprotein receptor-related protein LRP1B, such as uPA and PAI-1, are components of the uPA system, one of the major extracellular matrix-degrading proteinase systems. Extracellular matrix degradation is required for uninhibited local tumour growth and metastasis, mainly achieved by lactate-induced tissue acidification (aerobic glycolysis) and activation of matrix-degrading proteinase systems.

Urothelial tumours are characterized by various genetic alterations, which can be exploited for diagnostic reasons. Fluorescence *in situ* hybridization (FISH) is a technique that detects numerical or structural chromosomal abnormalities in tumour cells. This FDA-approved urinary test detects four different chromosomal alterations within the cell and provides a "multi-marker test" that may be more informative given the heterogeneity of the disease. In addition to the detection of bladder malignancies, FISH might offer a broad spectrum of other diagnostic possibilities, such as the specification of genetically unstable tumours, improved diagnosis of upper urinary tract tumours, and anticipatory positive results allowing a very early diagnosis of recurrent tumours (***Chapter 5***).

The sequencing of the human genome and identification of oncogenes have advanced rapidly; however, this progress in gene expression profiling has rarely been transferred to routine clinical use. Because relevant genetic alterations will typically lead to changes in the proteome and proteins are among the main actors in all physiological and pathological processes, comprehensive protein profiling technologies, such as 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and ProteinChips®, have attracted significant interest. We evaluated modern protein profiling techniques in urothelial tumours, detecting differential protein expression patterns between normal, non-muscle-invasive and muscle-invasive carcinomas. High-risk tumours pT1 G3 displayed a strikingly similar protein expression to ≥pT2 tumours, and both tumour groups significantly overexpressed galectin-3 (***Chapter 6***), leading to a comprehensive galectin screening in urothelial tumours (Chapter 8).

ProteinChip® technology is efficient in detecting biomarkers in human serum, which is a preferred media for the detection of tumour markers. The technical management of protein serum analyses can now be performed

more easily because of improvement in fractionating devices and introduction of various and advanced platforms (***Chapter 7***). Data regarding specific protein expression patterns in patients with urothelial carcinomas, especially in muscle-invasive disease, promise to be challenging.
As described above (chapter 6), galectin-3 protein is overexpressed in high-risk and muscle-infiltrating tumours. Galectins are a family of carbohydrate-binding proteins and play a pivotal role in tumourigenesis by regulating apoptosis, angiogenesis, tumour immune escape, and tumour metabolism. Our analysis determined significant upregulation for galectins-2, -3, and -8 when comparing non-muscle-invasive and muscle-invasive tumours.
Disease-dependent mortality was significantly correlated with the expression of galectins-2 and -8 (***Chapter 8***). Galectin-3, one of the best investigated galectins, demonstrates strong antiapoptotic activity, and one of the key involvements is the activation of the PI3K/Akt pathway. This pathway promotes malignant transformation, enhances aerobic glycolysis, and renders cells dependent on glycolysis for survival. Activation of PI3K and Akt results in stimulation of glucose uptake, glycolysis, and inhibiting β-oxidation of fatty acids, which are the most fundamental alterations in tumour-specific metabolism. The increased dependence of cancer cells on the glycolytic pathway for ATP generation enhanced our study of cancer-specific deregulations in glucose degradadation and its possible impact on therapeutic strategies.

Concerning the transketolase-dependent, non-oxidative pentose phosphate pathway (PPP) as the most relevant key position in energy metabolism of tumours, we focused on the impact of the different transketolase enzymes in cancer tissues. For the first time, a tumour-specific upregulation of a transketolase-like-1 enzyme, TKTL1, other than the transketolase (TKT), was demonstrated in progressing tumours. The detection of TKTL1 explains many known and concomitant but unexplained enzymatic alterations, bringing them together like the pieces of a jigsaw puzzle (***Chapter 9***). Tumour cells with overexpression of TKTL1 and Akt are glucose addicted and die in the case of glucose withdrawal, which can be achieved by substrate limitation. A direct transfer of these basic research data to clinic has resulted in the use of TKTL1 as progression marker (Institute of Pathology, University of Freiburg, Germany), in the ongoing development of an anti-TKTL1 small compound (Tavargenix GmbH, Würzburg, Germany), and a TKTL1-adjusted diet (substrate limitation) for patients with progressing tumours under cytotoxic therapy (Department of Urology, AMC, University of Amsterdam, Netherlands).

Samenvatting

Bij de behandeling van urotheelkanker van de blaas is duidelijk een vooruitgang geboekt in de endoscopie en operatieve technieken. Ondanks deze verbeteringen zijn de belangrijkste problemen zoals invasieve diagnostiek, hoge recidief frequentie na resectie, moeilijk te voorspellen kans op progressie bij niet-spierinvasieve tumoren en de hoge mortaliteit bij spierinvasieve tumoren, niet verbeterd. De totale overlevingswinst van 5 tot 13% bij patiënten met een gemetastaseerde blaaskanker, behandeld met adjuvante of neoadjuvante chemotherapie is teleurstellend. Betere kennis van de carcinogenese en toepassing van moleculare screening in de heterogene groep van urotheel tumoren is nodig om selectieve diagnostiek en de individuele patiënt een gerichte follow-up en therapie te bieden.

Het eerste deel van het manuscript richt zich op uitdagingen bij de behandeling van urotheelcel kanker. De hoge recidief frequentie van niet-spierinvasieve tumoren is multifactorieel bepaald, en uit experimentele data blijkt dat de behandeling (TURT) zelf ook van invloed lijkt te zijn op de recidief frequentie. Onze analyse van twee verschillende resectie technieken aangaande recidief frequentie, progressie en incidentie van rest tumor bij niet-spierinvasieve blaastumoren, laat een duidelijke voordeel zien voor de gedifferentieerde resectietechniek. Hierbij wordt significant minder frequent een rest tumor gezien. Een verschil in recidief en progressie percentage was echter niet aantoonbaar voor de twee recectie technieken. Dit lijkt van belang omdat in eerder gepubliceerde series slechte resultaten zijn beschreven bij patiënten met vroege recidieven of progressieve tumoren (***hoofdstuk 2***).

Een gemetastaseerde urotheel kanker heeft een slechte prognose met een lange termijn overleving van minder dan 10%. Het doel van adjuvante polychemotherapie is verbetering van kanker specifieke overleving, maar gaat gepaard met ernstige toxiciteit voor de patiënt. Het gebruik van combinaties van chemotherapeutische middelen als cisplatin/methotrexaat of momenteel meer gebruikt cisplatin gemcitabine heeft geleid tot minder bijwerkingen en minder toxiciteit. Significante verlenging van de algehele overleving werd niet aangetoond voor de laatste combinatie chemotherapie (***hoofdstuk 3***).

Moleculaire markers voor progressie en marker profielen zijn nodig voor een betere patiënten selectie en verbeterde tumor gerichte therapie. Kanker wordt beschouwd als een ziekte veroorzaakt door genen die zorgen voor een disregulatie van de cellulaire proliferatie, apoptose en biochemische processen, resulterend in een abnormaal tumorcel metabolisme. Met behulp van genoom analyse hebben wij een potentieel tumor supressor gen geï-

dentificeerd op 2q21.2. Het chromosoom gebied is in detail onderzocht waarbij deleties op het LRP1B gen frequenter werden gezien in hoog-gradige carcinomen (***hoofdstuk 4***).
Een ligand van het low density lipoproteine receptor-gerelateerde eiwit LRP1B zoals uPA en PAI-1 is een component van het uPA systeem, één van de belangrijke extracellulaire matrix degraderende proteinase systemen. Extracellulaire matrix degradatie is vereist voor ongeremde lokale tumorgroei en metastasering welke voornamelijk worden bereikt door lactaat geïnduceerde weefsel verzuring (aerobe glycolyse) en activatie van matrix degraderende proteinase systemen.
Urotheelcel tumoren worden gekarakteriseerd door verschillende genetische veranderingen, die voor diagnostische doeleinden kunnen worden toegepast. Fluorescentie *in situ* hybridisatie (FISH) is een techniek welke chromosomale afwijkingen (aantal en structureel) in tumorcellen aantoont. Deze US FDA goedgekeurde urinetest, die tegelijkertijd 4 verschillende chromosomale veranderingen kan detecteren in de cel, lijkt hierdoor een goede multimarker test te zijn voor deze heterogene tumor. FISH kan naast de detectie van blaaskanker een breed spectrum van diagnostische mogelijkheden bieden zoals identificatie van genetisch instabiele tumoren en betere diagnostiek van tumoren van de hoge urinewegen. Daarnaast is eerdere diagnostiek van recidieven mogelijk door anticiperende positieve resultaten (***hoofdstuk 5***).
Er is een belangrijke ontwikkeling in de analyse en identificatie van het humane genoom en oncogenen, echter deze vooruitgang heeft nog geen ingang in de kliniek gevonden. Omdat relevante genetische veranderingen altijd leiden tot veranderingen in het proteoom, en eiwitten belangrijke hoofdrolspelers zijn in alle fysiologische en pathologische processen komen eiwit profileringstechnieken, zoals 2D polyacrylamide gelelectropherese (2D-PAGE) en ProteinChips®, steeds meer in de belangstelling. Wij hebben moderne eiwit profileringstechnieken geëvalueerd bij urotheelcel tumoren, waarbij verschillende eiwit expressie patronen zijn gevonden tussen normaal urotheel, niet-spierinvasieve en spierinvasieve carcinomen. Hoog risico (pT1G3) tumoren tonen een gelijke eiwit expressie als ≥pT2 gegradeerde tumoren. Beide tumor groepen laten ook een significante overexpressie zien van galectine-3 wat zou kunnen leiden tot een galectine screening bij urotheelcel tumoren (***hoofdstuk 6***).
Een efficiënte biomarker detectie in humaan serum kan plaatsvinden met de ProteinChip® technologie, één van de meest geschikte media voor tumor marker detectie. De technische uitvoering van proteïne serum analyses is tegenwoordig een stuk eenvoudiger door verbeterde fractionering

apparatuur en introductie van verschillende en geavanceerde technieken (***hoofdstuk 7***).
De gegevens met betrekking tot specifieke eiwit expressie patronen bij patiënten met urotheelcel carcinomen, met name bij spier-invasieve tumoren, zijn veelbelovend.
Zoals eerder beschreven (hoofdstuk 6) is er een overexpressie gevonden van het eiwit galectine-3 bij hoog-gradige en spier-invasieve urotheelceltumoren. Galectinen behoren tot een familie van koolhydraat bindende eiwitten en spelen een centrale rol in de tumor genese door regulatie van de apoptose, angiogenese, immunologische tumor escape en tumor metabolisme. Onze analyse laat een significante overexpressie zien van galectine-2, -3 en -8 bij spier-invasieve tumoren vergeleken met niet-spier-invasieve tumoren. Er was een significante correlatie tussen ziekte gerelateerde mortaliteit en expressie van galectine-2 en -8 (***hoofdstuk 8***).
Galectine-3, één van de best onderzochte galectinen, laat een sterk anti-apoptotische activiteit zien. Daarnaast zorgt galectine-3 voor de activatie van de P13K/Akt cascade. Deze cascade stimuleert maligne transformatie, versterkt de aërobe glycolyse en zorgt voor cel afhankelijkheid wat betreft de glycolyse voor overleving. Activatie van P13K en Akt resulteert in stimulatie van de glucose opname, aërobe glycolyse en remming van de β- oxidatie van vetzuren, welke het meest fundamenteel veranderd zijn bij het tumor-specifieke metabolisme. De toegenomen afhankelijkheid van kankercellen van de aërobe glycolyse voor het verkrijgen van ATP, ondersteunt onze studie over kanker-specifieke disregulatie van glucose afbraak en de mogelijke invloed op therapeutische strategieën.
Wat betreft de transketolase-afhankelijke, non-oxidatieve pentose phosphate pathway (PPP) als belangrijke stap voor het energie metabolisme voor tumoren, hebben wij gekeken naar de invloed van de drie verschillende transketolase enzymen in tumorweefsels. Voor het eerst is een tumor specifieke toename van de transketolase-like-1 enzym (TKTL-1), anders dan het transketolase (TKT) aangetoond in progressieve tumoren. Het aantonen van het TKTL1 verklaart meerdere bekende en tegelijk voorkomende, maar onverklaarde enzym veranderingen, zoals stukjes uit een legpuzzel (***hoofdstuk 9***). Tumor cellen met overexpressie van TKTL1 en Akt zijn glucose afhankelijk en sterven af in afwezigheid van glucose. Dit kan worden bereikt door beperking van het substraat. Een directe vertaling van dit basale onderzoek naar de praktijk heeft geresulteerd in het gebruik van TKTL1 als een progressie marker (Instituut voor Pathologie, Universiteit van Freiburg, Duitsland), de ontwikkeling van een anti-TKTL1 klein molecuul (Tavargenix GmbH, Würzburg, Duitsland) en een TKTL1 aangepast

dieet (beperking van het substraat) voor patiënten met progressieve tumoren onder cytotoxische therapie (Afdeling Urologie en Oncologie, AMC, Universiteit van Amsterdam, Nederland).

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"Leider läßt sich eine wahrhafte Dankbarkeit
mit Worten nicht ausdrücken"
JW v Goethe (1749-1832)

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„Und so lebte sie glücklich und zufrieden..., en zij leefde nog lang en gelukkig..."

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