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Original Article

Clinical role of serum expression of miR-222 as a novel non-invasive predictor for bladder cancer

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Abstract: In this study, we aimed to characterize miR-222 expression in serum as a non-invasive biomarker for diagnosis of bladder cancer (BC) and differentiation of muscle invasive bladder cancer (MIBC) from non-muscle invasive bladder cancer (NMIBC). Serum samples were obtained from 98 BC patients and 100 healthy individuals. Serum levels of miR-222 were detected by qRT-PCR analysis and further associated with the clinicopathological characteristics and prognosis of BC patients, and also compared between patients with MIBC and NMIBC. Receiver operating characteristic (ROC) curve analysis was conducted to assess the diagnostic value of serum miR-222 in BC screening and differentiation of MIBC from NMIBC. We found that serum miR-222 levels in BC patients were significantly higher in comparison with that in healthy controls, and the levels of serum miR-222 in patients with MIBC were significantly higher than that in patients with NMIBC. Upregulation of serum miR-222 was greatly associated with aggressive clinicopathological features and poor prognosis in BC patients. According to ROC curve analysis, between BC patients and healthy controls, the area under the curve (AUC) of serum miR-222 was 0.857, and its specificity and sensitivity were 0.745 and 0.920 at a diagnostic threshold of 2.035. Between patients with MIBC and NMIBC, the AUC of ROC curve of serum miR-222 was 0.799, and its specificity and sensitivity were 0.743 and 0.841 at a diagnostic threshold of 3.705. Serum miR-222 can be utilized as a promising non-invasive molecular marker for BC screening and prognosis, as well as differentiation of MIBC from NMIBC.

Keywords: Circulating microRNA, miR-222, biomarker, bladder cancer, muscle invasion, diagnosis, prognosis

Introduction

Bladder cancer (BC) is characterized as one of the most malignant neoplasms worldwide, which causes a large body of cancer-related death [1]. Although great improvements of treatment strategies and management have been achieved in the past several decades, the prognosis of BC, especially survival outcomes, remains dismal. According to clinical diagnosis, BC could be divided into non-muscle invasive bladder cancer (NMIBC) and muscle invasive bladder cancer (MIBC). MIBC, which might lead to an extreme cancer-specific mortality, was accounted for approximately 30% of the total BC cases [2, 3]. Therefore, it is of crucial importance for us to explore the underlying mechanisms of bladder carcinogenesis, which may contribute to the discovery of a novel diagnostic biomarker for BC in the near future.

MicroRNAs (miRNAs) are a series of short, single stranded and noncoding molecules (18-24 nucleotides), which participate in regulating gene expression through binding with the target mRNAs [4]. Recent articles indicated that dysregulated miRNAs expression was associated with a great number of malignancies, such as prostate cancer [5], breast cancer [6], gastric cancer [7] and BC [8]. Moreover, increasing studies indicated that circulating miRNAs, detected in the body fluids, including plasma, serum and urine, could be applied in diagnosis of various cancers [9, 10]. However, there were limited studies about serum miRNAs in BC.

MiR-222 was previously reported to be upregulated in human cervical cancer [11]. Zhang et al. indicated that miR-222 overexpression was detected in BC tissues and involved in the poor prognosis of BC patients [12]. However, the

Table 1. Demographic characteristics of BC group and control group

Characteristics	BC group (n = 98)	Control group (n = 100)	*P* value
Age (year)			0.795
< 65	39	38	
≥ 65	59	62	
Gender			0.799
Male	81	84	
Female	17	16	
Smoking status			0.905
Yes	41	41	
No	57	59	
Drinking status			0.667
Yes	52	50	
No	46	50	

diagnostic values of serum miR-222 in BC remain to be further elucidated. Thus, the present study aimed to explore whether the dysregulated expressions of serum miR-222 could reflect bladder carcinogenesis and differentiate MIBC from NMIBC from clinical view. Our investigation would evaluate the potential role of serum miR-222 as a biomarker for diagnosis of BC.

Material and methods

Patients and serum samples

Serum samples were collected from 98 BC patients who received transurethral or partial cystectomy in Minhang Hospital (n = 61) and First People's Hospital (n = 37). Patients who had received radiotherapy or chemotherapy prior to blood sampling were excluded from this trial. Patients with other prior systemic diseases, including diabetes mellitus, liver cirrhosis and cardiovascular diseases were also excluded. For each case, the diagnosis and the histological grade were confirmed by two experienced pathologists. All tumors were classified according to the 2002 Union for International Cancer Control (UICC) TNM classification [13] and World Health Organization (WHO) 2004 grading system [14]. Fasting peripheral blood (5 mL) was obtained from each patient prior to surgery. The control group consisted of 100 healthy volunteers who had never received a diagnosis of malignancy previously. As recorded in **Table 1**, there was no significant difference of gender (P = 0.795), age (P = 0.799), smoking status (P = 0.905) and drinking status (P = 0.667) between the two groups.

Immediately after collection, the blood samples were centrifuged at 3,000 rpm for 10 min at 4°C; and then, the supernatant fluids were centrifuged at 15,000 rpm for 10 min at 4°C in another Eppendorf tubes. Supernatants were recovered, frozen in 0.2 mL aliquots and stored at -80°C until further analysis. Written informed consent for the present study was obtained from all of the participants (both patients and controls) before sample collections, and the experimental procedures were approved by the Ethical Review Committee of Minhang Hospital and First People's Hospital.

RNA isolation and qRT-PCR

RNA purity and concentration were investigated using a NanoDrop 1000 ultraviolet photometric machine (Thermo Scientific, Wilmington, DE, USA). The purity of RNA was investigated through detecting the absorbances of samples at 260 and 280 nm and calculating the 260/280 ratio (acceptable range 1.77-1.92). The differentially expressed amounts of miR-222 in serum samples were validated in triplicate by qRT-PCR. Briefly, 2 μg of total RNA was reverse-transcribed into cDNA using a miScript Reverse Transcription kit (Qiagen, Germany); the cDNA then served as the template for PCR amplification of PCR with sequence-specific primers (Sangon Biotech, Shanghai, China) using the SYBR PrimeScript miRNA RT-PCR kit (Takara Biotechnology Co. Ltd, Dalian, China) on the 7500 Real-Time PCR systems (Applied Biosystems, Carlsbad, CA, USA). The sequences of the primers were listed as follows: miR-222, RT: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGAGACC-3', forward primer: 5'-AGCTACATCTGGCTACTG-3' and reverse primer: 5'-GTGCAGGGTCCGAGGT-3'. Synthetic *Caenorhabditis elegans* miRNA (Cel-miR-39, Ambion, Austin, TX, USA) was used as the internal control for normalization [15]. The cycle threshold (CT) value was calculated. The $2^{-\Delta CT}$ ($\Delta CT = CT_{microRNA} - CT_{Cel\text{-}miR\text{-}39}$) method was used to quantify the relative amount of serum miR-222 expression.

Statistical analysis

Statistical analyses were performed using SPSS 13.0 soft-ware (Chicago, IL, USA) and

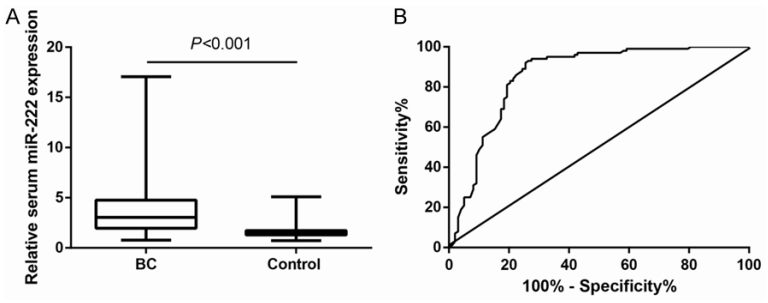

Figure 1. Serum miR-222 was upregulated in BC patients. A. Serum expression of miR-222 in the BC patients and the controls. B. ROC curve for detecting the diagnostic value of serum miR-222 for BC patients.

Table 2. Association between serum expression of miR-222 and clinicopathological characteristics of BC patients

Characteristics	Total number	Serum miR-222 expression		*P* value
		Low (n = 71)	High (n = 27)	
Age (years)				0.205
< 65	39	31	8	
≥ 65	59	40	19	
Gender				0.850
Male	81	59	22	
Female	17	12	5	
Smoking status				0.747
Yes	41	29	12	
No	57	42	15	
Drinking status				0.882
Yes	52	38	14	
No	46	33	13	
Tumor size (cm)				0.067
< 3.5	51	41	10	
≥ 3.5	47	30	17	
Tumor stage				< 0.001
Ta + T1 (NMIBC)	63	54	9	
T2 + T3 (MIBC)	35	17	18	
Tumor grade				0.063
G1	27	22	5	
G2	28	23	5	
G3	43	26	17	
Lymph node status				0.166
N0 + N1	84	63	21	
N2 + N3	14	8	6	
Distant metastasis				0.018
Yes	27	9	18	
No	71	62	9	

GraphPad Prism 5 (GraphPad Software Inc., CA, USA). A Chi squared test or independent samples t test was conducted when appropriate. Receiver-operating characteristic (ROC) curves were produced to evaluate the potential values of using serum miR-222 expression as a diagnostic biomarker for predicting BC and differentiating MIBC from NMIBC. When the Youden index (Youden index = sensitivity + specificity-1) reaches the maximum value, the matched threshold value will generate the highest sum of sensitivity and specificity. The survival curves were obtained by Kaplan-Meier analysis, and compared using the log-rank test. The logistic regression analysis was performed to identify factors that were independently associated with OS. For all statistic = al tests in this research, $P < 0.05$ was considered significant.

Results

Serum miR-222 was upregulated in BC patients

As shown in **Figure 1A**, the expression of serum miR-222 in the BC group was found to be significantly higher than approximately 2.55-fold in comparison with that in the control group ($P < 0.001$). The ROC curve analysis was thus performed to investigate the diagnostic accuracy of serum miR-222 for BC diagnosis. The AUC of ROC curve of serum miR-222 was 0.857 (95% CI: 0.802-0.913), and its specificity and sensitivity were 0.745 and 0.920 at a diagnostic threshold of 2.035, respectively (**Figure 1B**).

Serum miR-222 expression was associated with clinicopathological features of BC patients

The correlations between serum miR-222 levels and clinicopathological characteristics of BC patients were recorded in **Table 2**. The results indicated that increased serum miR-222

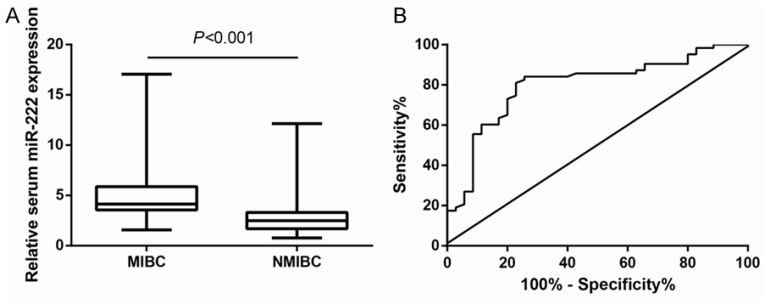

Figure 2. Serum miR-222 was upregulated in MIBC patients. A. Serum expression of miR-222 in the patients with MIBC and NMIBC. B. ROC curve for detecting the diagnostic value of serum miR-222 for differentiating MIBC from NMIBC.

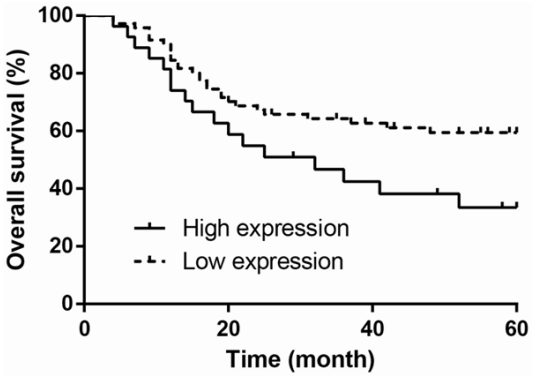

Figure 3. Serum miR-222 expression was associated with OS of BC patients. *P* value was calculated by log-rank test.

expression was closely related to tumor stage ($P < 0.001$) and distant metastasis ($P = 0.018$). However, no significant differences were found in age ($P = 0.205$), gender ($P = 0.850$), smoking status ($P = 0.747$), drinking status ($P = 0.882$), tumor size ($P = 0.067$), tumor grade ($P = 0.063$) and lymph node status ($P = 0.166$).

Serum miR-222 was upregulated in MIBC patients

As shown in **Figure 2A**, the expression of serum miR-222 in MIBC patients was found to be significantly higher than that in NMIBC patients ($P < 0.001$). The ROC curve analysis was further performed to investigate the diagnostic accuracy of serum miR-222 for differentiating MIBC from NMIBC. The AUC of ROC curve of serum miR-222 was 0.799 (95% CI: 0.706-0.892), with a sensitivity of 0.841, a specificity of 0.743 at a diagnostic threshold of 3.705, respectively (**Figure 2B**).

Serum miR-222 expression was associated with prognosis of BC patients

To assess the association of serum miR-222 with the prognosis of BC patients, Kaplan-Meier estimator analysis and the log-rank test were performed. As shown in **Figure 3**, BC patients with high serum expression of miR-222 had a significantly reduced OS compared with those with low serum expression of miR-222. In univariate analysis, advanced tumor stage ($P = 0.001$), distant metastasis ($P = 0.004$) and high serum miR-222 expression ($P = 0.040$) were associated with unfavorable prognosis of BC patients (**Table 3**). Multivariate logistic regression analysis revealed that serum miR-222 expression ($P = 0.048$) was an independent prognosis factor for OS of BC patients (**Table 4**).

Discussion

To date, miRNAs were considered as key regulators of gene expression, which were found to be involved in a large number of biological processes, including epigenetic regulation, cell cycle and cell differentiation [16-18]. With the rapid development of diagnostic technology, expressions of circulating miRNAs could be easily detected in a wide variety of biofluid samples, including plasma, serum and urine. Identification of noninvasive and invasive characteristics is of critical importance to select appropriate clinical management for BC patients [19]. In the present study, comparison to healthy individuals, we found that serum expression of miR-222 was obviously upregulated in BC patients. Our further investigations indicated that the expression of serum miR-222 was significantly higher in MIBC patients compared with NMIBC patients.

MiR-222 has also been reported to be overexpressed in multiple kinds of neoplasms [20-22]. Puerta-Gil et al. [23] indicated that miR-222 expression was significantly correlated to tumor grade, tumor size, presence of carcinoma *in situ*, and clinical outcome end points in BC patients. Similar articles uncovered that

Table 3. Univariate logistic regression analysis of factors associated with OS of BC patients

Characteristics	Univariate analysis		
	Risk ratio	95% CI	*P* value
Age (years)			
< 65 vs. ≥ 65	1.977	0.862-4.535	0.108
Gender			
Male vs. Female	0.427	0.138-1.323	0.140
Smoking status			
Yes vs. No	0.693	0.309-1.553	0.373
Drinking status			
Yes vs. No	0.704	0.316-1.566	0.389
Tumor size (cm)			
< 3.5 vs. ≥ 3.5	2.030	0.899-4.584	0.088
Tumor stage			
Ta + T1 vs. T2 + T3	4.773	1.966-11.585	0.001
Tumor grade			
G1 + G2 vs. G3	0.675	0.402-1.134	0.137
Lymph node status			
N0 + N1 vs. N2 + N3	2.400	0.741-7.776	0.144
Distant metastasis			
Yes vs. No	0.243	0.093-0.633	0.004
Serum miR-222 expression			
High vs. Low	0.383	0.153-0.956	0.040

Table 4. Multivariate logistic regression analysis of factors associated with OS of BC patients

Characteristics	Multivariate analysis		
	Risk ratio	95% CI	*P* value
Tumor stage			
Ta + T1 vs. T2 + T3	0.222	0.082-0.601	0.003
Distant metastasis			
Yes vs. No	0.555	0.260-1.186	0.128
Serum miR-222 expression			
High vs. Low	0.476	0.228-0.993	0.048

high miR-222 level in BC tissues was closely associated with tumor grade and tumor stage, which could be regarded as a predictor for BC patients [12]. In this study, we found a consistent conclusion that high-expressed miR-222 in serum samples was significantly associated with tumor stage and distant metastasis of BC patients.

MIBC was regarded as an extremely malignant type in BC, often with a poor prognosis for patients. Compared to patients with tumors of Ta and T1 stage, patients with MIBC often had lower body mass index, lower haemoglobin concentration, longer history of haematuria and longer time interval from first symptom to diagnosis [24]. Massari et al. [25] showed that patients with low expression of beta-tubulin-3/c-Myc had statistically significant better 2-year disease-free survival than those with mixed expression or double high expression, indicating that Beta-Tubulin-3 and c-Myc were prognostic markers in MIBC. Silvers et al. [26] demonstrated that periostin, an extracellular matrix protein overexpressed in many human cancers, was associated with poor clinical outcomes of patients with MIBC through affecting the surrounding tumor microenvironment and subsequently promoting cancer progression. The present study showed that serum miR-222 expression in MIBC patients was significantly increased than that in NMIBC patients, indicating that serum miR-222 can be considered as a promising non-invasive marker for differentiation of MIBC from NMIBC.

In conclusion, in spite of the above limitations, this study provided promising evidences that serum expression of miR-222 was obviously higher in BC patients than those in healthy controls. Besides, we also found that high expression of serum miR-222 was significantly correlated to poor clinicopathological features and prognosis of BC patients. Intriguing, subsequent investigations illustrated that the expression of serum miR-222 was markedly upregulated in MIBC patients compared with NMIBC patients. From clinical perspective, these results verified that serum miR-222 might function as a potential biomarker in the diagnosis and prognosis of BC, as well as a diagnostic tool for differentiating MIBC from NMIBC in the near future.

Disclosure of conflict of interest

None.

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References

[1] Cahn DB, Ristau BT, Ghiraldi EM, Churilla TM, Geynisman DM, Horwitz EM, Uzzo RG and Smaldone MC. Bladder preservation therapy: a review of the literature and future directions. Urology 2016; 96: 54-61.

[2] Prout GR and Marshall VF. The prognosis with untreated bladder tumors. Cancer 1956; 9: 551-558.

[3] Murta-Nascimento C, Schmitz-Drager BJ, Zeegers MP, Steineck G, Kogevinas M, Real FX and Malats N. Epidemiology of urinary bladder cancer: from tumor development to patient's death. World J Urol 2007; 25: 285-295.

[4] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-297.

[5] Kojima S, Goto Y and Naya Y. The roles of microRNAs in the progression of castration-resistant prostate cancer. J Hum Genet 2017; 62: 25-31.

[6] Asghari F, Haghnavaz N, Baradaran B, Hemmatzadeh M and Kazemi T. Tumor suppressor microRNAs: targeted molecules and signaling pathways in breast cancer. Biomed Pharmacother 2016; 81: 305-317.

[7] Sun W, Yang Y, Xu C, Xie Y and Guo J. Roles of long noncoding RNAs in gastric cancer and their clinical applications. J Cancer Res Clin Oncol 2016; 142: 2231-7.

[8] Martinez-Fernandez M, Rubio C, Segovia C, Lopez-Calderon FF, Duenas M and Paramio JM. EZH2 in bladder cancer, a promising therapeutic target. Int J Mol Sci 2015; 16: 27107-27132.

[9] Letelier P, Riquelme I, Hernandez AH, Guzman N, Farias JG and Roa JC. Circulating microRNAs as biomarkers in biliary tract cancers. Int J Mol Sci 2016; 17.

[10] Gyoba J, Shan S, Roa W and Bedard EL. Diagnosing lung cancers through examination of micro-RNA biomarkers in blood, plasma, serum and sputum: a review and summary of current literature. Int J Mol Sci 2016; 17: 494.

[11] Yang Y, Zhao X and Li HX. MiR-221 and miR-222 simultaneously target ARID1A and enhance proliferation and invasion of cervical cancer cells. Eur Rev Med Pharmacol Sci 2016; 20: 1509-1515.

[12] Zhang DQ, Zhou CK, Jiang XW, Chen J and Shi BK. Increased expression of miR-222 is associated with poor prognosis in bladder cancer. World J Surg Oncol 2014; 12: 241.

[13] Sano T, Coit DG, Kim HH, Roviello F, Kassab P, Wittekind C, Yamamoto Y and Ohashi Y. Proposal of a new stage grouping of gastric cancer for TNM classification: international gastric cancer association staging project. Gastric Cancer 2017; 20: 217-225.

[14] van Rhijn BW, Musquera M, Liu L, Vis AN, Zuiverloon TC, van Leenders GJ, Kirkels WJ, Zwarthoff EC, Boeve ER, Jobsis AC, Bapat B, Jewett MA, Zlotta AR and van der Kwast TH. Molecular and clinical support for a four-tiered grading system for bladder cancer based on the WHO 1973 and 2004 classifications. Mod Pathol 2015; 28: 695-705.

[15] Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, Lin DW, Urban N, Drescher CW, Knudsen BS, Stirewalt DL, Gentleman R, Vessella RL, Nelson PS, Martin DB and Tewari M. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A 2008; 105: 10513-10518.

[16] Margaillan G, Levesque E and Guillemette C. Epigenetic regulation of steroid inactivating UDP-glucuronosyltransferases by microRNAs in prostate cancer. J Steroid Biochem Mol Biol 2016; 155: 85-93.

[17] Wang Y and Blelloch R. Cell cycle regulation by microRNAs in stem cells. Results Probl Cell Differ 2011; 53: 459-472.

[18] Stevanato L and Sinden JD. The effects of microRNAs on human neural stem cell differentiation in two- and three-dimensional cultures. Stem Cell Res Ther 2014; 5: 49.

[19] Clark PE, Agarwal N, Biagioli MC, Eisenberger MA, Greenberg RE, Herr HW, Inman BA, Kuban DA, Kuzel TM, Lele SM, Michalski J, Pagliaro LC, Pal SK, Patterson A, Plimack ER, Pohar KS, Porter MP, Richie JP, Sexton WJ, Shipley WU, Small EJ, Spiess PE, Trump DL, Wile G, Wilson TG, Dwyer M and Ho M. Bladder cancer. J Natl Compr Canc Netw 2013; 11: 446-475.

[20] Ying X, Wu Q, Wu X, Zhu Q, Wang X, Jiang L and Chen X. Epithelial ovarian cancer-secreted exosomal miR-222-3p induces polarization of tumor-associated macrophages. Oncotarget 2016; 7: 43076-43087.

[21] Aragon Han P, Kim HS, Cho S, Fazeli R, Najafian A, Khawaja H, McAlexander M, Dy B, Sorensen M, Aronova A, Sebo TJ, Giordano TJ, Fahey TJ 3rd, Thompson GB, Gauger PG, Somervell H, Bishop JA, Eshleman JR, Schneider EB, Witwer KW, Umbricht CB and Zeiger MA. Association of BRAF (V600E) mutation and microRNA expression with central lymph node metastases in papillary thyroid cancer: a prospective study from four endocrine surgery centers. Thyroid 2016; 26: 532-542.

[22] Rinnerthaler G, Hackl H, Gampenrieder SP, Hamacher F, Hufnagl C, Hauser-Kronberger C, Zehentmayr F, Fastner G, Sedlmayer F, Mlineritsch B and Greil R. miR-16-5p is a stably-expressed housekeeping microRNA in breast cancer tissues from primary tumors and from metastatic sites. Int J Mol Sci 2016; 17.

[23] Puerta-Gil P, Garcia-Baquero R, Jia AY, Ocana S, Alvarez-Mugica M, Alvarez-Ossorio JL, Cordon-Cardo C, Cava F and Sanchez-Carbayo M. miR-143, miR-222, and miR-452 are useful as tumor stratification and noninvasive diagnostic biomarkers for bladder cancer. Am J Pathol 2012; 180: 1808-1815.

[24] Poletajew S, Biernacki R, Buraczynski P, Chojnacki J, Czarniecki S, Gajewska D, Pohaba T, Sondka J, Skrzypczyk M, Suchojad T, Wojtkowiak D, Zaforemski B, Zapala L, Zemla A and Radziszewski P. Stage of bladder cancer in central europe-polish perspective. Neoplasma 2016; 63: 642-647.

[25] Massari F, Bria E, Ciccarese C, Munari E, Modena A, Zambonin V, Sperduti I, Artibani W, Cheng L, Martignoni G, Tortora G and Brunelli M. Prognostic value of beta-tubulin-3 and c-Myc in muscle invasive urothelial carcinoma of the bladder. PLoS One 2015; 10: e0127908.

[26] Silvers CR, Liu YR, Wu CH, Miyamoto H, Messing EM and Lee YF. Identification of extracellular vesicle-borne periostin as a feature of muscle-invasive bladder cancer. Oncotarget 2016; 7: 23335-45.