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Increased expression of urinary exosomal LncRNA TUG-1 in early bladder cancer

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ABSTRACT

Background: Bladder cancer (BC) is known as one of the most common cancers in the world with high mortality rate. The diagnosis of BC is highly reliable on invasive methods which is not pleasant for patients. Exosomal Long non-coding RNAs (lncRNAs) have been widely studied in the last few years as a potential biomarkers and different studies show pleasing results in this regard. The aim of this study is to evaluate the expression level of two oncogenic lncRNAs (MALAT-1 and TUG-1) in the urinary exosomes of early stages of BC.
Material and methods: A total of 40 men enrolled in this study which classified as 30 BC patients and 10 healthy individuals. The fresh urine samples were collected from subjects and after the validation of exosome existence, exosome were extracted. The total RNA of exosomes was extracted base on silica-gel based membrane. Expression level of MALAT-1 and TUG-1 were determined using qRT-PCR. The relationship between their levels and clinicopathological factors of patients with bladder cancer was explored. A receiver operating characteristic (ROC) curve was constructed for differentiating bladder cancer from healthy subjects.
Result: TUG-1 and MALAT-1 differential expression levels between tumor and non-tumorous urine samples were 8.7 and 1.4, respectively. There was no significant correlation between the expression level of MALAT-1 and TUG-1 and clinicopathological characteristics. Also no significant difference was found between the expression of lncRNAs among stage I and stage II.
Conclusion: Taken together this study has shown that TUG-1 is significantly upregulated in early stages of bladder cancer and could be a potential non-invasive urinary exosome-based biomarker.

1. Introduction

Bladder cancer (BC) is one of the most common cancer types with the diagnostic and mortality rate of 549.393 and 199.922 respectively all around the world (Bray et al., 2018) with 79,030 and 16,780 in the united states (Siegel et al., 2017). According to the reports from American cancer society and Atlas of genetics and cytogenetics in oncology and hematology WHO reports (Compérat et al., 2018) BC is classified in three different categories, each of them are sub-divided into different classes (Kamat et al., 2016; Sanli et al., 2017). Moreover, BC can be classified according to the gene expression pattern into basal and luminal type, like breast cancer, which is explained in details by The Cancer Genome Atlas Research Center and Damrauer JS et al. (Network CGAR, 2014; Damrauer et al., 2014).

Detection of BC is one of the most important challenges in the treatment and survival time of patients as well as in other malignancies. Cystoscopy and CT urography are the gold standard methods for BC detection with high sensitivity and specificity. However, these two methods are invasive and unpleasant for patients (Helenius et al., 2015). Cytology is another method for BC detection which has high specificity (>90%), but it has low sensitivity (<50%) specially in the detection of low grade tumors (Schmitz-Dräger et al., 2015). Besides the current methods, there are also some urine biomarker that can be used for BC detections including BTA (bladder tumor antigen), NMP22 (nuclear

Abbreviations: (BC), bladder cancer; (lncRNAs), Long non-coding RNAs; (ROC), receiver operating characteristic; (BTA), Bladder tumor antigen; (NMP22), Nuclear mitotic apparatus; (EVs), extracellular vesicles; (DLS), Dynamic light scattering; (EM), Electron microscopy; (SEM), scanning electron microscopy; (TEM), transmission electron microscopy; (qRT-PCR), Quantitative real time PCR.

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mitotic apparatus), UroVysion (looking for chromosomal instability in urine cells) and ImmunoCyt (uses three kinds of monoclonal immunofluorescence antibody against membrane marker of urothelial tumor cells in urine), diagnostic performance of these biomarker is ranged from 50% to 80% for sensitivity and 70% to 90% for specificity (Proctor et al., 2010). But there is still no proper biomarker/methods that enable to diagnose BC as much as cystoscopy does.

Long non-coding RNAs (lncRNAs) is a sub-class of non-coding RNAs which characterize by their length with >200 nucleotides (Mercer et al., 2009). The classification of lncRNAs is based on different characteristics including genomic location, expression pattern in a particular tissues, molecular mechanism, and their localization in cells (Ma et al., 2013). lncRNAs can play pivotal role in cancer as a tumor suppressors or oncogenic molecules which influence various aspect of tumor cellular behavior of cells such as cell proliferation, cellular signaling, metastasis and EMT process, apoptosis (Maruyama and Suzuki, 2012; Li and Chen, 2016). lncRNAs mediates their function through interaction with diverse biomolecules in different cell types, including lncRNA-RNA interaction (specially microRNAs), lncRNA-DNA/chromatin complex interaction and lncRNA-protein interaction (Ferre et al., 2015; Quinodoz and Guttman, 2014).

TUG-1 and MALAT-1 are oncogenic lncRNAs consists of approximately 7 K to 8 K nucleotides (different variants have different number of nucleotides) and are located on the 11q13.1 and 22q12.2 respectively (Iliev et al., 2016; Lin et al., 2016). The functions of these transcripts have widely studied in recent years. These lncRNAs have many oncogenic functions which dysregulated in different malignancies including osteosarcoma, lung cancer, bladder cancer, esophageal squamous cell carcinoma, colorectal cancer and breast cancer (Zhang et al., 2013; Xu et al., 2015; Zhang et al., 2014; Chang and Hu, 2018; Jadaliha et al., 2016; Zuo et al., 2017; Mei et al., 2018) Besides, the studies show that these two lncRNAs also upregulated in BC (Iliev et al., 2016; Xie et al., 2017; Li et al., 2017). For example, Liang Ling et al. have demonstrated that MALAT-1 is highly overexpressed in tumor tissue in compare to healthy tissue (Ying et al., 2012). Robert Iliev and colleagues also determine the expression pattern of TUG-1 in bladder cancer, they found out that TUG-1 is highly overexpressed in tumor tissues (Iliev et al., 2016). Based on these studies, overexpression of MALAT-1 and TUG-1 is positively correlated with tumor metastasis and migration of cancer cells. Moreover, these two lncRNAs can be detectable in body fluids including blood and urine, freely or insides EVs which make them to be considered as a potential biomarkers (Duan et al., 2016; Yazarlou et al., 2018). Therefore, the aim of this study is to evaluate the expression pattern of these lncRNAs and to determine the sensitivity and specificity of them.

2. Methods

2.1. Patients and sample collection

This study was performed on 40 first voided urine samples which collected from two groups, 30 urine samples were collected from early stage BC patients and 10 urine samples were collected from normal control. We obtained the written consent from each individuals and all protocols were approved by ethics committee of Tehran University of medical sciences. After urine collection, the samples were centrifuged at 3000*g* for 20 min in canonical tubes in order to eliminate any cells, cellular debris and bacteria to prevent releasing cellular content such as proteins and RNAs into the urine. Then the supernatant was transfered to another canonical tube and was kept at −80C° until further process.

2.2. Validation tests to examine exosomes in urine samples

2.2.1. Dynamic light scattering (DLS)

Sample for DLS measurement was prepared according to the manufacturers protocol using the "urine exosomal RNA isolation kit" (Norgen Biotek, Thorold, Canada, Cat. 47100) Appendix B. DLS measurement was conducted using a 3D cross correlation spectrometer from the Malvern instrument (UK) and confirmed that the average size of nanoparticle is about 200 nm.

Table 1
Primers sequence.

MALAT-1	Forward	GAC GAG TTG TGC TGC TAT CTT
	Reverse	GAT TCT GTG TTA TGC CTG GTT AG
TUG-1	Forward	TAG CAG TTC CCC AAT CCT TG
	Reverse	CAC AAA TTC CCA TCA TTC CC
5srRNA	Forward	GCC CGA TCT CGT CTG ATC T
	Reverse	AGC CTA CAG CAC CCG GTA TT

2.2.2. Western blot

For investigation of CD-63, exosomal protein marker, total protein from the urine was extracted according to the manufacturers protocol using the "urine exosomal RNA isolation kit" (Norgen Biotek, Thorold, Canada, Cat. 47100) Appendix A. The SDS PAGE and western blot analysis was performed based on standard protocols.

2.2.3. Electron microscopy (EM)

To analyze the structure and the morphology of exosome scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were conducted. For SEM three different concentrations of exosomes on lam were prepared as follows: 3 drop of urine on the three different lamsup to dry up and then two drop of urine on the two of three previous lams were added, we allowed them to dry up and then add one drop to one of the three previous lam and allowed to dry up, which finally provided three different concentrations of urine for SEM measurement and dried drops were examined under scanning electron microscopy (Sem made by FEI Company of USA model Quanta 200).

To study the structures of exosomes by TEM the urine sample according to the manufacturers protocol was prepared using the "urine exosomal RNA isolation kit" (Norgen Biotek, Thorold, Canada, Cat. 47200). 50 μl of sample was loaded on formvar carbon grid followed staining by uranyl acetate 2% for 90 s. The formvar carbon grid was dried by filter paper, stained again by uranyl acetate 2% for 2 min and then dried. Finally, exosomes were examined under transmission electron microscopy (Zeiss, EM10C, 100kv, Germany).

2.3. Quantitative real time PCR (qRT-PCR)

Total exosomal RNAs were extracted from urine according to the manufacturer's protocol using the "Urine Exosome RNA Isolation Kit" (Norgen Biotek, Thorold, Canada, Cat. 47200) which first isolate the exosomes and then the total RNA was extracted using silica-gel based membrane. The extracted RNA were reverse transcribed using prime script RT reagent kit (TAKARA, japan, Cat. RR037Q). Then qRT-PCR was performed using the SYBR green PCR kit (TAKARA). 5srRNA was used as internal control. The primer pairs used in this study are listed in Table 1. qRT-PCR was performed in a total reaction volume of 10 μl, including 1 μl cDNA, 0.5 μl reverse primer and 0.5 μl forward primer of target genes and internal control, 5 μl SYBR green and 3 μl double-distilled water. The reactions were performed by using the ABI StepOnePlus™ real time PCR system (applied biosystems, USA) in duplicate to confirm reproducibility. The average value of each duplicate was used to calculate the relative amount of MALAT-1 and TUG-1 using the comparative ΔCt method.

2.4. Statistical analysis

Date were analyzed using SPSS statistic 17 software (IBM Corporation, Armonk, NY). The level of MALAT-1 and TUG-1 expression were compared between two groups using independent *t*-test and also independent t-test was used for relation between MALAT-1 and TUG-1

Table 2
Demographic characteristic of patients.

Study group (number of sample)	Variables	Values
Bladder cancer patients (30)	Age (mean ± SD (range))	62.67 ± 11.96 (37–81)
	Stage Ta-T1	20 (66.7%)
	Stage T2	10 (33.3%)
	Low grade	13 (43.3%)
	High grade	17 (56.7%)
Controls (10)	Age	57.4 ± 5.7 (47–67)

expression and clinicopathological characteristic. The Receiver Operating Characteristic (ROC) curve was applied to determine the diagnostic value of the target genes using GraphPad prism version 8.0.2. *P*-value <0.05 was considered to be statistically significant.

3. Results

3.1. Subjects characteristics

A number of total 10 normal control and 30 Non-muscle invasive bladder cancer were enrolled in this study which according to the

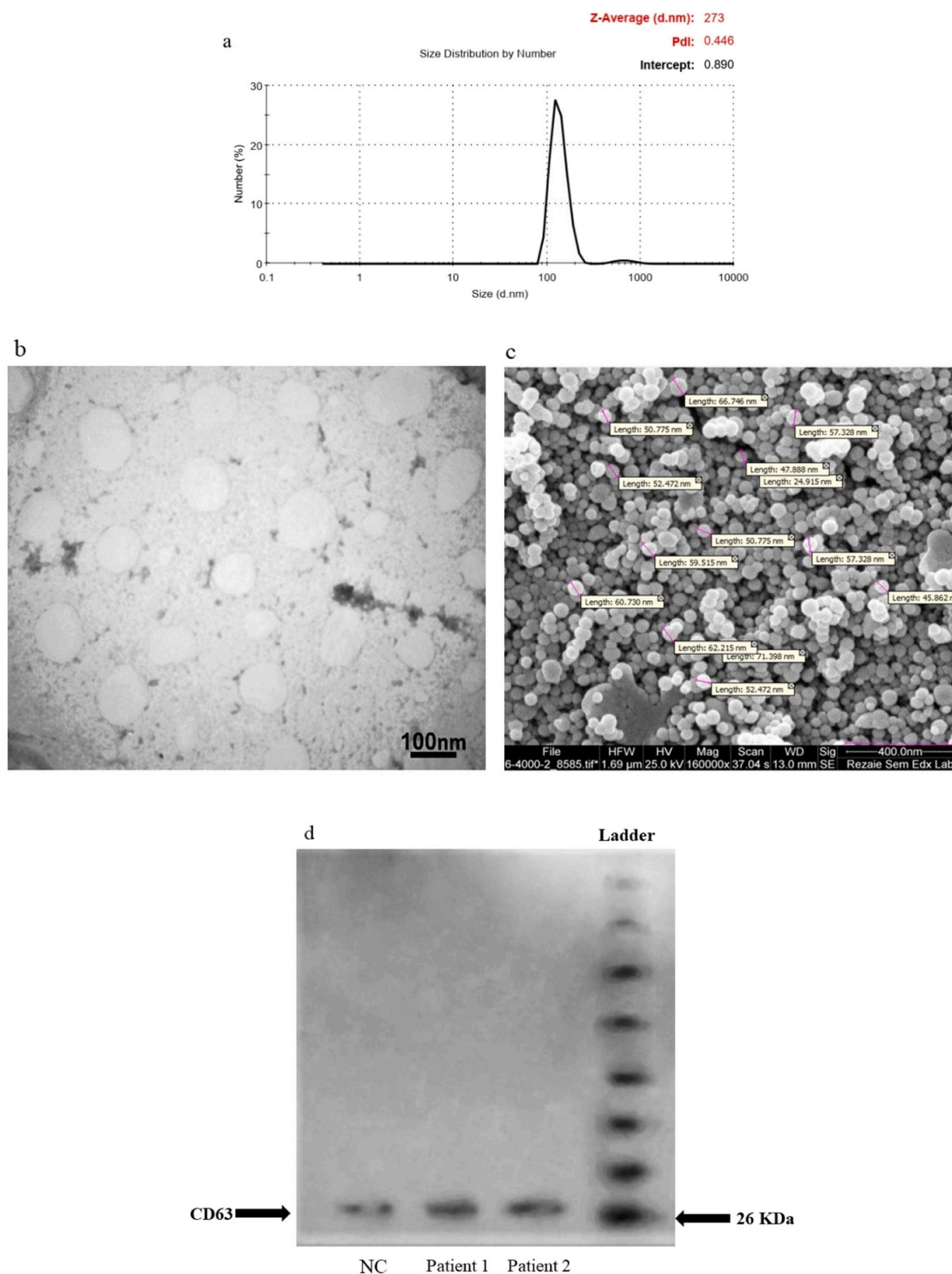

Fig. 1. Urinary exosomes were assessed by a. dynamic light scattering (DLS), b. Transmission electron microscopy (TEM), c. Scanning electron microscopy (SEM) and d. western blot analysis also confirmed the existence of exosomes in urine samples. NC (Normal Control).

Table 3
Relation between the expression of MALAT-1 and TUG-1 with clinicpathological reports.

LncRNA	Variables	P-value	Correlation coefficient
MALAT-1	Stage	0.143	–
	Grade	0.198	–
	Recurrence	0.7	–
	Age	0.243	−0.345
	Tumor size <3 cm	0.14	0.513
	Tumor size>3 cm	0.805	0.131
TUG-1	Stage	0.9	–
	Grade	0.145	–
	Recurrence	0.5	–
	Age	0.3	−0.183
	Tumor size <3 cm	0.15	0.655
	Tumor size≥3 cm	0.393	0.441

pathology and cystoscopy reports divided into two stages (stage I and stage II, 20n and 10n, respectively), low grade (17n) and high grade (13n) patients. Demographic characteristics is shown in Table 2.

3.2. Characterization of urinary exosomes

Exosomes were isolated and then characterized by using TEM, SEM and DLS. The size distribution was mainly between 50 and 200 nm (Fig. 1a, b and c). Western blot analysis of Exosomal marker, CD-63, further confirmed the existence of exosomes in urine (Fig. 1d).

3.3. Relation between Exosomal expression of MALAT-1 and TUG-1 with clinicpathological features

We assessed the relationship between the expression of MALAT-1 with TNM stage, grade, recurrence, tumor size and age. There was no relation between MALAT-1 and stage ($p = 0.143$), MALAT-1 and grade ($p = 0.198$) and MALAT-1 and recurrence ($p = 0.7$). However, the expression of MALAT-1 had a negative correlation with age but it was not significant (correlation coefficient: −0.345, $p = 0.243$). As the cut off for tumor size in BC is 3 cm (Babjuk et al., 2017), we classified the tumor size of the patients into two groups (tumor size<3 cm and tumor size>3 cm), after examination there was no correlation between MALAT-1 expression and tumor size (tumor size<3 cm: correlation coefficient = 0.513, $p = 0.140$, and tumor size>3 cm: correlation coefficient = 0.131, $p = 0.805$).

There was no correlation between TUG-1 and stage ($p = 0.9$), TUG-1 and grade ($p = 0.145$), TUG-1 and recurrence ($p = 0.5$), TUG-1 and age (correlation coefficient: −0.183, $p = 0.3$), and also there was no correlation between TUG-1 and tumor size (tumor size<3 cm: correlation coefficient = 0.655, $p = 0.15$, and tumor size>3 cm: correlation coefficient = 0.441, $p = 0.393$) (Table 3).

3.4. The level of TUG-1 is significantly different between patient and control groups

Expression levels of MALAT-1 and TUG-1 were measured by RT-qPCR in early stages of BC. The result demonstrated that there was no significant difference between the levels of MALAT-1 in BC patients and control ($p > 0.05$). However, there was a significant difference between the level of TUG-1 in the BC patients and healthy individuals ($p = 0.002$) (Fig. 2a). The results also indicate that even though the TUG-1 was significantly upregulated in BC patients, but there is no difference between the level of TUG-1 in stage I and stage II, as well as the MALAT-1 also has the same result in stage I and stage II ($p > 0.05$). (Fig. 2b). The fold change was calculated using $2^{-\Delta\Delta Ct}$ method and the results indicate a fold change of 8.7 and 1.4 for TUG-1 and MALAT-1, respectively.

3.5. Diagnostic significance of urine Exosomal MALAT-1 and TUG-1 for BC patients

Receiver operating characteristic (ROC) analysis have been shown that Exosomal MALAT-1 is not capable to diagnose BC patients from healthy individuals, at least in early stages, with the area under the curve (AUC) value of 0.6 (95% CI: 0.40–0.8420) (Fig. 3). On the other hand, TUG-1 has better accuracy to diagnosis BC patients from healthy

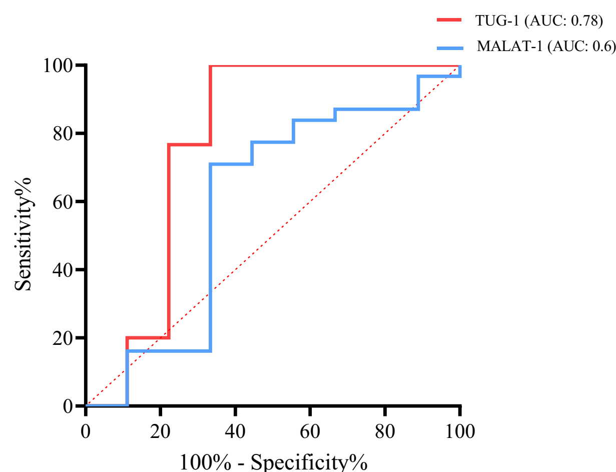

Fig. 3. Receiver operating characteristic (ROC) curve analysis for MALAT-1 and TUG-1 demonstrated that despite MALAT-1, TUG-1 has a proper AUC value to distinguish BC patients from healthy individuals.

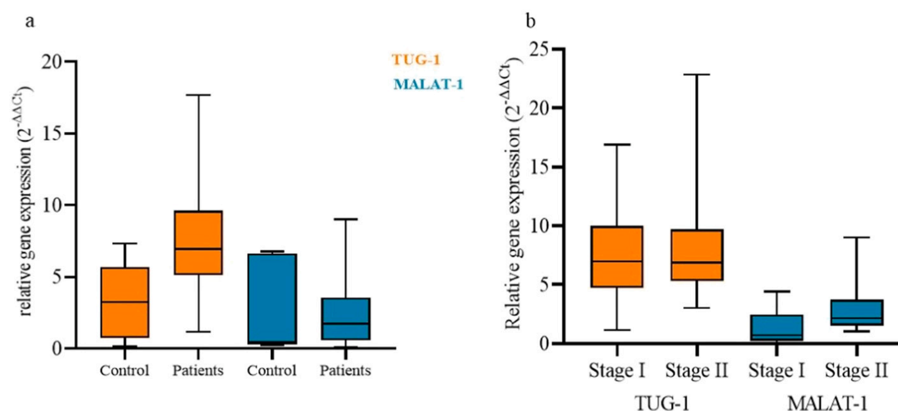

Fig. 2. There was a significant difference between the levels of TUG-1 in two groups. a: relative gene expression of TUG-1 and MALAT-1 between two groups was calculated as 8.7-fold and 1.4-fold respectively, b: relative TUG-1 and MALAT-1 expression between stage I and stage II groups.

individuals with an area under the curve (AUC) value of 0.78 (95% CI: 0.55–0.969). The sensitivity and specificity of TUG-1 was 76.67% and 77.78% respectively.

4. Discussion

Diagnosis of bladder cancer based on current methods and biomarkers don't have much performance except for cystoscopy which is expensive and unpleasant for patients, and also require several cystoscopies each year to check for recurrence. LncRNAs display high organ and cell specificity, can be found in the blood, urine, tumor tissue, or other tissues or bodily fluids of some patients with cancer. Urinary exosomes are excreted from bladder muscle can be served as a reliable new source of biomarkers for detection and diagnosis of bladder diseases.

In this study we evaluated the difference of expression of two oncogenic lncRNAs, MALAT-1 and TUG-1, in the urinary exosomes of BC and normal control. Both MALAT-1 and TUG-1 were not significantly different between the patients and control but the fold change for TUG-1 is higher than MALAT-1, 8.7-fold and 1.4-fold respectively. It has been shown that these lncRNAs are upregulated in many cancer and related to cancer progression. MALAT-1 upregulated in many cancer types and positively related to the cancer development, progression and metastasis but in our study there were no correlation between MALAT-1 and clinicpathological features which might be due to the early stages (stage I and II) of patients. In a recent study performed on ovarian carcinoma by Michael A Gordon et al. they also showed that MALAT-1 was not significantly upregulated in early stages but higher expression of MALAT-1 is associated with end stages (stage III and IV) as well as with recurrence (Gordon et al., 2019). Moreover, Chao Li et al. showed that the MALAT-1 expression was significantly higher in patients with advanced histological grade in compared to low grade BC patients ($p = 0.023$) (Li et al., 2017). MALAT-1 has an important role in cancer progression through different mechanism especially metastasis and migration which mediate through processes such as EMT, cell signaling and extracellular matrix degradation (Sun and Ma, 2019). As the migration and invasion occurs in end stages that's might be the reason that MALAT-1 is not overexpressed in early stages of cancers. Besides, the role described for MALAT-1 is dependent on the cancer types for example, while numerous studies indicated that MALAT-1 upregulated in many tumors including BC (Ying et al., 2012), but some studies showed that this RNA downregulated in some cancer e.g. multiple myeloma, glioma and breast cancer (Hu et al., 2018; Han et al., 2016; Kwok et al., 2018). Therefor according to numerous studies it seems that MALAT-1 has both oncogenic and tumor suppressive functions which subsequently affected its expression.

Over the last few years, many studies evaluate that whether MALAT-1 can be considered as a potential biomarker for cancer diagnosis or not. In a recent meta-analysis, the AUC for MALAT-1 was calculated as 0.85 with the sensitivity and specificity of 0.74 and 0.83, respectively (Mei et al., 2018). Furthermore, MALAT-1 was evaluated in the serum of non-small cell lung cancer (NSCLC) and BC patients which the results demonstrated that use of MALAT-1 with a panel of non-coding RNAs give better accuracy to distinguish cancer than use of MALAT-1 alone (Duan et al., 2016; Peng et al., 2016). In another study, the expression pattern of MALAT-1 was evaluated in blood of NSCLC patients and indicate that MALAT-1 is not a good biomarker for diagnosing lung cancer due to the low accuracy (Weber et al., 2013).

Similarly, different study has proven the oncogenic function of TUG-1 in different malignancies which mainly mediated by microRNA processing and microRNA sponging (Yang et al., 2019; Zhou et al., 2018). Previous studies showed that this lncRNA is highly upregulated in muscle invasive bladder cancer and associated with poor prognosis in BC patients (Iliev et al., 2016; Liu et al., 2017). In the present study we evaluate the expression of TUG-1 in early stage of BC patients, which the results indicate that despite MALAT-1, TUG-1 was significantly upregulated in early stage. Also, the overexpression of TUG-1 in early stages has been reported in colon adenocarcinoma along with mir-153-3p (Liu et al., 2018). In another study, the combination uses of 4 lncRNA (including TUG-1) has an AUC of 0.8 to distinguish gastric cancer patients (Esfandi et al., 2019) and in another meta-analysis study high expression of TUG-1 associate with poor prognosis of cancer patients (Li et al., 2018). Generally, according to a recent consensus article, extracellular RNAs, including lncRNAs, can be used as a potential biomarker in cancer detection and prognosis, but there are several issues that can affected the qualification and quantification of lncRNAs (Qi and Zhou X-y, 2016) as follows: 1-sample choice, 2-extraction methods, 3-low level of extracellular RNAs in body fluids, 4-data normalization The advances in wide range of potential biomarker in this field which hope that in the next years the scientists can improve the diagnosis methods in order to early diagnosis of cancer. Therefore, more studies need to be carried out to make the gapes.

5. Conclusion

Highly sensitive and specific determination of urine exosomal TUG-1 LncRNAs level and overall acceptable AUC (0.78) has been appeared to provide a simple, noninvasive diagnostic test to identify patients with bladder cancer. The accuracy of Exosomal TUG-1 lncRNA for BC diagnosis is high, and Exosomal lncRNAs could be considered as promising candidates for BC diagnosis.

CRediT authorship contribution statement

EK designed and supervised the study and also edited the manuscript. MS and MA designed, performed experiments, wrote the manuscript and prepare tables and figures.

Declaration of competing interest

The authors declare there is no conflict of interest.

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