Nuclear overexpression levels of MAGE-A3 predict poor prognosis in patients with prostate cancer

Azadeh khalvandi

Iran University of Medical Sciences

Maryam Abolhasani

Iran University of Medical Sciences

Zahra Madjd

Iran University of Medical Sciences

Mehdi Shekarabi

Iran University of Medical Sciences

Masoumeh Kourosh Arami

Iran University of Medical Sciences

Monireh Mohsenzadegan (✉ monirehmohsenzadegan@gmail.com)

iran university of medical sciences <https://orcid.org/0000-0003-2434-9728>

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Abstract

Melanoma antigen gene A3 (MAGE-A3) is one of the most immunogenic cancer- testis antigens and is common in various types of cancers. Due to its high immunogenicity and oncogenicity, it has been a candidate for cancer immunotherapy and used as a cancer vaccine in clinical trials of various types of cancers. In this study, for the first time, we performed immunohistochemical analysis to evaluate the expression of MAGE-A3 in 153 formalin-fixed paraffin-embedded prostate tissue samples including 112 cases of prostate cancer (PCa), 22 cases of benign prostatic hyperplasia (BPH) and 19 cases of high-grade prostatic intraepithelial neoplasia (HPIN). Both nuclear and cytoplasmic expressions of MAGE-A3 with different staining intensities were observed among the tissues. Increased expression of MAGE-A3 was significantly found in PCa tissues compared to both HPIN and BPH tissues (nuclear expression at $P = 0.011$, and cytoplasmic expression at $p = 0.034$; for both comparisons $P < 0.0001$, respectively). A significant correlation was observed between higher nuclear and cytoplasmic expressions of MAGE-A3 with Gleason score ($P < 0.0001$ and 0.006 , respectively). Increased expression of MAGE-A3 was associated with shorter biochemical recurrence-free survival (BCR-FS) and disease-free survival (DFS) of patients ($P = 0.042$ and $P = 0.0001$, respectively). In multivariate analysis, nuclear expression of MAGE-A3 and Gleason score (≤ 7 vs > 7) were independent predictors of the DFS (both; $P = 0.019$). Nuclear expression of MAGE-A3 was also significantly related to BCR-FS ($P = 0.015$). MAGE-A3 can be considered as a valuable predictor for poor prognosis and an attractive option for vaccine immunotherapy in patients with PCa.

Introduction

Prostate cancer (PCa) is the second most common cancer among the world's male population and has been identified as the sixth leading cause of cancer deaths in the world. In 2019, about 174,650 new cases of this cancer were diagnosed (1, 2). The growth of the tumor is slow and asymptomatic in many patients and in some cases progresses over time and spreads to other parts of the body, including bone and lymph nodes. Common treatments for patients with localized PCa include surgery (radical prostatectomy) and radiotherapy (brachytherapy or external beam radiotherapy), and patients are monitored after an initial treatment. After early treatments, increased levels of PSA (prostate specific antigen) in the blood are assessed as biochemical recurrence (BCR) which occurs in about 35% of patients for whom androgen deprivation therapy is used in combination with one of the chemical or surgical methods. Although the use of androgen ablation is initially associated with high efficacy, it has significant side effects and, for some patients whose life span is prolonged, the disease eventually spreads to advanced metastatic castration-resistant PCa (mCRPC). MCRPC has poor prognosis and is almost untreatable. Due to the challenging conditions of mCRPC patients, it is important to develop therapeutic strategies with higher efficacy and lower side effects (3, 4).

Having gained much enthusiasm in recent years, immunotherapy, through its many types, has a great impact on various cancers and is considered a good option in the treatment of advanced PCa. In this

regard, the identification of a molecule that is highly elevated in PCa could be a good option for cancer immunotherapy (4).

The melanoma antigen gene A (MAGE-A) family is a member of the cancer testis antigens (CTAs) whose genes are clustered on the q28 region of the X chromosome. Members of this family normally express only the placenta and germ cells of the testis and are not present in other tissues. Increased expression of MAGE-A family has been observed in various types of tumors and malignancies. Members of the MAGE-A family have oncogenic function and support tumor growth and are usually associated with poor prognosis, metastasis and invasion in cancer patients. Several studies have shown that the MAGE-A family increase the viability of tumor cells and exert their function by preventing apoptosis (5, 6). This family acts as transcriptional regulators and their functions include the following:

1- The regulator of Krüppel-associated box *Protein 1* (*KAP1*) activity as a transcriptional activator via ubiquitin stimulation and KRAB zinc finger (KZNF) degradation. 2- The regulator of p53 activity as a co-transcription repressor by stimulating p53 degradation, blocking p53 binding to the p53 target gene promoter, calling histone deacetylase 3 (HDAC3) to the p53 promoter and suppressing gene expression. 3- Affecting androgen receptor (AR) to increase its activity (observed in MAGE-A11 in PCa) (7).

MAGE-A3 is a member of the MAGE-A family that has been shown to be effective in tumorigenesis through the inhibition of tumor cell apoptosis. Among members of the MAGE-A family, MAGE-A3 is common in various types of cancers, and higher levels of mRNA and protein have been observed in many cancers including melanoma (8), breast (9), ovarian (10), brain (11) and lung cancers (12, 13). It is also one of the most immunogenic CTAs (14) and is one of the top 10 antigens of the CTA family in terms of immunogenicity and oncogenicity according to National Cancer Institute (15). MAGE-A3 has the potential to stimulate the cellular and humoral immune response (14). Studies have shown that MAGE-A3 specifically binds to *KAP1* and forms a complex with E3 RING ubiquitin ligase, results in the degradation of p53 and AMPK α 1 and ultimately inhibits apoptosis in tumor cells of non-small cell lung carcinoma (NSCLC) and hepatocellular carcinoma (7). In addition, due to the high immunogenicity of this antigen and its high expression in various tumors, it has been a candidate for cancer immunotherapy in recent years and used as a cancer vaccine in clinical trials of various types of cancers (16).

Before a successful immunotherapy against the PCa can be established, it is important to know the expression pattern of the MAGE-A3 protein in prostate tissues as well as its association with the prognosis of the PCa. We examined, for the first time, tissue microarray (TMA) and needle biopsy specimens from PCa, as well as tissue samples from high-grade prostatic intraepithelial neoplasia (HPIN) and benign prostatic hyperplasia (BPH) for the expression of MAGE-A3 by immunohistochemistry analysis. Then, we evaluated the relationship between the expression of the MAGE-A3 with the clinic-pathological parameters, BCR-free survival (BCR-FS) and disease-free survival (DFS).

Material And Methods

Patient characteristics

A total of 153 paraffin-embedded prostate tissue samples were used for immunohistochemical analysis. Of these, 112 were cases of PCa, 22 cases of BPH and 19 cases of HPIN diagnosed by pathological hematoxylin and eosin (H&E) staining. In this retrospective study, we obtained paraffin-embedded blocks of prostate tissues from the Hasheminejad Kidney Center- a major university-based referral urology center in Tehran, Iran. These tissues belonged to patients who underwent surgery between 2006 and 2015 (radical prostatectomy and simple prostatectomy) as well as prostate biopsies before receiving current treatments including radiotherapy, hormone therapy or chemotherapy. Clinical reports of patients including age and serum PSA level (before surgery or treatment) were obtained from their records. Histopathologic data were also collected, which includes pathologic tumor stage (pTNM staging), tumor volume, Gleason score (Gs) and extra prostatic extension (EPE). Data of tumor invasion to lymph nodes, bladder neck, seminal vesicles and vasa deferentia, vascular invasion, perineural invasion and surgical margins were also collected. The pathologic tumor stage was determined based on the AJCC / UICC TNM staging system and the tumor grade (Gs) was defined according to the International Scoring System Society of Urological Pathology (ISUP) consensus (17, 18).

Regarding available PCa patients, we collected data on BCR (increase of serum PSA level to above 0.2 ng/ml with a steady increase), time of clinical symptoms, treatments received after surgery, and from those who were refractory or susceptible to treatment. We also collected data on cause of death from the year of surgery or primary treatment until the end of 2019. BCR was defined as the time from radical prostatectomy to the date of rise in serum PSA in PCa. DFS rates in PCa were calculated from the date of radical prostatectomy to clinical symptoms, metastasis or PCa-related death during treatment with ADT, radiotherapy and chemotherapy.

Ethical approval and consent

Tissue samples were obtained from patients with informed consent. Patient information was anonymously used in order to respect patients' rights and maintain ethical principles. This research is approved by the Ethics Committee of Iran University of Medical Sciences.

Immunohistochemistry (ihc)

Immunohistochemical analysis was performed on PCa, HPIN, and BPH tissues. From 153 prostate tissue samples fixed with formalin, paraffin-embedded 5-µm slices were prepared for Immunohistochemistry staining on charged slides (Superfrost Plus, Thermo Scientific, Germany). TMA was performed on tissues obtained from prostatectomy including PCa, BPH and HPIN samples (19).

The best area of paraffin-embedded tissue was determined through investigating H &E slides by a professional pathologist for TMA preparation. Tissue cores were prepared for each prostatectomy

specimen using the Tissue Arrayer MiniCore (ALPHELYS, Plaisir, France). To enhance accuracy, 3 cores were selected and evaluated for every specimen of microarray tissues. The average scoring of all 3 cores was considered as the final score. In addition, needle biopsy specimens of PCa with high grade malignancy were also collected and their whole sections were prepared.

Slides were incubated at 60 ° C (for 20 minutes) and then immersed in xylene to be deparaffinized. Subsequently, the slides were rehydrated with increasing ethanol dilution. They were exposed to methanol containing 3% hydrogen peroxide (H₂O₂) to inhibit intracellular peroxidase (20 min at room temperature). Slides were autoclaved (10 min) in citrate buffer (pH = 6) for antigen retrieval by heat. Primary antibody (anti-MAGE-A3 antibody, Biorbyt, UK) was added with different dilutions (1/100, 1/500, 1/1000) (overnight at 4 ° C) to obtain the best dilution. The primary antibody with dilution of 1/500 was selected as optimal dilution. Secondary antibody (EnVision™ + / HRP, Dual Link Rabbit / Mouse, Dako, Denmark) was added to tissues and were incubated for 60 min at room temperature. In this study, secondary antibody (EnVision) with standard chain polymer-conjugated technique was used to enhance the quality of tissue staining. 3,3'-diaminobenzidine (DAB; Dako) as substrate was added to the slides to visualize the antigen reaction with the antibodies and then hematoxylin staining was performed for counterstaining. Finally, the slides were dehydrated by decreasing ethanol dilution, clarified with xylene and then mounted. In this study, PBS buffer was used instead of the primary antibody as the negative control.

Scoring System

The expression pattern of MAGE-A3 on TMA and needle biopsy tissues was evaluated by a semi-quantitative scoring system. A professional pathologist, unaware of the clinical and pathological information of the patients, observed IHC-stained samples by optical microscope. Initial evaluations were performed at 10x magnification and subsequently positive cells with higher magnification were observed. Scoring was separately performed for MAGE-A3 cytoplasmic and nuclear expression patterns. Tissue staining intensity was classified as follows: 0 (no expression), 1 (weak), 2 (moderate) and 3 (strong). Histochemical score (H-score) was calculated for each tissue sample separately and used for statistical analysis of clinic and pathologic parameters, BCR-FS and DFS. Ranging from 0 to 300, the H-score is the product of multiplying the staining intensity by the percentage of staining of cells.

Statistical analysis

Statistical analysis was performed using SPSS software version 25 (SPSS, Chicago, IL, USA). Independent sample t-test was run to differentiate MAGE-A3 expression in PCa, HPIN and BPH groups. Pearson's χ^2 test was run to evaluate the association of MAGE-A3 expression with clinic and pathological parameters. One-way analysis of variance (ANOVA) procedure was employed to compare various Gs with MAGE-A3 expression. Receiver operating characteristic (ROC) curve analysis was performed to determine the sensitivity, specificity and differentiation of MAGE-A3 expression among PCa, HPIN and BPH groups.

To evaluate MAGE-A3 as a marker of prognosis, survival analysis was performed by Kaplan-Meier method (with log-rank test). To predict MAGE-A3 expression, clinical and pathological parameters as independent factors for survival, Cox regression analysis were assessed. Multivariate analysis was performed based on the results of univariate analysis. P-value of less than 0.05 was considered significant. Charts were drawn using Prism™ version 8.3.0 software (Graph Pad Inc., San Diego, CA, USA) Graphs.

Results

Study population

The median age of patients in PCa, BPH and HPIN groups was 66 years (range 48–90), 69 years (range 56–89) and 65 years (range 49–79), respectively. The overall median age of these three groups was 66 years, which is the range from 48 to 90 years. Serum PSA levels in PCa patients (before surgery and receiving systemic treatment) were divided into three groups: less than 4 ng / ml, between 4 and 10 ng / ml, and more than 10 ng / ml (range 6-352, median :9 ng/mL) (20). Of the 90 PCa patients whose PSA level information was available, 6 cases (6.7%) had a PSA of < 4 ng / ml, 46 cases (51.1%) 4 to 10 ng / ml and 38 cases (42.2%) > 10 ng / ml. Of the 91 PCa cases with available tumor volume information, the median tumor volume percentage was 30 (range 5–100%). Based on the cut-off median point, tumor volume percentages were categorised into two groups: values of smaller than 30 (52 cases, 57.1%) and values of greater than 30 (39 cases, 42.9%).

The pathological grade of the tumor was assessed by Gs and classified as follows (21):

1. Tumors with Gs 6 or well-differentiated were observed in 40 (35.7%) tissues.
2. Tumors with Gs 7 (3+4) or moderately differentiated were seen in 33 (29.5%) tissues.
3. Tumors with Gs 7 (4 + 3) or moderately-poorly differentiated were found in 15 (13.4%) tissues.
4. Tumors with Gs 8 or poorly differentiated were found in 11 (9.8%) tissues.
5. Tumors with Gs 9-10 or undifferentiated were seen in 13 tissues (11.6%).

As evident in the patient's data available for pTNM staging, 49 (60.5%) had PT2 and 32 (39.5%) pT3.

H-score median was considered as cut-off (100 for nuclear expression and 200 for cytoplasmic expression). Patients were classified into high and low expressions according to the H-score median. We considered values above median as high expression and values below median as low expression.

All the information on age, pathologic features of tumors, and tumor invasion into the surrounding tissues are summarized in Tables 1 and 2.

Table 1

Association between MAGE-A3 expressions (intensity, percentage of positive cells and H-score) and clinic-pathological parameters of PCa cases (P-value, Pearson's chi-square test)

Patient and tumor characteristics	Total samples N (%)	Nuclear expression of MAGE-A3			Cytoplasmic expression of MAGE-A3		
		Intensity	% of positive cells	H-score (cut-off = 100)	Intensity	% of positive cells	H-score (cut-off = 200)
age	59(52.7%)	0.247	0.201	0.930	0.570	0.198	0.581
≤ 66	53(47.3%)						
> 66	112						
Total							
PSA(ng/mL)	6(6.7%)	0.349	0.574	0.152	0.252	0.557	0.356
≤ 4	46(51.1%)						
4–10	38(42.2%)						
> 10	90						
Total							
Gleason scores	40(35.7%)	> 0.0001	0.051	> 0.0001	> 0.0001	0.571	0.006
Gs 6	33(29.5%)						
Gs 7(3 + 4)	15(13.4%)						
Gs7(4 + 3)	11(9.8%)						
Gs 8	13(11.6%)						
Gs 9–10	112						
Total							
Tumour volume (%)	52(57.1%)	0.038	0.458	0.239	0.540	0.817	0.437
≤ 30	39(42.9%)						
> 30	91						
Total							

Patient and tumor characteristics	Total samples N (%)	Nuclear expression of MAGE-A3			Cytoplasmic expression of MAGE-A3		
		Intensity	% of positive cells	H-score (cut-off = 100)	Intensity	% of positive cells	H-score (cut-off = 200)
pTNM system	49(60.5%)	0.938	0.763	0.928	0.197	0.845	0.408
pT2	32(39.5%)						
pT3	0						
pT4	81						
Total							
Gs: Gleason score, H-score: histological score, PCa: prostate cancer, PSA: prostate specific antigen, pTNM: pathologic tumor stage.							

Table 2

Association between MAGE-A3 expression (intensity of staining, percentage of positive cells and H-score) with surgical margin, regional lymph node, perineural invasion, seminal vesicles, vasa deferentia, vascular invasion and bladder neck involvement (P-value, Pearson's chi-square test).

Patient and tumour characteristics	Total samples N (%)	nuclear expression of MAGE-A3			cytoplasmic expression of MAGE-A3		
		Intensity	% of positive cells	H-score (cut-off = 109)	Intensity	% of positive cells	H-score (cut-off = 165)
Extra Prostatic Extension	16(23.2%)	0.001	0.204	0.003	0.637	0.424	0.485
Present	53(76.8%)						
Absent	69						
total							
Regional lymph node involvement	7(8.3%)	0.964	0.878	0.788	0.416	0.203	0.661
Present	77(91.7%)						
Absent	84						
total							
Perineural invasion	97(95.1%)	0.701	0.696	0.334	0.750	0.309	0.922
Present	5(4.9%)						
Absent	102						
total							
Vascular invasion	3(4.2%)	0.976	0.572	0.839	0.565	0.405	0.312
Present	68(95.8%)						
Absent	71						
total							
Surgical margins	32(39%)	0.711	0.928	0.423	0.885	0.536	0.621
Present	50(61%)						
Absent	82						
total							

Patient and tumour characteristics	Total samples N (%)	nuclear expression of MAGE-A3			cytoplasmic expression of MAGE-A3		
		Intensity	% of positive cells	H-score (cut-off = 109)	Intensity	% of positive cells	H-score (cut-off = 165)
Seminal vesicles and vasa deferentia	16(19.5%)	0.547	0.848	0.598	0.044	0.369	0.012
Present	66(80.5%)						
Absent	82						
total							
Bladder neck involvement	5(11.4%)	0.795	0.635	0.940	0.387	0.660	0.979
Present	39(88.6%)						
Absent	44						
total							

Nuclear and cytoplasmic expressions of MAGE-A3 in terms of H-score between PCa, HPIN and BPH groups

Expression pattern of MAGE-A3 protein in 153 prostate tissue samples (112 PCa samples, 22 BPH and 19 HPIN) was analysed by IHC. Immunohistochemical analysis showed the pattern of both nuclear and cytoplasmic expressions of MAGE-A3 in all prostate tissue epithelial cells (Fig. 1).

Among PCa and BPH tissues, negative staining of nuclear and cytoplasmic expressions was not observed, except in one case of HPIN tissues. It should be noted that in HPIN and BPH tissues, strong staining was not observed for both nuclear and cytoplasmic patterns.

Intensity of nuclear staining for 112 PCa tissues was as follows: 57 cases (50.9%) weak, 41 cases (36.6%) moderate, 14 cases (12.5%) strong and for cytoplasmic staining: 24 cases (21.4%) weak, 65 cases (58%) moderate and 23 cases (20.6%) strong. For 19 HPIN tissues, the nuclear and cytoplasmic staining intensities were similar: one case (5.3%) no staining, 17 cases (89.5%) weak, one case (5.3%) moderate and no case with strong staining. For 22 BPH tissues, the nuclear and cytoplasmic staining intensities revealed similar statistics: 21 cases (95.5%) weak and one case (4.5%) moderate (Fig. 1).

Statistical analysis was performed by independent sample t-test which compared the pattern of nuclear and cytoplasmic expressions of MAGE-A3 in PCa, HPIN and BPH groups. There was a significant

difference for nuclear expression between the PCa and HPIN ($P = 0.011$) and PCa and BPH ($P = 0.034$) groups. A significant difference was also observed between the cytoplasmic expression of PCa and HPIN ($P < 0.0001$) and PCa and BPH ($P < 0.0001$). P-values for nuclear and cytoplasmic expression patterns of MAGE-A3 were 0.294 and 0.654 between HPIN and BPH groups, respectively (Fig. 2, A and B). These results indicated the high expression of MAGE-A3 in PCa and represented differences of staining intensity for both cytoplasmic and nuclear expressions in cancerous tissues compared to BPH and HPIN.

According to the ROC curve analysis, the AUC values (area under the curve) with 95% confidence interval (CI) for the MAGE-A3 nuclear expression between the PCa and HPIN groups was 0.654 (95% CI: 0.56–0.74), and between PCa and BPH 0.623 (95% CI: 0.52–0.71). The AUC value for cytoplasmic expression between the PCa and HPIN groups were 0.876 (95% CI: 0.80–0.94) and for the PCa and BPH 0.874 (95% CI: 0.80–0.94). The AUC values of 0.556 (95% CI: 0.37–0.73) was calculated for nuclear expression and 0.516 (95% CI: 0.33–0.69) for cytoplasmic expression between HPIN and BPH (Fig. 2, C-H). These analyses demonstrated that there are distinct expressions of MAGE-A3 between PCa versus BPH and HPIN.

Correlation of MAGE-A3 expression in terms of H-score with clinic-pathological features

The association of MAGE-A3 nuclear or cytoplasmic expression with clinical and pathological features was evaluated using Pearson Chi-Square analysis. A significant relationship was found between MAGE-A3 nuclear expression with EPE ($P = 0.003$). The results also showed that there was a significant relationship between MAGE-A3 cytoplasmic expression pattern with tumor invasion to seminal vesicles ($P = 0.012$) (Table 2).

We observed a significant positive relationship between Gs and nuclear and cytoplasmic expression patterns of MAGE-A3 ($P < 0.0001$ and 0.006, respectively) (Table 1). The Gs was also divided into low and high grades; Gs 6 and 7 (3 + 4) as low grade, and Gs 7 (4 + 3) and 8–10 as high grade. A significant positive correlation between classified Gs and the MAGE-A3 nuclear and cytoplasmic expressions was also observed ($P < 0.0001$ and $P = 0.022$, respectively). These findings indicated that MAGE-A3 expression increase with higher grade of malignancy in PCa tissues (Fig. 3, A and B). ANOVA (post-hoc comparisons; Scheffe) analyses were also performed to compare various Gleason scores with MAGE-A3 expression. For nuclear expressions, a significant relationship was observed between Gs 6 with Gs 8, 9 and 10 ($P = 0.004$, 0.006, 0.002, respectively), Gs 7 (3 + 4) with Gs 8 and 9 ($P = 0.017$, 0.024, respectively) and Gs 7 (4 + 3) with Gs 8, 9 and 10 ($P = 0.019$, 0.027, and 0.039, respectively). A significant relationship was observed for cytoplasmic expression between Gs 6 with Gs 10 ($P = 0.018$) (Fig. 3, C and D).

Except for the above mentioned, there was no significant relationship between cytoplasmic and nuclear expression patterns of MAGE-A3 with other clinical and pathological features listed in Tables 1 and 2.

Prognostic significance of MAGE-A3 expression in terms of H-score in PCa

Kaplan-Meier analysis (with log-rank test) was used to investigate the association between MAGE-A3 expression with BCR-FS and DFS. Based on the median H-score, nuclear and cytoplasmic expressions of MAGE-A3 were divided into high and low groups. BCR and DFS information were obtained from the time of surgery or primary treatment until the end of 2019 (for patients whose information was accessible; N = 51). The mean follow-up period was 82.15 months (SD = 4.54), median 93 months, and range was 5-153 months. Table 3 shows the main characteristics of patients included for survival analysis.

Table 3
The main characteristics of PCa patients included for survival analysis

Number of patients (n)	51
Duration of follow up time (mean, SD)	82.15 months, 4.54
Duration of follow up time (median)	93 months
Number of BCR (%)	33(64.7%)
Number of patients with resistance to treatment (%)	6(11.7%)
Number of metastasis patients (%)	3(5.8%)
Number of alive patients without any complication (%)	28(54.9%)
Number of death related to PCa (%)	7(13.7%)
BCR: biochemical recurrence, SD: standard deviation, PCa: prostate cancer	

There was a significant discrepancy between nuclear expression of MAGE-A3 with BCR-FS and DFS of patients ($P = 0.042$, $P = 0.0001$ respectively) (Fig. 4, A and B). The mean DFS for patients with low and high MAGE-A3 nuclear expression was 130 (SD = 7.04) and 83 (SD = 11.07) months, respectively. The mean BCR-FS for patients with low and high MAGE-A3 nuclear expression was 130 (SD = 7.67) and 86 (SD = 11.78) months, respectively. There was no significant association between cytoplasmic expression of MAGE-A3 with BCR and DFS of patients ($P = 0.504$, $P = 0.115$, respectively) (Fig. 4C and D). These results showed that BCR-FS and DFS rates were significantly longer for patients whose tumors were classified as low expression for nuclear MAGE-A3 expression, as compared with patients whose tumors were classified as high expression for nuclear MAGE-A3.

Significant parameters affecting both DFS and BCR-FS in univariate analysis included nuclear expression of MAGE-A3 and Gs stratified as ≤ 7 vs > 7 or 6, 7(3 + 4) vs 7(4 + 3)-10. In multivariate analysis, nuclear expression of MAGE-A3 and GS (≤ 7 vs > 7) were significantly related to DFS (for both; $P = 0.019$). Nuclear expression of MAGE-A3 was also significantly related to BCR-FS ($P = 0.015$). Based on the results of this study, high MAGE-A3 expression is an independent marker of poor prognosis in PCa (Table 4).

Table 4

Univariate and multivariate Cox regression analyses of potential prognostic factors for DFS and BCR-FS in patients with PCa.

	DFS		BCR-FS	
Covariate	Univariate analysis	Multivariate analysis	Univariate analysis	Multivariate analysis
	HR (95% CI) p value	HR (95% CI) p value	HR (95% CI) p value	HR (95% CI) p value
Nuclear Hscore of MAGE-A3	3.04(1.24–7.42) 0.015	3.38(1.22–9.36) 0.019	3.75(1.39–10.13) 0.009	4.17(1.31–13.24) 0.015
Low vs high				
TNM stage	4.7(0.9-24.56) 0.066		4.36(0.79-23.92) 0.09	
T2 vs T3				
Gleason score	7.54(3.03–18.75) <0.001	3.87(1.09–13.79) 0.019	7.51(2.87–19.63) <0.001	
≤ 7 vs > 7				
6, 7(3 + 4) vs 7(4 + 3)-10	4.05(1.67–9.84) 0.002		4.7(1.73–12.74) 0.002	
Tumor volume (%)	0.66(0.19–2.32) 0.527		1.07(0.28–4.06) 0.915	
≤ 30% vs > 30%				
Age	1.08(0.44–2.68) 0.856		0.78(0.3–2.01) 0.6	
≤ 66 vs > 66				
BCR-FS: biochemical recurrence -free survival, DFS: disease-free survival, CI: confidence interval, HR: hazard ratio, H-score: histological score, PCa: prostate cancer.				

Discussion

In this study, for the first time, we performed immunohistochemical analysis to evaluate the expression pattern of MAGE-A3 in 153 prostate tissue samples including PCa, HPIN and BPH. Highly sensitive for staining tissue, EnVision method was employed and almost all studied tissues were stained with various staining intensities. The results were analysed with the clinical and pathological parameters of the patients with PCa (age, EPE, pTNM, tumor volume, Gs and invasion of the tumor toward the surrounding regions of the prostate gland (). Survival analysis was also performed to investigate the vitality of the MAGE-A3 as a potential prognostic factor.

Hudolin et al. performed immunohistochemical analysis by staining multiple MAGE-A on 30 samples in patients with penile carcinoma and 92 PCa. They found cytoplasmic expression of multiple MAGE-A in 97% of penile carcinoma samples (22) and 85.9% of PCa (23). Most previous studies on different types

of cancers have not drawn a distinction between nuclear and cytoplasmic expression pattern of MAGE-A family and have not examined the association of MAGE family subcellular expression pattern with disease prognosis. Based on a study by Tinguely and colleagues on myeloma patients, expression of MAGE-C1 antigen was examined, suggesting that sub-cellular localization of this marker is associated with disease prognosis. It was found that patients with only cytoplasmic expression of MAGE-C1 antigen had better prognosis compared to patients who had only nuclear expression or concomitant nuclear and cytoplasmic expression of this antigen. It was also shown that nuclear expression was significantly correlated with poor prognosis and more aggressive form of the tumor. The researchers suggested that the biological function of the tumor cells may be related to the subcellular expression pattern of the MAGE-C1 antigen and since members of the MAGE family function as transcriptional regulators, MAGE-C1 activity may be regulated by localization in the nucleus; the high expression of this antigen in the nucleus is also associated with higher tumor malignancy and poor prognosis (24).

Another study by Laban and his colleagues examined the MAGE-A family nuclear and cytoplasmic expression pattern in patients with head and neck cancers. The researchers noted that the subcellular expression pattern of MAGE family members is important in prognosis. Simultaneous expression of cytoplasmic and nuclear MAGE family antigens in patients rendered significant and direct relationships with clinical outcomes and overall survival of patients. It was hypothesized that the pattern of subcellular expression of these antigens could indicate a difference in their biological activity (25).

However, our findings indicated both nuclear and cytoplasmic expressions of MAGE-A3 in the studied groups (PCa, HPIN and BPH). There was a significant difference between nuclear and cytoplasmic expressions of MAGE-A3 in PCa, HPIN, and BPH groups. The nuclear and cytoplasmic expressions of MAGE-A3 in PCa samples significantly increased compared to BPH and HPIN. HPIN is a pre-cancerous step, a condition where some epithelial prostate cells begin to behave abnormally (26). We found no difference in nuclear and cytoplasmic MAGE-A3 expression between BPH and HPIN implying that alterations in the low expression of the MAGE-A3 are insignificant in BPH and HPIN, and that they need to be transformed into malignant cells to elevate the expression of the MAGE-A3. Additionally, BPH tissues were weakly stained with MAGE-A3 antibody because of two reasons: first, we used sensitive EnVision method; and second, non-cancerous prostate tissues may also poorly express the MAGE-A3, although further studies are needed.

A significant direct correlation was observed between MAGE-A3 nuclear and cytoplasmic expressions and Gs, so that with increasing tumor grade, the expression of this protein increased. Consistently, when Gs patients were classified as low and high grades, a direct correlation was also found between both MAGE-A3 nuclear and cytoplasmic expressions. In line with these findings, we analysed tumor involvement into areas surrounding the prostate tissue with MAGE-A3 expression; except for EPE and invasion to seminal vesicles, no significant correlation was found between the MAGE-A3 expression and the spread of tumor toward the surrounding regions of the prostate. As an explanation, information regarding tumor involvement into areas surrounding the prostate tissue for all patients was not available and further investigations are needed for this matter.

As previously mentioned, the MAGE family members are transcriptional regulators which regulate the activity of KAP1, p53 and AR, thereby they translocate into nuclear and affect gene transcription (7). Given that the CTAs are functionally silent in adult normal cells, but upregulated in malignant cells (27), it can be concluded that tumors with higher grade of malignancy increased the MAGE-A3 nuclear expression level. Since this protein is a transcriptional regulator and functions in the cell nucleus, it seems reasonable that the high Gs in the patients has a strong significant relationship with the high nuclear expression of MAGE-A3.

Previous studies on various cancers (such as non-small cell lung carcinoma (28), gastric cancer (29), urothelial bladder cancer (30), cutaneous squamous cell carcinoma (31) and lymphoma (32)) have shown a direct relationship between MAGE-A3 expression and poor prognosis. However, no study has been conducted to investigate the association between this marker and the prognosis of PCa. In our study, survival analysis was performed and the association of either nuclear or cytoplasmic expression of MAGE-A3 with BCR-FS and DFS was determined. Univariate analysis indicated that nuclear MAGE-A3 expression had a significant association with BCR-FS and DFS, which was later confirmed in multivariate analysis. The BCR-FS and DFS were longer in patients with nuclear poor expression of the MAGE-A3; however, no association was observed for cytoplasmic expression of the MAGE-A3. Additionally, nuclear expression of MAGE-A3 and Gs (classified into ≤ 7 vs > 7) were independent prognostic indicators for DFS in patients with PCa. Overall, these findings suggested a significant effect between elevated expression of nuclear MAGE-A3 and poor prognosis, and MAGE-A3 could be potentially considered an independent indicator for prognosis in patients with PCa. We interpreted these results based on MAGE-A3 function as a transcriptional regulator and its high nuclear expression in patients with high Gs. It means that, at high malignancy, where the MAGE-A3 nuclear expression is increased, disease prognosis is poor.

Although current treatments for cancer such as chemotherapy and radiotherapy are effective for some patients, they are inadequate for patients with advanced PCa. Immunotherapy as cancer vaccine has always been an attractive option in the treatment of patients with PCa. Most clinical trials have used the MAGE-A family as an immunogen in cancer vaccines. On the other hand, Sipuleucel-T is autologous cell-based immunotherapy in which antigen presenting cells (APCs) are activated by prostatic acid phosphatase antigen and granulocyte-macrophage colony-stimulating factor (GM-CSF), and the response of specific T cells arises in vivo conditions. Sipuleucel-T is the only cell-based cancer vaccine for PCa approved by Food and Drug Administration (FDA), which is used to treat patients with mCRPC who are asymptomatic or minimally symptomatic (33). However, as studies have shown, it does not prolong progression-free survival and causes rapid PSA progression (34). One of the ways to enhance strong immune responses against tumor cells and prolong the survival of patients is to use tumor immunogenic antigens in vaccination. Accordingly, the introduction of a marker with high immunogenicity is an attractive proposition for vaccine-mediated immunotherapy in patients with PCa. Based on a study conducted in phase II clinical trial on patients with melanoma (35), they were treated with recombinant MAGE-A3 protein and two immunostimulants. Humoral and cellular immune responses were observed and the data from this study provided a good basis for the onset of larger clinical trials based on the MAGE-A3 vaccine. In another clinical study conducted on multiple-myeloma patients, the safety and

efficacy of MAGE-A3-stimulated T cells in ex-vivo environment were evaluated and the majority of patients showed a clinical response to MAGE-A3. This response was induced by specific T cells against MAGE-A3 which were also well tolerated in patients (36). Several clinical trials are currently underway with the aim of using MAGE-A in vaccine immunotherapy. In the current study, we demonstrated a high expression of MAGE-A3 in tissues of PCa patients with high malignancy and its association with poor prognosis. The results of this study could be the basis for the establishment of vaccination by this antigen in PCa.

Totally, it can be inferred that the expression level of the MAGE-A3 increased with a higher degree of tumor malignancy. BCR-FS and DFS were shortened with elevated nuclear expression of MAGE-A3, making MAGE-A3 a potential prognostic indicator in PCa. According to the information mentioned above and the results of this study, the high expression of MAGE-A3 in PCa tissues could make this protein as a suitable candidate for vaccine immunotherapy of PCa. It could be a breakthrough for future researchers and a step toward introducing new therapeutic approaches in patients with PCa.

Declarations

Acknowledgment

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Role of the funding source

The funding source is a college institute and has sponsored the project financially and approved.

Ethical approval

All procedures performed in the study were in accordance with the ethical standards of the institution at which the study was conducted.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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Figures

Figure 1

Total staining pattern of MAGE-A3 expression in cores (original magnification of cores $\times 100$) and needle biopsies (original magnification $\times 400$) of PCa tissues (A-D) with pathological different grades of tumor, BPH (E), HPIN cores (F) and PCa tissue without primary antibody (negative control; G). A, weak staining for both nuclear (100% positive cells) and cytoplasmic (100% positive cells) expressions (low grade, Gs 3 + 3); B, weak staining for nuclear expression (30% positive cells) and strong staining for cytoplasmic

expression (80% positive cells) (low grade, Gs 3 + 4); C, strong staining for nuclear expression (70% positive cells) and moderate staining for cytoplasmic expressions (100% positive cells) (high grade, Gs 4 + 4); D, moderate staining for both nuclear and cytoplasmic expressions (for both; 70% positive cells) (high grade, Gs 5+4); E, weak staining for both nuclear (90% positive cells) and cytoplasmic (100% positive cells) expressions of BPH tissue; F, weak staining for both nuclear and cytoplasmic expressions (for both; 100% positive cells) of HPIN tissue ; and G, no staining of PCa tissue. Left upper corner of each core is the same figure with higher magnification.

Fig 2

Figure 2

Different expression of MAGE-A3 in PCa, HPIN and BPH groups. Nuclear (A) and cytoplasmic (B) expression of MAGE-A3 among PCa, HPIN and BPH groups with immunohistochemical analysis (independent sample t test). ROC curve analysis represents nuclear expression of MAGE-A3 in PCa vs HPIN (C, AUC = 0.654, 95%CI :0.56-0.74), nuclear expression of MAGE-A3 in PCa vs BPH (D, AUC = 0.623, 95% CI:0.52-0.71), cytoplasmic expression of MAGE-A3 in PCa vs HPIN (E, AUC = 0.876, 95% CI: 0.80-0.94), cytoplasmic expression of MAGE-A3 in PCa vs BPH (F, AUC = 0.874, 95%CI:0.80-0.94), nuclear expression of MAGE-A3 in HPIN vs BPH (G, AUC = 0.556, 95% CI:0.37-0.73), and cytoplasmic expression of MAGE-A3 in HPIN vs BPH (H, AUC = 0.516, 95% CI:0.33-0.69)

Fig 3

Figure 3

Relationship between Gs and MAGE-A3 expression. Immunohistochemical analysis of nuclear (A, $P < 0.0001$) and cytoplasmic (B, $P = 0.022$) MAGE-A3 expression with pathological grade of tumor (Gs) (Pearson's chi-square test). Immunohistochemical analyses of nuclear (C) and cytoplasmic (D) MAGE-A3 expressions with pathological grade of tumor (Gs) (One-way ANOVA). For comparison between nuclear expression and Gs, P values were as follows: Gs 6 with Gs 8, 9 and 10 ($P = 0.004$, 0.006 , 0.002 , respectively), Gs 7 (3+4) with Gs 8 and 9 ($P = 0.017$ and 0.024 , respectively), Gs 7 (4 + 3) with Gs 8, 9 and 10 ($P = 0.019$, 0.027 , and 0.039 , respectively) and for cytoplasmic expression, p-value for Gs 6 and Gs 10 was 0.018

Fig 4

Figure 4

Survival analysis for MAGE-A3 expression in PCa patients. Kaplan-Meier curves of MAGE-A3 nuclear expression for BCR-FS (A, $P=0.042$) and DFS (B, $P=0.0001$), and cytoplasmic expression for BCR-FS (C, $P=0.504$) and DFS (D, $P=0.115$).