Original Article
Expression and clinical significance of cancer-testis genes in clear cell renal cell carcinoma

Bo Yin[1], Yu Zeng[2], Xiaosong Wang[3], Gang Liu[1], Mo Zhang[1], Yongsheng Song[1]

[1]Department of Urology, Shengjing Hospital of China Medical University, Shenyang 110004, China; [2]Department of Urology, The First Hospital of China Medical University, Shenyang 110001, China; [3]Dan L. Duncan Cancer Center, Department of Medicine, Baylor College of Medicine, Houston, TX 77030, USA

Abstract: Cancer-testis (CT) antigens, which are encoded by CT genes, have been recognized as a group of highly attractive targets for cancer immunotherapy. However, the expression and clinical relevance of CT genes in clear cell renal cell carcinoma (ccRCC) remains largely unknown. The present study aims to analyze the expression profile of 6 individual CT genes including MAGE-A1, MAGE-A3, MAGE-A12, cTAGE-1, cTAGE-2, and NY-ESO-1 in ccRCC and further investigate their possible correlations with clinicopathologic characteristics. The mRNA expressions of these CT genes were detected using reverse transcriptase-polymerase chain reaction (RT-PCR) in 105 ccRCC tissue samples (T_{1-2} in 70 samples, T_{3-4} in 35 samples; G_{1-2} in 65 samples, G_{3-4} in 40 samples) as well as the paired adjacent normal tissues. The most frequently expressed CT gene was MAGE-A3 (27.6%), followed by MAGE-A12 (23.8%), NY-ESO-1 (21%), MAGE-A1 (20%), cTAGE-1 (17.1%), and cTAGE-2 (14.3%). In contrast, no expression of CT genes was detected in the paired adjacent normal tissues. Furthermore, the MAGE-A3 protein expression was determined by Western blot and immunohistochemistry. MAGE-A3 protein was expressed in 21.9% ccRCC samples with a cytoplasmic staining pattern. No MAGE-A3 protein expression was found in the paired adjacent normal tissues. There was a significant correlation between MAGE-A3 expression at both mRNA (P =0.045) and protein (P = 0.03) levels with advanced stages of the disease. Taken together, CT genes may serve as promising targets of specific immunotherapy for ccRCC and particularly, MAGE-A3 may serve as a potential prognostic marker for ccRCC patients.

Keywords: Cancer-testis (CT) gene, clear cell renal cell carcinoma, immunotherapy, prognosis

Introduction

Renal cell carcinoma (RCC) represents the most common malignancy of the adult kidney and comprises 2-3% of all malignant tumors in adults [1]. Annual estimates of newly diagnosed cases of RCC have been increasing gradually over recent years [2]. Clear cell RCC (ccRCC) accounts for approximately 85% of RCC [3]. However, ccRCC possesses a high metastatic potential, and the prognosis of ccRCC patients is very poor [4]. Furthermore, ccRCC is resistant to conventional therapies such as radiation and chemotherapy [5], underscoring the urgent need for novel prognostic biomarkers and therapeutic targets.

RCC is regarded as one of the most immune responsive cancers and has well-documented responses to some cytokines such as interleu-

kin-2 and interferon-α [6, 7]. However, the overall efficacy of such nonspecific immunotherapy is limited; therefore, cancer-specific immunotherapy may become a promising strategy for treating patients with RCC [8].

A key step in the development of cancer-specific immunotherapy for RCC is the implementation of vaccination strategies allowing for the consistent induction of immune responses to tumor antigens. In this respect, the choice of appropriate antigens, based on both the frequency and the specificity of their expression in cancer tissues, is of paramount importance. Among tumor antigens identified to date, cancer-testis (CT) antigens have been recognized as a group of the most promising targets for cancer vaccines. CT antigens are encoded by CT genes. To date, more than 100 CT genes

Table 1. Primers and conditions of RT-PCR analysis

Genes	Primers from 5' to 3'	Annealing temperature	Cycle no.	Product size (bp)
MAGE-A1	f: GGAGCACCAAGGAGAAGA	57°C	35	291
	r: TGATGGTAGTGGGAAAGG			
MAGE-A3	f: AGTCCGAGTTCCAAGCAG	53°C	35	239
	r: GCAGGTGGCAAAGATGTA			
MAGE-A12	f: GGAAGATGGCTGAGTTGG	53°C	35	214
	r: AGGCAGGTGACAAGGATG			
cTAGE-1	f: GGAGACCTACCGAAAGCG	53°C	35	303
	r: TCAGATGAAGGCCAACCC			
cTAGE-2	f: GGAGACCTACCGAAAGCG	53°C	35	303
	r: TCAGATGAAGGCCAACCC			
NY-ESO-1	f: GAGCCGCCTGCTTGAGTT	53°C	35	245
	r: AGCACTGCGTGATCCACATC			
β-actin	f: GTGGGGCGCCCCAGGCACCA	53°C	35	498
	r: CTCCTTAATGTCACGCACGATTTC			

have been identified [9]. CT antigens are named after their pattern of expression, as they are found in various types of cancers but only in testicular germ cells of normal adult tissues [10]. Because of this tumor-associated expression pattern, CT antigens have been the focus of attention as potential targets in immunotherapy for cancer. Several therapeutic cancer vaccine trials of CT antigens such as MAGE-A3 and NY-ESO-1 have demonstrated the ability to induce cellular and humoral immune responses as well as an excellent safety profile without evidence of clinical autoimmunity [11, 12]. The persistence of vaccine-induced B- and T-cell memory responses years after booster immunization has also been demonstrated [12]. Consequently, the National Cancer Institute in the USA has placed two CT antigens, MAGE-A3 and NY-ESO-1, among the top 10 category of the Project for the Prioritization of Cancer Antigens [13].

Despite the attractiveness of CT genes in cancer-specific immunotherapy, the majority of them have not been investigated in ccRCC yet. Here, we analyzed the expressions of 6 CT genes, MAGE-A1, MAGE-A3, MAGE-A12, cTAGE-1, cTAGE-2, and NY-ESO-1 in 105 ccRCC tissue samples to suggest these genes as potential vaccine targets in this immune responsive tumor. We also sought to assess the correlation between CT gene expression and clinicopathologic characteristics to determine if any of these genes had potential prognostic value.

Materials and methods

Patients and tissue samples

Tissue samples from 105 ccRCC patients were collected in the Department of Urology of Shengjing Hospital of China Medical University between 2009 and 2013. All the patients, including 42 women and 63 men, were diagnosed with ccRCC for the first time and received surgical treatment. The median age of patients was 58 years (range 31-83). The pathologic stage of tumors was established in accordance with the tumor-node-metastasis (TNM) system recommended by the American Joint Committee on Cancer, which showed T_{1-2} in 70 cases and T_{3-4} in 35 cases. Using the Fuhrman grading system we classified tumor grade as follows: 65 cases were Fuhrman grade 1-2 (G_{1-2}) and 40 were Fuhrman grade 3-4 (G_{3-4}). All tumor samples and the paired adjacent normal tissues that were more than 5 cm away from the tumor margin were freshly obtained during surgery, subjected to snap-frozen in liquid nitrogen immediately after resection and then stored at -80°C until further use. The rest of the samples were subjected to routine pathology examination. Informed consent was obtained from each patient. The study protocol was approved by the institutional review board of Shengjing Hospital of China Medical University.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from 100 μg tissue samples using Trizol reagent (Invitrogen, CA, USA), and subsequently treated by DNase. The RNA samples were then stored at -80°C until further use. RT reaction was carried out in a 20 μl volume system, containing 1 μg RNA, 10 μl 2× buffer, 4 μl 25 mM $MgSO_4$, 1 μl 10 mM dNTPs, 1 μl 22 u/μl AMV, 1 μl 50 μM Oligo-dT, and 0.5 μl 40 u/μl RNase inhibitor.

The PCR system contained 3 μl cDNA, 2.5 μl 10× PCR buffer, 2 μl dNTPs (2.5 mM), 0.2 μl Taq enzyme (5 u/μl), 0.2 μl of each primer, and

Table 2. The mRNA expressions of 6 individual CT genes correlated with disease stage and grade of ccRCC

	Total	MAGE-A1 positive	MAGE-A3 positive	MAGE-A12 positive	cTAGE-1 positive	cTAGE-2 positive	NY-ESO-1 positive
ccRCC	105	21 (20%)	29 (27.6%)	25 (23.8%)	18 (17.1%)	15 (14.3%)	22 (21%)
Stage							
T_{1-2}	70	15 (14.3%)	15 (14.3%)	18 (17.1%)	12 (11.4%)	10 (9.5%)	16 (15.2%)
T_{3-4}	35	6 (5.7%)	14 (13.3%)*	7 (6.7%)	6 (5.7%)	5 (4.8%)	6 (5.7%)
Grade							
G_{1-2}	65	13 (12.4%)	17 (16.2%)	15 (14.3%)	11 (10.5%)	9 (8.6%)	14 (13.3%)
G_{3-4}	40	8 (7.6%)	12 (11.4%)	10 (9.5%)	7 (6.7%)	6 (5.7%)	8 (7.6%)

*$P = 0.045$.

16.9 µl ddH$_2$0. The primers and the reaction conditions are shown in **Table 1**. Primers for target genes were designed based on GenBank sequences. PCR was carried out under the following conditions: initial denaturation for 3 min at 94°C followed by denaturation for 40 s at 94°C, annealing for 1 min at variable temperatures (**Table 1**), elongation for 1 min at 72°C, totally 35 cycles, and ending with a final 7-min elongation at 72°C. Electrophoresis was done by loading 10 µl of each sample on a 2% agarose gel, and visualized by ethidium bromide staining using the Bio-Imaging System (Ultra-Violet Products, Cambridge, UK). Positive expression was defined as those bands that electrophoresed with the expected size. ß-actin was used as a loading control.

Western blot

Tissue samples were lysed, homogenated and centrifuged for 15 min at 12,000 rpm at 4°C. The supernatant was defined as total protein and the protein concentration was determined by the Bradford assay (BioRad, CA, USA). Then, 25 µg of total protein was separated on a 10-20% SDS–PAGE and transferred onto a PVDF membrane (Millipore, MA, USA). The membrane was incubated with primary antibody overnight at 4°C followed by incubation with horseradish peroxidase-conjugated secondary antibody, and developed with the Super Signal West Dura Extended Duration Substrate kit (Pierce, IL, USA). The primary antibody to MAGE-A3 (mouse monoclonal antibody; Santa Cruz Biotechnology, CA, USA) was incubated at 1:500 overnight then 1:2000 secondary antibody (horse anti-mouse antibody; Santa Cruz Biotechnology) was incubated for 2 h at room temperature. ß-actin was used as a loading control.

Immunohistochemistry (IHC)

Briefly, formalin-fixed paraffin-embedded tissue sections were deparaffinized in xylene, rehydrated through graded concentrations of ethanol to distilled water, and treated in hydrogen peroxide for 10 min at room temperature to block the endogenous peroxidase activity. The sections were then subjected to antigen retrieval by microwave heating for 15 min in 10 mM citrate buffer, pH 6.0. Polyclonal antibody LS-B884 (LifeSpan BioSciences, WA, USA) against MAGE-A3 was used as the primary antibody. The sections were incubated overnight with anti-MAGE-A3 antibody at a concentration of 10 µg/ml at 4°C, followed by detection using PowerVision two-step histostaining reagent (Zhongshan Biotechnology, Beijing, China).

Statistical analysis

Statistical analysis was done with SPSS 16.0 (SPSS Inc., IL, USA). Pearson x^2 test was performed to compare the correlation between disease stage, grade, and CT genes expression. $P < 0.05$ was considered statistically significant.

Results

The mRNA expressions of CT genes and the relationship with tumor characteristics

The mRNA expressions of 6 individual CT genes were analyzed in tumor samples and the paired adjacent normal tissues by RT-PCR. The most frequently expressed CT gene in ccRCC was MAGE-A3 (27.6%), followed by MAGE-A12 (23.8%), NY-ESO-1 (21%), MAGE-A1 (20%), cTAGE-1 (17.1%), and cTAGE-2 (14.3%); (**Table 2**). Examples of positive mRNA expression of each CT gene are shown in **Figure 1**. In con-
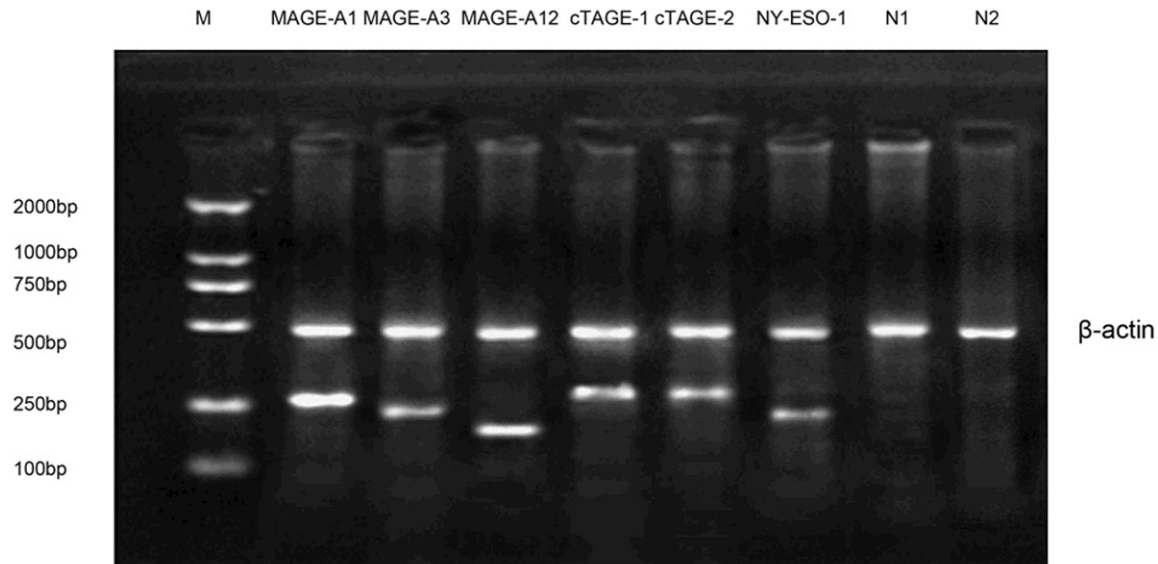
Figure 1. Examples of positive expression of each CT gene detected by RT-PCR in ccRCC. M: marker; N, N2: adjacent normal tissues.
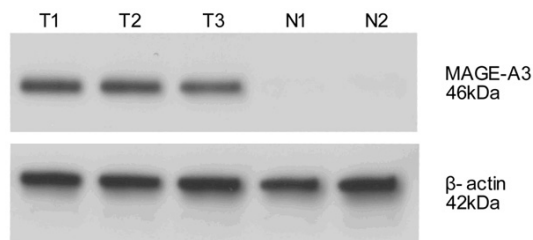

Figure 2. Expression of MAGE-A3 protein detected by Western blot in ccRCC. T1-T3: cancer tissues; N1, N2: adjacent normal tissues.

Table 3. MAGE-A3 protein expression by Western blot correlated with disease stage and grade of ccRCC

	Total	MAGE-A3 protein positive
ccRCC	105	23 (21.9%)
Stage		
T_{1-2}	70	11 (10.5%)
T_{3-4}	35	12 (11.4%)*
Grade		
G_{1-2}	65	13 (12.4%)
G_{3-4}	40	10 (9.5%)

*$P = 0.03$.

trast, no CT gene expression was found in the paired adjacent normal tissues. In all 105 ccRCC patients, 37 patients (35.2%) expressed at least one of 6 CT genes tested. Among them, 5 patients (4.8%) expressed one CT gene. The other 32 patients (30.5%) expressed multiple CT genes.

The relationship between CT gene expression and the clinicopathologic characteristics of the ccRCC samples in terms of tumor stage and degree are shown in **Table 2**. CT genes were expressed across all tumor stages and grades. Among 35 cases of T_{3-4} tumors, there were 14 cases with positive MAGE-A3 mRNA expression, while only 15 out of 70 cases of T_{1-2}. MAGE-A3 mRNA–positive cases were primarily found in advanced-stage (T_{3-4}) tumors in a statistically significant fashion ($P = 0.045$). The

other CT genes studied did not show any significantly different distribution between tumor stages. We did not detect any significant correlation between CT gene expression and tumor grade ($P > 0.05$).

Protein expression of MAGE-A3 by Western blot and the relationship with tumor characteristics

Western blot analysis revealed that MAGE-A3 protein was expressed in 23 (21.9%) ccRCC samples (**Figure 2**). As expected, all of these samples were previously found to be positive for MAGE-A3 mRNA expression. There was no MAGE-A3 protein expression found in the paired adjacent normal tissues.
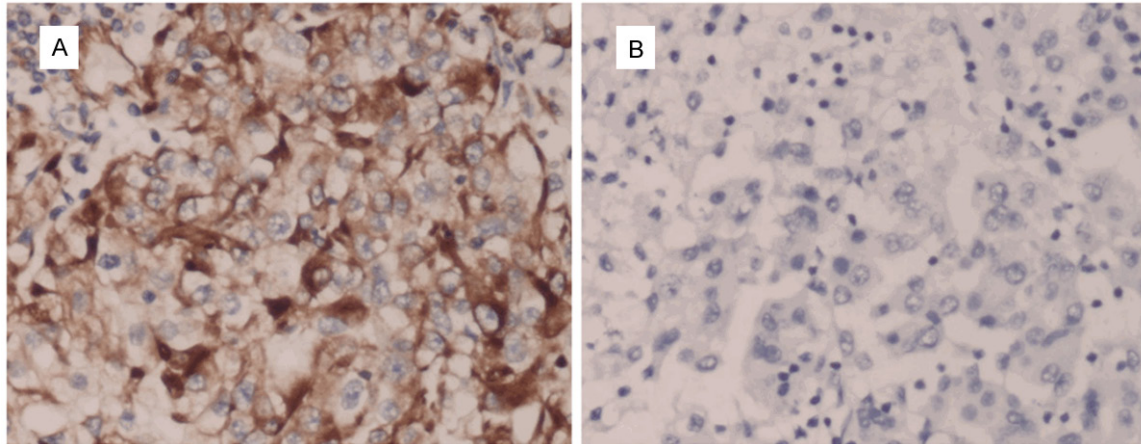
Figure 3. Immunohistochemical staining of MAGE-A3 protein in ccRCC. Panels refer to representative sections showing positive cytoplasmic staining (A) and negative staining (B). Original magnification is ×200.

The expression pattern of MAGE-A3 protein is summarized in **Table 3**. We detected a significant correlation between MAGE-A3 protein expression and advanced stages of the tumor ($P = 0.03$): tumor samples from advanced stages (T_{3-4}) showed a higher expression frequency of MAGE-A3 protein. We did not detect any significant correlation between MAGE-A3 protein expression and tumor grade ($P > 0.05$).

MAGE-A3 protein expression confirmed by IHC

Five ccRCC samples that had positive MAGE-A3 mRNA/protein expression and 5 samples that had negative mRNA/protein expression were stained by IHC. The expression pattern was heterogeneous, ranging from focal to diffuse immunoreactivity. As shown in **Figure 3**, MAGE-A3 expression showed an exclusively cytoplasmic staining pattern in all of the 5 samples with positive MAGE-A3 expression, and as expected, no expression was observed in the other 5 negative samples.

Discussion

In this study, we determined the expression profile of 6 CT genes including MAGE-A1, MAGE-A3, MAGE-A12, cTAGE-1, cTAGE-2, and NY-ESO-1 in 105 ccRCC tissue samples as well as the paired adjacent normal tissues. Using RT-PCR, we found that the mRNA expressions of 6 individual genes can be detected with various frequencies across all tumor stages and grades in ccRCC samples but not in the adjacent normal tissues, which was similar to the

results of some previous reports [14, 15]. Over 1/3 patients expressed at least one CT gene tested. There was a tendency that the mRNA expressions of these 6 CT genes appeared to be clustered. Among 37 CT mRNA–positive cases, 32 expressed multiple CT mRNAs simultaneously. This pattern was also found in melanoma, breast cancer, colon cancer, non-small-cell lung cancer and bladder cancer [16-19]. This clustering phenomenon may be related to the activation/de-repression process for CT genes in cancer. For instance, hypomethylation state in cancer cells might induce the activation of otherwise silent CT genes [17]. It is also possible that activation of a single CT gene could be the switch for activating other CT genes [16].

Among 6 CT genes studied, we were particularly intrigued by the expression of MAGE-A3. The MAGE-A3 gene encodes a 46 kDa protein that has been mapped to q28 of the X-chromosome. MAGE-A3 is of particular interest in tumor immunology since it is expressed in a wide variety of cancers and in a very restricted number of normal tissues. Lonchay et al. found that MAGE-A3 gene was one of the most commonly expressed CT genes in malignancies [20]. In the present study, we also found that MAGE-A3 showed the highest mRNA expression frequency (27.6%) in ccRCC samples among the 6 CT genes studied. Furthermore, we observed that the mRNA expression of MAGE-A3 in T_{3-4} ccRCC samples was much higher than that in T_{1-2} samples. There was a significant correlation between MAGE-A3 mRNA expression and

advanced stages of the disease (P = 0.045). This is consistent with previous reports that increased CT antigen expression was associated with more aggressive diseases, or advanced stages of disease [21, 22]. Therefore, the expression of MAGE-A3 may have potentially prognostic as well as therapeutic value for ccRCC patients.

Since the expression of tumor antigen at protein level is a prerequisite for design of a therapeutic vaccine, we further explored the protein expression of MAGE-A3 gene using Western blot and immunohistochemistry. This is of particular importance, as discrepancies have been reported between mRNA and protein expression levels in previous studies of CT genes [23, 24]. In the present study, we detected that the MAGE-A3 protein was also expressed in 23/105 (21.9%) ccRCC samples with a cytoplasmic staining pattern and was primarily found in advanced-stage (T_{3-4}) tumors in a statistically significant fashion (P = 0.03), which was consistent with its mRNA expression pattern. However, the microarray study by Jungbluth et al. showed that none of MAGE-A genes examined was expressed in RCC [25]. Some authors pointed out that they preferred full sections of tumor tissue rather than tissue microarray samples because of the heterogeneous expression of CT genes in different tumor areas [24]. Therefore, these conflicting data can be explained by differences in the sensitivities of the techniques employed or patient groups, emphasizing the need for additional studies to evaluate the potential of CT genes as biomarkers and candidate vaccine antigens for ccRCC.

Immunogenicity of MAGE-A3 has been demonstrated in clinical trials, including the induction of both cellular and humoral immune responses and the persistence of memory responses years after booster immunization [11, 12]. Furthermore, some recent data suggested that CT antigens may be predominantly expressed on cancer stem cells [26, 27]. Therefore, elimination of CT antigen-positive cells may be effective at eradicating the tumor. These unique features of MAGE-A3, such as its frequent expression in tumors but very restricted expression in normal tissues, as well as its spontaneous immunogenicity, rank it among the most attractive targets for cancer vaccines. Multiple clinical trials using MAGE-A3 antigen have been carried out, with promising results observed in several studies [12, 28, 29].

However, the high disease burden and associated immunosuppressive tumor environment are believed to represent the important obstacles limiting the efficacy of the induced immune response from cancer vaccines. Multi-antigen vaccines might be a benefit in this case by eliciting more effective T-cell responses and limiting the potential for positive-selection of antigen-negative tumor cells that may 'escape' CTL killing [10, 16, 30]. The characterization of CT antigens expression pattern in a particular type of tumor may be of critical importance in developing multi-antigen vaccines for active specific immunotherapy. In this study, instead of analyzing selected antigen, we evaluated a broad panel of CT genes. Our data about the expression profile of CT genes in ccRCC could be particularly useful for the targeting of therapeutics in the form of multi-antigen vaccine preparations in active specific immunotherapy for ccRCC.

Taken together, the present study determined the expression profile of 6 individual CT genes in tissue samples of ccRCC. No expression of CT genes was detected in the paired adjacent normal tissues. Therefore, CT genes may serve as promising targets of specific immunotherapy for ccRCC, especially based on multi-antigen vaccine preparations. Furthermore, we observed a significant correlation between MAGE-A3 expression at both mRNA and protein levels with advanced stages of the disease. MAGE-A3 thus may serve as a potential prognostic marker for ccRCC patients.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yongsheng Song, Department of Urology, Shengjing Hospital of China Medical University, 36 Sanhao Street, Shenyang 110004, China. Tel: +86-24-96615-34211; E-mail: yinbowzf@163.com

References

[1] Escudier B. Emerging immunotherapies for renal cell carcinoma. Ann Oncol 2012; 23 Suppl 8: viii35-40.

[2] Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer J Clin 2012; 62: 10-29.

[3] Karumanchi SA, Merchan J, Sukhatme VP. Renal cancer: molecular mechanisms and newer therapeutic options. Curr Opin Nephrol Hypertens 2002; 11: 37-42.

[4] Neumann E, Engelsberg A, Decker J, Störkel S, Jaeger E, Huber C, Seliger B. Heterogeneous expression of the tumor-associated antigens RAGE-1, PRAME, and glycoprotein 75 in human renal cell carcinoma: candidates for T-cell-based immunotherapies? Cancer Res 1998; 58: 4090-5.

[5] Motzer RJ, Russo P. Systemic therapy for renal cell carcinoma. J Urol 2000; 163: 408-17.

[6] Clark JI, Atkins MB, Urba WJ, Creech S, Figlin RA, Dutcher JP, Flaherty L, Sosman JA, Logan TF, White R, Weiss GR, Redman BG, Tretter CP, McDermott D, Smith JW, Gordon MS, Margolin KA. Adjuvant high-dose bolus interleukin-2 for patients with high-risk renal cell carcinoma: a cytokine working group randomized trial. J Clin Oncol 2003; 21: 3133-40.

[7] Messing EM, Manola J, Wilding G, Propert K, Fleischmann J, Crawford ED, Pontes JE, Hahn R, Trump D; Eastern Cooperative Oncology Group/Intergroup trial. Phase III study of interferon alfa-NL as adjuvant treatment for resectable renal cell carcinoma: an Eastern Cooperative Oncology Group/Intergroup trial. J Clin Oncol 2003; 21: 1214-22.

[8] Nishizawa S, Hirohashi Y, Torigoe T, Takahashi A, Tamura Y, Mori T, Kanaseki T, Kamiguchi K, Asanuma H, Morita R, Sokolovskaya A, Matsuzaki J, Yamada R, Fujii R, Kampinga HH, Kondo T, Hasegawa T, Hara I, Sato N. HSP DNAJB8 controls tumor-initiating ability in renal cancer stem-like cells. Cancer Res 2012; 72: 2844-54.

[9] Simpson AJ, Caballero OL, Jungbluth A, Chen YT, Old LJ. Cancer/testis antigens, gametogenesis and cancer. Nat Rev Cancer 2005; 5: 615-25.

[10] Sharma P, Shen Y, Wen S, Bajorin DF, Reuter VE, Old LJ, Jungbluth AA. Cancer-testis antigens: expression and correlation with survival in human urothelial carcinoma. Clin Cancer Res 2006; 12: 5442-7.

[11] Valmori D, Souleimanian NE, Tosello V, Bhardwaj N, Adams S, O'Neill D, Pavlick A, Escalon JB, Cruz CM, Angiulli A, Angiulli F, Mears G, Vogel SM, Pan L, Jungbluth AA, Hoffmann EW, Venhaus R, Ritter G, Old LJ, Ayyoub M. Vaccination with NY-ESO-1 protein and CpG in Montanide induces integrated antibody/Th1 responses and CD8 T cells through cross-priming. Proc Natl Acad Sci U S A 2007; 104: 8947-52.

[12] Atanackovic D, Altorki NK, Cao Y, Ritter E, Ferrara CA, Ritter G, Hoffman EW, Bokemeyer C, Old LJ, Gnjatic S. Booster vaccination of cancer patients with MAGE-A3 protein reveals long-term immunological memory or tolerance depending on priming. Proc Natl Acad Sci U S A 2008; 105: 1650-5.

[13] Cheever MA, Allison JP, Ferris AS, Finn OJ, Hastings BM, Hecht TT, Mellman I, Prindiville SA, Viner JL, Weiner LM, Matrisian LM. The prioritization of cancer antigens: a national cancer institute pilot project for the acceleration of translational research. Clin Cancer Res 2009; 15: 5323-37.

[14] Yamanaka K, Miyake H, Hara I, Gohji K, Arakawa S, Kamidono S. Expression of MAGE genes in renal cell carcinoma. Int J Mol Med 1998; 2: 57-60.

[15] Kulkarni P, Shiraishi T, Rajagopalan K, Kim R, Mooney SM, Getzenberg RH. Cancer/testis antigens and urological malignancies. Nat Rev Urol 2012; 9: 386-96.

[16] Yin B, Liu G, Wang XS, Zhang H, Song YS, Wu B. Expression profile of cancer-testis genes in transitional cell carcinoma of the bladder. Urol Oncol 2012; 30: 886-92.

[17] Li M, Yuan YH, Han Y, Liu YX, Yan L, Wang Y, Gu J. Expression profile of cancer-testis genes in 121 human colorectal cancer tissue and adjacent normal tissue. Clin Cancer Res 2005; 11: 1809-14.

[18] Sahin U, Türeci O, Chen YT, Seitz G, Villena-Heinsen C, Old LJ, Pfreundschuh M. Expression of multiple cancer/testis (CT) antigens in breast cancer and melanoma: basis for polyvalent CT vaccine strategies. Int J Cancer 1998; 78: 387-9.

[19] Scanlan MJ, Altorki NK, Gure AO, Williamson B, Jungbluth A, Chen YT, Old LJ. Expression of cancer-testis antigens in lung cancer: definition of bromodomain testis-specific gene (BRDT) as a new CT gene, CT9. Cancer Lett 2000; 150: 155-64.

[20] Lonchay C, van der Bruggen P, Connerotte T, Hanagiri T, Coulie P, Colau D, Lucas S, Van Pel A, Thielemans K, van Baren N, Boon T. Correlation between tumor regression and T cell responses in melanoma patients vaccinated with a MAGE antigen. Proc Natl Acad Sci U S A 2004; 101 Suppl 2: 14631-8.

[21] Kurashige T, Noguchi Y, Saika T, Ono T, Nagata Y, Jungbluth A, Ritter G, Chen YT, Stockert E, Tsushima T, Kumon H, Old LJ, Nakayama E. Ny-ESO-1 expression and immunogenicity associ-

ated with transitional cell carcinoma: correlation with tumor grade. Cancer Res 2001; 61: 4671-4.

[22] Dhodapkar MV, Osman K, Teruya-Feldstein J, Filippa D, Hedvat CV, Iversen K, Kolb D, Geller MD, Hassoun H, Kewalramani T, Comenzo RL, Coplan K, Chen YT, Jungbluth AA. Expression of cancer/testis (CT) antigens MAGE-A1, MAGE-A3, MAGE-A4, CT-7, and NY-ESO-1 in malignant gammopathies is heterogeneous and correlates with site, stage and risk status of disease. Cancer Immun 2003; 3: 9.

[23] Sugita Y, Wada H, Fujita S, Nakata T, Sato S, Noguchi Y, Jungbluth AA, Yamaguchi M, Chen YT, Stockert E, Gnjatic S, Williamson B, Scanlan MJ, Ono T, Sakita I, Yasui M, Miyoshi Y, Tamaki Y, Matsuura N, Noguchi S, Old LJ, Nakayama E, Monden M. NY-ESO-1 expression and immunogenicity in malignant and benign breast tumors. Cancer Res 2004; 64: 2199-204.

[24] Adams S, Greeder L, Reich E, Shao Y, Fosina D, Hanson N, Tassello J, Singh B, Spagnoli GC, Demaria S, Jungbluth AA. Expression of cancer testis antigens in human BRCA-associated breast cancers: potential targets for immunoprevention? Cancer Immunol Immunother 2011; 60: 999-1007.

[25] Jungbluth AA, Busam KJ, Kolb D, Iversen K, Coplan K, Chen YT, Spagnoli GC, Old LJ. Expression of MAGE-antigens in normal tissues and cancer. Int J Cancer 2000; 85: 460-5.

[26] Yawata T, Nakai E, Park KC, Chihara T, Kumazawa A, Toyonaga S, Masahira T, Nakabayashi H, Kaji T, Shimizu K. Enhanced expression of cancer testis antigen genes in glioma stem cells. Mol Carcinog 2010; 49: 532-44.

[27] Gedye C, Quirk J, Browning J, Svobodová S, John T, Sluka P, Dunbar PR, Corbeil D, Cebon J, Davis ID. Cancer/testis antigens can be immunological targets in clonogenic CD133+ melanoma cells. Cancer Immunol Immunother 2009; 58: 1635-46.

[28] Marturano J, Longhi R, Casorati G, Protti MP. MAGE-A3(161-175) contains an HLA-DRbeta4 restricted natural epitope poorly formed through indirect presentation by dendritic cells. Cancer Immunol Immunother 2008; 57: 207-15.

[29] Ait-Tahar K, Liggins AP, Collins GP, Campbell A, Barnardo M, Lawrie C, Moir D, Hatton C, Banham AH, Pulford K. Cytolytic T-cell response to the PASD1 cancer testis antigen in patients with diffuse large B-cell lymphoma. Br J Haematol 2009; 146: 396-407.

[30] Errington JA, Conway RM, Walsh-Conway N, Browning J, Freyer C, Cebon J, Madigan MC. Expression of cancer-testis antigens (MAGE-A1, MAGE-A3/6, MAGE-A4, MAGE-C1 and NY-ESO-1) in primary human uveal and conjunctival melanoma. Br J Ophthalmol 2012; 96: 451-8.