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ORIGINAL ARTICLE

Cancer-Testis Antigens as New Candidate Diagnostic Biomarkers for Transitional Cell Carcinoma of Bladder

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Abstract To evaluate the diagnostic potential of 23 candidate genes, belonging to a category of tumor-specific antigens known as cancer-testis antigens (CTAs), in transitional cell carcinoma (TCC) patients. The expression of 16 known candidate CTAs and seven testis restricted/selective genes, predominantly expressed in the testis, was evaluated by reverse transcriptase-polymerase chain reaction (RT-PCR). Urinary exfoliated cells (UECs) and cancerous tissues of 73 TCC patients were used as cases, while **25** tumor-free adjacent bladder tissue specimens along with bladder tissue specimens and UECs of five non-TCC individuals were analyzed as controls. Among the known CTAs only MAGEA3, MAGEB4, TSGA10, PIWIL2, OIP5, and ODF4 were expressed specifically in TCC tissues and UEC samples. ACTL7A, AURKC, and CGB2 were testis-restricted/selective genes that indicated specific expression in cases in comparison to controls. MAGEA3, MAGEB4, and ODF4 mRNA was detectable in more than 50% of both TCC tissues, and UEC samples. Slight differences were detected in the mRNA expression pattern of candidate genes between the UEC samples and tumor tissues. Different panels formed by combinations of these genes can show up to 95.9% and 94.5% of positivity in TCC tissues and UEC samples, respectively, suggesting their diagnostic and surveillance potential. Meanwhile the RT-PCR assay of at least MAGEA3, MAGEB4, and ODF4 may be particularly useful for diagnostic and surveillance of TCC in the form of a multi-biomarker panel.

Keywords Cancer testis antigen · Clinical markers · Transitional cell carcinoma · Urinary bladder neoplasms

Introduction

Identifying new and specific candidate diagnostic biomarkers for transitional cell carcinoma (TCC) is a priority in uropathology. With 429,800 new cases in the year 2012, TCC was considered the ninth most common type of cancer and the 13th most common cause of cancer deaths worldwide (165,100 deaths) [1]. Based on calculated ASRs (age-standardized rates), TCC is amongst the five most frequent male cancers especially in the southern parts of Iran (6.8 per 100,000) [2]. TCC patients require regular and lifelong surveillance, currently managed by using the combination of urine cytology and cystoscopy [3]. Although quite invasive and costly, standard white light cystoscopy is currently the gold standard and reference diagnostic procedure for TCC with a sensitivity of 90% [4]. Voided urine cytology, on the other hand, lacks adequate sensitivity especially in low-grade tumors, regardless of its 99% specificity [5]. Despite significant advances in biomarker development, the diagnostic and

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surveillance clinical approaches have not improved remarkably over the past decade.

Genes or antigens exclusively or preferentially expressed in cancerous tissues are potential TCC biomarkers for diagnostic and clinical surveillance approaches. Recently a category of tumor-specific antigens known as cancer-testis antigens (CTAs) was identified. These antigens are normally only expressed in human germ line cells. However, it has been shown that they are frequently expressed in different types of cancers as well [6]. Their restricted expression profile in normal tissues made them potential candidates for gene specific diagnosis in cancerous cells [7].

The present catalog of CTAs contains more than 100 gene families divided into 200 distinct genes (ctdatabase, http://www.cta.lncc.br/index.php). Based on their frequent expression in bladder carcinoma [8–11]. a previous study has suggested that specific members of the CTA catalog may have both predictive and prognostic values in this cancer [12]. Transcripts of tumor-specific CTAs can be detected by the highly sensitive reverse transcriptase-polymerase chain reaction (RT-PCR) method. Although the expression patterns of some CTAs (HSP105, CT7 & 10, BAGE, GAGE, MAGE-A-1,-2, −3, −4, −8, −9, −10 & -12, MAGEC1, CTAGE-1 (LAGE-1) &-2, NY-ESO-1 (CTAG1B), BAGE-1, −4 & -5, SSX-1, −2 & -4, HOM-TES-85, SCP-1, PRAME) have been previously determined in bladder tumors [10, 13–20], there are still many other CTAs that have not been evaluated in TCC up to now. To the best of our knowledge, no expression analyses of CTAs have been done on urinary exfoliated cells (UECs) isolated from the urine of TCC patients to evaluate the possibility of applying them as urinary TCC biomarker.

This study provided a comprehensive mRNA expression analysis of 16 known CTA and seven testis-restricted/selective genes in both UECs and tumor tissues among 73 TCC patients, in comparison to 25 tumor-free adjacent bladder tissues, along with bladder tissue specimens and UECs of 5 non-TCC individuals. The aim of this study was to identify the most eligible and potential CTAs associated with TCC, which could be applied as new candidate diagnostic biomarkers using a small amount of sample cells in a RT-PCR assay.

Methods

Selection of Genes

Selection of the most promising known CTAs for subsequent evaluation of their expression in TCC tissues and UECs was conducted with a semi-systematic review of the data available in the literature. We also evaluated previously provided high-throughput expression data (EST, MPSS, CAGE, and RT-PCR experiments), as well as published data on CTA mRNA expression in cancer cell lines (database, http://www.cta.lncc.br/index.php) [21]. Subsequently, CTA expression patterns in bladder carcinoma were mined from publicly available data on SAGE Anatomic Viewer and its Virtual Northern tool (HTTP://cgap.nci.nih.gov/SAGE/anatomicviewer), plus next bio (http://www.nextbio.com) databases. Eventually, those CTA expression patterns were selected which showed different expression in TCC vis-à-vis healthy bladder samples, annotated with their official symbol and merged based on the shared National Center for Biotechnology Information RefSeq nucleotide identifiers (Fig. 1).

For selecting candidate genes which are predominantly expressed in the testis, three distinct EST pools were derived. The first pool contained ESTs of most normal adult tissue cDNA libraries except for testis, ovary, placenta, pooled normal tissues, and normal tissues of unknown origin. The second pool included ESTs from libraries of any cancer type except for testicular cancer, whereas the third pool contained libraries from normal testis. The inclusion criteria for genes was "[(predominant expression in the testis) AND (at least one cancer-associated tissue OR belonging to at least one EST/cDNA library with testis and cancer annotation)]" AND "[no expression level above 5% of that observed in the normal testis in any other tissue except for the placenta, ovary, and brain]". All hypothetical proteins, predicted genes, and genes with multiple publications indicating expression in somatic tissues were excluded, and the remainder was applied for further RT-PCR analysis (Fig. 1).

Patients and Samples

From March 2014 to December 2015, 73 TCC patients (including three females) and five pathologically non-TCC patients who underwent cystoscopy due to bladder stones or prostate malignancies at the Imam-Khomeini hospital complex, Medical Sciences/Tehran University, Tehran, Iran, were enrolled in this study. None of the patients had received prior cytotoxic or radiation therapy during the previous 2 years. The first-morning complete urine samples were collected before the cystoscopy biopsy. A portion of each urine specimen was set aside for cytology, and the remainder was stored at 4 °C for a maximum of 4 h. All patients underwent local, organ-sparing transurethral resection of their bladder tumor (TURBT) in the Department of Urology, Imam Khomeini General Hospital, Medical Sciences/Tehran university. Cystoscopy biopsy samples were freshly obtained from each TCC tumor and its adjacent normal tissue (distance to the edge of the suspected TCC tumor >5 cm). All samples were snap-frozen in liquid nitrogen within 30 min, and stored at −80 °C for a maximum of one month before testing. Testis tissue

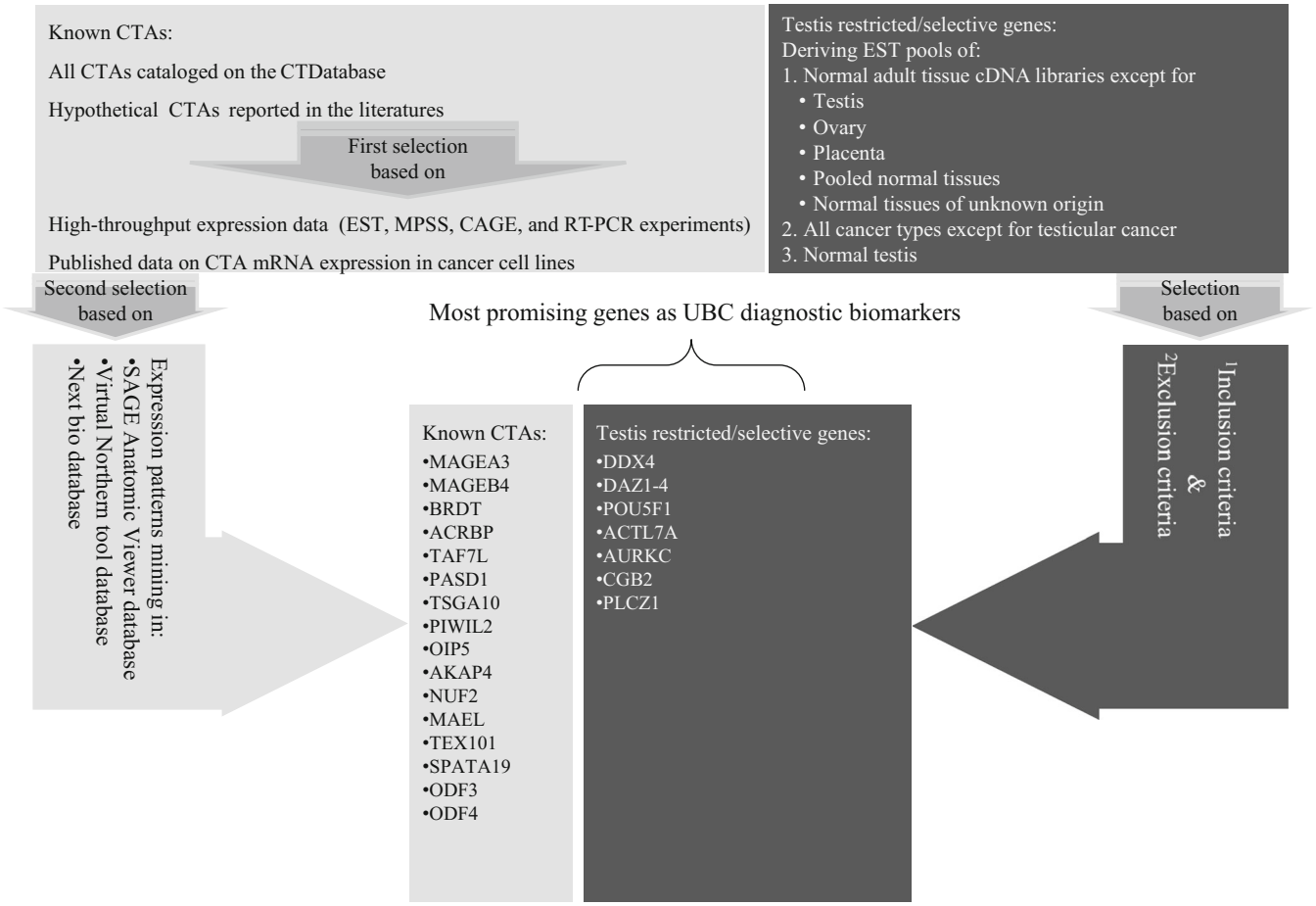

Fig. 1 Approaches and steps taken to select the most promising known cancer-testis antigens (CTAs) and testis selective/restricted genes. 1. Inclusion criteria: [predominant expression in testis AND (at least one cancer-associated tissue OR belonging to at least one EST/cDNA library with testis and cancer annotation)]" AND "[no expression level above 5% of that observed in normal testis in any other tissue except for placenta, ovary, and brain]". 2. Exclusion criteria: Hypothetical proteins, predicted genes, and genes with multiple publications indicating expression in somatic tissues

(provided from the prostate cancer patient who underwent orchiectomy and radical prostatectomy) was used as the positive control.

The clinical diagnosis was confirmed pathologically and patients were ultimately divided into high grade and low grade TCCs based on the World Health Organization system [22]. Tumor tissue and UEC samples were then used as cases, while tumor-free adjacent bladder tissue specimens along with non-TCC sample sets were analyzed as controls.

Urine samples were centrifuged at 800 g for 10 min at 4 °C. Cell pellets were then treated with TriPure isolation reagent (Roche, Germany) and stored at −80 °C for a maximum of one month before testing.

Total RNA Extraction, Integrity Assessment, and Complementary DNA (cDNA) Synthesis

Total RNA was isolated from approximately 100 mg of each frozen tissue sample and whole urinary cell pellets, using TriPure isolation reagent (Roche, Germany) according to the protocol provided by the manufacturer. The quality and purity of each RNA sample were measured with a nanodropnd-2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and sample integrity was confirmed by electrophoresis on 1.0% agarose gel (UltraPureTM Agarose; Invitrogen). High-quality RNA samples with no degradation indications were stored at −80 °C for further analyses.

RNA elution corresponding to 500 ng was primed with an Oligo (dt) and subjected to cDNA synthesis at a total volume of 10 μl according to the manufacturer's instructions (PrimeScript™RT reagent kit, TaKaRa, cat#RR037A).

RT-PCR Amplification of CTAs

A PCR assay with specific primers for the gene ribosomal protein S13 (RPS13) was performed to verify RNA existence and DNA contamination (Supplementary Table 1). To detect any false positive result related to genomic DNA contamination in the RNA preparations, the primers used for RPS13 PCR amplification were designed to encompass the intron

between exon sequences so that they amplified both the DNA and mRNA template with different sized amplicons of 443 bp and 187 bp, respectively (Supplementary Table 1). The PCR amplification was carried out under an initial heating for 3 min at 95 °C and 30 cycles of amplification, followed by a final extension of 8 min at 72 °C. Only the cDNAs, which amplified 187 bp of the mRNA template, were used for further analysis and those that amplified the DNA template as well were excluded. This procedure was only designed for four genes for which designing an intron spanning primer was impossible (i.e., intronless genes: MAGEB4 and ACTL7A, also MAGEA3, and POU5F1 which share a high similarity in their sequences compared to their other gene family).

The mRNA expression of the candidate genes was investigated by RT-PCR, using previously published oligonucleotide primers (Supplementary Table 1). Amplifications were carried out by adding 2 μl of the 1:5 dilution cDNA of each sample to 10 μl of the PCR master mix (Ampliqon, Denmark), 8 μl of nuclease free water, and 0.5 μl of each forward/reverse primer (10 pm). Primers were designed to target all known variants of each gene in RefSeq, and their specificity was previously checked by aligning them against the Genome (chromosome from all organisms) and RefSeq mRNA in the GenBank database, using the Primer-BLAST web-based tool (NCBI). Primers used for DAZ1, DAZ2, DAZ3, and DAZ4 in this study were not able to discriminate the expression of these genes since they share a high similarity in their sequences. Primer dimer formation also has been tested using the Gene Runner Software package (Gene Runner Version 3.00 Hasting Software Inc., Hastings, NY). PCR reactions were performed in an ABI thermal cycler (Applied Biosystems, USA) under the following conditions: after an initial denaturation for 3 min at 95 °C, samples were subjected to 35 cycles of amplification, followed by a final extension of 8 min at 72 °C.

No reverse-transcriptase sample was included in each run to double check any false positive result related to genomic DNA contamination in the RNA preparations. Samples with no RNA template and no cDNA template were run as negative controls. Ultimately electrophoresis of 8 μl PCR products and 1 μl DNA molecular weight markers was conducted on a 2.5% agarose gel (Ultrapure™ Agarose; Invitrogen). We used SYBR safe staining and the digital photography (Gene Genius bioimaging system, Syngene, Frederick, MD, USA). Cases with a detectable band were considered positive, while those with very low transcript levels (fainter bands) were scored positive only if the result was reproducible with a repeated RT-PCR session.

Statistical Analysis

We performed statistical analyses of samples using Stata version 14.1 (STATA Corp, Inc., College Station, TX). To compare the results of the RT-PCR for the CTAs in the TCC tissues and UEC samples vs. tumor-free adjacent bladder tissue, Fisher's exact test, with a statistically significant level of $p < 0.05$ was applied. Fisher's exact test was also used to assess the relationships between categorical variables (disease grade and candidate gene expression at mRNA level) with same statistically significant level.

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (+LR), negative likelihood ratio (−LR), ROC area, and diagnostic odds ratio (DOR) of each CTA found in the UEC and TCC tissues were calculated compared to the tumor-free adjacent bladder tissue specimens, using the Diagt command.

Ethical Consideration

All participants received a detailed description of the purpose and procedures of this study and gave written informed consent. The Ethics Committee of Tehran University of Medical Sciences approved the study protocol.

Results

Gene Selection

CTAs selected for assessment of gene expression in the TCC tissues and UECs included MAGEA3, MAGEB4, BRDT, ACRBP, TAF7L, PASD1, TSGA10, PIWIL2, OIP5, AKAP4, NUF2, MAEL, TEX101, SPATA19, ODF3, and ODF4. DDX4, DAZ1–4, POU5F1, ACTL7A, AURKC, CGB2, and PLCZ1 were also selected due to their restricted expression patterns in testis tissue and their mRNA absence in normal tissues including normal bladder (Fig. 1 and Supplementary Table 1).

Expression of Candidate Genes in TCC Tissues and UEC Samples

Positive results of the cytology test among 73 malignant cases accounted for only 67%. Due to the high number of selected genes and the limitation in the RNA quantity, a pilot study was conducted to verify the expression status of all candidate genes in 25 clinically/pathologically documented TCC tissues and UEC samples in comparison to their tumor-free adjacent bladder tissue and a sample set from the five non-TCC subjects (bladder tissue and their corresponding UEC samples). The mRNA expression of 23 candidate genes was successfully assessed in all sample set, using RT-PCR analysis. Non-TCC controls were the key controls to eliminate all the genes that could be detected in non-TCC bladder tissues by simple RT-PCR. We excluded NUF2, POU5F1, and TAF7L due to their low specificity indicated by their expression in at least one of the Non-TCC controls or mRNA detection in more than 20% of the tumor-free adjacent bladder tissue specimens (Fig. 2). Among the remaining candidate genes, mRNAs of PLCZ1, SPATA19, PASD1, TEX101,

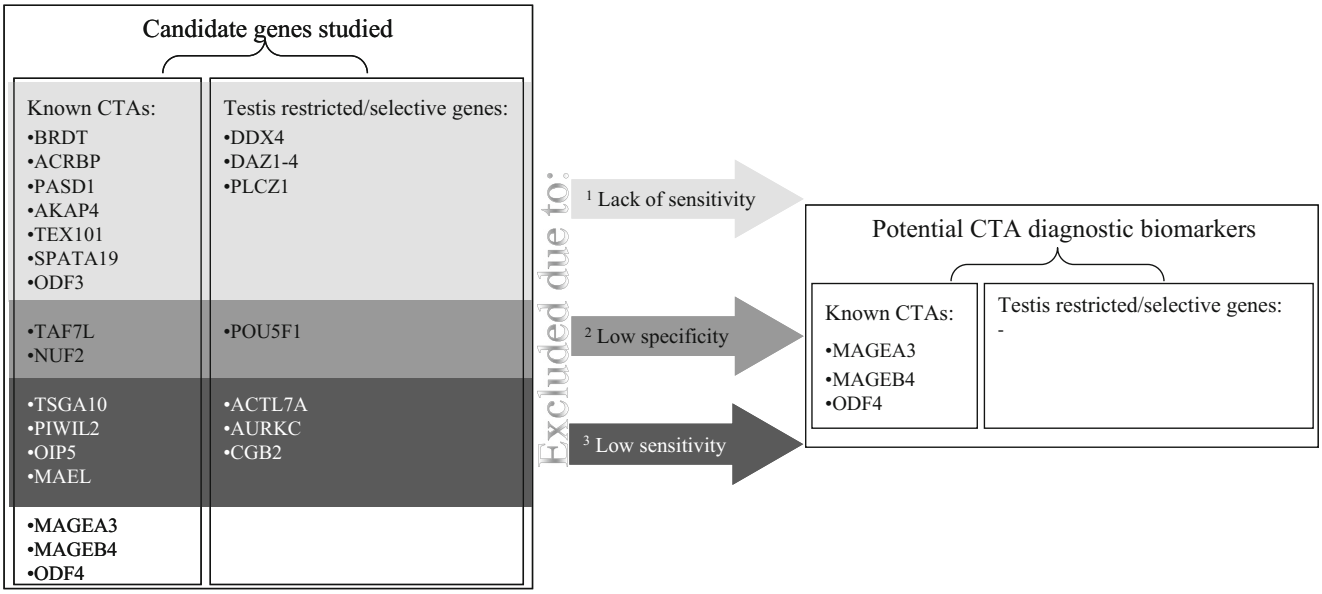

Fig. 2 The rational behind selecting MAGEA3, MAGEB4, and ODF4 as having the most potential as cancer-testis antigens (CTAs) diagnostic biomarkers. 1. Lack of sensitivity: Not being detected in tumoral tissues and urinary exfoliated cells isolated from transitional cell carcinoma (TCC) patients. 2. Low specificity: Detection in more than 20% of the tumor-free adjacent bladder tissues. 3. Low sensitivity: Detected in less than 50% of both tumoral tissues and urinary exfoliated cells isolated from TCC patients

ACRBP, DDX4, DAZ1–4, BRDT, AKAP4, and ODF3 were detected in neither TCC tissues nor UEC samples with RT-PCR. Therefore, they were set aside from further analysis (Fig. 2). Further mRNA expression assessments of the remaining candidate genes were then evaluated in the rest of the TCC tissues and their corresponding UEC samples, only.

Ninety-five point 9 % of the TCC tissues and 94.5% of the UEC samples showed expression of at least one of the gene transcripts. Considering cytology results, as RT-PCR adjunct conventional diagnostic test, the positivity rate increases to 97.3% in both TCC tissues and UEC samples. Significantly higher positive rates of ODF-4, MAGEA3, MAGEB4, MAEL, TSGA10, PIWIL2, ACTL7A, AURKC, and CGB2 were revealed in both TCC tissues and UEC samples compared to the tumor-free adjacent bladder tissues ($p < 0.05$, Fisher's Exact Test) (Supplementary Table 2). A slight difference was observed in the expression patterns of the studied CTAs and testis restricted/selective genes between the TCC tissues and UEC samples (Table 1). MAGEA3 and ODF4 had the highest incidence of mRNA positivity among the TCC tissues with the frequency of 65.7%, followed by MAGEB4, and PIWIL2 (64.3%) (Table 1). AURKC and TSGA10 exhibited the highest expressed CTA among the UEC samples with frequencies of 73.9 and 72.6%, respectively, while MAGEA3 and MAGEB4 mRNAs were detected in 63% and 53.4% respectively (Table 1). MAGEA3, MAGEB4, and ODF4 mRNA were detectable in more than 50% of both TCC tissues and UEC samples.

The sensitivity of the RT-PCR results in the TCC tissue candidate genes to the tumor-free adjacent bladder tissue specimens ranged from 28.8%–65.8%, whereas it ranged between 11%–74% in the UECs to tumor-free adjacent bladder tissue specimens (Table 2). The specificity of TCC tissues and UECs to tumor-free adjacent bladder tissue specimens ranged from 58.2% to 96% and 66.3% to 96%, respectively (Table 2). The DOR score ranking order of examined genes in the TCC tissues to tumor-free adjacent bladder tissue specimens were ACTL7A, ODF4, MAGEB4, PIWIL2, MAGEA3, AURKC, MAEL, TSGA10, CGB2, and OIP5, from highest to lowest (Table 2). The DOR score ranking order differed slightly in the UEC to tumor-free adjacent bladder tissue specimens in comparison to the TCC tissues, with AURKC as the highest followed by TSGA10, ODF4, MAGEB4, MAGEA3, ACTL7A, MAEL, OIP5, CGB2, and PIWIL2, respectively (Table 2). Other diagnostic test performance indicators including the positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (+LR), negative likelihood ratio (−LR), and ROC area are presented in Supplementary Table 3.

No significant correlation was observed between CTA expressions and disease grade ($p > 0.05$, Fisher's exact test) except for MAGEA3 in which a higher mRNA expression was indicated in the high grade groups in comparison to the low grade TCC samples (16 positive TCC tissues among 31 low grade samples and 32 positive TCC tissues among 42 high grade samples, with $p < 0.05$, Fisher's exact test).

Discussion

In the present study, we determined the expression of 16 known CTAs and seven testis restricted/selective candidate

Table 1 Expression of selected cancer testis antigen genes at the mRNA level in cancerous tissues and urinary exfoliated cells isolated from 73 urothelial bladder carcinoma patients

Known CTA genes					Testis restricted/selective genes				
Gene symbol	Tissues		Urinary exfoliated cells		Gene symbol	Tissues		Urinary exfoliated cells	
	Positive	Negative	Positive	Negative		Positive	Negative	Positive	Negative
MAGEA3	48 (65.7)	25 (34.3)	46 (63)	27 (37)	ACTL7A	38 (52.5)	35 (47.57)	19 (26.1)	54 (73.9)
MAGEB4	47 (64.3)	26 (35.7)	39 (53.4)	34 (46.6)					
TSGA10	21 (28.7)	52 (71.3)	53 (72.6)	20 (27.4)	AURKC	29 (39.7)	44 (60.3)	54 (73.9)	19 (26.1)
PIWIL2	47 (64.3)	26 (35.7)	8 (10.9)	65 (89.1)					
OIP5	22 (30.1)	51 (69.9)	37 (50.6)	36 (49.4)	CGB2	33 (45.2)	40 (54.8)	32 (43.8)	41 (56.2)
MAEL	30 (41.1)	43 (58.9)	26 (35.6)	47 (64.4)					
ODF4	48 (65.7)	25 (34.3)	40 (54.7)	33 (45.3)					

Values indicated are numbers followed by (percentage in brackets)

genes in 73 TCC tissues and their corresponding UEC samples in comparison to 25 tumor-free adjacent specimens and five non-TCC tumor tissues and UECs, using RT-PCR. The high frequency of examined CTA expression in both TCC tissues and UEC samples present the burden of lacking a rapid, effective, and noninvasive diagnosis and surveillance clinical test. The expression profiles of MAGEA3, MAGEB4, and ODF4 have been detected in more than 50% of both TCC tissues and UEC samples. According to our dataset, both studied MAGE genes mRNAs were absent in only 16.4% of the UEC samples and 13.7% of the TCC tissues (Table 3), a result comparable to the findings previously reported on TCC tissues [23, 14]. Adding ODF4 can lead to mRNA positivity of at least one of these three CTAs in 83.6% of the UEC samples and 90.4% of the TCC tissues. This is even more interesting when we compare it to the cytology test that showed only 67% positivity among the 73 studied malignant cases. The genes RT-PCR results for all three MAGEA3, MAGEB4, and ODF4 along with cytology reports, as a combined multi-biomarker test, increases the positivity rate to 93.1% in the UEC samples and 95.9% in the TCC tissues.. Therefore these CTAs seemed to be particularly useful for diagnosis and surveillance of TCC in the form of a multi-biomarker panel and especially as an adjunct to conventional diagnostic test, cytology.

The result which particularly intrigued us was the slight difference in studied CTAs and testis restricted/selective genes in TCC tissues in comparison to UECs (Table 1). For instance PIWIL2 and ACTL7A could be detected less in UEC samples. Conversely, mRNAs of TSGA10, OIP5, and AURKC were detected in UEC samples rather than cancerous tissues. The additional ability to detect mRNA in TCC tissues might be simply the result of mRNA instability and gene repression phenomenon due to cell detachment. Interestingly among three other studied genes that showed additional mRNA positivity in UECs in comparison to TCC tissues, OIP5

Table 2 The sensitivity, specificity, and diagnostic odds ratio (DOR) of each cancer testis antigen (CTA) gene found in urinary exfoliated cells and tissues isolated from TCC tissues to the tumor-free adjacent bladder tissue specimens

Classification	Gene symbol	Tissues			Urinary exfoliated cells (UEC)		
		Sensitivity	Specificity	DOR	Sensitivity	Specificity	DOR
Known CTA genes	MAGEA3	65.8	84.0	10.1	63.0	84.0	8.9
	MAGEB4	64.4	92.0	20.8	53.4	92.0	13.2
	TSGA10	28.8	92.0	4.6	72.6	92.0	30.5
	PIWIL2	64.4	92.0	20.8	11.0	92.0	1.4
	OIP5	30.1	58.2	0.6	50.7	73.5	2.8
	MAEL	41.1	88.0	5.1	35.6	88.0	4.0
	ODF4	65.8	92.0	22.1	54.8	92.0	13.9
Testis restricted/selective genes	ACTL7A	52.1	96.0	26.1	26.0	96.0	8.4
	AURKC	39.7	92.0	7.5	74.0	92.0	32.7
	CGB2	45.2	67.3	1.7	43.8	66.3	1.5

Table 3 Clustered mRNA expression of MAGEA3, MAGEB4, and ODF4 genes in cancerous tissues and urinary exfoliated cells (UEC) isolated from 73 urothelial bladder carcinoma patients

	UEC	Tissue
Expression of none of studied MAGE genes	12 (16.4)	10 (13.7)
Expression of MAGEA3 only	18 (24.7)	16 (21.9)
Expression of MAGEB4 only	11 (15.1)	15 (20.5)
Expression of both studied MAGE genes	28 (38.4)	32 (43.8)
Expression of both studied MAGE genes and ODF4	20 (27.4)	27 (37.0)
Expression of none of studied MAGE genes and ODF4	12 (16.4)	7 (9.6)

Values indicated are numbers followed by (percentage in brackets)

and AURKC both encode proteins that are localized to centromeres in the cell nucleus. OIP5 is predicted to be involved in the recruitment of CENP-A through the mediator Holliday junction recognition protein while AURKC organizes microtubules in relation to centrosome/spindle function during mitosis [24, 25]. Although the role of TSGA10 is still quite uncertain, some believe it may be involved in cell division, differentiation, and migration [26]. Therefore it seems that their additional activation/de-repression process after cell detachment might be related to the migration preparation and invasion process, which make them an attractive target for immunotherapy. Apart from the reasons causing the difference in the mRNA expression of the candidate genes, it is worth mentioning that evaluation of the diagnostic significance of each candidate gene for TCC cases would be more practical if it focused on the expression analysis of the UEC samples rather than the original tissues.

ODF4 and MAGEA3 revealed the highest sensitivity in the RT-PCR results of the candidate genes in the TCC tissues to the tumor-free adjacent bladder tissue specimens (65.8%), whereas AURKC indicated the highest sensitivity in the RT-PCR of the candidate genes in TCC tissues to the tumor-free adjacent bladder tissue specimens (74%) (Table 2). The specificity of TCC tissues and UECs to the tumor-free adjacent bladder tissue specimens ranged from 58.2% to 96% and 66.3% to 96%, respectively (Table 2). The DOR score, calculated using the equation $\mathrm{DOR} = \frac{\mathrm{TP}}{\mathrm{FN}} / \frac{\mathrm{TP}}{\mathrm{FN}} = \frac{\mathrm{TP}}{\mathrm{FN}} / \frac{\mathrm{TP}}{\mathrm{FN}}$, ranges from 0 to infinity, and shows the ratio of odds of disease in the test positives per the odds of disease in the test negatives. As can be interpreted from the formula, it does not depend on the prevalence, while higher values of sensitivity and specificity increase its value. Higher values of the DOR score represent the better performance of diagnostic tests. The DOR score ranking order of candidate genes in our study differed slightly in the UEC to the tumor-free adjacent bladder tissue specimens in comparison to the score seen in TCC tissues to tumor-free adjacent bladder tissue specimens. Based on the DOR score, ACTL7A, ODF4, MAGEB4, PIWIL2, and MAGEA3 were the top five best TCC markers, whereas AURKC, TSGA10, ODF4, MAGEB4, MAGEA3 were considered to be the top five best UEC markers. Common genes between these two groups are ODF4, MAGEB4, and MAGEA3, which subsequently reconfirmed their potential to be used as TCC biomarkers.

Among all studied CTAs only MAGEA3 indicated a significant correlation with the disease grade ($p < 0.05$, Fisher's exact test), which is in concordance with the previous findings [23]. MAGE-A3 is among the most commonly expressed CTAs in different malignancies. It showed the highest mRNA expression frequency among TCC tissue samples in our experience as well. A previous study indicated that MAGE-A3 protein expression is consistent with its mRNA expression status [18]. MAGE-A3 proteins bind to and activate RING E3 ubiquitin ligases. It seems that its interaction with p53 proteins may block the association of p53 with its cognate sites in chromatin [27]. This active characterization in anti-apoptotic processes made it an attractive target for immunotherapy. Its frequent expression in TCC tissues and UECs plus limited expression in normal tissues as indicated in our study could additionally rank it among the most eligible candidate for TCC biomarker discovery studies.

Finally, we must mention that the sample size we used here was enough to examine the expressions pattern of candidate genes and evaluate their potentials as TCC biomarkers. On the other hand, further investigations are needed to precisely predict if CTA RT-PCR can add any additional clinical value to the currently applied gold standard and reference TCC diagnostic procedure. Additionally, the diagnostic value of only the most promising CTAs indicated in this study must be evaluated in a larger sample size, preferably from other ethnicities with different genetic, epigenetic, environmental and lifestyle risk factors, using UECs only. Unfortunately due to the high number of selected genes and the need for evaluating TCC tissues and tumor-free adjacent bladder tissue as well, it was not possible in this study.

Conclusion

Based on this result, we are quite optimistic that some of our examined CTAs, especially MAGEA3, MAGEB4, and ODF4, might be useful diagnostic and surveillance biomarkers for TCC. Because not only were they expressed in more than 50% of both TCC tissues and UEC samples,

but also they were among the top five best TCC markers in both UEC samples and tumor tissues, based on the main diagnostic test performance indicators (sensitivity, specificity, and DOR). Moreover, considering the mRNA expression status of MAGEA3, MAGEB4, and ODF4 as a multi-biomarker panel could lead to 83.6% and 90.4% positivity in UEC samples and TCC tissues, respectively, which is higher than the cytology test that showed only 67% positivity among the 73 studied malignant cases. Adding cytology reports to MAGEA3, MAGEB4, and ODF4 multi-biomarker panel can lead to 93.1% and 95.9% positivity rate in the UEC samples and the TCC tissues, respectively, suggesting they can be particularly helpful as a combined multi-biomarker diagnostic test for TCC cases.

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