Prediction of recurrence of non muscle-invasive bladder cancer by means of a protein signature identified by antibody microarray analyses

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About 70% of newly diagnosed cases of bladder cancer are low-stage, low-grade, non muscle-invasive. Standard treatment is transurethral resection. About 60% of the tumors will recur, however, and in part progress to become invasive. Therefore, surveillance cystoscopy is performed after resection. However, in the USA and Europe alone, about 54 000 new patients per year undergo repeated cystoscopies over several years, who do not experience recurrence. Analysing in a pilot study resected tumors from patients with (n = 19) and without local recurrence (n = 6) after a period of 5 years by means of an antibody microarray that targeted 724 cancer-related proteins, we identified 255 proteins with significantly differential abundance. Most are involved in the regulation and execution of apoptosis and cell proliferation. A multivariate classifier was constructed based on 20 proteins. It facilitates the prediction of recurrence with a sensitivity of 80% and a specificity of 100%. As a measure of overall accuracy, the area under the curve value was found to be 91%. After validation in additional sample cohorts with a similarly long follow-up, such a signature could support decision making about the stringency of surveillance or even different treatment options.

Keywords:
Antibody microarrays / Bladder cancer / Immunoassay / Protein arrays / Proteome analysis / Recurrence

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1 Introduction

Bladder cancer is the fourth most common type of cancer in men and the ninth most common cancer in women [1]. Some 70% of newly diagnosed bladder cancer cases are non muscle-invasive. Even after an initially successful treatment by apparently complete surgical resection of the tumor, some 60% of the tumors will recur, and 10–30% will progress to become a muscle-invasive disease [2]. Most tumors recur within 5 years after surgical resection of the primary tumor. Since no dependable marker exists to predict recurrence, a regular
surveillance cystoscopy and urine cytology is recommended every 3 months during the first 2 years after resection, at longer intervals over the next 2 years, and annually thereafter [3]. The lifetime cost for each bladder cancer patient varies from US$ 99 000 to US$ 121 000 [4]. Bladder cancer costs are the seventh highest among all cancers [5]. Even more important is the stress that patients are under. They live in a constant state of uncertainty and are confronted with the possibility of yet another cancer diagnosis every 3 months.

The disease’s impact on both patients and health care costs could be dramatically alleviated by an effective prediction of recurrence. Currently, any prognostic assessment of non muscle-invasive bladder cancer employs several features of the tumors such as multifocality, clinical grade, size, and stage [6]. In addition, a few immunohistochemical biomarkers have been proposed, but they have had only little impact in practice [7]. Transcriptional profiling studies yielded variations at the mRNA level. The identified transcripts include proto-oncogenes, proliferation activators, cadherins, and cell adhesion molecules [6, 8]. Also, a lower level of galectin-8 in tumors as compared to the normal urothelium was described and proposed as a recurrence marker [9]. Methylation changes at the promoter of RUNX3 were recently proposed as a predictor of tumor progression [10]. Mutations of FGFR3 have been reported to be associated with a low risk of recurrence [11]; conversely, others found an increased risk instead [12]. Similarly, some TP53 mutations are associated with a higher risk of recurrence [13–15]. However, while a combination of apoptosis related markers such as Caspase 3 (CASP3), BCL2, and P53 permit a prediction of the outcome of bladder cancer [16], no dependable marker or marker set exists at current for a prediction of recurrence for non muscle-invasive bladder cancer [6, 17, 18].

In our study, we used a complex antibody microarray [19] for a protein analysis on bladder cancer tissue samples obtained by transurethral resection. Antibody microarrays are a relatively new tool for an analysis of protein abundance in a parallel and highly multiplex manner [20]. Studies on protein extracts from cells or tissues have been rare to date due to technical aspects [21]. However, thoroughly assessed and detailed protocols were established recently that permit such analyses with documented high accuracy and reproducibility [22, 23], and sensitivities sufficient for the detection of single molecule interactions have been reported [24].

Our analysis of the protein content of a limited number of resected bladder tumors with a long-term follow-up revealed proteins with significantly differential abundance in recurrent and non-recurrent tumors. This set of proteins provides novel insights into processes involved in recurrence and disease progression. In addition, this pilot study yielded a protein signature, which could permit a highly accurate classification of patients into a group that will experience recurrence and a group that will not.

2 Materials and methods

2.1 Array production

Antibody microarrays were produced and used according to protocols and strict quality control procedures as reported earlier in very detail [22, 23]. For the analysis, a set of 668 target proteins was selected on the basis of transcriptional studies of different cancer entities. Affinity-purified polyclonal antibodies were produced in rabbits by Eurogentec (Seraing, Belgium). An additional 145 antibodies were purchased from different sources or provided by collaborating partners. Last, five other proteins—for example, receptor molecules fused to Fc-parts of antibodies—were added that are known to exhibit specific binding to particular ligands. A complete list of binders is provided in the Supporting Information (Supporting Information Table 1). The specificity of the antibodies had been verified by different techniques, such as Western blotting, immunohistochemistry, or fluorescence imaging by us or their providers. Also, this particular set has been used in the analysis of a total of about 2000 samples of different types (tissues, cell cultures, serum, urine), also providing information about their quality. A considerable set of these studies has been published (e.g. [19, 21–23]). Also, antibodies that target different epitopes of the same protein were used, thereby cross-validating findings.

The array composition and layout can be accessed in the public repository ArrayExpress (A-MEXP). All molecules were immobilized by contact printing on epoxysilane slides (Schott Nexterion, Jena, Germany) at a concentration of 1 mg/mL in spotting buffer made of 10 mM sodium borate, pH 9.0, 125 mM MgCl₂, 0.005% w/v sodium azide, 0.25% w/v dextran, and 0.0005% w/v (octylphenoxy)polyethoxyethanol, using a MicroGrid robot (BioRobotics, Cambridge, UK) and SMP3B pins (Telechem, Sunnyvale, USA). All arrays used in this study were from a single production batch of more than 1000 microarrays. Each array comprised 1800 features. All antibodies were spotted at least twice in a randomized pattern in different array sectors. For control purposes, antibodies against the proteins beta-actin, human IgM, glyceraldelyde-3-phosphatase dehydrogenase, and albumin were spotted. For the same purpose, a polyclonal antibody directed against whole human serum protein was added. Negative controls consisted of spotting buffer as well as further control antibodies, for example, molecules directed against mouse Ig gamma. All these controls were spotted in 8 to 18 copies across the entire array to ensure good distribution. After printing, the slides were kept at 4°C in a humidity-free environment until use.

2.2 Protein samples

Primary tumors were resected from patients with low-grade (stage Ta, G1 or G2) non muscle-invasive bladder cancer at the
Department of Urology and Pathology of Henri Mondor Hospital in Paris, France. Written informed consent was obtained from all subjects. The analysis was approved by the local ethics committee. All experiments conformed to the principles set out in the WMA Declaration of Helsinki. Only samples were considered with a patient follow-up of at least 5 years post the surgical resection of the primary tumor. Nineteen patients experienced recurrence while six patients did not. In addition, three normal bladder tissue samples were available as controls. Age and gender were equally distributed in all sample sets and none of the patients did receive any cancer-related therapy before sampling. The frozen tumor tissue samples were grounded to a fine powder in liquid nitrogen prior to an isolation of the total protein content by a one step protocol with the Tissue Protein Extraction Reagent (T-PER, Pierce Biotechnology, Inc., Rockford, IL, USA).

2.3 Sample labeling

The total protein concentration was measured by the BCA assay (Thermo Fisher Scientific). The protein concentration was adjusted to 1 mg/mL and samples were labeled with 0.4 mg/mL of the NHS-esters of the fluorescence dyes Dy-549 and Dy-649 (Dyomics, Jena, Germany), respectively, in 100 mM sodium bicarbonate buffer, pH 8.5, 1% w/v Triton-100 on a shaker at 4°C. After 1 h, the reaction was stopped by addition of 10% glycerol. Unreacted dye was removed 30 min later and the buffer changed to PBS using Zeba Desalt columns (Thermo Fisher Scientific). Subsequently, the Complete Protease Inhibitor Cocktail (Roche, Mannheim, Germany) was added as recommended by the manufacturer. All labeled protein samples were stored in aliquots at -20°C until use.

For the competitive dual color incubations, a reference sample was produced by pooling the same amount of all protein samples. The same reference sample was used throughout the analysis. For an identification of dye-specific effects that may introduce a bias during labeling, the dyes were also swapped between the individual and reference samples in all experiments.

2.4 Incubation

Homemade incubation chambers were fixed to the array slides prior to blocking for 4 h with 10% w/v skimmed milk powder and 0.05% w/v Tween-20 in PBS on a Slidebooster instrument (Advalytix, Munich, Germany) [19]. After 4 h, the blocking buffer was removed and a mixture of 10 μL each of a labeled patient sample and the corresponding, differently labeled reference sample were mixed with 580 μL blocking buffer supplemented with 0.05% w/v Triton-100 and 1x Complete Protease Inhibitor Cocktail. Incubation was done on the Slidebooster instrument for 16 h. Subsequently, the slides were thoroughly washed with PBSTT (PBS supplemented with 0.05% w/v Triton-X 100 and 0.05% w/v Tween-20), rinsed with 0.1x PBS and water and dried in a stream of air. The samples were incubated in five batches with recurrent and non-recurrent samples equally distributed to the batches. The normal samples were analyzed within the same batch.

2.5 Data analysis and statistical testing

Slide scanning was done on a ScanArray 4000 XL unit (Packard, Billerica, USA) using identical instrument laser power and PMT for all measurements. Spot segmentation was performed with GenePix Pro 6.0 (Molecular Devices, Union City, USA). Resulting data were analyzed using the LIMMA package [25] of R-Bioconductor after uploading the mean signal intensities. For normalization, a specialized invariant Lowess method was applied as described before [26], taking advantage of the always present reference sample. In the analyses, duplicate spots were taken into consideration [27]. For differential analysis of protein expression, a one-factorial linear model was fitted with LIMMA resulting in a two-sided t-test based on moderated statistics. All p-values were adjusted for multiple testing by controlling the false discovery rate (FDR) according to Benjamini and Hochberg [28]. For hierarchical clustering, the control antibodies were filtered; the euclidean distance and the complete linkage algorithm were used for agglomeration. Intra-array replicates were averaged preceding. A random forest classifier [29] was built and cross-validated by building different test and training sets defined by the leave-one-out algorithm in a reiterated outer loop. Within each training set, a classifier was built using the twenty proteins with the highest significance in a two-sided t-test analysis. Leave-one-out cross-validation was performed by applying the framework of the R package CMA (CMA: a comprehensive Bioconductor package for supervised classification) [30]. Receiver operating characteristics curve and area under the curve are based on the classification results derived from the respective leave-one-out-loops. The array data and experimental layout were submitted to the public repository ArrayExpress [31] and can be accessed at E-MEXP-3625.

3 Results

3.1 Differentially expressed proteins in recurring bladder tumors

For a better understanding of the proteins involved in tumor recurrence and for the definition of a prognostic signature, we studied expression variations at the protein level of 25 primary, non-muscle-invasive bladder tumor tissue samples (stage Ta) from patients that had been followed for at least 5 years after resection of the initial tumor. Patients are being considered cured of bladder cancer, if there is no recurrence after 5 years. For yielding protein signatures that fit to this standard, only samples were studied, for which
appropriate long-term follow-up information was available. This limited the number of samples in the analysis drastically. However, we considered it important to focus on such samples in order to account also for slow recurrence. An overview about the information resulting from the analysis is given in Fig. 1.

For analysis, we used an array of 818 antibodies targeting 724 cancer-related proteins (Supporting Information Table 1). In earlier analyses, the overall analysis process had been studied with respect to various performance parameters, meeting or even exceeding the quality characteristics of DNA-microarrays that have been approved by regulatory agencies for diagnostic applications [22, 23]. For normalization, the incubations of the individual patient samples were performed in presence of a common reference sample that had been made up by pooling equal amounts of all protein samples. The reference was labeled with a second fluorescent dye. The Pearson’s correlation coefficients of the two detection channels were in the range of 0.92 \( \leq r \leq 0.98 \) throughout. To assess the effect of different incubation dates, four samples were repeatedly incubated during the entire period of the study. All samples were incubated with the dyes swapped between the individual patient samples and the common reference sample. No variations could be observed that indicate a significant influence of labeling, incubation date, or any other parameter. All incubations with identical samples produced results that match closely in an unsupervised hierarchical clustering (Supporting Information Fig. 1).

From the 724 proteins that were analyzed, 255 exhibited a significantly differential abundance (FDR < 0.05) between samples from patients with or without recurrence (Supporting Information Table 2). While 105 proteins were found to be more abundant in recurrent tumors, 150 proteins exhibited lower abundances. The relatively high number indicates substantial differences in tumor biology in recurring and non-recurring tumors. Comparison of very few normal and the cancerous bladder samples (Supporting Information Fig. 2; Supporting Information Table 3) found other proteins to be differentially expressed than those that differ between recurrent and non-recurrent samples. However, a possible overlap may be missed because of experimental limitations. Far fewer proteins could be identified at an FDR < 0.05, since the low number of healthy samples resulted in low statistical power and thereby a higher FDR.

From the 255 proteins, 102 were identified at an FDR of less than 0.003 (Fig. 2). About 56% of these proteins had been reported before to be involved in the recurrence of various cancer types. However, for 80% of them, there was no report found concerning recurrence in bladder cancer. As a matter of fact, 53% of the 102 proteins had not been reported in any context of bladder cancer before. To assess the homogeneity of the results and thus the significance of the findings for individual patients, we looked at expression variations individually. Lamin A (LMNA), transcription factor AP-1 (JUN) and Y Box Transcription Factor-1 (YBOX1) were the three proteins with the strongest overrepresentation in recurrent samples, while \( \beta \)-Selectin (LYAM1), Cyclin-Dependent Kinase Inhibitor 1A (CDN1A), and Mothers Against Decapentaplegic Homolog 3 (SMAD3) were the most underrepresented ones (Fig. 3).

### 3.2 Accurate prediction of recurrence by protein signature

In order to assess the prognostic potential of the proteins that exhibited differential abundance, we performed a
multivariate analysis. We established a classification rule based on the random forest method [29] and the 20 proteins that exhibited the most significant differences (Table 1). Classification of the training set was possible at a sensitivity of 100% and specificity of 100%. To assess the transferability to other test sets, we performed leave-one-out cross-validations. The classification results for the different test sets in the cross-validation steps are summarized as a receiver operating characteristics curve (Fig. 4). The respective overall misclassification rate for the cross-validation was 20% (SD 0.08). The area under the curve value of 0.91 demonstrates the very high accuracy of discriminating between patients with or without recurrence. The corresponding sensitivity is 80% at a specificity of 100%.

3.3 Functional annotation

For identifying possible mechanisms that may be responsible for the difference between recurrence and non-recurrence, the proteins that exhibited expression differences (Supporting Information Table 2) were studied with respect to functional aspects using the web-based analysis platform DAVID [32]. Interestingly, proteins known to be involved in the development of bladder cancer, such as p14ARF (CD2A2), p53, p21 (CDN1A), P16Ink4a (CD2A1), VEGFA, MMPs, and IL8, were expressed at a lower level in recurrent tumors. About 56% of the proteins that were of higher abundance in recurrent tumors are located inside cells, while 61% of the less abundant proteins are known to be found in the extracellular space. A pathway analysis with KEGG [33] revealed a strong repression of the TGF-beta signaling pathway (Fig. 5; Supporting Information Fig. 4) in recurrent cancer. The signaling factors IFNG, TNFA, TGFB, and THBS1 were expressed less, abundance of the inhibitor MAPK3 (also known as ERK1) was higher, while SMAD2, SMAD3, and SMAD4 were again significantly underrepresented. Consistently, also the protein SP1 was less expressed in recurrent samples. Additionally, apoptosis was one of the pathways with a strong regulation (Supporting Information Fig. 5). The effectors Caspase 3 (CASP3) and 9 (CASP9), in their activated form, as well as the regulator Bcl-2–associated X (BAX) protein were higher expressed in recurrent samples. This could be mediated by the Trail-receptor, which was also found to be stronger expressed in recurrent samples, while FAS was expressed less. An overview of observed protein expression variations in all cancer pathways is given in Supporting Information Fig. 6.

The KEGG findings were supported by GO analyses, which revealed a high percentage of regulated proteins that are either involved in apoptosis or cellular proliferation. Proteins more abundant in recurrent samples had a much higher percentage of pro-apoptotic and antiproliferative effects. For
Table 1. List of the 20 proteins with the most significant expression variations between recurrent and non-recurrent tumors

<table>
<thead>
<tr>
<th>Protein Uniprot entry name</th>
<th>Uniprot accession</th>
<th>log2 fold change</th>
<th>Reported studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMNA_HUMAN</td>
<td>P02545</td>
<td>3.3 × 10⁻⁰⁹</td>
<td>0.72</td>
</tr>
<tr>
<td>YBOX1_HUMAN</td>
<td>P67809</td>
<td>1.9 × 10⁻⁰⁸</td>
<td>0.52</td>
</tr>
<tr>
<td>JUN_HUMAN</td>
<td>P05412</td>
<td>1.3 × 10⁻⁰⁷</td>
<td>0.50</td>
</tr>
<tr>
<td>AKT3_HUMAN</td>
<td>Q9Y243</td>
<td>2.5 × 10⁻⁰⁷</td>
<td>0.48</td>
</tr>
<tr>
<td>YETS2_HUMAN</td>
<td>Q9ULM3</td>
<td>5.2 × 10⁻⁰⁶</td>
<td>0.32</td>
</tr>
<tr>
<td>CAC1_HUMAN</td>
<td>P12830</td>
<td>1.2 × 10⁻⁰⁵</td>
<td>0.33</td>
</tr>
<tr>
<td>TIA1_HUMAN</td>
<td>P31483</td>
<td>2.1 × 10⁻⁰⁷</td>
<td>−0.39</td>
</tr>
<tr>
<td>SMAD3_HUMAN</td>
<td>P84022</td>
<td>2.1 × 10⁻⁰⁷</td>
<td>−0.58</td>
</tr>
<tr>
<td>PABP1_HUMAN</td>
<td>P11940</td>
<td>2.1 × 10⁻⁰⁷</td>
<td>−0.36</td>
</tr>
<tr>
<td>CDN1A_HUMAN</td>
<td>P38936</td>
<td>3.4 × 10⁻⁰⁷</td>
<td>−0.52</td>
</tr>
<tr>
<td>LYAM1_HUMAN</td>
<td>P14151</td>
<td>9.6 × 10⁻⁰⁷</td>
<td>−0.51</td>
</tr>
<tr>
<td>AKTIP_HUMAN</td>
<td>Q9H8T0</td>
<td>5.4 × 10⁻⁰⁶</td>
<td>−0.34</td>
</tr>
<tr>
<td>PRI1_HUMAN</td>
<td>P48642</td>
<td>1.0 × 10⁻⁰⁵</td>
<td>−0.35</td>
</tr>
<tr>
<td>HSP7C_HUMAN</td>
<td>P11142</td>
<td>1.0 × 10⁻⁰⁵</td>
<td>−0.34</td>
</tr>
<tr>
<td>RSSA_HUMAN</td>
<td>P08865</td>
<td>1.1 × 10⁻⁰⁵</td>
<td>−0.35</td>
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<tr>
<td>GRM1A_HUMAN</td>
<td>Q96CP6</td>
<td>1.1 × 10⁻⁰⁵</td>
<td>0.28</td>
</tr>
<tr>
<td>TPA_HUMAN</td>
<td>P00750</td>
<td>1.1 × 10⁻⁰⁵</td>
<td>0.23</td>
</tr>
<tr>
<td>ZBT17_HUMAN</td>
<td>P13473</td>
<td>1.1 × 10⁻⁰⁵</td>
<td>0.26</td>
</tr>
<tr>
<td>JUN_HUMAN</td>
<td>P05412</td>
<td>1.3 × 10⁻⁰⁵</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Log fold changes in expression and the related FDR are shown. Also, literature is listed in which the protein or respective gene was reported in connection with bladder cancer (BC), recurrence of bladder cancer (RBC), recurrence of other cancer forms (RC), or cancer overall (C).

the identification of key players, strongly regulated proteins were additionally studied with the pathway analysis software STRING 9.1 [34]. Among the highly abundant proteins in recurrent tumors, Nuclear Factor NF-kappa-B1 (NFκB1), transcription factor AP-1 (JUN), DNA Topoisomerase 2 alpha (TOP2A), Krueppel-Like Factor 5 (KLF5), and Cadherin-1 (CADH1) are interacting through protein–protein binding and activation of expression (Supporting Information Fig. 7). Also proteins that were less abundant in recurrent tumors—Mothers Against Decapentaplegic Homolog-3 (SMAD3), Matrix Metalloprotease 1 (MMP1), Tissue Inhibitor of Metalloproteinases 1 (TIMP1), Vascular Endothelial Growth Factor A (VEGFA), and transcription factor SP1—were reported to interact. Interestingly, the highly abundant JUN is known to suppress the expression of the less abundant protein TIMP1. Also, JUN and NFκB1 interact with SP1.

4 Discussion

A prognosis on patients diagnosed with non muscle-invasive bladder cancer could have immediate clinical consequences. In this study, we set out to look for molecular variations that may permit prediction of tumor recurrence, focussing on proteins as molecular markers since this molecule class is directly responsible for many cellular activities and thus likely to represent best functionally relevant differences. In earlier protein studies of bladder cancer, body fluids had been analyzed. An antibody microarray was used to profile serum samples from bladder cancer patients [35]. Besides a differentiation of healthy and cancer patients, also a separation of the patients in high- and a low-risk groups was achieved on the basis of four proteins. In a study analyzing urine, peptides were identified by MS that predicted muscle invasive disease with a sensitivity of 92% and a specificity of 68% [36]. While these studies used samples from patients with both high- and low-grade bladder cancers, we focused entirely on cases of low-grade, low-stage bladder tumors, and the prediction of recurrence. Also, we insisted on a 5-year follow-up, although this limited substantially the number of samples in the analysis. The proteome of the primary tumors was analyzed, since patient prognosis depends strongly on the likelihood of local recurrence. Clinically, the required tumor material is

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available for analysis, since transurethral resection is routinely performed upon initial diagnosis.

Comparing non muscle-invasive tumors with a long-term follow-up on recurrence, we identified 255 proteins with significant expression differences. The large number is surprising, since most proteins targeted by the antibodies had originally been selected on the basis of transcriptional variations observed in pancreatic, colon, and breast cancer in comparison to the related healthy tissue. One would not have expected to see so many differences between two tumor types of a similar stage in the same organ. The many variations in protein levels indicate considerable differences in the tumor biology of recurrent and non-recurrent tumors. Several proteins that were more abundant in recurrent tumors are known key players in oncogenesis, such as JUN, TOP2A, or NFκB1. Elevated levels of JUN had been identified as a prognostic marker for high risk and recurrence of prostate cancer [37]. At transcript level, its upregulation in bladder cancer is part of a four-transcript signature associated with bad outcome [8]. Among the proteins that were less abundant in recurring tumors was transcription factor SP1. In high-grade and muscle-invasive bladder cancer, SP1 has been reported as a potential therapeutic target [38-40].

Strikingly, proteins that are important for apoptosis, such as active CASP3, CASP9, or BAX, were more abundant in recurrent than non-recurrent tumors, while CASP3 levels are known to be low in higher-grade bladder tumors [41]. Also in other cancer entities, an overexpression of CASP3 was associated with recurrence and higher cancer mortality [42-44]. The increased rate of recurrence in apoptotic tumors after radio or chemotherapy was explained by the ability of CASP3 to repopulate tumor cells through prostaglandin E2 [43]. Apoptotic tumor cells lead to increased levels of arachidonic acid in the extracellular milieu through CASP3-mediated iPLA2 activation. This causes an activation of prostaglandin E2 through prostaglandin synthase H (PGH2, also known as COX2), a downstream product of arachidonic acid and a key regulator of tumor growth. Noticeably, Huang et al. [43] could correlate the level of active CASP3 with a higher rate of tumor recurrence.

Figure 5. KEGG analysis of the TGF-beta signaling pathway. Affected proteins are labeled in green or red if their expression was lower or higher, respectively, in recurrent rather than non-recurrent cancer. The original KEGG representation is shown in Supporting Information Fig. 4.
recurrence in head and neck squamous cell carcinoma and breast carcinoma. In our study, we identified active CASP3 and CASP9 as well as PGH2 at higher levels in the recurrent tumors. This indicates a similar mechanism of apoptosis-activated tumor repopulation via PGH2 for the recurrence of bladder cancer.

Triggering apoptosis via p53 and the TGF-β pathway was significantly reduced in recurrent compared to non-recurrent bladder cancer samples. For the TGF-beta signaling pathway, the activators IFN-γ, TNF-α, TGF-β, the SMAD proteins as well as the transcription factor SPI1 were found at reduced levels in recurrent samples, while the repressor MAPK3 was elevated. Such a downregulation of TGF-β/SMAD signaling had been observed in different cancers before and is associated with a higher risk of recurrence or a poor outcome [45–47].

Overall, the study revealed a 20-protein signature that allows for a prediction of bladder cancer recurrence with high accuracy. The values of 80 and 100%, respectively, for sensitivity and specificity compare well with the accuracy of the approved NMP22 assay for bladder cancer diagnosis [48], which exhibits a sensitivity of merely 55.7% and specificity of 85.7%. Unfortunately, not many samples were available for which a 5-year follow-up had been done. Therefore, additional analyses on more tissue samples are required for confirmation; however, collection will take time. Furthermore, as most of the significantly regulated proteins are secreted, non-invasive analysis may be possible using plasma or urine samples of bladder cancer patients. Identified in an immuno-based system, these marker proteins may be translated relatively easily to standard immuno-based assay platforms that are more suited to routine application in a clinical setting. Such a test could help to adjust the treatment scheme and the rigidity of surveillance. This could dramatically reduce surveillance cost and simultaneously improve the patients’ outcome substantially.

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H.S., J.D.H., M.S.S.A., and C.S. designed the microarray experiments; D.V., Y.A., and F.R. selected patients and provided protein extracts; H.S. performed microarray experiments; M.S. and C.S. contributed to data analyses; H.S., C.S., and J.D.H. interpreted the data and prepared the manuscript.

Potential conflict of interest: Harish Srinivasan, Christoph Schröder, François Radvanyi, and Jörg D. Hoheisel filed a patent application for a prognostic use of the marker set described within this manuscript. Christoph Schröder and Jörg D. Hoheisel are founders of the spin-off company Scimics GmbH. It offers services based on the use of antibody microarrays.

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