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TISSUE BIOMARKERS IN CANCER OF THE URINARY BLADDER AND KIDNEY

High-throughput tissue microarrays in the study of urinary tract malignancies

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Academic Dissertation

To be publicly discussed with permission of the Medical Faculty of the University of Helsinki, in auditorium 3 of the Biomedicum Helsinki, Haartmaninkatu 8, on June 7th, 2003, at 12 noon.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals.

I Rao JY, Seligson D*, Visapää H*, Horvath S, Eeva M, Michel K, Pantuck A, Belldegrun A, Palotie A. Tissue microarray analysis of cytoskeletal actin-associated biomarkers gelsolin and E-cadherin in urothelial carcinoma. *Cancer* 95:1247-1257, 2002

II Visapää H, Seligson D, Eeva M, Gaber F, Rao JY, Belldegrun A, Palotie A. 8q24 amplification in transitional cell carcinoma of bladder. *Applied Immunohistochemistry & Molecular Morphology* 11:33-36, 2003

III Visapää H, Seligson D, Huang Y, Rao JY, Belldegrun A, Horvath S, Palotie A. Ki-67, gelsolin and PTEN expression in sarcomatoid renal tumors. *Urological Research* 30:387-389, 2003

IV Visapää H, Bui M, Huang Y, Seligson D, Tsai H, Pantuck A, Figlin R, Rao JY, Belldegrun A, Horvath S, Palotie A. Correlation of Ki-67 and gelsolin expression to clinical outcome in renal clear cell carcinoma. *Urology* 61:845-850, 2003

*These authors contributed equally to the respective work.

ABBREVIATIONS

AJCC	American Joint Committee on Cancer
CCRCC	clear-cell renal cell carcinoma
cDNA	complementary DNA
CGH	comparative genomic hybridization
DNA	deoxyribonucleic acid
FISH	fluorescence in situ hybridization
IHC	immunohistochemistry
mRNA	messenger RNA
p	short arm of the chromosome
PRCC	papillary renal cell carcinoma
q	long arm of the chromosome
RCC	renal cell carcinoma
RNA	ribonucleic acid
TCC	transitional cell carcinoma
TMA	tissue microarray
UICC	Union Internationale Contre le Cancer
VHL	von Hippel-Lindau disease

ABSTRACT

The carcinogenic events leading to urinary bladder and kidney malignancy are incompletely understood. This study examined protein expression of potential biomarkers in cancers of the urinary bladder and kidney, utilizing the tissue microarray (TMA) technique, which is a novel high-throughput tool for molecular studies of cancer.

The results indicate that urinary bladder tumors display distinct expression profiles for the actin-associated proteins gelsolin and E-cadherin. Gelsolin and E-cadherin expression was decreased in premalignant and malignant lesions, when compared with that of benign samples. With an increase in tumor grade and stage, however, gelsolin expression increased, unlike E-cadherin expression. An increased gelsolin expression in high-grade bladder tumors was associated with an increased risk for progression and recurrence. Expression of biomarkers p53 and Ki-67 increased from the level in benign lesions to levels in malignant lesions, and then rose with increasing tumor grade. Additionally, genomic amplification in chromosomal region 8q24 occurred in a subgroup of predominantly high-grade bladder tumors and in a more significant group of distant metastases. That the tumors and distant metastases carrying the 8q24 amplification over-expressed Ki-67 supports the hypothesis that 8q24 amplification contributes to these tumors' malignant potential. None of the regional lymph node metastases carried 8q24 amplification.

Renal tumors having sarcomatoid features were studied for the expression of tissue biomarkers Ki-67, gelsolin, and PTEN, and compared with that in clear-cell and papillary tumors. The distinct expression profiles in all these tumor types support the hypothesis that in the development of renal malignancies various molecular pathways are involved. Gelsolin expression in clear-cell and sarcomatoid tumors was low, but high in papillary tumors, which suggests that gelsolin may play multiple roles within these renal tumor types. A more detailed analysis of the most common renal malignancy, renal clear-cell carcinoma, revealed that Ki-67 expression independently predicts prognosis. Additionally, simultaneous increased Ki-67 expression and diminished gelsolin expression may indicate poor prognosis in grade 2 tumors. The most significant predictor of cancer-specific survival in renal clear-cell carcinoma was, however, tumor stage.

In summary, this study shows that urinary tract tumors have distinct biomarker expression profiles. The results also indicate, in cancers of the urinary bladder and kidney, that these expression profiles may be used to predict prognosis. Additionally, that gelsolin expression was transiently down-regulated in these cancer types suggests that gelsolin may play multiple roles in the development of urinary tract cancer and its progression. These results demonstrate that the TMA

technique effectively facilitates study of urinary tract malignancies and provides an opportunity for multimarker analysis in discovery-based cancer profiling.

REVIEW OF THE LITERATURE

High-throughput techniques in cancer research

Cancer development involves multiple genetic and epigenetic alterations in those cells undergoing malignant transformation (Hanahan and Weinberg 2000). Until recently, cancer research has mainly progressed through studies of one or only a very few molecules, events, or samples at a time. Sequencing of the human genome (International Human Genome Sequencing Consortium 2001; Venter et al. 2001) and the simultaneous rapid development of high-throughput techniques for the study of cellular events has had an enormous impact on cancer research (Weber 2002). For example, promising results have come from microarray techniques to classify breast cancer and lymphoma more accurately according to their molecular status (Perou et al. 2000; Garcia et al. 2002). These new techniques enable a global discovery-based approach to events leading to cancer and may provide a more comprehensive view of the carcinogenic process. These novel techniques are also useful in target identification, pinpointing the molecules and pathways essential to cancer development. At the other end of the spectrum, the amount of novel information is sufficiently large to allow for the recognition of molecular relationships at organism level. It is likely that such discovery-based approaches will become the frontiers of cancer research, enabling the identification of truly new, unpredicted findings (Lee 2001).

Systematic analysis of the carcinogenic events – oncogenomics – can be performed combining data from several different sources. Several novel laboratory methods – cDNA expression arrays, oligonucleotide arrays, comparative genomic hybridization (CGH), TMAs, protein arrays, cell arrays, and single cell arrays – facilitate studies of various aspects of cancer, ranging from the expression of a single gene to complex cell dynamics (Schena et al. 1995; Sapolsky and Lipshutz 1996; Kallioniemi et al. 1992; Kononen et al. 1998; Madoz-Gurpide et al. 2001; Ziauddin and Sabatini 2001; Levsky et al. 2002) .

Tissue microarrays

Traditionally, tissue-level protein expression in cancer has been studied in one tumor, or a few tumors, at one time. Immunohistochemical (IHC) methods can be time-consuming, with inter-experimental variability in the results, due to variability in staining conditions. Costs of such experiments can be high, especially with a large number of tissues. Thus, novel methods free of these limitations should accelerate discovery in studies of large numbers of tumors for protein expression (Kononen et al. 1998).

The multitumor tissue block introduced in 1986 was one of the first steps in the development of a more efficient technique for IHC studies of protein expression (Battifora 1986). This tissue block was designed to permit economical and rapid screening for tissue-specific monoclonal antibodies and simultaneous selection of those antibodies that perform well in tissue sections. Assessment of protein expression in individual samples was, however, impossible. The checkerboard tissue block, which was developed a few years after the multitumor tissue block, allowed the study of up to one hundred tissue samples in a single experiment, simultaneously allowing evaluation of each individual sample (Battifora and Mehta 1990). It was designed to permit rapid and inexpensive screening of new histologic reagents, thus facilitating intra- and interlaboratory quality control, and was mainly designed to answer questions regarding reagents, not regarding the tissue samples themselves.

The most important step thus far in the development of high-throughput techniques for tissue-level molecular pathology studies was the development of the tissue microarray (TMA) technique by Kallioniemi and colleagues (Kononen et al. 1998). The TMA technique allows protein-expression studies of hundreds of individual tissue samples in a single experiment, while allowing several – up to 200 – consecutive experiments on the same set of samples with different antibodies. Furthermore, the TMA technique also enables DNA- and RNA-level studies of the same TMA blocks used for protein expression analyses. Compared to traditional methods, TMA experiments save costs, time, and precious archived tissue samples. TMA and cDNA techniques are complementary high-throughput techniques; TMA is applicable to targeted population screening (1 gene / 1000 tissues), whereas the cDNA microarray is applicable to genome screening (1 tissue / 5000 genes).

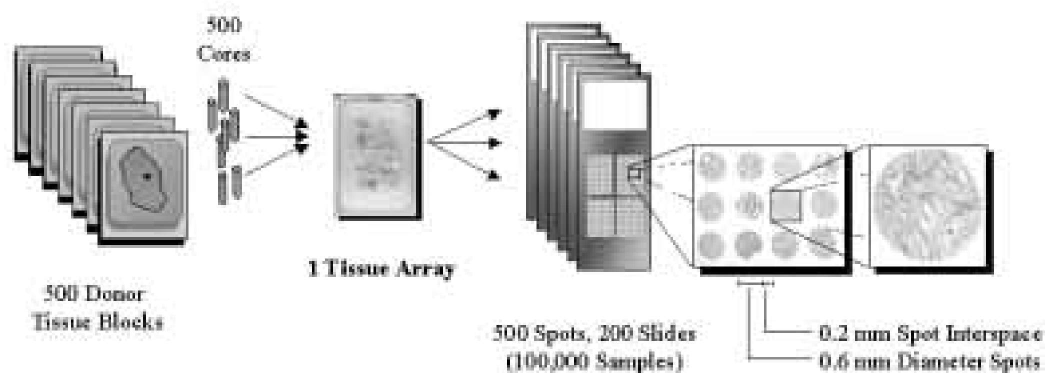


Figure 1. TMA construction.

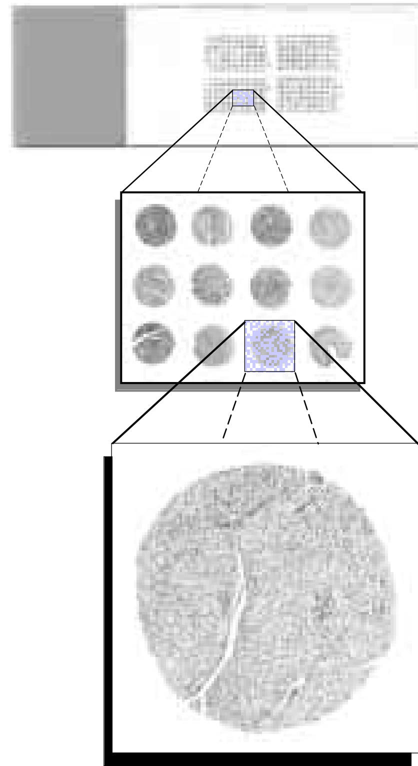
Construction of TMAs

Construction of TMAs is a multistep process. The part requiring the most time and efforts in the construction of TMA is the search, organization, pathological review, and processing of the specimens to be included in the array (Kallioniemi et al. 2001). After the formalin-fixed, paraffin-embedded original specimens have been selected and reviewed by the pathologist, core biopsies – usually 0.6 mm in diameter – are taken from areas representing the desired morphology, be it normal-appearing, dysplastic, or malignant, and then inserted into a TMA block. The sufficient number and qualities of cores taken per sample has been a question of debate. Cores selected should reflect the properties of the whole tissue without compromising rapid analyses. Since the degree of heterogeneity varies between tumor types, the number of cores required depends on tissue type (Gillett et al. 2000). TMA validation studies for cancer tissues show that the optimal number of cores to capture reliably the heterogeneity of tumors varies from three in breast to four in prostate cancer (Camp et al. 2000; Rubin et al. 2002). In bladder cancer, correlations between clinicopathological parameters and Ki-67 antibody expression in a whole-tissue analysis can be accurately reproduced with TMA. The TMA technique can also be applied to studies of heterogeneous tumors, e.g., lymphomas (Garcia et al. 2002). Despite some discrepancies between matched whole-tissue and TMA samples, overall results are strikingly similar (Nocito et al. 2001). TMA construction, illustrated in Figure 1, is a straightforward process in a technical sense. Existing TMAs have been constructed by use of a manual first-generation arrayer (Kononen et al. 1998). Once the TMA block has been finished, thin – usually 5 µm – sections are cut by microtome to generate TMA slides for molecular analyses (Figure 2). An adhesive-coated tape-sectioning system serves to keep the orientation of the tissue spots intact.

Detection of targets on TMA slides

DNA-level TMA studies utilize a fluorescence in situ hybridization (FISH) technique. This technique was first introduced in 1969, when radioactive labels were the standard (Gall and Pardue 1969). An in situ hybridization technique based on fluorescent labeling was introduced in 1986 (Pinkel et al. 1986), and FISH was used to detect chromosomal aberrations in bladder cancer for the first time in 1989 (Hopman et al. 1989). FISH has been successfully applied in studies of amplifications of defined genomic regions in several types of cancers (Richter et al. 2000; Kallioniemi et al. 2001; Simon et al. 2001; Simon et al. 2002). The hybridization procedure has been improved since the introduction of the TMA technique, in order to address the challenge of varying hybridization properties between individual samples on a single slide (Andersen et al. 2001).

Figure 2. Urothelial cancer tissue microarray slide stained with hematoxylin and eosin. Original magnification: x1, x20 and x100 (top to bottom).



The TMA analysis of mRNA expression is performed with labeled oligodeoxynucleotide probes recognizing mRNA (Kononen et al. 1998). Investigations of mRNA expression using TMAs have limitations, however, due to the unpredictable degree of degradation of the RNA before and during the paraffin process (Hoos and Cordon-Cardo 2001).

TMA analyses of protein expression are performed by standard IHC methods (Kononen et al. 1998). Conditions for IHC analyses on TMA slides need, however, to be adjusted, due to the adhesive that remains on the final TMA slide. Variation in staining conditions is small within the samples on a single slide. Moreover, slides from several different TMA blocks can be stained simultaneously for the selected antibody, reducing the variation created by staining at different time-points. When consecutive sections of a TMA block are analyzed, variation in the morphological features of the individual samples is small, allowing reliable comparison of several molecular targets in practically identical, well-defined regions of the tissues (Kallioniemi et al. 2001). Analysis of the stained TMA slides

is performed by a trained researcher using a regular light microscope, following the guidelines for IHC analyses of the tissue type on the slide. Histological verification of each core is usually by a hematoxylin and eosin staining, and is repeated after a certain number of slides, e.g., once for every 40 slides (Kononen et al. 1998).

TMA technique in cancer research

Most studies utilizing the TMA technique concern human cancer; several focus on the expression of cancer-related proteins as well as on potentially cancer-related proteins in a large number of arrayed tumors (breast, prostate, bladder, kidney) (Bubendorf et al. 1999a; Bärlund et al. 2000; Kallioniemi et al. 2001; Nocito et al. 2001; Schraml et al. 2001). Many of these studies have shown the ability of TMA to serve as a large-scale screening tool for the expression of proteins involved in carcinogenesis. The initial studies for each tumor type also serve to validate the unique TMA-related characteristics of an individual cancer type. These studies thus establish a standardized manner of conducting TMA work on a given cancer, so that analyses of their results reveal the properties of the whole tumor as closely as possible and allow comparison of the results of different studies (Bubendorf et al. 1999a; Camp et al. 2000; Richter et al. 2000; Sallinen et al. 2000; Chung et al. 2001; Hoos et al. 2001; Schraml et al. 2001; Hedvat et al. 2002; Sugita et al. 2002).

A potentially effective means of validating new tissue biomarkers of cancer is the combination of cDNA microarrays and TMAs. In this two-phase strategy, the first phase would involve the use of cDNA microarrays to identify genes whose expression differs in cancer tissues from that of normal tissues. The second phase would involve investigating the expression of protein products of genes identified in the first stage (Mohr et al. 2002). This method can reveal genes and their protein products involved in malignant processes and improve prognostic methods. This is exemplified by the fact that hereditary breast cancers exhibit distinct gene and protein expression profiles (Hedenfalk et al. 2001). In these studies fully exploiting the efficiency of TMA as a screening and validation tool, molecular information is analyzed together with comprehensive clinical data (Manley et al. 2001). Once the often laborious process to set up the TMA and clinical database is completed, comparing biomarker expression to parameters already in the database will then be rapid. A recent report combined oligonucleotide and tissue microarrays in a study of lung carcinoma (Sugita et al. 2002). Another application of this strategy to discover novel biomarkers combined CGH, cDNA microarrays, and TMAs, and showed that elongin C is a potential target gene of amplification at chromosomal region 8q in prostate cancer (Porkka et al. 2002); the advantage of this approach is that larger genome-level changes at defined locations can be taken into account in interpreting cDNA microarray results, and the additional information provided by

CGH can further guide the selection of proteins to be validated by TMA. Perhaps the most effective way to validate TMA findings prior to clinical research is functional validation: a recent report showed that EZH2 protein expression increases during tumor progression in prostate cancer, allowing the repressor effect of EZH2 on a specific cohort of genes to be studied at cellular level. This led to the conclusion that suppression of gene expression by EZH2 may be essential for a tumor to acquire metastatic potential, and that EZH2 may serve to evaluate which prostate tumors are most likely to progress (Varambally et al. 2002).

Thus far, the TMA technique has mainly been used in research settings. Clinical applications also have been proposed, however. TMA can serve as an internal quality control for antibody staining, if a small array is stained simultaneously – and on the same slide – with a whole-tissue sample being analyzed for clinical purposes (Packeisen et al. 2002). TMA can also serve as a tool for inter-laboratory quality control; staining conditions and interpretation of the results can be standardized, which should have obvious benefits for clinical patient care (Parker et al. 2002).

Limitations

The TMA technique was introduced as a rapid screening method enabling studies of tumors at DNA, RNA, and protein level (Kononen et al. 1998). Thus far, the majority of TMA studies have investigated protein-level events, whereas DNA- and RNA-level studies have been rare. Optimization of the FISH technique for TMAs can be challenging, due to autofluorescence and weak hybridization; this has been the greatest restraint in DNA-level analysis (Andersen et al. 2001). RNA-level TMA studies suffer from the unpredictable degree of degradation of RNA molecules before and during the paraffin process (Hoos and Cordon-Cardo 2001). A problem frequently encountered in TMA studies is that spots are often missing on the slide, because the cores are not always of the same length and not always oriented at a similar depth in the TMA block (Hoos and Cordon-Cardo 2001). To overcome this, more than one thin core from the tumor area can be piled on top of another in the tissue array (Hoos and Cordon-Cardo 2001). Furthermore, results from the TMA technique, accurate as they may be at population level, cannot be extrapolated to describe the molecular pathology of any individual tumor, due to the small amount of tumor mass in the array and the heterogeneity of the tumor. For example, the sample may not come from a representative area of the tumor, or in the tumor the heterogeneous areas may be unevenly located (Nocito et al. 2001). Another point is that the assessment of protein-level events alone does not provide a comprehensive view of each tumor’s biology (Ginestier et al. 2002). Further setting limits to applicability of the TMA technique, the resolution of traditional IHC methods used in TMA may not always be sufficiently accurate to capture

subtle differences in protein expression. This drawback can at least partially be reduced by staining methods such as quantitative fluorescence image analysis (QFIA) that are capable of capturing quantitative differences in an individual protein's expression more accurately (Rao et al. 2002).

The TMA technique is essentially a tool for retrospective studies. Molecular analyses can be reliably performed for archived formalin-fixed paraffin-embedded samples dating back at least 60 years (Camp et al. 2000). TMA results should, however, be verified in large prospective studies analyzing whole-tissue samples before any results are applied clinically (Torhorst et al. 2001).

Tumor profiling

Traditionally, potential biomarkers for cancer have been investigated one biomarker at a time. With recent developments in laboratory methods, classifying tumors more precisely according to their molecular status has become more feasible. A recent report showed that breast tumors can be classified into distinct subclasses based on gene expression profiles (Perou et al. 2000). Furthermore, these subclasses were later shown to have clinical implications, since outcomes differed among subclasses (Sorlie et al. 2001). Another study examined the expression profiles of several cell cycle-regulatory and proliferation markers in early-stage rectal adenocarcinomas utilizing TMAs; this study demonstrated that down-regulation of the p27 protein expression may be associated with survival, while other markers (p53, Mdm2, Ki-67) show no association with recurrence or survival (Hoos et al. 2002). Similarly, distinct prognostic subgroups of renal clear-cell carcinoma have been described by cDNA microarrays (Takahashi et al. 2001). Perhaps somewhat ironically, despite all the technical developments in biomedicine, the traditional evaluation of tumor behavior by grade and stage still remains the most applicable in day-to-day clinical decision processes. Emerging techniques, however, hold great promise, illustrated by reports describing gene-expression profiles and validating biomarkers in breast cancer based on cDNA microarrays and TMAs (Perou et al. 2000; Hedenfalk et al. 2001). It is likely that in the near future, extensive basic research assisted by high-throughput oncogenomics will lead to discoveries of biomarkers having clinical use jointly with existing methods such as grade and stage. Molecular classification of tumors may in many cancers eventually replace or at least supplement traditional methods. Thus far, however, classification schemes based solely on molecular phenotypes have not been introduced to clinical practice, for several reasons such as lack of prospective randomized studies, lack of standardization of laboratory methods, and most importantly, lack of specific treatment options for individual molecular phenotypes (Ring and Ross 2002). As soon as specific treatments with well-characterized

effects and molecular targets become available, selection of treatment for an individual patient ultimately may be based on the tumor's molecular phenotype. The current applications of molecular phenotyping, such as defining adjuvant treatment for breast cancer on the basis of estrogen receptor and c-erbB2 status of the tumor, have shown the usefulness of this molecular approach (Mohr et al. 2002).

Biomarkers

An optimal tumor marker for cancer would be specific, sensitive, easy to interpret, and applicable to the general population. Thus far, the only tumor marker widely used in urological practice is the prostate-specific antigen (PSA), used for the detection and follow-up of prostate cancer (Takeuchi et al. 1983; Killian et al. 1985; Van Brussel and Mickisch 1999a). Many of such potential biomarkers are encoded by oncogenes (Ras, c-erbB2) or tumor suppressor genes (p53, retinoblastoma); functions of the biomarkers are often related to cell cycle control (p53, retinoblastoma), cell proliferation (Ki-67), or cell adhesion (E-cadherin) (Byrne et al. 2001; Knowles 2001).

The various biological properties of urinary bladder cancer create special requirements for a marker for diagnostic, prognostic, or follow-up purposes. Low-grade bladder cancer is often a chronic recurrent illness that rarely progresses, whereas high-grade bladder cancer can be a notorious disease progressing rapidly to a non-curable state. Thus, a marker for bladder cancer would optimally identify cancer and make a distinction between these two prognostically different entities. Additionally, since the urinary bladder is an organ cystoscopically easily accessed and biopsied, a good bladder cancer marker is difficult to find, if we are to exceed the high specificity and sensitivity of cystoscopy and bladder biopsy (Van Brussel and Mickisch 1999b; Droller 2002).

Renal cancer is often non-curable at the time of diagnosis, due to its ability to remain symptomless while progressing to an advanced stage at which treatment options are very limited. A tumor marker capable of detecting renal malignancy at an early stage would therefore be potentially life-saving (Van Brussel and Mickisch 1999b).

The simultaneous study of several aspects of carcinogenesis in these tumors could aid in characterizing the carcinogenic process, which is incompletely understood in cancers of the urinary bladder and kidney.

Actin-associated proteins

Mutations or reversible defects in actin-associated proteins such as E-cadherin have been associated with many malignancies. These molecules may act

as both tumor suppressors and invasion suppressors (Nollet et al. 1999). Alterations in actin cytoskeleton remodeling have been associated with various aspects of urothelial carcinoma carcinogenesis, including cellular differentiation (Rao et al. 1990), transformation (Rao et al. 1991; Rao et al. 1993), and apoptotic control (Rao et al. 1999). Monitoring changes in actin polymerization status may provide a means of predicting tumor recurrence (Hemstreet et al. 1999). Because the cytoskeletal actin network is also crucial to cell motility and adhesion, it has long been assumed that alterations in the actin network may also be involved in tumor invasiveness. Studying several actin-associated proteins (c-erbB2, vinculin, gelsolin) involved in different aspects of actin function simultaneously in actual tumor samples may improve our overall understanding of how actin is involved in the carcinogenic process. It may also determine whether an actin-based molecular profiling analysis can supplement traditional histopathological markers (tumor grade and stage) in predicting tumor properties such as tumor invasion and recurrence (Feldner and Brandt 2002; Rao 2002).

Gelsolin

Gelsolin, a well-characterized member of the actin-binding protein family, is linked to the carcinogenesis of several organs such as the bladder, prostate, breast, and lung (Tanaka et al. 1995; Lee et al. 1999; Shieh et al. 1999; Thor et al. 2001; Winston et al. 2001). In normal human cells, gelsolin is involved in dynamic changes in the actin cytoskeleton, thus affecting cell motility. A major actin regulatory protein, it is involved in regulating the actin polymerization process by severing and capping F-actin, the polymerized filamentous form of actin (Kwiatkowski 1999). It also has functions related to signaling pathways and apoptosis. Mutations in the gelsolin gene, located at chromosomal region 9q33, cause familial amyloidosis (Finnish type), an autosomal dominant form of familial amyloid polyneuropathy (Levy et al. 1990; O'Brien et al. 1993). Familial amyloidosis (Finnish type) is a slowly progressing disorder leading to the accumulation of amyloid protein in several organs. Despite mutation in the gelsolin gene, the patients are at no increased risk for developing malignant tumors (Kiuru 1992). In the normal human adult kidney, gelsolin is widely expressed in the distal tubules and collecting ducts (Lueck et al. 1998). During carcinogenesis, both up- and down-regulation of gelsolin occurs in a number of solid tumor types (Kwiatkowski 1999). Most reports have shown a decreased expression of gelsolin in solid tumors, suggesting a tumor-suppressive role in malignancies of the prostate and breast (Tanaka et al. 1995; Lee et al. 1999; Winston et al. 2001). In contrast, up-regulation and a negative effect on prognosis have been reported in breast cancer and non-small-cell lung cancer (Shieh et al. 1999; Thor et al. 2001). A recent in vivo study of a highly metastatic murine melanoma cell line suggested a

metastasis-suppressive function for gelsolin (Fujita et al. 2001). The mechanisms by which gelsolin exerts its function in cancer development are still uncertain. In vitro studies suggest that loss of gelsolin expression plays a critical role in urothelial carcinogenesis in cell lines, because the introduction of authentic gelsolin cDNA into a human bladder cancer cell line reduces its colony-forming ability and tumorigenicity (Tanaka et al. 1995). Furthermore, gelsolin is also involved in the regulation of cellular apoptotic processes by functioning as a candidate for caspase activity (Kothakota et al. 1997). When adenovirus-mediated gelsolin gene therapy was attempted in nude mice with orthotopic bladder cancer, tumor size in the gelsolin treatment group decreased markedly (Sazawa et al. 2002).

A recent in vitro study showed that invasion induced by gelsolin is dependent on Ras activity, establishing a connection between gelsolin and the Ras oncogenic signaling pathway (DeCorte et al. 2002). When cancer cells are treated with Compound 2, a novel potential therapy for human cancer based on inhibition of deacetylases, gelsolin expression increases (Fournel et al. 2002). These findings further support the assumption that gelsolin is a carefully regulated downstream target of carcinogenic events.

E-cadherin

E-cadherin is an actin-binding protein belonging to the cadherin family of transmembrane glycoproteins, which are the prime mediators of intercellular adhesion (Behrens et al. 1989). It mediates the selective adhesion of epithelial cells and is required for the interaction and maintenance of normal epithelial integrity (Takeichi 1991). Loss of E-cadherin expression leads to dissociation of cells from cohesive tissues and, in a variety of solid tumors, generates de-differentiation and invasiveness, demonstrating the role of E-cadherin as a suppressor of tumor invasion and metastasis (Frixen et al. 1991; Vleminckx et al. 1991). Although a number of studies have observed decreased E-cadherin expression in patients with urothelial and renal carcinoma, the independent clinical value of E-cadherin immunostaining is controversial (Bornman et al. 2001; Shariat et al. 2001; Shimazui et al. 1997; Fischer et al. 1999; Nakopoulou et al. 2000; Popov et al. 2000). Furthermore, a broad clinical survey showed E-cadherin to be transiently downregulated in prostate cancer, and to be strongly expressed in hormone-refractory and metastatic prostatic tumors (Rubin et al. 2001).

Markers of proliferation

An inherent characteristic of malignant tumors, cell proliferation, can be measured by a variety of methods. Mitotic indices widely serve as an element of various tumor grading methods. Cell proliferation can be measured by specifically

assaying enzymes involved in DNA synthesis, such as thymidine-kinase. Immunohistochemical determination of proliferation indices is an expanding area of research, based on detection of antigens present during cell proliferation; the widely used Ki-67 now has been replaced by the MIB-1 antibody, which has a similar epitope selectivity but recognizes its target also in paraffin-embedded tissues. No consensus exists as to the best proliferation index, or as to the optimal methodology, reagents, or data interpretation. Flow cytometry is widely used, but MIB-1 assays are increasingly popular because of their minimal tissue requirements and suitability for routinely fixed tissues (Spyratos et al. 2002).

Ki-67

Ki-67 is a large protein with an established structure. It has a complex localization pattern within the cell nucleus and undergoes phosphorylation and dephosphorylation during mitosis. Regulation of Ki-67 seems to be tightly controlled. Despite the vast amount of information on Ki-67, its function is still unclear. Ki-67 is vital for cell proliferation, since removal of Ki-67 protein prevents proliferation. Because its protein structure is unique, its function cannot be elucidated by comparison with the structure of other proteins (Brown and Gatter 2002). Ki-67, present in all cycling human cells and a marker of active cell proliferation, can identify cells in the proliferating pool (G1, S, and G2 phases) of human tumor cells, a fact which has been widely documented for a variety of tumors (Elias 1997). IHC staining of Ki-67 (MIB-1) provides an index that estimates the growth fraction of a population of cells. Increased Ki-67 expression indicates active cell proliferation. The diagnostic and prognostic potential of Ki-67 protein expression has been studied in cancers of organs such as breast, lung, brain, prostate, bladder, and kidney (Brown and Gatter 2002). The labeling indices of Ki-67 correlate with grade and clinical outcome in bladder cancer and renal cell carcinoma (RCC), and provide additional prognostic indication of biological aggressiveness (Jochum et al. 1996; Pfister et al. 1999; Rioux-Leclercq et al. 2000). While some studies indicate that Ki-67 expression level is an independent prognostic factor in RCC, others suggest that Ki-67 expression does not contribute additional prognostic information unless incorporated into a prognostic index with other factors (Hofmockel et al. 1995; Aaltomaa et al. 1997; Gelb et al. 1997).

Tumor suppressor proteins

For development and growth of multicellular organisms, regulation of cell survival and cell death is essential. The induction or inhibition of cell death is fundamental to shaping and organizing tissues during development and for permitting adult organisms to respond to and survive environmental stresses that are not overwhelmingly damaging to normal cell function. The balance between

signals that promote or impair cell survival defines tissue homeostasis and may underlie aging and diverse pathologies. Inappropriate loss of cells can lead to degenerative diseases and autoimmune disorders, whereas failure to eliminate mutated cells that have escaped the constraints of normal growth regulation can lead to cancer. Thus, to ensure quality control within cells and ensure the organism's viability, life and death signals work in coordination (Mayo and Donner 2002). Several tumor-suppressor proteins have been linked to the carcinogenesis of urothelial carcinoma and RCC (VHL, fumarate hydratase, retinoblastoma 1, p53) (Zambrano et al. 1999; Knowles 2001; Tomlinson et al. 2002).

p53 and PTEN

The p53 and PTEN tumor suppressors are functionally linked (Mayo and Donner 2002); p53, a short-lived, non-abundant protein in normal cells, plays a major role in regulating the response of mammalian cells to stress and damage, in part through the transcriptional activation of genes involved in cell-cycle control, DNA repair, senescence, angiogenesis, and apoptosis. Disruption of any of these processes can allow cells to escape from normal growth constraints such as apoptosis, allowing passage of mutations from one cell generation to the next, which may permit the development of cancer. In approximately half of all cancers, the p53 gene is mutated. Its prognostic significance has been evaluated in numerous cancers (breast, colon, lung, bladder, kidney) (Soussi and Beroud 2001).

The PTEN tumor-suppressor protein is a dual-specificity phosphatase. It functions as a tumor suppressor by inhibiting activation of PtdIns3-kinase and its downstream target, Akt. Mutations of PTEN have been described in cancers of several organs such as brain, breast, prostate, and kidney (Li et al. 1997; Steck et al. 1997). PTEN and p53, together with the oncoprotein Mdm2, form a tumor suppressor-oncoprotein network. The capacity of PTEN to inhibit PtdIns3-kinase-Akt signaling allows it to block nuclear entry of Mdm2. Recent observations confirm this supposition and additionally show that Mdm2, when restricted to the cytoplasm, is degraded. Expression of PTEN in PTEN-null glioblastoma cells increases the expression of p53 target genes, including those associated with cell cycle arrest. PTEN thus protects p53 from survival signals emanating from growth-factor receptors by inhibition of PtdIns3-kinase-Akt signaling and nuclear entry of Mdm2. Demonstration of this PTEN-p53 connection establishes the fact that tumor suppressors need not function individually. Rather, tumor suppressors, growth signaling pathways, and oncoproteins are networked, and the function of these growth inhibitory/growth stimulatory networks is essential for homeostasis. These observations also suggest that, during the progression of cancer, loss or mutation of one tumor suppressor can undermine the function of another (Mayo and Donner 2002).

Urinary bladder cancer

Overview

Urothelial carcinoma, or transitional cell carcinoma (TCC), is the most common cancer type of the lower urinary tract. More than 57,000 new cases and 12,000 deaths are predicted for the United States in the year 2003, with the incidence of TCC in the United States remaining stable (Jemal et al. 2003). In Finland, 900 new cases are predicted in the year 2003 (Finnish Cancer Registry 2003). Like other malignant neoplasms, urothelial carcinoma develops through multiple genetic and epigenetic changes that lead to alterations in growth, differentiation, and apoptotic control (Brandau and Böhle 2001). These tumors provide a useful model system to study carcinogenic processes, because they have a well-defined progression of disease: from premalignant dysplasia to preinvasive carcinoma in situ (CIS), to superficial carcinoma, and, finally to invasive carcinoma. In addition, it is relatively easy to access the entire organ system through urine cytologic and cystoscopic examination (Droller 2002). TCC is a unique tumor type. Morphologically, low-grade papillary tumors, which are predominantly noninvasive at the time of initial presentation, have a high recurrence rate, with over two-thirds of them recurring. In addition, high-grade tumors are more invasive at initial presentation, although not all high-grade tumors are initially invasive (Jordan et al. 1987). Because tumor grade alone may fail to predict the behavior of an individual tumor, additional biomarkers that can predict tumor recurrence and the invasive behavior will be useful clinically.

Several predisposing factors have been identified for TCC. Among the most common are exposure to aromatic amines and other chemical carcinogens through tobacco use or industrial exposure. Genetic factors contribute to the carcinogenesis of sporadic bladder cancer, although the majority of patients with TCC have no family history of TCC of the urinary tract. No TCC-causing syndromes have been described (Kiemeney and Schoenberg 1996). Cystoscopy and urine cytology are the current standard diagnostic methods, and molecular genetic methods and several potential bladder tumor tests may assist in its diagnosis. The current treatment options are surgery, immunotherapy, and chemotherapy. No chemoprevention exists; however, several promising molecules such as cyclo-oxygenase 2 inhibitors are under investigation (Patton et al. 2002). The prognosis of patients with bladder TCC is rather favorable, with the 5-year cancer-specific survival being over 70% (Finnish Cancer Registry 2003).

Pathological classification

TCC is by far the most common histologic variant of bladder cancer, accounting for approximately 90% of cases. The next most common histologic variants are squamous cell carcinoma and adenocarcinoma, at 7% and 2% (Izawa and Grossman 2000). Urothelial cancers develop along two biologically distinct pathways – low-grade superficial papillary and high-grade flat CIS – with different clinical prognoses (Patton et al. 2002). TNM classification from 1997 is used for bladder cancer staging (Table 1), with the World Health Organization classification currently used for tumor grading (Table 2) (Mostofi et al. 1973; Sobin et al. 1997; Cheng and Bostwick 2000).

Pathophysiology

Various carcinogens and risk factors contribute to the generation of bladder carcinomas: beta-naphthylamine, a compound of cigarette smoke, is a well-known carcinogen, and smokers have a 4-fold risk for developing urothelial carcinoma. An elevated incidence of urothelial carcinomas also appears among workers in the rubber industry who are heavily exposed to beta-naphthylamine and other amines (Clavel et al. 1989; Vineis and Pirastu 1997).

In bladder cancer, as in most types of cancer, the transformation of a normal into a malignant cell involves a multistep mechanism. Sequentially, the expression of various classes of genes – oncogenes, tumor-suppressor genes, cell-cycle genes, and DNA-repair genes – is altered. These alterations involve mutations or chromosomal aberrations such as translocation, insertion, amplification, and deletion (Brandau and Böhle 2001). Ras, c-erbB2, and epidermal growth factor receptor (EGFR) are among the oncogenes characterized in TCC inducing carcinogenesis through activation mechanisms, whereas the retinoblastoma gene and p53 are among the tumor-suppressor genes involved in bladder carcinogenesis (Brandau and Böhle 2001).

Bladder cancer shows frequent chromosomal alterations. The most frequent gains are in chromosomal regions 1q21-q24, 8q21-q22, and 17q, whereas the most common deletions are in chromosomal regions 11p15-p14, 8pter-p22, 9pter-p21, and 9q (Simon et al. 2000). Loss of heterozygosity (LOH) also occurs in bladder cancer, contributing to carcinogenesis through inactivation of tumor-suppressor genes such as p53 (Brandau and Böhle 2001).

Markers associated with bladder malignancies

Current pathological and clinical parameters such as TNM classification provide essential prognostic information yet still have limited ability to predict the true malignant potential of most bladder tumors. In the recent years, investigation of basic mechanisms involved in carcinogenesis and tumor progression by

Table 1. TNM staging system for bladder cancer (Sobin et al. 1997).

Primary Tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Ta	Noninvasive papillary carcinoma
Tis	Carcinoma *in situ*: "flat tumor"
T1	Tumor invades subepithelial connective tissue
T2	Tumor invades muscle
T2a	Tumor invades superficial muscle (inner half)
T2b	Tumor invades deep muscle (outer half)
T3	Tumor invades perivesical tissue
T3a	microscopically
T3b	macroscopically (extravesical mass)
T4	Tumor invades any of the following: prostate, uterus, vagina, pelvic wall, abdominal wall
T4a	Tumor invades prostate, uterus, vagina
T4b	Tumor invades pelvic wall, abdominal wall
Regional Lymph Nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single lymph node, 2 cm or less in greatest dimension
N2	Metastasis in a single lymph node, more than 2 cm but not more than 5 cm in greatest dimension, or multiple lymph nodes, none more than 5 cm in greatest dimension
N3	Metastasis in a lymph node more than 5 cm in greatest dimension
Distant metastasis (M)	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Table 2. Histologic grading of papillary urothelial carcinoma (Mostofi et al. 1973).

Grade	Description
Grade 1	Well differentiated
Grade 2	Moderately differentiated
Grade 3	Poorly differentiated / undifferentiated

molecular biology has provided a host of tumor markers of potential diagnostic or prognostic value for bladder carcinoma (Tiguert et al. 2002). These markers may serve as tools for early and accurate prediction of tumor recurrence and progression, and for development of metastases and prediction of response to therapy. Precise prediction of tumor behavior would facilitate treatment selection of patients who may benefit from various treatments including surgery and adjuvant therapy. Currently, no single marker is able accurately to predict the clinical course of bladder tumors and thus to serve as a reliable prognostic marker. A combination of prognostic markers could predict which superficial tumors require an aggressive form of therapy. Several urine and serum tests have been developed in the attempt to discover a marker useful for the detection or prognostication of bladder cancer. Despite these numerous research efforts, no diagnostic marker with a specificity and sensitivity comparable to cystoscopy currently exists. Some of the potential tissue markers of bladder cancer are listed in Table 3, among the most promising being Ki-67 and p53 (Kausch and Böhle 2001; Kausch and Böhle 2002).

Marker	**Biological function**	**Potential prognostic value**
ABO	Blood group antigen	Diagnostic marker, association with progression
Ras, c-MYC, c-erbB2	Oncogenes	Prognosticators of disease recurrence (Ras) or survival (c-erbB2), correlation with tumor grade (c-erbB2, c-MYC) or metastasis (c-erbB2)
Rb, p53	Cell cycle regulators	Correlation with progression and survival (Rb), marker of progression, or recurrence and survival (p53)
Ki-67, PCNA	Proliferation-associated antigens	Marker of recurrence, progression, and survival (Ki-67), correlation with tumor grade (PCNA)
E-cadherin	Cell adhesion molecule	Association with metastasis, tumor grade, stage, and survival
VEGF	Peptide growth factor	Correlation with tumor stage, grade, and recurrence
EGFR	Growth factor receptor	Correlation with recurrence and progression

Table 3. Tissue markers of bladder cancer (Kausch and Böhle 2002).

Renal cell carcinoma

Overview

RCC is, in the US, the fourteenth leading cause of cancer mortality, with 32,000 new cases and 12,000 deaths predicted in 2003 (Jemal et al. 2003). Incidence has been increasing in the United States during the last 20 years (Tsui et al. 2000). In Finland, 800 new cases are predicted in the year 2003 (Finnish Cancer Registry 2003). One-third of patients have metastatic disease at diagnosis; approximately 50% of those undergoing surgical resection for less advanced disease eventually relapse (Bui et al. 2001). The 5-year cancer-specific survival of kidney cancer is 60% (Finnish Cancer Registry 2003). Several predisposing factors include cigarette smoking, obesity, and hypertension. Genetic factors contribute to the carcinogenesis of sporadic kidney cancer, and some inherited syndromes predispose to kidney cancer (Godley and Kim 2002).

For RCC, the best available prognostic indicator is stage, but the current prognostic factors: grade, and performance status, as well as stage, are insufficient in predicting patient outcome and cancer aggressiveness (Rioux-Leclercq et al. 2000; Tsui et al. 2000; Pantuck et al. 2001; Zhou and Rubin 2001; Zisman et al. 2001). Identification of biomarkers that provide further prognostic information would thus be vital for defining optimal treatment and outcomes (Elias 1997). Before the availability and widespread use of abdominal ultrasonography and computerized tomography, the usual presentations of renal lesions were symptoms and/or signs of urinary tract pathology. Now, due to the improved imaging techniques and their good availability, renal masses are most frequently incidental findings (Nicol 2000). Therapeutic options for renal cancer are surgery and systemic therapy. Thus far, for localized kidney cancer nephrectomy is the treatment of choice. For advanced kidney cancer, cytotoxic chemotherapy has been disappointing, but immunotherapy has remained the mainstay of treatment. The most commonly used immunotherapeutic agents are interleukin-2 and interferon-alpha (Godley and Kim 2002). Cytotoxic agents such as vinblastine have also been used in advanced RCC in combination with immunotherapeutic agents (Pyrhönen et al. 1999). Gene therapy for RCC is under investigation (Pulkkanen et al. 2001; Haviv et al. 2002). Attempts to create more accurate staging systems for RCC include integration of molecular and clinical information into the system along with the current prognostic factors (Pantuck et al. 2001; Zisman et al. 2001).

Pathological classification

The UICC/AJCC classification of RCC serves to classify it, with morphological and genetic facts integrated (Table 4) (Storkel et al. 1997; Pantuck et al. 2001). Sarcomatoid differentiation appears in all RCC subtypes, and when

Table 4. UICC/AJCC classification of renal cell carcinoma (Sobin et al. 1997).

Benign neoplasms
Papillary adenoma
Renal oncocytoma
Metanephric adenoma
Malignant neoplasms
Clear cell carcinoma
Papillary renal cell carcinoma
Chromophobe renal cell carcinoma
Collecting duct carcinoma
Renal cell carcinoma, unclassified

Table 5. Genetic classification of renal cell carcinoma (Pantuck et al. 2001).

Tumor type	**Tissue origin**	**Genetic alteration**
Clear cell	Proximal tubule	3p (VHL tumor suppressor)
Papillary	Proximal tubule	+7q (c-Met oncogene),+17,-Y
Chromophobe	Collecting duct	-1, -2, -6, -10
Collecting duct	Medullary collecting duct	-11
Oncocytoma	Distal tubule	-1, -Y, 11q
Papillary adenoma	Proximal tubule	+7, +17, -Y

present, indicates poor prognosis. RCC can also be classified based on genetic information alone (Table 5) (Pantuck et al. 2001). Familial adult renal neoplasias constitute a distinguishable entity among all renal neoplasias (Table 6) (Takahashi et al. 2002). The Fuhrman system is used for histological grading (Table 7) (Fuhrman et al. 1982), and the TNM classification proposed by UICC in 1997 is the staging system most commonly used (Table 8) (Sobin et al. 1997).

Pathophysiology

Although the etiology of RCC remains elusive, a number of studies have investigated potential environmental and genetic risk factors. Cigarette smoking, obesity, hypertension, and acquired polycystic kidney disease have been consistently associated with RCC. Von Hippel-Lindau (VHL) disease is probably the best characterized of the hereditary syndromes, with 23 to 45% of afflicted patients developing RCC in their lifetime. The protein product of the VHL gene is thought to regulate the transcription of RNA polymerase II and regulate vascular endothelial growth factor expression at the posttranscriptional level. Treatment for these patients is difficult because several hundred carcinomas may be present in a given VHL kidney. Tuberous sclerosis is associated with hamartomas in various

Table 6. Familial adult renal neoplasias (Takahashi et al. 2002; Tomlinson et al. 2002).

Disease	**Molecular genetics**		**Clinical features**	
	Gene location / translocation	*Gene*	*Main lesions*	*Other associated lesions*
Von Hippel-Lindau disease (VHL)	3p25	*VHL*	Retinal hemangioma, CCRCC, cerebellar haemangioblastoma, pheochromocytoma	Endocrine pancreatic tumors, epididymal cystadenoma
Familial non-VHL CCRCC with chromosome 3 translocation	3p14 3q13.3 3q21	*FHIT, TRC8?* Unknown Unknown	CCRCC	Thyroid, bladder, pancreatic, and gastric cancer
Familial non-VHL CCRCC without chromosome 3 translocation	Unknown	*TRC8?*	CCRCC	
Hereditary papillary cancer (HPRC)	7q34	*c-Met*	PRCC (type 1)	Breast, pancreatic, biliary tract, and lung cancer, malignant melanoma
Hyperparathyroidism-jaw tumor	1q21-q32	Unknown	Parathyroid adenoma/carcinoma, ossifying jaw tumors	Renal cysts, renal hamartoma, adult Wilms tumors, PRCC (type 1)
Tuberous sclerosis complex	9q34 16p13.3	*TSC1* *TSC2*	Renal angiomyolipoma, rhabdomyoma	CCRCC
Birt-Hogg-Dube syndrome	17p12-q11.2	Unknown	Multiple fibrofolliculoma, trichodiscoma, renal oncocytoma, chromophobe RCC	PRCC (type 1), CCRCC, multiple lipoma, pulmonary cysts
Hereditary leiomyomatosis and renal cell cancer	1q42-q44	*Fumarate hydrase (FH)*	Uterine leiomyoma, PRCC (type 2)	Uterine leiomyosarcoma, skin leiomyoma, breast cancer, bladder cancer
Familial papillary thyroid carcinoma-papillary renal neoplasia	1q21	Unknown	Papillary thyroid carcinoma	PRCC (type 1), papillary renal adenoma, renal oncocytoma
Familial oncocytoma	Unknown	Unknown	Renal oncocytoma, renal cyst	
Hereditary non-polypotic colorectal cancer (HNPCC)	2p22-p21 3p22-p21 2q31-q33 7p22 2p16	*hMSH2* *hMLH1* *hPMS1* *hPMS2* *hMSH6*	Colorectal cancer, endometrial cancer	Gastric, ovarian, hepatobiliary, renal pelvic, ureteral, and small intestine cancer

Table 7. Fuhrman grading system for renal cell carcinoma (Fuhrman et al. 1982).

Grade 1	Round uniform nuclei
Grade 2	Larger, irregular nuclei and visible nuclei under high power (x400)
Grade 3	Larger, irregular nuclei and large nucleoli under low power (x200)
Grade 4	Bizarre, often multilobed nuclei and chromatin clumps

organs, and although more commonly associated with renal angiomyolipomas, shows an increased risk for RCC as well. The tuberous sclerosis genes TSC1 and TSC2 are tumor-suppressor genes that affect cellular proliferation indirectly. Other hereditary RCC-causing syndromes, such as hereditary papillary renal carcinoma, familial renal oncocytoma, and the Birt-Hogg-Dube syndrome, have also been described and their genetic loci identified (Moyad 2001; Godley and Kim 2002; Takahashi et al. 2002).

Markers associated with renal malignancies

Currently, no clinically useful diagnostic or prognostic marker for RCC exists. Clinical diagnosis of RCC is usually confirmed by imaging studies, although benign renal lesions sometimes pose a diagnostic challenge. None of the serum diagnostic markers studied has reached clinical practice (Zhou and Rubin 2001).

Prognostic factors measure tumor aggressiveness and host response both natural and therapy-induced. Any factor involved in tumor proliferation, invasion, or metastasis, as well as the patient's specific and nonspecific response to the tumor, can contribute to prognosis. Among numerous factors studied for their prognostic potential (Table 9), several molecules are promising, but none has reached clinical practice (Zhou and Rubin 2001). The role of gelsolin in RCC remains to be studied.

Future prospects

Major technical advances are expected to occur during the next few years that will transform the TMA technique from an institution-limited screening tool to a medium that will enable multi-institutional collaborative studies. Possibly all the data produced will also be available to the scientific community more widely through the internet, resembling the current practice in the Human Genome Project and in some expression-array centers, where freely available genomic sequence databases have considerably accelerated the rate at which discoveries are made (Manley et al. 2001). Technical improvements most likely to occur in the near future include automated arraying and staining methods, as well as an automated image-capturing system allowing remote analysis of the samples in silico (Bova et

Table 8. TNM staging system for renal cell carcinoma (Sobin et al. 1997).

Primary Tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Tumor 7 cm or less in greatest dimension limited to the kidney
T2	Tumor more than 7 cm in greatest dimension limited to the kidney
T3	Tumor extends into major veins or invades adrenal gland or perinephric tissues but not beyond Gerota's fascia
T3a	Tumor invades adrenal gland or perinephric tissues but not beyond Gerota's fascia
T3b	Tumor grossly extends into renal vein(s) or vena cava below diaphragm
T3c	Tumor grossly extends into vena cava above diaphragm
T4	Tumor invades beyond Gerota's fascia
Regional Lymph Nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single regional lymph node
N2	Metastasis in more than one regional lymph node
Distant Metastasis (M)	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

al. 2001; Camp et al. 2002). These developments will further accelerate the discovery process, simultaneously placing new demands on database construction and maintenance, since this novel way to do TMA research will be more dynamic than the traditional method with data laboriously generated by one or very few researchers at a time (Manley et al. 2001).

The challenge that researchers currently encounter – and are likely to encounter more often in the future – is how to extract essential information from the enormous amount of data being produced by TMAs. Despite developments in several aspects of the TMA technique, statistical methods applicable to TMAs have not yet undergone detailed study (Manley et al. 2001). The majority of the methods will be similar to epidemiological studies in which a large amount of data is analyzed. There are, however, features inherent to the TMA technique that must be addressed specifically. First, if the results are to be generally utilized in the scientific community, data-recording methods must be standardized. Second,

Table 9. Potential molecular prognostic markers for renal cell carcinoma.

Marker	**Suggested effect**
Acute phase reactants	
C-reactive protein	Predicts prognosis (Ljungberg et al. 1995)
Haptoglobin	Predicts prognosis (Ljungberg et al. 1995)
Ferritin	Predicts prognosis (Ljungberg et al. 1995)
Uromucoid	Predicts prognosis (Ljungberg et al. 1995)
Alpha1-antitrypsin	Predicts prognosis (Ljungberg et al. 1995)
Angiogenesis	
VEGF	Serum level correlates with tumor volume (Sato et al. 1999)
BFGF	Serum level increased in disseminated RCC (Dosquet et al. 1997)
HGF/scatter factor	Serum level increased in RCC (Dosquet et al. 1997)
Cell adhesion molecules and proteases	
Urokinase plasminogen activator	Predicts early relapse (Hofmann et al. 1996)
Plasminogen activator inhibitor	Predicts prognosis (Hofmann et al. 1996)
CD44	Correlates with progression or recurrence (Gilcrease et al. 1999)
Alpha-catenin	Predicts prognosis (Shimazui et al. 1997)
Metallothionein	Correlates with invasive growth pattern (Zhang and Takenaka 1998)
Nm-23	Predicts prognosis (Nakagawa et al. 1998)
Cell cycle molecules	
cyclin A	Predicts prognosis (Aaltomaa et al. 1999)
Glycolytic and other enzymes	
Aldolase A, gamma-enolase	Simultaneous elevated expression predicts prognosis (Takashi et al. 1993)
Calpain 1	Correlates with advanced grade (Braun et al. 1999)
Glutathione S transferase	Predicts prognosis (Grignon et al. 1994)
Cytokines	
IL-10	Serum level predicts prognosis (Wittke et al. 1999)
Growth factors	
Erythropoietin	Serum level predicts prognosis (Ljungberg et al. 1992)
EGF-receptor	Predicts prognosis (Moch et al. 1997)
Proliferation markers	
Ki-67	Predicts prognosis (Rioux-Leclercq et al. 2000)
PCNA	Predicts progression (Fischer et al. 1999)
TPS	Predicts progression (Hobarth et al. 1996)
Tumor suppressor/oncogenes	
p53	Predicts progression (Rioux-Leclercq et al. 2000)
Genetic markers	
LOH of 9p	Predicts progression (Moch et al. 1996)

incorporation of TMA data with data from other sources, e.g., from cDNA microarrays and cell biology studies, is a constantly evolving field not yet thoroughly explored.

The need to overcome the adverse effect of paraffin-embedding on the integrity of RNA, of lipids and of some proteins, has led to a modification of TMA, cryoarray (Fejzo and Slamon 2001; Hoos and Cordon-Cardo 2001). The cryoarray technique uses frozen tissue as target of molecular analyses. Undoubtedly more accurate in some analyses, especially RNA analyses, this novel TMA application has the disadvantage that collection of the fresh-frozen tissues required for array construction is often slower and more difficult than is use of archived paraffin-embedded samples.

Application of the TMA technique to non-neoplastic tissues is a frontier yet to be explored. Although several arrays from different institutions, including ours, contain non-neoplastic components (Hoos et al. 2001; Rubin et al. 2001), those tissues serve as a source of additional information assisting in the interpretation of molecular events in malignancy. Expansion of the TMA technique to solely non-neoplastic cerebral tissues has recently occurred (Goldstine et al. 2002), but the applicability of TMAs in study of benign tissues and diseases remains to be fully determined by studies including several different target histologies.

Potential applications of TMA are many: longitudinal arrays with samples collected at different time-points from selected patients, lymph node or metastasis arrays enabling studies of events leading to invasion and metastasis, cascade-based approaches with a selected signalosome comprehensively studied, replicate arrays multiplying the number of molecules that can be analyzed from each individual tumor. Most likely, combination of data from several sources (TMAs, cDNA microarrays, cell arrays) will become more common, assisting efforts to develop a comprehensive view of cellular events leading to cancer. The recent observation that genomic aberrations at different sites of the disease-causing pathway in polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL) can lead to an identical disease phenotype encourages efforts also to profile cancer tumors in order to reveal novel molecular pathways (Paloneva et al. 2002). Furthermore, the discovery of a specific treatment for metastatic gastrointestinal tumors involving a tyrosine kinase inhibitor shows that there exist underlying pathologies in cancer that can be discovered and exploited to develop novel treatments (Joensuu et al. 2001). Compared to cDNA microarray data, sensitive to technique-related variation such as temperature variation during sample collection, the replicability of TMA findings is good, an advantage in assessing their reliability (Dash et al. 2002).

Today's medicine is revealing the pathoetiology of disease and discovering novel specific treatments at a constantly increasing rate. TMAs may in future serve as a tool accelerating drug-target discovery and also as a tool to evaluate the effects of new drugs on target tissues (Bubendorf 2001; Katsuma and Tsujimoto 2001; Anzick and Trent 2002).

AIMS OF THE STUDY

Molecular tumor profiling has the potential to extend our knowledge of carcinogenic processes by comprehensively portraying molecular events within individual tumors. The novel tissue microarray technique may serve as a high-throughput tool to accelerate these profiling efforts; the TMA technique may also serve to validate biomarkers potentially useful in the detection, prognostication, or follow-up of malignant tumors.

The aims of this study were:

1) To apply the tissue microarray technique in molecular profiling of urinary tract malignancies (I-IV).

2) To study the expression of actin-associated biomarkers gelsolin and E-cadherin in urothelial carcinoma (I).

3) To study the amplification of chromosomal region 8q24 in bladder cancer (II).

4) To profile renal tumors based on Ki-67, on gelsolin, and on PTEN protein expression (III).

5) To define the prognostic significance of Ki-67 and gelsolin expression in renal clear-cell carcinoma, the most common renal tumor type (IV).

MATERIALS AND METHODS

Patient samples

Urothelial cancer

The urothelial cancer TMA includes archival bladder carcinoma tissue samples from 202 cases (146 patients) dated between 1985 and 1995. The material came from the Department of Pathology and Laboratory Medicine at the University of California, Los Angeles (UCLA) Medical Center, following approval by the UCLA Institutional Review Board. Table 10 shows the clinicopathologic data for the 146 patients included in the final analysis. For patients with multiple cases, data from either the patient's first procedure of any type or their cystectomy case, if performed within one month of the first procedure, were used for all subsequent analyses. The patients ranged in age from 33 to 94 years, with a mean age of 67 years. The male-to-female ratio was 3.6:1. There were 57 Tis/Ta/T1 noninvasive or superficially (lamina propria) invasive tumors, and the remaining 89 were deeply invasive, including 44 T2, 35 T3, and 10 T4 tumors. There were 8 CIS, 6 grade 1, 40 grade 2, and 92 grade 3 tumors. Detailed demographic, pathologic, and clinical information including treatment and follow-up data for at least 5 years was incorporated into a correlative database linked to the tissue specimens. In addition, data from the original pathology reports were utilized for analysis. Tumor registry data including treatment, recurrence, and survival data came from the UCLA Cancer Program of the Jonsson Comprehensive Cancer Center.

Renal cancer

For the renal cancer TMA, a total of 355 formalin-fixed paraffin-embedded primary renal carcinoma specimens came from the Department of Pathology and Laboratory Medicine at the University of California, Los Angeles (UCLA) Medical Center, from patients undergoing surgery for RCC between the years 1987 and 1999, following approval by the UCLA Institutional Review Board. A total of 257 representative clear-cell carcinoma specimens were chosen for detailed analyses; 215 of these were radical nephrectomy and 42 partial nephrectomy specimens. Table 11A illustrates the distribution of the specimens representing clear-cell morphology according to stage and grade. The clinical characteristics of this study population are illustrated in Table 11B. For the other renal tumor subtypes, the specimens were analyzed solely according to tumor morphology, excluding grade and stage from the analyses. Survival data for the clear-cell RCC tumors were obtained by reviewing the hospital records after approval by the UCLA Institutional Review Board. Outcome assessment was based on chart review of

Table 10. Characteristics of 146 bladder cancer patients by age, gender, tumor grade, stage, and surgical procedure.

Characteristics	**Number of patients (%)**
Gender	
male	114 (77.7)
female	32 (22.3)
Age, years (mean)	67 (range 33-94)
Grade	
1	6 (4.1)
2	40 (27.4)
3	92 (63.0)
CIS	8 (5.5)
Stage	
Tis	8 (5.5)
Ta, T1	49 (33.6)
T2	44 (30.1)
T3	35 (24.0)
T4	10 (6.8)
Procedure	
Transurethral resection	58 (39.7)
Cystectomy	88 (60.3)

Table 11. Characteristics of clear-cell RCC study population.

A) Tumor grade and stage distribution.

	Grade 1	Grade 2	Grade 3	Grade 4	Total
Stage 1	10	59	17	0	86
Stage 2	2	36	11	2	51
Stage 3	0	43	35	8	86
Stage 4	0	3	4	2	9
Total	12	141	67	12	232*

*Stage information missing for 25 of 257 tumors.

B) Clinical data for the 257 clear-cell RCC patients.

Gender, male/female	167/90
Age, years (mean)	68 (range 33-92)
Follow-up, months (mean)	34 (range 0-138)
Alive*	108
Deceased / RCC	95
Deceased /other causes	37

*Follow-up data missing for 17 patients.

demographic, clinical, and pathologic data. Patients were evaluated from histological diagnosis to the last known follow-up.

Tissue microarrays

For the urothelial cancer TMA, the original hematoxylin and eosin (H&E)-stained case slides were reviewed utilizing the 1997 TNM classification (Sobin et al. 1997). During this review, slides containing tumor, adjacent dysplasia, and distant benign fields were selected and marked as such by designated colored ink. TMA blocks were constructed following the original technique described by Kononen and colleagues (Kononen et al. 1998). Where available, four representative tissue samples from each selected area were included in the array. Analysis was limited to urothelial carcinoma cases, including 140 from the bladder (2 of which showed small cell differentiation, one showed signet ring features, and one accompanied concomitant renal pelvis urothelial carcinoma), 3 from the renal pelvis alone, and 3 from the ureter alone. Metastatic tumors and tumors showing exclusive squamous cell carcinoma and adenocarcinoma differentiation were excluded from analysis. Final analysis included 146 patients, 202 cases, and 1208 tissue spots.

For each case, attempts were made to obtain not only tumor areas, but also adjacent dysplastic areas and distant benign fields. These field samples, which include progressive changes from benign, to adjacent dysplasia, and finally to CIS, provided a mechanism to study how marker expressions are altered in the early stage of the malignant process. The benign fields came from the ureter or urethral resection margins (for cystectomy specimens) or from benign-appearing urothelium at least 5 mm distant from the sampled tumor (for transurethral resection). A total of 81 benign field, 8 adjacent dysplasia, and 40 informative CIS samples were included.

For the renal cancer TMA, the original tumors were staged according to TNM classification, graded according to Fuhrman, and histologically subtyped according to the recommendations of the UICC (Fuhrman et al. 1982; Sobin et al. 1997). Four core tissue biopsies 0.6 mm in diameter were taken from selected morphologically representative regions of each paraffin-embedded renal tumor and precisely arrayed by use of a custom-built instrument as previously described (Kononen et al. 1998). For each tumor, three of the biopsies were taken from tumor areas, and one biopsy from a benign region of the sample.

Sections 4 µm thick of the resulting tumor TMA blocks were transferred to glass slides by the paraffin sectioning aid system (Instrumedics Inc., Hackensack, NJ, USA).

Immunohistochemistry

H&E-stained array sections were evaluated histopathologically in blinded fashion to validate the diagnostic morphology of each array spot. Commercial antibody preparations were utilized for the analyses of p53 (Dako Corporation, Carpenteria, CA, USA), E-cadherin (Zymed, San Francisco, CA, USA), Ki-67 (Dako), PTEN (Zymed), and gelsolin (Sigma, St. Louis, MO, USA).

For IHC staining of gelsolin, a standard two-step indirect avidin-biotin complex (ABC) method was used (Vector Laboratories, Burlingame, CA, USA). Tissue array sections (4 µm thick) were cut immediately before staining and were heated to 56 °C for 20 minutes, followed by deparaffinization in xylene. These sections were rehydrated in graded alcohols and endogenous peroxidase quenched with 3% hydrogen peroxide in methanol at room temperature. The sections were then placed in a 95 °C solution of 0.01 M sodium citrate buffer (pH 6.0) for antigen retrieval. Protein blocking was accomplished through application of 5% normal horse serum for 30 minutes. Endogenous biotin was blocked with sequential application of avidin D, then biotin (A/B blocking system). Primary mouse anti-gelsolin monoclonal IgG1 antibody was applied at a 1:750 dilution for 60 minutes at room temperature. After washing, biotinylated horse antimouse IgG was applied for 30 minutes at room temperature. The ABC complex was applied for 25 minutes with diaminobenzidine (DAB) used as the chromogen. Phosphate-buffered saline (10 mM), pH 7.4, was used for all intermediate wash steps, and a moist humidity chamber was used for prolonged incubations. The sections were counterstained with Harris hematoxylin, followed by dehydration and mounting. For staining of p53, E-cadherin, and Ki-67, the DakoEnvision biotin-free dextran peroxidase staining system was used, as was the Dako Autostainer automated staining system, and for PTEN, the manual Vector Elite avidin-biotin conjugate kit.

Tissue samples with known expression for each marker served as positive controls (Ki-67 and p53 for high-grade breast carcinoma tissue sections; gelsolin and E-cadherin for normal prostate tissue sections). Negative controls were sections treated as described above, but with the primary antibody replaced with pooled nonimmune mouse IgG of the same concentration, or with the primary antibody omitted as a null slide. All sections were analyzed with a BX-40 brightfield microscope (Olympus, Tokyo, Japan) under x10-20 objectives. When questions arose concerning tissue morphology, H&E-stained sections were reviewed for confirmation.

Fluorescence in situ hybridization

The bacterial artificial chromosome (BAC) clone RP11-184M21 (GenBank accession number AC090798) residing in the center of the reported amplified region served as a specific probe to detect copy number alterations in the 8q24 chromosomal region. This probe was random primed with Spectrum Orange. FISH was performed for the TMAs following the protocol recently described by Andersen and colleagues (Andersen et al. 2001).

Evaluation of protein expression

Quantitative assessment of antibody staining in the bladder cancer TMA and renal cancer TMA was performed blinded to clinicopathologic variables. The intensity of DAB brown chromogen staining (Max) was graded. For the nuclear-staining markers p53 and Ki-67, the 0 to 3 scale was: 0, negative; 1, weak staining; 2, moderate staining; 3, strong staining. For non-nuclear staining of gelsolin and E-cadherin, the 0 to 4 scale was: 0, negative; 1, weak staining; 2, weak but distinct staining; 3, moderate staining; 4, strong staining. Gelsolin and PTEN have a cytoplasmic staining pattern, whereas E-cadherin has a membrane staining pattern. The proportion of analyzed cells staining positively (Pos) was also recorded. For each marker, the Max, Pos, and the product of both (MaxPos), obtained by multiplying Max with Pos, were determined. For PTEN, Pos value was the only parameter analyzed. The median value of repeated core spots representing each area of interest for each sample was used for the final analysis.

Evaluation of genomic amplification

Evaluation of amplification at chromosomal region 8q24 was performed with a Leica DMR fluorescence microscope workstation for all patients demonstrating specific hybridization in at least one morphologically representative tumor spot. High-copy amplification was defined as the presence of at least 10 signals or tight clusters of at least 5 signals per cell in at least 5% of tumor cells. The main reasons for failed scoring of a spot were unrepresentative spot morphology, weak signal, and autofluorescence.

Statistical analyses

For the bladder cancer TMA, the association of marker expression versus clinicopathologic parameters was demonstrated in several ways. First, mean ±standard error of MaxPos was analyzed against progressive field changes, tumor

grade, and stage. The Student t-test, analysis of variance (ANOVA), and the nonparametric Kruskal-Wallis tests were used to test whether the immunoreactivity of each marker differed between groups defined by the clinicopathologic parameters grade and stage. To analyze the recurrence risk of multiple clinicopathologic and marker expression variables, multivariate logistic regression was used. The adjusted odds ratios (relative risks) and their respective p-values were determined. Kaplan-Meier curves were used to estimate recurrence-free time curves, and the log rank test served to test whether curves differed between groups. The Cox proportional hazards model was used to assess which covariates affected recurrence-free time. For each covariate, relative hazard rate and associated p-value were reported. For all analyses, a p-value less than 0.05 was considered significant, and analyses were performed with the software package R (url:http://cran.r-project.org/).

For analysis between Ki-67 or p53 expression and 8q24 amplification in the bladder cancer TMA, each spot was given a score from 0 to 300 for the biomarker analyzed, by multiplying the maximal intensity of staining (scale 0-3) by the percentage of positively staining tumor cells (scale 0-100). The median biomarker expression score of each tumor was calculated from the available 1 to 4 spots per tumor and used in further analyses. The standard deviation of biomarker expression was calculated from the mean expression of each biomarker. The Mann-Whitney test was performed to compare median biomarker expression between amplified and non-amplified tumors, and to compare times of removal between metastases.

For clear-cell RCC TMA analyses, the association between biomarker outcome measures and the various pathological parameters was studied by mean and confidence interval plots and the Kruskal-Wallis test. The recursive partitioning method (RPART function in S-Plus, Insightful Corporation, Seattle, WA, USA) served for survival-tree analysis to find appropriate cutoffs for classifying patients by Ki-67 and gelsolin expression. Kaplan-Meier curves were used to estimate survival, and the log rank test and the Wilcoxon test to test whether curves differed between groups. For the Kaplan-Meier curves, the tumor specimens were categorized into two groups (Ki-67-negative and Ki-67-positive) according to Ki-67 status, with all tumors presenting no Ki-67 staining considered negative, and all others positive. Furthermore, the specimens were also categorized into two groups (gelsolin-positive and gelsolin-negative). The gelsolin-positive group consisted of tumors with gelsolin staining in every tumor cell, and the gelsolin-negative group of all other tumors, thus including tumors with partial or non-perceptible gelsolin staining. To assess which covariates predicted survival, univariate and multivariate Cox proportional hazards models were used. Covariates included Ki-67 and gelsolin expression, grade, stage (pT), gender, and age at diagnosis. The proportional hazards assumption was confirmed by Schoenfeld

residuals. The relative hazard rate and the associated p-values were determined for each covariate. For all analyses, a p-value of less than 0.05 was accepted as significant, and the analyses were carried out with the software package SAS. After fitting a multivariate regression model, the default SAS stepwise procedure was used to eliminate insignificant covariates from the model. Patients with other covariates or biomarker outcome measures missing were eliminated from analysis. Cancer-specific survival was defined as time from nephrectomy to last follow-up contact (alive/dead), taking cause of death into account (died of RCC/ died of other causes). The Pearson Chi-square test was used to assess the relationship between dichotomized staining phenotypes of the tumors for Ki-67 and gelsolin. To compare the median expression of the biomarkers gelsolin, Ki-67, and PTEN between the renal tumor subtypes, the median biomarker expression value constructed from the expression values of the three cancer tissue spots for each tumor was used in analyses. For sarcomatoid tumors, only the tissue spots with sarcomatoid morphology were included in analyses. The Wilcoxon two-sample test was performed to analyze biomarker expression between tumor types.

RESULTS AND DISCUSSION

Biomarker expression in bladder cancer (I-II)

Understanding the oncogenic processes leading to recurrence and progression in urothelial carcinoma would potentially lead to more accurate prognostication and to development of novel specific treatments for this malignancy. Although much progress has been made in recent years in identifying molecular events that lead to development of cancer, the exact mechanisms underlying the evolution of malignant progressive phenotypes remain poorly understood. For instance, the exact biochemical events leading to cancer invasion or recurrence have yet to be determined. Understanding these mechanisms may have an impact on the development of markers that can be used to define an individual's risk for tumor progression and to customize appropriate management strategies.

Changes in cellular motility occur frequently in malignancies with invasive potential, such as bladder cancer. In general, it has been assumed that alterations in the cytoskeletal protein actin, a major factor in the structure and motility of the cell, play an important role in cell invasion and metastasis, even though the exact mechanisms remain to be fully elucidated. The actin network is a complex structural and functional system of all eukaryotic cells (Pollard and Cooper 1986). Molecular mechanisms underlying actin remodeling involve several oncogenic signal transduction pathways, the most notable being the small GTPase of the Ras superfamily of proteins: Rac, Rho, and Cdc42 (Nobes and Hall 1995; Olson et al. 1995). In addition, the actin polymerization process is regulated by numerous actin-binding and actin-regulatory proteins (Way and Weeds 1990; Singer 1992). Many of these proteins (gelsolin, E-cadherin, vinculin) have tumor-suppressor functions individually (Vleminckx et al. 1991; Rodriguez Fernandez et al. 1992; Prasad et al. 1993; Tanaka et al. 1995). A comprehensive and simultaneous analysis of all actin-related molecules, rather than one isolated molecule at a time, will therefore be necessary for a clearer understanding of how actin is associated with malignant phenotypic changes. One genomic region involved in the development of a relatively large fraction of urothelial cancers is 8q24 (Sauter et al. 1995; Fadl-Elmula et al. 2001). Less is known about the relationship of 8q24 amplification and the expression of biomarkers p53 and Ki-67, which in urothelial cancer are frequently overexpressed (Lipponen 1993; Pfister et al. 1999).

The TMA technique provides a convenient high-throughput tissue-based tool for in situ gene dosage and protein expression studies (Kononen et al. 1998). This technique, utilized to rapidly profile a number of molecular markers, produces results comparing well with those by standard methods (Bubendorf et al. 1999b;

Bärlund et al. 2000; Camp et al. 2000; Gillett et al. 2000). In our study, the two most notable actin-binding proteins, gelsolin and E-cadherin, were evaluated as markers for urothelial carcinoma recurrence and progression utilizing a bladder cancer TMA. Their expression patterns were also compared with clinicopathologic characteristics of tumor progression and with the expression patterns of two other well-studied markers, Ki-67 and p53. Amplification of chromosomal region 8q24, harboring several potentially cancer-related genes, was also studied, and the findings were correlated with Ki-67 and p53 expression.

Biomarker analyses

Ki-67 and p53 showed an exclusively nuclear staining pattern, gelsolin showed a cytoplasmic staining pattern, and E-cadherin a membrane staining pattern. Gelsolin stained not only the urothelium, but also stromal tissue, including some smooth muscle cells and endothelium. For each marker, the maximum intensity of staining (Max), the proportion of analyzed cells staining (Pos), and the product of both (MaxPos), obtained by multiplying Max by Pos, were determined. Both Ki-67 and p53 showed a similarly progressive increased MaxPos from benign, to adjacent dysplasia, to CIS, and from low-grade to high-grade tumors. Gelsolin MaxPos decreased in the dysplastic and CIS field lesions compared with levels in benign urothelium ($p<0.05$ by ANOVA). Although still lower in tumor samples than in the benign urothelium, gelsolin MaxPos increased with increase in tumor grade ($p<0.05$ by ANOVA). E-cadherin MaxPos was also lower in the adjacent dysplastic and CIS lesions compared with levels in benign urothelium, and there occurred a gradual decrease in MaxPos with increase in tumor grade, contrary to the trend seen with gelsolin. This decrease was also statistically significant ($p<0.05$ by ANOVA). It is noteworthy that E-cadherin expression, but not gelsolin expression, was lower in CIS lesions than in grade 1 lesions ($p<0.05$ by Student t-test). This is consistent with the fact that CIS is a high-grade noninvasive lesion, supported by higher Ki-67 and p53 MaxPos in CIS than in low-grade tumors.

Table 12 compares the MaxPos of each marker in different tumor stages, from noninvasive (Ta/Tis) to superficially invasive (limited to the lamina propria, T1) to deeply invasive (invasion to or beyond the muscularis propria, T2 and above). One hundred cases had informative data across all markers. Both gelsolin and p53 showed a significantly increased MaxPos, with progression of tumor invasion ($p<0.05$ by ANOVA). E-cadherin showed a marginal association ($p<0.1$ by ANOVA), and Ki-67 showed no significant association with tumor stage. Correlation matrix analysis for the interrelationship of these markers using the MaxPos value showed that, except for some associations between Ki-67 and p53 ($r=0.28$), all other marker matrices had an r-value less than 0.20, indicating that these markers were expressed rather independently in individual patient samples.

Table 12. Comparison of marker expression (MaxPos, mean±SE) among noninvasive (Ta/Tis), superficially invasive (T1), and deeply invasive (T2-T4) urothelial tumors (p-values calculated by ANOVA) (n=130).

Biomarkers	**Ta/Tis** (n=33)	**T1** (n=18)	**T2-T4** (n=79)	**p-value**
Gelsolin	90.0±9.5	126.5±12.0	153.0±13.9	< 0.05
E-cadherin	138.7±13.8	161.0±14.6	120±10.8	> 0.05
p53	32.1±4.6	38.6±4.3	68.8±8.5	< 0.05
Ki-67	35.7±5.7	40±5.2	31.5±8.0	> 0.05

ANOVA = analysis of variance. MaxPos = maximum staining intensity multiplied by percentage of positively staining cells.

Gelsolin is associated with the regulation of actin cytoskeleton dynamics. It severs assembled actin filaments and caps the fast-growing plus-end of a free or newly severed filament (Kwiatkowski 1999). It is a prominent substrate of caspase-3 activity in vitro and is therefore an important effector of cellular apoptosis (Kothakota et al. 1997). Gelsolin expression is down-regulated in 60 to 90% of breast, bladder, prostate, and lung carcinomas (Asch et al. 1996; Tanaka et al. 1995; Lee et al. 1999; Shieh et al. 1999). Tanaka and colleagues demonstrated in the majority of mice tested that the addition of retroviral gelsolin cDNA constructs results in marked and reproducible tumor growth inhibition and prolonged survival time (Tanaka et al. 1999). These findings demonstrate the tumor-suppressor effect of the gelsolin gene and its potential for treatment of human urinary bladder carcinomas. No in vivo study of gelsolin expression in actual human bladder carcinoma samples exists, however. Our study on urothelial carcinoma TMA revealed a distinctive biphasic gelsolin expression pattern in urothelial carcinoma. Compared with the normal field, expression decreased in both premalignant and malignant lesions, as would be expected from in vitro observations. This decreased expression is more striking in the noninvasive lesions, including dysplasia, low-grade tumor, and even CIS. There did, however, occur a trend toward increased expression from noninvasive (Ta/T0) to superficially invasive (T1) and to deeply invasive (T2 and above) tumor. A higher gelsolin labeling index (Max or MaxPos) was an independent marker for tumor recurrence and progression, particularly for high-grade tumors. Acquiring a certain level of gelsolin expression might therefore facilitate the conversion of a noninvasive tumor to an invasive one. This is consistent with the fact that cytoskeletal proteins play an important role in tumor cell motility and invasion.

A similar gelsolin expression pattern has appeared in other carcinoma types such as non-small-cell lung carcinoma and breast carcinoma (Shieh et al. 1999; Thor et al. 2001). Thor and colleagues showed that by univariate and multivariate

analyses overexpression of gelsolin, epidermal growth factor receptor (EGFR), and c-erbB2 significantly predicted poor clinical outcome (Thor et al. 2001). In lymph node-positive patients, coexpression of all three markers was associated with median disease-specific survival of 3 years (compared with c-erbB2-positive, EGFR-positive, and gelsolin-negative patients with their median survival of 6 years). The biphasic expression pattern of gelsolin is intriguing. First, it suggests that epigenetic mechanisms may be involved in the regulation of gelsolin gene expression. Second, the increasing expression from noninvasive to invasive tumors supports the hypothesis that tumor invasion is attributable to cytoskeletal actin-associated motility machinery. Third, in view of our positive association between increased gelsolin expression and tumor progression, caution is needed when developing therapeutic interventions based on gelsolin regulatory strategies.

Our study confirmed observations from a number of recent studies that decreased expression of E-cadherin occurs in urothelial carcinoma, primarily in the high-grade tumors (Nakopoulou et al. 2000; Popov et al. 2000; Bornman et al. 2001; Shariat et al. 2001). Our study also showed, however, that decreased expression of E-cadherin is associated with tumor recurrence in low- to intermediate-grade tumors. Bornman and colleagues showed that one of the potential mechanisms for decreased expression of E-cadherin in urothelial carcinoma is related to DNA methylation (Bornman et al. 2001). Altered methylation is evident in the nontumor areas of older patients. In their study, however, altered methylation of E-cadherin did not perfectly correlate with decreased expression in the nontumor areas. This finding suggests that in bladder carcinoma, DNA methylation may not be specific for down-regulation of E-cadherin. In many previous studies, abnormal E-cadherin expression was defined arbitrarily by the complete absence of immunoreactivity or by the heterogeneous staining of tumor areas. Although our study used semiquantitative Max and MaxPos values, overall findings were similar.

The association between marker expression and tumor recurrence was analyzed by both univariate and multivariate approaches. Table 13 shows the results of univariate analysis, in which the mean of staining parameters (Max, Pos, and MaxPos) of each marker was compared for patients with tumor recurrence versus those without recurrence, after stratification by tumor grade (grade 1-2 vs. grade 3). Information on tumor/no tumor recurrence was analyzed for 113 patients, 38 of whom had grade 1 to 2 tumors, and 75 of whom had grade 3. Patients with grade 1 and grade 2 tumors who experienced subsequent tumor recurrence had significantly higher Ki-67 MaxPos and p53 Max, as well as lower E-cadherin MaxPos, than did patients with no tumor recurrence ($p<0.05$ for all). For patients with grade 3 tumors, however, the gelsolin Max value was significantly higher in those with tumor recurrence than in those with none ($p<0.05$).

Table 13. Marker expression vs. recurrence status in urothelial tumors stratified by tumor grade (grade 1-2 vs. grade 3)(n = number of tumors).

Recurrence status		Ki-67			p53			Gelsolin			E-cadherin		
	n	*Max*	*Pos*	*MP*	*Max*	*Pos*	*MP*	*Max*	*Pos*	*MP*	*Max*	*Pos*	*MP*
Grade 1-2													
No recurrence	12	1.2	12	20	0.4	17	31	1.6	56	124	2.3	81	192
Recurrence	26	1.6	17	**37**	**1.0**	21	39	1.7	71	137	1.8	66	**124**
Grade 3													
No recurrence	39	1.3	16	29	1.2	30	59	1.4	65	135	1.7	65	139
Recurrence	36	1.3	18	31	1.4	27	66	**2.1**	72	162	1.6	61	116

Max = maximum staining intensity; Pos = percentage of positively staining cells; MaxPos=MP=Max multiplied by Pos. p-value determined by Student t-test. Bold numbers indicate $p < 0.05$.

In multivariate analysis for those with complete follow-up and informative data across all markers (n=68), initially in each marker analyzed, including p53 and Ki-67, Max outperformed Pos or MaxPos in predicting tumor recurrence. In multivariate analysis (Table 14), the significant predictors for probability of tumor recurrence were low tumor grade (grade 1-2 of 3) and higher gelsolin and p53 Max. Tumor stage and expression of Ki-67 and E-cadherin were not significantly associated with probability of tumor recurrence. Association of tumor recurrence with low tumor grade is not surprising, because low-grade tumors tend to recur. In addition, patients with low-grade tumors are less likely to be treated with definitive therapy (cystectomy) than are patients with high-grade tumors. In our study, 12% of patients with low-grade tumors received a cystectomy, compared with 88% of patients with high-grade tumors ($p<0.001$ by chi-square test).

In Cox regression analysis of correlation of marker expression with tumor recurrence-free time, all tumor markers reached statistical significance (Table 15). Again, similar findings were seen with the Pos or MaxPos value. For tumor markers, the strongest association was increased Ki-67, followed by increased gelsolin, increased p53, and decreased E-cadherin expression, in decreasing order of significance. Patient's age and tumor grade were negatively associated with early tumor recurrence.

Because negative association between tumor grade and tumor recurrence-free time may be related to treatment method, Cox regression analysis was performed separately in cases stratified for low or intermediate grade (grade 1-2) versus high grade (grade 3) and for noninvasive tumors or those superficially invasive (Tis/T0/T1) versus deeply invasive (T2-T4). For low- to intermediate-grade tumors (grade 1-2), both E-cadherin and p53 were significant markers in addition to patient age and tumor stage. However, for the high-grade tumors,

Table 14. Multivariate analysis for urothelial tumors: significant factors associated with recurrence status by logistic regression analysis (n=68).

Factors	OR	95% CI	p-value
Stage	1.76	0.88-3.5	0.11
Grade	**0.12**	0.03-0.42	**0.001**
Ki-67 Max	1.21	0.58-2.51	0.62
p53 Max	**2.66**	1.19-5.95	**0.017**
Gelsolin Max	**2.60**	1.22-5.51	**0.013**
E-cadherin Max	1.04	0.55-1.95	0.914

OR = odds ratio; CI = confidence interval; Max = maximum staining intensity.
Bold numbers indicate significant factors.

increased gelsolin was the strongest indicator for early tumor recurrence (p=0.0025, Table 15), followed by Ki-67 (p=0.031). When superficial (Ta/T0/T1) and deeply invasive (T2-T4) tumors were analyzed separately, Ki-67 was the only marker significant for the former, with gelsolin the only marker significant for the latter.

A Kaplan-Meier curve of tumor recurrence time versus staining intensity of gelsolin in high-grade tumors showed no tumor recurrence in tumors with negative gelsolin staining. The median recurrence time for weak (1+) Max staining was 10 years, for moderate (2+) staining 7 years, for strong (3+) staining 4 years, and for strongest (4+) staining less than one year. These data show that increased gelsolin staining intensity in tumor areas is the most important predictor of decreased tumor recurrence-free time in high-grade tumors.

To further test whether abnormal marker expression in the primary tumor predicts the behavior of an individual tumor, expression levels (MaxPos value) of markers in tumors with and without progression were compared longitudinally. Among 146 patients, 21 had more than one tumor specimen collected at intervals of at least one month and had an adequate amount of tissue to be analyzed. These samples were included in TMA analysis. For these 21 cases, paired tissue samples from the first and last visit were analyzed for any relationship between tumor progression and marker expression. Tumor progression was defined as increase in either grade (grade 1 to grade 3) or stage (stage 1 to 4). Patients with tumor progression (n=14) had an average follow-up time of 25 months, and patients without (n=7), an average follow-up time of 21 months. Patients with tumor progression showed a significantly higher level of expression for gelsolin than did patients without tumor progression in the primary tumor samples (p=0.007), indicating that for patients with recurrent tumors increased gelsolin in a primary tumor sample predicts progressive behavior. Although Ki-67 and p53 expression

Table 15. Multivariate analysis of marker expression versus recurrence-free time in urothelial tumors (OR and 95% CI) by Cox regression analysis.

Characteristics		Grade		Stage	
	All tumors (n=68)	**1-2** (n=29)	**3** (n=41)	**Ta/T0/T1** (n=33)	**≥T2** (n=37)
Patient age	**0.73** (0.66-0.80)	**0.64** (0.52-0.78)	**0.74** (0.63-0.87)	**0.67** (0.55-0.81)	**0.75** (0.66-0.86)
Grade	**0.07** (0.02-0.21)	-	-	0.30 (0.05-1.79)	**0.14** (0.02-0.88)
Stage	**2.07** (1.12-3.62)	**4.45** (1.42-14.0)	**2.58** (1.06-6.31)	-	-
Ki-67	**2.55** (1.45-4.47)	2.28 (0.69-7.52)	**3.10** (1.11-8.67)	**3.76** (1.38-10.3)	1.43 (0.57-3.55)
p53	**1.84** (1.26-2.69)	**2.08** (1.06-4.11)	1.58 (0.92-2.70)	1.96 (0.92-4.20)	1.38 (0.86-2.21)
Gelsolin	**2.08** (1.31-3.31)	1.09 (0.37-3.21)	**3.21** (1.51-6.83)	1.76 (0.73-4.25)	**2.17** (1.15-4.12)
E-cadherin	**0.62** (0.43-0.89)	**0.45** (0.24-0.85)	1.15 (0.61-2.18)	0.32 (0.29-1.02)	1.00 (0.50-2.00)

OR = odds ratio; CI = confidence interval. Bold numbers indicate $p < 0.05$; Max values (maximum staining intensity) used.

was increased, and E-cadherin expression was decreased in progressing tumors, these differences did not reach statistical significance. No statistically significant difference appeared in marker expression between primary and recurrent tumor samples.

Although a wide spectrum of lesions were analyzed, the small sample size for some of these lesions (only six grade 1 tumors) precluded comprehensive analysis. Although this was a limitation of our study, the analysis provided strong support for the use of gelsolin as a potential prognostic marker for high-grade urothelial carcinomas. Most bladder tumors (70-80%) are classified as superficial (Bane et al. 1996). Between 50 and 70% of patients with superficial tumors will develop new superficial TCC, often within 12 months of diagnosis. In 10 to 20% of these patients, these tumors will infiltrate the muscular propria (T2-T4) (Heney et al. 1982). Because only the progressive tumors will eventually lead to cancer-related death, it is crucial to identify these patients early so that more aggressive treatment methods can be applied.

Ki-67 and p53 stainings were also studied after conversion to a positive/negative schema using the threshold reported in the literature (20% of cells staining for either marker) (Cordon-Cardo et al. 1997; Popov et al. 1997). Ki-67 was positive in 75% of CIS lesions and in 17% of grade 1, 55% of grade 2, and

50% of grade 3 tumors; p53 was positive in 38% of CIS lesions and in 17% of grade 1, 38% of grade 2, and 53% of grade 3 tumors. These findings are similar to those in the literature. When marker expression was analyzed against tumor recurrence, however, the Max value of each marker provided stronger associations than did Pos or MaxPos values. In our analysis of marker expression versus tumor recurrence, the results were similar, regardless of the values (Max, Pos, or MaxPos) used. This finding differs slightly from those of other investigators, and emphasizes the fact that the results of such studies depend on the quantitative method (Cordon-Cardo et al. 1997; Popov et al. 1997).

Findings regarding these four markers demonstrate that expression patterns for the cytoskeletal proteins gelsolin and E-cadherin are distinctive from those of Ki-67 and p53 in bladder cancer. Gelsolin and E-cadherin show an overall lower expression than do corresponding benign fields. However, gelsolin is low mainly in premalignant and preinvasive lesions. In tumors, gelsolin expression generally increases with increasing grade and stage. Elevated expression of gelsolin relative to that in premalignant lesions is a strong indicator for the probability of tumor recurrence, early tumor recurrence, and tumor progression, particularly for high-grade tumors. Conversely, E-cadherin decreases as tumor grade increases. This finding provides in vivo evidence to support the theory that distinctive patterns of actin family gene expression may occur in different stages of the malignant transformation process. Whereas decreased expression of gelsolin occurs at the early stages of malignant transformation, increased gelsolin expression may play a role in converting the less aggressive superficial tumor to a more aggressive invasive tumor. This study shows the value of examining the utility of these markers as prognostic indicators for urothelial carcinomas.

8q24 amplification

We evaluated the frequency of 8q24 amplification in urothelial carcinoma, focusing on high-grade tumors and metastases, using primary tumor samples from 131 patients (Table 16). Of these patients, 42 had low- to intermediate-grade (grade 1-2) disease, and 89 had high-grade (grade 3) disease. Metastases were available from 39 patients. The majority of primary tumors and metastases were represented by three cancer tissue spots in the array. High-copy amplification (at least 10 signals or tight clusters of at least 5 signals/cell in at least 5% of tumor cells) occurred in either a primary tumor or a metastasis in 10% (16/159) of the patients. Concordance of 8q24 amplification between the spots for each individual tumor and metastasis was 100%. Of the primary tumors, amplification was most frequently observed in high-grade tumors (10%), with low- to intermediate-grade tumors amplified less frequently (7%). None of the grade 1 tumors was amplified. Interestingly, one of the patients with bladder TCC had recurrent TCC in the renal

Table 16. 8q24 amplification in primary bladder tumors and metastases.

	Patients with 8q24 amplification (n amplified / n total (%))
Grade 1	0/5 (0)
Grade 2	3/37 (8)
Grade 3	9/89 (10)
Primary tumors (total)	12/131 (9)
Lymph node metastasis	0/28 (0)
Metastasis (other sites)	4/11 (36)
Metastatic patients (total)	4/39 (10)

pelvis 5 years later. Whereas this bladder TCC showed no amplification at 8q24, the renal pelvic TCC did. The small number of tumors harboring the 8q24 amplification has to be taken into account in assessing the importance of these findings.

These findings support the previous notion that the frequency of chromosomal region 8q24 amplification increases with progressing tumor grade, being highest in high-grade TCC tumors. The proportions of low to intermediate grade (grade 1-2) and high-grade (grade 3) tumors showing amplification (7% and 10%, correspondingly) in this study are concordant with those in the literature (Kallioniemi et al. 1995; Sauter et al. 1995; Richter et al. 1997). Furthermore, the finding that 8q24 is not amplified in grade 1 tumors fits well with the hypothesis that genomic amplifications are more abundant in tumors of higher grade, yet our small sample size has to be taken into account in assessing the significance of this observation.

As metastatic lesions commonly occur in urothelial cancer, we studied the frequency of 8q24 amplification in metastatic lesions to discover how it relates to the amplification frequency in primary tumors. Amplification was detected in 4 of 39 (10%) of the patients having metastases (Table 17). None of 30 lymph node metastases and 6 of 16 (38%) metastases at other sites demonstrated amplification (Table 17). Of the 4 patients whose metastases showed 8q24 amplifications, 2 had matching primary tumor tissue available, neither of whom showed amplification in their primary tumor, whereas their metastases to the labium and lung (Figure 3) did show amplification. In the remaining 2 cases, no primary tumor was available, but patient histories indicated that both had grade 3 primary tumors 5 and 6 years earlier. The follow-up times between lymph node metastases and other types of metastases differed greatly, with 90% of lymph node metastases removed during cystectomy or other surgical treatment for the primary tumor, whereas the

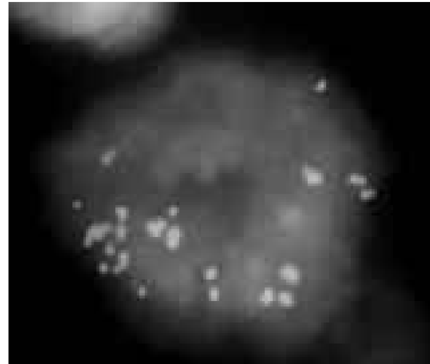
Table 17. 8q24 amplification status of bladder tumor metastases.

	Amplified metastases	All metastases
Regional metastases		
Lymph node	0	28
Distant metastases		
Lymph node	0	2
Vagina	0	4
Lung	2	3
Labium	2	2
Liver	1	2
Bowel	0	2
Retroperitoneum	1	1
Pelvic soft tissue	0	1
Calf	0	1
All metastases	6	46

metastases of other sites were usually removed long after treatment for the primary tumor. Median times of removal were 0 months for lymph node metastases and 26 months for metastases at other sites ($p<0.0001$). The elapsed median time between removal of the primary tumor and of the 8q24-amplified distant metastases was 42 months, longer than the median of 22 months for non-amplified distant metastases, a difference not statistically significant ($p=0.30$).

The finding of amplification in metastatic lesions but not in the corresponding primary lesions of the same patient is intriguing. In our study, 33% of distant metastases showed amplification, whereas no regional metastases did. Since genetic amplifications are likely to accumulate during the carcinogenic process, it is possible that the later the metastases are removed, the more likely they possess genomic amplifications of 8q and other genomic regions. The alternative explanation is that the area of 8q may harbor genes essential in driving the potential for tumors to metastasize to distant sites. Our results showing amplification in a defined chromosomal region in a subgroup of bladder tumors and metastases parallel the findings of the c-erbB2 proto-oncogene amplification status of breast tumors and their corresponding metastases (Gancberg et al. 2002). Furthermore, that a molecular signature of metastasis in primary solid tumors has been reported for several tumor types shows that a subset of primary tumors may resemble metastases with respect to this gene-expression signature (Ramaswamy et al. 2003). Our own findings imply the existence of molecularly heterogeneous subgroups of bladder tumors and metastases defined by 8q24 amplification status.

Figure 3. 8q24 amplification of a transitional cell carcinoma lung metastasis, visible as multiple hybridization signals (Spectrum Orange) within a tumor cell nucleus (DAPI). Original magnification: x100.



Since p53 and Ki-67 protein expression correlates with tumor grade, as does 8q24 amplification, we studied the possible correlation between 8q24 amplification and expression of these biomarkers in the same patient group, using the same arrays that we analyzed for the 8q24 amplification. In tumors carrying 8q24 amplification, expression of each biomarker varied widely. Similar wide variation in expression was noted also in tumors lacking this amplification (Table 18). The expression of Ki-67 was, however, significantly higher in tumors possessing the amplification than in tumors without it ($p<0.005$). Expression of p53 showed a similar trend, but due to wide variation this did not reach statistical significance ($p=0.19$). Although a correlation between amplification at 8q24 and expression of Ki-67 was detectable ($p<0.005$), a causative relationship between 8q24 amplification and increased Ki-67 expression can obviously not be suggested based on this study alone. Since Ki-67 is a marker of active cell proliferation, however, this finding parallels the assumption that those urothelial tumors showing 8q24 amplification are also more proliferative and thus potentially more aggressive. The location of the MDM2-binding protein (MTBP) in chromosomal region 8q24 and its potential involvement in the p53 apoptotic pathway made the p53 expression analysis interesting (Mayo et al. 2002). Although p53 expression showed a correlative trend, it did not reach statistical significance ($p=0.19$). Studies with larger sample size may confirm this tentative finding.

Our results show that in a subgroup of primary bladder tumors and metastases, high-copy amplification of the 8q24 chromosomal region occurs. The primary tumors and metastases carrying 8q24 amplification frequently overexpressed Ki-67. It remains to be seen whether a more detailed analysis of the 8q24 region in urothelial carcinoma will reveal specific alterations in genes, and whether these may be useful for tumor classification or as specific targets of treatment.

Table 18. Biomarker expression in primary bladder tumors.

	Non-amplified tumors (n=119)			Amplified tumors (n=12)			
	Median score	Range	SD	Median score	Range	SD	p
p53	5	0-300	75	25	0-240	98	0.19
Ki-67	10	0-210	40	55	10-240	63	<0.005

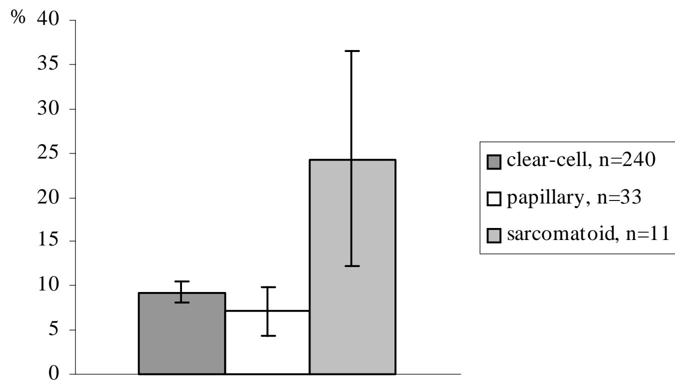
Biomarker expression in renal cell carcinoma (III-IV)

RCC subtypes

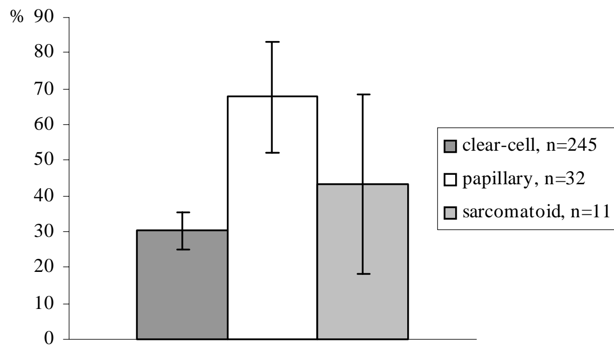
Ki-67, gelsolin, and PTEN staining levels were first assessed in the RCC subtypes within the TMA. As expected, Ki-67 expression was higher in sarcomatoid than in clear-cell (Figure 4, $p<0.05$) or papillary ($p<0.005$) tumors. Interestingly, gelsolin expression was higher in papillary tumors than in clear-cell tumors ($p<0.0001$) or in sarcomatoid tumors ($p<0.05$). PTEN expression did not significantly differ between sarcomatoid and clear-cell or papillary tumors. However, PTEN expression was lower in clear-cell than in papillary tumors ($p<0.005$). Study of the protein expression of Ki-67, gelsolin, and PTEN revealed distinct biomarker expression profiles for each of the RCC subtypes. As the prognosis of sarcomatoid tumors is worse than that of other renal tumors (Pantuck et al. 2001), one might expect that the malignant phenotype would be reflected in the tumor's biomarker expression, as well. Ki-67, a marker of active cell proliferation, is indeed highly expressed in sarcomatoid tumors, in concordance with the aggressive nature of this tumor type (Delahunt 1999; Moch et al. 2000), and confirms the reports of increased Ki-67 expression in sarcomatoid tumors (Renshaw et al. 1998; Kanamaru et al. 1999; Kuroiwa et al. 2001). On the other hand, gelsolin expression is low in clear-cell and sarcomatoid tumors, but high in papillary tumors. This is intriguing in the light of reports of a tumor-suppressive role for gelsolin in cancers of the bladder, prostate, and breast (Tanaka et al. 1995; Lee et al. 1999; Winston et al. 2001), because our results showing differences in gelsolin expression suggest that gelsolin may play multiple roles within renal tumor types. The tumor-suppressor protein PTEN was highly expressed in both papillary and sarcomatoid tumors, whereas PTEN expression was significantly lower in clear-cell than in papillary tumors. In all of these tumor types, however, PTEN was generally expressed in 80 to 90% of tumor cells, suggesting that PTEN expression is not severely affected in any of these tumor types, and the difference between clear-cell and papillary tumors may be a bystander phenomenon. Furthermore, we also observed that all but two normal kidney tissue samples in our array expressed PTEN in every cell. Further studies controlling for additional

Figure 4. Biomarker expression in clear-cell, papillary, and sarcomatoid renal tumors, with percentage of positively staining tumor cells on y-axis. Values are mean±SD.

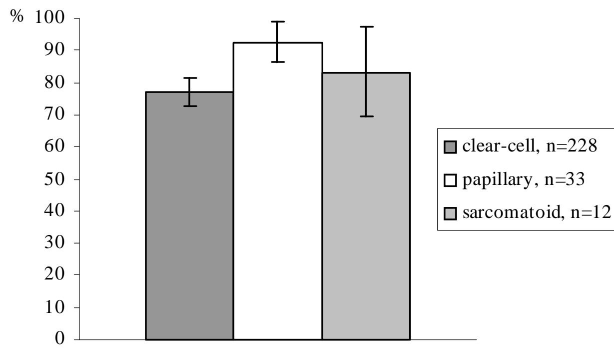
Ki-67



Gelsolin



PTEN



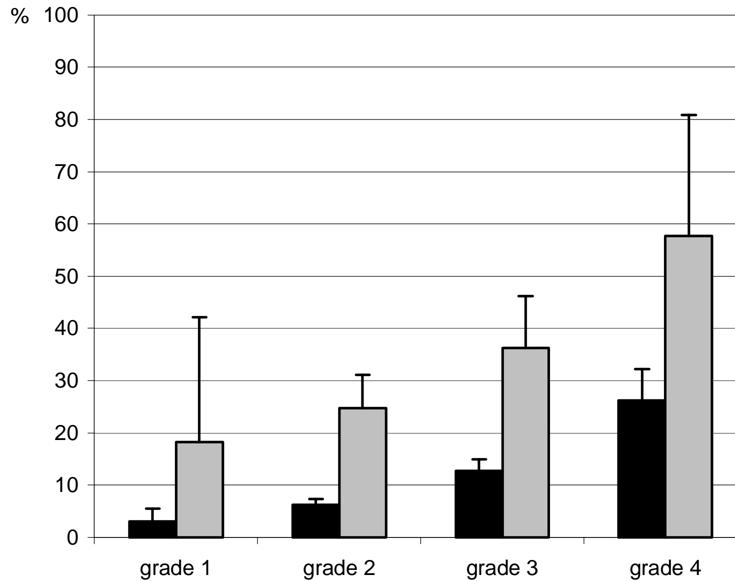
variables such as grade, stage, and intensity of staining may fully characterize these findings.

The results thus demonstrate that, compared with clear-cell and papillary tumors, sarcomatoid renal tumors have a distinct protein expression profile. Ki-67 expression in sarcomatoid tumors is significantly higher than in clear-cell or papillary tumors, whereas gelsolin expression is lower in sarcomatoid than in papillary tumors, and PTEN is highly expressed in all these tumor types. This supports the evidence of Ki-67 as a biomarker for renal tumors, whereas the roles of gelsolin and PTEN in the carcinogenesis of various renal tumor types remain to be elucidated. It must be emphasized that although differences in biomarker expression were evident when each tumor subtype was analyzed as a single group, differences may exist in expression within these groups when the tumors are classified according to clinicopathological parameters such as grade and stage. Our detailed analysis of Ki-67 and gelsolin expression in clear-cell RCC already shows that comprehensive analysis may reveal additional information concerning the stages of the carcinogenic process.

Clear-cell RCC

Ki-67 and gelsolin expression was further evaluated in the 257 tumors representing clear-cell morphology. Ki-67 expression was detected in 175 of 241 (73%) of the evaluable tumors (mean expression 9% of tumor cells, median expression 5%, SD 0.63). Gelsolin expression was detected in 106 of 246 (43%) of the evaluable tumors (mean expression 30% of tumor cells, median expression 0%, SD 2.67). Ki-67 showed an increase in staining with increasing tumor grade (Figure 5, $p<0.0001$), as did gelsolin ($p=0.0037$). Although both biomarkers showed similar trends of increased staining at higher grades, no correlation appeared between gelsolin and Ki-67 expression ($\rho=0.087$). We also performed survival analyses for the clear-cell RCC specimens to clarify the value of Ki-67 and gelsolin as predictors of survival. When these biomarkers were compared to the conventional predictors in univariate and multivariate settings, with cancer-specific survival as the outcome, multivariate Cox regression analysis for cancer-specific survival indicated that stage (pT) was a significant prognostic factor ($p<0.0001$), and Ki-67 expression was also a significant prognostic factor ($p=0.0216$), when all covariates were included in the analysis. Higher stage and increased Ki-67 expression predicted poor survival. A total of 180 patients were included in the multivariate analyses after exclusion of patients with missing values. The SAS stepwise procedure indicated that stage was the most important predictor ($p<0.0001$), followed by Ki-67 ($p=0.0016$). In a multivariate analysis that included Ki-67 and grade along with stage, Ki-67 was statistically significant ($p=0.0309$), but grade was not ($p=0.1951$), suggesting that Ki-67 has predictive

Figure 5. Relationship of tumor grade and biomarker expression in clear-cell RCC. Grade on x-axis, percentage of positively staining tumor cells on y-axis as mean value +SD. Ki-67 = black bars. n=13 (grade 1), n=144 (grade 2), n=69 (grade 3), n=15 (grade 4). Gelsolin = gray bars. n=11 (grade 1), n=149 (grade 2), n=71 (grade 3), n=15 (grade 4). n=number of tumors.



value independent of grade. A correlation between Ki-67 and grade occurred (ρ=0.52). It is possible that one of the reasons why grade was nonsignificant is that relatively few grade 1 and 4 tumors were included. The analyses used the pT staging variable, in which stage is defined for the primary tumor by a pathologist. It remains to be seen whether further studies controlling for patients' nodal and metastatic status will reveal novel correlations in subgroups of this population.

To evaluate the potential prognostic significance of Ki-67 and gelsolin expression in a clinically useful setting, we categorized the patients into groups with dichotomized values for the biomarkers to discover prognostic factors. Dichotomized Ki-67 and gelsolin expression values were inversely related (Pearson X^2 statistic p=0.0017). Kaplan-Meier plots with cancer-specific survival time revealed that patients with Ki-67-negative tumors did better than those with Ki-67-positive tumors (Figure 6A, p=0.0006). Gelsolin did not significantly affect survival time (Figure 6B, p=0.33) in the same patient group. When the analysis was restricted to grade 2 tumors, Ki-67 expression showed no evidence of differing survival distributions (p=0.0833), whereas gelsolin expression showed a trend

toward differing survival distributions (logrank p=0.0566), in favor of the group with positive gelsolin staining. The fact that Ki-67 and gelsolin had been stained in the same set of samples made it possible to study whether a combined expression profile of these biomarkers would provide additional prognostic information within each grade. Patients with Ki-67-positive staining and gelsolin-negative staining were compared to patients with Ki-67-negative staining and gelsolin-positive staining. Due to the large variation in gelsolin staining in grade 1 and 4 tumors and its contribution to noise in the statistical analysis, the sample was stratified to include only one grade. In grade 2, analysis with a combined expression profile of Ki-67 and gelsolin revealed a difference in cancer-specific survival distributions between groups (Figure 6C, log rank statistic p=0.0507), in favor of the group with Ki-67-negative/gelsolin-positive tumors. This may suggest that Ki-67 and gelsolin are at least partially affected by the same carcinogenic pathways. Other grades showed no significant difference in survival. In our study, the regional and distant metastatic status of the tumors was never taken into account. However, when patients with metastases were excluded from analysis, the same trend was observed for grade 2 tumors, but results were not significant, most likely due to small sample size (n=55).

In conclusion, this renal cancer TMA study shows that renal tumor profiling utilizing the TMA technique is feasible. Furthermore, these profiles can serve as sources of new information on renal carcinogenesis, and, once clinical information is incorporated into the analysis, also as validation tools for potential prognostic biomarkers. Increased Ki-67 expression predicts poor prognosis both as a continuous variable and as a categorized variable at a cutoff value for Ki-67 staining. For grade 2 tumors, the combined expression profile of the two biomarkers may predict survival. As a prognostic biomarker for RCC, Ki-67 shows great promise. These findings also suggest an important role for gelsolin in carcinogenesis of RCC and should stimulate further cellular study. Future research will elucidate the events that cause some of the grade 2 tumors to become more aggressive and will assist in development of more effective treatment options for advanced disease potentially targeting Ki-67- and gelsolin-related pathways.

Figure 6. Kaplan-Meier curves demonstrating probability of cancer-specific survival for clear-cell RCC patients grouped according to biomarker expression.

- **A)** Ki-67 staining among all grades. a = Ki-67-negative patients (n=62). b = Ki-67-positive patients (n=167).
- **B)** Gelsolin staining among all grades. a = gelsolin-negative patients (n=180). b = gelsolin-positive patients (n=53).
- **C)** Combined Ki-67 and gelsolin staining profile in grade 2. a = patients with negative Ki-67 staining and positive gelsolin staining (n=15). b = patients with positive Ki-67 staining and negative gelsolin staining (n=80).

Figure 6A

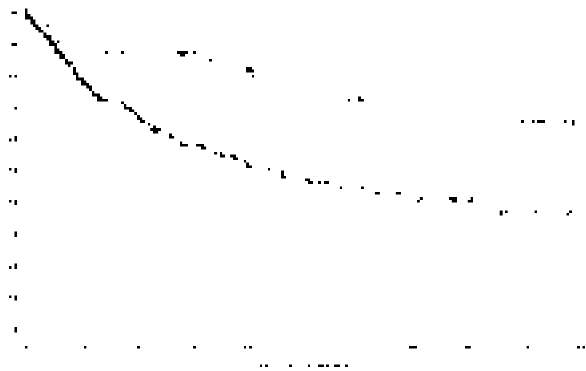


Figure 6B

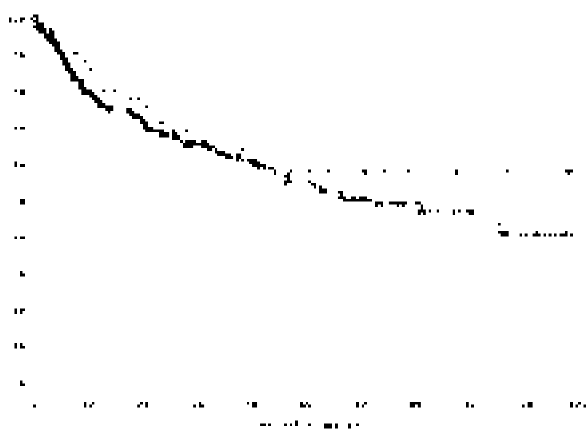
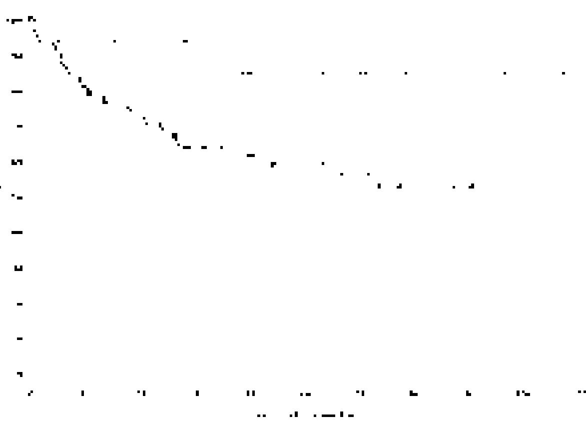


Figure 6C



Technical aspects of tissue microarrays

Several issues need to be taken into account during a TMA analysis. Selection of the samples to be arrayed is a crucial step; for the future experiments to be successful, the review process of the archived samples prior to TMA construction has to be done very carefully. Otherwise, if non-representative tissue areas are selected, future analyses may be limited, and the results potentially misleading. In this respect, RCC TMA is easier to construct than urothelial TMA, since renal tumors are often large, with well-defined boundaries, which makes it easier to obtain the correct tissue type during TMA construction. In comparison, benign uroepithelium and urothelial tumors are often relatively thin macroscopically and more difficult to hit with the arrayer. Thus, there likely are more empty spots or spots representing non-desired morphology in urothelial TCC TMA than in RCC TMA. Cancer type affects the number of cores needed to reliably capture the heterogeneity of the tumor; the more heterogeneous the tumor, the more cores needed. RCC and TCC are relatively homogeneous tumors compared to, for instance, prostate cancer, allowing reliable analyses with a fewer cores per tumor. Evaluation of stained slides mainly involves the same issues as does the specific staining technique in question, i.e., the issues in IHC TMA analysis parallel the issues in IHC analysis on regular tissue slides.

Recurring malignancies pose another challenge for TMA studies: urothelial TCC often recurs, and study of molecular events leading to recurrence or progression requires samples from recurrent tumors to enable longitudinal studies of biomarker expression, whereas for RCC, tissue collection is often straightforward and the course of the disease often shorter, with nephrectomy often the primary treatment and repeated operations rare. Analyzing metastases from all urinary tract malignancies would potentially benefit the study of processes leading to invasion and metastasis, but removing metastases from patients for therapeutic or palliative purposes is rarely indicated, and removing metastases strictly for research purposes is uncommon.

A vast amount of data can be rapidly produced in high-throughput fashion utilizing the TMA technique. A consecutive analysis of up to 200 molecules in hundreds of specimens makes great demands on statistical analyses of TMA data. Statistical approaches are being developed to enable an analysis in which both molecular and clinical information can be efficiently analyzed on the same basis. Our study applied modern statistical methods successfully. In interpreting results, accurate clinical information on the patients turned out to be essential.

Based on our study, the TMA technique benefits the study of urothelial carcinoma and RCC as a time- and tissue-preserving, high-throughput screening tool.

CONCLUDING REMARKS

The tissue microarray technique was developed as an attempt to provide a high-throughput tool for studies of cancer tissues. Several studies utilizing the TMA technique have investigated malignant tumors for genomic alterations and protein expression, providing clues to the pathways associated with malignancy. TMA studies focusing on molecular profiling of tumors have, however, thus far been rare.

We studied biomarker expression in cancers of the urinary bladder and kidney and showed that bladder tumors had distinct expression profiles for the actin-associated proteins gelsolin and E-cadherin, and that increased gelsolin expression in high-grade bladder tumors was associated with increased risk for progression and recurrence. The traditional biomarkers p53 and Ki-67 also had distinct expression profiles in the same samples. Additionally, genomic amplification in the chromosomal region 8q24 commonly amplified in bladder cancer occurred in a subgroup of predominantly high-grade tumors and in a more significant group of distant metastases. The tumors and metastases carrying the 8q24 amplification frequently over-expressed Ki-67, supporting the hypothesis that 8q24 amplification contributes to the malignant potential of bladder tumors. Our results also indicate that renal tumors having sarcomatoid features display distinct expression profiles for the tissue biomarkers Ki-67, gelsolin, and PTEN, when compared with profiles in clear-cell and papillary renal tumors. The distinct expression profiles in all these tumor types support the hypothesis that renal malignancy may develop through various molecular pathways. We also demonstrated that in the most common renal malignancy, renal clear-cell carcinoma, Ki-67 expression increased with tumor grade and independently predicted prognosis, providing further evidence of the prognostic significance of Ki-67 expression.

We studied in detail the expression of gelsolin, an actin-associated protein suggested to have tumor suppressing properties in various cancers. Gelsolin expression was down-regulated in low-grade renal tumors, showing increasing expression with grade. This expression pattern, together with a similar expression pattern in bladder cancer, suggests that gelsolin may play multiple roles in cancer development and progression. Additionally, increased Ki-67 expression and diminished gelsolin expression in individual tumors may indicate poor prognosis in grade 2 renal clear-cell carcinoma, suggesting that the molecular signature of renal tumors may be useful in prognostication.

This study shows that urinary tract tumors have distinct expression profiles for these biomarkers, and provides evidence that molecular tumor profiling utilizing the TMA technique has the capacity to extend our comprehension of the

carcinogenic process. Our results also indicate that such expression profiles may be useful in predicting clinical outcomes in cancers of the urinary bladder and kidney. Based on these results, the TMA technique is an effective screening tool for studies of molecular events during carcinogenesis of urinary tract malignancies.

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