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Original Article

Hypomethylation of *MAGE-A1* in clear cell renal cell carcinoma

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Abstract: Introduction: *Melanoma Antigen Family A1* (*MAGE-A1*) geneis a cancer-testis antigen (CTA) gene and its methylation status remains unknown in renal cell carcinoma (RCC). This study aimed to investigate the methylation status of the *MAGE-A1* promoter and the effect of *MAGE-A1* hypomethylation on the tumor progression and survival outcome in RCC patients. Materials and Methods: Paraffin-embedded specimens from 42 RCC patients were analyzed for *MAGE-A1* methylation by methylation-specific polymerase chain reaction. The distribution of TNM/AJCC stages and survival outcome were compared among patients with unmethylated (U), partially methylated (P), and methylated (M) promoter of the MAGE-A1 gene. Results: There were 6 cases (14.3%) with methylation (M), 15 cases (35.7%) with partial methylation (P) and 21 cases (50%) with unmethylation (U) of the *MAGE-A1* promoter. Significances were found in T (P=0.042) and AJCC (P=0.032) stage distributions among M, P, and U groups. Kaplan-Meier survival analysis showed that the survival outcome was higher in M group as compared with U and P groups although the difference did not reach a statistical significance. Conclusion: Our findings suggested that hypomethylation of *MAGE-A1* is not rare in RCC, and methylation status of *MAGE-A1* promoter could influence the disease stage as well as the survival outcome of RCC patients.

Keywords: Renal cell carcinoma, methylation, *MAGE-A1,* cancer-testis antigen (CTA)

Introduction

Renal cell carcinomas (RCC) is a malignant tumor originated from renal cortex and is the most common adult renal malignancy. It has been shown that RCC has an annual incidence of newly diagnosed cases of > 200,000 worldwide, accounting for approximately 2% of all malignancies [1]. In China, the annual incidence of RCC is about 540 per million people [2]. The prognosis of RCC is markedly dependent on the tumors stages. RCC patients at American Joint Committee on Cancer (AJCC) stage I have a 5-year survival rate over 80%, while the 5-year survival rate for patients at stage IV is less than 10% [3]. According to the WHO classification, RCC can be classified into several major subtypes. Among them, clear cell RCC (ccRCC) is the most predominance one, accounting for about 75% of all RCCs [4].

Melanoma Antigen Family A1 (*MAGE-A1*) gene, originally identified on the surface of melanoma cells [5], belongs to the MAGE-A gene family-comprising 12 members (*MAGE-A1*-A12) and locating at chromosomal Xq28 region [6]. *MAGE-A1* is mainly silent innormal somatic tissues except for testicular germ cells (spermatogonia and primary spermatocytes) and placentabut has found to be transcriptionally activated in a variety of human cancers, such as melanomas [5], oral squamous cell carcinoma [7], hepatocellular carcinoma [8], breast cancer [9], renal cell carcinoma [10], ovarian cancer [11] and colorectal cancer [12]. Hence, *MAGE-A1* was termed as a cancer-testis antigen (CTA) gene or cancer-germline (CG) gene [13], and its coding protein could be recognized by T cells and used as a target for T cell-mediated cancer immunotherapy. As for the biological function, *MAGE-A1* has been shown to be a

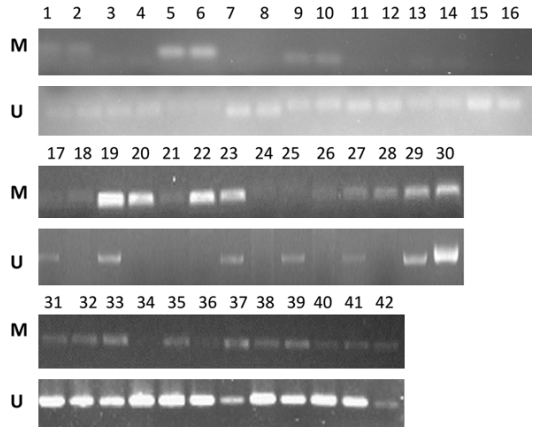

Figure 1. Methylation status of the MAGE-A1 promoterin clear cell renal cell carcinoma tissues was determined by methylation-specific polymerase chain reaction. The number 1-42 indicates the patient number. M, methylated amplified PCR fragment, U, unmethylated amplified PCR fragment.

potent transcriptional repressor through association with Ski-interacting protein and histone deacetylase 1 (HDAC1) [14].

In normal somatic tissues, epigenetic inactivation, such as DNA methylation and recruitment of methylated DNA-binding proteins, are the common mechanisms which inhibit the expression of CAT genes [15]. In cancer cells, however, these epigenetic silences are reduced, which consequently result in the production of tumor-specific antigens [16]. It has been known that this epigenetic reactivation mechanism accounts for the expression of *MAGE-A1* in cancer cells. Previous studies showed that promoter hypomethylation can reactivate expression of *MAGE-A1* in human breast cancer, prostate cancer, colon cancer cell lines [17] and melanoma cancer cell lines [18]. In the cancer tissue specimens, hypomethylation of *MAGE-A1* promoter has been shown in breast cancer [19], colorectal cancer [20] and gastric cancer [22]. In addition, these epigenetic alterations are usually associated with expression of *MAGE-A1* in cancer tissues.

At present, studies on *MAGE-A1* in RCC are still limited. Yamanaka *et al*. have reported that *MAGE-A1* gene was expressed in 11 (22%) of RCC samples but not in normal kidney tissues [23]. Another study demonstrated that *MAGE-A1* mRNA was detected in 20% ccRCC samples but not in the paired adjacent normal tissues [10]. Even though *MAGE-A1* has been shown to express in RCC, however, the hypomethylation status in RCC remains unknown. In addition, the hypomethylated *MAGE-A1* promoter has been revealed in several cancer types, but the methylation status of *MAGE-A1* in RCC remains to be investigated. Therefore, the purpose of this study was to investigate the methylation status of the *MAGE-A1* promoter by using methylation-specific polymerase chain reaction (MSP) technique with 42 ccRCC paraffin-embedded tissue specimens. We also elucidated the effect of *MAGE-A1* hypomethylation on the tumor progression and survival outcome in ccRCC patients.

Methods

Patients and data collection

This study analyzed 42 ccRCC paraffin-embedded tissue specimens from patients underwentsurgical resection oftumor in our center from February 1999 to August 2009. The inclusion criterion was patients diagnosed of ccRCC, and the exclusion criterion included patients who cannot be followed up. This study was approved by the Institutional Review Board of the First Affiliated Hospital of Sun Yat-sen University. Informed consent was obtained from all patients. Patients' demographic data and clinical data including the tumor-node-metastasis (TNM)/American Joint Committee on Cancer (AJCC) staging, and postoperative follow-up data were recorded.

Methylation-specific PCR (MSP)

Paraffin-embedded tissues were sectioned and dried for 2 hours at 60°C or overnight at 37°C. The slices were immersed in xylene twice for 15 min, and then gradually rehydrated through gradient ethanol into water. DNA samples were extracted from the tissue samples by using a QIAamp DNA FFPE Tissue Kit (Qiagen, USA), and then the DNA samples were subjected to DNA bisulfite conversion using EZ DNA Methylation Kit (ZYMO Research, Irvine, CA, USA) according to the manufacture's protocol. After which, the bisulfite-modified DNA samplewas used as a template for MSP analysis. Primers specific for methylated sequence were forward 5'-TTCGGGTGTTCGGATGTGAC-3' and reverse 5'-CCTAAATCAAATTCTTCGACCG-3', and primers for unmethylated sequence were forward 5'-TTTGGGTGTTTGGATGTGAT-3' and reverse 5'-CCTAAATCAAATTCCTTCAACCA-3'. The amplified PCR fragments were 122 bps and were analyzed on a 1% agarose gel (Beijing

Table 1. Demographic and clinical data of ccRCC patients with methylated (M), partially methylated (P), and unmethylated (U) *MAGE-A1* promoter (n=42)

	M (n=6)	P (n=15)	U (n=21)	*p* value
Gender, n (%)				0.019*
Male	2 (33.3)	8 (53.3)	18 (85.7)	
Female	4 (66.7)	7 (46.7)	3 (14.3)	
Age, years (SD)	53.3 (16.8)	48.0 (13.0)	46.0 (14.6)	0.253
Height, cm (SD)	155.3 (6.5)	160.1 (10.1)	166.5 (6.4)	0.008**
Body weight, kg (SD)	55.2 (8.8)	61.7 (14.9)	64.0 (11.4)	0.308
BMI, kg/m^2 (SD)	23.0 (4.6)	23.8 (3.7)	23.0 (3.2)	0.659
Blood type, n (%)				0.308
A	1 (16.7)	6 (40.0)	4 (22.2)	
B	4 (66.7)	3 (20.0)	4 (22.2)	
AB	0	0	1 (5.6)	
O	1 (16.7)	6 (40.0)	9 (50.0)	
Tumor diameter, cm (SD)	7.4 (3.0)	7.8 (2.9)	5.9 (2.4)	0.099
Following time, month (SD)	131.0 (23.0)	71.7 (44.4)	72.0 (44.7)	0.017*

*p < 0.05, **P < 0.01.

Hengao Biotechnology, Beijing, China). The images were captured using a UV gel imaging system (UVP Imaging system EC3). The PCR products with a single band only in the methylated or unmethylated fragment were defined as both alleles of methylated (M) or unmethylated (U) status, respectively. The presence of both methylated and unmethylated fragmentswas-defined as partial methylation (P).

Statistical analysis

Statistical analyses were performed using IBM SPSS Version 20 (SPSS Statistics V20, IBM Corporation, Somers, New York). Continuous data are presented as the mean ± standard deviation (SD). Significance was assessed using analysis of variance (ANOVA) followed by Tukey Honestly Significant Difference test for all baseline characteristics and hetmatological parameters. Categorical data including TNM stage, AJCC stage, blood type, and gender were analyzed by Fisher's exact test. Kaplan-Meier Survival curves of patients in the three groups were plotted and the differences were compared by log-rank test. All significant level was set at $P < 0.05$.

Results

Hypomethylation of MAGE-A1 promoter in ccRCC

Toevaluate the methylation status of the *MAGE-A1* promoter in ccRCC, we included 42 ccRCC samplesfor MSP analysis. Acording to the MSP data (**Figure 1**), there were 6 cases (14.3%) with methylation (M), 15 caseswith (35.7%) partial methylation (P) and 21 cases (50%) with unmethylation (U)of the *MAGE-A1* promoter. The results showed half of ccRCC samples with hypomethylation at the *MAGE-A1* promoter.

Patient demographic and clinical data

To determine if hypomethylation at *MAGE-A1* has an effect on the disease progression and survival outcome of ccRCC patients, we divided the 42 patients into three groups based on their methylation status of the *MAGE-A1* promoter. **Table 1** summarized the demographic and clinical data of patients among 3 groups. In general, except for gender, height and the following time, there was no significant difference in all the othercharacteristics among 3 groups. In addition, there was nosignificant difference in the 27 hematological parameters among groups (**Table 2**). These results indicated that the three groups are comparable. The correlation coefficient analysis showed that the methylation status was correlated with the tumor diameter (r=-0.312, P=0.047), which is consistent withthe observation that the U group had descriptively smaller tumor diameter than M and P groups (P=0.099, **Table 1**).

Association between MAGE-A1 promoter methylation and TNM/AJCC staging

Next, we analyzed the distributions of TNM and AJCC stage among the three groups. As shown in **Table 3**, significances were found in T (P=0.042) and AJCC (P=0.032) stage distributions among M, P, and U groups, suggesting that methylation status of the *MAGE-A1* promoter was associated with T and AJCC tumor stage in RCC. The correlation coefficient analysis also showed that there was a significant correlation between methylation status and N stage (r=0.307, P=0.048), suggesting that a

Table 2. Hematological parameters of ccRCC patients with methylated (M), partially methylated (P), and unmethylated (U) *MAGE-A1* promoter (n=42)

	M	P	U	*p* value
K^+, mmol/L (SD)	4.0 (0.0)	4.3 (0.3)	4.4 (0.6)	0.485
Na^+, mmol/L (SD)	139.0 (0.0)	139.5 (3.6)	141.7 (3.5)	0.230
Cl^+, mmol/L (SD)	103.1 (5.2)	103.8 (6.4)	105.9 (5.1)	0.582
Ca^{2+}, mmol/L (SD)	2.3 (0.2)	2.3 (0.4)	2.3 (0.4)	0.916
AST, U/L (SD)	27.3 (15.8)	20.5 (8.0)	23.0 (11.8)	0.460
ALT, U/L (SD)	23.0 (16.3)	20.5 (14.1)	26.4 (20.5)	0.630
TBA, mmol/L (SD)	6.2 (3.1)	6.1 (5.2)	4.9 (2.1)	0.529
ALP, U/L (SD)	54.7 (15.2)	70.8 (37.7)	75.6 (24.2)	0.307
GGT, U/L (SD)	19.7 (6.4)	31.8 (28.7)	40.2 (33.2)	0.308
LDH, U/L (SD)	200.5 (82.2)	203.3 (50.2)	167.4 (62.1)	0.191
AFU, nmol/ml • h (SD)	10.0 (4.0)	10.7 (4.9)	10.5 (6.4)	0.970
ALB, g/L (SD)	38.8 (4.3)	43.0 (3.6)	41.3 (4.5)	0.113
GLO, g/L (SD)	31.5 (5.2)	31.8 (5.5)	28.9 (5.4)	0.245
DBIL, μmol/L (SD)	2.2 (1.9)	3.3 (1.0)	3.7 (2.6)	0.311
IBIL, μmol/L (SD)	8.9 (6.2)	9.6 (3.5)	10.9 (8.6)	0.752
BUN, mmol/L (SD)	6.0 (1.6)	4.3 (1.4)	5.4 (2.1)	0.086
CRE, μmol/L (SD)	92.3 (14.3)	89.8 (21.8)	98.8 (28.4)	0.548
UA, μmol/L (SD)	336.8 (140.8)	348.9 (109.7)	333.3 (105.2)	0.917
CHO, mmol/L (SD)	4.7 (1.2)	4.5 (1.2)	4.6 (0.8)	0.911
TG, mmol/L (SD)	1.8 (1.2)	1.6 (1.0)	1.5 (1.1)	0.751
GLU, mmol/L (SD)	5.3 (0.7)	5.1 (0.8)	4.9 (0.7)	0.502
HDL, mmol/L (SD)	1.3 (0.4)	1.2 (0.3)	1.2 (0.3)	0.725
LDL, mmol/L (SD)	3.1 (1.0)	2.7 (1.0)	2.9 (0.8)	0.608
WBC, 10^9/L (SD)	8.1 (1.8)	8.1 (2.4)	8.4 (2.5)	0.920
RBC, 10^{12}/L (SD)	4.0 (1.0)	4.5 (0.7)	4.9 (0.8)	0.056
Hb, g/L (SD)	119.3 (31.3)	134.0 (24.9)	134.1 (15.9)	0.322
PLT, 10^9/L (SD)	219.8 (103.1)	276.3 (116.7)	270.7 (79.6)	0.466

higher methylation status was associated with a higher N stage (from N0 to N2).

MAGE-A1 promoter methylation and survival

Next, we investigated whether the methylation status has an effect on the survival outcome of RCC patients. Kaplan-Meier survival analysis showed that the survival rate was higher in M group as compared with U and P groups, however, the difference did not reach a statistical significance (log-rank test, P=0.248, **Figure 2A**). Further analyses were conducted to paired-wisely compare the survival outcome. We found that M group consistently possessed the best survival rate in all comparison (**Figure 2B**, **2C**), and the difference between M and P group reached marginal significance (**Figure 2B**, P=0.074). Nevertheless, there was no significance between M and U groups (**Figure 2C**) (P= 0.210) as well as P and U groups (**Figure 2D**) (P= 0.516).

Discussion

In this study, we investigated the methylation status of the *MAGE-A1* promoter and its correlation with tumor stage and survival outcome in 42 ccRCC patients. The results showed that half of ccRCC samples had a hypomethylated promoter at *MAGE-A1*. In addition, the methylation status of the *MAGE-A1* promoter was associated with T and AJCC tumor stage in RCC. Furthermore, patients with a fully methylated *MAGE-A1* had the best survival outcome. Our findings suggested that hypomethylation of *MAGE-A1* is not rare in RCC, and methylation status of *MAGE-A1* promoter could influence the disease stage as well as the survival outcome of RCC patients.

RCC is considered as an immunotherapy responsive malignancy [24] and cancer-specific immunotherapy may be a promising strategy for RCC treatment. Understanding the epigenetic alteration of CTA gene may facilitate development for gene-specific strategies of immunotherapy. Hypomethylation of *MAGE-A1* promoter seems a common event and has already been described in several other cancer types. The previous study on breast cancers showed that mean hypomethylation rate of the *MAGE-A1* promoter is around 33% (n=37) [19], while in gastric cancer, the *MAGE-A1* promoters were hypomethylated in 29% (n=84) specimens [22]. In colorectal cancer, promoter hypomethylation of *MAGE-A1* was observed in 43% specimens (n=87) [20], and in NSCLC, hypomethylation of *MAGE-A1* was detected in 41.8% (n=67) of the specimens [21]. As for RCC, although mRNA

Table 3. TNM and AJCC staging based on methylation status of *MAGE-A1* promoter (n=42)

	M (n=6)	P (n=15)	U (n=21)	*p* value
T stage, n (%)				0.042
T1	2 (33.3)	5 (33.3)	13 (61.9)	
T2	4 (66.7)	9 (60.0)	3 (14.3)	
T3	0	1 (6.7)	3 (14.3)	
T4	0	0	2 (9.5)	
N stage, n (%)				0.229
N0	6 (100.0)	12 (80.0)	13 (61.9)	
N1	0	2 (13.3)	4 (19.0)	
N2	0	1 (6.7)	4 (19.0)	
M stage, n (%)				0.490
M0	6 (100.0)	13 (86.7)	19 (90.5)	
M1	0	2 (13.3)	2 (9.5)	
AJCC stage, n (%)				0.032
I	2 (33.3)	4 (26.7)	9 (42.9)	
II	4 (66.7)	8 (53.3)	3 (14.3)	
III	0	0	3 (14.3)	
IV	0	3 (20.0)	6 (28.6)	

expression of *MAGE-A1* has been reported previously [10, 23], the methylation status of *MAGE-A1* promoter has not been investigated yet. Our results showed that 50% RCC tissue sample had a hypomethylated promoter at *MAGE-A1* gene (n=42). To our knowledge, this is the first study reporting the frequency of hypomethylation at *MAGE-A1* in RCC. The finding suggested that incidence of hypomethylation of *MAGE-A1* is not arare event as compared to the other cancer types, and *MAGE-A1* could be considered as a potential target for cancer-specific immunotherapy against RCC.

Our results showed that the distributions of TNM and AJCC stage were significantly different among the three groups, indicating methylation status of the *MAGE-A1* promoter was associated with tumor stage in RCC.Patients with methylated *MAGE-A1* (M group, n=6) mainly distributed in the first two stages of TNM and AJCC staging system, andhad no regional lymph node and distant metastasis (N1, N2 and M1). In contrast, patients with hypomethylated *MAGE-A1* (U group, n=21) were mainly in the first stage of TNM and AJCC, however, there were some patients in U group were of advanced stages. Notably, asmall fraction of patients in U group was of regional lymph node and distant metastasis. In addition, there was a significant correlation between methylation status and N stage. However, the patient distribution differences in N and M stages did not significantly different among three groups due to the small sample size. Similarly, the effect of hypomethylated *MAGE-A1* on the tumor progression has also been reported in gastric cancer. Honda *et al*. showed that hypomethylation of both *MAGE-A1* and *-A3* promoters was more frequently in gastric cancer patients with advanced clinical stages, and these patients also had a higher incidence of lymph node metastasis than those without hypomethylation [22].

In the present study, the Kaplan-Meier survival analysis showed that patients with methylated *MAGE-A1* (M group) had the best survival outcome among 3 groups although the difference did not reach a statistical significance. The difference between M and P group reached marginal significance. It should be mentioned that there were only 6 patients in M group, which should be main reason leading to thestatistical insignificance of survival analyses. Interestingly, the prognostic potential of the methylation status of *MAGE-A1* has also been described in previous studies. Yamanaka *et al*. have reported that patients with high stage RCCs had a higher incidence of the expression of plural MAGE genes as compared with patients with low stage RCC [23]. A study by Honda *et al*. on gastric cancer reported that patients with hypomethylation *MAGE-A1* tended to have a worse prognosis [22]. Another study on NSCLC also showed that patients with hypomethylated *MAGE-A1* had a worse prognosis [21]. Therefore the prognostic value of methylation status of *MAGE-A1* is worthy to be further investigated.

It should be noted that there are several limitations in this study. Firstly, the sample size of this study was relatively small, especially the 42 patients were unevenly distributed among three groups, making the results of statistical analyses not significant. A well-design prospective study with large sample size should be conducted to further confirm the findings of this study. In addition, we did not assess the expression of *MAGE-A1* in these cancer tissue samples to further confirm if hypomethylation of *MAGE-A1* is associated with reactivation of *MAGE-A1*. Furthermore, we did not simultaneously assess the adjacent normal tissues. However, our data still provide preliminary re-

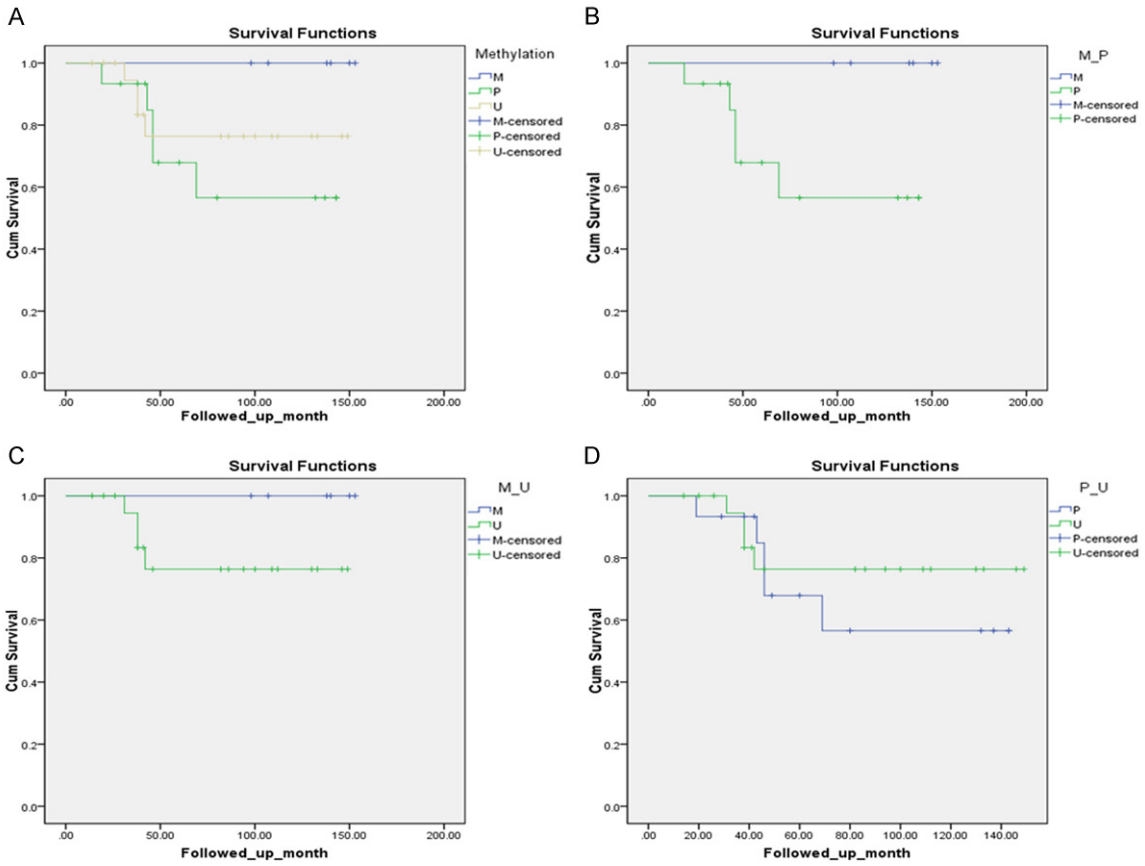

Figure 2. Kaplan-Meier survival curves for overall survival in ccRCC patients with different *MAGE-A1* promoter methylation status. (A) M vs. P vs. U, (B) M vs. P, (C) M vs. U, (D) P vs. U.

sults regarding the status and roles of hypomethylation of *MAGE-A1* in the RCC. All these limitations should be considered and further addressed in the following study.

Conclusion

Our data with 42 ccRCC samples showed that hypomethylation of *MAGE-A1* is not rare in RCC and the methylation status of *MAGE-A1* may affect the disease progression and survival outcome of RCC patients. These preliminary findings should be helpful for better understanding the hypomethylation of *MAGE-A1* in RCC.

Disclosure of conflict of interest

None.

Abbreviations

AJCC, American Joint Committee on Cancer; ccRCC, clear cell renal cell carcinoma; CG, cancer-germline; CTA, cancer-testis antigen; M, methylated; *MAGE-A1*, *Melanoma Antigen Family A1*; MSP, methylation-specific polymerase chain reaction; NSCLC, non-small cell lung cancer; P, partially methylated; RCC, renal cell carcinoma; U, unmethylated.

Ethics statement

This study was approved by the Institutional Review Board of the First Affiliated Hospital of Sun Yat-sen University. Informed consent was obtained from all patients.

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