

Urine exosome gene expression of cancer-testis antigens for prediction of bladder carcinoma

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Background: Exosomes have been regarded as emerging tools for cancer diagnosis. Tumor-derived exosomes contain molecules that enhance cancer progression and affect immune responses.

Material and methods: In the present study, we evaluated expression of seven cancer-testis antigens (CTAs) that are regarded as putative biomarkers and immunotherapeutic targets along with *NMP22* in urinary exosomes of bladder cancer patients, healthy subjects and patients affected with nonmalignant urinary disorders.

Results: Exosomal expression of *MAGE-B4* was significantly higher in bladder cancer patients compared with normal samples (expression ratio=2.68, $P=0.01$). However, its expression was lower in bladder cancer patients compared with benign prostate hyperplasia (BPH) patients (expression ratio=0.17, $P=0.01$). Exosomal expression of *NMP22* was significantly higher in bladder cancer patients compared with BPH patients (expression ratio=9.22, $P=0.02$). Expressions of other genes were not significantly different between bladder cancer patients and normal/nonmalignant samples. We found significant correlation between *MAGE-A3* and *MAGE-B4* expressions in exosomes obtained from controls. In addition, *TSGA10* expression was correlated with expression of *NMP22* in both cancer patients and controls.

Conclusion: The present study provides evidences for differential expression of CTAs in urinary exosomes of bladder cancer patients and urogenital disorders and warrants further studies for assessment of their significance in cancer diagnosis and immunotherapeutic approaches.

Keywords: cancer-testis antigen, bladder cancer, exosome

Introduction

Exosomes as natural extracellular nanoparticles have been detected in nearly all biofluids including urine. Assessment of exosome contents has led to identification of pathophysiological features of the corresponding tissues. Consequently, extensive efforts have been made to apply urinary exosome content as biomarkers with the hope to replace the invasive tissue biopsy.¹ The lipids, mRNA, and microRNA content of exosomes might participate in the pathogenesis of human disorders through altering intercellular communication.² In addition, cancer cell-derived exosomes can change tumor microenvironment in favor of cancer cell growth and arrange subsequent steps in the promotion of metastasis and chemoresistance.³ Bladder cancer cell-originated exosomes have been shown to suppress tumor cell apoptosis via induction of Akt and ERK pathway genes.⁴ Moreover, expression of an extracellular matrix-associated glycoprotein in urinary exosomes of bladder cancer patients has been demonstrated to enhance invasive properties of cancer cells and endothelial cells.⁵ Cancer-testis

antigens (CTAs) are among putative biomarkers in bladder cancer patients whose expressions are associated with clinical features of the corresponding tumors.⁶ They are also involved in the critical aspects of tumor proliferation, migration, and invasion.⁷ These properties along with their selective expression in tumoral tissues despite their absence in normal tissues make them appropriate targets in cancer immunotherapy.⁸ Consequently, their expression in cancer-derived exosomes might be involved in the pathogenesis of cancer on one hand and might be applied as therapeutic target on the other hand. The latter is enforced by the growing application of exosomes in specific delivery of drugs and other therapeutic agents to cancer cells.³ Based on our previous detection of CTA expression in urinary exfoliated cells (UEC) of patients with transitional cell carcinoma (TCC),⁶ we selected seven CTAs to assess their expression in urinary exosomes isolated from TCC patients and controls. As NMP22 is regarded as a marker of urothelial cell death whose expression is increased in the urine of TCC patients,⁹ we also assessed expression of NMP22 in urinary exosomes of all study participants.

Materials and methods

Sample collection

The Ethical Committee of Tehran University of Medical Sciences approved this study (ethical code: 23968-51-03-93). The study participants provided written informed consent, which was conducted in accordance with the Declaration of Helsinki. Random urine samples were collected from 59 patients with TCC, 24 healthy volunteers, 11 patients with bladder stone, six patients with obstructive uropathy, and eight patients with benign prostate hyperplasia (BPH). Urine samples were stored at 4°C until further assessments.

Urine exosome isolation

Urine exosomes were isolated by spin column protocol as provided in Norgen's Urine Exosome RNA Isolation Kit (BIOTEK Corporation, Thorold, ON, Canada). This kit establishes an all-in-one system for the exosome isolation and isolation of exosomal RNA from urine.

Urine exosome confirmation

The size and shape of exosomes were confirmed by western blotting, dynamic light scattering (DLS) assessments, and electron microscopy.

Western blotting

Urinary exosome were subjected to western blot analysis with antibodies against exosomal marker protein CD63. In

brief, the protein concentration of the preparations was evaluated using Bradford protein assay. BSA was applied as the standard sample. Samples were incubated for 5 minutes at 37°C and separated on 10% precasted gel. Subsequently, they were transferred to nitrocellulose membranes and blocked overnight (5% milk and 0.05% Tween-20 in PBS). Then they were incubated with primary antibody (Santa Cruz Biotechnology, Dallas, TX, USA.) for 1 hour, washed by PBS, and finally incubated with secondary HRP-conjugated antibody (SinaClon, Tehran, Iran). The corresponding immunoreactive bands were visualized using chemiluminescent detection system. The molecular weights of proteins were assessed using the prestained protein ladder (SinaClon).

DLS assessments

The exosomes were sized by DLS using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) according to the company guidelines.

Electron microscopy

A portion of the purified exosomes was fixed in 2.5% glutaraldehyde, dehydrated with mounting grades of ethanol, vacuum dried on a glass surface, and sputter coated with gold. The size and shape of the exosomes were evaluated using scanning electron microscopy (SEM) (The QUANTA SEM system, FEI Company, Hillsboro, OR, USA).

Exosomal RNA isolation

RNA was isolated from urine exosomes using Urine Exosome RNA Isolation Kit (Norgen, BIOTEK Corporation) according to manufacturer's instructions. This kit uses an all-in-one system for the concentration and isolation of exosomal RNA from biological samples. After binding of the urinary exosomes to a patented resin, RNA is refined from the exosome by means of a column-based method.

Quantitative real-time PCR analysis

The first strand cDNA was synthesized from RNA samples using PrimeScript™ RT reagent Kit (Takara, Tokyo, Japan). The relative transcript levels of CTA genes in urine exosomes were quantified in the rotor gene 6000 Corbett Real-Time PCR System using RealQ Plus 2x Master Mix Green (Ampliqon, Odense, Denmark). Gene expression analyses were performed in a total volume of 30 µL. 5S rRNA was used to normalize expression levels. All experiments were performed in duplicates. A cDNA pool was prepared for primary assessment of CTA gene expressions. CTA genes with no expression in this sample were excluded from further

studies. The nucleotide sequence of primers used in expression analyses are shown in Table 1.

Statistical methods

SPSS v.18.0 (IBM Corp., Armonk, NY, USA) was used for statistical analyses. The relative expression of each gene was quantified using $\text{Efficiency}^{\Delta\text{CT}}_{\text{target gene}} / \text{Efficiency}^{\Delta\text{CT}}_{\text{normalizer gene}}$ equation. The magnitude of expression of each gene between cancerous and control samples was described as fold change value and was calculated by dividing the obtained values in cancerous sample to the corresponding value of control sample. The association between clinicopathological data and relative expression of genes in TCC patients was calculated using independent *t*-test. Correlation between expression levels of genes in urine samples was assessed using Pearson correlation coefficient. The level of significance was set at $P < 0.05$ in all analyses. The receiver operating characteristic (ROC) curve was designed to appraise the suitability of gene expression levels for differentiation of TCC status from non-

malignant conditions. The Youden index (*j*) was applied to obtain the most difference between sensitivity (true-positive rate) and $1 - \text{specificity}$ (false-positive rate). The validity of transcript level of each gene for diagnosis of bladder cancer was described through calculation of the area under curve (AUC) values. AUC values were judged using the following method: 0.90–1=excellent (A), 0.80–0.90=good (B), 0.70–0.80=fair (C), 0.60–0.70=poor (D), and 0.50–0.60=fail (F).

Results

General data of patients

The general characteristics of study participants are summarized in Table 2.

Confirmation of exosome size and morphology

Assessment of isolated particles with SEM confirmed that we could effectively isolate exosomes with acceptable quality regarding their size range and morphology (Figure 1).

Table 1 The nucleotide sequence of primers used in expression analyses

| Gene name | Forward primer | Reverse primer |
|-----------------|------------------------|-----------------------|
| <i>NMP22</i> | AGATGACACTCCACGCCACC | TGCTGTCCCTCTTCAGTGCC |
| <i>MAGE-A3</i> | GTCGTCGGAAATTGGCAGTAT | TGGGGTCCACTTCCATCA |
| <i>MAGE-B4</i> | ACGAAGATGTTAGTGACGTTCC | GTGCGCTGAGAGACTTTCC |
| <i>TSGA-10</i> | ATGAGCGCCATTTGGCAGAA | GGGTAATTTCTTCTGTGCCTG |
| <i>AKAP4</i> | TAACGTGCCCATGCTCTACT | ACCTCCTACACTGTACCCCT |
| <i>SYCP1</i> | ACGGGAAGAAACCAGGCAAG | GGAATTCTCAGCTTGACACG |
| <i>OY-TES-1</i> | TGCTCCAACCTCCCTTATGC | CGTGGTGGGTGAGACTTCAG |
| <i>NY-ESO-1</i> | TCAGGGCTGAATGGATGCTG | TATGTTGCCGGACACAGTGA |
| <i>5S rRNA</i> | GCCCGATCTCGTCTGATCT | AGCCTACAGCACCCGGTATT |

Table 2 The general characteristics of study participants

| Study groups | Number of samples | Variables | Values |
|--------------|----------------------|-----------------------|---|
| TCC patients | 59 | Age (mean±SD [range]) | 61.28±13.01 (33–84) |
| | | Smoking | Negative 16 (27.1%) |
| | | | Positive 43 (72.8%) |
| | | Opium addiction | Negative 30 (50.8%) |
| | | | Positive 29 (49.1%) |
| | | Grade | High grade 34 (57.6%) |
| | | | Low grade 25 (42.3%) |
| Controls | Total | 49 | Age (mean ± SD [range]) 64.42±15.53 (31–88) |
| | | Smoking | Negative 30 (61.2%) |
| | | | Positive 19 (38.7%) |
| | Normal | 24 | Age (mean ± SD [range]) 68±13.56 (39–88) |
| | Obstructive uropathy | 6 | Age (mean ± SD [range]) 42.5±8.26 (33–52) |
| | Bladder stone | 11 | Age (mean ± SD [range]) 55.42±15.55 (31–84) |
| | BPH | 8 | Age (mean ± SD [Range]) 73±7.48 (54–81) |

Abbreviations: BPH, benign prostate hyperplasia; TCC, transitional cell carcinoma.

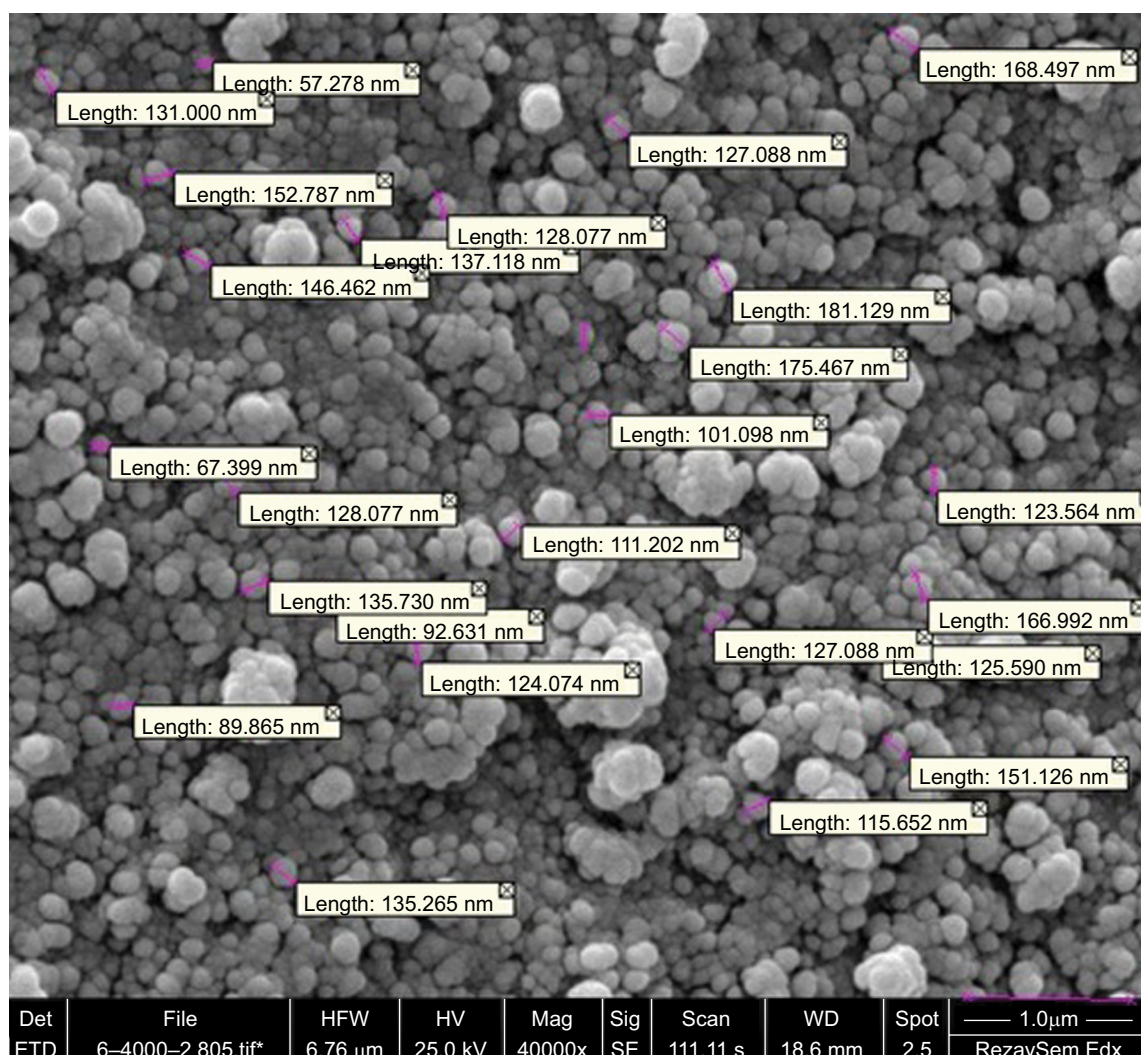


Figure 1 Scanning electron micrographs of fixed and dehydrated exosomes isolated from urine samples.

The results were also confirmed by DLS assessment (Figure 2). In addition, western blot analysis of samples showed the expression of CD63 as a common marker of exosomes.

Expression of CTA genes in urinary exosomes

After confirmation of exosome isolation through SEM and western blot analyses, we assessed the expression of seven CTAs along with *NMP22* in all samples. *SYCP1*, *OY-TES*, *NY-ESO-1*, and *AKAP4* expressions were not detected in the cDNA pool prepared from all exosomal samples, and so these genes were excluded from further steps. Other genes were detected in exosomes of both TCC patients and nonmalignant conditions. Exosomal expression of *MAGE-B4* was significantly higher in TCC patients compared with normal samples

(expression ratio=2.68, $P=0.01$). However, its expression was lower in TCC patients compared with BPH patients (expression ratio=0.17, $P=0.01$). Exosomal expression of *NMP22* was significantly higher in TCC patients compared with BPH patients (expression ratio=9.22, $P=0.02$). Expressions of other genes were not significantly different between TCC patients and normal/nonmalignant samples. Table 3 shows relative expression of genes in urinary exosomes of TCC patients compared with nonmalignant conditions.

Associations between exosomal expression of CTA genes and patients' clinicopathological features

No significant association was found between relative expression of genes in urinary exosomes of TCC patients and clinicopathological data (Table 4).

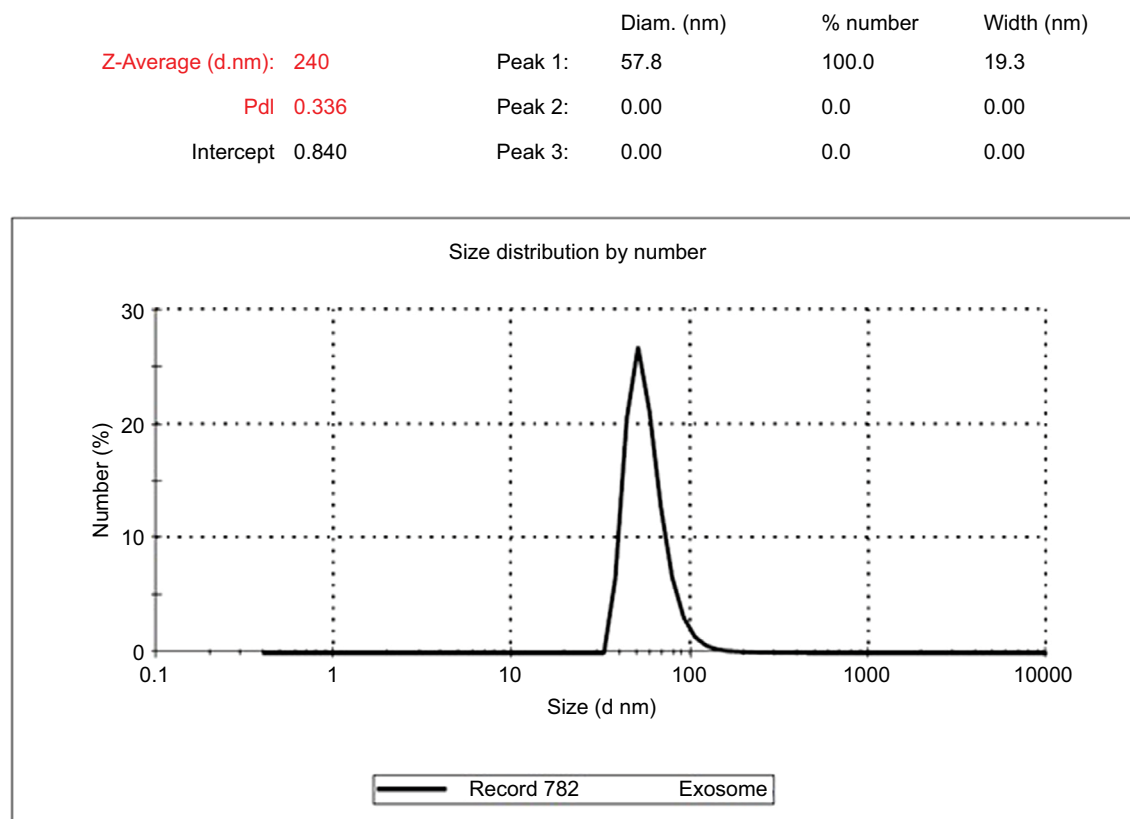


Figure 2 Size distribution of exosomes by volume as demonstrated by Zetasizer Nano ZS (Malvern Instruments).

Table 3 Relative expression of genes in urinary exosomes of TCC patients compared with nonmalignant conditions

| Gene names | TCC (n=59) vs nonmalignant conditions (n=49) | TCC (n=59) vs normal samples (n=24) | TCC (n=59) vs obstructive uropathy (n=6) | TCC (n=59) vs bladder stone (n=11) | TCC (n=59) vs BPH (n=8) |
|------------------|--|-------------------------------------|--|------------------------------------|-------------------------|
| <i>MAGE-A3</i> | | | | | |
| Expression ratio | 1.26 | 1.39 | 1.1 | 1.17 | 1.11 |
| P-value | 0.32 | 0.25 | 0.87 | 0.64 | 0.82 |
| <i>MAGE-B4</i> | | | | | |
| Expression ratio | 1.3 | 2.68 | 0.71 | 1.09 | 0.17 |
| P-value | 0.43 | 0.01 | 0.6 | 0.87 | 0.01 |
| <i>TSGA10</i> | | | | | |
| Expression ratio | 1.32 | 2.18 | 0.04 | 2.86 | 1.1 |
| P-value | 0.37 | 0.11 | 0.25 | 0.35 | 0.7 |
| <i>NMP22</i> | | | | | |
| Expression ratio | 0.9 | 0.47 | 0.37 | 1.09 | 9.22 |
| P-value | 0.78 | 0.15 | 0.28 | 0.92 | 0.02 |

Abbreviations: BPH, benign prostate hyperplasia; TCC, transitional cell carcinoma.

Correlations between exosomal expressions of CTA genes

We assessed correlations between expression levels of mentioned genes in urinary exosomes of both TCC patients and nonmalignant conditions. We found significant correlation between *MAGE-A3* and *MAGE-B4* expressions in exosomes obtained from controls. In addition, *TSGA10* expression was

correlated with expression of *NMP22* in both TCC patients and controls. Table 5 shows the results of correlation analysis between relative expressions of genes in urinary exosomes.

ROC curve analysis

As we demonstrated significant overexpression of *MAGE-B4* in urinary exosomes of TCC patients compared with normal

Table 4 Associations between relative expression of genes in urinary exosomes of TCC patients and clinicopathological data

| Demographic data | MAGE-A3 | P-value | MAGE-B4 | P-value | TSGA10 | P-value | NMP22 | P-value |
|-------------------|-----------|---------|-------------|---------|---------------|---------|--------------|---------|
| Age | | 0.62 | | 0.14 | | 0.23 | | 0.22 |
| <60 years | 0.02±0.03 | | 0.01±0.02 | | 107.34±442.58 | | 0.03±0.12 | |
| ≥60 years | 0.03±0.14 | | 0.005±0.009 | | 0.02±0.11 | | 0.0007±0.001 | |
| Cigarette smoking | | 0.47 | | 0.91 | | 0.58 | | 0.07 |
| Yes | 0.03±0.12 | | 0.008±0.01 | | 57.02±322.58 | | 0.0006±0.001 | |
| No | 0.01±0.01 | | 0.007±0.01 | | 0.06±0.18 | | 0.04±0.04 | |
| Opium addiction | | 0.32 | | 0.95 | | 0.37 | | 0.29 |
| Yes | 0.05±0.17 | | 0.009±0.02 | | 101.38±530.11 | | 0.0006±0.001 | |
| No | 0.01±0.02 | | 0.009±0.01 | | 0.003±0.01 | | 0.03±0.12 | |
| Grade | | 0.31 | | 0.27 | | 0.44 | | 0.2 |
| High grade | 0.04±0.14 | | 0.006±0.009 | | 72.99±364.96 | | 0.001±0.002 | |
| Low grade | 0.01±0.01 | | 0.01±0.02 | | 0.003±0.01 | | 0.03±0.13 | |

Note: Relative expression levels were calculated using $\text{Efficiency}^{\Delta\text{CT}}_{\text{normalizer gene}} / \text{Efficiency}^{\Delta\text{CT}}_{\text{target gene}}$ formula and data are presented as mean±SD.

Abbreviation: TCC, transitional cell carcinoma.

Table 5 Pearson correlation coefficient values between relative expression of genes in urinary exosomes of TCC patients and controls

| Demographic data | NMP22 | TSGA10 | MAGE-B4 |
|------------------|-------|--------|---------|
| MAGE-A3 | | | |
| TCC samples | 0.02 | 0.03 | 0.04 |
| Controls | 0.007 | 0.01 | 0.23** |
| MAGE-B4 | | | |
| TCC samples | -0.02 | -0.02 | |
| Controls | 0.05 | -0.02 | |
| TSGA10 | | | |
| TCC samples | 0.12* | | |
| Controls | 0.12* | | |

Note: Relative expression levels were calculated using $\text{Efficiency}^{\Delta\text{CT}}_{\text{normalizer gene}} / \text{Efficiency}^{\Delta\text{CT}}_{\text{target gene}}$ formula. * $P < 0.05$, ** $P < 0.01$.

Abbreviation: TCC, transitional cell carcinoma.

males, we assessed the performance of transcript levels of this gene in diagnosis of cancer status using ROC curve analysis (Figure 3). Transcript levels of this gene could be used as a diagnostic tool for bladder cancer with sensitivity and specificity of 71.7% and 66.7%, respectively (Table 6).

Discussion

In the present study, we isolated and purified urinary exosomes from samples obtained from TCC patients as well as other urogenital disorders. Size, morphology, and expression of CD63 as a protein hallmark in these exosomes were similar to those of exosomes obtained from other biofluids.^{4,10} We further assessed expression a mini-set of CTAs in the isolated exosomes and demonstrated expression of three CTAs namely *MAGE-A3*, *MAGE-B4*, and *TSGA10* in urinary exosomes of TCC patients and nonmalignant conditions. The unique

expression pattern of CTAs in immune-privileged sites and their re-expression in a wide range of cancers have potentiated them as a cancer vaccine candidate.¹¹ Moreover, most of CTAs are considered as intrinsically disordered proteins (IDPs). Contrary to typical proteins that have unique three-dimensional folding, IDPs could adopt different conformations to expand their interacting partners. This flexibility let them to be key players of various cellular processes acting as a hub in signaling pathways and cell cycle progression or act as a platform to assemble other protein compartments.¹²

MAGE-A3, *MAGE-B4*, and *TSGA10* were among the most specifically expressed CTAs in TCC samples as revealed in our previous study.⁶ Moreover, *MAGE-A3* expression and spontaneous immune responses against the encoded protein have been detected in several human malignancies. Such observations have led to establishment of several clinical trials of *MAGE-A3* vaccines in cancer patients with preliminary promising results.⁸

On the other hand, we could not detect *SYCP1*, *OY-TES*, *NY-ESO-1*, and *AKAP4* expressions in urinary exosomes isolated from TCC patients or nonmalignant conditions. *NY-ESO-1* has been among the most frequently expressed CTAs in bladder cancer tissues.¹³ The existence of spontaneous humoral and cellular immune responses in cancer patients has placed this CTA in the top of the list of CTAs being applied in immunotherapeutic clinical trials.⁷ We could not previously detect expression of *AKAP4* in either UECs or tumoral tissues of TCC patients by conventional RT-PCR.⁶ However, to the best of our knowledge expressions of *SYCP1*, *OY-TES*, and *NY-ESO-1* have not been assessed in UECs of TCC patients yet. Considering the importance of urinary exosomes and UECs as two separate tools for noninvasive

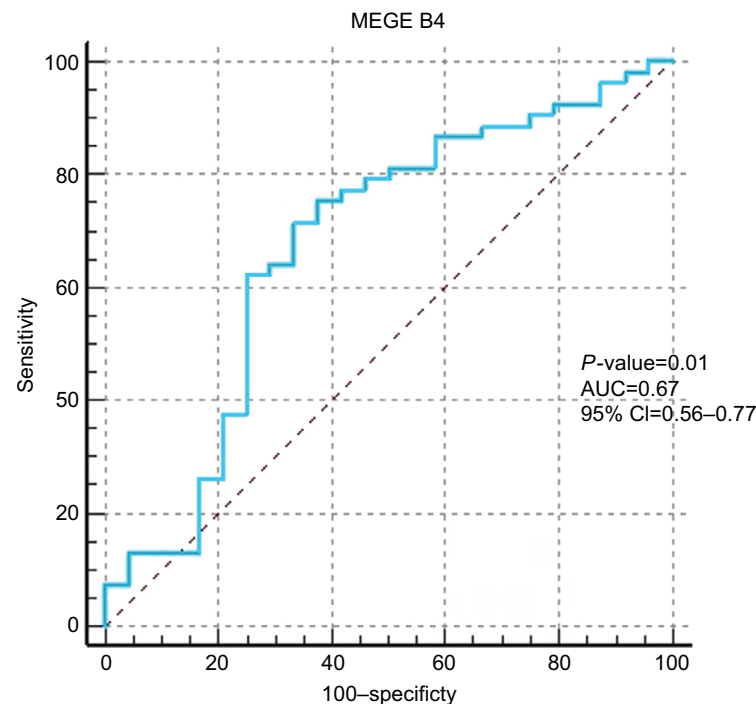


Figure 3 The results of ROC curve analysis of performance of *MAGE-B4* transcript levels in urinary exosomes for diagnosis of bladder cancer.

Abbreviations: AUC, area under the curve; ROC, receiver operating characteristic.

Table 6 The results of ROC curve analysis

| Gene name | Estimate criterion | AUC | J ^a | Sensitivity | Specificity | 95% CI | P-value ^b |
|----------------|--------------------|------|----------------|-------------|-------------|-----------|----------------------|
| <i>MAGE-B4</i> | ≤11.02 | 0.67 | 0.38 | 71.7 | 66.7 | 0.56–0.77 | 0.01 |

Note: ^aYouden index, ^bsignificance level of *P* (area=0.5), estimate criterion: optimal cutoff point for gene expression.

Abbreviations: AUC, area under curve; ROC, receiver operating characteristic.

detection of bladder cancer, parallel assessment of these two sources might facilitate design of novel diagnostic panels.

We demonstrated higher expression of *MAGE-B4* in urinary exosomes of TCC patients compared with normal samples, which is in line with the proposed role for MAGE proteins in inhibition of apoptosis and enhancement of tumor cell survival.¹⁴ However, *MAGE-B4* expression was lower in TCC patients compared with BPH patients. We previously demonstrated specific expression of *MAGE-B4* in TCC tissues and UEC samples of patients compared with noncancerous tissues and control UEC samples.⁶ Consequently, *MAGE-B4* seems to have a parallel expression in both UECs and exosomes, which facilitates its application as a diagnostic biomarker. In spite of frequent expression of *MAGE* genes in tumoral tissues, the evaluation of function of *MAGE* genes in normal development has been precluded by absence of domains with noticeable homology to a definite motif or domain with acknowledged

function.¹⁵ Necdin, as one putative member of the MAGE family, is expressed in neurons where it interacts with viral transforming proteins and the transcription factor E2F1 leading to inhibition of E2F1-dependent transcription.^{16,17} The observed different pattern of *MAGE-B4* expression between TCC and BPH samples might be attributed to low expression of E2F1 in BPH¹⁸ in spite of its activation in bladder cancer cells.¹⁹ Few studies have reported a causal link between the presence of viral transforming proteins and development of both primary urothelial carcinoma and distant metastases.²⁰ Future studies should focus on assessment of interactions between *MAGE-B4* and viral transforming proteins and the significance of it in immunotherapy of TCC.

We also detected higher exosomal expression of *NMP22* in TCC patients compared with BPH patients, which is in line with the frequently reported elevated levels of NMP22 protein in urine samples of TCC patients.²¹

In spite of the observed significant upregulation of *MAGE-B4* in TCC exosomes, transcript levels of this gene had the sensitivity and specificity values of 71.7% and 66.7%, respectively. Future studies are necessary to assess its suitability to be included in a panel of urinary exosome transcripts for diagnosis of bladder cancer.

Finally, we found significant correlation between *MAGE-A3* and *MAGE-B4* expressions in exosomes obtained from controls, which is consistent with the presence of a common epigenetic mechanism for expression of *MAGE* genes.¹⁴ In addition, *TSGA10* expression correlated with expression of *NMP22* in both TCC patients and controls. *TSGA10* is a CTA with widespread expression in human malignancies. However, its forced expression in HeLa cells has disrupted HIF-1 α axis and suppressed tumor angiogenesis and metastasis.²² On the other hand, *NMP22* is a nuclear matrix protein, which is released from cells during apoptosis.²³ The observed correlation between *TSGA10* and *NMP22* expressions in both cases and controls might reflect a functional link between these two transcripts, which should be evaluated in future studies.

In brief, in the present study we could demonstrate expression of three CTA transcripts in urinary exosomes and assess their potential in TCC diagnosis. Based on the differential expression of some CTAs in urological disorders, they might be applied as diagnostic markers in these kinds of disorders. However, the relative small sample size has been a limitation of our study. Future studies are needed to evaluate this hypothesis in larger sample sizes.

Acknowledgments

The current study was supported by a grant from Tehran University of Medical Sciences.

Disclosure

The authors report no conflicts of interest in this work.

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