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Differential proteomic analysis of nuclear matrix in muscle-invasive bladder cancer: Potential to improve diagnosis and prognosis *

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Abstract. *Introduction:* Although several molecular markers for bladder cancer have been identified, at present little information on prognostic biomarkers is available in the literature. Prognostication of this tumor is largely based on clinicopathological characteristics. Our aim was to identify nuclear matrix (NM) proteins that might serve to better characterize the phenotype of the invasive bladder cancer and to investigate their diagnostic and prognostic roles.

Methods: NM proteins expressed in normal ($n = 3$) or non-tumoral ($n = 9$) tissue specimens and muscle-invasive bladder cancer ($n = 21$) specimens were analyzed by two dimensional (2D) gel electrophoresis. PDQuest image analysis software was used to generate a comparative NM proteome analysis. Selected spots were characterized by liquid chromatography coupled to tandem mass spectrometry and Western blot.

Results: We detected over 800 protein spots in each 2D map and 43 spots were identified. 30 proteins were differentially expressed by bladder tumor cells; among these, 19 proteins were detected in bladder tumoral tissues but not in normal and non-tumoral tissues and seven proteins correlated with tumor stage. One protein ($p54^{nrb}$) was strongly correlated with vascular invasions and appeared to be also significantly ($P < 0.0001$) associated with a decreased probability of survival.

Conclusion: Important alterations in NM proteins occur in muscle-invasive bladder cancer. The differentially expressed proteins include biomarkers potentially useful for disease diagnosis, progression and prognosis. Our findings beyond improving the understanding of the biology of bladder cancer, could help to stratify patients into different prognostic subgroups and to select those who might be better candidate to multimodal therapeutic approaches.

Keywords: Bladder carcinoma, nuclear matrix proteins, proteomics, biological markers, diagnosis, prognosis

1. Introduction

Bladder cancer is the fourth most commonly diagnosed cancer in men and the ninth in women and ranks second only to prostate cancer among genitourinary tumors [24]. In western countries, approximately 90% of all patients present transitional cell carcinomas (TCCs) and, at the time of initial presentation, 70% of tumors are superficial (Ta, T1) and 30% are lesions at a higher grade and/or stage; moreover, approximately 50% of patients with a superficial TCC will develop an invasive disease. Radical cystectomy is the standard therapy for patients with muscle-invasive bladder cancer; however, in these patients, the five-year survival rate is less then 50% [36]. The major challenge today is to identify the subset of muscle-invasive disease patients that have a worse prognosis and should receive adjuvant therapies. Among the clinicopathological factors, tumor stage and vascular invasion appear to be promising in predicting recurrence and progression [22] as well as some biomarkers (Ki-67 and p53), but results

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are still heterogeneous [19]. Therefore, an improved understanding of the molecular biology of this tumor should increase the likelihood of detecting new diagnostic and prognostic markers.

It has long been recognized that the process of cancerogenesis involves several alterations of nuclear morphology and the amount and distribution of heterochromatin [33]. The condensed state of the chromatin domains, which encompass repressed genes, is determined by the complex balance of the conformational effects arising from the binding of trans-acting factors. The most important trans-acting factors come from the post-translational modifications of the histone complement and from the interaction of chromatin with the nuclear matrix (NM), the protein scaffold that provides the structural framework for organizing chromatin; furthermore, this subnuclear structure plays a pivotal role in the spatial and temporal coordination of gene activation events [41]. Actually, several studies from different laboratories have identified some NM proteins whose expression is significantly related to the occurrence of different tumors and recently some of these tumor-associated proteins were employed in clinical diagnosis or in preclinical studies [12]. Getzenberg and coworkers [18] reported the first evidence that NM composition is able to differentiate bladder cancer tissue from normal bladder tissue. Interestingly, some NM proteins were detected in the urine and blood and indeed non-invasive urine-based tests were developed for the diagnosis of bladder cancer [30]. For this reason, the proteinaceous components of the NM are good candidates to become new biomarkers with potential prognostic and therapeutic value. We have recently shown that the development of human prostate cancer is characterized by an increase in the complexity of the protein pattern of the NM [1,2]; moreover, a few newly expressed proteins were significantly correlated with the risk of biochemical progression [5].

Here we report the proteomic analysis of the NM isolated from healthy, non-tumoral and tumoral bladder tissues; the major proteins that are differentially expressed were identified. Additionally, we have analyzed whether these epigenetic alterations are correlated with clinicopathological characteristics and with follow-up of the patients in order to determine their possible prognostic role.

2. Materials and methods

2.1. Patients and samples

Studies were performed on TCC bladder tumor tissue specimens obtained from 21 patients who had undergone radical cystectomy. Nine samples of adjacent, non-tumoral (NT) tissue were also analyzed. The main characteristics of the patients studied are summarized in Table 1. Healthy urothelium (HU) specimens were collected from three unaffected individuals. All patients gave informed consent. Fresh tissues were immediately frozen in liquid nitrogen until sample preparation. All tissues were histologically examined and diagnosis was confirmed by hematoxylin and eosin stain. The tumors were classified according to the TNM system [38].

Table 1
Patient demographics and tumor characteristics

Characteristics	n (%)
Median age (range), years	72.0 (54.0–84.0)
Gender	
Male	17 (81.0)
Female	4 (19.0)
Stage	
pT2	4 (19.0)
pT3a	4 (19.0)
pT3b	6 (28.6)
pT4	7 (33.4)
Involved nodes	
pN0	11 (52.4)
pN1	2 (9.5)
pN2	4 (19.0)
pNx	4 (19.0)
Grade	
2	7 (33.4)
2–3	4 (19.0)
3	10 (47.6)
Tumor multicentric	
Absent	20 (95.2)
Present	1 (4.8)
Lymphatic invasion	
Absent	2 (9.5)
Present	6 (28.6)
Undetermined	13 (61.9)
Vascular invasion	
Absent	16 (76.2)
Present	5 (23.8)
Perineural invasion	
Absent	9 (42.7)
Present	11 (52.5)
Undetermined	1 (4.8)
Carcinoma *in situ*	
Absent	1 (4.8)
Present	18 (85.7)
Undetermined	2 (9.5)

2.2. Isolation of the NM

The NM was isolated according to Barboro et al. [3], with minor modifications. Tissue specimen of 2–4 g were minced into 1 mm^3 pieces, homogenized in 10 ml of 75 mM NaCl and 24 mM Na_2EDTA pH 7.8, using a potter with Teflon pestle and sieved through a 230 mesh stainless steel grid to trap the fibrous elements of the tissue. This procedure removes muscle and stromal aggregates and yields an almost homogeneous preparation of urothelial cells as judged from observations with a light microscope equipped with phase-contrast optics [2]. Nuclei were then isolated on a sucrose cushion. The nuclear pellet was resuspended in digestion buffer consisting of 10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.8) (all from Sigma, St. Louis, MO) and 2 mM vanadyl ribonucleoside complex (BioLabs, New England), to prevent the activation of endogenous RNase, and digested for 1 h at 25°C with 1,000 U/ml RNase-free DNase (Calbiochem, Darmstadt, Germany). Chromatin fragments were extracted by adding $(NH_4)_2SO_4$ to a final concentration of 0.25 mM. The NM was recovered by centrifugation at 6,500 $\times$ g for 15 min and extracted again with a large excess of digestion buffer containing 0.25 M $(NH_4)_2SO_4$.

2.3. High resolution two dimensional polyacrilamide gel electrophoresis (2D-PAGE)

NM samples isolated from bladder tissues were reduced, alkylated and separated from nucleic acids as described by Candiano et al. [8]. Protein concentration was determined using the Bio-Rad protein microassay with bovine serum albumin as a standard. The NM proteins extracted per g of tissue were 116 $\pm$ 23 μg (mean $\pm$ SEM) for HT and NT and 527 $\pm$ 144 μg for TCC.

Immobiline Dry-Strips (Amersham Biosciences) (pH 3.0–10.0 NL, 18 cm) were rehydrated for 18 h. The protein samples (120 μg) were loaded at the anode and isoelectric focusing was run for 110 kVh at 18°C, using a Pharmacia Multiphor II system. Eight strips were processed in parallel; at least one HT sample and one NT sample were always present. After isoelectric focusing, the strip gels were equilibrated with a solution containing 50 mM Tris-HCl (pH 8.3), 6 M urea, 4% SDS, 30% glycerol and 1% dithiothreitol (all from Sigma) for 15 min twice and once again in the same buffer containing 2.6% iodoacetamide instead of dithiothreitol and 0.4% bromophenol blue (BioRad, München, Germany). The equilibrated strips were embedded in 1% agarose containing 0.8% bromophenol blue on top of a stacking gel and separated in the second dimension according to their molecular mass using 8–14% linear gradient sodium SDS-PAGE at 40 mA for 5 h in a 13 $\times$ 16 cm gel system (BioRad); four gels were run in parallel. The gels were silver stained [20] for protein patterns analysis or processed for mass spectrometry (MS) analysis. 2D-PAGE was performed from two up to four times for each sample to ensure reproducibility. For one sample of TCC was not possible duplicate gels because an insufficient amount of NM was extracted.

2.4. Image analysis of 2D-PAGE gel spot patterns

All of the silver-stained 2D gels were digitized with a GS-800 densitometer (BioRad) using the same scanning conditions. Spot detection and gel alignment were performed using the software package PDQuest (Ver. 7.3.0, BioRad). 2D gels were grouped in four sets according to type of tissue: HU, NT and TCC at lower (pT2–pT3a) and higher (pT3b–pT4) stage. The gels were matched and for each matched set of gels, a single master gel, which represented the best pattern of spots in the samples, was generated by the software. Few additional spots consistently present in some gels were also added to each master gels representing the sample tissue. The analyses were performed, compared the four master gels thus obtained, in higher-level MatchSets according to PDQuest. The master gel corresponding to TCC at higher stage (pT3b–pT4) were used as the reference. The total density in a gel image was used to normalize each spot volume.

2.5. Protein identification

Protein identification was carried out using both MS and 2D gel Western blotting (WB). For MS analysis, unfixed gels were stained using a sensitive colloidal Coomassie Blue G-250 stain, spots of interest were excised and subjected to in-gel trypsin digestion [8]. Analysis of the resulting peptide mixtures was performed by LCQ-DECA MS/MS ion trap mass spectrometer coupled to an HPLC Surveyor (Thermo Finnigan) and equipped with a 1 $\times$ 150 mm column, Vydac C_{18}, 5 μm, 300 Å (Dionex) as described [6]. Computer analysis of peptide MS/MS spectra was performed using the TurboSEQUEST software version 1.2 (University of Washington, licensed to ThermoFinnigan Corp.) and searched against the National

Table 2
Specificity of the antibodies used in this study

	Host	Clone	Source	Dilution
Primary antibodies				
Lamin A/C	Goat	N-18	Santa Cruz Biotechnology	1:200
Lamin B	Goat	C-20	Santa Cruz Biotechnology	1:350
Vimentin	Mouse	Vim 3B4	Boehringer Mannheim	1:600
Desmin	Mouse	D33	DAKO	1:300
Actin	Mouse	AC-40	Sigma	1:400
Intermediate filaments	Mouse	IFA	Hybridoma TIB 131 cell line (American Type Culture Collection, Manassas, VA)	1:1,500
$p54^{nrb}$	Mouse	$3/p54^{nrb}$	BD Transduction Laboratories	1:2,000
Secondary antibodies peroxidase-conjugated				
Anti-goat	Donkey		Santa Cruz Biotechnology	1:1,000
Anti-mouse	Goat		DAKO	1:1,000

Center for Biotechnology Information (NCBI) human protein database.

For 2D WB, proteins were transferred to a Hybond-P membrane (Amersham Biosciences) and immunodetection was carried out as already described [2] using the antibodies reported in Table 2.

2.6. Statistical analysis

Associations among the variables in the study were investigated by using Pearson-correlation [16]. Overall survival was defined as the time from radical cistectomy to death. Curves for protein $p54^{nrb}$ were constructed using Kaplan-Meier method and compared with log-rank test [26]. The analyses were performed using SPSS version 14.0.1 for Windows. The comparison of the relative amounts of NM proteins identified in HT and NT vs. TCC was performed using t-test within the OriginPro 7.5 software exporting the single value of each gel. All of the P-values reported are two sided, $P \leqslant 0.05$ was considered statistically significant.

3. Results

3.1. Proteome of NM isolated from bladder tissues

NM proteins isolated from HU, NT and TCC tissues were analyzed by 2D-PAGE. Representative silver-stained gel patterns are reported in Fig. 1. The overall expression profiles are rather similar, but tumor cells were characterized by a more complex NM protein pattern when compared to HU and NT. On average 770 protein spots were detected in NM from HU (range 613–880) and 901 (range 679–988) from NT. This number increased with tumor stage: 1172 (range 879–2196) from pT2–pT3a and 1266 (range 850–2051) from pT3b–pT4, respectively. 191 spots occur in 100% HU, 170 in 100% NT, 150 in 100% TCC (pT2–pT3a) and 133 in 100% TCC (pT3b–pT4), respectively. 103 spots are detected across all gels. When we matched the proteins that occur in 100% of HU, with those present in almost 50% of NT and 50% of TCC we observed that 15 spots were detected in HU but not in NT or TCC, 34 in NT but not in HU or TCC and 108 in TCC but not in HU or NT. Of the last ones, only 14 were expressed in 100% of TCC tissue specimens. These observations indicate an elevated tumor heterogeneity that in turn represents a major engine of tumor progression [29] and suggests that the stages of tumor development could hardly be characterized using a single marker protein.

3.2. Identification of bladder cancer-associated NM proteins

MS and WB analyses were used to identify the spots that show major qualitative and quantitative alterations, among different tissues. We selected 85 of such spots for identification by MS. Out of the 85 protein spots picked and processed, 43 (51%) were unambiguously assigned identities, including redundancies due to proteolysis and post-translational modifications; about 40% of these were identified also by 2D immunoblot analysis. The majority of the proteins that were picked, but could not be identified, correspond

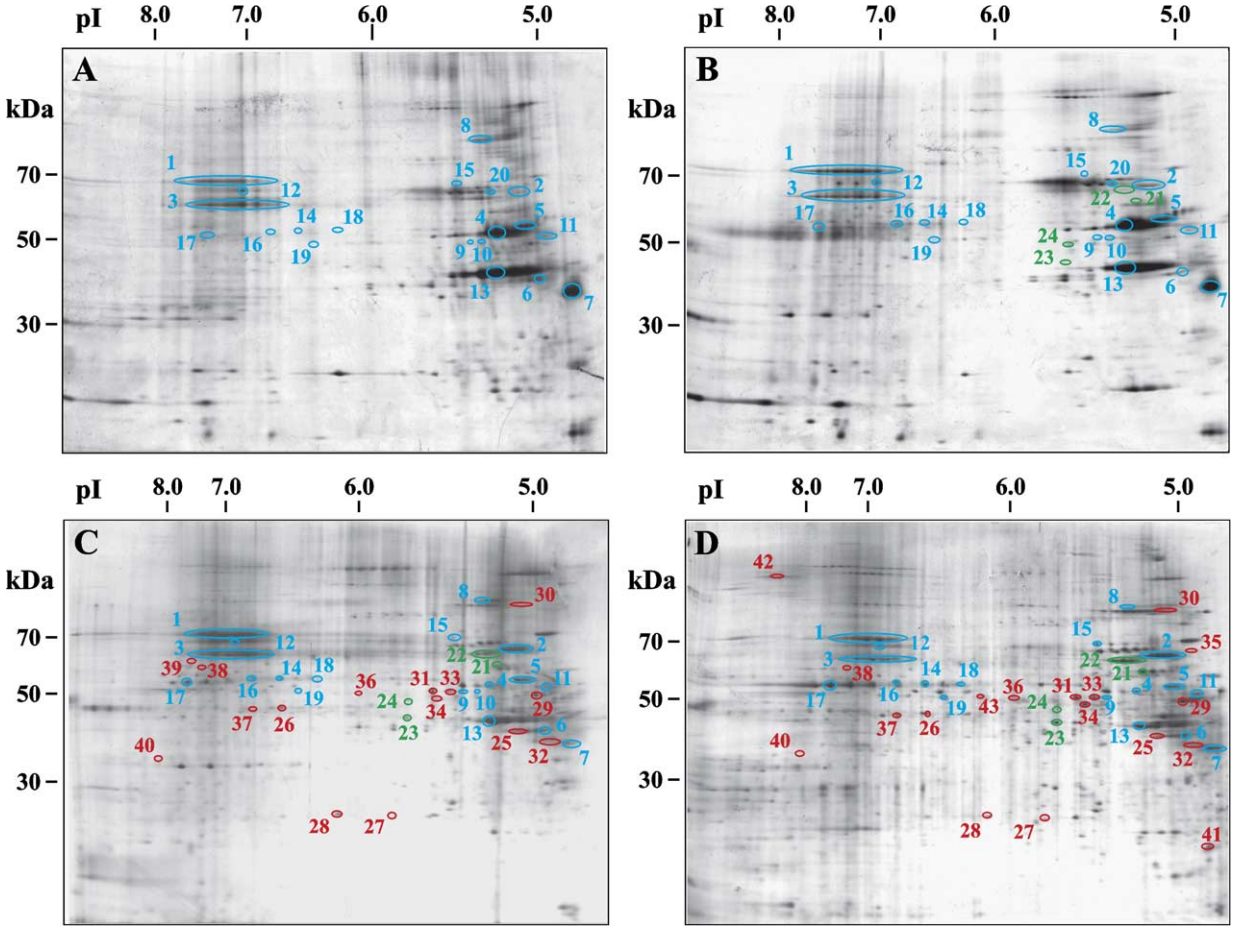


Fig. 1. Representative 2D silver-stained gel maps of the NM proteins extracted from different bladder tissues. (A) HU, (B) NT, (C) and (D) TCC at different clinical stages (pT2 and pT4, respectively). The 43 proteins identified are highlighted in circles and are marked with the same number used in Tables. The color denotes the tissue in which the spots have been detected first (blue HU, green NT, red TCC).

to low abundant spots occurring at levels below detection limit of MS. The proteins identified are shown in the representative sample set in Fig. 1. Their characteristics (molecular mass and pI), function and frequency in the different tissues are reported in Tables 3 and 4; most of detected proteins had high sequence coverage. As expected, the proteins identified include structural and heat shock proteins, enzymes and proteins of the RNA machinery. The presence of intermediate filaments and of their fragments is in line with evidence suggesting that these proteins are stably and tightly associated to the NM [37,40].

Based on the differences in the expression level in the tissues studied, the 43 proteins identified can be divided into 5 patterns. Pattern A groups 19 proteins present in all the tissues examined, of which 13 were expressed at the same level and 6 were significantly deregulated in the TCC (Fig. 2). The up-regulated proteins were fibrinogen β chain (spot n°18) and lamin B (spot n°2) while the down-regulated proteins included actin, desmin, vimentin and a vimentin fragment. Pattern B includes only one protein (serum albumin) that was identified in HU and NT specimens but that was absent in tumor tissues. Pattern C groups four proteins observed both in NT and TCC but not in HU tissue; one of these proteins is a basic isoform of lamin B (spot n°22) and is present in 100% of the pathological tissues (NT and TCC). Patterns D and E include 19 proteins detected in bladder tumor tissues but not in HU and NT tissues; pattern D groups 16 proteins found in all cancer tissues, irrespective of tumor stages, while pattern E is present in the samples obtained from cancers with a higher stage (pT3b–pT4).

The relationship between any individual protein and clinicopathological variables were analyzed; only the

Table 3
Proteins identified by MS and WB

Spot no.*	Protein name	Accession number	Function	Method of identification	Score	Peptides matched	Sequence cov. (%)	Observed		Calculated MW (kDa)
								pI	MW† (kDa)	
				Pattern A						
1	Lamin A	gi: 224901	Structural	MS, WB	428.3	27	46	7.5–6.7	77.8	74
2	Lamin B1	gi: 15126742	Structural	MS, WB	368.3	21	35	5.1	66.4	66.3
3	Lamin C	gi: 307108	Structural	WB	n.d.‡	n.d.	n.d.	7.2–6.7	64.8	n.d.
4	Desmin (mutant)	gi: 11907570	Structural	MS, WB	170	15	43	5.2	52.1	53
5	Vimentin	gi: 47115317	Structural	MS, WB	1180	33	57	5.1	53.6	57
6	Cytokeratin 19	gi: 45709960	Structural	MS, WB	180	17	49	5.0	39.5	44
7	Vimentin (fragment)	gi: 47115317	Structural	MS	248	15	32	n.d.	36.5	49.6
8	Actinin α 1	gi: 30585329	Structural	MS	156	12	25	5.3	103.2	103
9	Cytokeratin 7	gi: 20178293	Structural	MS, WB	258.3	19	42	5.4	49.8	51.2
10	Cytokeratin 7	gi: 20178293	Structural	MS, WB	108.3	8	19	5.3	49.4	51.2
11	Tubulin β-1 chain	gi: 135448	Structural	MS	350.3	16	50	4.9	51.1	50.3
12	Lamin A (fragment)	gi: 224901	Structural	MS, WB	526	23	27	6.9	72.7	79
13	Actin β	gi: 14250401	Structural	MS, WB	122	6	35	5.2	42.5	41.8
14	Cytokeratin 5 (fragment)	gi: 9739163	Structural	MS, WB	90.4	4	9	6.1	54.4	62.4
15	Heat shock 70 kDa protein 9 (HSP70)	gi: 21040386	Heat shock protein	MS	226.3	20	37	5.4	73.8	71
16	Fibrinogen β chain preproprotein	gi: 11761631	Plasma protein	MS	30.3	3	8	6.6	54.1	55.9
17	Fibrinogen β chain precursor	gi: 399492	Plasma protein	MS	316	12	48	7.7	54.1	56
18	Fibrinogen β chain	gi: 7924018	Plasma protein	MS	280.3	14	30	6.1	54.4	23.9
19	Fibrinogen β chain	gi: 7924018	Plasma protein	MS	100.3	10	24	6.1	49.1	23.9
				Pattern B						
20	Serum albumin	gi: 23307793	Plasma protein	MS	124	8	16	5.2	69.0	69
				Pattern C						
21	Heat shock 60 kDa protein 1 (HSP60)	gi: 31542947	Heat shock protein	MS	288	14	36	5.2	59.5	55
22	Lamin B1	gi: 30583721	Structural	MS, WB	332	18	31	5.3	64.2	67.7
23	Lamin A/C (fragment)	gi: 21619981	Structural	MS	164	12	18	5.7	42.8	53
24	Fibrinogen β chain precursor	gi: 399492	Structural	MS	316	15	53	5.7	47.7	37
				Pattern D						
25	Cytokeratin 19	gi: 7594732	Structural	MS, WB	192	23	62	5.1	39.3	44
26	Nuclear matrix protein 265 (hNMP 265)	gi: 20532400	mRNA processing	MS	50	5	12	6.1	46.8	46.8
27	Heat shock 27 kDa protein 1 (HSP27)	gi: 15126735	Heat shock protein	MS	70	5	31	5.7	22.8	23
28	Heat shock 27 kDa protein 1 (HSP27)	gi: 15126735	Heat shock protein	MS	75	7	41	6.0	22.8	23

Table 3
(Continued)

Spot no.*	Protein name	Accession number	Function	Method of identification	Score	Peptides matched	Sequence cov. (%)	Observed		Calculated MW (kDa)
								pI	MW† (kDa)	
29	ATP synthase, H+ transporting, F1 complex, β mitochondrial subunit precursor	gi: 32189394	Enzyme	MS	220.3	13	37	5.0	48.9	51.9
30	Fibrinogen γ chain, isoform γ-B precursor	gi: 14488402	Plasma protein	MS	404.3	17	32	5.1	99.1	51.5
31	Cytokeratin 8	gi: 39645331	Structural	MS, WB	70	4	10	5.6	49.9	53
32	Cytokeratin 1 (fragment)	gi: 1346343	Structural	MS	102.4	8	15	n.d.	37.4	65.8
33	Cytokeratin 1 (fragment)	gi: 1346343	Structural	MS, WB	134.4	11	18	5.5	50.0	53
34	Cytokeratin 20	gi: 542923	Structural	MS, WB	70	1	3	5.5	48.4	48.5
35	Lamin B1	gi: 15126742	Structural	MS, WB	78.3	10	19	4.9	67.0	66
36	Heterogeneous nuclear ribonucleoprotein H′ (hnRNP H′)	gi: 9845253	mRNA processing	MS	72	2	6	6.0	49.3	49.2
37	Elongation factor Tu, mitochondrial precursor (EF-Tu)	gi: 1706611	mRNA translation	MS	180.3	12	35	6.6	46.1	49.5
38	p54^{nrb}	gi: 2808511	mRNA processing	MS, WB	30.3	3	9	7.39	61.0	54
39	p54^{nrb}	gi: 2808511	mRNA processing	MS, WB	30.3	2	7	7.6	63.3	54
40	Glyceraldehyde-3-phosphate dehydrogenase	gi: 30584593	Enzyme	MS	172.4	14	54	8.6	36.2	36
			Pattern E							
41	Heterochromatin protein-1 β (HP1 β)	gi: 12803555	Chromatin structure	MS	84.3	5	29	4.9	21.0	21
42	Pro α 1 (I) collagen	gi: 4755085	Structural	MS	30.3	2	9	8.5	138.0	138
43	Fibrinogen β chain	gi: 7924018	Plasma protein protein	MS	74.3	7	22	6.0	50.2	23.9

*Spot no. is the same number used in Fig. 1 to identify a 2D gel spot; †Molecular weight; ‡Not determined.

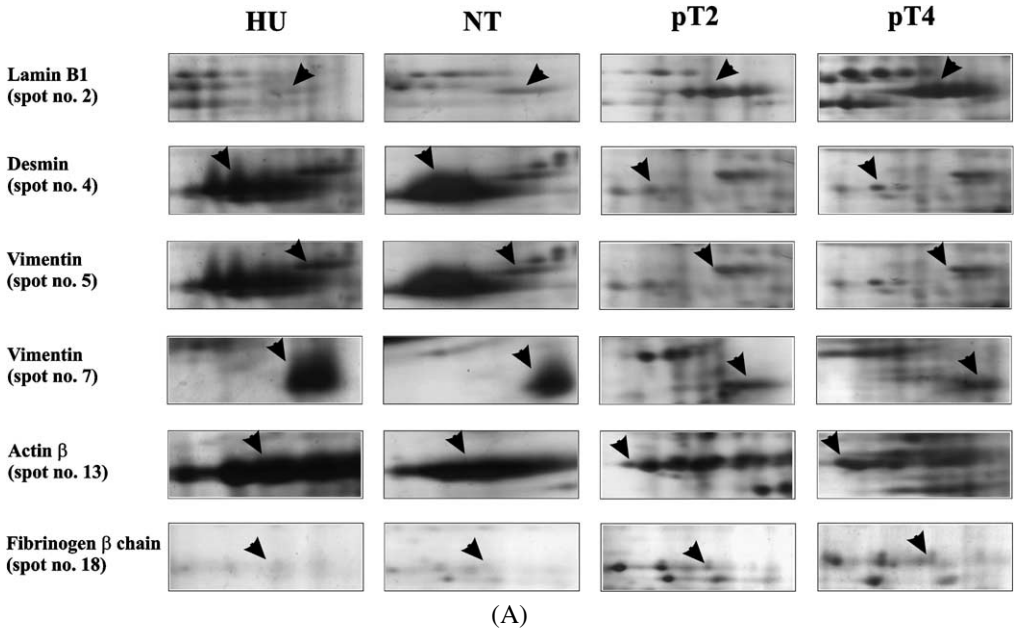
Table 4
Frequency of expression of identified proteins in normal, non tumoral and tumoral tissues at different stages

Spot no.*	Protein name	Accession number	Frequency (%)†			
			HU $n = 3$	NT $n = 9$	pT2-3a $n = 8$	pT3b-4 $n = 13$
		Pattern A				
1	Lamin A	gi: 224901	100	100	100	100
2	Lamin B1	gi: 15126742	100	100	100	100
3	Lamin C	gi: 307108	100	100	100	100
4	Desmin (mutant)	gi: 11907570	100	100	100	100
5	Vimentin	gi: 47115317	100	100	100	100
6	Cytokeratin 19	gi: 45709960	100	100	100	100
7	Vimentin (fragment)	gi: 47115317	100	100	75	62
8	Actinin α 1	gi: 30585329	100	100	88	69
9	Cytokeratin 7	gi: 20178293	100	100	50	54

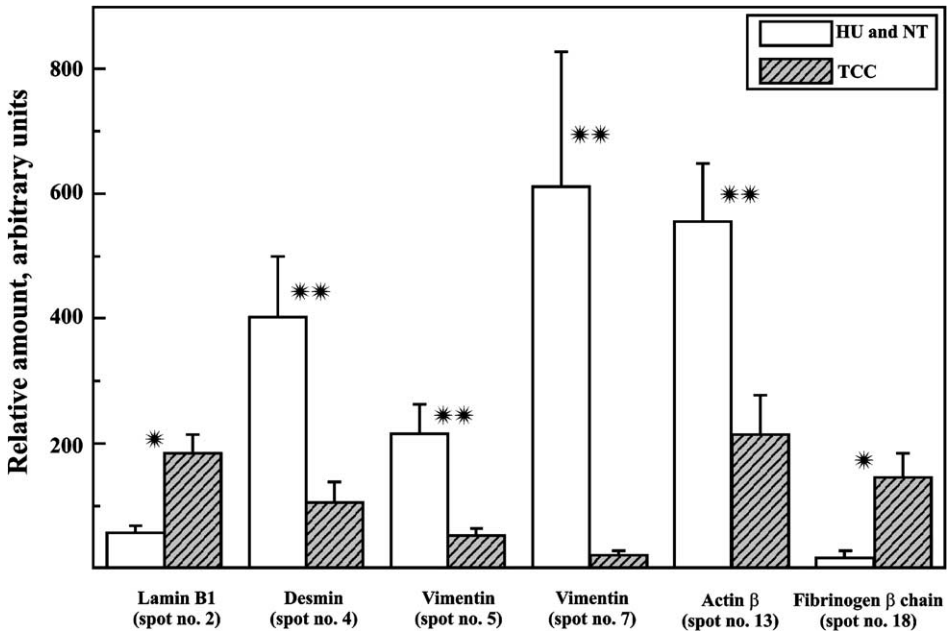
Table 4
(Continued)

Spot no.*	Protein name	Accession number	Frequency (%)†			
			HU	NT	pT2-3a	pT3b-4
			$n = 3$	$n = 9$	$n = 8$	$n = 13$
10	Cytokeratin 7	gi: 20178293	100	100	63	31
11	Tubulin β-1 chain	gi: 135448	100	67	88	77
12	Lamin A (fragment)	gi: 224901	100	67	100	92
13	Actin β	gi: 14250401	67	100	100	100
14	Cytokeratin 5 (fragment)	gi: 9739163	67	67	100	100
15	Heat shock 70 kDa protein 9 (HSP70)	gi: 21040386	100	50	88	92
16	Fibrinogen β chain preproprotein	gi: 11761631	67	100	100	92
17	Fibrinogen β chain precursor	gi: 399492	67	50	100	100
18	Fibrinogen β chain	gi: 7924018	67	100	88	85
19	Fibrinogen β chain	gi: 7924018	67	100	50	85
	Pattern B					
20	Serum albumin	gi: 23307793	67	83	0	0
	Pattern C					
21	Heat shock 60 kDa protein 1 (HSP60)	gi: 31542947	0	50	75	100
22	Lamin B1	gi: 30583721	0	100	100	100
23	Lamin A/C (fragment)	gi: 21619981	0	67	63	92
24	Fibrinogen β chain precursor	gi: 399492	0	33	38	92
	Pattern D					
25	Cytokeratin 19	gi: 7594732	0	0	100	100
26	Nuclear matrix protein 265 (hNMP 265)	gi: 20532400	0	0	100	100
27	Heat shock 27 kDa protein 1 (HSP27)	gi: 15126735	0	0	38	100
28	Heat shock 27 kDa protein 1 (HSP27)	gi: 15126735	0	0	38	100
29	ATP synthase, H+ transporting, mitochondrial F1 complex, β subunit precursor	gi: 32189394	0	0	75	100
30	Fibrinogen γ chain, isoform γ-B precursor	gi: 14488402	0	0	88	69
31	Cytokeratin 8	gi: 39645331	0	0	88	77
32	Cytokeratin 1 (fragment)	gi: 1346343	0	0	88	85
33	Cytokeratin 1 (fragment)	gi: 1346343	0	0	63	77
34	Cytokeratin 20	gi: 542923	0	0	50	92
35	Lamin B1	gi: 15126742	0	0	25	23
36	Heterogeneous nuclear ribonucleoprotein H′ (hnRNP H′)	gi: 9845253	0	0	38	100
37	Elongation factor Tu, mitochondrial precursor (EF-Tu)	gi: 1706611	0	0	75	85
38	$p54^{nrb}$	gi: 2808511	0	0	75	100
39	$p54^{nrb}$	gi: 2808511	0	0	25	54
40	Glyceraldehyde-3-phosphate dehydrogenase	gi. 30584593	0	0	50	54
	Pattern E					
41	Heterochromatin protein-1 β (HP1 β)	gi: 12803555	0	0	0	100
42	Pro α 1 (I) collagen	gi: 4755085	0	0	0	85
43	Fibrinogen β chain	gi: 7924018	0	0	0	54

*Spot no. is the same number used in Fig. 1 to identify a 2D gel spot; †Frequency is the number of positive samples/number of samples examined.



(A)



(B)

Fig. 2. Identified NM proteins grouped in pattern A that exhibit a significant deregulation in TCC. (A) Magnified sections of 2D silver-stained gel maps reported in Fig.1; (B) comparison of the relative amounts. The ordinates represent the mean ± standard error (SEM). $^{*}P < 0.01$, $^{**}P < 0.005$.

proteins correlating at a P level of 0.05 or less ($P \leqslant 0.05$) with at least one parameter studied are reported in Table 5. Among the 43 identified proteins seven correlated with tumor stage and one with vascular invasion.

3.3. Prognostic value of identified proteins

We focused on the 19 proteins present only in bladder tumor tissues. In particular we focused on p54^{nrb} protein (spot n°39) because this protein was not ex-

Table 5
Correlation between identified proteins and clinicopathological variables

Spot no.*	Protein name	Stage	Grade	Involved nodes	Vascular invasion	Perineural invasion
27	HSP27					
	Pearson corr.	0.713	0.025	−0.139	0.050	0.135
	P	0.0005	0.9	0.5	0.8	0.6
28	HSP27					
	Pearson corr.	0.713	−0.295	−0.362	0.050	0.129
	P	0.0005	0.2	0.1	0.8	0.6
34	Cytokeratin 20					
	Pearson corr.	0.482	0.025	0.085	0.313	0.141
	P	0.03	0.9	0.7	0.2	0.5
36	hnRNP H′					
	Pearson corr.	0.713	0.025	0.085	0.050	0.129
	P	0.0005	0.9	0.7	0.8	0.6
39	$p54^{nrb}$					
	Pearson corr.	0.283	−0.131	0.138	0.645	0.261
	P	0.2	0.6	0.5	0.002	0.2
41	HP1 β					
	Pearson corr.	1	−0.339	−0.037	0.208	0.183
	P	0.0005	0.1	0.9	0.4	0.4
42	Pro α 1 (I) collagen					
	Pearson corr.	0.823	−0.139	−0.045	0.085	0.216
	P	0.0005	0.5	0.8	0.7	0.3
43	Fibrinogen β chain					
	Pearson corr.	0.615	−0.036	−0.159	0.022	−0.172
	P	0.003	0.9	0.5	0.9	0.5

*Spot no. is the same number used in Fig.1 to identify a 2D gel spot.

pressed at all in the tumor specimens of the 8 patients who at the time of the present analysis (median follow-up time: 41.5 months; 95% CI 38.5–45.0) were still alive, while it was expressed in tumor specimens of 9 of the 13 patients who had died. This $p54^{nrb}$ expression, beyond being strongly correlated with vascular invasion (Table 5) appeared to be also a strong predictor of patients survival following cistectomy ($P < 0.0001$) (Fig. 3).

4. Discussion

The prognosis of patients who have undergone radical cystectomy for muscle-invasive TCC is very heterogeneous; about 50% of the cases will develop metastatic disease. Therefore, it is important to identify patients at higher risk and select them for adjuvant therapy. As in other solid tumors, progression in bladder cancer seems to be a multifactorial process that involves genetic and epigenetic modifications. Several strategies for identifying phenotypic changes in the bladder cancer are being used and one of the most promising is the proteomic approach [10,15]. We focused on a molecular analysis of NM changes associated with tumor development in muscle invasive TCC, since it is known that the NM is involved in the epigenetic modulation of tumor progression [12]; besides two NM proteins, Nuclear Matrix Protein 22 (NMP22) and Bladder Cancer Antigen 4 (BLCA4), are used or have a potential use as clinical biomarkers [30].

We have identified 43 NM proteins and out of the 19 present in all tissues, 13 of these occur at the same level of expression and 6 down- or up-regulated. Although this group of proteins (pattern A) can provide relevant information on the molecular mechanisms of cancer development, they have no practical diagnostic utility and are not examined in this discussion. We will only examine the nature of more significant qualitative changes.

Four proteins, the basic isoform of lamin B1, HSP60, the low molecular weight fragment of lamin A and human fibrinogen β chain precursor, were absent in HU but present in NT and TCC (pattern C);

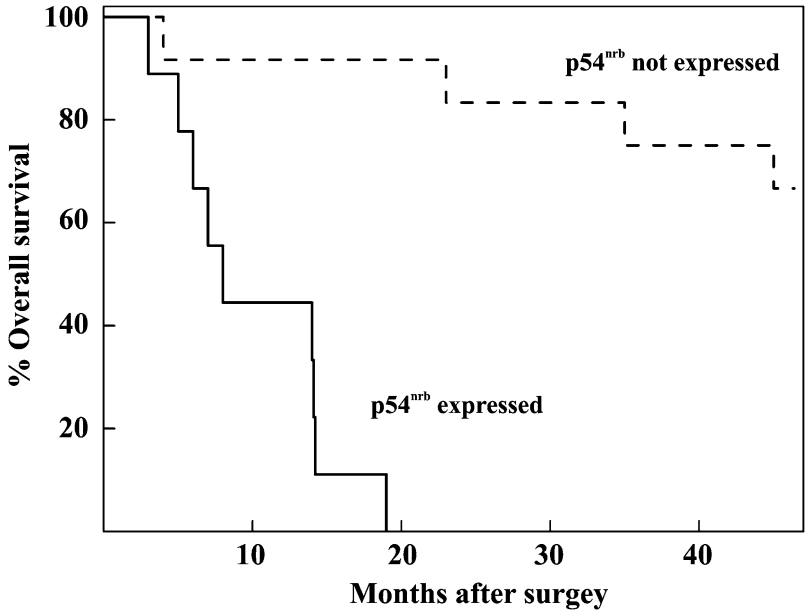


Fig. 3. Kaplan–Meier curves for overall survival according to $p54^{nrb}$ expression in the whole group of patients ($n = 21$) with muscle invasive TCC ($P < 0.0001$).

additionally, the first one was present in 100% of specimens. This observation indicates that the earliest preneoplastic changes might be detected in morphologically normal areas in patients with bladder cancer. This confirms previous results by Getzenberg and coworkers [7,27] who proposed the hypothesis that normal-appearing tissues adjacent to tumors have already undergone specific changes in NM proteins. In a recent study, we reported that, in addition to the nuclear lamina, lamins form an intranuclear web [3] that is expected to be a highly dynamic structure involved both in the transition between eu and heterochromatin and in RNA synthesis and processing. Moreover, in cancerous prostate tissues, the expression of lamin B changes in its isoform distribution and undergoes a significant increase that is correlated to the level of differentiation [13].

Pattern D identified the proteins neo-expressed in tumor tissues and among these several cytokeratins are present: CK8, CK20, CK1 fragments and a basic isoform of CK19. The correlation between the over expression of a particular type of CK and bladder cancer is well documented and several studies have shown that cytokeratins and their degradation products may be a useful marker in urothelial bladder cancer. In this study, we found that a basic isoform of CK19 was present only in tumor tissues and the expression of CK20 was correlated with tumor stage (Table 5). These results are in line with the findings that CK19 and its soluble fragments (CYFRA 21) are strongly associated with malignant bladder phenotype [31] and that CK20 expression depends upon the degree of differentiation [11].

Among the neo-expressed proteins found in this study, hNMP265 was present in 100% of the tumor specimens examined. This protein is a NM protein homologous to eukaryotic translation initiation factor 4A and could be involved in processing of preribosomal RNA [21]. No data are available about the role of this protein in the cancerogenesis process and in bladder carcinomas. More interestingly, three proteins grouped in pattern D also correlated with stage: two isoforms of HSP27 and hnRNP H′. HSP27 is constitutively expressed at low levels in the cytosol and after induction undergoes rapid post-translation phosphorylation and moves from the cytoplasm to the nucleus. Phosphorylated isoforms of HSP27 may be a general molecular marker of tumor cells and in many types of cancer, HSP27 overexpression has a strong prognostic value [17]. In bladder carcinoma, the role of HSPs is controversial depending on the stage of the tumors analyzed. Storm et al. [39] previously found that in 24 bladder cancer specimens from patients who had undergone radical cystectomy, 50% overexpressed HSP27 but expression did not correlate with clinicopathological characteristics. More recently, Lebret et al. [28] found a strong correlation between the loss of HSP27 and disease stage. Finally, Celis et al.

[9], using proteomics and immunohistochemistry techniques, showed that HSP28 (also denoted HSP27) is up-regulated in 19 out of 24 of invasive TCC. Taken together, these findings support the view that HSP27 expression in bladder cancer is heterogeneous depending on tumor stage. hnRNP H′ is one member of the hnRNP large family. hnRNP proteins are among the most abundant components of the NM and are involved in the organization of the internal NM along with RNA and lamins [3]. hnRNPs have been found associated with actively proliferating cells [34] and in prostate carcinoma work carried out in our laboratory provided evidence that hnRNP K is significantly correlated with the risk of biochemical progression [4,5]. In particular, the proteins belonging to the hnRNPs H group have been implicated in several steps of pre-mRNA processing and are up-regulated in a few cancer tissues [23].

The proteins grouped in pattern E are HP1 β, pro α 1 (I) collagen and fibrinogen β; the first is present in 100% of the tumors at higher stages. HP1 β is one of three human HP1 family members; they are highly conserved proteins that regulate chromatin organization. Improper expression of HP1 has been associated with tumor progression: HP1 α is down-regulated in breast cancer cells with an invasive metastatic phenotype [25] but is increased in all cases of acute myeloid leukaemia [35]. Variation in HP1 expression in different tumors can be attributed to differences in the role of this protein in different tissues; De Lucia et al. [14] reported that HP1 can act as a transcriptional repressor in heterochromation, while it can act as positive transcriptional regulator in euchromatin. Here we show a clear association between over expression of HP1 β and tumor stage in bladder cancers; it has yet to be established whether up-regulation of HP1 β is an intrinsic feature of tumor progression or merely is a consequence of the cellular dedifferentiation.

Finally, we have shown, for the first time, that p54^{nrb} expression was strongly correlated with patient mortality. p54^{nrb} is a nuclear RNA- and DNA-binding protein implicated in transcription and splicing but whose specific functions are not known though the strong correlation found by us between the expression of this proteins and vascular invasion might be intriguing in this regard. It has been reported that loss or alteration of this protein in human breast cancer may contribute to tumor growth and progression [32]. The overexpression of p54^{nrb} could characterize a subgroup of TCC with higher risk of recurrence, and might helps to select patients who might benefit from adjuvant therapies. Work is in progress in our laboratory to test the prognostic value of this biomarker with respect to the other clinicopathological variables though multiparametric models including larger number of patients. Further studies are also needed to clarify its biological role in bladder cancer tissue.

5. Concluding remarks

Proteomic analysis is the most useful method to screen candidates for panel markers. Using this technology, we have identified 19 NM proteins that are newly expressed in muscle-invasive bladder cancer; seven of these proteins correlated with clinicopathological variables and one of them, proteins p54^{nrb}, also with survival. We feel that despite the relatively small size of this cohort, our data are sufficient to reach the preliminary but sound conclusion that these proteins might represent biomarkers potentially useful for disease diagnosis and prognosis. The identified proteins might also represent new therapeutic targets.

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