Simultaneous Proteomic and Genomic Analysis of Primary Ta Urothelial Cell Carcinomas for the Prediction of Tumor Recurrence

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Abstract. *Background: The prediction of tumor recurrence in patients with Ta urothelial cell carcinoma is inaccurate and new prognostic markers are desirable. Materials and Methods: Surface-enhanced laser-desorption ionization time-of-flight mass spectrometry (SELDI-TOF MS) was performed on 33 primary Ta tumors (16 and 17 tumors were from patients with long and short recurrence-free periods, respectively) and data were compared to previously obtained mRNA expression profiles of 49 genes. Results: The intensities of a protein peak at m/z 33331 varied most significantly between the two patient groups (p=0.0048). This was comparable to survivin, whose mRNA expression differed most significantly (p=0.0042) of the 49 genes. ROC analysis revealed an area under the curve for protein peak 33331 and survivin of 0.78 (95% CI, 0.62-0.94) and 0.79 (95% CI, 0.63-0.94), respectively. Protein peak 33331 and survivin identified 3 (17%) and 8 (47%) patients with a recurrence-free period of at least 4 years, respectively, without generating false-negatives. Conclusion: These findings indicate that SELDI-TOF MS and real-time Q-PCR analysis on the same tissue can result in the identification of markers with comparable differential expression. Such combined analyses may yield combinations of several markers that might improve disease prognosis.*

The most important form of bladder cancer is urothelial cell carcinoma (UCC). It originates in the urothelium which forms the inner lining of the bladder. About 70% of the patients with UCC are diagnosed with superficial tumors (Ta, T1), of which the depth of organ invasion is limited to the stromal layer below the urothelium. Therefore, these tumors can be removed relatively easily from the bladder by transurethral resection. In approximately 70% of the patients with superficial UCC, tumors recur after surgery. Since the prediction of the individual recurrence-free period is poor, frequent follow-up examination of the bladder of all patients by invasive cystoscopy is necessary. This also generates a substantial number of unnecessary bladder examinations in those patients that remain recurrence-free for several years or even for the rest of their lives. Accurate prediction of the individual recurrence-free period would, therefore, limit the number of redundant invasive cystoscopies and reduce patient burden.

Patients with Ta or T1 tumors differ with respect to the risk of recurrence after primary tumor resection (1, 2). Recurrence-free periods of several years are more frequently noted for patients with Ta UCC (3-5). The number of unnecessary control cystoscopies will therefore be highest among patients with Ta tumors. The prediction of recurrence in patients with Ta tumors mainly depends on pathological parameters such as tumor grade, tumor multiplicity and tumor size (5, 6). The assessment of these tumor characteristics, however, is subject to observer variability (7, 8). A more accurate test for the prediction of tumor recurrence is therefore required. In recent years, research has focused on the prognostic potential of molecular markers (9). To date, only a limited number of studies revealed a positive correlation between such markers and the recurrence pattern among patients with Ta UCC (10-15). Unfortunately, these potentially useful tests have not been investigated further and are not integrated in urological practice.

The present study aims to combine proteomics-based and genomics-based profiling of Ta tumors and evaluate its

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effect on the prediction of recurrence in patients with Ta UCC. To this end, the protein expression profiles of 33 primary Ta UCC's were generated by surface-enhanced laser desorption ionisation time-of-flight mass spectrometry (SELDI-TOF MS). The differential expression of 47 protein profiles was compared to that of the mRNA expression profiles of 49 genes that had previously been obtained from the same 33 Ta tumors using real-time quantitative PCR (16, 17). In addition, the performance of differentially expressed markers, identified by SELDI-TOF or real-time Q-PCR, with respect to the identification of patients with Ta tumors who remain recurrence-free for several years after primary surgery was investigated.

Materials and Methods

Tumor specimens. Thirty-three primary Ta UCC's (Table I) were included in this study. They were obtained from the bladder tumor biobank of the Department of Urology of the Academic Hospital in Uppsala (Sweden) and from bladder tumor collections of the Department of Urology of the Charles University in Prague (Czech Republic). Tumors were removed by transurethral resection and histopathological parameters were evaluated using the WHO criteria for grade (18) and TNM classification for stage (19). All tissues were stored at –80˚C. The tumors were collected after approval of the local ethics committees. The biopsies contained at least 60% tumor cells as assessed by hematoxylin and eosin staining.

The group of patients consisted of 21 men and 12 women, with an average age of 64.7 years (range 42-79 years). The distribution of tumor grade was: 4 GI, 25 GII and 4 GIII. The non-recurrence (NR) and recurrence (R) groups consist of 17 and 16 selected patients, respectively. Patients in the NR-group remained recurrence-free for 4 years, whereas those from the R-group had recurrences within the first 2 years after primary surgery. All patients received their first follow-up examination three months after primary surgery. These and subsequent bladder examinations were performed by means of cystoscopy and urine cytology. No patient received adjuvant treatment after tumor resection or presented with concomitant carcinoma *in situ*.

Table I. *Patient details with respect to tumor class and recurrence-free interval.*

Patient #	Recurrence group	Tumor class	Recurrence-free interval (months)
Pt1	NR	TaGI	96
Pt2	NR	TaGII	204
Pt3	NR	TaGII	180
Pt4	NR	TaGII	120
Pt5	NR	TaGII	108
Pt6	NR	TaGII	108
Pt7	NR	TaGII	108
Pt8	NR	TaGII	84
Pt9	NR	TaGII	84
Pt10	NR	TaGII	72
Pt11	NR	TaGII	72
Pt12	NR	TaGII	60
Pt13	NR	TaGIII	60
Pt14	NR	TaGI	48
Pt15	NR	TaGI	57
Pt16	NR	TaGIII	54
Pt17	NR	TaGII	51
Pt18	R	TaGII	11
Pt19	R	TaGII	3
Pt20	R	TaGII	14
Pt21	R	TaGII	4
Pt22	R	TaGIII	14
Pt23	R	TaGII	5
Pt24	R	TaGIII	6
Pt25	R	TaGII	12
Pt26	R	TaGII	15
Pt27	R	TaGII	8
Pt28	R	TaGII	4
Pt29	R	TaGII	23
Pt30	R	TaGII	7
Pt31	R	TaGI	7
Pt32	R	TaGI	4
Pt33	R	TaGII	22

Protein isolation. The assessment of the percentage of tumor cells in the tumor biopsies and the isolation of mRNA for real-time quantitative PCR analysis were described previously (16, 17). The proteins were isolated simultaneously with the mRNA from the same tumor sections using TRIzol® Reagent (Invitrogen C+C), according to the manufacturer's instructions. The pelleted proteins were dissolved in phosphate buffered saline (PBS) containing 1% sodium dodecyl sulphate (SDS). Protein concentrations were measured on a spectrophotometer (Gene Quant *pro*, Amersham Biosciences C+C) at OD_{280}. All samples were stored at –20˚C until use.

SELDI-TOF MS. To generate profiles of the isolated proteins by SELDI-TOF MS, the cation-exchange chip CM10 (Ciphergen Biosystems, Inc C+C) was used. This chip can be used to bind proteins dissolved in SDS, whereas SDS can be efficiently washed away in a second step avoiding SDS artefacts (20). Optimization experiments using TRIzol®-extracted tumor proteins revealed that passive binding of protein-SDS complexes by dry-inn experiments, followed by washing with 0.1 M ammonium acetate pH 3 washing buffer and laser desorption/ionization with a laser intensity of 185 yielded the highest abundance of different protein peaks. Artefacts due to SDS were only observed when <3 washing steps were used; these SDS patterns showed as repetitive peaks in the 2000-5000 Da range with intervals of ~150 Da (data not shown). When 5 washing steps were used and samples had a minimal protein concentration of 400 ng/μl, reproducible protein profiles were obtained without interference of SDS patterns (see Figure 1). Therefore, the following protocol was used to generate tumor protein profiles from all samples. First, 10 μl of the dissolved tumor protein extract was applied to a 0.1 M ammonium acetate (pH 3)-equilibrated CM10 chips, which were then allowed to air-dry. Next, spots were washed five times with equilibration buffer (to completely remove SDS) and allowed to air-dry. Finally, 0.8 μl of a saturated solution of sinapinic acid in 0.5% (v/v) trifluoroacetic acid and 50% (v/v) acetonitrile were applied to each spot surface, allowed to air-dry, and reapplied. Mass/charge (m/z) spectra were

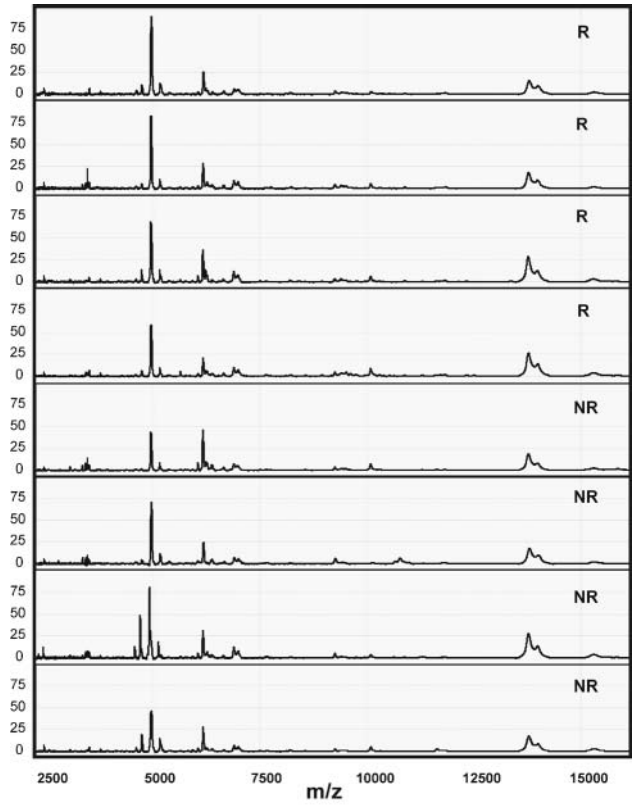

Figure 1. *Comparison of protein profiles from 8 TRIzol®-extracted protein fractions dissolved in SDS. Tumor protein extracts were applied to CM10 ProteinChips, using 0.1 M amonium acetate (pH 3) as washing buffer. Mass spectra of retained proteins were generated by SELDI-TOF MS with a laser intensity of 185 M/z. Peak intensities are in arbitrary units. R: recurrence group; NR: non-recurrence group.*

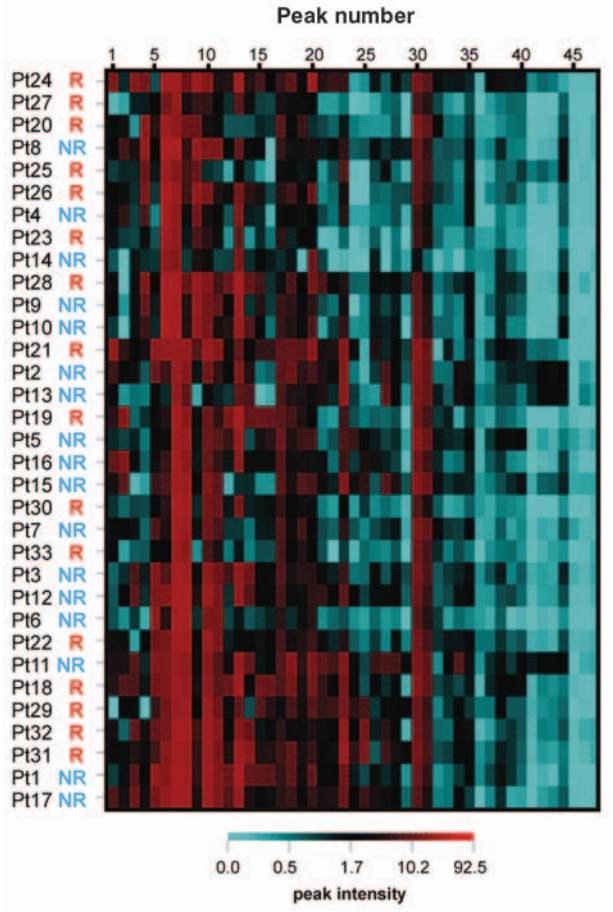

Figure 2. *Protein profiles of tumor protein extracts. SELDI-TOF MS spectra were generated (with laser intensity 185) from all 33 tumor protein samples. When a signal-to-noise ration of 5 was used, 47 potential biomarkers were detected. Notably, biomarker 44 corresponds to protein peak m/z 33331. Unsupervised clustering was used to visualize whether protein expression profiles correlated with bladder cancer recurrence. Relative high peak intensities are indicated in red; blue indicates low intensity. Absolute values of peak intensities are given in Supplementary Table S1.*

generated in a Protein Biology System II time-of-flight mass spectrometer (Ciphergen Biosystems, Inc). Laser intensity was set to 185 (profiling 2.5-50 kDa) or 230 (profiling 10-200 kDa) with detector sensitivity of 9, high mass to acquire 50 with an optimization range of 5-15 kDa, or 200 kDa with an optimization range 10-50 kDa; 400 laser shots were averaged to obtain the spectra. External calibration was performed using Hirudin BKHV (7033.6 Da), bovine cytochrome C (12230.9 Da), myoglobin (16951.5 Da), and bovine carbonic anhydrase (29023.7 Da) as standards (Ciphergen Biosystems, Inc). Spectra were normalized to total ion current before further analysis. ProteinChip binding and SELDI-TOF MS experiments were performed in two separate sessions with samples randomly distributed over ProteinChip arrays, and ProteinChip Software 3.0 was used for analysis of the mass spectra. The Biomarker Wizzard application of the ProteinChip Software was used for biomarker analysis. Only biomarker peaks present in at least 5 out of the 33 spectra with a signal-to-noise ratio of 5 were used for further analysis. Values of representative single measurements were used for statistical analysis. Color-coded clustered image maps, "heat maps", to represent protein expression profiles were generated using the web-based CIMminer algorithm (http://discover.nci.nih.gov/cimminer/index.jsp).

mRNA expression analysis. The real-time quantitative PCR (Q-PCR) analysis of mRNA expression and a detailed description of the 49 target genes, the housekeeping gene cyclophilin A (for expression normalization (21)) and the 18S ribosomal RNA subunit (for cDNA quality assessment) are described elsewhere (16, 17).

Statistical analysis. Differences in gene mRNA expression or protein intensities between the two different patient groups (non-recurrent (NR) and recurrent (R)) were analyzed using the Wilcoxon test, with a p-value <0.05 indicating statistical significance. Receiver-operating-characteristic (ROC) curves were created to indicate the performance of the markers with respect to the identification of patients with short or prolonged recurrence-free intervals. Specificity and sensitivity refer to the identification of patients with long (non-recurrence (NR) group) and short (recurrence (R) group), respectively. Markers were considered to have discriminative ability if the 95% confidence interval (95% CI)

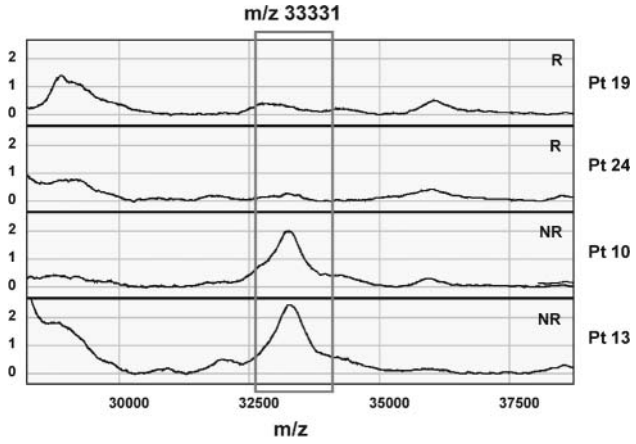

Figure 3. *Comparison of 33331 protein peak intensities in tumors from the non-recurrence (NR) and recurrence (R) groups. Cell lysates were applied to CM10 ProteinChips, using 0.1 M amonium acetate (pH 3) as washing buffer. Mass spectra of retained proteins were generated by SELDI-TOF MS. Only profiles from the four patients which contained the 33331 protein peaks (boxed) with lowest (Pt19 and Pt24) and higest (Pt10 and Pt13) intensities are shown. M/z, mass/charge ratio. Intensities are in arbitrary units.*

for the area under the curve (AUC) did not include 0.5. Statistical analyses were performed using SPSS software version 12.0.1.

Results

SELDI-TOF MS. To compare SELDI-TOF MS analysis of primary Ta tumor tissues with real-time Q-PCR analysis, the aim was to generate protein profiles from the same 44 Ta tumors used in the previous studies that investigated mRNA expression analysis (16, 17). Therefore, it was first tested whether TRIzol® extracted tumor proteins dissolved in SDS could be used to generate protein profiles. As shown in Figure 1, TRIzol® extraction and SELDI-TOF MS on 8 samples yielded comparable protein spectra in the 2.5-15 kDa region (Laser intensity 185), without interference of SDS, when 5 washing steps were performed after application of the samples to the ProteinChip (see Material and Methods section). Unfortunately, protein extraction from 11 tumors did not yield the critical protein concentration of 400 ng/μl needed to generate comparable protein profiles (see Material and Methods Section). SELDI-TOF MS analysis was therefore restricted to the remaining 33 Ta tumors (Table I). The non-recurrence (NR) and recurrence (R) group consisted of 17 and 16 patients, respectively. In the protein spectra from these 33 tumor protein samples, 47 biomarkers were detected with a signal-to-noise ratio of 5 (Supplementary Table S1) and were used for further analysis.

As shown in Figure 2, overall protein expression patterns in the 33 samples were quite similar and unsupervised clustering did not clearly separate the NR and R groups. Although a number of proteins were found to be differentially expressed in a small number of samples (Figure 2), only one was considered to be significantly different in both groups. The discriminative peak was identified at an m/z ratio of 33331 and representative profiles showing this peak with highest and lowest intensities are shown in Figure 3.

As shown in Figure 4A, the intensity, or expression, of this protein was significantly higher in the NR-group than in the R-group ($p=0.0048$), suggesting that reduced expression of this protein is correlated with a more aggressive nature of the tumor. Subsequently, the ability of this peak to identify patients with short or prolonged recurrence-free periods was investigated. The intensities of the protein peak for both patient groups were compared using ROC analysis. This yielded an AUC of 0.78 (95% CI, 0.62-0.94; Figure 2B). At an intensity cut-off level of 0.70, protein peak 33331 identified 76% and 75% of the patients with long or short recurrence-free periods, respectively. For the identification of patients who remain recurrence-free for at least 4 years without generating false-negatives (*i.e.* patients who will develop an early recurrence, but who will be labeled as non-recurring), the intensity cut-off level of the 33331 peak must be increased to 1.63. At this level the sensitivity is 100% and results in the correct identification of 3 patients (17.6%).

Protein peak 33331 might very well correspond to a double charged form of albumin. To investigate whether this was indeed the case, protein profiles from 10-200 kDa (including albumin of 66 kDa) were generated at laser intensity 230. As shown in Figure 5A, the albumin peak was clearly detectable in the samples with high m/z 33331 intensities, and the ProteinChip software assigned the latter peak as an double charged form of the albumin peak (data not shown). As shown in the lower panel of Figure 5A, albumin is one of the major peaks in a protein spectrum generated from a serum sample applied to the same ProteinChip; the sizes of the double charged form of albumin in serum and tumor protein extracts match exactly. To confirm the hypothesis that protein peak 33331 is a double charged form of albumin, the correlation of these two peaks in all 33 tumor samples was investigated. A very strong correlation (with a correlation coefficient of 0.93) between the intensities of the 33331 and 66662 peaks in all samples is shown in Figure 5B. Under the latter SELDI-TOF MS conditions, protein peaks 33331 and 66662 were higher in the NR group than in the R groups with similar significance of $p=0.013$ and $p=0.012$, respectively. Together, these data indicate that the discriminative peak m/z 33331 observed by SELDI-TOF MS corresponds indeed to a double charged form of albumin. Apparently, the double charged form of albumin, profiled at low laser intensities, has the highest discriminative power between the NR and R groups.

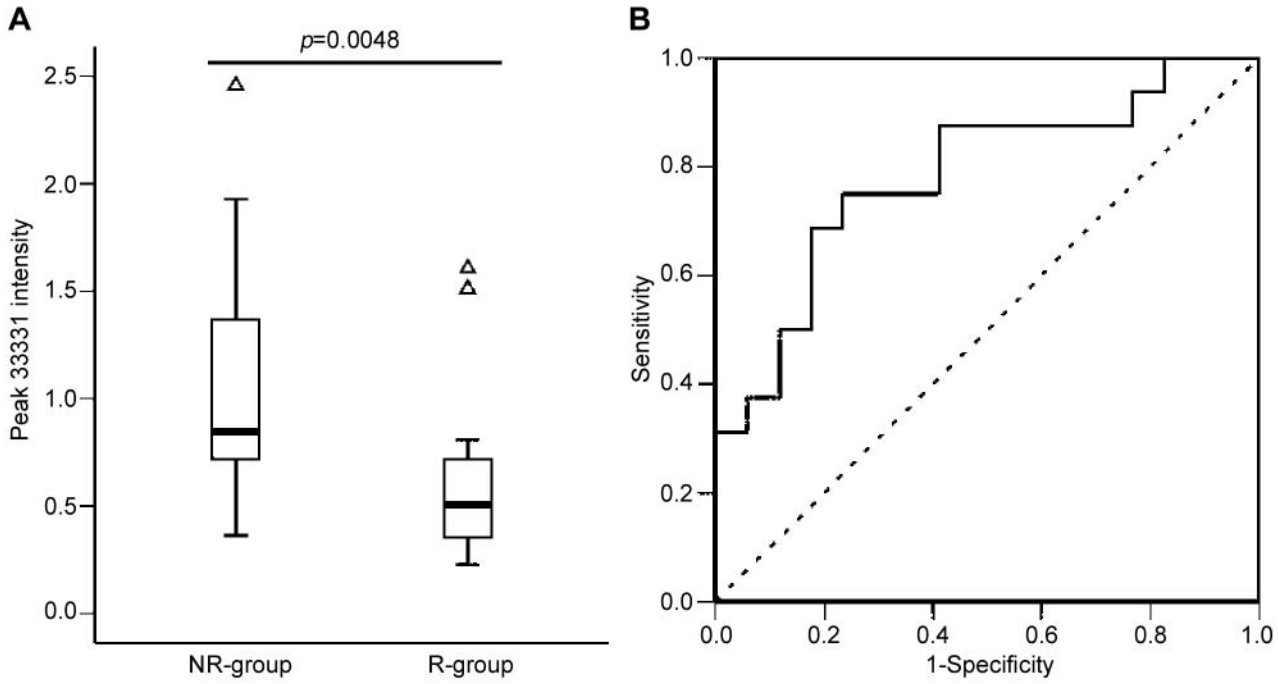

Figure 4. *Distribution of the 33331 protein peak intensities in the non-recurrence (NR) and recurrence (R) groups (A) and the test performance with respect to the identification of patients with short or long recurrence-free periods (B). A, The boxes represent the interquartile range of the 33331 protein peak intensity. Open triangles represent outliers. The median intensitiy level (black horizontal bar) in the NR and R groups are (range in parenthesis), respectively, 0.85 (0.36-2.46) and 0.51 (0.23-1.61). B, Comparison of the receiver-operating-characteristic curves for the 33331 peak intensities between patients with short or long recurrence-free periods revealed an area under the curve of 0.78 (95% CI, 0.62-0.94).*

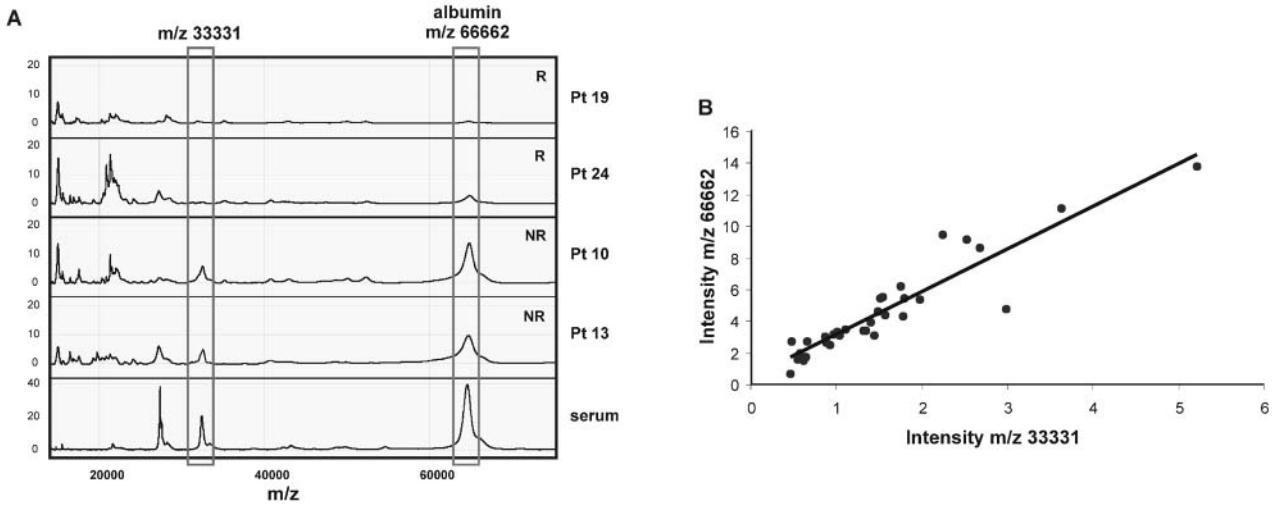

Figure 5. *Comparison of 33331 and 66662 protein peaks in tumors from the non-recurrence (NR) and recurrence (R) groups. (A) Cell lysates were applied to CM10 ProteinChips, using 0.1 M amonium acetate pH3 as washing buffer. Mass spectra of retained proteins were generated by SELDI-TOF MS with a laser intensity of 230. Only profiles from the four patients, which contained the 33331 protein peaks with lowest (Pt19 and Pt24) and higest (Pt10 and Pt13) intensities (see Figure 3), are shown. Spectra were compared with that of serum profiled under the same conditions (lower panel). Positions of protein peaks 33331 and 66662 (albumin) are indicated. M/z, mass/charge ratio. Intensities are in arbitrary units. (B) Correlation between intensities of protein peaks 33331 and 66662 (albumin) in all 33 investigated tumor samples. Correlation coefficient = 0.93.*

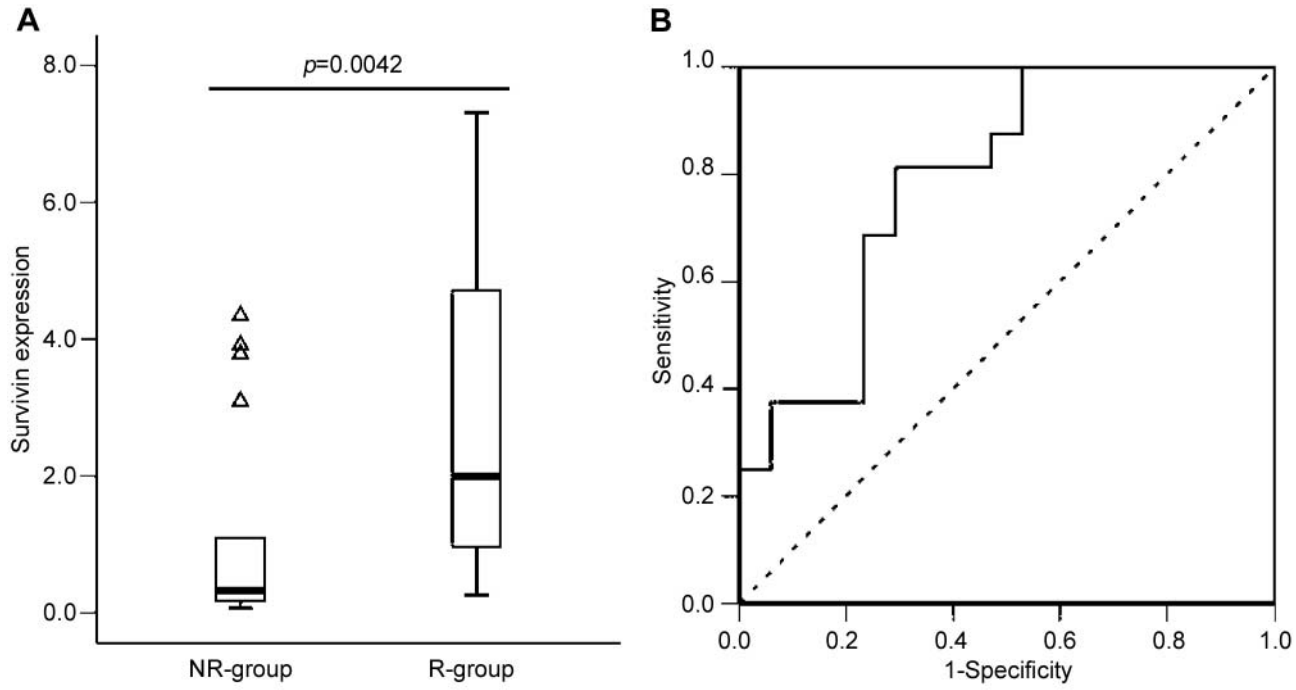

Figure 6. *Distribution of survivin mRNA expression in tumors of the non-recurrence (NR) and recurrence (R) groups (A) and the test performance with respect to the identification of patients with short or long recurrence-free periods (B). A, The boxes represent the survivin expression interquartile range. Open triangles represent outliers. The median survivin expression level (black horizontal bar) in the NR- and R-group are (range in parenthesis), respectively, 0.32 (0.07-4.35) and 1.99 (0.26-7.31). B, Comparison of the receiver-operating-characteristic curves for survivin expression between patients with short or long recurrence-free periods revealed an area under the curve of 0.79 (95% CI, 0.63-0.94).*

mRNA expression analysis. The mRNA expression profiles of the 49 genes in the 33 Ta tumors were already available (16, 17). Restriction of the Wilcoxon analysis to the 33 tumors for the identification of differentially expressed genes revealed that survivin was the most significantly differentially expressed gene between the two patient groups ($p=0.0042$; Figure 6A). Next, the performance of survivin in identifying patients with short or prolonged recurrence-free periods was investigated. ROC curves for survivin mRNA expression in both patient groups were compared and revealed an AUC of 0.79 (95% CI, 0.63-0.94; Figure 6B). At a cut-off level of 0.84, survivin identified 70.6% and 81.3% of the patients with long or short recurrence-free periods, respectively. If the cut-off level is lowered to 0.25, the test correctly identifies 8 patients (47%) with a recurrence-free period of 4 years, without generating false-negatives.

Discussion

Prediction of the recurrence-free period in patients with Ta UCC after primary surgery is still inaccurate and requires improvement. The present study was aimed at the comparison of the potential of SELDI-TOF MS and real-time Q-PCR to distinguish between patients with Ta tumors and short or prolonged recurrence-free intervals. To that purpose, TRIzol®-extracted RNA and proteins from the same tissue samples were used for analysis. Interestingly, a recent comparative study indicated that in certain cases UREA-CHAPS might be better in solubilizing TRIzol®-extracted proteins than SDS (22). Nevertheless, our SELDI-TOF MS analysis on SDS-dissolved proteins identified a marker that was differentially expressed between the two patient groups, with a significance comparable to that of survivin, which showed the best discriminative ability of the 49 mRNA markers. The performance of the protein peak identified by SELDI-TOF MS with respect to the identification of patients with Ta tumors with recurrence-free periods of at least 4 years was inferior to that of survivin.

The number of molecular markers that show a relationship with the time to tumor recurrence in patients with Ta tumors is limited (10-15). In two recent reports, we attempted to distinguish between patients with Ta UCC who experienced their first recurrence within two years and those who remained recurrence-free for at least 4 years (16, 17). Both reports included the 33 tumors used in the present study and revealed that survivin was the most differentially expressed gene. Therefore, it is not surprising that of all 49 genes, survivin expression varied most significantly in the present 33 Ta tumors.

Proteomic analysis of tissue for the identification of molecular markers involved in bladder tumorigenesis and progression is a growing field of research (23-28). Thus far, only one of these studies revealed an association between protein markers and tumor recurrence (28). SELDI-TOF MS has been used before to identify differentially expressed proteins in normal urothelium and tumor tissue (27). However, SELDI-TOF MS has not been employed for the prediction of tumor recurrence in patients with superficial UCC. The present study demonstrates for the first time the ability of SELDI-TOF MS to distinguish between patients with Ta UCC and short or prolonged recurrence-free periods. A peak with a m/z ratio of 33331 was detected and showed differential expression between the two patient groups, comparable to survivin mRNA expression.

Our analyses show that protein peak 33331 corresponds to a double charged form of albumin. Since albumin is a negative acute phase protein (29), the negative correlation of this peak with the time to recurrence may be mediated by the more aggressive nature of tumors from patients with early recurrences. Interestingly, the positive acute phase C-reactive protein was recently shown to be negatively correlated with the survival of patients with bladder cancer (30), confirming a possible predictive value of acute phase phenomena in bladder cancer prognosis. However, it is presently not clear what the molecular basis of a possible association of tumor-associated albumin and recurrence could be.

The investigation of differences in mRNA or protein expression in the same tumor biopsy by two different techniques yielded two interesting markers. The analysis of alterations in different macromolecules, such as mRNA, protein or DNA, isolated from the same tumor biopsy may increase the amount of prognostic information that can be obtained from valuable patient material. This may also result in the identification of a combination of markers that supplement each other and improve the prediction of the clinical course of patients with bladder cancer, as shown before (31). Unfortunately, ROC analysis of a combination of survivin and protein peak 33331 did not reveal a synergistic effect with respect to the prediction of tumor recurrence (data not shown). Future studies should demonstrate the ability of combinations of markers to accurately predict the length of the recurrence-free period in patients with Ta tumors. Survivin may represent an interesting candidate to include in such a panel of markers, since it showed good performance in the identification of patients who remained recurrence-free for at least 4 years (this study and (17)). The additional use of synergistic mRNA or protein markers might eventually result in a significant reduction of the number of unnecessary invasive cystoscopies in patients with Ta UCC.

Conclusion

The simultaneous analysis of the same tumor sections using a proteomics- and a genomics-based technique yielded two comparable discriminative markers. Despite the lack of a synergistic effect, the study of the same tumor material by different techniques may yield combinations of several markers that might improve disease prognosis.

Acknowledgements

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