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Profiles of urinal exosomal miRNAs derived from bladder cancer

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Introduction Exosomes contain nucleic acids and proteins inside of them. These are suggested as cell--cell communication materials and it is considered that they can modulate the status of other cells.
Material and methods To understand the bladder cancer (BC) related exosomal microRNAs (miRNAs), we compared the 752 urine exosomal miRNAs in healthy control (n = 7), low grade (LG) BC (n = 6) and high grade (HG) BC (n = 6) by RT-qPCR.
Results The differential expressing (DE) urine exosomal miRNAs (2 > fold regulation) were 96 and 78 in LG and HG, respectively. Our exosomal miRNAs profiles cover many miRNAs which have been reported in BC patients' tissues and other biofluids. Most DE exosomal miRNAs were up-regulated in the profiles. Seven up-regulated exosomal miRNAs in the LG group (miR-28-5p, miR-16-5p, miR-28-3p, miR-24-3p, miR-25-3p, miR-19b-3p and miR10b-5p) and 3 miRNAs in the HG group (miR-150-5p, miR-28-5p and miR28-3p) were found as directly *TP53* targeting. Twenty-two and 18 *PTEN* targeting miRNAs were observed in up-regulated miRNAs of LG and HG. The target genes of these exosomal miRNAs and their interaction network predicted that the *TP53* is the strongest hub gene in both BC groups exosomal miRNA networks. Several DE miRNAs were found that could potentially be used as biomarkers for the diagnosis of BC.
Conclusions Profiles of urinal exosomal miRNAs derived from BC manifested potentially epigenetic regulation of the *TP53* and *PTEN* genes as compared to other oncogenes and tumour suppressors.

Key Words: miRNAs ‹› bladder cancer ‹› biomarker ‹› liquid biopsy ‹› biofluids ‹› urine exosomes

INTRODUCTION

Bladder cancer (BC) is a prevalent global concern, ranking as the tenth most common cancer, and it is often diagnosed in advanced stages, contributing to high mortality rates [1, 2]. Existing diagnostic and follow-up methods, both noninvasive gold standards and invasive procedures like cystoscopy, have limitations, necessitating the search for new diagnostic markers [3]. Exosomal microRNAs (exomiRs) are potential candidates to improve the diagnosis of BC.

A recent study emphasised the crucial role of the tumour microenvironment (TME) in tumour development. The TME comprises not only tumour cells but also elements of the extracellular matrix and non-malignant cells [4]. Exosomes, small extracellular vesicles, play a pivotal role in TME signalling. Produced by various cells, including tumour cells, exosomes carry biologically active cargo molecules, such as proteins, transcription factors, mRNAs, and microRNAs (miRNAs) [4, 5], which are small, non-coding RNAs involved in post-transcriptional gene regulation [6].

MiRNAs play a significant role in tumorigenesis by binding to target mRNAs, leading to the degradation of the target mRNA or inhibiting its translation [6, 7]. Compared to mRNAs, miRNAs exhibit stability in various samples, including urine [8–10], making them potential diagnostic markers. Aberrant miRNA expression in cancer tissues has been reported, and the presence of these miRNAs in urine suggests a potential reflection of cancer cell miRNA composition [11, 12]. The shared environment of urinary bladder cells through urine implies communication through exosomes, contributing to heterogeneity and oncotransformation [13, 14].

To improve our understanding of the cell-to-cell communication of bladder cells in patients with low-grade (LG) and high-grade BC (HG) and the possible impact of the urine exomiRs on the oncotransformation of normal cells, profiles of differentially expressed urine exomiRs were analysed. Striking differences in miRNA cargo between BC and control groups were identified. These differences could be further exploited as a tumour biomarker for further diagnostics. Such a biomarker would not only be able to increase sensitivity and specificity compared to the currently available, inferior methods, but also allow an understanding of the tumour type, providing a wider view compared to a biopsy from a single site, thus providing timely and appropriate treatment.

MATERIAL AND METHODS

Between 2019 and 2021, freshly voided urine samples were prospectively collected from patients with BC and controls at Pauls Stradins University Hospital. Urine was collected before performing transurethral resection of bladder (TURB). Collected urine samples were centrifuged within 24 hours of collection at 3.0 rcf (4.4 rpm) for 5 minutes and 30 seconds at room temperature and then separated into supernatant that was stored at –80˚C until use. Patients were stratified into 3 groups: group 1 with histologically proven Ta or T1 low-grade tumour (n = 6), group 2 with T2 high-grade tumour (n = 6), and group 3 – a control group (n = 7). Patients were staged and graded according to TNM classification and WHO criteria.

Inclusion and exclusion criteria

The study included patients over the age of 50 years with pathohistologically proven primary or recurrent bladder tumour, who underwent TURB or cystectomy and had available medical records. In patients who did not have pathohistologically confirmed BC, a urine sample was not analysed, and they were excluded from the study. Patients with high-grade, non-muscle-invasive BC (NMIBC) and low-grade, muscle-invasive BC (MIBC) were not included in the analysis. The control group comprised patients of matched age, with no history of prior oncology, urine analysis without signs of inflammation or infection, and no clinically significant changes in blood tests. Patients with incomplete data in the medical records, history of other current oncological disease or previous upper urinary tract cancer, diabetes mellitus, autoimmune diseases, inflammation, and infections except for urinary tract infection in the case of BC were excluded from the study.

Exosome isolation and RT-qPCR

Exosome isolation prior to miRNA isolation was performed in accordance with the protocol by miRCRURY Exosome Cell/Urine/CSF Kit (Qiagen, Hilden, Germany, Cat. No./ID: 76743). The urine samples were thawed on ice and re-centrifuged for 10 min at 10.000 g at room temperature to remove residual cell debris. Subsequently, the exosomes were isolated from 1960 μl of urine supernatant divided into 2 tubes. Further miRNeasy Mini Kit (Qiagen, Hilden, Germany, Cat. No./ID: 17004) was used for miRNA and total RNA isolation from urine exosomes according to the manufacturer's protocol. Each sample was spiked with synthetic UniSp2, UniSp4, UniSp5 RNA mix (Qiagen, USA, Cat.No./ID: 339390), and purified RNA was eluted once in 50 μl of RNase-free water. The MiRCURY LNA RT Kit (Qiagen, USA, Cat.No./ID: 339340) was used for reverse transcription and polyadenylation of miRNA to cDNA according to the manufacturer's instructions. We used 4 μl of RNA template instead of 2 μl, then incubated at 42°C for 60 min and 95°C for 5 min, and then held at 4°C. Incubation was performed with a peqSTAR thermal cycler.

For reverse transcription, miRCURY LNA miRNA PCR Starter Kit (Qiagen, USA, Cat. No. / ID: 339320) and miRCURY LNA SYBR Green PCR Kit (Qiagen, USA, Cat. No. / ID: 339347) were used. Predesigned MiRCURY LNA miRNA miRNome PCR Panels (Qiagen, USA, Cat. No. / ID: 339322) were used for miRNA identification. RT-qPCR data using the miRNA panel was processed with Applied Biosystems® ViiA™ 7 Real-Time PCR according to the manufacturer's protocol. Automatic threshold and baseline were used for all the miRNAs to record the CT value.

RT-qPCR data analysis

For further data analysis of miRNA panels, miRCURY miRNA PCR Data Analysis v1.0 was used. Data normalisation was carried out using a global

mean normalisation method. The p-values of differences in miRNA expression levels between controls and cancers were calculated based on a Student's t-test of the replicate 2^(–ΔCT) values for each miRNA in the control group and cancer group. The p-value calculation used is based on a parametric, two-sample equal variance, unpaired, and two-tailed distribution calculation. GeNorm, which is an embedded module of miRCURY miRNA PCR Data Analysis v1, was used for the identification of internal control miRNAs with expression levels correlating with the global mean CT values, and that most resemble the mean CT value.

The differentially expressed miRNAs in LG and HG were compared with the control group and were filtered by the condition of the fold exchange and p-values.

Sample size calculation for miRNA panels to identify differentially releasing miRNAs in urine was performed using the referenced article [15]. Statistical power was set 90% to detect two-fold difference in 700 miRNAs. A 0.05 significance level was determined. The calculated minimal sample size is 5 vs. 5. All raw data are available upon request via the following link: https://doi.org/10.48510/FK2/MQ8K26.

MiRNA target analysis and pathway analysis

The list of the statistically significant differentially expressed miRNAs were applied to the miEAA (https://ccb-compute2.cs.uni-saarland.de/mieaa2/) [16] for miRNA enrichment analysis. The result was filtered by the FDR and picked up the subcategories with the top 5 highest number of observed miRNAs. Prediction of the miRNA targets was performed by miRTargetLink 2.0 (https://ccb-compute.cs.uni-saarland.de/mirtargetlink2) [17]. The interaction of predicted targets was analysed by STRING (https://string-db.org/) [18]. The hub genes were analysed by CytoNCA and CytoHubba, which are plugins of Cytoscape [19] with the network centrality of degree, betweenness, closeness and eigenvector (CytoNCA), and EPC, MCC, MNC, and stress (CytoHubba).

Bioethical standards

The study was conducted in accordance with the Declaration of Helsinki and approved by the Central Medical Ethics Committee of Latvia (Nr.1/19-02-12) on 12.02.2019. All patients involved in the study signed an informed consent form.

RESULTS

Patient groups

This study was performed to identify miRNAs of interest in the urinary exomes of bladder cancer patients. Twelve patients were identified in bladder cancer groups (LG group 6 men, median age 71.5 years old vs. HG group 2 men, 4 women, median age 71.5 years old) and 6 patients in the healthy control group (6 men and 1 woman, median age 66 years old). For a more detailed view of the cohorts, see Table 1.

Differentially expressed urine exosome miRNAs in bladder cancer patients

After comparison of the overall profiles of exomiRs in cancer patients' urine samples, differentially

Table 1. *Clinical-pathological parameters of the study cohorts*

Variable	Study groups		
	Control (n = 7)	LG (n = 6)	HG (n = 6)
Age (years)			
Median	66	71.5	71.5
Range	53–81	50–78	61–86
Sex			
Male	6	6	2
Female	1	0	4
Pathological stage			
≤pT1	–	6	0
≥pT2	–	0	6
Grade			
LG	–	6	0
HG	–	0	6

HG – high grade; LG – low grade

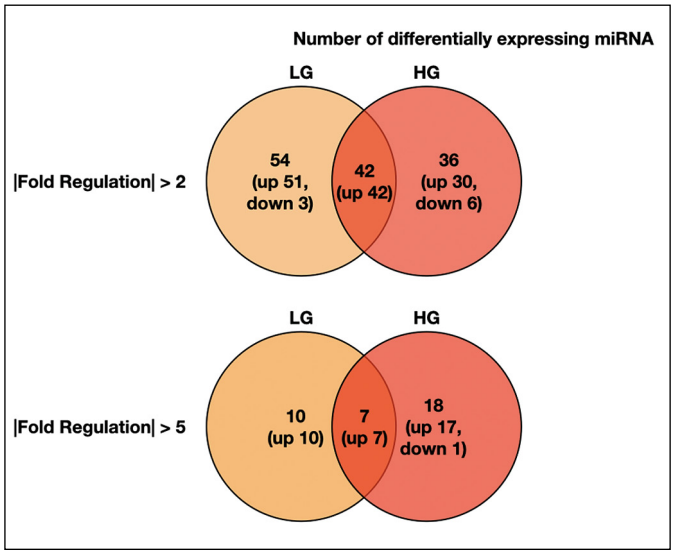

Figure 1. *Number of differentially expressed miRNA in low-grade and high-grade groups.*

Table 2. *Differentially expressed urine exosomal miRNAs and corresponding target genes in low grade bladder cancer patients group*

miRNA	Fold regulation	p-value	Target gene
Up-regulated			
has-miR-21-3p	14.25	0.016	*PTEN*
has-miR-324-5p	12.24	0.007	
has-miR-31-3p	11.27	0.018	
has-miR-30b-3p	8.57	0.001	
has-miR-188-5p	8.50	0.034	
has-miR-769-5p	8.15	0.033	
has-miR-132-3p	7.55	0.040	*EGFR*
has-miR-628-3p	7.30	0.004	
has-miR-126-3p	5.62	0.003	*AKT1*
has-miR-221-3p	5.62	0.028	*PTEN*
has-miR-455-3p	5.51	0.005	
has-miR-34a-3p	5.42	0.007	*AKT1, CTNNB1, MYC*
has-let-7f-2-3p	5.37	0.047	
has-miR-28-5p	5.30	0.039	*TP53*
has-miR-210-3p	5.29	0030	
has-miR-20a-3p	5.11	0.011	*PTEN, MYC*
has-miR-10b-3p	5.01	0.043	
has-miR-196b-3p	4.94	0.005	
has-miR-652-3p	4.84	0.018	
has-miR-582-5p	4.73	0.036	
has-miR-15b-5p	4.64	0.010	
has-miR-27b-3p	4.49	0.034	
has-miR-29a-5p	4.39	0.018	
has-miR-193b-5p	4.36	0.019	
has-miR-301a-3p	4.36	0.033	*PTEN*
has-miR-450a-5p	4.35	0.018	
has-miR-29c-5p	4.34	0.025	
has-miR-135b-5p	4.33	0.037	
has-miR-21-5p	4.32	0.018	*PTEN, EGFR*
has-miR-152-3p	4.18	0.017	*PTEN*
has-miR-16-5p	4.05	0.012	*TP53*
has-miR-200a-5p	3.99	0.037	*PTEN, CTNNB1*
has-miR-320b	3.94	0.002	
has-miR-151a-3p	3.90	0.009	
has-miR-20b-3p	3.87	0.012	
has-miR-18a-5p	3.82	0.009	*PTEN*
has-miR-425-5p	3.82	0.009	*PTEN*
has-miR-148b-3p	3.78	0.034	
has-miR-31-5p	3.75	0.036	
has-miR-15a-5p	3.74	0.027	
has-miR-129-5p	3.73	0.049	
has-miR-27a-3p	3.71	0.015	
has-miR-376c-3p	3.71	0.018	
has-miR-101-3p	3.67	0.022	
has-miR-197-3p	3.65	0.039	
has-let-7e-3p	3.46	0.015	
has-miR-24-2-5p	3.46	0.049	
has-miR-181a-3p	3.45	0.044	
has-miR-664a-3p	3.44	0.007	
has-miR-106b-3p	3.30	0.043	*PTEN*
has-miR-23a-3p	3.28	0.006	
has-miR-331-3p	3.24	0.044	
has-miR-185-5p	3.23	0.027	*AKT1*
has-miR-99b-5p	3.18	0.026	
has-miR-28-3p	3.17	0.007	*TP53*
has-miR-501-3p	3.16	0.018	
has-miR-191-5p	3.12	0.004	
has-miR-502-3p	3.04	0.024	
has-miR-151a-5p	3.00	0.011	
has-miR-378a-3p	2.99	0.004	
has-miR-200a-3p	2.97	0.034	*PTEN, CTNNB1,*
has-miR-324-3p	2.97	0.037	*EGFR*
has-miR-532-3p	2.93	0.022	
has-miR-500a	2.80	0.045	
has-miR-222-3p	2.80	0.050	*PTEN*
has-miR-141-3p	2.62	0.011	*PTEN*
has-miR-24-3p	2.61	0.045	*TP53, MYC*
has-miR-19a-3p	2.60	0.024	*PTEN*
has-miR-22-3p	2.59	0.017	*AKT1*
has-miR-25-3p	2.59	0.028	*TP53*
has-miR-200c-3p	2.55	0.014	*PTEN*
has-miR-200b-3p	2.54	0.013	
has-miR-125a-5p	2.51	0.050	*AKT1*
has-miR-320c	2.50	0032	
has-miR-423-5p	2.50	0.037	
has-miR-33a-5p	2.48	0.025	*MYC*
has-miR-429	2.47	0.012	*PTEN*
has-miR-320d	2.43	0.023	
has-miR-26b-5p	2.43	0.038	*PTEN*
has-miR-181a-5p	2.42	0.015	*PTEN*
has-miR-19b-3p	2.42	0.038	*TP53*
has-miR-320a	2.40	0.046	*PTEN*
has-miR-424-5p	2.38	0.046	
has-miR-10b-5p	2.38	0.047	*TP53*
has-miR-203a-3p	2.29	0.004	
has-miR-107	2.26	0.048	
has-miR-374b-5p	2.24	0.031	
has-miR-934	2.17	0.001	
has-miR-361-5p	2.17	0.0150	
has-miR-93-5p	2.15	0.014	*PTEN*
has-miR-29c-3p	2.15	0.024	*PTEN*
has-miR-103a-3p	2.14	0.047	*PTEN*
has-miR-628-5p	2.09	0.046	
Down-regulated			
hsa-miR-1247-5p	-2.31	0.049	
hsa-miR-492	-2.60	0.035	PTEN
hsa-miR-135b-3p	-2.84	0.045	

presented miRNAs with a cutoff of over 2-fold regulation (FR) or under –2 FR as compared to controls, the number of miRNAs corresponding to these cutoff values was 96 miRNAs in the LG group (93 up-regulated and 3 down-regulated) and 78 in the HG group (72 up-regulated and 6 down-regulated), respectively (see Figures 1, 2, and Tables 2, 3). Among these differentially presented miRNAs in the exosome, 42 miRNAs were found in both the LG and HG groups, and these miRNAs were all up-regulated in cancer patients.

TP53- and *PTEN*-related exosomal miRNAs

The majority of miRNAs in the profiles of LG and HG groups are up-regulated. It suggests the possibility that many of these miRNAs can be involved in down-regulation of tumour suppressors (TS). Then we picked up *TP53* targeting and *PTEN* targeting miRNAs (Table 4).

Among up-regulated exomiRs, *TP53* targeting miRNAs, which were found in the miRTargetLink database with robust validation, comprised 5 miRNAs in the LG group and 2 miRNAs in the HG group, and 4 miRNAs in the both the LG and HG groups (Table 4).

Table 3. *Differentially expressed urine exosomal miRNAs and corresponding target genes in low grade bladder cancer patients group*

miRNA	Fold regulation	p-value	Target gene
Up-regulated			
hsa-miR-150-5p	26.15	0.044	*TP53, CTNNB1*
hsa-miR-126-3p	21.35	0.015	*AKT1*
hsa-miR-205-5p	12.37	0.038	*PTEN*
hsa-miR-324-5p	11.47	0.009	
hsa-miR-628-3p	11.42	0.005	
hsa-miR-28-5p	9.95	0.001	*TP53*
hsa-miR-18a-5p	9.14	0.005	*PTEN*
hsa-miR-21-3p	9.10	0.018	*PTEN*
hsa-miR-24-1-5p	8.57	0.003	
hsa-miR-342-3p	8.49	0.018	
hsa-miR-130b-3p	8,24	0.038	*PTEN*
hsa-miR-197-3p	7.54	0.028	
hsa-miR-376c-3p	7.41	0.029	
hsa-miR-182-5p	7.38	0.008	*PTEN*
hsa-miR-20a-3p	7.36	0.006	*PTEN*
hsa-miR-590-3p	7.30	0.003	
hsa-miR-21-5p	6.38	0.035	*PTEN, EGFR*
hsa-let-7f-2-3p	6.31	0.015	
hsa-miR-200b-5p	6.26	0.010	
hsa-miR-374a-5p	6.05	0.001	
hsa-miR-7-1-3p	5.56	0.018	*EGFR*
hsa-miR-130b-5p	5.44	0.014	
hsa-miR-505-5p	5.14	0.002	
hsa-miR-183-5p	5.01	0.042	
hsa-miR-320b	4.97	0.029	
hsa-miR-105-5p	4.91	0.035	*AKT1*
hsa-miR-27b-3p	4.85	0.021	
hsa-miR-450a-5p	4.81	0.008	
hsa-miR-454-3p	4.79	0.030	
hsa-miR-28-3p	4.73	0.000	*TP53*
hsa-miR-146b-5p	4.71	0.009	
hsa-miR-425-5p	4.66	0.018	*PTEN*
hsa-miR-191-5p	4.65	0.008	
hsa-miR-301a-3p	4.62	0.014	*PTEN*
hsa-miR-151a-3p	4.47	0.004	
hsa-miR-320c	4.43	0.012	
hsa-miR-18a-3p	4.43	0.018	
hsa-miR-148b-3p	4.27	0.041	
hsa-miR-342-5p	4.12	0.042	
hsa-miR-664a-3p	4.10	0.016	
hsa-miR-455-3p	4.09	0.038	
hsa-miR-574-3p	4.06	0.002	*EGFR*
hsa-miR-618	3.89	0.040	
hsa-miR-365a-3p	3.81	0.003	
hsa-miR-200b-3p	3.74	0.000	
hsa-miR-181d-5p	3.69	0.034	
hsa-miR-193b-5p	3.68	0.028	
hsa-miR-200c-3p	3.50	0.001	*PTEN*
hsa-miR-23b-3p	3.48	0.024	*PTEN*
hsa-miR-374b-5p	3.37	0.002	
hsa-miR-92a-3p	3.29	0.021	*PTEN*
hsa-miR-320d	3.26	0.014	
hsa-miR-429	3.19	0.000	*PTEN, MYC*
hsa-miR-151a-5p	3.15	0.003	
hsa-miR-339-3p	3.15	0.023	
hsa-miR-26b-5p	3.12	0.023	*PTEN*
hsa-miR-99b-5p	3.11	0.016	
hsa-miR-489-3p	3.03	0.024	
hsa-miR-361-5p	2.92	0.014	
hsa-miR-320a	2.91	0.033	*PTEN*
hsa-miR-34a-3p	2.79	0.022	*AKT1, CTNNB, MYC*
hsa-miR-26a-5p	2.78	0.021	*PTEN*
hsa-miR-187-5p	2.77	0.033	
hsa-miR-218-5p	2.66	0.045	*EGFR*
hsa-miR-532-3p	2.66	0.047	
hsa-let-7d-3p	2.63	0.034	
hsa-let-7d-5p	2.61	0.019	
hsa-miR-125a-5p	2.43	0.012	*AKT1*
hsa-miR-106b-5p	2.30	0.026	*PTEN*
hsa-let-7g-5p	2.29	0.033	
hsa-miR-423-3p	2.17	0.011	
hsa-miR-423-5p	2.15	0.026	
Down-regulated			
hsa-miR-548m	-2.02	0.035	
hsa-miR-1247-5p	-2.39	0.020	
hsa-miR-492	-2.52	0.030	*PTEN*
hsa-miR-302b-3p	-2.60	0.032	
hsa-miR-135b-3p	-2.75	0.041	
hsa-miR-1-3p	-6.99	0.035	

Table 4. *Differentially expressed urine exosomal miRNAs that target TP53 and PTEN*

miRNA	Low grade		High grade	
	Fold regulation	p-value	Fold regulation	p-value
TP53 targeting miRNAs				
hsa-miR-126-3p	5.62	**0.002687**	21.35	**0.015415**
hsa-miR-18a-5p	3.82	**0.008685**	9.14	**0.004716**
hsa-miR-28-5p	5.30	**0.039468**	9.95	**0.000754**
hsa-miR-28-3p	3.17	**0.006807**	4.73	**0.000163**
hsa-miR-25-3p	2.59	**0.028475**	6.82	0.157505
hsa-miR-24-3p	2.61	**0.044578**	3.67	0.078445
hsa-miR-19b-3p	2.42	**0.038066**	2.54	0.063060
hsa-miR-10b-5p	2.38	**0.047099**	2.14	0.069420
hsa-miR-16-5p	4.05	**0.012415**	8.65	0.166509
hsa-miR-146b-5p	2.35	0.112566	4.71	**0.008794**
hsa-miR-150-5p	3.11	0.179476	26.15	**0.044293**
PTEN targeting miRNAs				
hsa-miR-18a-5p	3.82	**0.008685**	9.14	**0.004716**
hsa-miR-21-5p	4.32	**0.017528**	6.38	**0.034562**
hsa-miR-200c-3p	2.55	**0.014434**	3.50	**0.001310**
hsa-miR-21-3p	14.25	**0.015740**	9.10	**0.017916**
hsa-miR-20a-3p	5.11	**0.011170**	7.36	**0.005637**
hsa-miR-221-3p	5.62	**0.028214**	1.42	**0.309503**
hsa-miR-130b-3p	3.73	**0.053148**	8.24	**0.037600**
hsa-miR-182-5p	2.80	**0.214419**	7.38	**0.008211**
hsa-miR-205-5p	15.55	**0.078348**	12.37	**0.038030**

PTEN targeting miRNAs found in the miRTargetLink database with robust validation included 1 miRNA in the LG group and 3 miRNAs in the HG group, and 5 miRNAs in both the LG and HG groups (Table 4).

Table 5. *Key target gene analysis of miRNAs in CytoNCA and CytoHubba in low-grade bladder cancer patients group*

CytoNCA						CytoHubba								
Betweenness		Betweenness		Degree		Eigenvector		EPC		MCC		MNC		Stress	
TP53	139671.39	*TP53*	0.02640	*TP53*	347	*TP53*	0.17362	*TP53*	169.75	*RPL4*	1.30079E+27	*TP53*	346	*TP53*	2459550
CTNNB1	77327.39	*GAPDH*	0.02635	*AKT1*	289	*GAPDH*	0.16015	*GAPDH*	169.39	*RPSA*	1.30079E+27	*AKT1*	289	*GAPDH*	1670310
GAPDH	71795.57	*AKT1*	0.02634	*GAPDH*	277	*AKT1*	0.15996	*MYC*	167.50	*RPS8*	1.30079E+27	*GAPDH*	277	*CTNNB1*	1548678
AKT1	68962.64	*MYC*	0.02634	*MYC*	277	*MYC*	0.15962	*AKT1*	167.21	*RPS3A*	1.30079E+27	*MYC*	276	*AKT1*	1513244
HSP90AA1	66738.03	*CTNNB1*	0.02632	*CTNNB1*	266	*CTNNB1*	0.15120	*CTNNB1*	164.75	*RPL27*	1.30079E+27	*CTNNB1*	265	*MYC*	1483944
MYC	64594.49	*HSP90AA1*	0.02632	*HSP90AA1*	242	*PTEN*	0.14304	*PTEN*	160.78	*RACK1*	1.30079E+27	*HSP90AA1*	241	*HSP90AA1*	1465830
EGFR	64423.48	*EGFR*	0.02631	*PTEN*	231	*EGFR*	0.13506	*HSP90AA1*	158.23	*RPS2*	1.30079E+27	*PTEN*	230	*UBC*	1293598
UBC	53594.86	*PTEN*	0.02630	*EGFR*	231	*HSP90AA1*	0.13330	*EGFR*	156.94	*RPL6*	1.30079E+27	*EGFR*	228	*EGFR*	1265514
UBA52	46076.90	*SRC*	0.02628	*JUN*	199	*JUN*	0.13301	*JUN*	152.81	*RPS3*	1.30079E+27	*JUN*	197	*UBA52*	1155906
CDC42	41823.67	*UBC*	0.02627	*UBC*	197	*HIF1A*	0.12128	*HIF1A*	148.84	*RPL9*	1.30079E+27	*UBC*	195	*PTEN*	953772
PTEN	41206.15	*JUN*	0.02627	*SRC*	194	*KRAS*	0.12069	*ESR1*	147.75	*EIF3I*	1.30016E+27	*UBA52*	193	*HSPA8*	866314
SRC	38859.36	*UBA52*	0.02627	*UBA52*	193	*SRC*	0.12068	*NOTCH1*	147.52	*EEF1A1*	1.29952E+27	*SRC*	191	*SRC*	822966
HSPA8	37711.25	*HSPA8*	0.02625	*ESR1*	177	*ESR1*	0.12008	*SRC*	145.35	*EIF3B*	1.29830E+27	*ESR1*	175	*CDC42*	774068
ESR1	34233.53	*ESR1*	0.02625	*KRAS*	177	*NOTCH1*	0.12001	*KRAS*	144.20	*RPL14*	1.29805E+27	*KRAS*	175	*ESR1*	706210
BRCA1	30645.92	*MTOR*	0.02625	*NOTCH1*	170	*MTOR*	0.11088	*MTOR*	142.91	*PA2G4*	1.29701E+27	*NOTCH1*	170	*JUN*	665702
NOTCH1	28079.76	*HIF1A*	0.02624	*HSPA8*	168	*VEGFA*	0.11065	*UBC*	142.63	*CCT3*	1.29457E+27	*HSPA8*	167	*NOTCH1*	631136
TNF	27541.57	*NOTCH1*	0.02624	*HIF1A*	165	*CDH1*	0.10870	*UBA52*	141.74	*RUVBL1*	1.29268E+27	*HIF1A*	165	*BRCA1*	626702
JUN	26984.02	*KRAS*	0.02623	*MTOR*	161	*CDKN2A*	0.10762	*CDH1*	140.53	*PSMD2*	1.29268E+27	*MTOR*	161	*TNF*	600260
MTOR	25403.38	*TNF*	0.02623	*TNF*	160	*TNF*	0.10525	*CDKN2A*	140.12	*CCT8*	1.27880E+27	*TNF*	159	*MTOR*	591232
CDK1	24630.37	*CDC42*	0.02623	*CDH1*	155	*SIRT1*	0.10045	*VEGFA*	139.27	*CCT6A*	1.27707E+27	*CDH1*	153	*CDK1*	590886
RAB5A	23991.12	*CDH1*	0.02622	*VEGFA*	150	*UBC*	0.09826	*TNF*	138.57	*RPL7A*	1.26842E+27	*VEGFA*	150	*CDH1*	540048
KRAS	23939.88	*CDKN2A*	0.02622	*CDC42*	149	*CREB1*	0.09559	*SIRT1*	137.28	*RPL35A*	1.26829E+27	*CDC42*	149	*KRAS*	538298
CDH1	23554.02	*CDK1*	0.02622	*CDKN2A*	148	*PIK3CA*	0.09547	*HSPA8*	135.16	*TCP1*	1.26164E+27	*CDKN2A*	147	*RACK1*	491350
POLR2A	20306.50	*BRCA1*	0.02622	*BRCA1*	145	*SMAD4*	0.09393	*PIK3CA*	134.88	*HSPA8*	1.25727E+27	*BRCA1*	142	*HIF1A*	489466
RACK1	19890.60	*SIRT1*	0.02620	*SIRT1*	140	*FOXO3*	0.09306	*EZH2*	134.34	*RAN*	8.73154E+26	*PIK3CA*	139	*SMARCA4*	444384
SMARCA4	19504.41	*VEGFA*	0.02620	*PIK3CA*	139	*EZH2*	0.09269	*CREB1*	130.53	*PSMD11*	8.72352E+26	*SIRT1*	138	*RAB5A*	442400
HIF1A	19449.99	*RACK1*	0.02620	*CDK1*	137	*BRCA1*	0.09232	*BRCA1*	130.10	*RPL12*	8.64560E+26	*CDK1*	137	*SIRT1*	441248
CDKN2A	18716.99	*SMAD4*	0.02619	*EZH2*	134	*UBA52*	0.09134	*SMAD3*	127.12	*PSMD4*	4.36227E+26	*EZH2*	134	*POLR2A*	438488
SIRT1	18499.02	*PIK3CA*	0.02619	*RACK1*	129	*SMAD3*	0.08997	*GSK3B*	126.31	*RPS5*	4.27637E+26	*RACK1*	128	*CDKN2A*	436768

Table 5. *Continued*

CytoNCA										CytoHubba					
Betweenness		Betweenness		Degree		Eigenvector		EPC		MCC		MNC		Stress	
CUL1	17619.86	*CREB1*	0.02619	*CREB1*	127	*GSK3B*	0.08909	*FOXO3*	125.73	*RPS24*	4.26909E+26	*CREB1*	126	*GRB2*	392492
ADAM12	17324.98	*APP*	0.02619	*SMAD4*	122	*SMAD2*	0.08823	*FN1*	125.15	*HSP90AA1*	5.52272E+25	*SMAD4*	122	*FN1*	380422
APP	16838.32	*GSK3B*	0.02618	*SMARCA4*	122	*HSPA8*	0.08600	*SMAD4*	124.21	*AIMP1*	3.41505E+25	*SMARCA4*	120	*APP*	367420
GRB2	16784.52	*EZH2*	0.02618	*FN1*	121	*FN1*	0.08288	*CDK1*	123.73	*GAPDH*	3.06873E+25	*FN1*	120	*CUL1*	356552
FN1	16772.13	*GRB2*	0.02618	*FOXO3*	120	*CDC42*	0.08201	*PIK3R1*	121.13	*TP53*	3.04799E+25	*FOXO3*	119	*EZH2*	342406
PAAT	16589.31	*SMAD3*	0.02618	*GRB2*	118	*CDK1*	0.08123	*SMAD2*	120.99	*CTNNB1*	3.04799E+25	*SMAD3*	117	*VEGFA*	334088
VCP	15717.12	*SMARCA4*	0.02618	*SMAD3*	118	*PIK3R1*	0.07941	*CDC42*	118.30	*MYC*	3.04799E+25	*GSK3B*	117	*VCP*	330860
RAB1A	15064.00	*SMAD2*	0.02618	*GSK3B*	118	*SMARCA4*	0.07718	*SMARCA4*	118.12	*PTEN*	3.04798E+25	*POLR2A*	116	*SRSF1*	325274
COPS5	14224.77	*FN1*	0.02617	*POLR2A*	117	*NFKBIA*	0.07684	*RACK1*	117.18	*ESR1*	3.04770E+25	*GRB2*	116	*SMAD3*	323756
PCDHA10	13836.00	*FOXO3*	0.02617	*PIK3R1*	117	*FGF2*	0.07640	*GRB2*	116.48	*KRAS*	3.04692E+25	*PIK3R1*	115	*PIK3CA*	321294
PIK3R1	13459.14	*PIK3R1*	0.02617	*SMAD2*	114	*RPS6KB1*	0.07617	*CCNA2*	114.38	*SRC*	3.04324E+25	*SMAD2*	113	*DICER1*	316540
SEC61A1	13308.79	*ACTG1*	0.02617	*RUVBL1*	108	*FOXO1*	0.07551	*RPS6KB1*	114.13	*VEGFA*	3.04146E+25	*RUVBL1*	108	*PIK3R1*	314746
SMAD3	13284.94	*DICER1*	0.02616	*KAT2B*	105	*GRB2*	0.07440	*FOXO1*	113.55	*NOTCH1*	3.03517E+25	*KAT2B*	104	*COPS5*	310048
EZH2	13271.90	*VCP*	0.02616	*PABPC1*	102	*CCNA2*	0.07255	*CDK6*	113.55	*HIF1A*	3.01924E+25	*PABPC1*	102	*RUVBL1*	298730
SRSF1	13234.46	*CCNA2*	0.02615	*DICER1*	102	*CDK6*	0.07156	*SP1*	113.27	*JUN*	2.91634E+25	*DICER1*	102	*ADAM12*	295146
PHB1	12975.83	*KAT2B*	0.02615	*RPS3*	100	*TGFB1*	0.07102	*FGF2*	113.19	*CDKN2A*	2.91570E+25	*RPS3*	98	*SMAD4*	293032
RUVBL1	12473.54	*POLR2A*	0.02615	*EFTUD2*	99	*DNMT1*	0.06930	*NFKBIA*	112.98	*CDH1*	2.80904E+25	*CCNA2*	98	*CREB1*	276004
VEGFA	12360.18	*FBXW7*	0.02615	*CCNA2*	98	*MCL1*	0.06890	*KAT2B*	111.86	*MTOR*	2.77055E+25	*EFTUD2*	97	*GSK3B*	270180
PIK3CA	12281.99	*NFKBIA*	0.02614	*APP*	98	*KAT2B*	0.06883	*DICER1*	111.64	*SMAD4*	2.71931E+25	*APP*	95	*PAAT*	266302
HSP90B1	11969.34	*FGF2*	0.02614	*VCP*	95	*PTGS2*	0.06855	*MCL1*	111.59	*AKT1*	2.71467E+25	*H2BC21*	95	*H2BC21*	265714
FMR1	11795.68	*HTT*	0.02614	*H2BC21*	95	*DICER1*	0.06821	*DNMT1*	109.47	*EGFR*	2.69060E+25	*NFKBIA*	95	*PABPC1*	261368

EPC – Edge Percolated Component; MCC – Maximal Clique Centrality; MNC – Maximum Neighborhood Component

Table 6. *Key target gene analysis of miRNAs in CytoNCA and CytoHubba in high-grade bladder cancer patients group*

CytoNCA								CytoHubba					
Betweenness		Closeness		Degree		Eigenvector		EPC		MCC		MNC		Stress	
TP53	43318.11	*TP53*	0.08692	*TP53*	400	*TP53*	0.14084	*TP53*	36.96	*TP53*	4.64876E+37	*TP53*	400	*TP53*	818632
AKT1	34899.65	*AKT1*	0.08683	*AKT1*	390	*AKT1*	0.13901	*CTNNB1*	35.28	*CTNNB1*	4.64876E+37	*AKT1*	390	*AKT1*	718546
CTNNB1	33186.80	*CTNNB1*	0.08656	*CTNNB1*	363	*CTNNB1*	0.13490	*AKT1*	34.75	*STAT3*	4.64876E+37	*CTNNB1*	363	*CTNNB1*	677670
MYC	24278.85	*MYC*	0.08639	*MYC*	348	*MYC*	0.13356	*EGFR*	34.49	*MYC*	4.64876E+37	*MYC*	348	*MYC*	575480
EGFR	17742.88	*EGFR*	0.08604	*EGFR*	311	*STAT3*	0.12497	*JUN*	33.75	*HRAS*	4.64876E+37	*EGFR*	310	*EGFR*	449474
JUN	15688.20	*JUN*	0.08592	*JUN*	297	*JUN*	0.12306	*HIF1A*	33.42	*VEGFA*	4.64872E+37	*JUN*	297	*JUN*	402476
SRC	14826.75	*PTEN*	0.08586	*PTEN*	294	*EGFR*	0.12272	*PTEN*	33.29	*PTEN*	4.64871E+37	*PTEN*	294	*SRC*	369210
IL6	12890.36	*STAT3*	0.08582	*STAT3*	290	*PTEN*	0.12237	*MYC*	33.28	*HIF1A*	4.64870E+37	*STAT3*	290	*IL6*	362580
EP300	12564.87	*SRC*	0.08564	*SRC*	272	*HRAS*	0.11447	*STAT3*	32.55	*CCND1*	4.64867E+37	*SRC*	271	*PTEN*	344868
PTEN	12175.20	*IL6*	0.08557	*HRAS*	264	*VEGFA*	0.11438	*HRAS*	31.71	*CASP3*	4.64862E+37	*HRAS*	264	*STAT3*	323652
CDH1	11612.66	*HRAS*	0.08557	*IL6*	258	*SRC*	0.11318	*IL6*	31.31	*SRC*	4.64825E+37	*IL6*	258	*EP300*	304670
HSP90AA1	10878.28	*VEGFA*	0.08550	*VEGFA*	256	*CCND1*	0.11273	*CASP3*	31.15	*JUN*	4.64515E+37	*CCND1*	256	*CDH1*	292526
STAT3	10463.25	*CCND1*	0.08549	*CCND1*	256	*HIF1A*	0.11113	*CCND1*	31.03	*KRAS*	4.64328E+37	*VEGFA*	256	*HRAS*	291064
HRAS	9998.29	*NOTCH1*	0.08537	*NOTCH1*	245	*NOTCH1*	0.11058	*SRC*	30.96	*MTOR*	4.61065E+37	*NOTCH1*	245	*HSP90AA1*	286176
ESR1	9833.07	*CASP3*	0.08534	*HIF1A*	241	*IL6*	0.10920	*NOTCH1*	30.25	*AKT1*	4.60476E+37	*HIF1A*	240	*ESR1*	276576
HIF1A	9642.17	*HIF1A*	0.08534	*CASP3*	237	*CASP3*	0.10908	*KRAS*	30.06	*IGF1R*	4.58689E+37	*CASP3*	237	*CCND1*	275162
CCND1	8931.99	*ESR1*	0.08529	*KRAS*	236	*KRAS*	0.10757	*VEGFA*	28.77	*CDKN2A*	4.52123E+37	*CDH1*	236	*VEGFA*	260862
FN1	8405.20	*CDH1*	0.08526	*CDH1*	236	*ESR1*	0.10533	*ESR1*	28.60	*CDH1*	4.51446E+37	*KRAS*	236	*FN1*	254858
VEGFA	8071.93	*KRAS*	0.08525	*ESR1*	235	*CDH1*	0.10354	*CDH1*	28.45	*IL6*	4.46168E+37	*ESR1*	235	*HIF1A*	253298
CASP3	7862.63	*ERBB2*	0.08516	*EP300*	226	*ERBB2*	0.10037	*FN1*	27.72	*EGFR*	4.39080E+37	*EP300*	226	*NOTCH1*	242982
NOTCH1	7647.77	*EP300*	0.08513	*FN1*	225	*FN1*	0.09700	*ERBB2*	27.67	*NOTCH1*	4.35771E+37	*FN1*	225	*IL1B*	227416
ERBB2	7125.52	*FN1*	0.08511	*ERBB2*	224	*IL1B*	0.09356	*MTOR*	27.51	*IGF1*	4.16343E+37	*ERBB2*	223	*CASP3*	220624
RHOA	6872.49	*HSP90AA1*	0.08510	*HSP90AA1*	219	*CDKN2A*	0.09339	*IL1B*	27.12	*HSP90AA1*	3.95631E+37	*HSP90AA1*	219	*ERBB2*	207914
IL1B	6710.04	*IL1B*	0.08496	*IL1B*	211	*HSP90AA1*	0.09333	*HSP90AA1*	26.35	*MAPK8*	3.78193E+37	*IL1B*	211	*SMAD3*	207866
HDAC2	6475.09	*CDKN2A*	0.08486	*CDKN2A*	196	*MTOR*	0.09170	*CDKN2A*	26.27	*CDKN1A*	3.54946E+37	*CDKN2A*	196	*RHOA*	204496
SMAD3	6356.20	*MTOR*	0.08478	*MTOR*	190	*IGF1*	0.09033	*CREB1*	25.68	*ERBB2*	3.49330E+37	*MTOR*	189	*SMAD2*	195824
SMAD2	6248.53	*SMAD3*	0.08475	*SMAD3*	183	*EP300*	0.08876	*IGF1*	25.44	*FN1*	3.48111E+37	*SMAD3*	183	*KRAS*	195774

Table 6. *Continued*

CytoNCA										CytoHubba					
Betweenness		Closeness		Degree		Eigenvector		EPC		MCC		MNC		Stress	
MTOR	5964.37	*SMAD4*	0.08467	*SMAD4*	176	*SMAD4*	0.08460	*SMAD3*	25.40	*SMAD2*	3.24648E+37	*SMAD4*	176	*SOX2*	177378
SOX2	5825.79	*IGF1*	0.08463	*IGF1*	173	*SMAD3*	0.08439	*EP300*	25.11	*SMAD4*	3.17361E+37	*MAPK1*	172	*MTOR*	169344
KRAS	5246.49	*CREB1*	0.08463	*SMAD2*	172	*PIK3CA*	0.08390	*FOXO3*	24.55	*ESR1*	3.16477E+37	*EZH2*	172	*EZH2*	169208
PPARG	5108.88	*PPARG*	0.08463	*PPARG*	172	*CREB1*	0.08213	*PIK3CA*	24.35	*SMAD3*	3.13466E+37	*PPARG*	172	*HDAC2*	162266
EZH2	4958.64	*SMAD2*	0.08460	*EZH2*	172	*PPARG*	0.08166	*SIRT1*	24.19	*IL1B*	3.03260E+37	*IGF1*	172	*PPARG*	160972
CREB1	4872.41	*MAPK1*	0.08460	*MAPK1*	172	*SMAD2*	0.08122	*RHOA*	24.15	*MMP2*	2.67257E+37	*CREB1*	171	*SMAD4*	149714
MAPK1	4640.42	SIRT1	0.08460	RHOA	171	MAPK1	0.08043	IGF1R	24.11	RHOA	2.57949E+37	RHOA	171	MAPK1	147868
AR	4350.69	RHOA	0.08459	CREB1	171	SIRT1	0.07987	STAT1	23.91	PPARG	1.48338E+37	PIK3CA	170	CREB1	145682
SMAD4	4252.57	PIK3CA	0.08458	PIK3CA	170	FOXO3	0.07965	MAPK1	23.84	CDKN1B	1.17501E+37	SMAD2	170	CDKN2A	142236
CDH2	4097.98	FOXO3	0.08454	SIRT1	169	STAT1	0.07936	KIT	23.76	FOXO3	1.15523E+37	SIRT1	169	CDK2	135550
BDNF	4096.94	EZH2	0.08452	FOXO3	166	RHOA	0.07857	PTGS2	23.54	BCL2L1	1.10569E+37	FOXO3	166	FOXO3	127036
FOXO3	4087.12	SOX2	0.08451	SOX2	163	GSK3B	0.07788	BCL2L1	23.22	SIRT1	1.10102E+37	SOX2	163	STAT1	126868
GRB2	4063.63	MAPK14	0.08444	STAT1	160	MAPK14	0.07698	SMAD4	23.21	CASP8	1.07225E+37	STAT1	160	AR	124004
PTPN11	4011.15	MAPK8	0.08439	MAPK14	155	MAPK8	0.07665	TLR4	23.19	AR	9.87754E+36	MAPK14	155	BDNF	117584
CDKN2A	3870.89	STAT1	0.08439	GSK3B	152	IGF1R	0.07567	EZH2	22.99	PTGS2	6.65197E+36	GSK3B	152	SIRT1	114494
CDK2	3835.93	GSK3B	0.08437	MAPK8	152	BCL2L1	0.07505	MAPK14	22.66	MAPK1	6.64063E+36	MAPK8	151	MAPK8	113312
STAT1	3782.77	KDR	0.08431	PIK3R1	150	KIT	0.07490	KDR	22.65	ATM	6.31194E+36	PIK3R1	150	GRB2	113286
MAPK8	3758.49	PIK3R1	0.08430	ATM	149	CDKN1A	0.07454	GSK3B	22.63	HGF	5.61595E+36	KIT	149	CDH2	106958
LRRK2	3439.97	CDKN1A	0.08429	KIT	149	EZH2	0.07435	NFKBIA	22.59	BCL2L11	4.97005E+36	NFKBIA	148	PIK3CA	106326
HNF4A	3412.08	ATM	0.08429	NFKBIA	148	JAK2	0.07383	ATM	22.49	TGFB1	4.91611E+36	ATM	148	MAPK14	105788
IGF1	3400.95	KIT	0.08429	BCL2L1	147	SOX2	0.07369	HGF	22.49	STAT1	3.31280E+36	BCL2L1	147	PTPN11	105016
KDR	3383.64	IGF1R	0.08429	TLR4	146	PIK3R1	0.07275	PPARG	22.26	KDR	3.25620E+36	TLR4	146	ITGB1	104348
DICER1	3267.78	NFKBIA	0.08428	CDKN1A	143	PTGS2	0.07271	MMP2	22.14	SOX2	3.20016E+36	CDKN1A	143	ATM	102894

EPC – Edge Percolated Component; MCC – Maximal Clique Centrality; MNC – Maximum Neighborhood Component

Pathway enrichment analysis of exosomal miRNAs

The main results of pathway enrichment analysis of miRNAs with over 2 FR in the LG and HG groups are listed in Tables 5, 6. The enriched localisation of miRNAs comprosed exosome, microvesicle, and circulated with high significance in both the LG and HG groups. In the KEGG pathway and MNDR disease categories, mainly cancer-related subcategories were found.

We could not find statistically significant enriched pathways using the list of down-regulated miRNAs due to the small numbers of miRNAs in each group.

Table 7. *Key target genes of urinal exosomal miRNAs in low-grade and high-grade groups*

Low grade	High grade
TP53	*TP53*
CTNNB1	*AKT1*
GAPDH	*CTNNB1*
AKT1	*MYC*
HSP90AA1	*EGFR*
MYC	*JUN*
EGFR	*SRC*
PTEN	*IL6*
SRC	*PTEN*
HSPA8	*CDH1*
ESR1	*HSP90AA1*
NOTCH1	*STAT3*
JUN	*HRAS*
MTOR	*ESR1*
KRAS	*HIF1A*
CDH1	*CCND1*
HIF1A	*FN1*
CDKN2A	*VEGFA*
VEGFA	*CASP3*
	NOTCH1
	ERBB2
	RHOA
	IL1B
	SMAD3
	MTOR
	KRAS
	PPARG
	MAPK1
	SMAD4
	FOXO3
	CDKN2A

Analysis of target genes of up-regulated miRNAs and their interactions

A total of 1,425 predicted target genes were identified for 91 up-regulated miRNAs in the LG group and 805 target genes for 71 up-regulated miRNAs in the HG group. Using these genes, regulatory gene interaction networks were constructed. To select hub genes, the top 50 ranking genes were compared in each 4 methods of CytoNCA and CytoHubba (Figure 3, Tables 5, 6). The common genes in the list of CytoNCA and CytoHubba were extracted (Table 7). Nineteen hub genes in the LG up-regulated miRNA target group and 32 in the HG up-regulated miRNA target group were extracted. The 17 target genes were overlapped in both groups.

In both hub gene analyses with each 4 methods, *TP53* is ranked first except MCC of CytoHubba (ranked at 34) and *PTEN* was ranked between 6 and 11 in the LG (Table 5) and between 7 and 10 in the HG (Table 6).

DISCUSSION

Urine exosomes contain miRNAs from cells in the urinary tract [20]. The profile of the urine exomiRs is considered to reflect the cell condition. These miRNAs are known to modulate other cells that receive the exosomes [7]. The aim of this study is to compare the urine exomiRs in the healthy control group, and the LG and the HG cancer patient groups to understand the cell-cell molecular information exchange and the cancer development.

During the study, several miRNAs that were not previously described as altered in BC were found.

These novel miRNAs are mir-let-7f-2-3p; mir-28-5p, mir-196b-3p; mir-450a-5p, mir-320b; mir-151a-3p, mir-20b-3p; mir-425-5p, mir-376c-3p; let-7e-3p, mir-24-2-5p, mir-181a-3p, mir-664a-3p, mir-331-3p, mir-99b-5p, mir-28-3p, mir-501-3p, mir-500a, mir-628-5p, mir-7-1-3p, mir-105-5p, mir-342-5p, mir-455-3p, mir-365a-3p, mir-181d-5p, mir-423-3p, and mir-548m. Moreover, the profile of differentially expressing exomiRs in BC in this study widely covers already reported BC-related miRNAs.

There are 2 patterns observed for up-regulated miRNAs relative to the control group: 1) HG > LG or HG alone and 2) LG > HG or LG alone.

In the first pattern, highly expressed miRNAs include miR-126-3p, detected in plasma exosomes of HG BC patients [21] and BC urine samples [22]. MiR-126-3p acts as both oncogenic and a tumour suppressor depending on the tissues and tumourigenesis timing. It was proposed that mir-126-3p secretion into exosomes accelerates tumour development by stimulating

angiogenesis and promoting oncotransformation of non-cancer cells [21].

Substantial up-regulation of miR-21-5p, miR-205-5p, and miR-141-3p in urine samples was detected in the case of BC and prostate cancer [23], which is similar to our results (Table 2).

Our results show upregulated miR-628-3p, which matched with studies on MIBC patients where elevated miR-628-3p levels in urine compared to plasma EVs was found, while no significant difference was noted in isolated MIBC tissues [21].

Up-regulation of miR-200 families in BC tissue has been reported and suggested as a marker of poor prognosis [24]. In this study, the miR-200 family were found as up-regulated exomiRs in both cancer groups.

In the case of the second regulatory model, the following results were found. MiR-210-3p overexpression in urine sediment from BC patients [25] contrasts with down-regulation in BC tissue, where it inhibits tumour growth and metastasis by acting on fibroblast growth factor receptor-like 1 [26].

Up-regulation of miR-31-3p and miR-31-5p, contrary to their previously reported down-regulation in urothelial BC tissues [27], is evident in our study. Juracek et al. found that urinary miR-31-5p levels were significantly higher in BC patients compared to healthy controls and patients with ccRCC. Moreover, miR-31-5p concentration decreased significantly in disease-free patients when urine was re-analysed 3 months after surgery compared to preoperative samples [28]. Overexpression of miR-31

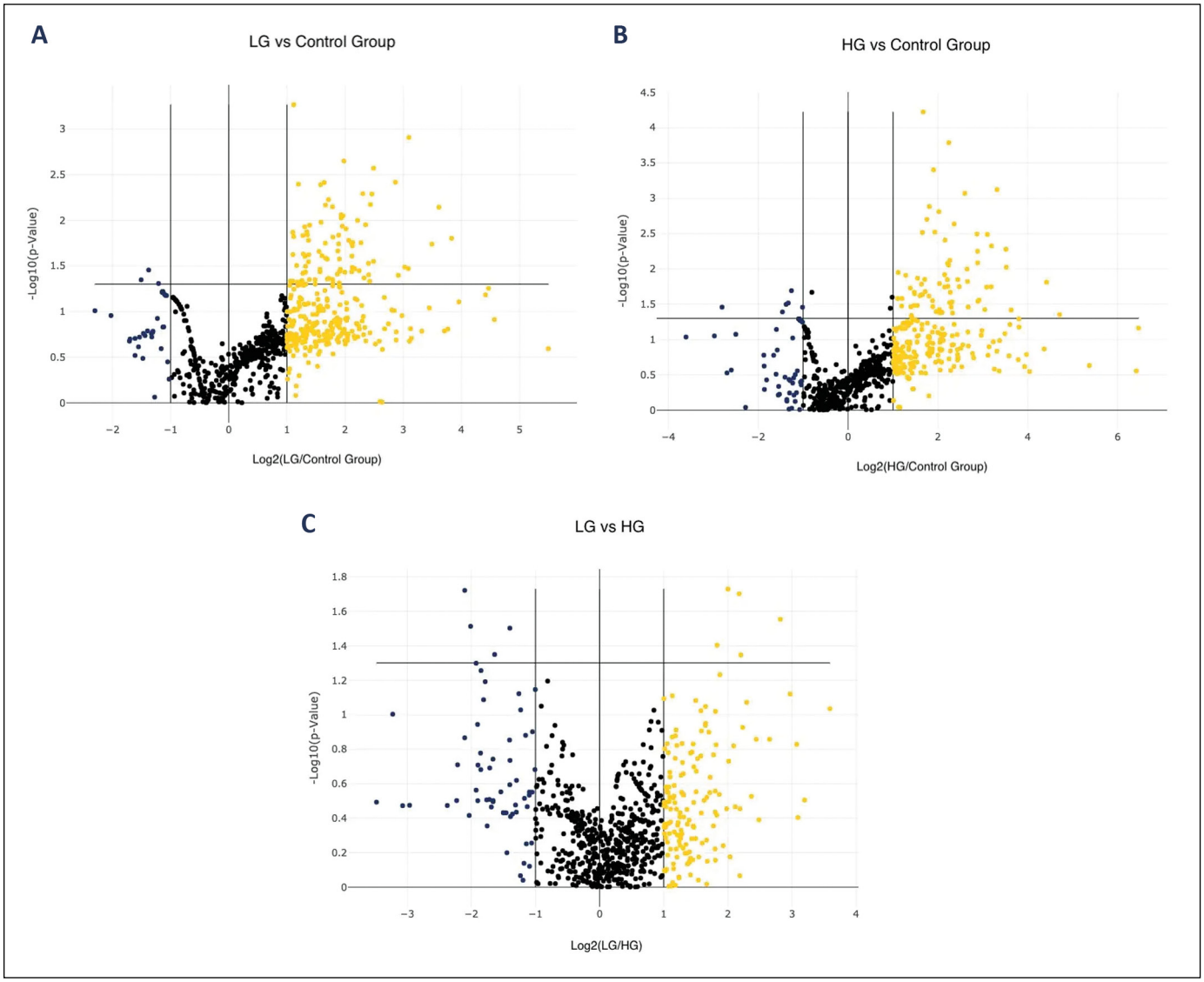

Figure 2. *Volcano plots of comparison.* **A)** *Wild type low-grade to control group;* **B)** *wild type high-grade to control group;* **C)** *wild type low-grade to high-grade group.*

in BC tissues was found to suppress BC cell proliferation by acting as a tumour suppressor [27].

Regarding miRNAs targeting *TP53* and *PTEN*, KEGG pathway analysis showed the gene targets by deregulated miRNAs and their role in biological processes. The results of this analysis proved the reliability of our results, because several of them are associated with different cancers. *TP53, AKT1, MYC*, and *PTEN* have been previously described as target genes in BC development [29], which was also reflected in our data (Table 7).

Previous investigations have highlighted correlation of *TP53* mutations with clinical features such as tumour grade, invasiveness, recurrence rate, and poor prognosis [30, 31].

In a meta-analysis of 7 studies, it was reported that *TP53* mutation was significantly higher in MIBC compared with NMIBC [32].

Comparing LG and HG profiles, miRNAs targeting *TP53* and *PTEN* exhibited higher fold regulation (FR) values in HG (Table 4), suggesting an accelerated cancer development state compared to LG BC. Considering the modulatory potential of exosomal miRNAs on cell conditions [7, 32], it is plausible that miRNAs targeting tumour suppressors, including *TP53* and *PTEN*, contribute to the oncotransformation of normal bladder cells.

Our hypothesis aligns with observations that BC patients often exhibit hyperplasia, dysplasia, and multiple synchronous cancers. Multiple synchronous and metachronous BC has been explained by the intraepithelial migration of tumour cells and intraluminal seeding from a primary cancer [33]. Lindgren et al. note that synchronous cancers may not consistently display the same genomic rearrangements or mutations in specific genes [34], implying an alternative mechanism for inducing oncotransformation in normal bladder cells affected by BC. Urine exomiR profiles in this study suggest the potential role of these miRNAs in repressing *TP53* and *PTEN*, contributing to oncotransformation in normal cells exposed to exosomes from developed BC.

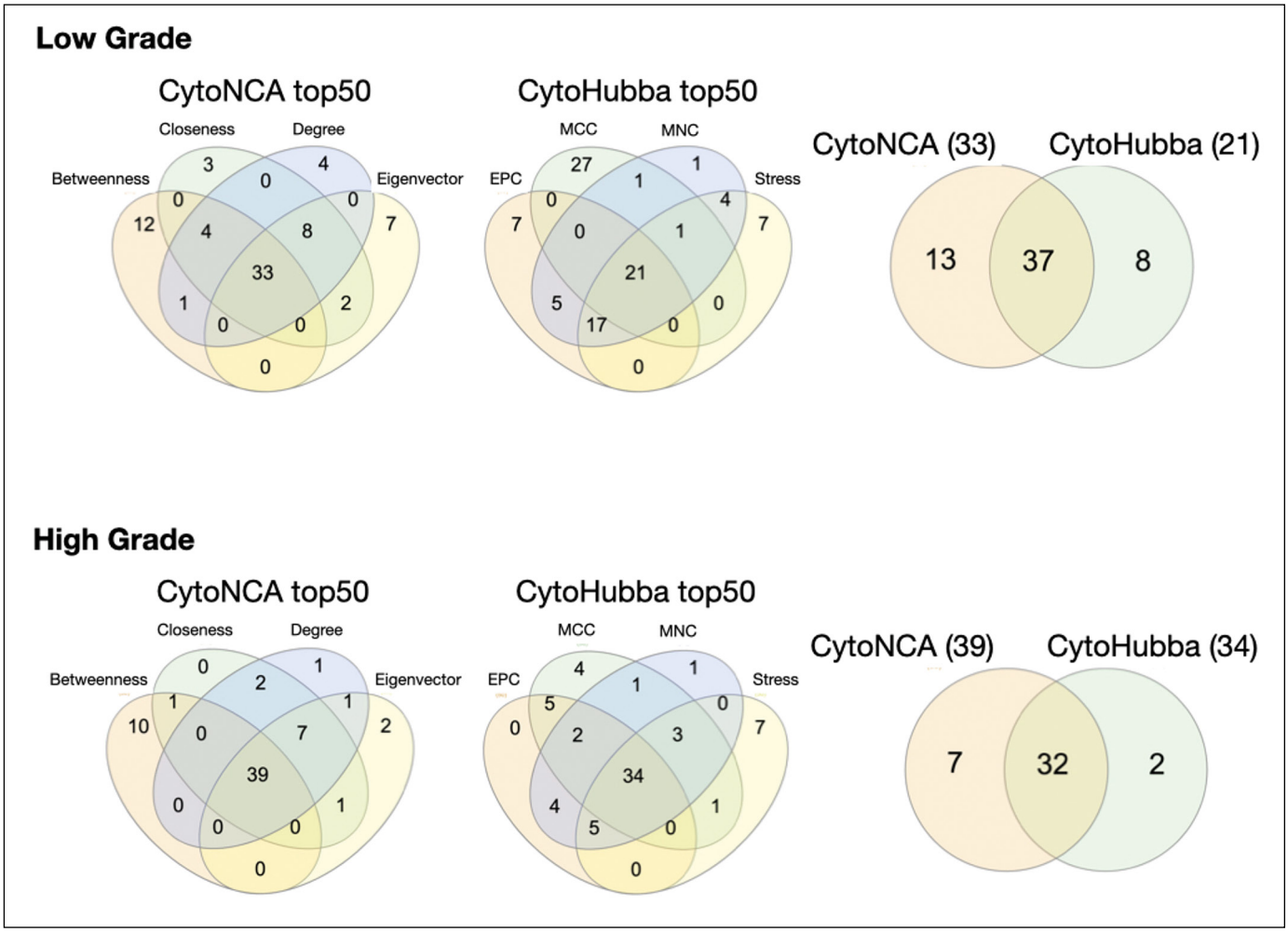

Figure 3. *Network of the target genes of up-regulated differentially expressed miRNAs in LG group and in HG group.*

A regulatory relationship between multiple miRNAs and *PTEN* has been reported [29], with miR-21, known for its significance in BC tumourigenesis, which regulates the proliferation and migration of cancer cells by its communication pathways with *PTEN* and *TP53*. Its overexpression has a suppressive effect on *TP53* [35, 36]. Furthermore miR-18a, miR-20a, miR-21, and miR-221 in network analysis were found to target *PTEN*, which in turn regulates miR-19a, miR-21, and miR-25, and as a consequence miR-25 targets *TP53* [37]. The strong connection of miR-205-5p with *PTEN* in the HG group further underscores the intricate regulatory networks involved (Table 4). However, the complexity of bladder tumours is evident because *PTEN* alterations alone may not consistently result in tumourigenesis [38, 39], emphasising the multifaceted changes in expression across various components, including the *AKT*/*PI3K*/*mTOR* pathway [40]. Our regulatory gene interaction networks highlight multiple interactions with the central hub gene *TP53* (Figure 3). These findings underscore the intricate molecular landscape of BC, emphasising the necessity for a comprehensive understanding of the interconnected pathways for improved diagnostic and therapeutic strategies.

The limitations of this study include the small number of patients. The association of specific miRNAs with specific genes was established using data from a database, but it lacked confirmation from biological studies. Nevertheless, our results demonstrate that the methods used were effective because many previously described miRNAs associated with BC were detected in our profile of BC exomiRs, as well as their downstream influence from both tumour suppressors and oncogenes. The majority of the urine exomiRs in BC patients were up-regulated, and their FRs compared with the control were high enough to distinguish between BC patients and healthy control groups. The identified miRNAs have potential value as biomarkers for BC detection.

Another issue of this study is that only urine samples were analysed, and neither serum nor urinary bladder tissues were examined. It would be worthwhile comparing the expression levels of differentially expressed miRNAs in BC tissues with urinary exosomes. The main challenge in identifying miRNAs that can serve as reliable biomarkers for BC is the widespread alteration of their expression in various conditions, including other malignancies and non-cancerous diseases [7, 23, 28, 32]. This lack of specificity hinders the validation and clinical utility of individual miRNAs as diagnostic or prognostic tools.

CONCLUSIONS

In this study, a number of differently expressed miRNAs, which can potentially be used as diagnostic biomarkers, were identified, but this needs further work in a validation phase. Profiles of urinal exomiRs derived from bladder cancer manifested potentially epigenetic regulation of the *TP53* and *PTEN* genes as compared to other oncogenes and tumour suppressors.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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ETHICS APPROVAL STATEMENT

The study was approved by the Central Medical Ethics Committee of Latvia (Nr.1/19-02-12) on 12.02.2019.

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