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ORIGINAL ARTICLE

Expression profile of Rho kinases in urinary bladder cancer

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Summary

Purpose: *To investigate the expression of RhoA, RhoB, RhoC, Rac1 and Cdc42 kinases in urothelial cell carcinoma (UCC) of the urinary bladder and determine the expression profile of 107 Rho-associated genes, including GTPases, GDIs, GAPs and GEFs.*

Methods: *Rho expression was investigated using microarrays, qPCR and Western blotting in 77 UCC specimens with paired normal urothelium. Computational analysis was also performed on Gene Expression Omnibus datasets. Further microarray analysis was carried out for the expression profiling of the Rho-associated genes.*

Results: *RhoB mRNA and protein levels were significantly lower in UCC, suggesting a tumour-suppressor role. On the contrary, mRNA of RhoC and protein levels of RhoA, RhoC and Cdc42, respectively, were significantly higher in UCC vs. normal tissue. High Cdc42 mRNA levels correlated with worse overall survival (p=0.027), whereas high RhoB mRNA levels correlated both with better overall (p=0.0258) and cancer-specific (p=0.0272) survival. Computational analysis verified the expression profile of Rho kinases among superficial UCCs, muscle-invasive UCCs and normal tissues.*

Conclusion: *The majority of the Rho-related genes showed over-expression in UCC vs. normal tissue. Alterations in RhoA, RhoB, RhoC, Rac1 and Cdc42 expression play a significant role in the genesis and progression of UCC of the urinary bladder.*

Key words: expression, profile, Rho kinases, urothelial cell carcinoma, urinary bladder

Introduction

Urinary bladder cancer (BC) is the most common malignancy of the urinary tract, responsible for significant mortality and morbidity worldwide [1]. Almost all BCs are UCCs. At presentation, 75-85% of tumours are restricted to the mucosa, or invade the lamina propria mucosae. Over 60% of the superficial tumours recur whereas 15% progress to less differentiated or invasive neoplasms. The most useful prognostic parameters are tumor grade, stage, size, prior recurrence rate and the presence of carcinoma *in situ* (CIS) [2].

Normal epithelial cells exhibit specific polarity and highly organized cell to cell junctions. Increased motility and stimulation of extracellular matrix remodeling are essential events for the oncogenic transformation of epithelial cells as well as for local invasion and metastasis [3]. Rho GTPases comprise a major subgroup of the Ras superfamily that mediate housekeeping aspects of cell biology. In particular, Rho GTPases play a fundamental role in the reorganization of actin and microtubule cytoskeleton [4]. Therefore, Rho proteins govern a variety of cytoskeleton-dependent processes including alterations in cell shape, polarity, adhesion, cell motility, as well as cell to cell and cell to matrix interactions. Furthermore, Rho proteins appear to be involved in gene transcription by activating SRF [5] and in the regulation of cell cycle progression, growth and apoptosis [6].

Rho members may affect the process of tumourigenesis either by over-expression of some members of the family with oncogenic activity or by down-modulation of other members with suggested tumour suppressor activity [7]. Since Rho GTPases regulate actomyosin-based contractility and extracellular matrix remodelling, these GTPases may be involved in the transmi-

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gration of cells, thereby facilitating local invasiveness and metastasis [8].

The present study investigated the mRNA and protein expression levels of the Rho family of GTPases, RhoA, RhoB, RhoC, Cdc42 and Rac1, in UCC of the urinary bladder and in non-neoplastic urothelium.

Methods

Patients and tumour samples

A total of 77 paired samples, consisting of tumour and normal urothelium, were obtained from 77 Greek patients with UCC of the urinary bladder and studied to determine the expression of RhoA, RhoB, RhoC, Cdc42 and Rac1. Written informed consent in accordance with the Institutional Committee for the Protection of Human Subjects was obtained from all patients. All patients were treated at the same Institution, between 2006 and 2010. Included were 68 males and 9 females; the mean age was 72.12 years (range 43-93). None of the patients had previously received systemic chemotherapy or external radiation therapy. Of the 77 patients, 56 had newly diagnosed BC and 21 had recurrent disease.

All tissue samples were immediately stored at -80° C until RNA extraction. Prior to RNA extraction an H&E slide was prepared for all tumour and normal urothelium specimens in order to verify the presence and absence of cancer cells, respectively. The histological grading was in accordance with the 1973 World Health Organization classification, while the pathological staging was performed according to the 2002 TNM Classification System. Forty-seven patients had non-muscle-invasive tumours (Tis, Ta and T1). The remaining had muscle-invasive disease (T2-T4a) and were treated with either radical cystectomy or radiotherapy, with or without systemic chemotherapy. Postoperative follow-up ranged from 6 to 48 months.

Microarray analysis

Oligo nucleotide microarray chips (~57k genes) were obtained from GE HealthCare (IL, USA) and Applied Microarrays (MA, USA). Hybridization was performed using the CodeLink RNA amplification and Labeling kit, with Cy5 fluorescent dye, as previously described [9-11]. The scanned images were processed with the CodeLink Expression Analysis Software v5.0 (Amersham Biosciences). Two-way (genes-against samples) average-linkage hierarchical clustering with Euclidian distance was applied and TreeView was used to visualize the results. Their expression profile was investigated in each BC sample vs. the mean expression of the 5 controls (Figure 1).

RNA extraction and reverse transcription

Tissue specimens were homogenized in TRIzol® reagent (Invitrogen, Carlsbad, CA) using a power homogenizer followed by chloroform addition and centrifugation. Total-RNA (2 μg) was reverse-transcribed using random primers with the "reverse transcription system" (Promega, Madison, WI) [12].

Real-time polymerase chain reaction (qPCR) analysis

Transcribed products were subjected to real-time PCR assay in an M×3000P programmable thermal controller apparatus (Stratagene, La Jolla, CA). For each target gene 1 μl of cDNA was amplified in a total volume of 20 μl containing GoTaq qPCR Master Mix (2×) (Promega, Madison, WI), CXR (ROX) as passive reference dye and 0.6 μM of each primer set. The housekeeping genes, *GAPDH* and *RPL13A,* were used as normalisers [13]. Primer sequences and their annealing conditions are listed in Table 1. The expression levels were calculated using the ΔΔCt method [14].

Total protein extraction and Western blot analysis

Following the addition of 250 μl ice-cold GST-Fish lysis buffer and a protease inhibitor cocktail the homogenised tissue samples were centrifuged at 14,000 g for 15 min at 4° C. The supernatant was then collected and stored at -80° C. Samples were dissolved in LDS sample buffer and heated at 70° C for 7 min. Thirty μg were electrophoresed in NuPAGE 4-12% Bis-Tris gel (Invitrogen, UK) and transferred to a nitrocellulose membrane. The membrane was immersed in 5% non-fat milk or BSA, 0.1% Tween 20 and dissolved in Tris-buffered saline to block the non-specific binding. The membranes were blotted with the following primary antibodies overnight at 4° C: mouse monoclonal anti-RhoA (diluted 1:1,000; Santa Cruz Biotechnology Inc., CA, USA), rabbit polyclonal anti-RhoB (diluted 1:500; Cell Signaling, Beverly, MA, USA), rabbit polyclonal anti-RhoC (diluted 1:500; Cell Signaling), mouse monoclonal anti-Cdc42 (diluted 1:500; BD Transduction Laboratories), and a mouse monoclonal anti-Rac1 (diluted 1:1,000; BD Transduction Laboratories). The membranes were then washed and incubated for 90 min at room temperature with a secondary antibody that included horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (diluted 1:1,500; Santa Cruz Biotechnology). Western blots were normalized using a monoclonal anti-β-actin

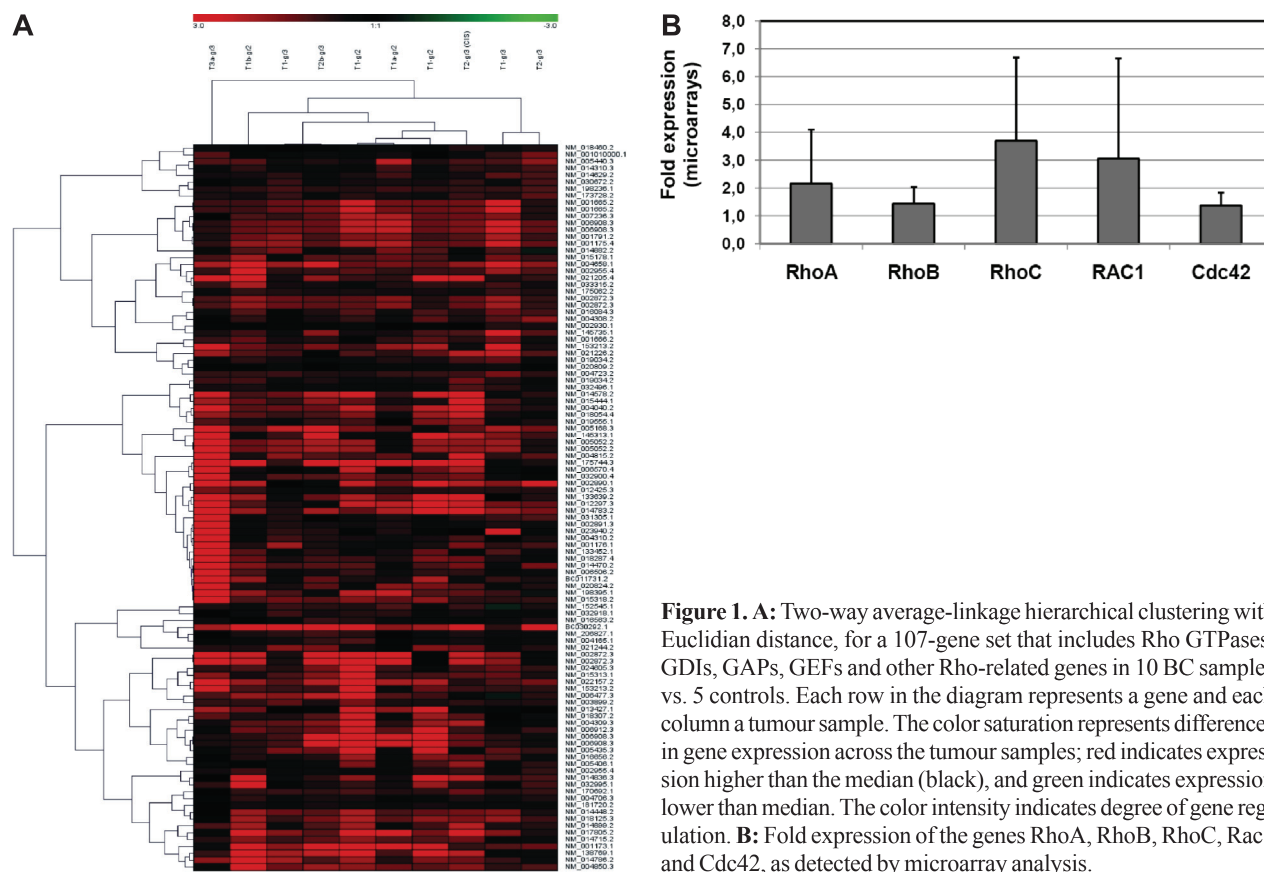


Figure 1. A: Two-way average-linkage hierarchical clustering with Euclidian distance, for a 107-gene set that includes Rho GTPases, GDIs, GAPs, GEFs and other Rho-related genes in 10 BC samples vs. 5 controls. Each row in the diagram represents a gene and each column a tumour sample. The color saturation represents differences in gene expression across the tumour samples; red indicates expression higher than the median (black), and green indicates expression lower than median. The color intensity indicates degree of gene regulation. **B:** Fold expression of the genes RhoA, RhoB, RhoC, Rac1 and Cdc42, as detected by microarray analysis.

antibody (diluted 1:5,000; Sigma Chemicals, St. Louis, MO, USA). The specific signals were visualized by ECL reagent following exposure to ECL film.

Computational analysis

Two publicly available Gene Expression Omnibus (GEO) datasets were analysed in order to further elucidate whether differences in the expression of Rho kinases occur among non-muscle-invasive UCCs with or without surrounding *in situ* lesions (CIS), muscle-invasive carcinomas (mTCC), cystectomy specimens, and normal tissue, as well as among low- and high-grade tumours and normal tissue [15,16]. The results were expressed as the mean levels of the $\log_2$ intensity and were statistically compared using the Mann-Whitney U test.

Table 1. Information of the primer sequences used for qPCR

Gene name	*GenBank AN*	*Primer sequence*	*Annealing position*	*Annealing temperature (° C)*	*PCR Length (bp)*
RhoA	NM_001664	5'-GGCTGGACTCGGATTCGTT-3' (**F**) 5'-CCATCACCAACAATCACCAGTT-3' (**R**)	249-267 296-317	60	68
RhoB	NM_004040	5'-GGACACCGACGTCATTCTCA-3' (**F**) 5'-ACCAGGATGATGGGCACATT-3' (**R**)	617-636 717-736	60	119
RhoC	NM_175744	5'-CAAGGTAGCCAAAGGCACTG-3' (**F**) 5'-CCCAACGTGCCCATCATC-3' (**R**)	855-874 724-741	60	150
Cdc42	NM_001039802	5'-TGAAAACGTGAAAGAAAAGTGGG-3' (**F**) 5'-GGCTTCTGTTTGTTCTTGGCA-3' (**R**)	562-584 679-699	60	137
Rac1	NM_006908	5'-AAAATGTCCGTGCAAAGTGGT-3' (**F**) 5'-CTCGATCGTGTCTTTATCATCCC-3' (**R**)	513-533 600-622	60	109
GAPDH	NM_002046.3	5'-GGAAGGTGAAGGTCGGAGTCA-3' (**F**) 5'-GTCATTGATGGCAACAATATCCACT-3' (**R**)	107-128 184-208	60	101
RPL13A	NM_012423.2	5'-CCTGGAGGAGAAGAGGAAAGAGA-3' (**F**) 5'-TTGAGGACCTCTGTGTATTTGTCAA-3' (**R**)	468-491 570-594	60	127

Table S1. Two-way average-linkage hierarchical clustering with Euclidian distance, for the 107-gene set, revealed that two groups of genes exhibited over-expression in T3, grade III tumours vs. normal tissue

A: The first group included the genes:

Gene name	*Gene symbol*	*Accession number*
Ras-GTPase-activating protein SH3-domain-binding protein	G3BP	NM_198395.1
ras homolog gene family, member C	RhoC	NM_175744.3
ras homolog gene family, member V	RhoV	NM_133639.2
RAS-like, family 11, member B	Rasl11B	NM_023940.2
Ras-GTPase activating protein SH3 domain-binding protein 2, transcript variant 3	cDNA clone MGC: 19504 IMAGE: 4337457	BC011731.2
Rho GTPase activating protein 21	ArhGAP21	NM_020824.2
Rho family GTPase 1	RND1	NM_014470.2
rho/rac guanine nucleotide exchange factor (GEF) 18	ArhGEF18	NM_015318.2
ras homolog gene family, member U	RhoU	NM_021205.4

B: The second group consisted of the genes:

Gene name	*Gene symbol*	*Accession number*
ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)	Rac2	NM_002872.3
ras homolog gene family, member A	RhoA	NM_001664.2
Ras-related GTP binding C	RRAGC	NM_022157.2
RasGEF domain family, member 1A	RasGEF1A	NM_145313.1
RAS p21 protein activator (GTPase activating protein) 1, transcript variant 1	RasA1	NM_002890.1
Ras-GTPase activating protein SH3 domain-binding protein 2	G3BP2	NM_012297.3
Rho GTPase activating protein 11A	ArGAP11A	NM_014783.2
Rho family GTPase 3	RND3	NM_005168.3
ras homolog gene family, member H	RhoH	NM_004310.2
Rho GDP dissociation inhibitor (GDI) gamma	ArhGDIG	NM_001176.1
RAVER1	RAVER1	NM_133452.1
RAS p21 protein activator 2	RasA2	NM_006506.2
Rho GTPase activating protein 12	ArhGAP12	NM_018287.4
Ras protein-specific guanine nucleotide-releasing factor 1, transcript variant 1	RasGRF1	NM_002891.3
Rho GTPase activating protein 24	ArhGAP24	NM_031305.1
ras homolog gene family, member D	RhoD	NM_014578.2
Ras-induced senescence 1	Ris1	NM_015444.1
ras-related C3 botulinum toxin substrate 3 (rho family, small GTP binding protein Rac3)	Rac3	NM_005052.2
Rho GTPase activating protein 22	ArhGAP22	NM_021226.2
Rho GTPase activating protein 29	ArhGAP29	NM_004815.2
Rho guanine nucleotide exchange factor (GEF) 19	ArhGEF19	NM_153213.2

Statistical analysis

The Kolmogorov-Smirnov test was used to determine whether the genes adhered to a normal distribution pattern. The Spearman rank correlation was used to examine both the correlation of the expression levels pairwise and their association with continuous variables. The Mann-Whitney U and Kruskal-Wallis H tests, were used to evaluate the GOI expression status with the various clinicopathological characteristics. The Kaplan-Meier method was used to estimate survival as a function of time, and survival differences were assessed by the Log-rank test. Numerical values are expressed as the mean±standard deviation (SD) or mean with 95%CI. Statistical significance was set at the 95% level ($p<0.05$).

Results

Microarray expression and clustering on a 107-gene set

We performed two-way average-linkage hierarchical clustering with Euclidian distance for a 107-gene set

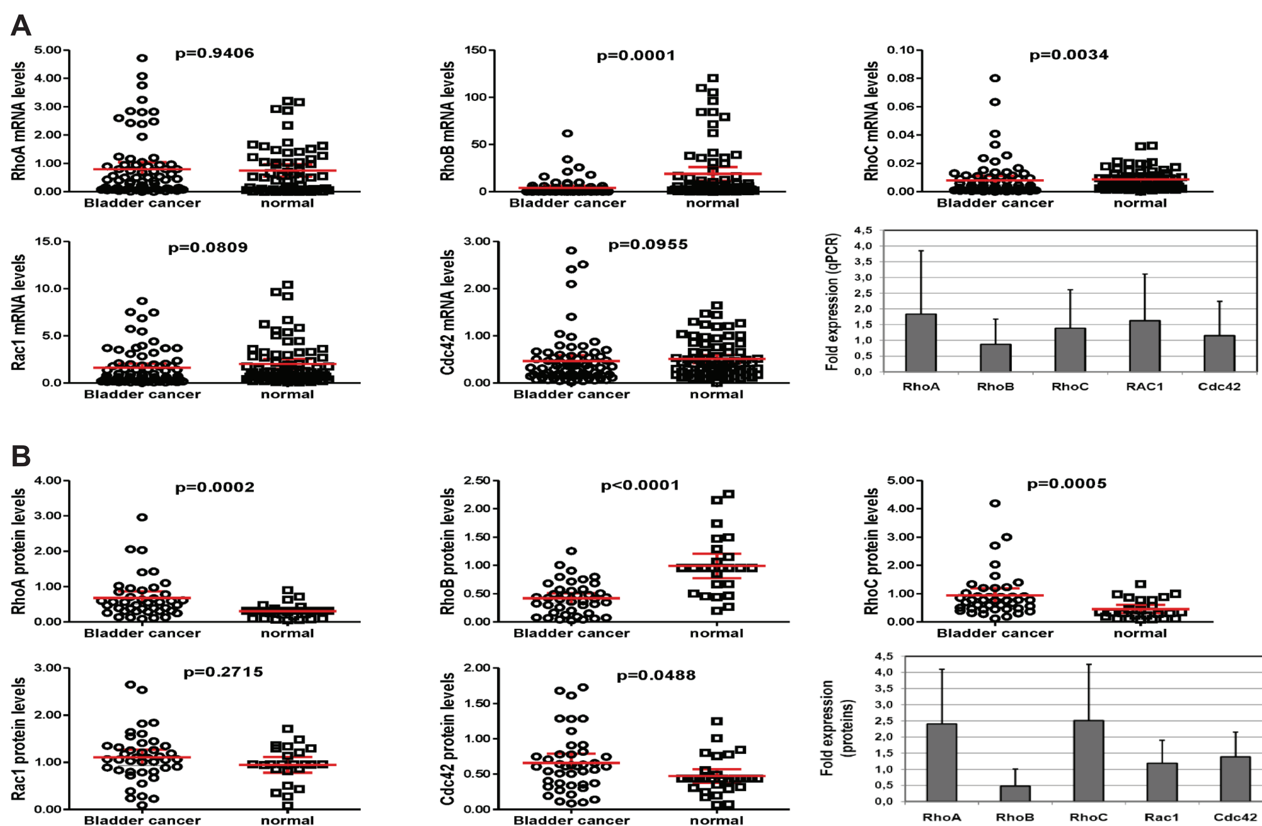


Figure 2. Scatterplots depict RhoA, RhoB, RhoC, Rac1 and Cdc42 mRNA (**A**) and protein (**B**) expression along with the corresponding fold difference in BC vs. normal tissue, as detected by qPCR and western blotting, respectively. Seventy-seven paired BC with normal tissue samples were investigated by qPCR and 44 by western blotting. The two groups were statistically compared using the Mann-Whitney U test. The bars indicate the mean with 95% CI values.

that included Rho GTPases, Rho dissociation inhibitors (GDIs), Rho GTPase-activating proteins (GAPs), Rho guanine exchange factors (GEFs), and other Rho-related genes, in 10 UCC samples vs. 5 controls. The $\log_2$ transformed fold expression pattern of the GOI was analysed and the tumours were divided into two main groups and several subgroups based on the differential expression of these 107 genes. Findings of our microarray analysis showed that the fold expression (mean±SD) of the genes RhoA, RhoB, RhoC, Rac1 and Cdc42 in TCC compared to the normal tissue was 2.2±1.9, 1.4±0.6, 3.7±3.0, 3.1±3.6 and 1.4±0.5, respectively (Figure 1).

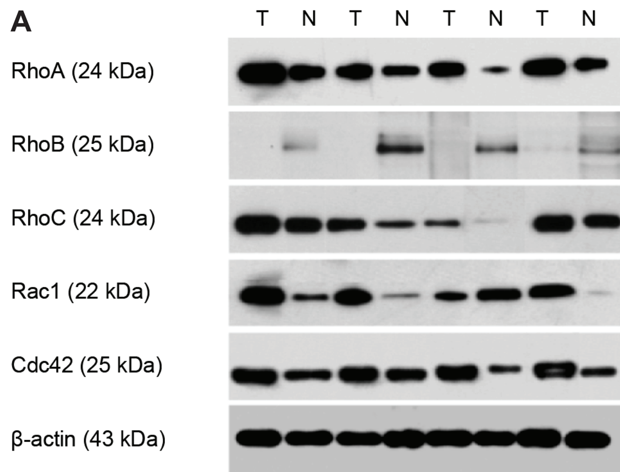
The majority of the Rho-related genes exhibited >1-fold expression in BC compared to controls. Interestingly, T3-grade III tumours exhibited over-expression of two groups of genes (Table S1).

Expression levels of Rho GTPases in urothelial carcinoma

The fold expression of RhoA and Rac1 was higher than 1.5-times, however, no statistically significant difference was noted (for RhoA: 1.8±2.0 fold change; for Rac1: 1.6±1.5 fold change, respectively). RhoB mRNA levels were significantly lower in UCC vs. normal urothelium (0.9±0.8 fold change, p=0.0001). RhoC mRNA levels were significantly higher in UCC vs. normal urothelium (1.4±1.2 fold change, p=0.0034). Cdc42 mRNA levels did not differ significantly between UCC and normal urothelium (1.1±1.1 fold change) (Figure 2). The protein levels of the genes RhoA, RhoC and Cdc42, as analysed by Western blotting (Figure 3), were significantly higher in UCC (mean-fold expression values were as follows: for RhoA, 2.40±1.70, p=0.0002; for RhoC, 2.51±1.74, p=0.0005; and for Cdc42, 1.39±0.76, p=0.0488, respectively). In contrast, RhoB exhibited significantly decreased protein levels in UCC vs. normal urothelium (mean fold expression was 0.48±0.53, p<0.0001). Rac1 protein levels did not show a significant difference (1.19±0.71) (Figure 2).

Rho GTPase mRNA and protein expression relative to clinicopathological characteristics

Stage 4 tumours showed higher RhoA mRNA levels vs. stage 1 (2.02±1.20 vs. 0.94±2.01, p=0.0146),



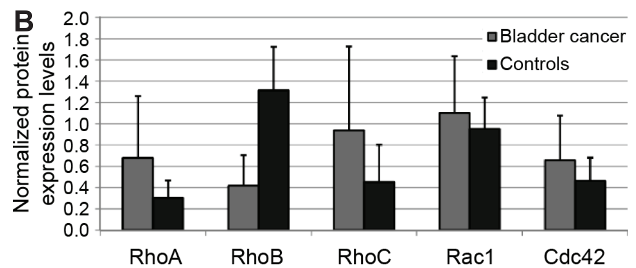


Figure 3. A: Representative Western blotting depicting the protein expression of RhoA, RhoB, RhoC, Rac1 and Cdc42 in bladder cancer (T) vs. normal tissue (N). **B:** Densitometric analysis of the Western blotting experiments depicting the mean ± SD protein expression levels of RhoA, RhoB, RhoC, Rac1 and Cdc42, in bladder cancer (n=44) and control tissue (n=44).

stage 2 (2.02±1.20 vs. 0.79±1.18, p=0.0316) and stage 3 tumours (2.02±1.20 vs. 0.77±1.04, p=0.0283). Moreover, stage 4 tumours exhibited higher RhoB mRNA levels compared to stage 1 (13.70±15.22 vs. 5.96±19.67, p=0.0161). Stage 4 tumours showed significantly higher Rac1 mRNA levels compared to stage 1 (4.33±2.69 vs. 2.09±3.81, p=0.0358), as well as stage 2 tumours (4.33±2.69 vs. 0.99±1.16, p=0.0176) (Figure 4). RhoA and RhoB exhibited higher levels in grade III vs. grade I/II tumours (for RhoA: 0.90±2.48, p=0.0191; for RhoB: 5.02±8.64 vs.1.03±1.93, p=0.0018) (Figure 4).

T4-grade III tumours exhibited significantly higher RhoA mRNA levels vs. controls (p=0.017), as well as vs. T1-grade I/II (p=0.004), T1-grade III (p=0.032), T2-grade III (p=0.028) and T3-grade II/III tumours (p=0.027). RhoB mRNA levels were significantly lower in T1-grade I/II (p<0.001), T1-grade III (p<0.001), and T2-grade III (p=0.030) UCCs compared to the controls. T4-grade III tumours exhibited higher Rac1 mRNA levels compared to the control tissue and vs. T2-grade III tumours, respectively (p=0.038 and p=0.016, respectively).

The correlation of the mRNA and protein levels of GOI was also examined in UCC and normal tissue, using the Spearman rank correlation test (Table 2).

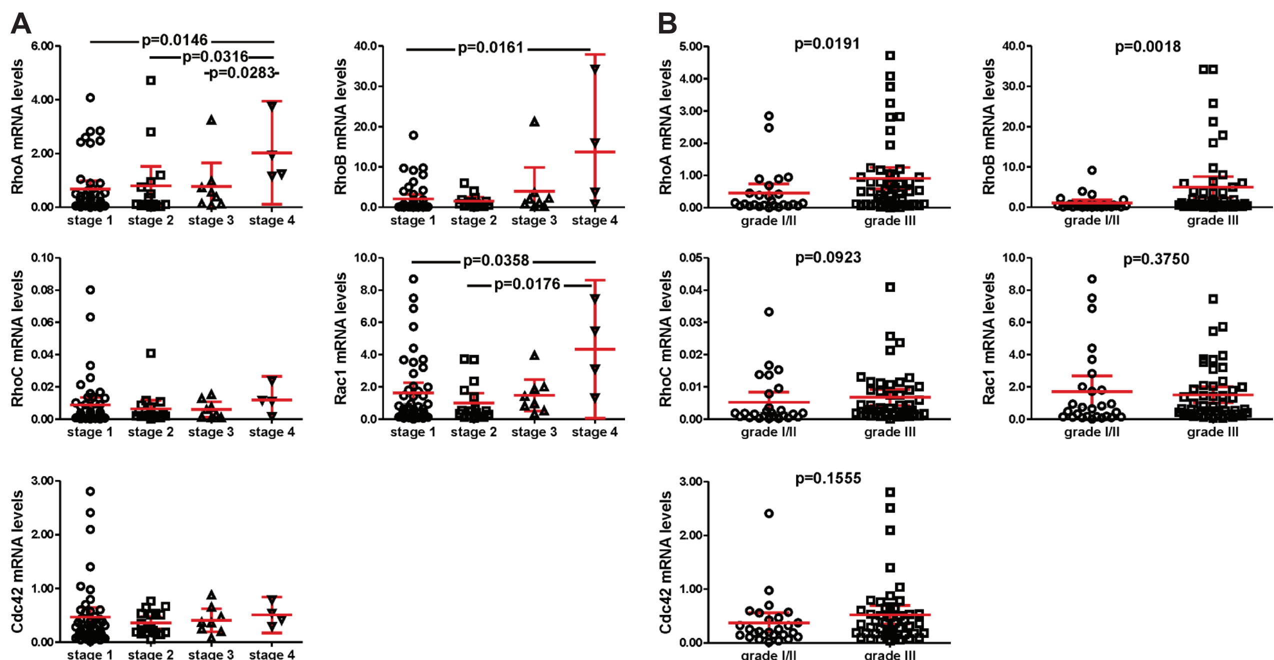


Figure 4. Scatterplots show the mRNA expression levels of RhoA, RhoB, RhoC, Rac1 and Cdc42, with regard to tumour stage (**A**) and grade (**B**). Group pairs were statistically compared using the Mann-Whitney U test. The bars indicate the mean with 95% CI values.

Table 2. Spearman rank correlation coefficient was used to determine the relationship between the mRNA expression levels of RhoA, RhoB, RhoC, Rac1 and Cdc42, in urinary bladder cancer (**A**) and normal tissue (**B**); and between the protein expression levels of RhoA, RhoB, RhoC, Rac1 and Cdc42, in urinary bladder cancer (**C**) and normal tissue (**D**). *Correlation is significant at the 0.05 level (2-tailed). **Correlation is significant at the 0.01 level (2-tailed)

A		*RhoA*	*RhoB*	*RhoC*	*Rac1*	*Cdc42*
RhoA	Correlation coefficient	1.000				
	Sig. (2-tailed)					
	N	72				
RhoB	Correlation coefficient	0.143	1.000			
	Sig. (2-tailed)	0.230				
	N	72	76			
RhoC	Correlation coefficient	0.233(*)	0.680(**)	1.000		
	Sig. (2-tailed)	0.049	0.000			
	N	72	74	74		
Rac1	Correlation coefficient	0.608(**)	0.550(**)	0.627(**)	1.000	
	Sig. (2-tailed)	0.000	0.000	0.000		
	N	72	75	74	75	
Cdc42	Correlation coefficient	0.134	0.557(**)	0.674(**)	0.412(**)	1.000
	Sig. (2-tailed)	0.263	0.000	0.000	0.000	
	N	72	76	74	75	76

B		*RhoA*	*RhoB*	*RhoC*	*Rac1*	*Cdc42*
RhoA	Correlation coefficient	1.000				
	Sig. (2-tailed)					
	N	60				
RhoB	Correlation coefficient	0.052	1.000			
	Sig. (2-tailed)	0.691				
	N	60	75			
RhoC	Correlation coefficient	-0.099	0.462(**)	1.000		
	Sig. (2-tailed)	0.463	0.000			
	N	57	68	68		
Rac1	Correlation coefficient	0.406(**)	0.306(**)	0.530(**)	1.000	
	Sig. (2-tailed)	0.001	0.008	0.000		
	N	60	75	68	75	
Cdc42	Correlation coefficient	-0.044	0.425(**)	0.633(**)	0.047	1.000
	Sig. (2-tailed)	0.738	0.000	0.000	0.689	
	N	60	75	68	75	75

C		*RhoA*	*RhoB*	*RhoC*	*Rac1*	*Cdc42*
RhoA	Correlation coefficient	1.000				
	Sig. (2-tailed)					
	N	42				
RhoB	Correlation coefficient	0.516(**)	1.000			
	Sig. (2-tailed)	0.000				
	N	42	42			
RhoC	Correlation coefficient	0.223	0.275	1.000		
	Sig. (2-tailed)	0.155	0.078			
	N	42	42	42		
Rac1	Correlation coefficient	0.775(**)	0.628(**)	0.106	1.000	
	Sig. (2-tailed)	0.000	0.000	0.506		
	N	42	42	42	42	
Cdc42	Correlation coefficient	0.528(**)	0.241	-0.042	0.496(**)	1.000
	Sig. (2-tailed)	0.000	0.125	0.791	0.001	
	N	42	42	42	42	42

D		*RhoA*	*RhoB*	*RhoC*	*Rac1*	*Cdc42*
RhoA	Correlation coefficient	1.000				
	Sig. (2-tailed)					
	N	42				
RhoB	Correlation coefficient	-0.245	1.000			
	Sig. (2-tailed)	0.117				
	N	42	42			
RhoC	Correlation coefficient	0.105	-0.168	1.000		
	Sig. (2-tailed)	0.617	0.422			
	N	25	25	025		
Rac1	Correlation coefficient	0.115	0.383(*)	-0.274	1.000	
	Sig. (2-tailed)	0.469	0.012	0.186		
	N	42	42	25	42	
Cdc42	Correlation coefficient	0.591(**)	-0.250	0.224	-0.124	1.000
	Sig. (2-tailed)	0.0000	0.110	0.282	0.433	
	N	42	42	25	42	42

Correlation between Rho mRNA/protein expression and survival rates of bladder cancer patients

A total of 77 urinary UCC cases were investigated for overall and cancer-specific survival rates. Those cases whose tumours exhibited high Cdc42 mRNA levels (>median value, 0.30) showed a worse overall survival rate than those expressing low Cdc42 mRNA levels (<median value, 0.30) (Log-rank, p=0.027). In the same manner, high RhoC mRNA levels exhibited a trend to

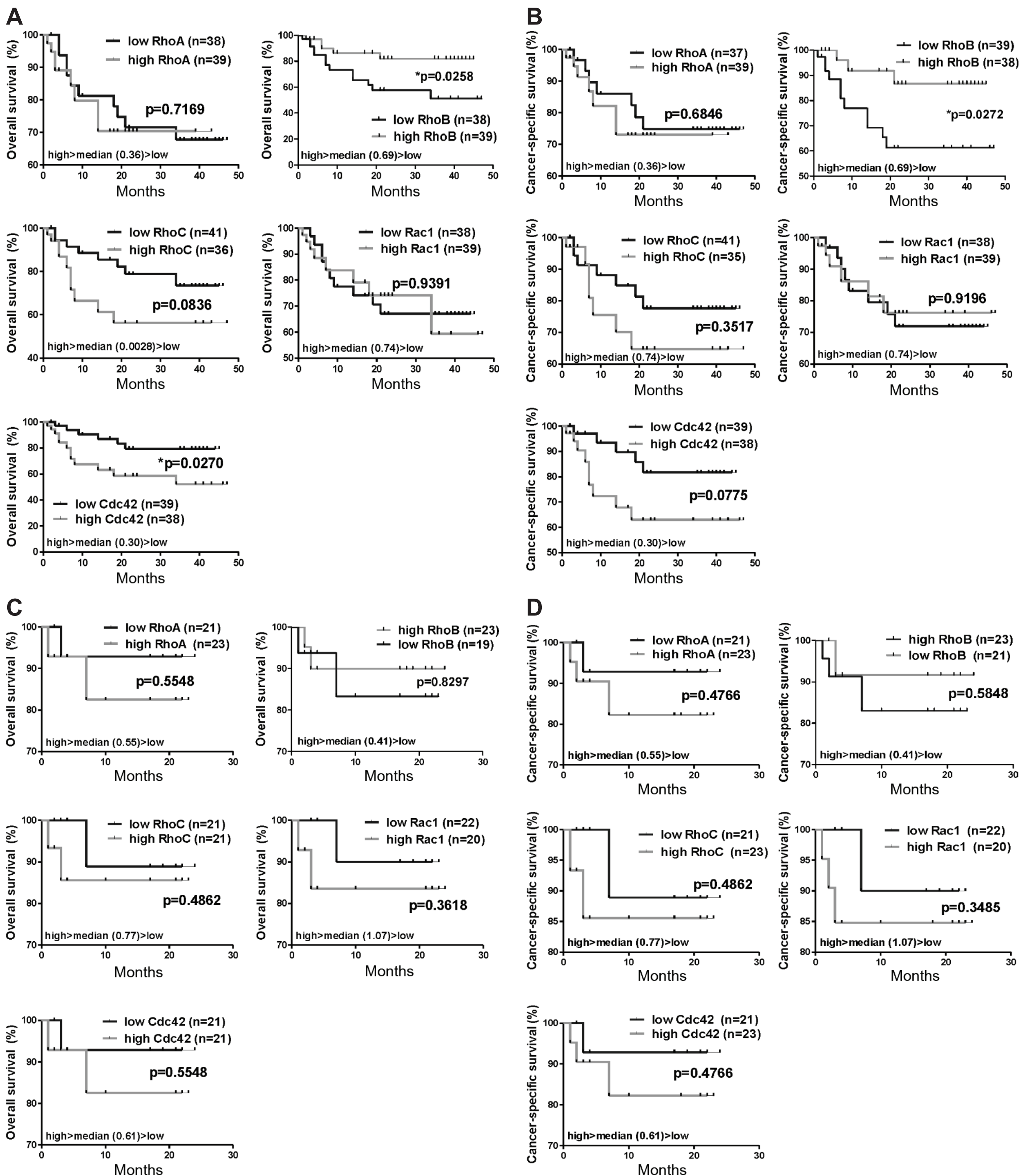


Figure 5. Kaplan-Meier curves show the overall (**A**) and cancer-specific (**B**) survival (%) of urinary bladder cancer patients, with regard to the mRNA expression as well as overall (**C**) and cancer-specific (**D**) survival (%) of urinary bladder cancer patients with regard to the protein expression of the genes RhoA, RhoB, RhoC, Rac1 and Cdc42, respectively. Survival differences were assessed using the Log-rank (Mantel Cox) test. Statistical significance was set at 95% (p<0.05).

correlate with worse overall survival, although no statistical significance could be obtained. On the contrary, the cases whose tumours exhibited increased levels of RhoB mRNA expression (>median value, 0.69) exhibited better overall as well as cancer-specific survival rates than the cases expressing decreased RhoB mRNA levels (<median value, 0.69) (Log-rank, p=0.0258 and p=0.0272, respectively) (Figure 5).

Computational analysis

Computational analysis of the expression profile of Rho kinases in UCC was performed in microarray data extracted from the GEO Datasets, GSE3167 [13] and GSE7476 [14]. Figure 6 depicts the computational analysis results, including all the statistically significant differences detected among superficial UCC with or without CIS, muscle-invasive UCC, cystectomy specimens and normal tissue.

Discussion

The present study investigated the expression of RhoA, RhoB, RhoC, Cdc42 and Rac1 using microarrays, qPCR and Western blot analysis in patients with all stages and grades of UCC of the urinary bladder. Our results showed an observable overlap between tumours and controls regarding the expression of Rho GTPases. Increasing evidence suggests that UCCs and "normal" urothelium often share common genetic and epigenetic alterations, probably representing the result of the field cancerization effect [17]. This mechanism, together with intraepithelial migration and intraluminal seeding, appear to be strongly responsible for multi-focal disease as well as for localised and distant recurrences within the bladder. Nevertheless, in our results we detected significantly decreased RhoB mRNA and protein levels in tumours vs. controls, whereas mRNA of RhoC and protein levels of RhoA, RhoC and

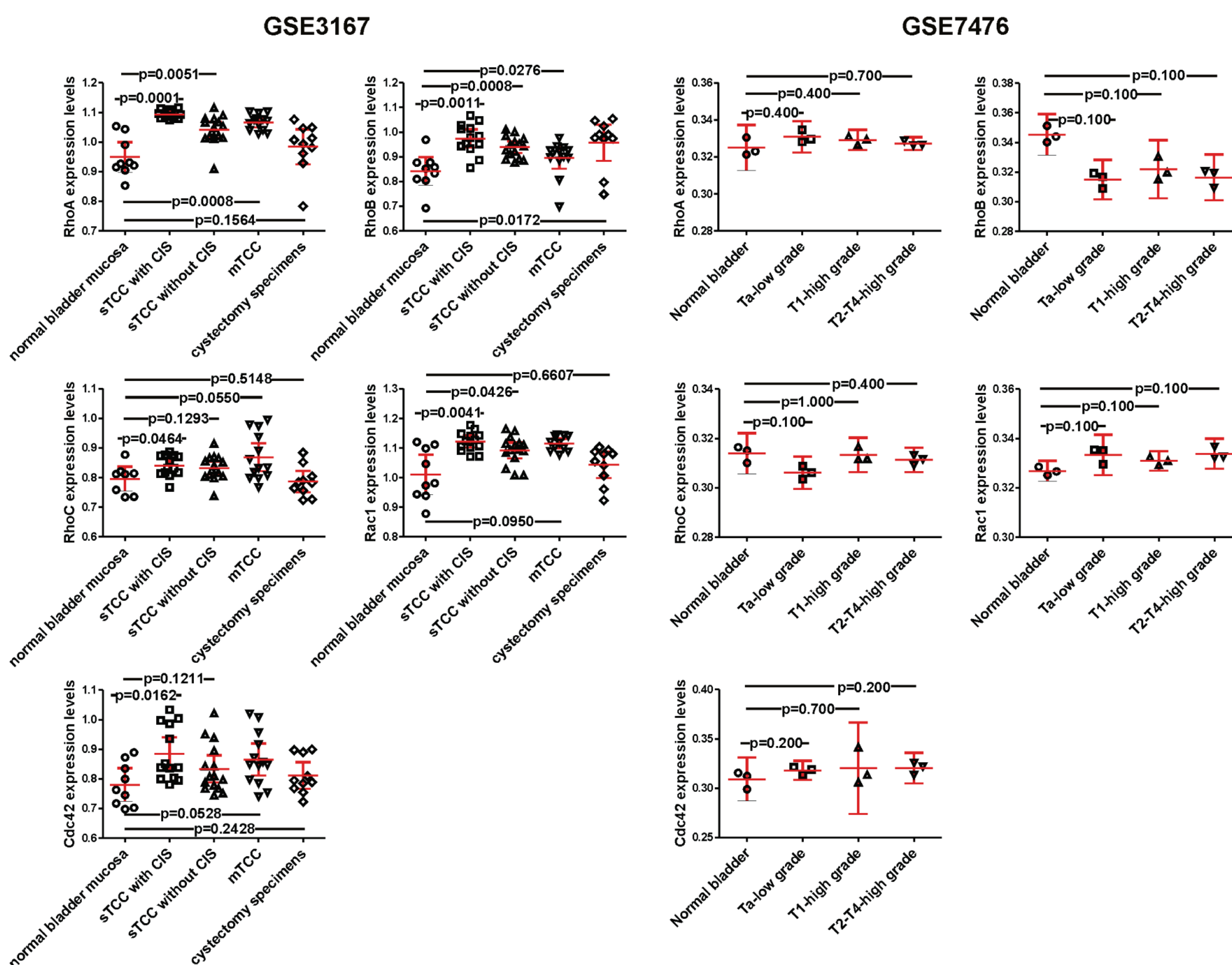


Figure 6. Two publicly available Gene Expression Omnibus (GEO) datasets were analysed using computational analysis; GSE3167 and GSE7476. The expression patterns of the Rho kinases RhoA, RhoB, RhoC, Rac1 and Cdc42 were extracted from the normalized datasets. The results were expressed as mean levels of the $\log_2$ intensity and were statistically compared using the Mann-Whitney U test. The bars indicate the mean with 95% CI values.

Cdc42 were significantly higher in UCCs.

Our results agree with a previous study documenting significantly higher RhoA and RhoC protein levels in Japanese patients with UCC of the urinary bladder [18]. Comparable patterns of expression have also been obtained from renal cell carcinoma [19] and in ovarian carcinoma [20]. Taken together, these data suggest that the over-expression of either RhoA and/or RhoC is involved in tumourigenesis irrespective of its origin.

The aforementioned studies also demonstrated an association between high RhoA/RhoC expression and poor histological differentiation. We noted significantly higher levels of RhoA in grade III and stage 4 tumours compared to grade I/II and stage 1 tumours, respectively. Many investigators support that high grade, invasive UCCs evolve via the occurrence of specific genetic alterations. Therefore, overexpression of RhoA may be one of the events leading to an aggressive phenotype, characterized by higher histological grade and more extensive local invasion.

High RhoA and RhoC protein levels were previously associated with poorer disease-free and overall survival in patients with BC [18]. Moreover, higher mRNA and protein expression of RhoA has also been correlated with shorter disease-free and overall survival in UCC of the upper urinary tract [21]. In accordance with these studies, we detected worse overall survival for patients with high RhoC expression [22].

In regard to RhoB, current evidence suggests a tumour-suppressor role [23-25]. Nevertheless, previous investigations evaluating the expression status of RhoB in genitourinary tract malignancies, including TCC of the urinary bladder, yielded contradictory results [18,19,22,26]. These data suggest that although the Rho genes exhibit strong homology with each other, different Rho GTPases play different roles in tumourigenesis and one Rho GTPase may have different roles in different tumour development. Our results clearly showed RhoB under-expression in UCC compared to normal tissue. Similarly, RhoB protein levels were more abundant in normal tissue. These findings confirm a tumour-suppressor role for RhoB, opposing the positive functions of RhoA and RhoC.

Previous studies related high RhoB protein expression to better overall survival in patients with BC [18]. In accordance to this, our results showed a significant correlation of high RhoB mRNA levels with better overall and cancer-specific survival. However, probably due to the smaller protein sample number, the statistical difference in the protein level of RhoB was not significant. One notable observation (that could affect the survival results) was the high RhoB mRNA levels in grade III and stage 4 tumours. In the literature, Rho GTPases have been reported with very low incidence of genetic alterations. Nonetheless, rearrangements of RhoH have been found in non-Hodgkin's lymphoma and in multiple myeloma [27]. Regarding RhoB, point mutations in cysteine 192 and cysteine 193 have been shown to abolish its functions [28]. High grade, muscle-invasive UCCs are characterised by major genetic divergence and by the accumulation of genetic alterations. Therefore, an unspecified yet genetic rearrangement may be responsible for the elevated RhoB levels. Unfortunately, since the literature lacks a satisfactory amount of relevant reports, this observation remains controversial.

Over-expression of Rac1 has been found to correlate with the progression of testicular [29], gastric [30] and breast cancer [31]. Similarly, Cdc42 was described as over-expressed in breast and testicular cancer [29], where Cdc42 gene expression correlated with tumour progression. Since Cdc42 cross-activates Rac in many cell types, it is possible that Cdc42 contributes to tumour formation and progression by activating Rac.

To our knowledge, this study is the first to evaluate the expression levels of Rac1 and Cdc42 in human samples of UCC of the urinary bladder. Our data did not show any significant difference in the mRNA levels between tumour and normal urothelium, apart from stage 4 tumours which demonstrated significantly higher Rac1 mRNA levels. At protein level, Cdc42 was more abundantly expressed in tumour compared to non-tumour samples. What is more, we detected a significant correlation of low Cdc42 mRNA levels with better overall survival.

Current evidence suggests that both Rac1 and Cdc42 regulate hypoxia inducible factors in response to hypoxia in human cancers [32]. Nevertheless, the results of a recent study demonstrated that the expression of either activated Rac1 or Cdc4 is not sufficient for malignant transformation of human fibroblasts. However, it was noted that the expression of a number of highly significant cancer-related genes requires the activities of Rac1 and/or Cdc42. Evidence indicates that under physiological conditions the sequential activation of Cdc42→Rac1→RhoA is crucial for the coordinated control of cell motility. In a similar manner, Rac1 and Cdc42 may cross activate RhoA and/or RhoC in various cell types and may contribute to tumour formation and progression. More importantly, preliminary evidence suggests that Cdc42 silencing leads to growth suppression in human bladder cancer cells [33].

A principal aspect of the regulatory possibilities of Rho GTPase signaling is their ability to regulate each other's activity via cross-talk and sequential activation. This regulation occurs among different Rho GTPase subfamilies as well as within subfamilies. In the pres-

ent study, we determined the relationships among all 5 genes, both at the mRNA and protein levels. Significant correlations were detected at both the mRNA and protein levels in tumour and normal tissue samples (Table 2). The above-mentioned findings support the previously proposed sequential activation molecular model. Similar results have been published regarding the expression of RhoA, Rac1 and Cdc42 in testicular cancer [29].

In conclusion, the present study showed that the mRNA and protein levels of RhoC and the protein levels of RhoA and Cdc42 were significantly higher in UCC compared to normal tissue. RhoB mRNA and protein levels exhibited an inverse expression profile, supporting its role as tumour-suppressor gene. Elucidating the regulatory mechanisms that modulate Rho-mediated signaling pathways in tumours may therefore provide novel targets for small-molecule therapeutic agents against cancer.

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